Stable Integration Vector for Nutrient Broth-Based Selection of Attenuated 
*Listeria monocytogenes* Strains with Recombinant Antigen Expression

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Recombinant *Listeria monocytogenes* strains induce strong cellular immune responses and may prove useful for antigen delivery for the vaccination of humans. However, the genetic systems currently available for the stable expression of recombinant antigens by *L. monocytogenes* rely on the use of antibiotic resistance genes. We report on a derivative, pPL2dalGlnA, of the *Listeria monocytogenes* pPL2 integration vector that completely lacks drug resistance genes. The selectable markers in pPL2dalGlnA are glutamine synthetase (GlnA) and alanine racemase (Dal). This novel vector was stably maintained in auxotrophic *L. monocytogenes* strains that normally require n-alanine. The pPL2dalGlnA vector also partially restored the ability of an *L. monocytogenes* Δdal Δdat strain to colonize the spleens and livers of infected mice. A novel, highly attenuated strain of *L. monocytogenes* with quadruple deletions was also engineered by deleting the *L. monocytogenes* actA and plcB virulence genes from a Δdal Δdat strain. Infection of mice with recombinants of this mutant strain that express the antigen from pPL2dalGlnA were shown to elicit CD8+ T-cell responses to human immunodeficiency virus Tat. This vector system is thus useful for stable antigen expression and vaccination studies.

*Listeria monocytogenes* is a gram-positive bacterium and facultative intracellular parasite of humans and animals. The bacterium expresses a hemolysin (LLO) that enables it to invade and replicate within the cytosol of eukaryotic cells. Similar to the proteins produced by viral infection, the proteins produced by cytosolic *L. monocytogenes* are processed and presented to the vertebrate immune system by the endogenous major histocompatibility complex (MHC) class I antigen presentation pathway. Peptide-loaded class I MHC molecules that reach the surface of specialized antigen-presenting cells prime naive CD8+ T cells to expand in number and differentiate into mature long-lived memory cell populations. Several endogenous *L. monocytogenes* antigens are known to trigger CD8+ T-cell responses that can protect mice from subsequent infections (15). In addition, recombinant proteins expressed by *L. monocytogenes* can access the endogenous MHC class I presentation pathway and prime protective CD8+ T-cell responses (12, 17). Thus, recombinant *L. monocytogenes* strains show promise as vaccination vehicles for the generation of CD8+ T-cell responses in humans.

The widespread use of live organisms for antigen delivery in humans currently faces several hurdles. These include the need to engineer attenuated strains that safely and stably express and deliver the foreign antigens of interest. To date, most systems used for recombinant antigen expression in *L. monocytogenes* have used plasmids with antibiotic resistance markers either to maintain the antigen-encoding plasmids in the cytoplasm of the bacteria or as selectable markers to aid with the isolation of chromosomal integrants of the antigen expression construct (8, 9, 12, 16, 17). While these strategies demonstrate the ability of recombinant *L. monocytogenes* strains to elicit cell-mediated immune responses to viral and cancer antigens, the currently used plasmid vectors are unstable without constant selection pressure and may be lost from the vaccine bacterial strain during replication within the host. Furthermore, vaccine strains that use antibiotic resistance markers for selection purposes have the potential to transmit resistance genes to other pathogens, undermining the therapeutic usefulness of the respective antibiotics.

The development of genetic systems to integrate antigen expression constructs into the *L. monocytogenes* genome circumvents several of the problems associated with plasmid instability. However, construction of such vaccine strains traditionally required the time-consuming process of allelic exchange. More recently, a novel integration vector has been developed to permit chromosomal insertions in a single step. This vector, pPL2, has previously been used to stably integrate a variety of genes at a specific site in the chromosome of *Listeria monocytogenes* (13). However, as with other genetic systems, the engineering of recombinant strains with pPL2 has entailed the use of antibiotic resistance genes as selectable markers.

Here we report on the development of a second-generation pPL2 vector that can be used to engineer recombinant *L. monocytogenes* vaccine strains in the complete absence of antibiotic resistance markers. This new vector, pPL2dalGlnA, is stably maintained in *L. monocytogenes* strains cultured in broth and can be used to express antigens. Furthermore, CD8+ T-cell responses specific for human immunodeficiency virus (HIV) Tat antigens were generated upon infection with a novel attenuated *L. monocytogenes* vaccine strain expressing the HIV Tat antigen from pPL2dalGlnA. Hence, the novel genetic system developed here may prove useful for the vaccination of humans.
MATERIALS AND METHODS

Chemicals, reagents, and animals. Molecular biology reagents were purchased from New England Biolabs (Ipswich, MA) and Promega (Madison, WI). Brain heart infusion (BHI) broth was used for L. monocytogenes cultures and was purchased from Difco Laboratories (a subsidiary of BD Biosciences, San Jose, CA). Components for Luria broth (LB) were purchased from Thermo Fisher Scientific (Pittsburg, PA). Six- to 8-week-old C57BL/6 mice were used for the competitive index infection studies and were purchased from Harlan (Indianapolis, IN). BALB/c mice were used for the immunization studies and were from National Cancer Institute (Frederick, MD). All animal studies were approved by the Institutional Care and Use Committees at the National Jewish Medical and Research Center and the University of Pennsylvania.

Engineering of pPL2dalGlnA vector. To generate pPL2dal, the gram-positive bacterial chloramphenicol transferase (cat) gene of pPL2 was deleted by digestion with KpnI and PvuI and replaced with the L. monocytogenes alanine racemase (dal) gene. The dal gene was amplified by PCR with primers Updal2 (GGGCGATGTTTCTTAAGTGTTTCCTACATATTCTCACATG) and Downdal2 (GGGCGATGTTTCTTAAGTGTTTCCTACATATTCTCACATG). These primers included a KpnI or a PvuI site (the two sites are underlined in the sequences of primers Updal2 and Downdal2, respectively). Subsequently, pPL2dalGlnA was engineered by removing the gram-negative bacterial cat gene from pPL2dal by using NotI and BstBI and replacing this with the glnA gene cloned from Escherichia coli K-12 strain Top10. The glnA gene was amplified with the primers EcGlnAfor (GGGCGATGTTTCTTAAGTGTTTCCTACATATTCTCACATG) and EcGlnArev (GGGCGATGTTTCTTAAGTGTTTCCTACATATTCTCACATG) and EcoRI and BamHI sites. These restriction sites were introduced with the primers EcGlnAfor and EcGlnArev, respectively in the vector pUC18. 

Generation of E. coli ΔglnA strains for cloning of pPL2dalGlnA. An E. coli ΔglnA strain for cloning of pPL2dalGlnA was engineered by deletion of the glnA gene and its promoter by the method of Datsenko and Wanner (6). The primers used for the deletion were ATCACAACATCCTCCGCAAACAAGTATTGCAA and MG1655 GAGTGTGTAGGCTGGAGCTGCTTCG and ACAGGCGAAAAGTTTCCACGAGTGTGTAGGCTGGAGCTGCTTCG and ACAGGCGAAAAGTTTCCAC. These restriction sites for BstBI and NotI are underlined in the sequences of primers EcGlnAfor and EcGlnArev, respectively. In primer EcGlnArev, 17 additional nucleotides from the p15A origin of replication are encoded upstream of the glnA promoter.

Growth and stability of pPL2dalGlnA strains. For the stability of pPL2dalGlnA strains, overnight cultures were started from single colonies of each strain for cloning of pPL2dalGlnA. The livers and spleens were harvested at 36 h postinfection (hpi), and serial dilutions of the lysates from these organs were plated on LB agar (strains 10403S and 10403S::pPL2) or BHI broth supplemented with D-alanine for L. monocytogenes strain 10403S::pPL2dalGlnA) or LB agar plus 100 μg/ml t- alanine (L. monocytogenes strain 10403S::pPL2dalGlnA) to enumerate the bacterial burdens.

Cloning of SIV Nef and HIV Tat into pPL2dalGlnA. An antigen expression cassette was made by cloning FLAG-tagged simian immunodeficiency virus (SIV) Nef (deleted of its first 11 amino acids) and vesicular stomatitis virus (VSV)-tagged HIV Tat downstream of DNA containing the glnA gene. The TCA-precipitated proteins were then blotted onto nitrocellulose membranes, blocked in BLOTTO buffer (1% Tween 20, 5% nonfat milk in PBS) for 2 h, incubated with a primary anti-tag antibody (anti-VSV antibody for Tat protein and anti-FLAG antibody for Nef protein) at 4°C overnight and then with horse-radish peroxidase–labeled immunoglobulin G (HRP) at 4 °C at room temperature, followed by lysis of red blood cells with ACK lysis buffer (Invitrogen). The TCA-precipitated proteins from 1 ml of spent medium were dissolved in sample buffer (62.5 mM Tris-Cl, pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, 5% (β-mercaptoethanol, 0.1% bromophenol blue) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were transferred to nitrocellulose membranes, blocked in BLOTTO buffer and incubated with a primary anti-tag antibody (anti-VSV antibody for Tat protein and anti-FLAG antibody for Nef protein) at 4°C overnight and then with horse-radish peroxidase–labeled immunoglobulin G for 1 h at room temperature, and detected with an enhanced chemiluminescence Western blotting analysis system and exposure to film.

Immunoblots. The recombinant L. monocytogenes Δdat ΔactA plaB Nef Tat strain and the control L. monocytogenes Δdat ΔactA plaB strain were grown in BHI broth (supplemented with t-alanine for L. monocytogenes Δdat ΔactA plaB) to mid-exponential phase, and the overnight cultures were diluted 1:20 and grown at 36°C for 4 h. After centrifugation, the proteins in the supernatant fractions of these cultures were precipitated with trichloroacetic acid (TCA). The TCA-precipitated proteins from 1 ml of spent medium were dissolved in sample buffer (62.5 mM Tris-Cl, pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, 5% (β-mercaptoethanol, 0.1% bromophenol blue) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were transferred to nitrocellulose membranes, blocked in BLOTTO buffer (1% Tween 20, 5% nonfat milk in PBS) for 2 h, incubated with a primary anti-tag antibody (anti-VSV antibody for Tat protein and anti-FLAG antibody for Nef protein) at 4°C overnight and then with horse-radish peroxidase–labeled immunoglobulin G (HRP) at 4°C at room temperature, followed by lysis of red blood cells with ACK lysis buffer (Invitrogen). The TCA-precipitated proteins from 1 ml of spent medium were dissolved in sample buffer (62.5 mM Tris-Cl, pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, 5% (β-mercaptoethanol, 0.1% bromophenol blue) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were transferred to nitrocellulose membranes, blocked in BLOTTO buffer (1% Tween 20, 5% nonfat milk in PBS) for 2 h, incubated with a primary anti-tag antibody (anti-VSV antibody for Tat protein and anti-FLAG antibody for Nef protein) at 4°C overnight and then with horse-radish peroxidase–labeled immunoglobulin G for 1 h at room temperature, and detected with an enhanced chemiluminescence Western blotting analysis system and exposure to film.
second day, the plates were washed and blocked for 2 h with RPMI 1640 containing 10% fetal bovine serum (Gemini BioProducts) and 1% of a 10,000-U/ml penicillin-streptomycin solution. Suspensions containing 5 × 10^7 splenocytes in RPMI 1640 were added to each well and were stimulated in duplicate, with or without 25 μg/ml of a complete set of HIV type 1 clade B Tat synthetic 15-amino-acid-long peptides, each of which overlapped by 11 amino acids (AIDS Research and Reference Reagent Program), or 25 μg/ml of concanavalin A (Sigma Chemical Co.). After incubation at 37°C in a 5% CO₂ incubator for 24 h, the plate was washed and incubated with biotinylated anti-mouse IFN-γ detection antibody (1:60 in 1% bovine serum albumin; R&D Systems) and incubated overnight at 4°C. The wells were then incubated with streptavidin-conjugated alkaline phosphatase (1:60 in 1% bovine serum albumin) for 2 h at room temperature, washed with PBS, and incubated with 5-bromo-4-chloro-3-indolyl phosphate substrate to develop the color. The spots were counted with an automated ELISPOT reader (Cellular Technology, Ltd., Shaker Heights, OH).

RESULTS AND DISCUSSION

Engineering of pPL2dalGlnA. L. monocytogenes strains that retain the ability to invade and replicate in the host cell cytosol also retain immunogenicity and are showing promise as vaccine delivery vehicles (5). D-Alanine is an essential component of bacterial peptidoglycan and is also incorporated into teichoic acids. In L. monocytogenes, D-alanine is produced from L-alanine by an alanine racemase (Dal) and from D-glutamic acid plus pyruvate by a D-amino acid transferase (Dat). L. monocytogenes strains from which both dal and dat are deleted (Δdal Δdat strains) thus require exogenous D-alanine for continued replication. Furthermore, since only trace quantities of D-alanine are produced by mammals, the replication of D-alanine-deficient) medium. To determine the ability of L. monocytogenes Δdal Δdat to grow on selective (D-alanine-deficient) medium. To determine the stability of pPL2dalGlnA integration in the absence of selective pressure, we repeatedly passed the complemented L. monocytogenes Δdal Δdat strain under nonselective conditions. When colonies were plated after 13 to 18 serial passages in the absence of selective pressure, 96 to 100% of the colonies recovered on nonselective BHI agar plates (supplemented with D-alanine) were also recovered on selective (nonsupplemented) BHI agar
L. monocytogenes 10403S, 10403S transduced with pPL2 (10403S::pPL2), and numbers of CFU were recovered from each organ at this time point. The CI ratios reflect the relative in vivo fitness of the Erm-sensitive pPL2 or pPL2dalGlnA test strains following coinfection with a control Erm-resistant strain with equivalent virulence yields a CI value of 1. Circles and triangles, the ratios for isolates recovered from individual mice; bars, mean CI values for each group. The means were significantly different (P < 0.001), as judged by the t test. Between 2 × 10^4 and 2 × 10^5 total numbers of CFU were recovered from each organ at this time point.

plates. These data are consistent with previously published data on the stability of pPL2 integration (13) and indicate that the integrated pPL2dalGlnA vector is highly stable even in the absence of selection.

pPL2dalGlnA only partially complements the virulence of L. monocytogenes Δdal Δdat. To evaluate the effects of pPL2dalGlnA on the growth and virulence of the L. monocytogenes strains, we first compared the growth rate of an L. monocytogenes Δdal Δdat strain with integrated pPL2dalGlnA (L. monocytogenes Δdal Δdat::pPL2dalGlnA) to that of wild-type L. monocytogenes parental strain 10403S and a pPL2 integrant of 10403S (Fig. 2A). Equal numbers of each bacterial strain were diluted in parallel from overnight cultures into nonsupplemented BHI broth, and the growth of the respective strains was measured by hourly readings of the OD from each culture. As shown in Fig. 2, the growth rates of the three bacterial strains were identical. In the absence of pdPL2dalGlnA or medium supplemented with o-alanine, L. monocytogenes Δdal Δdat showed no growth at all (data not shown). Thus, these data suggest that the expression of alanine racemase from the integrated pPL2dalGlnA vector fully complements the growth of L. monocytogenes Δdal Δdat in culture. We thus next used a CI assay to quantify the effects of pPL2dalGlnA integration on bacterial growth in infected C57BL/6 mice. For these experiments, groups of three to five mice each were infected with equal ratios of the pPL2 integrant or the pPL2dalGlnA integrant and a wild-type Erm-resistant L. monocytogenes 10403S strain (strain DP-L3903). At 72 h after i.v. infection, homogenates of tissues were harvested from the infected mice and plated on BHI broth alone or BHI broth supplemented with 1 μg/ml Erm. On the basis of the colony counts, the ratios of the CI of the Erm-sensitive strain (the pPL2 integrant of strain 10403S or L. monocytogenes Δdal Δdat::pPL2dalGlnA) to the CI of the Erm-resistant strain (10403S) from the liver of each mouse were determined (Fig. 2B). In two of two experiments, the pPL2 integrant of 10403S showed only a slight competitive disadvantage (mean CI ratio, ~0.5) relative to that of wild-type strain 10403S. In contrast, the L. monocytogenes Δdal Δdat::pPL2dalGlnA strain showed a more substantial reduction in its ability to compete for growth in vivo (mean CI ratio, ~0.01). These data suggest that alanine racemase is not sufficient for full complementation of the growth of L. monocytogenes Δdal Δdat during whole-animal infection, and that the dat deletion restricts the growth of these bacteria. In addition, one may speculate that the L-alanine substrate for alanine racemase may be limiting at sites of L. monocytogenes replication in infected mice.

It is known that L. monocytogenes must persist in the tissues of infected mice for over 24 h in order to generate robust protective T-cell responses (14). We thus also determined whether the attenuated L. monocytogenes Δdal Δdat::pPL2dalGlnA strain was capable of persisting for >24 h after monotypic infection of C57BL/6 mice. The results of this experiment are shown in Table 1. In mice given doses containing as many as 10^6 L. monocytogenes Δdal Δdat bacteria, we failed to recover any CFU from infected spleens and livers by 36 hpi. Conversely, when the mice were given the L. monocytogenes Δdal Δdat::pPL2dalGlnA strain, bacteria were still recovered from the tissues of infected mice at this time point. With the low-dose infection (10^4 CFU), the rate of recovery of L. monocytogenes Δdal Δdat::pPL2dalGlnA bacteria was ~20- to 250-fold less than that seen with low-dose infection with wild-type strain 10403S. However, when the mice were inoculated with a higher dose of L. monocytogenes Δdal Δdat::pPL2dalGlnA (10^6 CFU), bacteria were recovered in numbers only two- to fourfold lower than the numbers of wild-type strain 10403S recovered. These data indicate that the stable integration of pPL2dalGlnA into the chromosome of L. monocytogenes Δdal Δdat restores the ability to establish infection of mouse tissues that persists beyond 24 hpi. These data further suggest that pPL2dalGlnA-based antigen expression constructs engineered in
In addition, the attenuated 100-fold reduced growth, respectively, by the CI assay (11, 18). With 20 healthy human volunteers (1). Doses up to 10^9 CFU studied in a phase I dose escalation safety study of oral delivery which the actA active for bacterial cell-cell spread and had attenuated vaccine strain.

\[
\text{L. monocytogenes } \text{H9004}
\]

10^4 3.1 \times 10^4

10^2

<10^2 <10^2

\begin{table}
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\begin{tabular}{|c|c|c|c|}
\hline
Strain & Inoculum (CFU/mouse) & No. of CFU recovered from: & \\
& & Spleens & Livers \\
\hline
10403S & \(5 \times 10^5\) & 6.1 \times 10^5 & 2.6 \times 10^5 \\
L. monocytogenes \(\Delta \text{dal}\) & \(10^6\) & 1.5 \times 10^5 & 1.1 \times 10^5 \\
L. monocytogenes \(\Delta \text{dat}\) & \(10^4\) & 3.1 \times 10^4 & \\
L. monocytogenes \(\Delta \text{dat}\) & \(10^6\) & <10^2 & <10^2 \\
\hline
\end{tabular}
\caption{Transformation of \textit{L. monocytogenes} \(\Delta \text{dal} \Delta \text{dat}\) with pPL2dalGlnA restores its ability to persist during monotypic infection of mice.}
\end{table}

\footnotesize
\text{Mice were infected i.v. with the indicated strain and dose. Tissues were harvested from two to three mice per group for CFU quantification at 36 h after infection.}

\textbf{L. monocytogenes }\Delta \text{dal} \Delta \text{dat} \text{ strains should adequately restore colonization to immunize protective T-cell responses.}

\textbf{An attenuated \textit{L. monocytogenes} vaccine strain for use with pPL2dalGlnA.} The results presented above clearly indicate that the expression of \textit{dal} is not sufficient to fully complement the virulence of \textit{L. monocytogenes} \(\Delta \text{dal} \Delta \text{dat}\) in healthy mice. However, growth of the complemented strain was reduced by only \(\sim 100\)-fold. We thus sought to generate a more highly attenuated vaccine strain. \textit{L. monocytogenes} mutants that individually lacked the \textit{actA} or the \textit{pleC} virulence gene are defective for bacterial cell-cell spread and had \(\sim 10,000\)-fold and 100-fold reduced growth, respectively, by the CI assay (11, 18). In addition, the attenuated \textit{L. monocytogenes} 10403S from which the \textit{actA} and \textit{pleC} genes were deleted has also been studied in a phase I dose escalation safety study of oral delivery with 20 healthy human volunteers (1). Doses up to \(10^6\) CFU were well tolerated. In the previous study, there were no serious adverse events from systemic infection, suggesting that attenuated \textit{L. monocytogenes }\Delta \text{actA-pleC}\ is a reasonable strain for use as a start in the engineering of a human vaccine vector. Thus, to generate a more highly attenuated strain for use with pPL2dalGlnA-based vaccines, we deleted both of these linked pathogenicity genes from an \textit{L. monocytogenes }\Delta \text{dat} \Delta \text{actA-pleC}\ strain. The resulting strain is defined as \textit{L. monocytogenes }\Delta \text{dal} \Delta \text{dat} \Delta \text{actA-pleC}.\)

\textbf{Antigen expression and immunization with pPL2dalGlnA.} To demonstrate the usefulness of pPL2dalGlnA and \textit{L. monocytogenes }\(\Delta \text{dat}\) \(\Delta \text{actA-pleC}\) for the expression and delivery of foreign antigens, we engineered constructs for the expression of the SIV Nef and the HIV Tat antigens (Fig. 3A). Genes for these antigens were cloned downstream of DNA containing the \textit{L. monocytogenes }\textit{actA} and \textit{hly} promoters, respectively, and introduced into \textit{L. monocytogenes }\Delta \text{dat} \Delta \text{actA-pleC}. Culture supernatants from \textit{L. monocytogenes }\Delta \text{dal} \Delta \text{dat} \Delta \text{actA-pleC}\ transformants contained both the Nef and the Tat proteins (Fig. 3B), as detected by immunoblotting. When it was used to immunize BALB/c mice, the recombinant \textit{L. monocytogenes }\Delta \text{dat} \Delta \text{actA-pleC}\ Nef Tat strain elicited a clear response to the set of HIV type 1 Tat peptides (Fig. 3C). An IFN-\(\gamma\) ELISPOT assay revealed that \(\sim 1/10,000\) splenocytes responded to the Tat peptide at 10 days after immunization (Fig. 3C). Thus, we conclude that the pPL2dalGlnA vector system will be useful for the generation of safe vaccine strains of \textit{L. monocytogenes} by use of the novel strain \textit{L. monocytogenes }\Delta \text{dal} \Delta \text{dat} \Delta \text{actA-pleC}\ or other derivatives of \textit{L. monocytogenes }\Delta \text{dal} \Delta \text{dat}.

In conclusion, we have developed a novel genetic system for the stable expression of recombinant antigens from attenuated \textit{L. monocytogenes} in the absence of antibiotic resistance genes. We believe that our system is an improvement over that of Verch et al., who recently used \textit{dal} to maintain plasmids in auxotrophic \(\text{d-alanine-requiring } \text{E. coli}\) and \textit{L. monocytogenes} bacterial strains (21). Our novel system is instead based on modifications of the integrational vector pPL2. Like the parental pPL2 vector, pPL2dalGlnA is stably maintained in \textit{L. monocytogenes} even without selection. In addition, given the paucity of \(\text{d-alanine}\) in mammalian tissues, pPL2dalGlnA

\begin{figure}
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\includegraphics[width=\textwidth]{figure3}
\caption{T-cell response to exogenous antigens expressed from pPL2dalGlnA in a highly attenuated \textit{L. monocytogenes} strain. (A) Diagram of the SIV Nef and HIV Tat antigen expression cassette cloned into pPL2dalGlnA and integrated in \textit{L. monocytogenes }\Delta \text{dal} \Delta \text{dat} \Delta \text{actA-pleC}\ (Lmd\Delta \text{AB}). (B) Western blots showing FLAG-tagged Nef and VSV epitope-tagged Tat proteins in supernatants of the recombinant \textit{L. monocytogenes }\Delta \text{dat} \Delta \text{actA-pleC}\ Nef Tat strain. Shown are immunoblots of proteins precipitated with TCA from the supernatant of \textit{L. monocytogenes }\Delta \text{dat} \Delta \text{actA-pleC}\ Nef Tat strain (lanes 1 and 3) or a control \textit{L. monocytogenes }\Delta \text{dat} \Delta \text{dat}\ strain (lanes 2 and 4) probed with anti-FLAG tag or anti-VSV tag antibodies. (C) Frequency of IFN-\(\gamma\)-secreting cells responding to overlapping HIV Tat peptides. Shown are the mean number of responding IFN-\(\gamma\)-positive splenocytes detected by the ELISPOT assay 10 days after the immunization of mice with \textit{L. monocytogenes }\Delta \text{dat} \Delta \text{dat} \Delta \text{actA-pleC}\ Nef Tat. The results of the experiments shown are representative of those of at least two additional studies.}
should be positively selected to maintain antigen expression during immunization regimens. The development of safe, attenuated *L. monocytogenes* strains that express recombinant antigens in the absence of antibiotic resistance represents a major step toward the application of the *L. monocytogenes* antigen expression methodology for the vaccination of humans.

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