Vaccine 26 (2008) 6043-6052

Contents lists available at ScienceDirect

# Vaccine

journal homepage: www.elsevier.com/locate/vaccine

# Cytoplasmic delivery of antigens, by *Bacillus subtilis* enhances Th1 responses

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#### ARTICLE INFO

Article history: Received 17 June 2008 Received in revised form 28 August 2008 Accepted 4 September 2008 Available online 20 September 2008

Keywords: Bacillus subtilis Listeriolysin Intracellular survival Antigen delivery Th1 response

## ABSTRACT

Endospores of the Gram-positive bacterium, *Bacillus subtilis*, have been used successfully for delivery of antigens where the immunogen is expressed on the spore surface. In this work the spore has been engineered to deliver antigens to the cytoplasm of macrophages by expressing listeriolysin O (LLO) or a derivative,  $LLO_{L461T}$ , that is stable at neutral pH, from the *B. subtilis* vegetative cell. Following phagocytosis spores were shown to germinate in the phagosome enabling secretion of  $LLO/LLO_{L461T}$  and entry of the bacterium into the cytosol. We have shown that in the cytosol *B. subtilis* proliferates before eventually being destroyed. Immunisation of mice with spores that co-expressed LLO with Protective Antigen (PA) of *Bacillus anthracis* generated an increase in IgG2a against PA, toxin-neutralising activity coupled with specific IFN- $\gamma$  and IL-12 (and reduced IL-4) responses of splenocytes, both indicative of an enhanced Th1 response. Enhanced Th1 responses via LLO co-expression of antigen by *B. subtilis* spores may be a useful strategy to improve vaccine performance.

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#### 1. Introduction

The Gram-positive bacterium Bacillus subtilis has provided a valuable model system to study unicellular differentiation. In the last decade though, the way we consider this bacterium has changed considerably. Long thought of as a soil organism mounting evidence suggests that it can carry out its entire life cycle of spore germination, proliferation and re-sporulation within the gastrointestinal tract (GIT) [1,2]. Indeed, it is possible that it is a member of the gut microflora and, in part, this may help to explain its commercial use as a probiotic supplement [3]. Genetic engineering has been used to create B. subtilis spores that express heterologous antigens and it is here that the heat stable spore coupled with its ease of production makes it an attractive 2nd generation vaccine vehicle [4-6]. Studies have focused primarily on tetanus as a model disease. The highly immunogenic tetanus toxin fragment C (TTFC) from Clostridium tetani has been displayed on the spore surface and when delivered orally to mice protects against an otherwise lethal challenge with tetanus toxin [7-9]. Secretory IgA (sIgA) responses as well as TTFC-specific IgG responses were found showing that spore

delivery could be effective against mucosal pathogens. Protection has also been generated in mice immunised with recombinant spores expressing fragments of the Protective Antigen (PA) of *Bacillus anthracis*; although in this study spores were administered by the intra-peritoneal route [10]. In more recent studies spores have been used to orally vaccinate mice against challenge with a lethal dose of *Clostridium perfringens* alpha toxin and are to be evaluated as a poultry vaccine against necrotic enteritis [11].

The focus on spore vaccines to date has been on humoral responses though. Intriguingly, analysis of subclasses has revealed a marked bias in the nature of the T-helper cell produced following interaction of antigen with a CD4+ T cell. When using spore surface display of heterologous antigens the T helper response is predominately Th1 (T-helper 1) with the production of IFN- $\gamma$  [7,10,12]. This is relatively unusual and as reported by Mauriello et al. [13] the spore coat appears to induce a Th1 bias in contrast to most mucosal adjuvants that confer a Th2 bias. The precise nature of the cellular response is only just being determined but spore vaccines have been shown to induce IFN- $\gamma$  expression in splenocytes from mice orally immunised with TTFC [13].

Spores have been shown to enter the gut-associated lymphoid tissue (GALT) by M-cell translocation and in a rabbit model appear vital for robust development of the GALT [14]. Spores can be recovered from spleens and mesenteric lymph nodes and migration to





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<sup>0264-410</sup>X/\$ - see front matter © 2008 Elsevier Ltd. All rights reserved. doi:10.1016/j.vaccine.2008.09.024

the GALT has been determined *in vivo* [7]. Following phagocytosis spores are able to germinate and persist within the phagocytic cell for a number of hours [15]. This intracellular survival has raised the question of whether it is possible to engineer spores to enter the cytoplasm and, via this route, deliver antigens to the cytosol. Bielecki et al. [16,17] have shown that if *B. subtilis* is engineered to express listeriolysin O (LLO; a pore-forming, cholesterol-dependent, cytolysin from *Listeria monocytogenes*) then following phagocytosis in J774 macrophages *B. subtilis* could grow before eventually being cleared. They reasoned that LLO enabled escape from the phagosome and entry to the cytoplasm. If antigens could be expressed in the cytoplasm this may influence the balance of Th1/Th2 responses and the qualitative and quantitative nature of antibody responses.

In this paper we have developed a system for antigen delivery to the cytoplasm of a phagocytic cell line. We used spores engineered to express *B. anthracis* PA together with a wild type LLO protein, or a modified LLO that was stable at neutral pH. Our studies show clearly, that co-expression of LLO with PA confers a Th1 bias, enhancing induction of IFN- $\gamma$  and IL-12, together with increased titres of IgG2a antibodies and toxin-neutralising activity.

#### 2. Materials and methods

#### 2.1. Bacterial strains and cell lines

PY79 is a prototrophic, wild type (WT), strain of *B. subtilis* derived from the 168 type-strain [18]. For cytotoxicity studies, SC2329, a toxin producing strain of *B. cereus* [19] was used as a control. For invasive studies, a laboratory strain of *Listeria monocy-togenes* (NCTC 19115) was used as a control. DL507 (*cotB-pagA1b-3 rrnO-pagA*) and DL346 (*PrrnO-pagA*) have been described previously [10]. RAW264.7, Caco-2 and HEp-2 cell lines were obtained from the European Collection of Animal Cell Cultures. The mucus-secreting cell line, HT29-16E, has been described previously [20].

#### 2.2. Preparation of spores

Sporulation was induced in DSM (Difco-Sporulation Media) using the exhaustion method as described elsewhere [21]. Spore suspensions were lysozyme-treated and then heat-treated (68 °C, 1 h) to remove residual vegetative cells and titrated immediately for colony forming units (CFU)/ml before freezing aliquots at -20 °C.

#### 2.3. Construction of recombinant strains

- (i) JH27 (PrrnO-hlyA): The hlyA gene, that encodes LLO, was amplified from chromosomal DNA of L. monocytogenes (NCTC 19115) using forward and reverse primers (Supplementary Table). The complete *hlyA* gene, carrying the membrane secretion sequences (black box in Fig. 1A) was included. The 1587 bp hlyA amplicon was cut with XbaI and NotI, and then cloned downstream of the B. subtilis rrnO gene promoter and the ribosome binding site (RBS) of the sspA gene of plasmid pDL243 [10] resulting in plasmid pJH13 (Fig. 1A). To introduce the cloned DNA into the B. subtilis genome, pJH13 was linearised by digestion with Pvull and transformed into competent cells of PY79 with selection for chloramphenicol resistance (Cm<sup>R</sup>) resulting in strain JH27 (cited as PA to indicate expression of Protective Antigen). Cm<sup>R</sup> transformants would result from a stable, double crossover recombination at the *amyE* locus as described elsewhere [22,23].
- (ii) JH338 (*PrrnO-hlyA<sub>L461T</sub>*): The *hlyA<sub>L461T</sub>* gene was created using the QuikChange<sup>®</sup> Site-Directed Mutagenesis Kit (Stratagene).



**Fig. 1.** Construction of recombinants. Panel A shows, schematically, the chromosomal placement of the *pagA* gene and wild type and mutated *hlyA* genes in the *B. subtilis* chromosome. The *pagA* gene was inserted at the *thrC* locus and the *hlyA* genes at the *amyE* loci by double crossover recombinational events using vectors derived from pDL242 and pDL243, respectively, that have been designed for stable chromosomal insertion [22,23]. For both genes expression was controlled from the *rnO* promoter and *pagA* and *hlyA* signal sequences (black box) enabled membrane secretion of the encoded proteins Panel B shows expression levels in cell pellets (P) and culture supernatants (S) examined by SDS-PAGE (12%) followed by western blotting in strains expressing LLO and/or PA. 0.1 µg of purified PA or LLO protein, run in parallel, are shown as positive controls. Cultures were incubated till an OD<sub>600</sub> of 1.0 in LB was reached. For total cell lysates, 1 ml of culture was centrifuged and suspended in 100 µl of 1 × SDS-PAGE loading buffer and 20 µl loaded on a SDS-PAGE. For culture supernatants, 18 µl of supernatant mixed with 6 µl of 4× loading buffer was examined per lane.

The single amino acid changed *hlyA* was amplified from plasmid pJH13 using forward and reverse primers (Supplementary Data). The methylated, nonmutated, parental DNA template was digested with DpnI and the mutated plasmid transformed into XL1-Blue supercompetent cells and selected on LBampicillin agar plates containing 80  $\mu$ g/ml X-gal. The resulting plasmid pJH323 that carried the *rrnO-hlyA*<sub>L461T</sub> gene was linearised by PvulI and inserted at the *amyE* chromosomal locus of *B. subtilis* PY79 to produce strain JH338 (LLO<sub>L461T</sub>) as described above for JH27.

- (iii) JH287 (PrrnO-hlyA PrrnO-pag): This strain, referred to as JH287 (LLO PA) was constructed by transforming competent cells of DL346 (PrrnO-pagA) with linearised pJH13 and selection for Cm<sup>R</sup>. DL346 carries the *B. anthracis pagA* gene, that encodes Protective Antigen (PA), inserted at the *thrC* locus, and under the control of the *rrnO* promoter [10].
- (iv) JH342 (*PrrnO-hlyA<sub>L461T</sub> PrrnO-pagA*): This strain, referred to as JH342 (LLO<sub>L461T</sub> PA) was constructed by transforming competent cells of DL346 (*PrrnO-pagA*; or PA) with linearised pJH323 and selection for Cm<sup>R</sup>.

#### 2.4. Phagocytosis assay

The murine macrophage-like cell line RAW264.7 was cultured as monolayers in DMEM medium (Dulbecco's Modified Eagle Medium, Invitrogen) supplemented with 10% (v/v) foetal calf serum in an atmosphere of 90% humidity containing 5% CO<sub>2</sub> at 37 °C. Two days before use, the cells were detached by gentle scraping and seeded into 24-multiwell plates in the same medium at a density of approximately  $5 \times 10^4$  cells per well. *B. subtilis* spores were then added at a macrophage:spore ratio of 1:10 (approximately  $5 \times 10^5$ spores per well in DMEM medium without antibiotics). Phagocytosis was allowed to proceed at 37 °C in 5% CO<sub>2</sub>, for 2 h and halted by replacing the medium with DMEM containing 10 µg/ml gentamicin to kill any extracellular germinated spores or vegetative cells. At each time point, monolayers were washed four times in sterile 0.03 M PBS (pH 7.4). To quantify the total number of intracellular *B. subtilis*, monolayers were lysed by resuspension in 0.1% Triton X-100 in sterile distilled water for 5 min at 37 °C and serial dilutions of lysate from each well were prepared and plated on DSM agar. To evaluate spore counts, lysates were heated at 65 °C for 30 min to kill all heat-sensitive *B. subtilis*, prior to serial dilution and plating.

#### 2.5. Haemolytic activity assay

The haemolytic assay used was that described by Glomski et al. [24]. For the assay, bacterial strains were grown in LB (50 ml;  $37 \,^{\circ}$ C) and at an OD<sub>600</sub> of 1.0 the cell suspension was centrifuged and the supernatant removed. For one assay a 0.5% suspension of mouse erythrocytes was first prepared in haemolysis buffer (35 mM sodium phosphate, 125 mM sodium chloride, 0.5 mg/ml BSA) either at pH 7.4 or 5.5. Next, the bacterial supernatant was mixed with a mouse erythrocyte solution (prepared in Alsever buffer) at a 1:1 ratio and incubated at 37 °C for 1 h with gentle shaking. The absorbance at 450 nm for each sample was measured. Haemolytic activity was defined as the percentage of mouse red blood cells that had lysed. A positive control (100% haemolysis) was sheep erythrocytes mixed with Red Blood Cell Lysis Buffer (Sigma) and a negative control (0%) being erythrocytes alone.

#### 2.6. Invasion and cytotoxicity studies

The ability of live bacteria to invade HEp-2, Caco-2 or a mucussecreting cell line HT29-16E was as described by Hong et al. [20]. The cytotoxicity of filtered culture supernatants to lyse HEp-2 and HT29-16E cells using a colorimetric assay was performed as described previously [20].

#### 2.7. Immunofluorescence and confocal microscopy

RAW264.7 macrophages were cultured on sterile 13 mm diameter cover slips in 24-well culture plates as described for the phagocytosis assay. Phagocytosis was allowed to proceed at 37 °C in 5% CO<sub>2</sub>, for 2 h and halted by replacing the medium with DMEM containing 10  $\mu$ g/ml gentamicin to kill any extracellular germinated spores or vegetative cells. Incubation was continued for a further 2 h. Labelling steps were performed at room temperature (RT) as described by Duc et al. [15]. Samples were examined in a Nikon Eclipse fluorescence microscope equipped with a Bio-Rad Radiance 2100 laser scanning system. Anti-PA antibodies were used at 1:200 dilution and anti-CD107a at 1:50. Images were taken using LaserSharp software and processed with the Confocal Assistant programme. Sections were 0.3  $\mu$ m. Laser powers were 10% for Argon ion laser (488 nm) and 40% for Green HeNe laser, scanning speed was 50 lps. Image size was 40  $\mu$ m × 40  $\mu$ m.

#### 2.8. Immunisations for analysis of humoral responses

Groups of six mice (female, Balb/C, 6 weeks old) were immunised by the intra-peritoneal route with  $1 \times 10^9$  spores of PY79, JH27, JH338, DL346, JH287, JH342 or DL507 on days 1, 15 and 29. A naïve group received sterile water as a negative control. Final serum samples collected from sacrificed mice on day 43 were subjected to the toxin neutralising assay and indirect ELISA for detection of PA-specific serum antibodies as described in [10].

#### 2.9. Immunisations and spleen cells preparation

Groups of four mice (female, Balb/C, 6 weeks old; Harlan, UK) were immunised by the intra-peritoneal route with  $1\times 10^9$ 

spores of strain PY79, DL346, JH287 or JH342 on days 1 and 15. Spleens were removed from sacrificed mice on day 22 and crushed in 5 ml of PBS with the butt of a 10 ml syringe. A further 10 ml of PBS was added to the spleen and the resulting cell suspension was passed through a 70  $\mu$ M filter (Falcon) and centrifuged for 5 min at 800 × g. The supernatant was discarded and the pellet resuspended in 5 ml of ACK buffer (0.15 M NH<sub>4</sub>Cl, 1 mM KHCO<sub>3</sub>, 37.2 mg Na<sub>2</sub>EDTA, pH 7.2) and incubated at RT for 5 min 25 ml of PBS was then added and the sample centrifuged (5 min at 800 × g). The supernatant was discarded and the pellet resuspended in 5 ml of complete Alpha Modification Minimum Essential Medium Eagle ( $\alpha$ MEM) supplemented with L-glutamine (4 mM), penicillin (100 U/ml)/streptomycin (100  $\mu$ g/ml),  $\beta$ 2 mercaptoethanol (0.05 mM) (Gibco) and 10% heat inactivated FCS. Cells were counted using a Casy Counter (Scharfe).

#### 2.10. PA protein

Recombinant *B. anthracis* Protective Antigen was produced in *E. coli* BL21 (DE3 pLysS) from a pET28b expression vector (Novagen) that carried the *pagA* (with the signal peptide sequence deleted) and described elsewhere [10].

#### 2.11. Ex vivo IFN-γ spleen ELISPOT

PVDF membrane 96-well plates (Millipore) were coated with 5  $\mu$ g/ml of anti-mouse IFN- $\gamma$  antibody (Mabtech, Sweden) diluted in carbonate–bicarbonate buffer overnight at 4 °C. The following day plates were blocked for 1 h at 37 °C with complete media. 5 × 10<sup>5</sup> spleen cells were plated out in duplicate then serially diluted 1:2 two-times across the microtitre plate. Cells were stimulated at 37 °C in 5% CO<sub>2</sub> for 18–20 h with 10  $\mu$ g/ml of PA, 1  $\mu$ g/ml ConA (as a positive control) or with medium only. Plates were washed six times with PBS then incubated with biotin-conjugated anti-mouse IFN- $\gamma$  antibody (Mabtech, Sweden) for 2 h at RT. Plates were washed and incubated with 1  $\mu$ g/ml streptavidin alkaline phosphatase (Mabtech, Sweden) for 1 h at RT and then developed with an alkaline phosphatase developing kit (Biorad). Spots were counted using an ELISPOT plate reader (AID, Strassberg, Germany) and are represented as spot-forming cells (SFC) per million cells.

#### 2.12. Ex vivo cytokine assay

RNA extracted from  $1 \times 10^6$  spleen cells stimulated with PA at 10 µg/ml or unstimulated were carried out using an RNeasy Minikit (Qiagen) as described by the manufacturer. RNA was reverse transcribed to cDNA at 37 °C for 1 h using Omniscript reverse transcriptase (Qiagen) followed by incubation for 2 min at 97 °C to stop the reaction. Real time PCR was carried out using a light cycler (Roche). The PCR master mix was: 10 µl Qiagen PCR mix, 10 pmol of primers (Supplementary Table) diluted with PCR water. 19 µl of master mix and 1 µl of cDNA template was added to each light cycler tube (Roche) in duplicate. Gene expression was normalised to the mouse housekeeping gene hypoxanthine phosphoribosyl transferase (HPRT; Supplementary Table). The PCR programmes used were as follows: 94 °C for 15 min, 94 °C for 15 s, 60 °C for 15 s, 72 °C for 15 s; 45–60 cycles.

#### 2.13. IFN-y ELISA

 $5 \times 10^5$  spleen cells were seeded in 96-well cell culture plates in complete medium. Cells were stimulated with 10 µg/ml of PA antigen, 1 µg/ml ConA as a positive control or with medium only at 37 °C in a 5% CO<sub>2</sub>. Supernatants were harvested from cultures after 48 h of incubation. Microtitre plates (96-well; Maxisorb; Nunc)

# 6046 **Table 1**

Quantification of LLO and PA	expression in <i>B</i> subtilis <sup>a</sup>
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B. subtilis strain	Supernatant	Supernatant <sup>b</sup>		Cell pellet <sup>c</sup>	
	LLO	PA	LLO	PA	
PY79 (WT)	0	0	0	0	
JH27 (LLO)	$200\pm4$	0	0	0	
JH338 (LLO <sub>L461T</sub> )	$750\pm 66$	0	0	0	
DL346 (PA)	0	$490\pm93$	0	$0.66 \pm 0.24$	
JH287 (LLO PA)	$630\pm70$	$570\pm109$	0	$0.58\pm0.38$	
JH342 (LLO <sub>L461T</sub> PA)	$800\pm99$	$940\pm177$	0	$0.61\pm0.47$	

 $^{\rm a}\,$  Cells were grown at 37  $^\circ$ C in LB until an OD<sub>600</sub> of 1.0. Protein expression of LLO and PA determined by dot-blotting and densitometric analysis. Undetectable levels of protein are recorded as 0.

<sup>b</sup> Data shown as ng/ml.

<sup>c</sup> Data shown as ng/10<sup>6</sup> cells.

were coated with anti-mouse IFN- $\gamma$  monoclonal antibody (BD Biosciences) in 0.1 M carbonate-bicarbonate buffer overnight at 4 °C. Free binding sites were blocked with 10% FCS in PBS at RT for 1 h. Culture supernatants were tested in triplicate at RT for 2 h, and IFN- $\gamma$  was detected with a biotinylated anti-mouse IFN- $\gamma$ +streptavidin-horseradish peroxidase conjugate (BD Biosciences) at RT for 1 h. Recombinant mouse IFN- $\gamma$  (BD Biosciences) was used as a standard.

## 2.14. FACS analysis

Spleen cells were prepared as single cell suspensions. Staining of cell surface markers was performed for 20 min at RT. The following fluorescent conjugated mAbs were used: CD4-PE-Cy7, CD8-APC, CD25-PE, CD19-FITC, DX5-APC, CD69-FITC, CD3-PE, CD11c-PE, CD86-FITC and F4/80-APC (Becton Dickinson or eBioscience). Cells were incubated with individually optimised mAb concentrations, washed once, and resuspended in FACS buffer (5% FBS, and 0.1% sodium azide in PBS). Cells were analysed using a Becton Dickinson FACScalibur and data analysed using Cellquest software (BD).

#### 2.15. Transmission electron microscopy (TEM)

Infected RAW264.7 macrophages were fixed using 3% gluteradehyde prepared in a 0.1 M phosphate buffer and centrifuged at 3000 rpm for 5 min to pellet. After fixation for at least 24 h the cell pellets were then washed in 0.1 M phosphate buffer, post-fixed in 1% osmium tetroxide, dehydrated through a gradual series of alcohols up to 100% alcohol and placed in propylene oxide prior to embedding in araldite resin. The resin was polymerised at  $60 \,^{\circ}\text{C}$ 

Haemolytic and cytotoxic activity

B. subtilis strain	Haemoly	Haemolytic activity <sup>a</sup>		Cytotoxicity <sup>b</sup>	
	pH 5.5	pH 7.4	HEp-2	HT29-16E	
PY79 (WT)	0	0	$0\pm 5$	$2\pm16$	
JH27 (LLO)	$47\pm4$	0	$4\pm 6$	$0\pm4$	
JH338 (LLO <sub>L461T</sub> )	$62\pm 6$	$39\pm10$	$0\pm 8$	$5\pm8$	
DL346 (PA)	0	0	$14\pm13$	$0\pm11$	
JH287 (LLO PA)	$42\pm1$	0	$10\pm12$	$9\pm4$	
JH342 (LLO <sub>L461T</sub> PA)	$58\pm 6$	$29\pm8$	$0\pm10$	$4\pm 8$	
B. cereus SC2329	NT	NT	$70\pm10$	$68\pm5$	

<sup>a</sup> Bacterial cell culture supernatants were incubated (1 h at 37 °C) with a pH 5.5 or 7.4 buffered solution of 0.5% mouse erythrocytes. Haemolytic activity is defined as the percentage of red blood cells that had lysed. NT, not tested. Experiments were done in triplicate and values given with standard deviations (S.D.s).

<sup>b</sup> Shown as percentage of dead cells. Experiments were done in triplicate with S.D.s given.

Table 3			
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Bacterial strain	HEp-2	Caco-2	HT29-16E
PY79 (WT)	$0.24\pm0.05$	$0.06\pm0.01$	< 0.0001
H27 (LLO)	$0.46\pm0.01$	$0.12\pm0.01$	$0.02\pm0.001$
H338 (LLO <sub>L461T</sub> )	$0.83 \pm 0.03$	$0.15\pm0.01$	$0.04\pm0.002$
. monocytogenes (NCTC 19115)	$1.70\pm0.07$	$0.85\pm0.22$	$1.22\pm0.04$

<sup>a</sup> Expressed as percentage of bacteria that invaded the cultured cell line. Experiments were done in triplicate.

for 48 h. One-micron sections, stained with toluidine blue were prepared for light microscopy examination. Areas of cells were selected for ultrastructural examination. Ultrathin sections at 70–90 nm thickness were then prepared onto copper grids using a diamond knife, contrasted with uranyl acetate and lead citrate prior to examination using a Phillips CM10 TEM.

#### 3. Results

# 3.1. Construction of B. subtilis strains that express L. monocytogenes LLO and B. anthracis PA

LLO was expressed in B. subtilis in two forms, as the wild type LLO protein (strain JH27) and as a mutated form referred to as LLO<sub>L461T</sub> (strain JH338). The mutated form carried a leucine to threonine change at codon 461 in the gene, hlyA, that encodes LLO. LLO has an acidic pH optimum and changing leucine-461 residue to a threonine has been shown to increase, ten-fold, haemolytic activity of LLO at neutral pH [24]. In both cases the hlyA gene was placed under the control of the rrnO promoter of B. subtilis enabling high levels of expression in the vegetative cell [25]. In addition, the encoded LLO carried its own signal peptide for membrane secretion. Next, B. subtilis strains were constructed that carried the B. anthracis pagA gene placed in trans to either LLO (JH287) or LLO<sub>L461T</sub> (JH342). The pagA gene encodes the secreted 85 kDa Protective Antigen (PA) and has been used in immunisation experiments in *B. subtilis* [10] and so represented a suitable immunogen to evaluate using co-expression with LLO. In our constructions PA was designed to be secreted from the vegetative cell.

Quantification of PA and LLO confirmed that LLO was secreted efficiently with no protein detectable within the cell pellet (Fig. 1B and Table 1). By contrast, a significant level of PA was retained in the cell pellet indicating that the polypeptide had failed to be



**Fig. 2.** Phagocytosis of spores in murine macrophages. Murine RAW264.7 macrophages were infected with *B. subtilis* spores of strain PY79 ( $\Box$ ; WT), JH27 ( $\blacktriangle$ ; LLO) or JH338 ( $\blacksquare$ ; LLO<sub>L461T</sub>) *in vitro* at an infection ratio of 10 spores per macrophage. Phagocytosis was allowed to proceed for 2 h then halted by replacing the medium containing 10 µg/ml gentamicin to kill any extracellular germinated spores or vegetative cells. The data showed the number of vegetative cells inside macrophages at indicated time points. Data are presented as the mean  $\pm$  standard deviation.



**Fig. 3.** Laser scanning confocal micrographs showing phagocytosis of *B. subtilis* strain in murine RAW264.7 macrophages. RAW264.7 macrophages were infected with strain DL346 (PA) and JH342 (LLO<sub>L461T</sub> PA) spores as described in Section 2. Micrographs show an experiment using double immunofluorescence staining. Phagolysosomes were stained with an anti-CD107a serum and a FITC-conjugated antibody (green, Iane 1). Germinated spores were stained with an anti-PA serum and a TRITC-conjugated secondary antibody (red, Iane 3). Middle micrographs are double overlays (Iane 2).

translocated across the membrane or was retained in the cell wall. LLO<sub>I461T</sub> expression was found to be up to three-times higher than than that of LLO. Interestingly, in cells (JH287 and JH342) that coexpressed LLO/LLO<sub>I461T</sub> and PA the amount of PA that was secreted increased somewhat with the highest levels being obtained in cells (JH342) expressing LLO<sub>L461T</sub>. The potency of LLO and LLO<sub>L461T</sub> was assessed at neutral and acidic pH using a haemolytic assay (Table 2). Supernatants of JH338 (LLO<sub>L461T</sub>) and JH342 (LLO<sub>L461T</sub> PA) cells that secreted LLO<sub>L461T</sub> were active at both neutral and acidic pH whereas LLO secreted from either JH27 (LLO) or JH287 (LLO PA) cells was only active at acidic pH and was noticeably less haemolytic than LLO<sub>L461T</sub>. These results confirmed that the leucine to threonine modification in codon 461 of hlyA stabilised the LLO polypeptide. While it is clear that the levels of LLO<sub>L461T</sub> expression were higher than LLO the increased amounts of the mutant protein cannot solely account for the increase in activity. We reason this because at neutral pH, where LLO was completely inactive, the mutation caused a substantial increase in activity (39%). When compared to acidic conditions, the  $LLO_{L461T}$  mutation leads to a 50% increase in activity which could be accounted for by the increased levels of LLO.

We asked whether secretion of LLO from *B. subtilis* would enable vegetative cells to invade mammalian cells. A simple invasion assay was used to determine entry of live *B. subtilis* into three cell lines, HEp-2, Caco-2 and a mucus-secreting cell line, HT29-16E (Table 3). In HEp-2 and Caco-2 cell lines, LLO-secreting JH27 cells exhibited a two-fold increase in invasion compared to *B. subtilis* strain PY79 while JH338 cells, that secreted LLO<sub>L461T</sub>, showed even higher levels of invasion. Compared to equivalent levels of *L. monocytogenes* cells that were used in parallel assays, the levels of invasion exhibited by the three *B. subtilis* strains were significantly lower though. We wondered whether LLO or LLO<sub>L461T</sub> secreted from *B. subtilis* were

cytotoxic? Using a colorimetric assay to measure cell lysis we incubated cell free supernatants from cultures of *B. subtilis* expressing LLO and LLO<sub>L461T</sub> with or without co-expression of PA (Table 2). A positive control consisting of an enterotoxin producing strain of *B. cereus* (SC2329) demonstrated that there was no significant level (P>0.05) of cell lysis with HEp-2 or HT29-16E cells.

#### 3.2. Intracellular proliferation of B. subtilis expressing LLO

Murine, RAW264.7, macrophages were incubated with spores of PY79, JH27 (LLO) or JH338 (LLO<sub>L461T</sub>) at a macrophage/spore ratio of 1:10. After 2 h the cell culture medium was replaced with fresh medium containing gentamicin that would kill any residual bacteria that had adhered to macrophages. At appropriate time points the total number of viable cells (spores plus germinated spores) minus heat-resistant spore counts was determined. Fig. 2 shows that spores of all three strains had germinated within macrophages consistent with previous studies on the fate of phagocytosed spores [15-17]. JH27 (LLO) showed a very slight increase in cell numbers after which numbers fell to barely detectable counts after 10 h. With JH338 (LLO<sub>L461T</sub>) there was a significant increase in cell numbers during the first 5 h after which cells were cleared within 10 h. Counts of PY79 cells were considerably less with few remaining bacteria after 5 h. LLO then, enables prolonged survival within the macrophage and the modified LLO<sub>L461T</sub> enabled significant intracellular proliferation.

## 3.3. Phagocytic uptake of B. subtilis

RAW264.7 macrophages were infected with strain DL346 (PA) and JH342 (LLO<sub>L461T</sub> PA) spores and after 2 h of incubation phagocy-



**Fig. 4.** Electron microscopy of phagocytosed spores. Representative electron micrographs of RAW264.7 macrophages infected with spores of strain PY79 (WT), JH27 (LLO) and JH338 (LLO<sub>L461T</sub>). In Panel A clusters of PY79 spores are found in the phagolysosomal compartment. Panel B shows a JH338 spore contained within a phagosome. Panel C and D shows spores of PY79 (**C**) and JH27 (**D**) germinating within a phagosome. Panel E two vegetative cells (end-on) of JH27 one of which is within the cytoplasm, the other (arrowed) within a phagosome as determined by the dark layer of phagosomal membrane. Panel F shows residual fragments of coat material (arrowed) of JH338 spores within the cytoplasm. Bar markers are 200 nm (Panels B–D and F) or 1000 nm (Panels A and E).

tosis was arrested and the intracellular location of PA determined by double immunofluorescence staining. Phagolysosomes were located using an anti-CD107a serum that binds to lysosomalassociated membrane protein-1 (LAMP-1). Our confocal analysis of cells 2 h after termination of phagocytosis revealed that in DL346 infected cells PA expression was confined to the phagolysomal compartments (Fig. 3). Co-expression of LLO<sub>L461T</sub> with PA though in JH342 infected cells revealed dispersal of PA to the cytoplasm.

We also examined the intracellular fate of phagocytosed spores by electron microscopy (Fig. 4). Following phagocytosis significant numbers of spores could be found in phagosomes and phagolysosomes. Fig. 4A shows a cluster of PY79 spores contained within a phagolysosome. In Fig. 4C a PY79 spore is shown germinating and breaking free from the two-layered spore coat with a phagosome possibly entering into the cytoplasm. Fig. 4B shows a JH338 (LLO<sub>L461T</sub>) spore contained within the cytoplasm and Fig. 4D a JH27 (LLO) spore that had just germinated and was within the process of replicating as seen from the division septum. Vegetative cells were also found within the cytoplasm and Fig. 4E shows two end-on images of JH27 (LLO) cells one of which is contained within the cytoplasm and the other within a phagosome. Finally, we commonly found fragments of spore coat material within the cytoplasm as shown in Fig. 4F which shows residual coat fragments of a hatched JH338 (LLO<sub>L461T</sub>) spore. These results were surprising since we had not expected to find entry of intact spores into the cytoplasm.



**Fig. 5.** IgG1/IgG2a ratios following injection/TNA. Panel A: Groups of six Balb/C mice were immunised by the intra-peritoneal route with  $1 \times 10^9$  spores of recombinant *B. subtilis* strains on days 1, 15 and 29. Serum samples were collected on day 43 and tested by ELISA for PA-specific total-IgG, IgG1, IgG2a and IgG2b responses. Samples were also tested by the TNA for the ability to neutralise anthrax lethal toxin. Both assays were performed on individual mice and the data are presented as arithmetic means  $\pm$  standard deviations. Astericks indicate responses that were significantly (*P*<0.05) different to DL346 responses. Panel B: Serum samples collected on days 14, 28 and 43 were tested by ELISA for PA-specific IgG1 and IgG2a responses. Data show the IgG1 arithmetic mean: IgG2a arithmetic mean ratio.

#### 3.4. Co-expression of LLO elicits a Th1 bias

Groups of Balb/C mice were given three i.p. doses of  $1 \times 10^9$ spores of JH27 (LLO), JH338 (LLO<sub>L461T</sub>), DL346 (PA), JH287 (LLO PA) and JH342 (LLO<sub>L461T</sub> PA). Three control groups were included, one group receiving non-recombinant PY79 spores, a naïve group and a final group that received three i.p. doses of DL507 spores. DL507 spores express domains 1b-3 of PA fused to CotB on the spore surface as well as secreting full length PA from the germinated spore. Responses to DL507 have been shown to be protective in a challenge model [10]. Serum antibody responses were determined on days 14, 28 and 43. Fig. 5A shows the PA-specific IgG1, IgG2a and IgG2b subclasses on day 43. As expected JH27 and JH338 produced responses indistinguishable from the negative control groups. The four groups that received spores that secreted PA (DL346, DL507, [H287 and [H342] produced significant (P < 0.05) PA-specific titres. For DL346, IgG2b levels were higher than IgG2a. For JH287, JH342 and DL507 IgG2a levels were significantly (P < 0.05) higher than those observed in DL346 immunised mice. The ratios of IgG1:IgG2a over the course of the immunisation are shown in Fig. 5B. Mice immunised with DL346 spores showed a clear and incremental increase in the IgG1:IgG2a ratio indicating a marked Th2 bias. By contrast, mice receiving JH287 and JH342 spores showed clear differences. JH287 showed a marked drop in ratios after day 14 followed by a slight increase. For JH342 there was no significant increase in ratio at all. We interpret this as an evidence that coexpression of LLO or  $LLO_{L461T}$  together with vegetative expression of PA confers a Th1 bias.

We also measured the levels of neutralising IgG antibodies using the anthrax toxin neutralising assay (TNA). TNA levels (Fig. 5A) showed high levels in mice immunised with DL346, JH287 and JH342 spores. Only for JH342 and DL507, though, were these at levels significantly higher (P < 0.05) than those resulting from immunisation with DL346. For JH342 antibody titres were  $1.7 \times 10^3$ and twice those found in mice dosed with DL507. Neutralising antibody titres of  $1 \times 10^3$  have been shown to protect mice against 100 50% median lethal doses (MLD<sub>50</sub>) of *B. anthracis* STI spores administered by an intra-peritoneal route [10]. Therefore, we can predict that mice immunised with JH342 or DL507 spores will be protected to challenge with >100 MLD<sub>50</sub>s of *B. anthracis* STI spores.

#### 3.5. Cellular responses

Groups of mice were immunised by the intra-peritoneal route with  $1 \times 10^9$  spores of DL346 (PA), JH287 (LLO PA) and JH342 (LLO<sub>L461T</sub> PA) together with control groups. Splenocytes were extracted from sacrificed mice and re-stimulated with PA (10 µg/ml). After 2 days of stimulation culture supernatants were



**Fig. 6.** ELISPOT/ELISA. Groups of six Balb/C mice were immunised by the intraperitoneal route with  $1 \times 10^9$  spores of recombinant *B. subtilis* strains on days 1, 15. Splenocytes were extracted from sacrificed mice on day 22. Cells were stimulated at  $37 \degree C$  in a 5% CO<sub>2</sub> for 20 h with 10 µg/ml of PA antigen for IFN- $\gamma$  ELISPOT (Panel A) or 48 h for IFN- $\gamma$  ELISA (Panel B). Values represent the mean and standard deviation of duplicate of individual mouse. (\*) Significantly higher compared to naïve, PY79 and DL346 groups; (\*\*) significantly higher compared to naïve, PY79 and JL346 groups; (\*\*)

analysed for IFN- $\gamma$  levels by ELISA (Fig. 6A). DL346 immunised mice showed low levels of IFN- $\gamma$  but significantly (P<0.05) higher than the control groups (naïve and PY79 immunised mice). Splenic cells from JH287 and JH342 cells though, showed much higher levels of IFN- $\gamma$  with JH342 exhibiting significantly (P<0.05) higher levels than JH287. IFN- $\gamma$  levels were also evaluated using ELISPOT for splenic cells from naïve, DL346 and JH287 immunised mice. As shown in Fig. 6B both DL346 and JH287 showed production of IFN- $\gamma$  at significant levels with these being highest in JH287 splenic cells and at levels significantly (P<0.05) higher than DL346. Representative cytokine levels were also determined using Real Time PCR in PA stimulated splenic cells from mice immunised with DL346 and JH287 (Fig. 7). Most striking was the high levels of IL-12 observed in mice immunised with DL346



**Fig. 7.** PA-specific cytokines. RNA was extracted from  $1 \times 10^6$  spleen cells either stimulated with PA at  $10 \,\mu$ g/ml or unstimulated and cytokine expression levels determined from cDNA using real-time PCR. Results showed the fold increase over unstimulated cells.

and JH287. IFN- $\gamma$  and IL-17 were also induced but no induction of the pro-inflammatory cytokine, TNF- $\alpha$ . IL-4, a cytokine involved in stimulating antibody production was induced in DL346 cells but no expression was observed in JH287 cells. FACS analysis of spleen cells (Supplementary Figure) revealed significant increases in cell numbers of CD4 cells, B cells (CD19), dendritic cells (CD11c) and macrophages (F4/80) compared to un-immunised mice.

# 4. Discussion

Bacterial spores have been engineered as vaccines where the antigen is delivered on the spore coat and the vaccine administered orally or nasally [4,5]. In this work we have asked whether it is possible to engineer spores to deliver antigens to the host cell cytoplasm. This approach could be useful for a number of prophylactic and therapeutic purposes, for example, vaccines directed against intracellular pathogens, dendritic cell vaccination and for targeting of anti-cancer drugs. Our rationale was based on two sets of prior studies. First, following phagocytosis spores have been shown to be able to germinate within the phagosome or phagolysosome, although they appear unable to proliferate [15]. Second, live B. subtilis cells engineered to express LLO in the phagosome can invade the cytosol [16,17]. Accordingly, we have designed spores that following phagocytosis and germination within the phagosome are then able to secrete LLO and invade the cytosol. Since the phagosome is not highly acidic (pH of  $\sim$ 6.1), and LLO has an acidic pH optimum, we created a mutant form of LLO, LLO<sub>L461T</sub>, that has been shown to be more stable at neutral pH [24]. We reasoned this would enable efficient entry of live B. subtilis from the phagosome into the cytoplasm. This translocation should be markedly less than that from the phagolysosome where pH levels would fall to as low as 4.0.

Our results did show that  $LLO_{L461T}$  appeared more stable, and active, at neutral pH yet cells secreting this protein were not cytotoxic to mammalian cells. If either LLO or LLO<sub>L461T</sub> were cytotoxic then this would have precluded their future use of such a delivery system. A slight increase in invasion (compared to non-recombinant PY79 cells) was observed in cells expressing LLO/LLO<sub>L461T</sub> demonstrating little to no effect on the external cell membrane. Interestingly, considerably lower levels of invasion were observed with a mucus coated cell line and we assume that the live bacilli cannot penetrate this barrier. The non-recombinant strain, PY79, also invaded cells as has been observed previously [20,26], albeit at very low levels, and this may arise from secretion of surfactin, a lipopeptide that can permeabilise membranes [27]. In conclusion, our work shows that there is no obvious effect of LLO/LLO<sub>L461T</sub> secreted from live bacilli on the integrity of mammalian cells.

Following phagocytosis though, our assays demonstrate that cells secreting LLO/LLO<sub>L461T</sub> could grow and multiply. However, it is possible that the level of intracellular proliferation was actually higher than that observed by direct counting of CFU. As discussed elsewhere on the proliferation of *B. subtilis* within J774 cells [17] some gentamicin could enter the phagocytic cell via endocytosis reducing CFU somewhat, a possibility we cannot exclude. We also cannot exclude the possibility that LLO/LLO<sub>L461T</sub> secreted from the vegetative cell was adsorbed onto the spore surface and what, if any, this bound LLO/LLO<sub>L461T</sub> might make to the invasion and intracellular survival within phagocytes.

We could readily observe that, following phagocytosis, germinated spores expressing  $LLO_{L461T}$  were able to proliferate within the macrophage although cells were eventually killed. This is generally in agreement with other studies [16,17,28] but, as has been pointed out previously, the ability of *B. subtilis* to grow within mammalian cells is cell-specific [17] and probably also dependant on the stage in the cell cycle as well as other nutritional factors. Our data on the growth and proliferation of *B. subtilis* in RAW264.7 cells was not as prolific as that reported elsewhere using the J774 [16,17] or SV-BP-1 [28] cell lines, and which are thought to be the least bactericidal. Thus, the true outcome *in vivo*, where spores come into contact with primary macrophages, may be very different.

What induces the spore to germinate is unclear. The process of germination does not require de novo protein synthesis and is generally thought to occur following interaction of the spore with specific germinants such as L-alanine and L-asparagine [29]. It is entirely possible that these molecules might exist in the phagosome but acid and stress-induced germination has also been shown to occur with some species of Bacillus [30] and may be mimicked within the phagosome/phagolysosome. Electron microscopy revealed germination in the phagosome. Here, the acidity had not yet fallen to the levels that are found in the phagolysosome so we would argue that some other factor must be necessary to induce spore germination. B. anthracis, a known intracellular pathogen, germinates in the phagosome [31], and we might imagine this to be a more general mechanism for 'tricking' spores into germinating, thus, facilitating their eventual destruction. LLO and LLO<sub>L461T</sub> clearly enable entry of *B. subtilis* into the cytoplasm and this has been demonstrated convincingly using two imaging methods. Most interesting though, was electron microscopy, that showed striking images of the spore not only breaking open in the phagosome but in the process of replicating demonstrating permissive conditions for intracellular growth.

The aim of this work was not only to demonstrate that we could target B. subtilis to the cytoplasm, but also to show that we could co-express an antigen. Here, we chose the Protective Antigen (PA) of B. anthracis [32] that is part of the secreted anthrax toxin. By measuring anti-PA specific IgG it was clear that neutralising titres were greater when LLO<sub>I461T</sub> was co-expressed with PA, than when PA was secreted alone. Most importantly, expression of LLO or LLO<sub>1461T</sub> skewed the nature of the T helper immune response. Secretion of PA alone produced a Th2 bias while co-expression of LLO/LLO<sub>I461T</sub> introduced a Th1 bias, and thus evidence for a cellular immune response. A Th1 bias should only occur if the antigen or bacterium gains entry to the cytosol where class I MHC antigen presentation can occur. This was supported by the levels of IFN- $\gamma$  that showed clear evidence of a Th1 response (since Th1 cells, not Th2 cells, secrete IFN- $\gamma$ ). Further support was provided by the analysis of PAspecific cytokines. Here, IL-12 levels were higher in animals dosed with cells co-expressing LLO. IL-12 is a cytokine that promotes T cell and NK cells to secrete IFN- $\gamma$  and is uniquely stimulated by Gram-positive bacteria [33–35]. On the other hand, IL-4, a cytokine secreted by Th2 cells and required for the humoral response was markedly reduced in cells expressing LLO. A possible mechanism for the increased Th1 response associated with LLO expression could be increased survival of spores generating greater levels of available antigen together with cytoplasmic recognition of bacterial CpG motifs by the toll-like receptor, TLR9.

In conclusion then, we have shown that we can engineer spores of *B. subtilis* for transient survival within the mammalian cytoplasm and, when used as an antigen delivery system, was able to introduce a Th1 bias to responses against delivered immunogens.

#### Acknowledgements

This work was supported by EU grants NMP-2004-013523 and LSH-2005-036871 and the BBSRC (111/S19599) to SMC.

#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2008.09.024.

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