Expression of two *Listeria monocytogenes* antigens (P60 and LLO) in *Lactococcus lactis* and examination for use as live vaccine vectors

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Listeria monocytogenes is a food-borne intracellular pathogen that mainly infects pregnant and immunocompromised individuals. The pore-forming haemolysin listeriolysin O (LLO), the main virulence factor of *Listeria monocytogenes*, allows bacteria to escape from the harsh environment of the phagosome to the cytoplasm of the infected cell. This leads to processing of bacterial antigens predominantly through the cytosolic MHC class I presentation pathway. We previously engineered the food-grade bacterium *Lactococcus lactis* to express LLO and demonstrated an LLO-specific CD8⁺ response upon immunization of mice with the engineered *L. lactis* vaccine strains. In the present work, we examined the immune response and protective efficacy of an *L. lactis* strain co-expressing LLO and a truncated form of the listerial P60 antigen (tP60). Oral immunization revealed no significant protection against listeriosis with *L. lactis* expressing LLO, tP60 or the combined LLO/tP60. In contrast, intraperitoneal vaccination induced an LLO-specific CD8⁺ immune response with LLO-expressing *L. lactis* but no significant improvement in protection was observed following vaccination with the combined LLO/tP60 expressing *L. lactis* strain. This may be due to the low level of tP60 expression in the LLO/tP60 strain. These results demonstrate the necessity for improved oral vaccination strategies using LLO-expressing *L. lactis* vaccine vectors.

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INTRODUCTION

Listeria monocytogenes is a Gram-positive food-borne pathogen that primarily infects immunocompromised individuals. Pregnant women and newborns are particularly susceptible to listerial infection with disease culminating in miscarriage and meningitis, respectively (Meng & Doyle, 1997). Listeriolysin O (LLO) is a critical listerial virulence factor and immunodominant antigen. LLO facilitates the escape of *Listeria monocytogenes* from the phagosomal compartment to the cytoplasm of infected cells, a process mediated by membrane pore-forming activity (Vazquez-Boland *et al.*, 2001). LLO-mediated bacterial translocation into the cytoplasm leads to the processing of listerial antigens through the MHC class I antigen presentation pathway. This ultimately results in the

development of CD8⁺ cell-mediated immunity against the major immunodominant listerial antigens LLO and P60 (Pamer, 2004). P60 is a listerial protein that has important roles in cell division and in host cell invasion (Faith *et al.*, 2007; Kuhn & Goebel, 1989; Wuenschel *et al.*, 1993).

The unique phagosomal permeabilizing activity of LLO means that accompanying antigens can access the cytosolic antigen presentation pathway. This feature has been exploited by several investigators to develop or enhance cell-mediated immune responses against specific antigens by co-expression of these antigens with LLO in a live vaccine vector (Huang *et al.*, 2008; Radford *et al.*, 2002; Tvinnereim *et al.*, 2002).

Lactococcus lactis is a GRAS (generally regarded as safe) food-grade bacterium that has long been utilized in the food industry, especially in fermented dairy products. During the last two decades *L. lactis* has been investigated as a potential vaccine delivery platform for the delivery of various antigens (Bahey-El-Din *et al.*, 2010). *L. lactis* is non-pathogenic, non-invasive and non-commensal Gram-positive bacterium that

Abbreviations: APC, antigen presenting cell; Cm, chloramphenicol; GIT, gastrointestinal tract; GRAS, generally regarded as safe; IFN- γ , gamma interferon; IP, intraperitoneal; LLO, listeriolysin O; LOD, limit of detection; OVA, ovalbumin; RBS, ribosome-binding site; SOE, splicing by overlap extension; Th1, T helper type 1.

lacks the pro-inflammatory LPSs associated with Gram-negative bacteria. Although *L. lactis* has innate immunoadjuvant activity, it elicits only a weak immune response against itself, which is an obvious advantage as a vaccine live vector (Robinson *et al.*, 1997; Wells & Mercenier, 2008).

We have previously demonstrated that LLO-expressing *L. lactis* strains are capable of eliciting an antigen-specific CD8⁺ immune response and protection against *Listeria monocytogenes* challenge in the murine infection model (Bahey-El-Din *et al.*, 2008). The objective of the present work was to examine the protective efficacy of an *L. lactis* vaccine strain that dually expresses two listerial antigens: LLO and a truncated form of the P60 listerial antigen (tP60) to determine if it provides greater vaccination efficacy than either antigen expressed alone. We also hypothesized that the pore forming activity of LLO would facilitate the translocation of tP60 to the cytosol of infected cells with subsequent antigen presentation through the MHC class I pathway.

METHODS

Bacterial strains, plasmids and culture conditions. A summary of the bacterial strains and plasmids used in this study is shown in

Table 1. Luria–Bertani (LB) broth was used for *Escherichia coli* cultures, while M17 broth (Oxoid) supplemented with 0.5 % glucose (i.e. GM17) was used for *Lactococcus*. For *Listeria monocytogenes*, brain heart infusion (BHI) broth (Oxoid) was used. Technical agar (1.5 % w/v) (Merck) was added when solid media were required. Incubation temperatures were 30 °C for *L. lactis*, and 37 °C for *Listeria monocytogenes*, and *E. coli*. When required, ampicillin was used at a concentration of 100 µg ml⁻¹ for *E. coli*, while chloramphenicol (Cm) was used at 10 µg ml⁻¹ for *E. coli* and 5 µg ml⁻¹ for *L. lactis*. All cell culture media and reagents were obtained from Gibco unless otherwise stated.

Cloning of DNA encoding listerial antigens in pNZ8048-derivative plasmid vectors. Three plasmids were constructed for the expression of the two listerial antigens individually or in combination. Plasmid pNZPnisA:SEC-LLO was created for secreted LLO antigen, plasmid pNZPnisA:SEC-tP60 for secreted truncated P60 (tP60) antigen and plasmid pNZPnisA:SEC-LLO/tP60 for dually secreted LLO and tP60 antigens (Fig. 1). Plasmid pNZPnisA:SEC-LLO containing the *hly* gene encoding LLO was created as described in our previous work (Bahey-El-Din *et al.*, 2008) where LLO is secreted with the aid of the N-terminal Usp45 secretion signal upon nisin induction of the PnisA promoter. The Usp45 protein is a common lactococcal secreted protein (van Asseldonk *et al.*, 1990) and the use of the Usp45 secretion signal (here designated SEC) has been reported to direct the secretion of heterologous proteins in *L. lactis* (Steidler *et al.*, 2003; Wells *et al.*, 1993).

Table 1. Bacterial strains and plasmid vectors

Strain or plasmid name	Description	Reference or source
<i>E. coli</i> Top10	Chemically competent intermediate host, plasmid free	Invitrogen
<i>E. coli</i> NovaBlue	Chemically competent intermediate host	Novagen
<i>E. coli</i> M15(pREP4)	Expression host for pQE30-cloned genes, containing the repressor plasmid pREP4 (kanamycin resistant) for suppression of basal protein expression under uninduced conditions	Qiagen
<i>Listeria monocytogenes</i> EGDe serovar 1/2a	Wild-type <i>Listeria monocytogenes</i>	Glaser <i>et al.</i> (2001)
Muritized <i>Listeria monocytogenes</i> EGDm	<i>Listeria monocytogenes</i> EGDe <i>ΔintLA</i> where the <i>ΔintLA</i> locus was recreated in the chromosome with two amino acid mutations for enhanced murine gut invasion	I. R. Monk, C. Hill & C. G. M. Gahan, unpublished data
<i>Lactococcus lactis</i> subsp. <i>cremoris</i> MG1363	Plasmid-free <i>Lactococcus</i> strain	Gasson (1983)
<i>Lactococcus lactis</i> NZ9700	Nisin-producer strain	Kuipers <i>et al.</i> (1998)
<i>Lactococcus lactis</i> NZ9000 <i>ΔhtrA</i>	<i>L. lactis</i> subsp. <i>cremoris</i> MG1363 carrying <i>nisRK</i> on the chromosome with a chromosomal deletion in the <i>htrA</i> promoter and the 5'-end region of <i>htrA</i>	Lindholm <i>et al.</i> (2004)
pQE30	Expression vector using phage T5 promoter and adding an N-terminal six-His tag to the expressed protein, ampicillin resistant	Qiagen
pQE30/ <i>iap</i>	pQE30 vector with <i>iap</i> (without native secretion signal) gene cloned between <i>Bam</i> HI and <i>Kpn</i> I restriction sites	This study
pNZ8048	<i>E. coli</i> - <i>L. lactis</i> shuttle vector containing PnisA promoter and start codon in <i>Nco</i> I site, Cm ^R	Kuipers <i>et al.</i> (1998)
pNZPnisA:SEC-LLO	Modified pNZ8048 containing PnisA promoter (<i>Nco</i> I site eliminated) with downstream secretion signal of Usp45 protein (SEC) and His-tagged <i>hly</i> , Cm ^R	Bahey-El-Din <i>et al.</i> (2008)
pNZPnisA:SEC-tP60	pNZ8048 derivative containing PnisA promoter with downstream secretion signal of Usp45 protein (SEC) and His-tagged truncated P60-encoding gene, Cm ^R	This study
pNZPnisA:SEC-LLO/tP60	Modified pNZ8048 containing PnisA promoter (<i>Nco</i> I site eliminated) with downstream sequential His-tagged <i>hly</i> and truncated P60-encoding genes each with a secretion signal (SEC), Cm ^R	This study

Fig. 1. Diagrammatic representation of the three plasmids constructed in this study. PnisA, nisin-inducible promoter; RBS, ribosome-binding site; SEC, secretion signal of Usp45 protein; H, six histidine tag; T, transcription terminator. *hly* and *iap* are the genes encoding LLO and P60 antigens, respectively.

The pQE30 plasmid (Qiagen) was used as an intermediate cloning vector to create DNA encoding His-tagged P60 antigen for subsequent cloning into pNZ8048 derivatives. The *iap* gene (encoding P60) of *Listeria monocytogenes* EGDe (accession number AL591824) minus the secretion signal was PCR amplified from chromosomal DNA using primers P60-pQE30 (forward primer) and P60-pQE30 (reverse primer) (Table 2). This amplified *iap* gene was digested with *Bam*HI and *Kpn*I, and ligated using T4 DNA ligase (Roche) to a similarly digested pQE30 plasmid (Qiagen) that introduced an N-terminal six histidine tag to P60. The ligation reaction mixture was used to transform chemically competent *E. coli* NovaBlue (Novagen) by heat shock following the manufacturer's instructions and plated onto LB agar containing ampicillin. Colony PCR was used to identify positive colonies and plasmid (pQE30/*iap*) was extracted from *E. coli* NovaBlue using a miniprep kit (Qiagen). The integrity of the DNA sequence was confirmed by sequencing (Cogenics).

A construct composed of the SEC signal DNA [with a ribosome-binding site (RBS)] and DNA encoding a His-tagged truncated form of P60 was made using the splicing by overlap extension (SOE) technique (Horton *et al.*, 1990). The primers used in the PCRs are shown in Table 2. This construct was cloned downstream of *hly* in the

pNZPnisA:SEC-LLO plasmid between *Kpn*I and *Xba*I restriction sites (Fig. 1). Briefly, the SEC signal (including its native RBS) was amplified from the *L. lactis* MG1363 genome using primers 37 and 4. Using pQE30/*iap* as a template, primers 5 and 38 were used to amplify a DNA sequence corresponding to an N-terminus His-tag plus amino acids 26–402 of the native P60 protein (i.e. tP60). The two PCR products, i.e. SEC (with RBS) and truncated *iap* (encoding tP60), were SOE-spliced using primers 37 and 38. The spliced PCR product (designated RBS-SEC-tP60) was sequentially digested with *Kpn*I and *Xba*I, and ligated using T4 DNA ligase (Roche) to a similarly digested pNZPnisA:SEC-LLO. The ligation mix was used to transform commercial chemically competent *E. coli* Top10 (Invitrogen) following the manufacturer's instructions and plated onto LB agar containing Cm. After incubation at 37 °C for 24–48 h, positive colonies were detected by colony PCR. The created plasmid pNZPnisA:SEC-LLO/tP60 was extracted from *E. coli* Top10 using the miniprep kit (Qiagen) and the DNA sequence was confirmed by sequencing (Cogenics).

Plasmid pNZPnisA:SEC-tP60 was created for expression of tP60 alone (Fig. 1). The SEC signal was amplified (without its native RBS) from the *L. lactis* MG1363 genome using primers 34 and 4. Primers 5 and 38 were used to amplify DNA encoding a His-tagged tP60 from pQE30/*iap* plasmid as described above. These two PCR products (SEC and truncated *iap*) were spliced using primers 34 and 38 and the SOE technique (Horton *et al.*, 1990) resulting in the construct SEC-tP60. After sequential digestion of both SEC-tP60 and pNZ8048 (Kuipers *et al.*, 1998) with *Nco*I and *Xba*I, they were ligated and used to transform chemically competent *E. coli* Top10 (Invitrogen) following the manufacturer's instructions and plated onto LB agar containing Cm. After incubation at 37 °C for 24–48 h, positive colonies were detected by colony PCR and the DNA sequence of the created plasmid pNZPnisA:SEC-tP60 was confirmed by sequencing (Cogenics).

All PCRs were performed using the high fidelity KOD hot start DNA polymerase (Novagen) following the manufacturer's instructions.

Transformation of *Lactococcus* strains with the engineered plasmid vectors. *L. lactis* NZ9000 Δ *htrA* (Lindholm *et al.*, 2004) (kindly provided by Professor Airi Palva, Faculty of Veterinary Medicine, University of Helsinki, Finland) was used as a host for the three engineered plasmids. It should be noted that HtrA is an extracellular housekeeping protease (Poquet *et al.*, 2000) and HtrA-deficient *Lactococcus* mutants have been reported to be more efficient in secreting expressed protein (Le Loir *et al.*, 2005; Lindholm *et al.*, 2004). Electrocompetent *L. lactis* Δ *htrA* cells, prepared as described by Holo & Nes (1989), were transformed with the corresponding plasmid vectors using a Gene Pulser (Bio-Rad), plated onto GM17 agar containing Cm

Table 2. Oligonucleotide primers

Primer name	Primer sequence (5'→3')*
P60-pQE30 forward primer	GAAGGATCCAGCACTGTAGTAGTCGAAGC (<i>Bam</i> HI)
P60-pQE30 reverse primer	ATCTGGTACCTTATACGCGACCGAAGCCAA (<i>Kpn</i> I)
37	ATCTGGTACCATTCACCGAACTTAATGGGA (<i>Kpn</i> I)
4	TGATGTGTATCAGCGTAAACACCTGACAACG
5	TGTTTACGCTGATACACATCACCATCACCATCACGGA
38	CCATCTAGATTAAAAATACATATTTAGTGTAACC (<i>Xba</i> I)
34	TAGACCATGGGTAAAAAAGATTATCTCAGC (<i>Nco</i> I)

*Where applicable, recognition sites of restriction enzymes are underlined and the enzyme name is indicated in parentheses.

and incubated at 30 °C for 24 h. Colonies were checked by colony PCR and positive clones were preserved in glycerol stocks at -80 °C. In addition, plasmid pNZ8048 was transformed into *L. lactis* NZ9000 Δ htrA to create a negative control *Lactococcus* strain.

Investigation of LLO production by SDS-PAGE and Western blotting. Overnight cultures of the three engineered *Lactococcus* strains (i.e. containing pNZPnisA:SEC-LLO, pNZPnisA:SEC-tP60 or pNZPnisA:SEC-LLO/tP60) were subcultured (5% v/v) in fresh GM17 broth and grown statically at 30 °C to an optical density at 600 nm of 0.5. At this point, nisin, used as a filter-sterilized culture supernatant of the nisin-producer strain *Lactococcus* NZ9700 (Kunji *et al.*, 2003), was added at a concentration of 0.2% (v/v). These strains were allowed to grow for a further 3 h. Culture supernatants of the induced vaccine strains were collected and secreted proteins were precipitated using cold trichloroacetic acid precipitation (15% w/v final concentration) as described by (Piard *et al.*, 1997).

For Western blotting, SDS-PAGE gels were blotted onto nitrocellulose membranes (Hybond-ECL; Amersham Biosciences) and the membrane was then blocked overnight at 4 °C in 5% (w/v) skimmed milk in Tris-buffered saline buffer (0.8% w/v sodium chloride, 20 mM Tris/HCl, pH 7.6). Primary mouse anti-penta-His-tag antibody (Abgent) and secondary anti-mouse antibody (ECL Western blotting system) were used at 1/1000 and 1/1500 dilutions in 5 and 10% (w/v) skimmed milk, respectively. Western blot detection was performed using the Amersham ECL western blotting system (Amersham Biosciences) according to the protocol recommended by the manufacturer.

Assessment of LLO haemolytic activity. The haemolytic activity of LLO in induced culture supernatants was assessed using the method described by Kohda *et al.* (2002) with some modifications. Briefly, aliquots of 100 µl 0.5% (v/v) sheep red blood cells suspended in PBS (pH 5.9) were added to twofold dilutions (in PBS pH 5.9) of dialysed culture supernatants of the cultured induced strains, to a final volume of 1 ml. A positive control (distilled water) and negative controls (PBS pH 5.9 and culture supernatant of *L. lactis* NZ9000 Δ htrA(pNZ8048)) were also included. Tubes were incubated statically at 37 °C for 45 min after which they were centrifuged and the supernatants collected. Absorbance was measured at 415 nm and haemolytic activity was expressed in terms of complete haemolytic units (CHU), defined as the reciprocal of the highest dilution of supernatant showing complete haemolysis (Hess *et al.*, 2000).

Murine immunization protocols. Female BALB/c mice, 6–8 weeks of age, were used in all animal experiments. All animal procedures were reviewed and approved by the ethical assessment committee of University College Cork. Overnight cultures of *Lactococcus* vaccine and control strains were subcultured into fresh warm GM17 and incubated statically at 30 °C until an OD₆₀₀ of 0.5 was reached, whereupon nisin (0.2% v/v) was added and the cultures were incubated for a further 1 h. Cells were then pelleted by centrifugation, washed once with PBS and resuspended in PBS for intraperitoneal (IP) injection (10⁷ c.f.u. per dose) or resuspended in a bicarbonate buffer (0.2 M sodium bicarbonate, 5% casein hydrolysate, 0.5% glucose) (Robinson *et al.*, 2004) for oral inoculation (5 × 10⁹ c.f.u. per dose). In the oral vaccination experiment we used 5 µg Cm ml⁻¹ instead of the higher levels (10 µg ml⁻¹) reported previously (Bahey-El-Din *et al.*, 2008) for plasmid selection to decrease any inhibitory effect of Cm on acid tolerance in *Lactococcus* (Kim *et al.*, 2002). We postulated that this may increase lactococcal survival in the gastric acidic medium upon oral vaccination. As a positive control we vaccinated mice with a murinized *Listeria monocytogenes* (designated *Listeria monocytogenes* EGDm) strain (recently created in our lab with a mutated internalin A virulence factor to allow improved murine gut invasion by the oral route) (I. R. Monk, C. Hill & C. G. M. Gahan, unpublished data). An overnight culture of this strain was diluted in

the aforementioned bicarbonate buffer and inoculated orally (10⁶ c.f.u. per dose) in the oral vaccination experiments. Groups treated with a sublethal IP *Listeria monocytogenes* EGDe (2 × 10³ c.f.u. per dose) were also included as a positive control for the IP vaccination experiments. *L. lactis* NZ9000 Δ htrA(pNZ8048)-treated groups (IP and oral), and PBS-injected groups and bicarbonate buffer orally treated groups, were included as negative controls in the IP and oral vaccination experiments, respectively.

Groups were given seven doses of the vaccine or control strains by oral gavage on days 1, 2, 3, 28, 29, 30 and 37. This vaccine regimen is similar to a successful regimen utilized for *L. lactis*-based vaccination studies (Robinson *et al.*, 2004). For IP vaccination, three doses were given on days 1, 15 and 30. The final dose volume per mouse was 200 µl for both oral and IP inoculations.

Detection of LLO-specific CD8⁺ T cells by the enzyme-linked immunospot (ELISPOT) test. The ELISPOT test was used as described by Carvalho *et al.* (2001) to detect gamma interferon (IFN-γ)-secreting CD8⁺ cells specific to the H2 K^d-restricted LLO epitope LLO_{91–99}, GYKDGNEYI and the P60 epitope P60_{217–225}, KYGVSVDI (Peptide Protein Research) (Vijh & Pamer, 1997). Mouse mastocytoma line P815-1-1 cells (from the European Collection of Cell Cultures) were used as antigen presenting cells (APCs). P815-1-1 cells pulsed with 10⁻⁶ M LLO_{91–99} or P60_{217–225} peptides or non-pulsed were used to stimulate splenocytes from vaccinated and control mice. Briefly, MultiScreen 96-well plates (Millipore) were first coated with anti-mouse IFN-γ antibodies (clone R4-6A2) (Biolegend), and the splenocytes along with APCs were incubated in the coated wells for 20–24 h in a tissue culture incubator (37 °C and 5% CO₂ atmosphere). The wells were subsequently washed with PBS containing 0.05% (v/v) Tween 20 and treated with biotin anti-mouse IFN-γ antibodies (clone XMGL2) (Biolegend) followed by avidin-horseradish peroxidase (Biolegend). Spots corresponding to epitope-specific IFN-γ-secreting cells developed upon adding the peroxidase substrate 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma). The number of spots was determined using a stereomicroscope.

Murine challenge with *Listeria monocytogenes*. Listerial challenge was performed as previously described (Stack *et al.*, 2005). Briefly, mice were challenged with IP injection of 200 µl containing 2 × 10⁶ c.f.u. ml⁻¹ (4 × 10⁵ c.f.u. per mouse) *Listeria monocytogenes* EGDe. For the oral vaccination experiment, challenge was performed orally with the murinized *Listeria monocytogenes* EGDm (6 × 10⁹ c.f.u. per mouse) on day 42. Mice were euthanized 3 days post-challenge and the listerial burden was determined from the spleens by organ homogenization, serial dilutions and plating on BHI agar plates. Plates were incubated at 37 °C for 1–2 days and *Listeria* counts were calculated per spleen. The limit of detection (LOD) of *Listeria* was 50 c.f.u. per organ.

Interpretation of data and statistical analysis. Data satisfying the assumptions of normality were analysed using the Student's *t*-test. Data not showing normal distribution were analysed by Kruskal–Wallis one-way ANOVA by ranks with post hoc comparison using Dunn's method. In all cases, *P* values less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Production of LLO and tP60 by engineered lactococcal strains

SDS-PAGE followed by Western blotting using anti-penta-His-tag antibody (Abgent) revealed efficient production of

LLO and tP60 in the corresponding *L. lactis* NZ9000 $\Delta htrA$ strains (Fig. 2). However, it was observed that the level of tP60 production in NZ9000 $\Delta htrA$ (pNZPnisA:SEC-LLO/tP60) was significantly lower than its production level in NZ9000 $\Delta htrA$ (pNZPnisA:SEC-tP60) (Fig. 2). Assessment of the haemolytic activity of secreted LLO confirmed the secretion of biologically active haemolysin. Culture supernatants of NZ9000 $\Delta htrA$ (pNZPnisA:SEC-LLO) and *L. lactis* NZ9000 $\Delta htrA$ (pNZPnisA:SEC-LLO/tP60) showed 8 CHU, whereas no detectable haemolytic activity was observed with *L. lactis* NZ9000 $\Delta htrA$ (pNZ8048) or *L. lactis* NZ9000 $\Delta htrA$ (pNZPnisA:SEC-tP60).

Assessment of short-term immune responses

CD8⁺ T lymphocytes specific for the H2 K^d-restricted LLO_{91–99} epitope but not the P60_{217–225} epitope were elicited by the vaccine strains following IP immunization. The ELISPOT assay was used to analyse the development of epitope-specific CD8⁺ cells. Mouse mastocytoma P815-1-1 cells were used as APCs as they express restricted H2 K^d MHC class I molecules. As a result, only spots due to epitope-specific CD8⁺ cells are produced (Carvalho *et al.*, 2001). The short-term immune response was assessed on day 42 from the start of vaccination by the IP or oral routes. The two LLO-secreting strains, i.e. *L. lactis* NZ9000 $\Delta htrA$ (pNZPnisA:SEC-LLO) and *L. lactis* NZ9000 $\Delta htrA$ (pNZPnisA:SEC-LLO/tP60), demonstrated LLO_{91–99}-specific spots following a three-dose IP vaccination regimen (Fig. 3). No P60_{217–225}-specific spots were observed following this three-dose IP regimen with any of the tP60-secreting lactococcal vaccine strains whether in presence or absence of LLO co-expression (Fig. 3). Following oral vaccination, only the positive control murinized *Listeria monocytogenes* EGDm strain was found to elicit LLO_{91–99}- and P60_{217–225}-specific spots in the ELISPOT test (data not shown).

Fig. 2. Western blotting of culture supernatant protein precipitates following nisin induction. Mouse anti-His tag antibodies were used as primary antibodies to detect the His-tagged LLO and tP60. Lanes 1–4 represent trichloroacetic acid precipitates of the supernatants of nisin-induced cultures of NZ9000 $\Delta htrA$ (pNZPnisA:SEC-LLO) (lane 1), NZ9000 $\Delta htrA$ (pNZPnisA:SEC-LLO/tP60) (lane 2), NZ9000 $\Delta htrA$ (pNZPnisA:SEC-tP60) (lane 3) and NZ9000 $\Delta htrA$ (pNZ8048) (lane 4).

Fig. 3. ELISPOT test results following a three-dose IP vaccination regimen with the lactococcal vaccine strains. Mouse groups ($n=4$) were vaccinated by IP injection on days 1, 15 and 30 then examined by ELISPOT on day 42. Asterisks indicate a $P<0.05$ as compared to the negative control groups. Error bars represent the mean $\pm$ SEM. White bar, P60_{217–225}; black bars, LLO_{91–99}.

Protection against *Listeria monocytogenes* challenge following IP vaccination.

Mice were challenged intraperitoneally with *Listeria monocytogenes* EGDm on day 42 after the first vaccination dose. Three days following challenge, mice were euthanized and the *Listeria* counts were determined in the spleens. Spleen listerial counts following the three-dose IP vaccination revealed significant protection ($P<0.05$) with both *L. lactis* NZ9000 $\Delta htrA$ (pNZPnisA:SEC-LLO) and *L. lactis* NZ9000 $\Delta htrA$ (pNZPnisA:SEC-LLO/tP60) strains when compared with negative control groups or with groups vaccinated with *L. lactis* NZ9000 $\Delta htrA$ (pNZPnisA:SEC-tP60) (Fig. 4). No significant difference in protection was found between *L. lactis* NZ9000 $\Delta htrA$ (pNZPnisA:SEC-LLO) and *L. lactis* NZ9000 $\Delta htrA$ (pNZPnisA:SEC-LLO/tP60) (Fig. 4).

In oral vaccination experiments, no significant difference was observed between the orally vaccinated groups and the negative control groups for any of the three constructed vaccine strains after challenge with oral murinized *Listeria monocytogenes* EGDm (data not shown). This failure to protect by the oral route may be due to the rapid killing of *L. lactis* in the gastrointestinal tract (GIT) that has been reported by Kimoto *et al.* (2003) and by Vesa *et al.* (2000). This shows the necessity of an improved strategy to protect *L. lactis* during GIT transit in the murine model for enhanced presentation of the cloned antigens to the immune system. Other factors that may play critical roles upon oral vaccination are the vaccination regimen used, bacterial inoculation doses, amount and nature of the expressed antigens in lactococci, and the particular *L. lactis* strain utilized. Moreover, other harsh GIT conditions, such as the presence of bile, may play a significant role in

Fig. 4. Results of the challenge experiment following a three-dose IP vaccination regimen. Mouse groups ($n=5$) were IP-vaccinated on days 1, 15 and 30 then challenged with IP *Listeria monocytogenes* EGDe on day 42. Mice were euthanized 3 days later and the *Listeria* in the spleens counted. Asterisks indicate a $P<0.05$ as compared to negative control groups. Error bars represent the mean $\pm$ SEM. ND, Not detectable.

preventing the survival of *L. lactis*. A possible future approach to solve this problem may be the use of *L. lactis* harbouring BilE, the bile resistance system of *Listeria monocytogenes*, which allows better *L. lactis* survival and persistence in the murine gut (Watson *et al.*, 2008).

Assessment of long-term immune responses following an IP vaccination regimen

As seen above, the three-dose IP vaccination regimen did not show any protective difference between *L. lactis* dually expressing LLO and tP60, and *L. lactis* that expressed LLO alone. Moreover, we did not detect a significant shift in the immune response against tP60 towards a cell-mediated CD8⁺ response or T helper type 1 (Th1) response when LLO was co-expressed in NZ9000 $\Delta htrA$ (pNZPnisA:SEC-LLO/tP60). Hence, we decided to increase the frequency of IP doses to detect any weak immune response that might have been obscured due to suboptimal immunization. In addition, the immune response was assessed on day 69 rather than day 42 of vaccination to examine longer term immunity. Eight IP bacterial doses, according to a previously determined regimen from our work (Bahey-El-Din *et al.*, 2008), were injected by the IP route on days 1, 2, 7, 14, 21, 28, 35 and 36, and the immune response was assessed on day 69 from the start of vaccination. Fig. 5 summarizes the different vaccination regimens used in the present study.

ELISPOT results indicated significant LLO_{91–99}-specific responses in both mice treated with NZ9000 $\Delta htrA$ (pNZPnisA:SEC-LLO) or NZ9000 $\Delta htrA$ (pNZPnisA:

Fig. 5. Diagrammatic representation of the different vaccination regimens used in this study. The numbered arrows represent the days when vaccine inoculations were carried out. The numbered stars at the end (right side) of the regimen diagram represent the day of vaccine challenge or ELISPOT test.

SEC-LLO/tP60), but no significant difference was found between these two groups (Fig. 6). A P60_{217–225}-specific CD8⁺ response was detectable at low levels in only two out of five mice vaccinated with NZ9000 $\Delta htrA$ (pNZPnisA:SEC-LLO/tP60) but the overall P60_{217–225}-specific spots (evaluating all mice) were statistically insignificant as compared to negative control groups (Fig. 6). No P60_{217–225}-specific spots were observed in NZ9000 $\Delta htrA$ (pNZPnisA:SEC-tP60)-treated mice (Fig. 6). These results indicate that the effect of LLO on introducing tP60 to the MHC class I pathway was limited, perhaps

Fig. 6. ELISPOT test results following an eight-dose IP vaccination regimen (long-term investigation). Mouse groups ($n=5$) were vaccinated by IP injection on days 1, 2, 7, 14, 21, 28, 35 and 36 then examined by ELISPOT on day 69. Asterisks indicate a $P<0.05$ as compared to negative control groups. Error bars represent the mean $\pm$ SEM. White bar, P60_{217–225}; black bars, LLO_{91–99}.

due to the low levels of tP60 expression in NZ9000 $\Delta htrA$ (pNZPnisA:SEC-LLO/tP60) (Fig. 2).

Following IP challenge with *Listeria monocytogenes* EGDe on day 69, significant protection, as evidenced by splenic listerial counts, was achieved in mice treated with NZ9000 $\Delta htrA$ (pNZPnisA:SEC-LLO) or NZ9000 $\Delta htrA$ (pNZPnisA:SEC-LLO/tP60) as compared to negative control groups (Fig. 7). However, no statistical difference in protection was found between these two groups. In addition, no protection was observed in mice treated with IP NZ9000 $\Delta htrA$ (pNZPnisA:SEC-tP60). These results further confirm that tP60 expressed alone in *L. lactis* cannot afford protection against listeriosis and also the effect of LLO/tP60 combined expression in *L. lactis* does not offer a more pronounced protection than *L. lactis* expressing LLO alone.

Conclusions

Listeria monocytogenes is a food-borne pathogen that can cause miscarriage and meningitis in the pregnant and newborn, respectively. Several listerial outbreaks have been reported by Ramaswamy *et al.* (2007) and immunocompromised individuals are at a higher risk of infection. In a previous work, we demonstrated that LLO-expressing *L. lactis* vaccine vectors could elicit antigen-specific CD8⁺ immune responses and protection against *Listeria monocytogenes* challenge in the murine model (Bahey-El-Din *et al.*, 2008). The use of the GRAS *L. lactis* as a live vaccine vector

represents a safe and advantageous approach in current vaccine research, particularly in terms of the stimulation of mucosal immunity. In the present work, we co-expressed a truncated form of P60 antigen of *Listeria monocytogenes* along with LLO in an *L. lactis* strain and examined the vaccine potential of this construct in a murine model of listeriosis. Control *L. lactis* strains expressing LLO or tP60 individually were also included and examined to allow comparison between the single and dual expression vaccine candidates. Regardless of tP60 expression, we found that LLO expression by *L. lactis* is critical for successful vaccination against *Listeria monocytogenes*, most probably due to its reported immunodominance (Vijh & Pamer, 1997). Although P60 antigen is known to be immunogenic, LLO is considered more important for a protective cytotoxic CD8⁺ immune response, a phenomenon demonstrated by Hess and co-workers who used *Salmonella typhimurium aroA*⁻ attenuated strains as live vectors for LLO and P60 antigens (Hess *et al.*, 1996).

It is well known that the protective immune response against listeriosis is dependent on a cytotoxic CD8⁺ immune response (Pamer, 2004). Since cytoplasmic access favours antigen processing through the MHC class I presentation pathway, we hypothesized that LLO activity would facilitate this process for the accompanying tP60 antigen. However, it seems that the process was limited by the relatively moderate amount of tP60 expressed in *L. lactis* NZ9000 $\Delta htrA$ (pNZPnisA:SEC-LLO/tP60). The amount of antigen expressed in *L. lactis* vectors is reported to play a critical role in the resultant immune response and higher levels of antigen are usually associated with a better immune response (Wells & Mercenier, 2008). Although appreciably high anti-P60 IgG titres were observed in mice treated intraperitoneally with tP60-expressing strains, no significant difference was observed between serum anti-P60 IgG antibodies in the absence or presence of LLO co-expression (data not shown). Furthermore, examination of anti-P60 IgG1 (indicative of Th2 response) and IgG2a (indicative of Th1 response) subclasses did not show any shift towards a particular anti-P60 immune response (Th1 or Th2) when LLO was co-expressed with tP60 (data not shown). The reasons behind these results are not clear.

Surprisingly, the positive control *Listeria monocytogenes* IP-treated group showed a lower number of LLO₉₁₋₉₉-specific CD8⁺ T cells than those observed with NZ9000 $\Delta htrA$ (pNZPnisA:SEC-LLO/tP60) or NZ9000 $\Delta htrA$ (pNZPnisA:SEC-LLO) in the long-term IP regimen (Fig. 6). It is known that the pool of LLO₉₁₋₉₉-specific CD8⁺ T cells shrinks in time following parenteral administration of *Listeria monocytogenes* (Vijh & Pamer, 1997). Indeed we observed this phenomenon in a previous study (Bahey-El-Din *et al.*, 2008). Our observations may reflect the different rates of expansion and contraction of LLO₉₁₋₉₉-specific T lymphocytes. To examine this, it would be necessary to follow up the kinetics of the LLO₉₁₋₉₉-specific CD8⁺ response at different time intervals following a specific vaccination regimen.

Fig. 7. Results of the challenge experiment following an eight-dose IP vaccination regimen (long-term investigation). Mouse groups ($n=5$) were vaccinated by IP injection on days 1, 2, 7, 14, 21, 28, 35 and 36 then challenged with IP *Listeria monocytogenes* EGDe on day 69. Mice were euthanized 3 days later and the *Listeria* in the spleens counted. Asterisks indicate a $P<0.05$ as compared to negative control groups. Error bars represent the mean $\pm$ SEM.

We had two objectives in mind for the current study: first, to provide a safe bivalent *L. lactis* vaccine vector against listeriosis; and second, to examine the immunomodulatory effects of LLO on the accompanying antigen, tP60. LLO is known to direct the immune system towards cell-mediated immunity due to its ability to permeabilize the phagosomal membrane (Darji *et al.*, 1995; Dietrich *et al.*, 2001). When *E. coli* was engineered to express both LLO and ovalbumin (OVA), as a model antigen, the engineered strain could deliver an OVA K^b-restricted epitope for MHC class I presentation and mice were protected upon challenge with an OVA-expressing melanoma cell line (Radford *et al.*, 2002). More recently, *Bacillus subtilis* was genetically manipulated to express both LLO and PA (the protective antigen of *Bacillus anthracis*), and an enhanced Th1 immune response against PA was evident (Huang *et al.*, 2008). Nevertheless, it is clear that the use of the advantageous GRAS *L. lactis* as an LLO vector represents a potentially safe alternative platform for the cytosolic antigen presentation pathway.

Igwe and colleagues co-expressed large C-terminal portions of LLO and P60 antigens (LLO_{51–363} and P60_{130–477}, respectively) in an attenuated *Salmonella enterica* serovar Typhimurium strain (Igwe *et al.*, 2002). However, for cytoplasmic translocation of antigens they used the type III secretion system of *Salmonella* rather than the pore forming activity of LLO. Following oral vaccination in mice it was shown that the combined expression and translocation of the truncated LLO and P60 antigens offered better protection against wild-type *Listeria monocytogenes* challenge than either antigen alone (Igwe *et al.*, 2002). In our study, we could not find a significant difference in efficacy between LLO-expressing *L. lactis* and *L. lactis* expressing combined LLO/tP60 whether by the IP or the oral route. The apparent discrepancy between the findings of Igwe and co-workers and our data has a number of possible explanations. Firstly, *L. lactis* is non-invasive and is not adapted to survive intracellularly whether in the phagosome or intracytoplasmically (Bahey-El-Din *et al.*, 2008). In contrast *Salmonella* is an invasive bacterium that is able to persist in the phagosomal compartment, even when attenuated, which means a longer period of antigen production and presentation to the immune system (Carrol *et al.*, 1979). Also, in our hands the level of tP60 expression in NZ9000 Δ htrA (pNZPnisA:SEC-LLO/tP60) was found to be much lower than the tP60 level expressed by NZ9000 Δ htrA (pNZPnisA:SEC- tP60) (Fig. 2). Although the reason for this is not clear, it is likely to have an effect on the immunization potential of the combined LLO/tP60-expressing vaccine strain. Finally, the mechanism by which tP60 was intended to access the MHC class I presentation pathway in our study (LLO permeabilization) is different from the approach used by Igwe and co-workers (type III secretion system) (Igwe *et al.*, 2002). Despite these findings we believe that the safety profile of *L. lactis* suggests that this vector may be more applicable as a delivery vehicle in

humans and that further work is necessary to improve vaccine delivery using this platform.

In brief, we successfully created *L. lactis* vaccine strains that secrete the listerial antigens LLO and tP60 in combination or singly. In the murine model, no protective immunity was observed upon oral vaccination whilst IP immunization could elicit protective anti-listerial immunity with LLO-producing lactococcal strains. The immunodominant LLO antigen proved to be critical for CD8⁺ cell-mediated immunity and protection, while tP60 alone did not elicit protection and did not significantly improve the immune outcome upon combined expression with LLO in *L. lactis*. It is postulated that higher tP60 expression levels may be necessary for sufficient cytoplasmic access of the antigen upon LLO permeabilization of the phagosome.

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