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Intracellular fate and immunogenicity of *B. subtilis* spores

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Abstract

To support our work on the development of bacterial spores as oral vaccines we examined the immunogenicity and intracellular fate of *Bacillus subtilis* endospores in a murine model. Mice dosed orally with spores developed systemic IgG and mucosal sIgA responses. Analysis of IgG subclasses revealed a predominance of the IgG2a subclass during the early stages of immunisation. Analysis of cytokine mRNA in GALT and lymphoid organs showed early induction of IFN-γ, a Th1 cytokine, as well as the pro-inflammatory cytokine TNF-α. Significant levels of IgG antibodies were produced against vegetative bacilli following dosing with spores. This showed that spores could germinate in the GI tract. In vitro studies detailing the intracellular fate and persistence of spores in a macrophage-like cell line (RAW264.7) demonstrated that spores could germinate efficiently in macrophages, initiate gene expression as well as inducing pro-inflammatory cytokines.
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Keywords: *Bacillus subtilis*; Spores; Macrophages; Persistence

1. Introduction

The Gram positive soil bacterium *Bacillus subtilis* has been extensively studied as a model prokaryotic system with which to understand gene regulation and the transcriptional control of unicellular differentiation [1,2]. This organism is regarded as a non-pathogen and the spore form is currently being used as a probiotic for both humans and animals [3]. In recent work the *B. subtilis* spore has been exploited for delivery of heterologous antigens using tetanus as a model [4,5]. Fusion of tetanus toxin fragment C (TTFC) to the spore outer coat protein CotB followed by oral immunisation of mice with these spores resulted in protection against challenge with tetanus toxin. The use of spores as vaccines is persuasive providing a robust and heat-stable bio-particle that can be engineered to display more than one protein antigen on the spore coat. One potential drawback of using the spore coat for antigen presentation is whether a chimeric protein is stable, can fold properly and is subject to inadvertent proteolysis in the stomach. Addressing this, another approach has recently been identified [6] and is based upon the observation that *B. subtilis* spores germinate to some degree in the small intestine [7,8]. Here, the foreign antigen is expressed in the vegetative cell by use of a strong *B. subtilis* promoter that is expressed only during vegetative cell growth. Delivery of spores enables transit of the entire inoculum across the stomach followed by germination of a subpopulation (<1%) of spores in the small intestine. Germination could occur either in the lumen of the gastro-intestinal tract (GIT) or in the gut associated lymphoid tissue (GALT) and, supporting the latter, significant numbers of spores as well as germinated spores have been recovered in the Peyer's patches (PPs) and mesenteric lymph nodes (MLN) following oral dosing of mice [5]. Oral dosing of mice with spores carrying an antigen (*Escherichia coli* β-galactosidase) expressed only in the germinating spore was found to generate significant β-galactosidase-specific systemic IgG responses demonstrating that the germinating spore provides an additional, and attractive, route for oral immunisation [6].

The interaction of spores and germinating spores with the GALT is intriguing and shows that this soil organism is not simply a transient passenger of the gut. In this work we have examined the potential nature of this interaction with the GALT by examining the spectrum of immune responses raised against orally immunised spores as well as the fate of spores within antigen presenting cells.

2. Materials and methods

2.1. Strains

SC2362 has been described elsewhere [8] and carries the *rrnO-lacZ* gene as well as the *cat* gene encoding resistance to

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chloramphenicol (5 μg/ml). *rrnO* is a vegetatively expressed gene encoding a rRNA. In this strain the 5'-region of *rrnO* carrying the promoter had been fused to the *E. coli lacZ* gene. PY79 is the prototrophic and isogenic ancestor of SC2362 and is Spo^+ [9]. DL169 (*rrnO-lacZ gerD-cwlBΔ::neo*) carries the *rrnO-lacZ* reporter gene together with the *gerD-cwlB* mutation [6]. Deletion of this region of the chromosome results in a severe germination defect (reduced to 0.0015% when compared to that of an isogenic wild type strain PY79; E. Ricca; personal comm.).

2.2. Preparation of spores

Sporulation was induced in DSM (Difco-sporulation media) using the exhaustion method as described elsewhere [10]. Cultures were harvested 22 h after the initiation of sporulation. Purified suspensions of spores were prepared as described by Nicholson and Setlow [10] using lysozyme treatment to break any residual sporangial cells followed by successive washes in 1 M NaCl, 1 M KCl and water (two times). PMSF was included to inhibit proteolysis. After the final suspension in water spores were treated at 68 °C for 1 h to kill any residual cells, then titrated immediately for colony forming units (CFU)/ml before freezing aliquots at −20 °C.

2.3. Extraction of spore coat proteins and vegetative cell lysates for ELISA

Spore coat proteins were extracted from suspensions of spores of strain PY79 at high density (1×10^{10} spores/ml) using an SDS–DTT extraction buffer as described in detail elsewhere [10]. For vegetative cell lysates, strain PY79 was grown to an OD_{600} of 1.5 in LB medium and the cell suspension washed and then lysed by sonication followed by high-speed centrifugation. Extracted proteins were assessed for integrity by SDS-PAGE and for concentration using the Bio-Rad DC Protein Assay kit.

2.4. Immunisations

Groups of eight mice (female, C57 BL/6, 8 weeks) were lightly anaesthetised with halothane and then inoculated orally by intra-gastric gavage with a suspension of 1.5×10^{10} spores or 3×10^{10} vegetative cells of strain PY79 (in a volume of 0.2 ml) on days 0, 1, 2, 20, 21, 22, 36, 37 and 38. Serum and faecal samples were collected on days −1, 19, 35 and 62. A naïve, non-immunised control group of six animals was also included.

2.5. Detection of rrnO-lacZ expression

Cells carrying *rrnO-lacZ* (SC2362) were induced to sporulate in Difco-sporulation medium (DSM) using the exhaustion method [10]. Samples were removed at time points following the initiation of sporulation (hour 0) and cells pelleted, resuspended in 1 ml of Z buffer containing lysozyme (20 μg/ml) and β-galactosidase activity determined using the substrate ○-nitrophenyl β-D-galactopyranoside (ONPG) as described [10]. For Western blotting, total cell extracts were fractionated on 12% SDS-PAGE gels, transferred to nitrocellulose membranes and probed with a polyclonal antiserum to β-galactosidase protein (Sigma) at a dilution of 1:2000.

2.6. Indirect ELISA for detection of antigen-specific serum and mucosal antibodies

Plates (Nunc) were coated with 0.1 μg/well of either extracted spore coat protein or vegetative cell lysate in carbonate/bicarbonate buffer and left at room temperature overnight. After blocking with 0.5% BSA in PBS for 1 h at 37 °C serum samples were applied using a two-fold dilution series starting with a 1/40 dilution in ELISA diluent buffer (0.1 M Tris–HCl, pH 7.4; 3% (w/v) NaCl; 0.5% (w/v) BSA; 10% (v/v); sheep serum (Sigma); 0.1% (v/v) Triton X-100; 0.05% (v/v) Tween-20). Plates were incubated for 2 h at 37 °C before addition of anti-mouse HRP conjugates (Sigma). After a further incubation of 1 h at 37 °C enzymatic reactions were started using the substrate TMB (3,3′,5,5′-tetramethyl-benzidine; Sigma), and then stopped with 2 M H_2S0_4. Dilution curves were drawn for each sample and endpoint titres calculated as the dilution producing the same optical density as the 1/40 dilution of a pooled pre-immune serum. Statistical comparisons between groups were made by the Mann–Whitney *U*-test. A *P*-value of >0.05 was considered non-significant. For ELISA analysis of faecal IgA, we followed the procedure of Robinson et al [11] using approximately 0.1 g faecal pellets suspended in PBS with BSA (1%) and PMSF (1 mM) and incubated at 4 °C overnight prior to ELISA. For each sample the endpoint titre was calculated as the dilution producing the same optical density as the undiluted pre-immune faecal extract.

2.7. Preparation of antiserum against spores

New Zealand white rabbits were injected subcutaneously with 1×10^9 inactivated *B. subtilis* spores (incubated in 4% (v/v) formaldehyde overnight at 37 °C) of strain PY79 in Freund's incomplete adjuvant, followed by two boosters at 3-week intervals. Blood was removed from an ear vein. Whole serum was applied to a HiTrap Protein A HP column (Amersham Bioscience), and the IgG fraction was eluted as instructed by the supplier. Fractions were checked for purity by SDS-PAGE, then pooled and dialysed against PBS and stored frozen at −20 °C.

2.8. Macrophage cell culture and spore infection

The murine macrophage-like cell line RAW264.7 (obtained from the European Collection of Animal Cell Cultures (ECACC)) was cultured as monolayers in RPMI-1640 medium (Invitrogen) supplemented with 10% (v/v) fecal

bovine serum, 50 μg ml^{-1} penicillin and 50 μg ml^{-1} streptomycin (complete medium), in an atmosphere of 90% humidity containing 5% CO_2 at 37 °C. Two days before use, the cells were detached by gentle scraping and seeded into 24-multiwell disposable plates containing sterile 13-mm diameter cover slips in the same medium at a density of approximately 5×10^4 cells per well. The macrophage monolayers were then infected with *B. subtilis* spores (strain PY79, SC2362 or DL169) at a macrophage:spore ratio of 1:10 (approx. 5×10^5 spores per well) or when using vegetative cells a ratio of 1:60 (approximately 3×10^6 vegetative cells per well) in RPMI-1640 medium without antibiotics. Phagocytosis was allowed to proceed at 37 °C in 5% CO_2, for 2 h and halted by replacing the medium with RPMI-1640 containing 2.5 μg ml^{-1} gentamycin to kill any extracellular germinated spores or vegetative cells and 1×10^{-6} M cytochalasin which prevented phagocytosis. Background levels of spores physically bound to macrophages was determined by spore infection in the medium containing 1×10^{-6} M cytochalasin and 2.5 μg ml^{-1} gentamycin. At each time point, the cover slips with monolayers were removed and washed 5 times in sterile 0.03 M PBS (pH 7.4). Monolayers were evaluated at this point for counts or for immunofluorescence (see below). To quantify the total number of intracellular *B. subtilis*, monolayers were lysed by resuspension in sterile distilled water 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.

2.9. Immunofluorescence and confocal microscopy

Macrophage monolayers were infected with spores of strain SC2362 or DL169 as described above (Section 2.7). Labelling steps were performed at RT. Monolayers on cover slips were fixed by incubation in 3.7% (w/v) paraformaldehyde and 30 mM sucrose in PBS for 20 min. Free aldehyde groups were quenched by incubation for 10 min. with 50 mM NH_4Cl in PBS. The cells were washed once in PBS with 0.1% BSA and permeabilised by incubation for 5 min. in 0.2% Triton X-100 and 1% BSA in PBS. Immunofluorescence labelling was performed using 45 min. incubation with primary antibodies, four washes with PBS, and a further 45 min. incubation with fluocescein-labelled secondary antibodies. Specifically, dormant and germinated spores were detected by double indirect immunofluorescence staining using a rabbit polyclonal antibody directed against spores (Section 2.7, used at 1:400) and a mouse polyclonal antibody directed against β-galactosidase (Sigma, used at 1:200). A mixture of secondary antibodies, FITC-conjugated anti-rabbit IgG and TRITC-conjugated anti-mouse IgG (Sigma, used at 1:200) was subsequently added. The cells were washed again then mounted onto a microscope slide in SlowFade component A (Moleculer Probes). Samples were examined in a Nikon Eclipse fluorescence microscope equipped with a Bio-Rad Radiance 2100 laser scanning system. Images were taken using LaserSharp software and processed with the Confocal Assistant programme. Sections were 0.3 μm. Laser powers were 10% for Ar 488 nm and 40% for Green HeNe, scanning speed was 50 lps. Image size was 33 μm × 33 μm.

2.10. Electron microscopy

Macrophage monolayers in 24-multiwell disposable trays were infected with *B. subtilis* spores (PY79) as described above (Section 2.8). After 2 h, phagocytosis was stopped and monolayers were fixed for 1 h in 3% glutaraldehhyde in 0.1 M sodium cacodylate buffer (pH 7.4). The cells were washed in the same buffer, then post-fixed in 2% osmium tetroxide plus 1% potassium ferricyanide, dehydrated through a graded alcohol series, embedded in Epon, thin sectioned and finally stained with uranyl acetate and lead citrate before observation with a transmission electron microscope.

2.11. In vitro cytokine analysis

Macrophages (RAW264.7) were grown on 6-well cell culture plates in RPMI-1640 complete medium. Two-day-old macrophages were infected with *B. subtilis* spores strain PY79, DL169, or autoclaved PY79 spores, at a ratio of 10 spores per macrophage in complete medium, or with vegetative cells strain PY79 at the same ratio but in RPMI-1640 medium without antibiotics. Spore coats or cell walls extracted from spores or vegetative cells were also used to infect macrophages and the amount of extract used was adjusted to correspond to the equivalent number of spores or vegetative cells used in the parallel infection experiments. Cell walls were prepared as described previously [12]. At indicated time points, culture medium was removed, macrophages were washed and lysed in situ, homogenised by passing the cell extract five times through a 20-gauge needle, and total RNAs extracted and purified using an RNeasy mini kit as described by the manufacturer (Qiagen).

2.12. In vivo cytokine analysis

Specific pathogen free Balb/C mice (female, 8 weeks old) were inoculated with 1×10^{10} spores of *B. subtilis* strain PY79. A naïve, non-immunised group of mice was also included. Spleen, liver, mesenteric lymph nodes (MLN) and submandibular glands (SMG) were removed from sacrificed mice at indicated time points and frozen immediately at −80 °C until needed. To extract total RNAs, organs and tissues were thawed and disrupted by pressing between two glass slides, lysed in RLT buffer (Qiagen) containing 1% β-mercaptoethanol, homogenised by passing two times through a QIAshredder column (Qiagen). Total RNAs from lysates were extracted and purified as described by the manufacturer (Qiagen RNeasy mini kit).

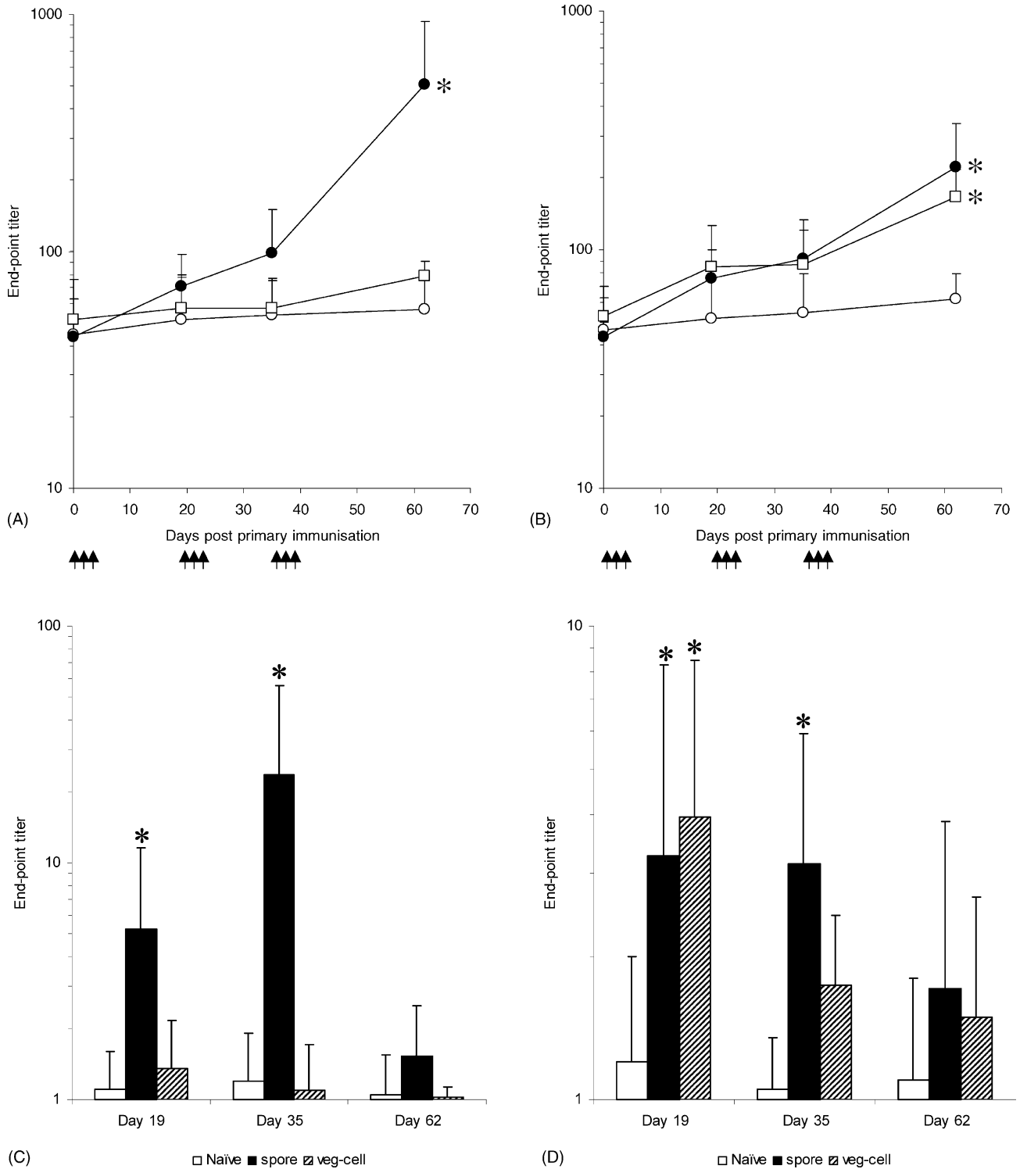

Fig. 1. Immune response to *B. subtilis* following oral administration of spores. Groups of eight C57 BL/6 mice were dosed (↑) orally with 1.5×10^{10} (●) spores or 3×10^{10} vegetative cells (□) of *B. subtilis*. Serum spore-specific IgG (A), vegetative cell-specific IgG (B), faecal spore-specific sIgA (C) and vegetative cell-specific sIgA (D) from naïve (○) and immunised groups was detected by ELISA. The end-point titer was calculated as the dilution of serum/faecal extract producing the same optical density as a 1/40 dilution of a pooled pre-immune serum/fecal extract. Asterisks show statistically significant differences compared to the naïve group at $P < 0.05$ using the Mann–Whitney *U*-test. Data are presented as the means ± standard deviations.

2.13. RT-PCR

Total RNAs were quantified by a GeneQuant spectrophotometer (Amersham Biosciences). RT-PCR was carried out using 1 μg of total RNA per reaction as described by the manufacturer (Amersham Biosciences ready-to-go RT-PCR beads). Primers specific for β-actin and various cytokines were detailed elsewhere [13]. Reaction conditions were first-strand cDNA synthesis at 42 °C for 15 min, reverse-transcriptase inactivation at 95 °C for 5 min, and PCR at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min. RT-PCR products were run on a 2% agarose gel, and subjected to UV visualisation and densitometric analysis with a Bio-Rad Gel Doc system.

3. Results

3.1. Immunogenicity of spores following oral administration in mice

To test for induction of local and systemic immunity, groups of eight inbred mice were immunised orally with either a suspension of wild type *B. subtilis* PY79 spores (1.5×10^{10} spores per dose) or 3×10^{10} vegetative cells of strain PY79. Analysis of anti-spore specific IgG titres following administration with spores (Fig. 1A) showed a clear systemic immune response together with seroconversion but not following dosing with vegetative cells. The response at day 62 was significantly ($P < 0.05$) above that of the naïve group and mice dosed with vegetative cells. Analysis of anti-vegetative cell-specific IgG titres (Fig. 1B) though, revealed low but significant immune responses above those of the naïve group ($P < 0.05$) when either spores or vegetative cells were used for dosing. Similarly, analysis of faecal IgA revealed clear anti-spore sIgA responses when spores were used for dosing but no significant ($P > 0.05$) levels of anti-spore specific sIgA responses when vegetative cells were used for dosing or in the naïve group (Fig. 1C). Finally, measurement of anti-vegetative cell-specific sIgA responses (Fig. 1D) showed seroconversion when either spores or vegetative cells were used for dosing and these levels were significantly above those in the naïve group ($P < 0.05$).

We also analysed the anti-spore specific IgG subclasses, IgG1, IgG2a and IgG2b, present in the serum. As shown in Fig. 2, we observed an immediate and rapid increase in the levels of the IgG2a subclass that, by day 20, had peaked and then were maintained at a steady level. In contrast, IgG1 titres increased steadily to their maximum by day 62. IgG2b levels also rose to significant levels but this increase followed that of IgG1.

3.2. Analysis of cytokine-specific mRNA in vivo

The predominance of the IgG2a subclass could indicate an early (Th1) T-cell response and the involvement of cellular

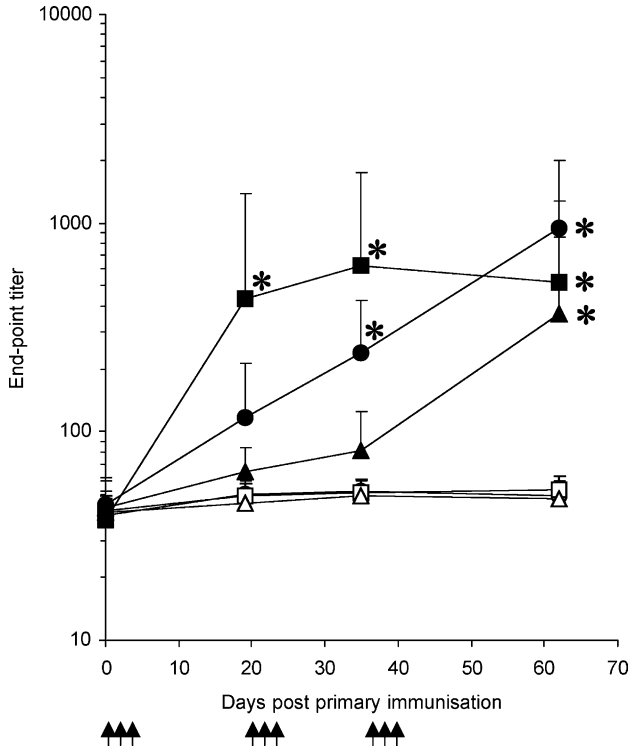

Fig. 2. Serum anti-spore IgG isotypes. Sera from naïve and immunised groups were taken at different days post-immunisation (↑) and analysed for IgG1, IgG2a and IgG2b isotypes. IgG subclasses from mice immunised with spores, IgG1 (●), IgG2a (■) and IgG2b (▲). Naïve groups, IgG1 (○), IgG2a (□) and IgG2b (△). Asterisks show statistically significant differences compared to the naïve group at $P < 0.05$ using the Mann–Whitney *U*-test. Data are presented as the means ± standard deviations.

immunity including CTL responses [14–17]. We analysed the profiles of seven cytokines, IL-1α, IL-2, IL-4, IL-5, IL-6, TNF-α, and IFN-γ, in the spleen, liver, MLN, submandibular glands (SMG) from mice given one oral dose of PY79 spores (Fig. 3). Induction of only two cytokines was apparent in the time course we followed, the pro-inflammatory cytokine TNF-α and the Th1 cytokine IFN-γ. Both cytokine mRNAs were induced early in the liver, SMG and MLNs with slightly higher levels of IFN-γ. Expression of both cytokines was highest in the MLNs and here IFN-γ expression was maintained at a steady level. Low but detectable levels of expression were found in the spleen. In control experiments using naïve mice no cytokine mRNA was detectable (not shown).

3.3. Intracellular survival of B. subtilis in macrophages

To analyse the intracellular survival of *B. subtilis* spores in macrophages we assessed the persistence of spores of strain PY79 within cultured macrophages. We infected the murine macrophage-like cell line RAW264.7 with spores at a macrophage/spore ratio of 1:10. Phagocytosis was allowed to continue for 2 h and then inhibited by addition

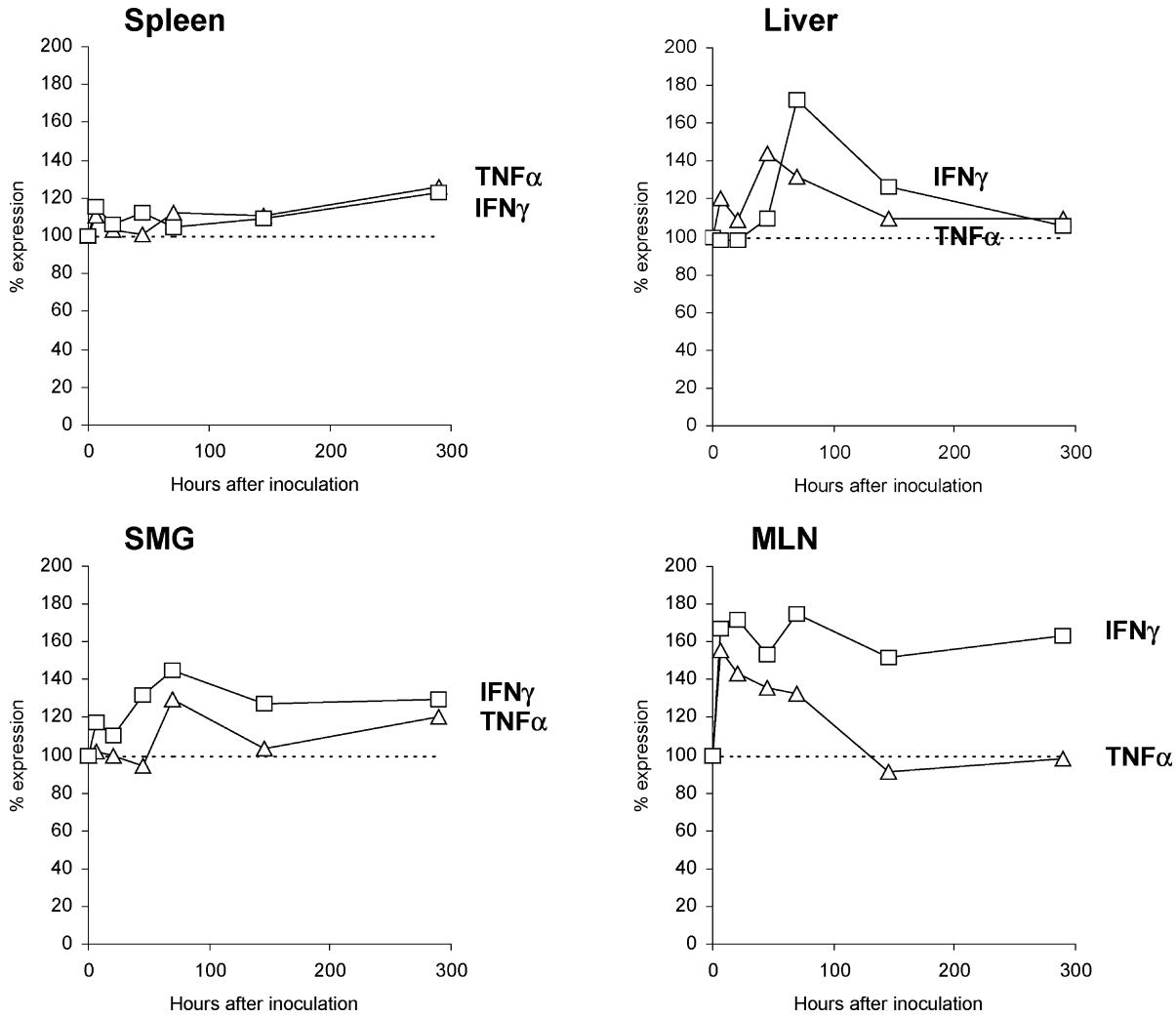

Fig. 3. Cytokine responses in vivo. Inbred Balb/C mice were inoculated with 1×10^{10} spores of *B. subtilis* strain PY79. At designated time points, cytokine expression in various organs and tissues of the animals was detected on total RNAs using RT-PCR. In each case two mice were examined and similar results obtained of which one is shown. Graphs show densitometric analyses of corresponding gel photographs, where % expression represents the relative abundance of each cytokine at each time point compared to that of β-actin. Dotted line represent relative abundance of each cytokine at hour 0 (100%). No expression was detected in naïve, non-immunised mice at any time point (not shown).

of cytochalasin (Fig. 4). Analysis of total and spore counts showed that spores were present in measurable numbers above background for 25 h following the arrest of phagocytosis. The lifespan of in vitro cultured macrophages in our experiment was about 24–30 h so spores appear to be present and viable for the same period. Interestingly, total counts were higher than the heat-treated, spore counts, for the first 8 h following inhibition of phagocytosis. In our assay phagocytosis was inhibited by replacement of the culture medium with fresh medium containing cytochalasin and gentamycin (2.5 μg/ml). Gentamycin at this concentration will kill any extracellular vegetative cells that might have arisen by spore germination yet is at a concentration that has been shown to have no effect on the survival of bacteria inside macrophages [18,19]. Infecting macrophages with vegetative *B. subtilis* cells (at a higher macrophage: cell ratio of 1:60) showed that vegetative bacteria were inactivated at a greater rate and no measurable numbers of bacteria could be detected 12 h after the inhibition of phagocytosis. Moreover, no increase in viable units was found showing that vegetative *B. subtilis* cannot replicate within a phagocytic cell.

3.4. Germination of spores in macrophages

Our in vitro analysis of viable counts suggested that spores might be capable of germinating within a macrophage since the total counts were higher than the spore counts. To test this directly we examined the fate and persistence of spores within cultured macrophages. In this experiment macrophages were infected with spores of strain SC2362 that carried the *rrnO-lacZ* gene. First, we examined expression of *rrnO-lacZ* and the presence of β-galactosidase in sporulating cells of SC2362. As shown in Fig. 5, initially, during the first 2 h of sporulation substantial levels

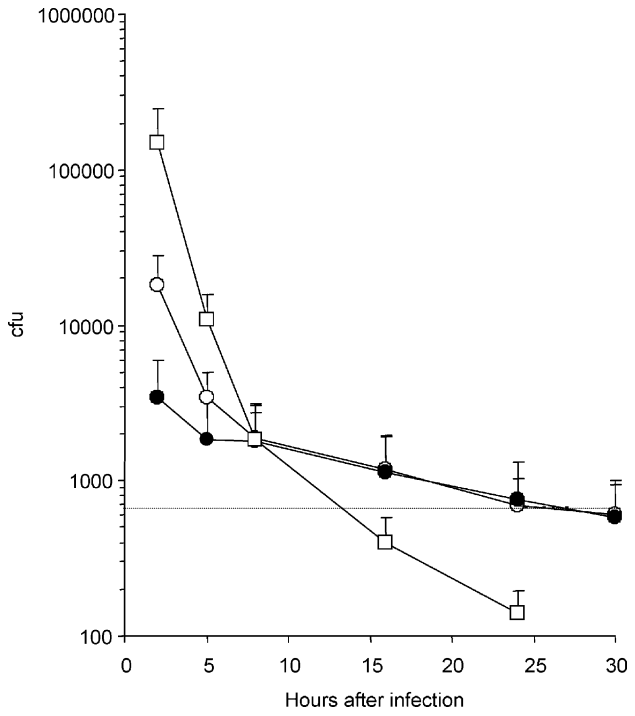

Fig. 4. Phagocytosis of *B. subtilis* spores. Murine RAW264.7 macrophages were infected in vitro at an infection ratio of 10 spores or 60 vegetative cells per macrophage. Phagocytosis was allowed to proceed for 2 h then arrested by addition of cytochalasin. Survival of *B.subtilis* spores (total (○), heat-resistant (●)) or vegetative cells (□) inside macrophages was determined. The dotted line is the background level of spores physically adhered to cytochalasin-treated macrophages. Data are presented as the mean ± standard deviation of eight independent experiments.

of active β-galactosidase were present in the developing cell but these levels rapidly declined as the cell became irreversibly committed to spore formation and vegetative expression was switched off. Importantly, by hour 6 when the immature spore is formed within the developing cell no β-galactosidase protein could be detected by Western blotting using an anti-β-galactosidase antibody. Since it was formally possible that a vegetatively expressed protein might be sequested into the spore if sufficiently high levels of the protein were made during vegetative cell growth this experiment demonstrated that SC2362 spores carried no detectable levels of β-galactosidase. Using an anti-spore coat polyclonal serum and a polyclonal anti-β-galactosidase serum we could specifically detect either spores or vegetative cells with no cross-reaction (results not shown) and using these reagents we could then examine the fate of phagocytosed spores within macrophages. Using double indirect immunofluorescence we examined in vitro the fate and persistence of spores within infected RAW264.7 macrophages in which phagocytosis was arrested after 2 h of infection (Fig. 6). Intact spores were readily detected inside the macrophage and the intensity of detection fell rapidly after 45 min. β-Galactosidase was also readily detectable within the macrophage after 45 min. To prove that the *rrnO-lacZ* expression was a result of spore germination we infected macrophages in parallel with spores of strain DL169 (Fig. 6) that carries *rrnO-lacZ* strain but is also germination defective. In this experiment no expression of *rrnO-lacZ* was detectable, moreover, spores were clearly seen inside the macrophage 5 h after infection in marked contrast to infection with SC2362 spores. Labelling of β-galactosidase could only occur if the *rrnO-lacZ* gene had been expressed and therefore proves that, in an in vitro cell line, *B. subtilis* spores can germinate and initiate outgrowth. Cells expressing *rrn0-lacZ* retained the ellipsoidal shape of spores and did not resemble the elongated rod-like shape of typical vegetative cells. This suggests that although spores had germinated they were unable to emerge from their spore coat and must therefore be blocked in outgrowth.

Analysis of electron micrographs of infected macrophages (Fig. 7) supported our confocal analysis. Spores were seen making contact with macrophages (Fig. 7A) which showed extending pseudopods [20] prior to engulfment of the intact spore within a phagosome (Fig. 7B). Two stages of spore germination could be detected. Cracking of the spore coats (Fig. 7C) in which the electron-dense outer layer of the spore coat was displaced from the rehydrating spore core, and germinated spores (Fig. 7D) which had lost their coats. We were unable to detect any rod-shaped bacilli though. Outgrowth is the first step before cell growth and replication and we interpret our direct counting, confocal and EM analysis as evidence that the spore can germinate, initiate protein synthesis but is unable to grow and replicate within the macrophage.

3.5. Analysis of cytokine-specific mRNA in vitro

RT-PCR was used to examine the expression of the pro-inflammatory cytokines, TNF-α, IL-6 and IL-1α in RAW264.7 macrophages infected with spores of the wild type strain PY79 (Fig. 8A). The most significant induction response was that of IL-6 which reached maximum levels 5–10 h after infection of macrophages after which the level of IL-6 began to decline. IL-1α and TNF-α responses were early and minimal. Since the spore can germinate within the macrophage we performed two additional controls in parallel. First, we infected macrophages with autoclaved spores (Fig. 8B) and second, infection with a germination defective strain, DL169 (Fig. 8C). With both controls the IL-6 responses were somewhat lower and did not decline but appeared stable after maximum levels had been reached. A further difference, although minor, was that IL-1α and TNF-α responses were more pronounced than with infection by wild type spores. To further dissect this profile of cytokine expression we performed infection experiments using purified spore coats (Fig. 8D), vegetative cells (Fig. 8E) and cell walls from vegetative cells (Fig. 8F). In each case IL-6 was the predominant cytokine expressed although less so using vegetative cells. IL-1α was expressed most significantly when macrophages were infected with vegetative cells and with cell lysates.

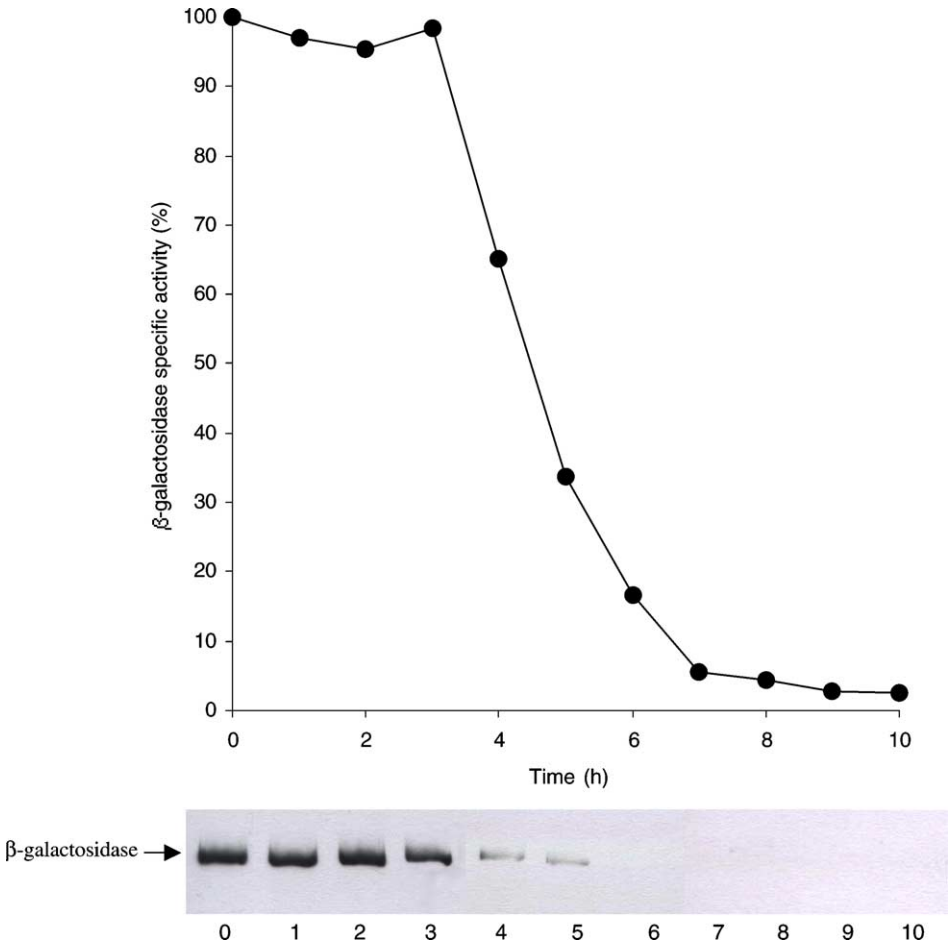

Fig. 5. Expression of *rrn0-lacZ*. Strain SC2362 (*rrn0-lacZ*) was induced to sporulate in Difco-sporulation medium (DSM) to induce synchronous sporulation. At hourly time points following the induction of sporulation (T_0) samples were taken for (1) analysis of β-galactosidase-specific activity and (2) detection of the 117 kDa β-galactosidase protein by Western blotting of SDS-PAGE fractionated whole cell extracts. β-Galactosidase activity is expressed as percentage of maximum activity. Numbers below the Western blot indicate the time after the initiation of sporulation. The forespore compartment first appears at hours 3–4 and the mature spore is released by lysis of the mother cell between hours 8 and 10. This experiment has been repeated a total of four times with the same result and the figure shows a representative profile of *rrnO-lacZ* expression.

4. Discussion

We have shown that *B. subtilis* spores are immunogenic when delivered orally to mice. This shows then, that although a resident soil organism, spores cannot be considered simply as a food, rather, they generate specific local and systemic immune responses. Our analysis of mucosal immune responses raises two important findings. Firstly, that spores generate not only humoral immunity but may also produce cellular immune responses. Secondly, that spores can germinate in the GALT. Evidence for cellular immunity comes from the predominance of the IgG2a subclass over IgG1 during the early stages of immunisation. Compelling evidence shows that a predominance of this subclass is indicative of a type 1 (Th1) T-cell response leading to CTL recruitment as well as IgG synthesis [11,14–17] The increase in IgG2b during the later stages of immunisation indicates a type 2 (Th2) T-cell response and would account for the sIgA/IgG1 response. Support for a Th1 response comes from our in vivo analysis of cytokine mRNA which showed early induction of a major effector of cellular immunity, IFN-γ, in the MLN, SMG and liver. These early responses suggest an innate immune response and secretion of IFN-γ by peripheral blood mononuclear cells. It is too early to speculate as to which cell type is responsible for IFN-γ synthesis but CD4+ natural killer (NK) T cells have been shown to produce early IFN-γ induction following infection with *Mycobacterium bovis BCG* [21] and *Listeria monocytogenes* [22]. Both NK cells and peritoneal macropohages have been shown to produce IFN-γ in experimentally induced bacterial peritonitis in mice [23]. In other work we have shown that spores can be found in the PPs, MLNs and SMGs following oral dosing [5]. Spores are approximately 1.2 μm in length so are of sufficient size to be taken up by M cells and then transported into the PPs where they could interact with macrophages, dendritic cells or B cells before being transported to the efferent lymph nodes. Our analysis of cytokine responses also showed early

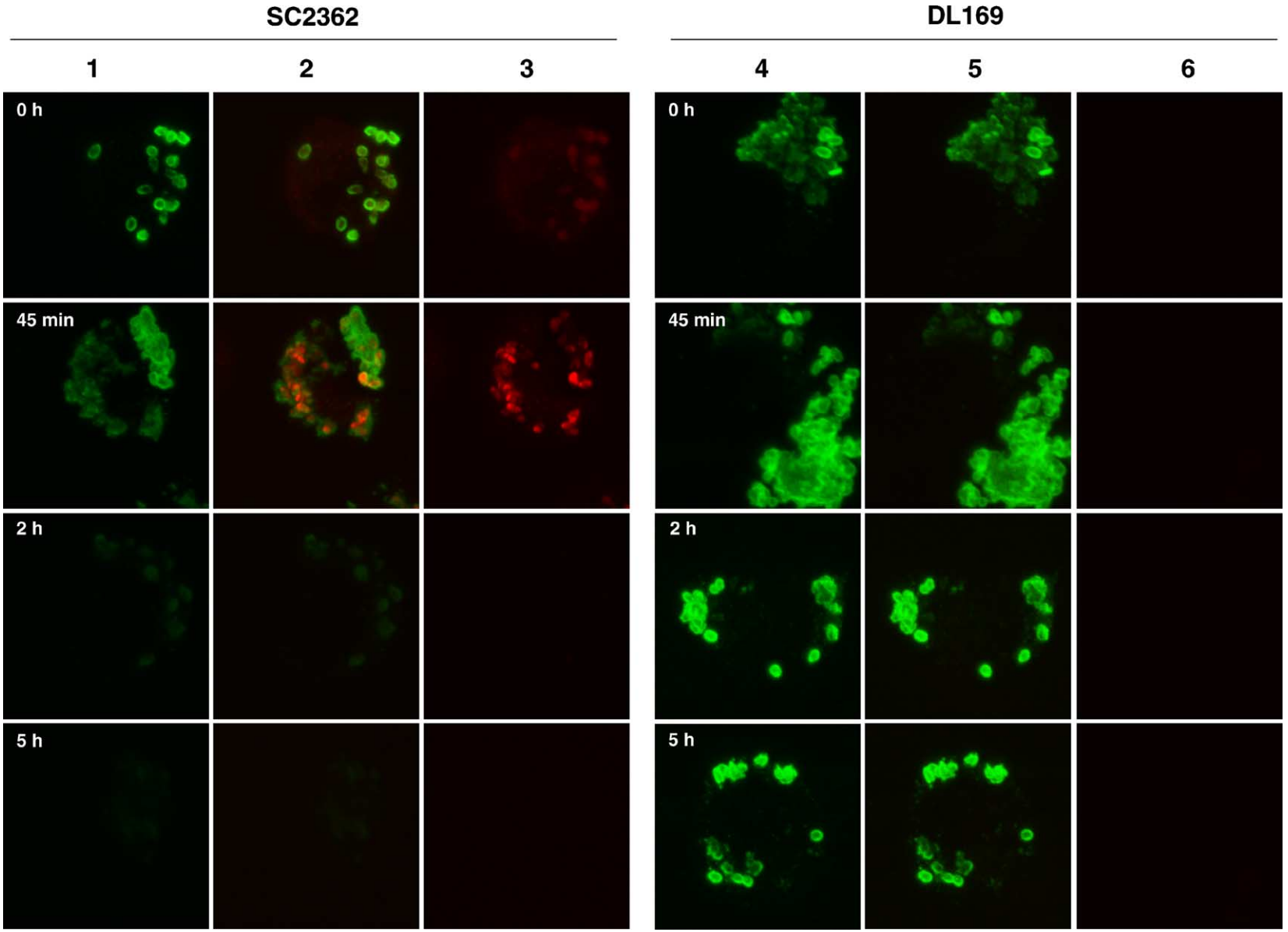

Fig. 6. Laser scanning confocal micrographs showing phagocytosis of *B. subtilis* in murine RAW264.7 macrophages. Micrographs show a representative time course experiment using double immunofluorescence staining. Macrophages were infected with spores of SC2362 (*rrnO-lacZ*) or DL169 (*rrnO-lacZ gerD-cwlBΔ*: :*neo*) and phagocytosis terminated as described in Section 2. At time points thereafter, dormant spores were stained with an anti-spore serum and an FITC-conjugated secondary antibody (green, lanes 1 and 4). Germinated spores were stained with an anti-β-galactosidase serum and a TRITC-conjugated secondary antibody (red, lanes 3 and 6). Lanes 2 and 5 are double overlays. This figure shows a representative infected macrophage of approximately 50 macrophages examined in total. This infection experiments has been repeated over five times under varying parameters.

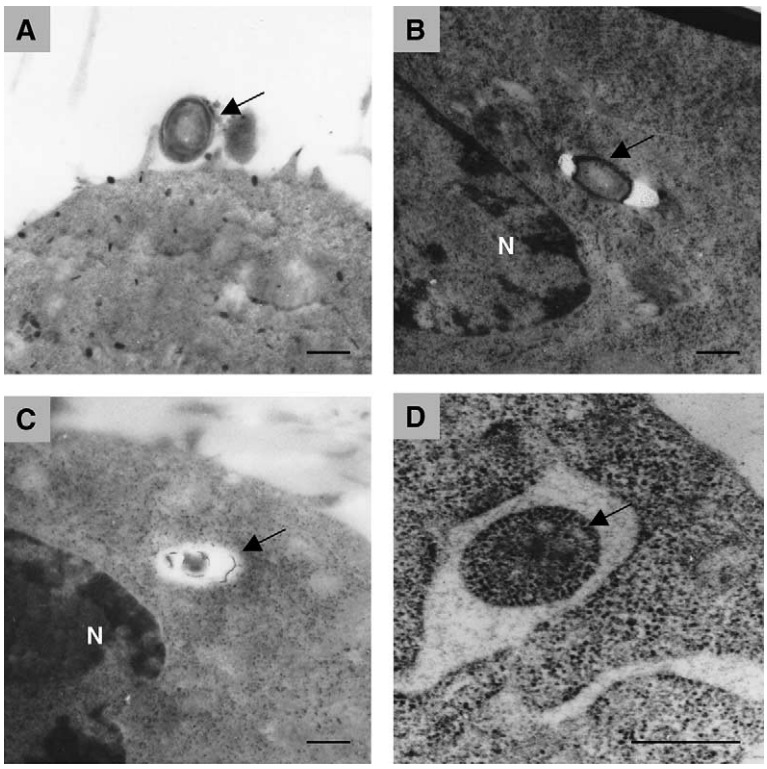

Fig. 7. Transmission electron microscopy of spore-infected RAW 264.7 macrophages. Macrophages were infected with PY79 spores (10 spores/macrophage) and incubated at 37 °C in RPMI-1640 containing 2.5 μg ml^{-1} gentamycin. Cells were harvested and prepared for examination using transmission electron microscopy. N, macrophage nucleus. (A) The arrow points to a spore being captured by a macrophage in which pseudopods are visible. (B) A spore captured inside a phagosome. (C) A spore within a phagosome showing breakage of the electron-dense outer coat. (D) A germinated spore inside a phagosome. Bar is 1 μm. Approximately 20 infected macrophages were examined in detail and shown to interact with spores in each of the stages outlined here.

induction of TNF-α which is a pro-inflammatory cytokine whose production by macrophages has been linked with chronic infections [24,25].

Our evidence for spore germination comes from anti-vegetative cell-specific IgG and sIgA responses when mice were dosed with a pure suspension of spores. To account for these responses when spores were used for dosing we propose that spores germinate in the GI tract since our inoculating dose was both lysozyme and heat-treated to remove any residual vegetative cells. While we cannot exclude the possibility of an extremely low level of contamination it would be surprising since dosing mice with a large dose (3×10^{10}) of vegetative cells gave similar responses to dosing with spores alone. Interestingly, when vegetative cells were used for dosing, anti-vegetative cell-specific responses were higher but in the case of sIgA declined more rapidly in comparison to spore dosing. This may indicate evidence of tolerance to vegetative cells since our immunisation regime involved multiple doses. Another possibility we cannot exclude is that spores and vegetative cells share some antigens. Dosing mice with vegetative cells generated no anti-spore responses (Fig. 1A) but dosing with spores did generate anti-vegetative cell responses (Fig. 1B). Therefore, we cannot exclude the possibility that breakage of the spore yields cross-reactive antigens sufficient to generate the anti-vegetative cell responses observed. In previous work we have used a molecular (RT-PCR) analysis to examine vegetative gene expression in the GI tract following oral dosing of spores [8]. In that study we detected germination in the jejunum and ileum and this supports our analysis here. The presence of spores as well as vegetative cells in the GALT would be explained if spores enter the PPs and germinate. Interestingly, we have also shown in a previous study that, following oral dosing, more spores were excreted in the faeces than were administered [7]. This somewhat surprising result implies that, irrespective of the fate of spores in the GALT, spores must be able to germinate in the GI tract lumen and undergo limited rounds of growth and replication. Since *B. subtilis* has been shown to be able to grow anaerobically [26] so this is a plausible explanation.

The immune responses to vegetative cells when mice were dosed only with spores and the presence of significant numbers of spores and vegetative cells in the GALT suggest that spores germinate in this important region of mucosal immunity. Previous work detailing the fate of *Bacillus anthracis* has shown that *B. anthracis* spores can germinate in alveolar macrophages [27]. *B. anthracis* is, of course, a potent pathogen and germination in macrophages is a necessary step for pathogenesis with the germinating spore ultimately gaining entry to the macrophage cytoplasm [28,29].

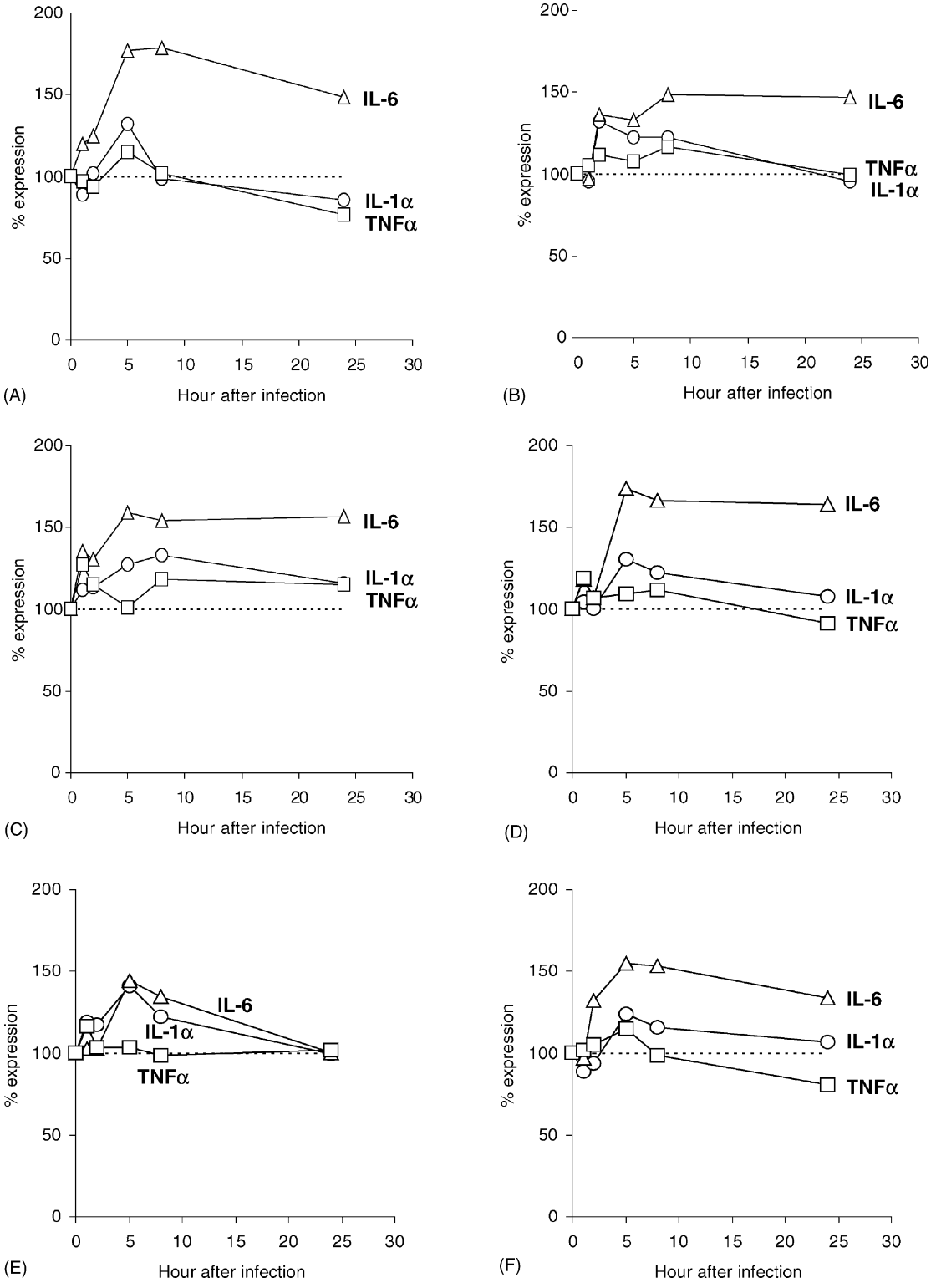

Fig. 8. Cytokine responses in vitro. RAW164.7 macrophages were infected with *B. subtilis* strain PY79 spores (A), autoclaved spores (B), germination defective spores of strain DL169 (C), spore coats (D), vegetative cells (E) and walls purified from vegetative cells (F). At designated time points, cytokine expression in macrophages was detected on total RNAs using RT-PCR. These experiments were repeated in their entirety four times and shown is a representative experiment. Graphs show densitometric analyses of corresponding gel photographs, where % expression represents the relative abundance of each cytokine at each time point compared to that of β-actin. Dotted line represent relative abundance of each cytokine at hour 0 (100%).

We reasoned that *Bacillus* spores, as metabolically dormant life forms, are likely to possess the same basic mechanisms for spore germination, a process that does not require de novo protein synthesis [30]. Analysis of the persistence of spores in macrophages together with confocal analysis of vegetative gene expression provided strong evidence that *B. subtilis* spores germinate and initiate gene expression but cannot grow. Spores are extremely robust bio-particles and there is no reason to assume that phagocytosis and ultimately engulfment within a phagolysosome will have any immediate effect on spore viability. Indeed, extensive research on the resistance properties of spores has shown that they can survive extremes of acidity, heat, irradation and noxious chemicals [31]. Our results do show however, that spores are not maintained within the macrophage but that they can germinate rapidly and it is this step that would lead to destruction of the vegetative cell. Spore germination is a well studied process and requires three steps in total, activation of the spore, germination per se (breakage of the spore coat), and outgrowth [30]. The germination step requires entry of specific germinants (e.g. L-alanine, L-asparagine) that trigger cracking of the coats. It is unlikely that the phagosome offers an nutritionally attractive environment but rather the phagosome/phagolysosome could somehow chemically mimic the conditions required for spore germination thus promoting germination and rapid destruction of the bacterial cell. This model has been proposed for *B. anthracis* [27] but we would argue that this may be a more general method, and indeed, an effective mechanism, for dealing with the destruction of resident spores. Limited survival of the vegetative *B. subtilis* cell in macrophages has been demonstrated previously [32] and is consistent with our findings here. However, although the vegetative cell is ultimately destroyed we have shown that there is sufficient time for the germinated spore to initiate vegetative gene expression. This may explain how in other work we have shown β-galactosidase-specific IgG and IgA responses following oral administration of SC2362 (*rrnO-lacZ*) spores to mice but not in mice dosed with non-germinating DL169 spores (*rrnO-lacZ gerD-cwlB Δ: :neo*). We reason then that the persistence of spores within macrophages must play a determining role in eliciting cellular immunity. Interestingly, cellular responses could provide a mechanism to account for the probiotic properties of these bacteria perhaps by serving as immunostimulant. As might be expected for an organism that can persist, albeit for a short time, within an antigen presenting cell, there is clear evidence of an early inflammatory response involving the inflammatory cytokines, TNF-α, IFN-γ and IL-6.

In conclusion, this study was designed to support the recent development of spore-based vaccines. We show that *B. subtilis* spores, although found normally in the soil, have a more intimate interaction with the GALT than might be initially predicted. They are not recognised as a food, they germinate in the GIT and, to account for the humoral responses, interact directly with the GALT presumably by uptake into the PPs. Importantly, we have also provided evidence for cellular and inflammatory responses. Cellular responses may be beneficial for the use of this organism as a vaccine vehicle but inflammatory responses are of potential concern with the use of this organism as a probiotic.

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