A mutant in the *Listeria monocytogenes* Fur-regulated virulence locus (*frvA*) induces cellular immunity and confers protection against listeriosis in mice

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*Listeria monocytogenes* is a Gram-positive intracellular pathogen that is responsible for listeriosis, a potentially fatal, food-borne illness. Due to its cytoplasmic location during infection, this pathogen can mediate a long-lasting cellular immune response, which makes attenuated strains strong candidates for vaccine development. Recently, our group identified and characterized *frvA* (Fur-regulated virulence factor A), and deletion of this gene resulted in disruption of iron homeostasis and a strong attenuation in virulence. Despite significant attenuation in the mouse infection model, the *frvA* mutant was capable of intracellular growth in antigen-presenting cells. Indeed, mice immunized with *L. monocytogenes* Δ*frvA* were able to effectively stimulate specific CD8+ T cells to the listerial epitopes LLO91–99 and P60217–225 at levels comparable with *L. monocytogenes* strain EGDe. Most notably, mice immunized with Δ*frvA* then subsequently challenged with the wild-type strain were completely protected from listerial infection. On the basis of these results, we advocate the use of Δ*frvA* as a live attenuated listerial vaccine, and propose that this mutant may serve as a platform for the development of a future vaccine delivery vehicle.

**INTRODUCTION**

*Listeria monocytogenes* is a Gram-positive intracellular bacterium that has been widely studied as a model intracellular pathogen. *L. monocytogenes* is capable of provoking strong cell-mediated immune responses against listerial proteins as well as co-expressed antigens. As this opportunistic pathogen is considered a model for the induction of major histocompatibility complex (MHC) class I-restricted immune responses, attenuated listerial strains make attractive candidates as carriers for vaccine antigens (Medina & Guzmán, 2001; Schoen et al., 2008).

The intracellular life cycle of *L. monocytogenes* is coordinated by several virulence genes under the regulatory control of PrfA, which facilitates invasion into a variety of phagocytes and non-phagocytic host cells via phagocytosis. Escape from the phagosome is mediated by listeriolysin O (LLO), a pore-forming haemolysin and significant listerial antigen. By virtue of this escape and replication in the cytosol, *L. monocytogenes* can direct antigens towards both the MHC class I and class II pathways, and in turn stimulate CD4+ and CD8+ T cell responses (Busch et al., 1998; Edelson & Unanue, 2000; Pamer, 2004). As cytotoxic T lymphocytes (CTLs) are considered a vital defence against tumours, viruses and intracellular bacterial pathogens, the ability of attenuated listerial strains to stimulate this type of immune response has broad clinical relevance (Kaufmann, 1993; Melief, 1992; Yap et al., 1978). To date, numerous live-attenuated *L. monocytogenes* strains have been investigated as potential vaccine carriers in various hosts, with a notable phase I human trial demonstrating that a ΔactA/plcB mutant can induce humoral, mucosal and cellular immune responses safely when administrated in high enough numbers (Angelakopoulos et al., 2002; Jiang et al., 2007; Yin et al., 2011).

The success of live, virulence-attenuated bacterial vaccines is based on the generation of abiding protective immunity
as well as the potential to induce cellular and humoral immune responses (Fouts et al., 2003; Kotton & Hohmann, 2004). But achieving maximal immunogenicity must be carefully balanced with safe attenuation of the carrier strain. While the design of rationally avirulent strains has been made possible with the availability of listerial genome sequences (Glaser et al., 2001; Nelson et al., 2004), attenuated strains, impaired in virulence gene expression, can exhibit greatly decreased efficacy of antigen delivery (Schoen et al., 2008). For instance, strains unable to express genes required for invasion, \textit{inlA} and \textit{inlB}, would be unable to enter non-phagocytic cells and therefore incapable of delivering antigens or antigen-encoding genes. Likewise, Pilgrim et al. (2003) demonstrated that loss of actA, the gene responsible for propulsion through mammalian host cells, resulted in significant loss of DNA transfer efficiency \textit{in vitro}. Consequently, it has been proposed that attenuated strains not impaired in virulence gene expression, such as auxotrophs or strains unable to maintain iron homeostasis \textit{in vivo}, could be most suitable as vaccine delivery vehicles (Álvarez et al., 2008; Kuklin et al., 2006; Schoen et al., 2008; Stritzker et al., 2004).

As iron is essential to the growth and survival of all cells, host sequestration of this metal provides a significant barrier to bacterial infection. This requirement for iron has driven the evolution of elegant systems by which pathogenic bacteria such as \textit{L. monocytogenes} can competitively obtain this element during host infection (McLaughlin et al., 2011). Recently we demonstrated that deletion of \textit{L. monocytogenes} \textit{frvA} (Fur-regulated virulence factor A) results in strong attenuation in both the \textit{Galleria mellonella} (greater waxmoth larvae) and murine models of infection (McLaughlin et al., 2012). Further analysis revealed that \textit{ΔfrvA} was impaired in growth under iron-limiting conditions and that \textit{frvA} was essential for resistance to haem toxicity as well as maintenance of iron homeostasis. Despite the significant attenuation in the murine infection model, the \textit{ΔfrvA} mutant was unimpaired in invasion or replication inside J774 macrophage cells, suggesting that this strain could be a promising vaccine vector for the presentation of antigens via the MHC class I pathway.

Here we demonstrate that immunization of mice with \textit{ΔfrvA} results in induction of protective CD8\textsuperscript{+} T lymphocytes to two listerial epitopes, LLO\textsubscript{91–99} and P60\textsubscript{217–225}, at levels comparable with the wild-type EGD\textit{e} strain after 5 weeks. Subsequently, we found that this immunization led to absolute protection against murine listeriosis. The work indicates that \textit{ΔfrvA} \textit{L. monocytogenes} is a suitable platform vector for stimulation of effective CD8\textsuperscript{+} responses.

**METHODS**

**Bacterial strains and culture conditions.** \textit{L. monocytogenes} strains were grown in Brain Heart Infusion (BHI) (Oxoid) broth at 37 °C with agitation at 200 r.p.m. Strains utilized for this study have been previously described (McLaughlin et al., 2012). For solid media, agar (Merck) was added at 1.5 % (w/v), and chloramphenicol (Cm) from Sigma was added at a concentration of 7.5 µg ml\textsuperscript{-1} for maintenance of the pPL2 plasmid in \textit{ΔfrvA}\textit{ApPL2::frvA}. Cell culture media and reagents were all obtained from Gibco.

**Growth of strains in human blood \textit{ex vivo}**. Male blood samples were collected in 10 ml glass tubes (BD Vacutainer, reference no. 366480) coated with the anticoagulant heparin (143 USP units). Five millilitre blood samples were inoculated with 1 × 10\textsuperscript{6} c.f.u. ml\textsuperscript{-1} of each of the tested strains: \textit{L. monocytogenes} \textit{ΔfrvA}, \textit{ΔfrvA}ApPL2::\textit{frvA} (McLaughlin et al., 2012) and wild-type EGD\textit{e}. Tubes were incubated at 37 °C with agitation over time. Bacterial growth of listerial strains was monitored over time by direct enumeration of c.f.u. in serial dilutions plated on BHI agar. All strains were monitored in triplicate. Some blood samples were left un inoculated to serve as negative controls.

**Immunization procedure.** Cultures grown overnight at 37 °C with agitation were centrifuged, washed twice and resuspended in an equal volume of PBS (Sigma). Female BALB/c mice (6–8 weeks old) were separated into three groups each containing nine mice. One group of mice was immunized intraperitoneally (i.p.) on days 1 and 14 with \textit{ΔfrvA} at 2 × 10\textsuperscript{6} c.f.u. in a total volume of 200 µl. Mice given 200 µl PBS on days 1 and 14 were included as a negative control group, whereas mice immunized i.p. with a sublethal dose of the wild-type \textit{L. monocytogenes} EGD\textit{e} (2 × 10\textsuperscript{4} c.f.u. per 200 µl) on the same days were included as a positive control group. On day 35, four mice from each group were euthanized for the ELISPOT assay and the remaining five mice from each group were utilized for the challenge assay.

**ELISPOT immunological assay.** Identification of antigen-specific CD8\textsuperscript{+} T cells by enzyme-linked immunospot (ELISPOT) assay was performed according to the protocol outlined by Carvalho et al. (2001). Briefly, the ELISPOT assay was used to detect CD8\textsuperscript{+} cells specific to two epitopes: an H2-K\texttextsuperscript{d} restricted epitope, LLO\textsubscript{91–99}, GYKDGNEYI, and a P60 epitope, P60\textsubscript{217–225}, KYGVSVQDI (Peptide Protein Research, UK) (Vijh & Pamer, 1997). The antigen-presenting cell line used for this assay was mouse mastocytoma P815-1-1 cells from the European Collection of Cell Cultures (ECACC). Splenocytes, isolated from mice following the immunization protocol mentioned above, were stimulated using P815-1-1 cells from the European Collection of Cell Cultures (ECACC). Splenocytes, isolated from mice following the immunization protocol mentioned above, were stimulated using P815-1-1 cells that had been pulsed with 10\textsuperscript{-5} M of LLO\textsubscript{91–99} or P60\textsubscript{217–225} peptides or non-pulsed. Epitope-specific interferon (IFN)-γ secreting CD8\textsuperscript{+} cells were detected by adding biotin anti-mouse IFN-γ antibodies (clone XMG1.2) (Biolegend) followed by avidin–horseradish peroxidase (Biolegend). Dark-brown spots corresponding to IFN-γ-secreting CD8\textsuperscript{+} cells were developed by adding the peroxidase substrate 3,3’-diaminobenzidine tetrahydrochloride (DAB) (Sigma). These spots were subsequently counted using a stereomicroscope. Final datasets represent the numbers of spots counted for antigen-pulsed cells minus the number of spots counted for non-pulsed cells (Carvalho et al., 2001).

**Challenge assay.** Following the immunization regime, on day 35, five mice from each group were challenged with 4 × 10\textsuperscript{5} c.f.u. per 200 µl of the wild-type \textit{L. monocytogenes} EGD\textit{e} strain via the i.p. route. Mice were euthanized 3 days post-challenge (day 38). Spleens and livers were harvested and homogenized in PBS. Bacteria were enumerated by plating the serial dilutions of organ homogenates on BHI agar left to incubate overnight at 37 °C.

**Statistical analysis and presentation of data.** Analysis of statistics was completed using one-way ANOVA with post hoc analysis by the Holm–Sidák method. Significance was considered at \textit{P}=0.05. All comparisons were performed using SigmaStat 3.5 (Systat software). Figures were compiled using Sigmaplot 10.0 graphing software (Systat Software).
RESULTS AND DISCUSSION

As outlined by Lieberman & Frankel (2002), *L. monocytogenes* represents an attractive vector for vaccine development for several reasons. (i) It has the ability to induce a strong and long-lasting host immunological response through the secretion of protein antigens in the cytosol of antigen-presenting cells which are presented to CD8\(^+\) CTLs via the MHC class I pathway. (ii) As the incidence of listeriosis is relatively low, pre-existing immunity to this bacterium should prove relatively low. (iii) It is reasonably easy to genetically engineer and can be used to carry foreign antigens encoded on plasmid DNA. (iv) From a safety point of view, it can be readily treated with antibiotics and is endotoxin-free. Additionally, studies have shown that murine models possessing pre-existing immunity to *Listeria* do not impact the ability to prime an effective immune response against foreign passenger antigens (Starks et al., 2004; Stevens et al., 2005). To date, several reports have described the successful use of attenuated *L. monocytogenes* to deliver DNA vaccines, antigen-encoding mRNA, as well as secreted and surface-located proteins to eukaryotic cells (Cabanes et al., 2002; Loeffler et al., 2006; Pilgrim et al., 2003; Schoen et al., 2005). More recently the clinical use of a live-attenuated *L. monocytogenes* strain was found to be safe for administration to patients with advanced carcinoma of the cervix (Maciag et al., 2009). Overall, an optimal *Listeria*-based vaccine or gene-delivery vector is likely to comprise a safe, highly attenuated strain that retains an ability to escape into the cytoplasm, to grow, and to mobilize between cells (Tangney & Gahan, 2010).

We propose that a mutant in the Fur-regulated virulence locus (*frvA*) has the key attributes of a *Listeria*-based delivery vehicle. Previous work, as well as work described herein, demonstrates that *ΔfrvA* is highly attenuated *in vivo*, is not impaired in survival or growth inside macrophages (McLaughlin et al., 2012), and grows well in human blood samples *ex vivo* (Fig. 1). This suggests that this strain can thrive in host niches associated with antigen presentation. Similarly, Dominguez-Bernal et al. (2008) demonstrate that *Salmonella enterica* serovar Choleraesuis strains harbouring deletions in *phoP* and *rpoS* are strongly attenuated in the porcine host yet still retain the ability to proliferate in macrophages and fibroblasts. As these cells are considered pivotal in modulating immune responses, those authors suggested that the ability to survive and proliferate in these cells would increase their suitability as vaccine candidates (Dominguez-Bernal et al., 2008).

*ΔfrvA* induces an antigen-specific CD8\(^+\) T cell response similar to the wild-type

In addition to being an important virulence factor for *L. monocytogenes* during infection, the LLO haemolysin is a major immunodominant listerial antigen and is regarded as one of the most antigenic secreted proteins in the context of stimulating specific CD8\(^+\) T cells (Pamer, 2004). Another antigen known to induce a significant CD8\(^+\) T cell response is P60, a murine hydrolase that is rapidly degraded in the host cytosol. It has been demonstrated that protective immunity against *L. monocytogenes* is dependent on induction of specific CD8\(^+\) T cells to the listerial epitopes LLO and P60 (Pamer, 2004). It is noteworthy that several studies have used live bacterial vectors, such as *Salmonella Typhimurium* (S. Typhimurium) and *Lactococcus lactis*, expressing LLO and/or P60 for vaccination against listeriosis (Bahey-El-Din et al., 2008, 2010; Hess et al., 1996). Here we show that mice immunized with *L. monocytogenes* *ΔfrvA* via the i.p. route were able to effectively stimulate specific CD8\(^+\) T cells against the listerial epitopes LLO\(_{91-99}\) and P60\(_{217-225}\) at levels comparable with the parental *L. monocytogenes* EGD\(_e\) strain.

Following two i.p. immunization doses on days 1 and 14, mice immunized with *ΔfrvA* elicited LLO\(_{91-99}\)-specific CD8\(^+\) T cells at levels comparable with the wild-type *L. monocytogenes* EGD\(_e\) on day 35 (Fig. 2a). No induction of LLO-specific CD8\(^+\) cells was observed for mice treated with PBS, as epitope-specific spots were not detected. Similarly, groups of mice immunized with either *ΔfrvA* or the wild-type *L. monocytogenes* EGD\(_e\) showed a statistically significant induction of P60\(_{217-225}\)-specific CD8\(^+\) T cells as compared with mice treated with PBS (*P*<0.05) (Fig. 2b).

**IP vaccination with *ΔfrvA* provides protection against subsequent wild-type *L. monocytogenes* challenge in mice**

As CTLs are considered a vital defence against intracellular bacterial pathogens and with confirmation that *ΔfrvA*
could successfully elicit an effective CD8+ T cell response, we examined the prospect of using this live attenuated strain as a vaccine against listeriosis in mice. Following i.p. booster inoculations on days 1 and 14, all groups were challenged with the wild-type \textit{L. monocytogenes} EGDe strain via the i.p. route on day 35. Three days post-challenge, mice were euthanized and bacterial counts were enumerated in the livers and spleens. Similar to mice injected with \textit{L. monocytogenes} EGDe, immunization with ΔfrvA offered mice significant protection from the listerial challenge compared with the PBS-treated group (\(P<0.05\)).

With a limit of detection of 100 c.f.u., no bacterial counts were recovered 3 days post-challenge from the livers or spleens of mice previously immunized with \textit{L. monocytogenes} EGDe or ΔfrvA (Fig. 3). Conversely, both livers and spleens of mice treated with PBS were shown to contain large listerial loads after 3 days, with over \(1 \times 10^5\) c.f.u. per organ (Fig. 3).

Live attenuated vaccine candidates harbouring deletions in genes involved in maintenance of bacterial iron homeostasis have previously been investigated in other pathogens. Mice immunized with an \textit{S. Typhimurium} triple mutant, containing mutations in three genes encoding receptors for catecholate siderophores, offered significant protection against subsequent challenge with the parental strain (Williams \textit{et al.}, 2006). Similarly, pigs vaccinated with a highly attenuated strain of \textit{Actinobacillus pleuropneumoniae} impaired in anaerobic metabolism and iron uptake due to the deletion of \textit{fur} were significantly protected from disease following challenge with the infectious parental strain (Maas \textit{et al.}, 2006). To our knowledge, the work herein is the first report of a live attenuated \textit{L. monocytogenes} vaccine candidate harbouring a deletion in a gene that is essential for maintenance of iron homeostasis. While our work demonstrates that ΔfrvA induces a specific CD8+ T cell response, we examined the prospect of using this live attenuated strain as a vaccine against listeriosis in mice. Following i.p. booster inoculations on days 1 and 14, all groups were challenged with the wild-type \textit{L. monocytogenes} EGDe strain via the i.p. route on day 35. Three days post-challenge, mice were euthanized and bacterial counts were enumerated in the livers and spleens. Similar to mice injected with \textit{L. monocytogenes} EGDe, immunization with ΔfrvA offered mice significant protection from the listerial challenge compared with the PBS-treated group (\(P<0.05\)).

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![Fig. 2. ELISPot assay. Results were obtained 35 days after the initial vaccination dose.](image1)

![Fig. 3. Challenge assay. All murine groups (\(n=5\)) were vaccinated via i.p. injection on days 1 and 14 prior to challenge with the wild-type \textit{L. monocytogenes} EGDe strain on day 35. Mice were euthanized 3 days post-challenge, when c.f.u. were enumerated from livers (a) and spleens (b). Bars represent mean ± SEM, and asterisks indicate statistical significance (\(P<0.05\)) compared with the negative control group to which PBS was administered. The limit of detection for this assay was 100 c.f.u.)](image2)
cell response and offers protection to mice against L. monocytogenes infection, future work will evaluate the optimal dose parameters required for protection.

In conclusion, we have investigated the use of ΔfrvA as a potential vaccine vector, with promising results. While frvA is absolutely essential for listerial infection in two model hosts, this ΔfrvA mutant retains potent immunogenicity. Moreover, immunization of mice with ΔfrvA was shown to offer complete protection against listeriosis. The ability of ΔfrvA to survive and proliferate inside antigen-presenting cells and in blood increases the suitability of this strain as a vaccine candidate. While future work is necessary to determine the success of ΔfrvA as a novel carrier system, continued research in fine-tuning candidate strains is imperative for the development of safe and effective live bacterial vaccine vectors.

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