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SHORT REPORT

Listeria monocytogenes mutants defective in gallbladder replication represent safety-enhanced vaccine delivery platforms

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ABSTRACT

The Gram positive intracellular pathogen *Listeria monocytogenes* represents a promising vaccine or therapeutic DNA delivery vector that has been successfully administered to humans in clinical trials. However in generating *Listeria* mutants with therapeutic potential it is important to balance safety attenuation with efficacy. Here we show that *L. monocytogenes* mutants with a reduced capacity for murine gallbladder replication are capable of stimulating T cell responses in mice and protecting vaccinated animals from secondary challenge. Mutation of *L. monocytogenes* genes *lmo2566* or *lmo0598* resulted in significant attenuation in the murine model yet mutants retained a capacity for intracellular growth and stimulation of T cell responses against key *Listeria* epitopes (LLO_{91-99} and $P60_{217-225}$). Importantly the mutants showed a reduced capacity for growth in the gallbladders of vaccinated mice as well as significantly reduced faecal shedding indicating that this approach generates live *Listeria*-based vector delivery systems with a reduced capacity for the spread of live genetically modified microorganisms into the natural environment.

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The Gram positive pathogen *Listeria monocytogenes* is capable of intracytoplasmic growth in a variety of mammalian cell types (including antigen-presenting cells and epithelial cells).[1,2] A number of features of this intracellular life-cycle recommend the use of attenuated *L. monocytogenes* strains as therapeutic vectors for either vaccine or nucleic acid delivery.[3,4] Within the host cell cytoplasm of antigen presenting cells the pathogen accesses the cytoplasmic antigen processing pathway and is therefore a potent inducer of major histocompatability complex (MHC) class I-restricted protective $CD8^+$ T cells.[5,6] The ability to proliferate within the cytoplasm significantly influences the magnitude of the resultant immune response as bacteria incapable of replication in this environment demonstrate markedly lower immunogenicity.[7,8] In addition, the ability to spread from cell to cell is likely to influence the efficacy of nucleic acid delivery to solid tumors and other tissues.[4,9] The pathogen is genetically tractable and it is possible to target specific genes in order to create mutants which are attenuated but retain vaccine potential.[10] Live attenuated *L. monocytogenes* delivery vectors expressing tumor-specific antigens have been administered to humans in phase I clinical trials and are generally safe and immunogenic.[11,12] Indeed, a recent clinical trial utilised an attenuated *L. monocytogenes* strain expressing the cancer antigen mesothelin as a component of a prime-boost immunotherapy in patients with pancreatic cancer with very promising results.[13] However there are risks associated with the use of live attenuated *Listeria* in this context as evidenced by the recent report of clinical disease (listeriosis) in a patient receiving the attenuated vaccine strain ADXS11-001.[14]

During systemic infection of mice *L. monocytogenes* grows readily in the gallbladder[15,16] and is shed into the faeces.[17] Interestingly, highly attenuated mutants of *L. monocytogenes* generated through mutation of classical virulence factors are still capable of efficient growth in the gallbladder during murine infection.[15] In humans the pathogen is known to be a cause of cholecystitis[18] and has been detected in human faeces.[19] We have previously examined the genetic systems required for *L. monocytogenes* to grow in *ex vivo* gallbladder bile.[20] Here we demonstrate that mutants in the expression of two 2 separate loci encoding proteins involved in biotin metabolism (*lmo0598*) and a putative lipoate protein ligase (*lmo2566*) are also defective for infection in mice. The attenuated mutants stimulate significant anti-listerial immune responses but have reduced potential for replication in gallbladders and demonstrate significantly reduced faecal shedding. We suggest that this represents a novel approach to create attenuated *Listeria* mutants which retain immunogenicity but have reduced potential for environmental spread.

We previously utilised a transposon mutagenesis approach to isolate *L. monocytogenes* mutants with reduced potential for growth in porcine gallbladder bile.[20] We performed significant *in vitro* characterization of these mutants and the study indicated that particular bacterial systems for amino acid metabolism, enzymatic activity and purine metabolism are necessary

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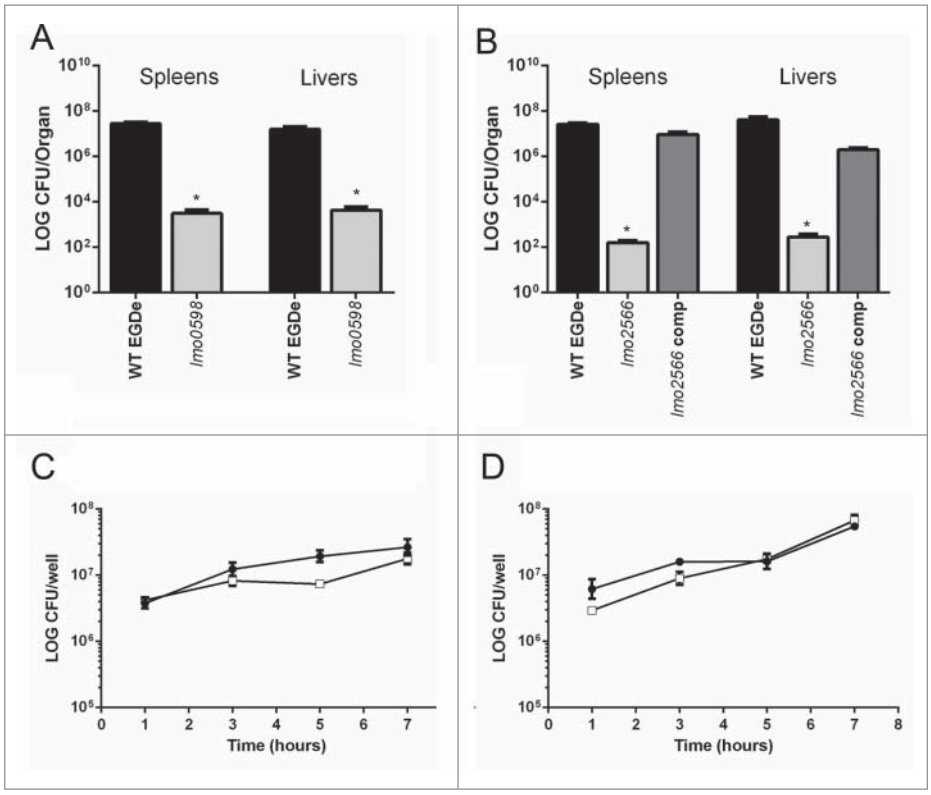

Figure 1. *Listeria*mutants are attenuated for infection of mice but retain an ability to replicate in macrophages. *Listeria monocytogenes* CFU from livers and spleens of infected mice 3 d post i.p. infection with 3×10^5 CFU. (A) Wild-type (EGDe) and biotin auxotroph Tn*lmo0598* levels in livers and spleens. (B) Wild-type (EGDe), Tn*lmo2566* and genetically complemented strain (*lmo2566* Comp) in livers and spleens. $^*P < 0.05$ by the Kruskal Wallis one way ANOVA, with post hoc comparison using Dunn's method. Differences are shown relative to the wild-type. Error bars represent the mean ± SEM (C) Intracellular growth of wild-type (●) and Tn*lmo0598* (□) in J774 macrophage cells using a gentamicin protection assay. (D) Intracellular growth of wild-type (●) and Tn*lmo2566* (□) in J774 macrophage cells using a gentamicin protection assay. No statistical significance observed.

for growth in this environment.[20] Further work demonstrated that 4 out of the 6 transposon mutants defined as deficient in growth in bile also demonstrated attenuated virulence in mice (unpublished data). Of these a mutant deficient in biotin metabolism (Tn*lmo0598*) demonstrated a 4-log reduction in capacity to replicate in murine liver and spleen (Fig. 1A). As Tn*lmo0598* can be chemically complemented through addition of biotin to bile or defined media we did not genetically complement this strain.[20] A mutant in a putative lipoate protein ligase (Tn*lmo2566*) demonstrated a 5-log reduction in virulence potential, a phenotype that can be restored through genetic complementation (Fig. 1B). Both strains carry a single copy of the transposon.[20] Interestingly both mutants were capable of intracellular growth in an *in vitro* J774 macrophage assay (Fig. 1C and D) that utilises a standard gentamicin protection protocol as described previously.[21] In contrast a mutant deficient for purine metabolism (Tn*purB*) was incapable of growth in macrophages (unpublished data).

Given that the mutants were attenuated *in vivo* yet capable of intracellular growth we tested the ability of these strains to vaccinate mice against secondary infection (as an index of immunogenicity). Animal studies were carried out following institutional review and under license from the Irish Department of Health. Balb/c mice were vaccinated using intra-peritoneal (i.p.) inoculation with 3×10^4 CFU of wild-type *L. monocytogenes* EGDe (a sub-lethal dose) or 1×10^6 CFU of the attenuated strains (Tn*lmo0598* or Tn*lmo2566*) or a sham inoculation (PBS) as a negative control (Fig. 2). Three days following injection of live bacteria 5 mice in each group were euthanized and analyzed for bacterial loads in the spleen, gallbladder or faeces (Fig. 3). The data indicate that even when utilised at a 100-fold higher

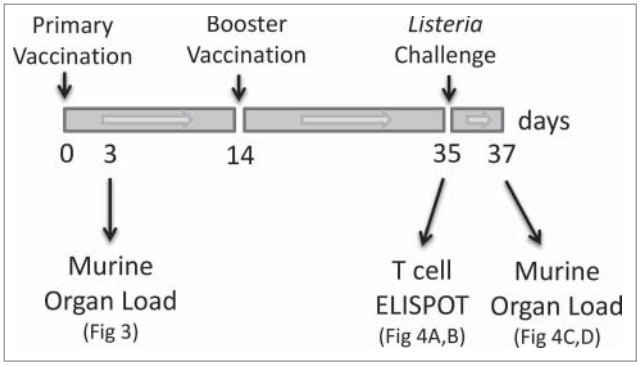

Figure 2. Overview of the vaccination study. Balb/c mice received an initial primary vaccination via the i.p. route with live wild-type bacteria (3×10^4 CFU) or attenuated vaccine strains (Tn*lmo0598* or Tn*lmo2566*) (1×10^6 CFU) at time 0. Mice received a vaccination booster on day 14 with the same vaccination doses. Sham inoculated control mice received sterile PBS. A subgroup of mice received a challenge dose of wild type (EGDe) bacteria at day 35. Subgroups of mice were analyzed at day 3 to determine the organ load of the initial vaccination dose and at day 35 for efficacy in generating T cells (by ELISPOT). Protection from secondary infection by *L. monocytogenes* was determined on day 37 in mice challenged with wild-type bacteria.

dose than the wildtype, the Tn*lmo2566* mutant in particular was significantly attenuated for replication in the spleen and demonstrated reduced capacity to replicate in the gallbladder and to be shed in faeces (Fig. 3).

The remaining mice were administered a vaccine booster at 14 days d following the primary inoculations and at day 35 were either analyzed for T cell responsiveness using an enzyme-linked immunospot (ELISPOT) assay or were challenged with a challenge dose of wild-type *L. monocytogenes* EGDe to determine levels of protective immunity (Fig. 2). We allowed 21 d between the vaccine booster and further immunological analyses in order to eliminate the possibility of non-specific immune effects associated with administration of the booster.[22] We used a standard ELISPOT approach to determine the levels of interferon gamma-producing $CD8^+$ T cells reactive to known *H2-K*$_d$ restricted epitopes of the major *L. monocytogenes* antigens LLO and P60 (epitopes $LLO_{91\text{-}99}$ and $P60_{217\text{-}225}$). The assay was carried out as described previously.[8,23,24] The data indicated that both Tn*lmo0598* and Tn*lmo2566* strains were capable of eliciting a potent *Listeria*-specific T cell response that was comparable to that elicited by the wild-type bacterium and significantly higher than that seen in naïve (sham inoculated) mice (Fig. 4A, B). In addition mice that had undergone the prime and boost vaccination were fully protected against challenge with wild-type *L. monocytogenes* EGDe (Fig. 4C).

Herein we demonstrate that *L. monocytogenes* mutants Tn*lmo0598* or Tn*lmo2566*, initially selected for an inability to grow in gallbladder bile,[20] are also attenuated for virulence during murine infection yet retain an efficient capacity for intracellular replication and stimulation of the host immune system. To our knowledge this is the first study that links reduced gallbladder replication (rather than reduced intracellular replication) to reduced virulence potential in mice. The work therefore demonstrates the importance of the gallbladder phase of infection for murine virulence. Importantly strain Tn*lmo2566* in particular was undetectable in the gallbladder or faeces of mice inoculated with a high vaccinating dose (1×10^6 CFU). We propose that the inability to replicate in the gallbladder results in significantly reduced faecal excretion of the vaccine strain, a hypothesis strengthened by previous studies.[17] While further study is required, we suggest that a mutant strain lacking *lmo2566* could have applications as a vaccine against listeriosis in domestic animals. Alternatively mutation of *lmo2566* could potentially be utilised alongside mutations in key virulence factors [10] to enhance safety and prevent gallbladder growth in existing live therapeutic *Listeria* vector systems. The current work therefore represents a novel approach to biological containment of live *Listeria*-based vector delivery systems with reduced capacity for the spread of live genetically modified microorganisms into the natural environment.

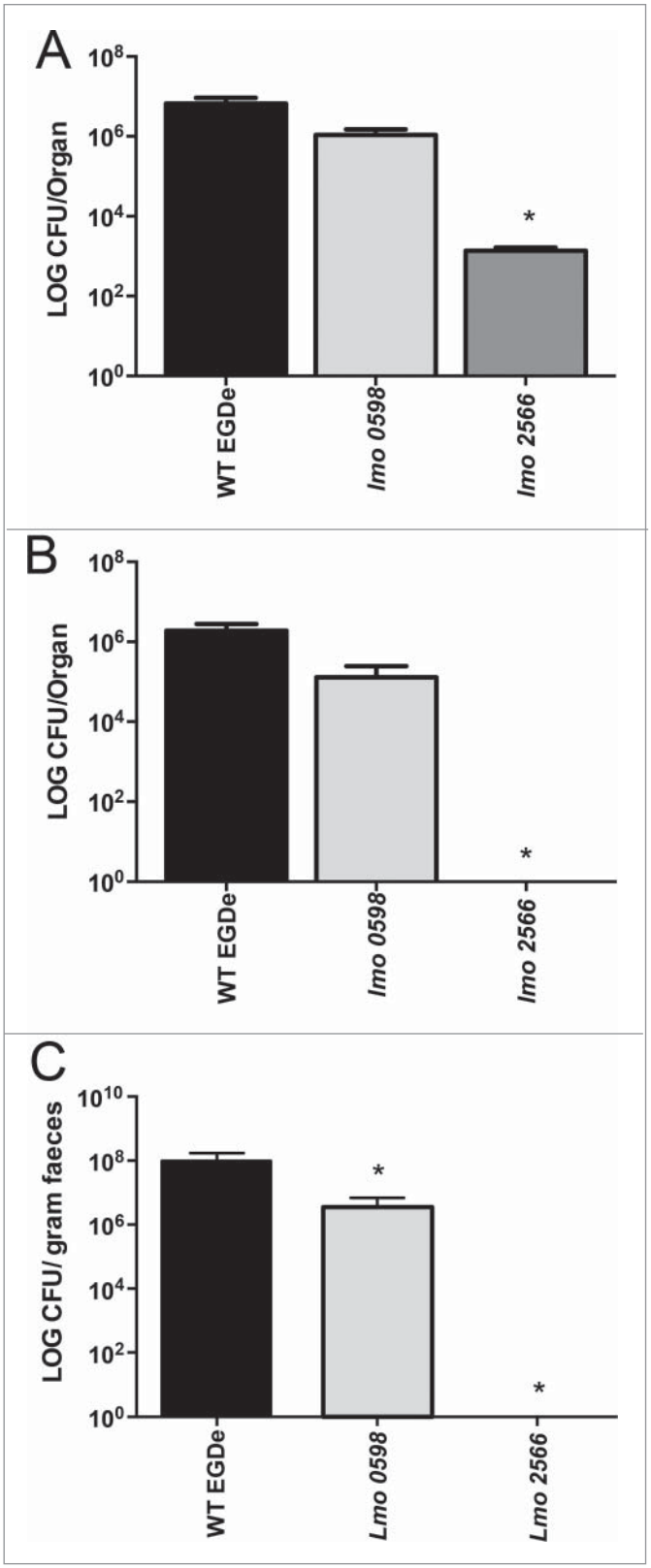

Figure 3. *L. monocytogenes* vaccine strains fail to significantly colonise mouse gallbladders and show reduced faecal excretion. Mice were inoculated via the i. p. route with wild-type (strain EGDe) bacteria (at 3×10^4 CFU) or attenuated vaccine strains (Tn*lmo0598* or Tn*lmo2566*) (1×10^6 CFU) and levels in internal organs were assessed 3 d later. (A) Levels of wild-type (strain EGDe) or attenuated strains Tn*lmo0598* or Tn*lmo2566* in spleens of infected mice. (B) Levels of wild-type (strain EGDe) or attenuated strains Tn*lmo0598* or Tn*lmo2566* in gallbladders of infected mice. (C) Levels of wild-type (strain EGDe) or attenuated strains Tn*lmo0598* or Tn*lmo2566* in faeces of infected mice. $^*P < 0.05$ by the Kruskal Wallis one way ANOVA, with post hoc comparison using Dunn's method. Differences are shown relative to the wild-type. Significant differences are indicated relative to the wild-type. Error bars represent the mean ± SEM.

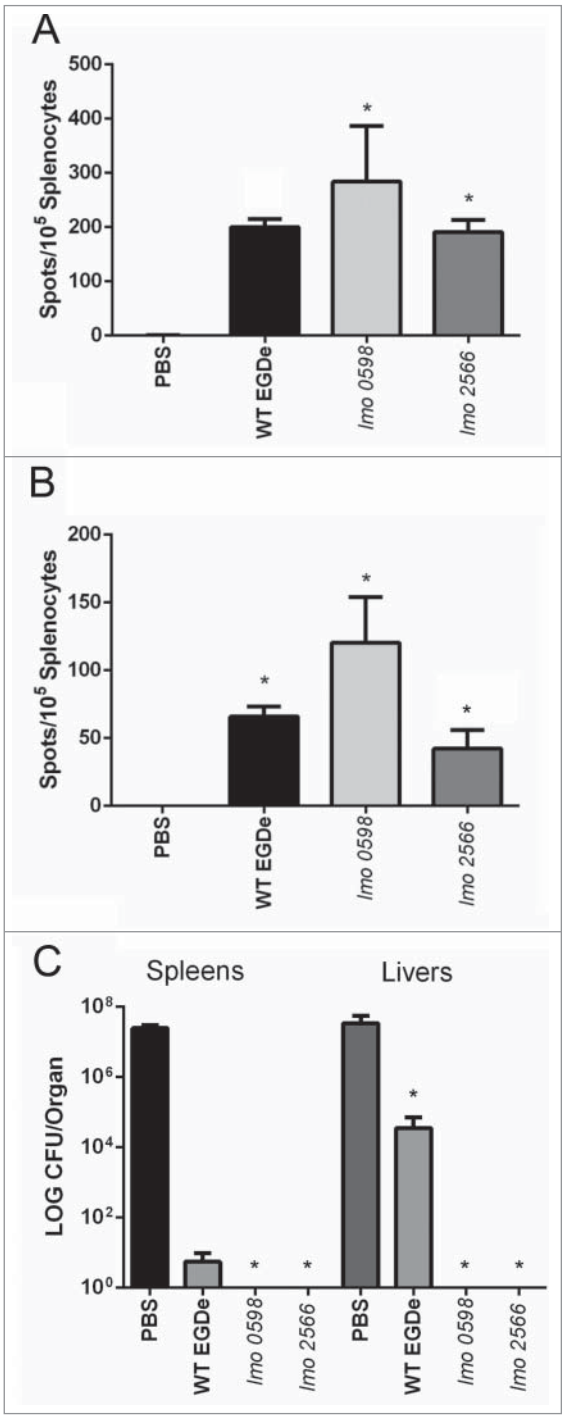

Figure 4. *L. monocytogenes* vaccine strains stimulate anti-*Listeria* T cells and protect mice against secondary infection. Mouse groups were vaccinated as outlined with a primary inoculation on day 0 and a booster at day 14. At day 35 mice were examined for T-cells responsive to (A) LLO_{91-99} or (B) $P60_{217-225}$ using peptide pulsed P815-1-1 cells. $^{*}P < 0.05$ by the Kruskal Wallis one way ANOVA, with post hoc comparison using Dunn's method. Significant differences are shown relative to the sham (PBS) inoculated animals. No significant differences were evident between Tn*lmo0598* or Tn*lmo2566* vaccinated animals. Error bars represent the mean ± SEM (C) A subgroup of vaccinated mice were challenged with *L. monocytogenes* wild type (EGDe) (3×10^5 CFU via the i.p. route). Mice vaccinated with wild-type (strain EGDe), attenuated strains Tn*lmo0598* or Tn*lmo2566* or sham-inoculated mice were analyzed for organ load 2 d post-infection. $^{*}P < 0.05$ by the Kruskal Wallis one way ANOVA, with post hoc comparison using Dunn's method. Significant differences are shown for protection relative to the sham (PBS) inoculated animals. Error bars represent the mean ± SEM.

Disclosure of potential conflicts of interest

The authors declare no conflict of interest

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