RESEARCH ARTICLE

Immunostimulatory activity of *Bacillus* spores

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Abstract

Bacillus species, typically *Bacillus subtilis*, are being used as probiotics and mounting evidence indicates that *Bacillus* species are important for development of a robust gut-associated lymphoid system (GALT). We used a number of gut isolates of *Bacillus* incorporating three species, *B. subtilis*, *Bacillus licheniformis* and *Bacillus flexus* to evaluate the nature of interaction between spores and the GALT. In mice orally administered with spores, evidence of cell proliferation was determined in the germinal centers of Peyer's patches. Stimulation of antigen-presenting cells and T lymphocytes was also markedly enhanced. Cytokines were shown to be induced in spleens and mesenteric lymph nodes of mice including the proinflammatory cytokines, tumour necrosis factor-α and IL-6. We also demonstrated that vegetative cells of *B. subtilis* can stimulate expression of the toll-like receptor (TLR) genes for TLR2 and TLR4. However, we were able to show that spores could not stimulate either and must, by default, interact with another TLR and by this mechanism help activate innate immunity.

Introduction

Bacillus subtilis has long been considered a nonpathogenic, spore-forming, soil microorganism that has provided a particularly suitable model for understanding the genetic control of unicellular differentiation. Over the last decade, the way in which we consider this Gram-positive bacterium has changed, with reports of natural isolates exhibiting behavioural traits very different from the laboratory strains used for genetic analysis (Nicholson, 2002). A number of studies have also revealed spore-forming *Bacillus* species as inhabitants of the gastrointestinal tracts of both vertebrates and invertebrates including humans (Hong *et al.*, 2005). This list includes not only mammals but also insects and aquatic animals, and molecular studies have demonstrated that *B. subtilis* can proliferate within the murine gastrointestinal tract (GIT) (Tam *et al.*, 2006). Commensal bacteria are known to play an important role in the development of the gut-associated lymphoid tissue (GALT) and animals lacking gut microbial communities are both nutritionally starved and lack a robust immune system (Macpherson & Harris, 2004). Commensals are important for both innate and adaptive immunity and in the case of the latter, experiments with germ-free mice have shown that when bacteria first colonize the GIT their interaction with the GALT leads to expansion of the germinal reaction centers between B and T cells within the Peyer's patches, which, in turn, leads to increased synthesis of IgA from B cells (Jiang *et al.*, 2004). Dendritic cells present in the gut epithelial layers process microorganisms present in the gut lumen and present them to B cells for production of sIgA (Macpherson & Harris, 2004). sIgA would help prevent the commensal population from crossing the mucosal epithelium. A further role for commensals would be in developing the preimmune antibody repertoire by somatic diversification of immunoglobulin genes in B cells that migrate to the GALT (Macpherson & Harris, 2004).

In one seminal study in rabbits in which the appendix had been surgically engineered to be germfree, development of the GALT was studied in response to specific members of the gut microbial communities (Rhee *et al.*, 2004). Remarkably, *B. subtilis* was shown to be of critical importance and found to be superior over other bacterial spore-forming species evaluated. *Bacillus subtilis* when administered to the appendix of these germfree rabbits was found to not only promote GALT development but also, in combination with *Bacteroides fragilis*, promoted somatic diversification of VDJ-Cm genes in appendix B cells. In agreement with studies showing that *B. subilis* could germinate and sporulate in the GIT of mice, the study using germfree rabbits also showed the

importance of sporulation, as opposed to vegetative cell growth, for this GALT development.

Studies demonstrating that *B. subtilis* can grow and sporulate in the gut, coupled with studies showing the unique importance of this species in promoting development of the GALT and preimmune antibody repertoire, support the idea that *B. subtilis* and indeed other *Bacillus* spore formers are in fact gut commensals. It is equally likely that ingestion of spores associated with food enables an early and rapid colonization of the newborn GIT. *Bacillus subtilis* is itself immunogenic; for example, inbred mice dosed with pure preparations of *B. subtilis* spores generate spore-specific sIgA as well as systemic IgG responses (Duc *et al.*, 2003b). Moreover, low levels of dissemination to target organs of the lymphoid system, including the Peyer's patches, have been documented (Spinosa *et al.*, 2000; Duc *et al.*, 2003b). Interestingly, the strain of *B. subtilis* that is used in preparing the Japanese staple, Natto, *B. subtilis* var. *natto* has been shown to promote proliferation of intestinal villi cells in chickens (Hosoi *et al.*, 2003). Therefore, the ability of *B. subtilis* to stimulate the GALT together with the production of specific sIgA responses suggests that ingestion of *B. subtilis* could have beneficial effects in strengthening the immune system and perhaps priming it for an adaptive immune response.

In this study we have examined some key immunological markers that result from oral dosing of mice with pure preparations of *B. subtilis* spores. We demonstrate that *B. subtilis* promotes active lymphocyte proliferation within the Peyer's patches accompanied by a marked increase in the expression of a number of cytokines. Finally, we show evidence for the first time of how this bacterium may interact with cellular components of the immune system by examining expression of Toll-like receptor (TLR) genes.

Materials and methods

General methods and preparation of spores

Methods for measurement of heat-resistant spores were as described (Cutting & Vander-Horn, 1990; Nicholson & Setlow, 1990). Spores for *in vivo* and *in vitro* experiments were prepared by the exhaustion method using Difco Sporulation Medium (DSM) (Duc *et al.*, 2003b). Spore suspensions were lysozyme-treated and then heat-treated (68 °C, 1 h) to remove residual vegetative cells and stored as aliquots at −20 °C before use. PY79 is a laboratory, or domesticated, strain and is Spo+, prototrophic, and derived from the type strain 168 (Youngman *et al.*, 1984). Other bacilli used in this study were obtained from a screen of *Bacillus* species found in human faeces described previously (Tam *et al.*, 2006). These were two strains of *B. subtilis*, HU58 and HU68, two isolates of *Bacillus licheniformis*, HU14 and HU53 and one isolate of *Bacillus flexus*, HU37.

Animal studies

Three animal experiments were performed in this work. In each case Balb/c (5–6-week-old, female, obtained from Harlan UK) were used. For each experiment suspensions of spores [in phosphate-buffered saline (PBS)] of six strains were used, *B. subtilis* strains PY79, HU58, and HU68, *B. licheniformis* strains HU14 and HU53 and *B. flexus* HU37. The inoculum was administered by oral gavage in a total volume of 0.2 mL of PBS. A naïve group receiving 0.2 mL of saline was used in each experiment. The three experiments were as follows:

(1) Determination of persistence in the murine GIT; animals (groups of four) received a dose of 1×10^9 spores. Faecal pellets were removed following dosing and the number of heat-resistant CFU g^{-1} in faeces determined as described previously (Duc *et al.*, 2004a).

(2) Histopathological and immunohistochemical analysis; animals (groups of three) were given a single dose of 1×10^{10} spores. At 30 days postinfection mice were sacrificed and segments of terminal ileum containing Peyer's patches collected and placed in 10% neutral buffered formalin and fixed for a minimum period of 24 h.

(3) Cytokine analysis; mice (groups of three) were administered a single dose of 1×10^{10} spores. Spleen and mesenteric lymph nodes (MLN) were removed at necropsy 3, 7 and 15 days and frozen immediately at −80 °C until needed.

Histopathology and immunohistochemistry (IHC) studies

Samples were placed directly into 10% neutral-buffered formalin for histopathology and immunohistochemistry studies. For histopathology, trimmed tissues were processed routinely to paraffin wax, and 4-μm sections were stained with Haematoxylin and Eosin (H&E). Slides were read using a Leica DMLB microscope with specific attention to the germinal centre size per follicle, number of mitoses per follicle and number of tingible bodies (macrophages containing pyknotic and karryorrhetic debris from apoptotic lymphocytes, most likely from lymphocytes that have undergone somatic hypermutation and not selected for further development) per lymphoid follicle.

Tissue sections, 4 μm thick, were cut and transferred to vectabonded glass slides (Vector Las, UK) before drying overnight at 37 °C. Before IHC, the sections were de-waxed in xylene, dehydrated in absolute alcohol and any endogenous peroxidase activity quenched with hydrogen peroxide/methanol blocking for 15 min. Slides were assembled onto Shandon coverplates to facilitate IHC using the Shandon Sequenza system (Shandon, UK). Primary antibody

cross-reactivity with tissue constituents was prevented using normal immune serum blockade. Primary antibodies were then applied overnight (18–20 h) and incubated at 4 °C. These were goat, anti-tumor necrosis factor (TNF)-α (Santa Cruz Biotechnology, SC 1351, 1/600 dilution), rat anti-mouse F4/80 (Serotec, MCA497R, 1/100) and rabbit anti-human CD3 (Dakocytomation, A0452, 1/500). Nonspecific binding was removed by three successive washes with TBS (5mM TBS, pH 7.6, 0.85% NaCl) before the secondary antibody was applied for 30 min. Three additional TBS washes were performed before and after the application of the avidin–biotin–peroxidase conjugate (ABC elite, Vector Labs) for 30 min. The IHC signal was detected using 3,3-diamninobenzidine (Sigma). The sections were then counterstained with Meyer's hematoxylin (Surgipath, UK), dehydrated in absolute alcohol, cleared in xylene and coverslipped. Slides were read using a Olympus CX41 microscope with specific attention to specific staining for CD3, F4/80 and TNF-α markers.

In vivo cytokine analysis by reverse transcriptase (RT)-PCR

To extract total RNAs, organs and tissues were thawed and disrupted by pressing between two sterile glass slides, lysed in Rneasy Lysis Buffer (RLT) buffer (Qiagen) containing 1% β-mercaptoethanol and homogenized by passing through a QIAshredder column (Qiagen) twice. 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 are shown in Table 1. 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 visualization and densitometric analysis with a Bio-Rad Gel Doc system and the intensity of the DNA bands was quantified by densitometric analysis with a Bio-Rad Gel Doc system.

In vitro cytokine analysis by RT-PCR

RAW264.7 macrophages were cultured and RNA extracted as described for TLR-expression analysis (described above). Spores (3×10^6) of six *Bacillus* strains were added to growing cells and at 2h, 4h and 6h total RNA was extracted. RT-PCR was carried out as described for *in vivo* cytokine analysis.

TLR expression using real-time PCR

Macrophages (RAW264.7; 3×10^5) were seeded and grown on six-well cell culture plates in complete Dulbecco's modified Eagle medium (DMEM). Two-day-old macrophages were infected with 3×10^6 spores or vegetative cells (in DMEM). To prepare vegetative cells, PY79 was grown in brain heart infusion (BHI) broth (Oxoid) at 30 °C for 16–18 h and washed two times with PBS. Vegetative cells or spores were suspended to *c.* 3×10^6 CFU mL^{-1} in complete culture medium supplemented with HEPES (using OD readings to approximate the CFU of vegetative cells or serial dilution on DSM agar plates for spore CFU). Controls used were (1) lipopolysaccharide 1 μg mL^{-1}; (lipopolysaccharide from *Escherichia coli* K235, Sigma); (2) peptidoglycan 100 μg mL^{-1} (from *B. subtilis*, Sigma) and (3) medium-only.

After 2 h, wells were washed three times with sterile 0.03 M PBS (pH 7.4) and the medium replaced with DMEM containing 10 μg mL^{-1} gentamicin to kill any extracellular germinated spores or vegetative cells. Two hours, 4 h and 6 h after infection the culture medium was removed, macrophages washed three times with sterile 0.03 M PBS (pH 7.4), lysed *in situ* and homogenized by passing the cell extract five times through a 20-gauge needle. Total RNAs were extracted and purified using an RNeasy mini kit as described by the manufacturer (Qiagen) and quantified using a GeneQuant spectrophotometer (Amersham Biosciences). Primers specific for β-actin, TLR2 and TLR4 are shown in Table 1. Real-time PCR was carried out as described by the Invitrogen SuperScript™ III RT/Platinum® protocols. Reaction conditions were first-strand cDNA synthesis at 48 °C for 20 min, reverse-transcriptase inactivation at 95 °C for 5 min, and PCR (40 cycles of 95 °C for 15 s and 50 °C for 30 s).

Table 1. PCR primer sequences

Gene	Forward 5′–3′	Reverse 5′–3′	cDNA (bases)
β-actin	TGGAATCCTGTGGCATCCATGAAAC	TAAAACGCAGCTCAGTAACAGTCCG	348
IL-1α	CTCTAGAGCACCATGCTACAGAC	TGGAATCCAGGGGAAACACTG	308
IL-5	ATGACTGTGCCTCTGTGCCTGGAGC	CTGTTTTTCCTGGAGTAAACTGGGG	242
IL-6	TGGAGTCACAGAAGGAGTGGCTAAG	TCTGACCACAGTGAGGAATGTCCAC	154
IFN-γ	AGCGGCTGACTGAACTCAGATTGTAG	GTCACAGTTTTCAGCTGTATAGGG	243
TNF-α	GGCAGGTCTACTTTGGAGTCATTGC	ACATTCGAGGCTCCAGTGAATTCGG	307
TLR2	TCTGGGCAGTCTTGAACATTT	AGAGTCAGGTGATGGATGTCG	321
TLR4	GATTATGTGAACATCAGAAATTCCT	TATCAGAAATGCTACAGTGGATACC	209

To quantify the total number of intracellular *B. subtilis*, monolayers were lysed by resuspension in 0.1% Triton X-100 in sterile distilled water (5 min at 37 °C) and serial dilutions plated on DSM agar to determine CFU. For infection with spores, spore counts were first determined by heating lysates at 65 °C for 30 min to kill all heat-sensitive *B. subtilis*, before serial dilution and plating. This number (spore CFU) was subtracted from total CFU to determine numbers of vegetative cells.

Statistics

Data were analysed using the Mann–Whitney *U*-test. For all tests, the level of significance was set at $P < 0.05$. Unless indicated otherwise, values in the text are means ± SEM.

Results

Persistence in the mouse GIT

Groups of inbred mice were administered a single oral dose of spores (1×10^9) of *B. subtilis* PY79 (a laboratory or domesticated strain) and five strains obtained from the human GIT. These were HU58 and HU68, both strains of *B. subtilis*, two strains of *B. licheniformis,* HU14 and HU53, and one of *B. flexus*, HU37. The numbers of spores shed in the faeces was determined by plate counting of heat-treated faeces at selected times following spore inoculation. As shown in Fig. 1, the numbers of spores present in faeces varied between strains with a minimum period of 15 days (PY79 and HU68) and reaching a maximum of 28 days (HU58).

Histopathological and IHC analysis of Peyer's patches

Groups (three) of inbred mice were given a single oral dose of spores of each of the six *Bacillus* strains together with a naïve group. Peyer's patches were examined for germinal centre size (Fig. 2a), mitosis (Fig. 2b) and tingible body macrophages per lymphoid follicle (Fig. 2c). Our analysis revealed no significant differences ($P > 0.05$) in germinal centre size in mice receiving spores amongst all groups. By contrast, with the exception of HU14, mice dosed with spores showed significant ($P < 0.05$) increases, compared with naïve animals, in the number of mitoses per follicle. Mice dosed with all six *Bacillus* spore types showed a significant increase in tingible body macrophages ($P < 0.05$).

Three immunohistochemical markers against CD3 (pan T lymphocytes), F4/80 (macrophages) and TNF-α were used (Fig. 3 shows staining from tissues of mice dosed with PY79 spores). Compared with naïve mice, HU58-, HU53- and HU37-dosed mice all showed significantly ($P < 0.05$) more staining for CD3. In PY79-, HU58-, HU68- and HU37-dosed mice significantly more ($P < 0.05$) cells stained positive for F4/80 than naïve mice. By contrast, no significant ($P > 0.05$) differences could be observed for TNF-α staining between treated and naïve mice. Taken together these results show that orally administered spores could stimulate proliferation of cells within the Peyer's patches.

Induction of cytokines *in vivo* in MLNs and spleens

The profiles of five cytokines, IL-1α, IL-5, IL-6, IFN-γ and TNF-α, were determined in MLNs and spleen tissues from

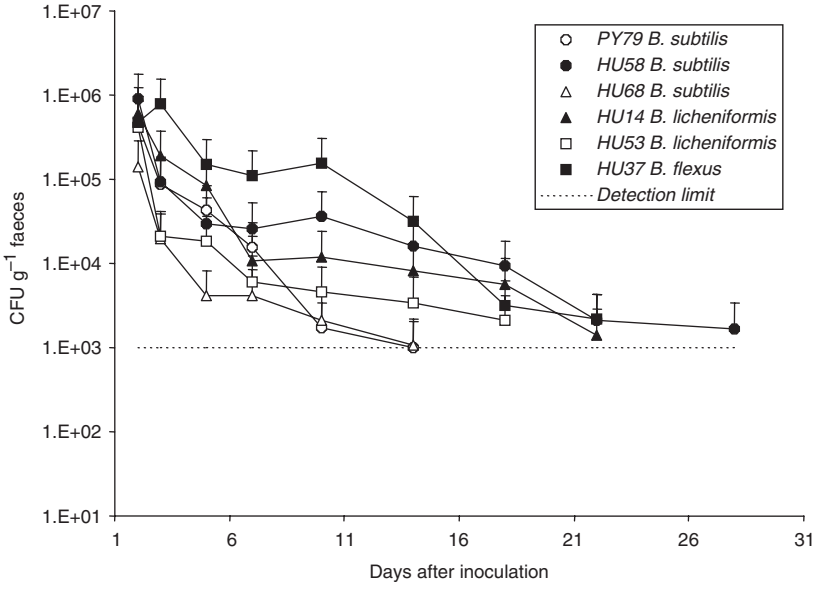

Fig. 1. Persistence of *Bacillus* species in the murine GIT. Counts of spores (as heat-resistant CFU) excreted in the faeces of mice following a single fixed oral dose (1×10^9 CFU) of spores.

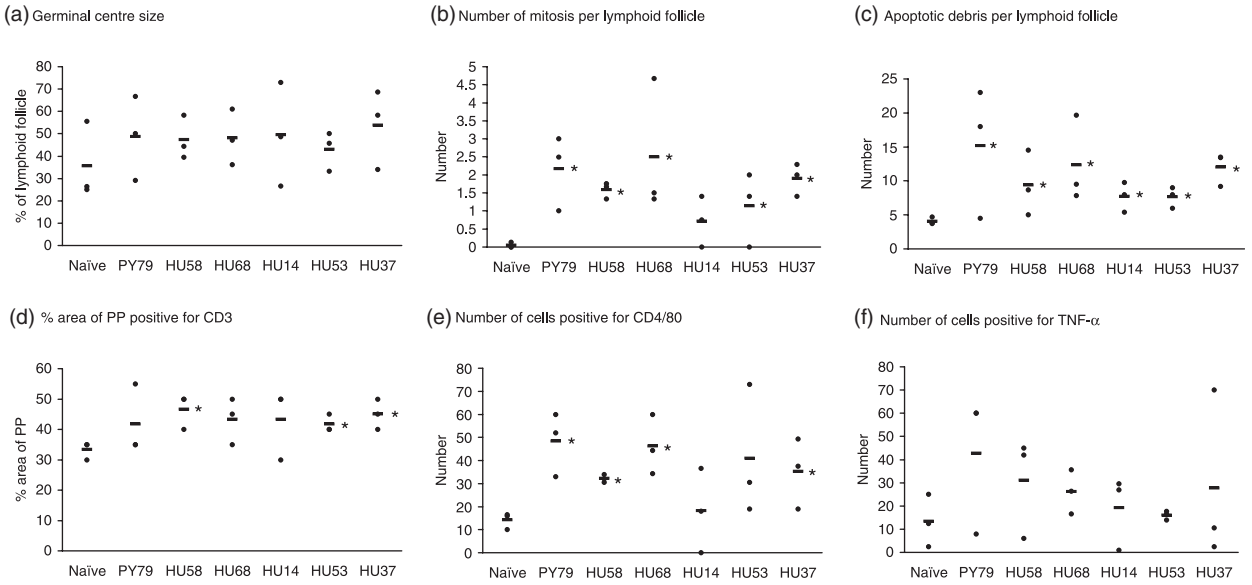

Fig. 2. Proliferative responses in Peyer's Patches. Mice were orally dosed with 1×10^{10} spores of six different *Bacillus* strains and on day 30 Peyer's patches examined for histopathology and IHC analysis. Groups that showed statistically significant differences compared with the naïve group ($P < 0.05$) are indicated with an asterisk.

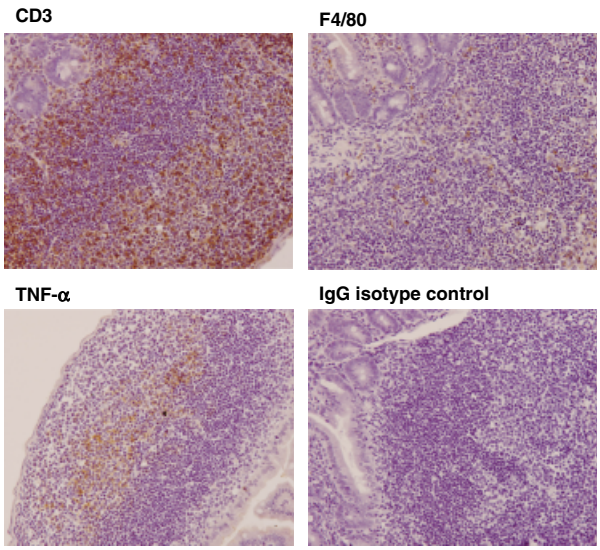

Fig. 3. IHC. Representative staining of sectioned Peyer's patches from mice dosed with *Bacillus subtilis* PY79 spores, × 200-magnification.

Table 2. Cytokine expression *in vivo**

	PY79	HU58	HU68	HU14	HU53	HU37
MLN						
IL-1α	+++§	++++†	++‡	++§	++‡	++‡
IL-5	++§	++†	+‡	++‡	++†	+†
IL-6	+++§	++++†	++++‡	+§	+†	+§
IFN-γ	++§	++†	++‡	++‡	++†	++§
TNF-α	+++§	++†	++‡	++§	+++†	+++§
Spleen						
IL-1α	–	–	–	–	–	–
IL-5	–	–	–	–	–	–
IL-6	–	–	–	–	–	–
IFN-γ	++†	++§	+†	+†	+‡	+§
TNF-α	++†	++§	–	+†	+‡	+†

*Expression levels were determined by semi-quantified by densitometric analysis of RT-PCR products on agarose gels. Expression was compared with samples obtained in naïve mice in each case. –, no expression; +, 1–50% expression; ++, 51–100%; +++, 101–150%; ++++, > 150%.
†Samples from day 3 exhibited the highest expression level.
‡Samples from day 7 exhibited the highest expression level.
§Samples from day 15 exhibited the highest expression level.

mice that received a single oral dose of spores of each of the six *Bacillus* strains. RT-PCR was used to quantify expression levels and Table 2 summarizes semi-quantitative results. Expression of all cytokines was detectable in MLNs and was stimulated by all six *Bacillus* strains. In the spleen only expression of IFN-γ and TNF-α was apparent and *B. subtilis* strains PY79 and HU58 were able to induce the highest levels of expression.

Induction of cytokines *in vitro*

We assessed the ability of all six *Bacillus* strains to induce the proinflammatory cytokines, TNF-α, IL-6 and IL-1α in cultured RAW264.7 macrophages. Using RT-PCR, semi-quantitative measurements of gene expression were determined (Table 3). The highest levels of induction were with TNF-α followed by IL-6, for which all strains induced expression. IL-1α, however, was only induced by *B. subtilis* PY79 and HU68 and *B. licheniformis* HU14.

Table 3. Cytokine expression *in vitro**

	Bacillus strain					
	PY79	HU58	HU68	HU14	HU53	HU37
IL-1α	+‡	–	+†	+†/‡	–	–
IL-6	++§	+‡	+†	+‡/§	++§	++§
TNF-α	++§	++§	++§	++‡	++§	++‡

*Semi-quantitative expression levels were determined by densitometric analysis of RT-PCR products on agarose gels. Expression was compared with samples obtained in control wells (medium only) in each case. –, no expression; +, 1–20% expression; ++, 21–40%.

†Samples from hour 2 exhibited the highest expression level.

‡Samples from hour 4 exhibited the highest expression level.

§Samples from hour 6 exhibited the highest expression level.

Induction of TLRs

The expression of TLR2 and TLR4 in RAW264.7 macrophages was determined by real-time PCR. Macrophages were coincubated with either spores or vegetative cells (VC) of *B. subtilis* PY79. Coincubation of cultured cells with either spores (3×10^6) or vegetative cells (3×10^6) showed a clear temporal increase in expression of both TLR2 and TLR4 expression over time (Fig. 4). Phagocytosis of spores or VC was also measured by determination of the number of VC present within macrophages as CFU. After 2 h incubation with VC a CFU of 9.4×10^5 representing 31% of the total inoculum added to macrophages had been phagocytosed. After 6 h incubation this number had fallen to 2.3×10^4 CFU. Similarly, for spores, after 2 h the numbers of VC present within macrophages was 4.1×10^4 CFU and must have originated from ingested spores that had been phagocytosed and then germinated. After 6 h the number of VC had fallen to just 1×10^3 CFU. To ensure that we could correctly determine TLR induction in parallel experiments cells were incubated with the ligands peptidoglycan and lipopolysaccharide. In agreement with previous work peptidoglycan was shown to induce TLR2 expression (Liu *et al.*, 2001) but not TLR4, and lipopolysaccharide induced a temporal increase in expression of both receptor genes (Faure *et al.*, 2000, 2001; Liu *et al.*, 2001; Nilsen *et al.*, 2004).

Closer inspection of TLR2 and TLR4 induction showed that for VC there was a progressive increase in expression over the 6 h time period we evaluated. For induction by spores there was a significant increase in expression only between hours 4 and 6. Note that lipopolysaccharide, used here as a control, also induced a temporal and progressive increase in TLR expression but this has been shown to result from induction by high doses of lipopolysaccharide (Liu *et al.*, 2001; Nilsen *et al.*, 2004). We addressed the temporal increases in gene expression by first determining whether *B. subtilis* could grow and replicate in cell culture medium in the presence of CO_2. As shown in Fig. 5 *B. subtilis* could grow in this medium, albeit slowly. The same approach

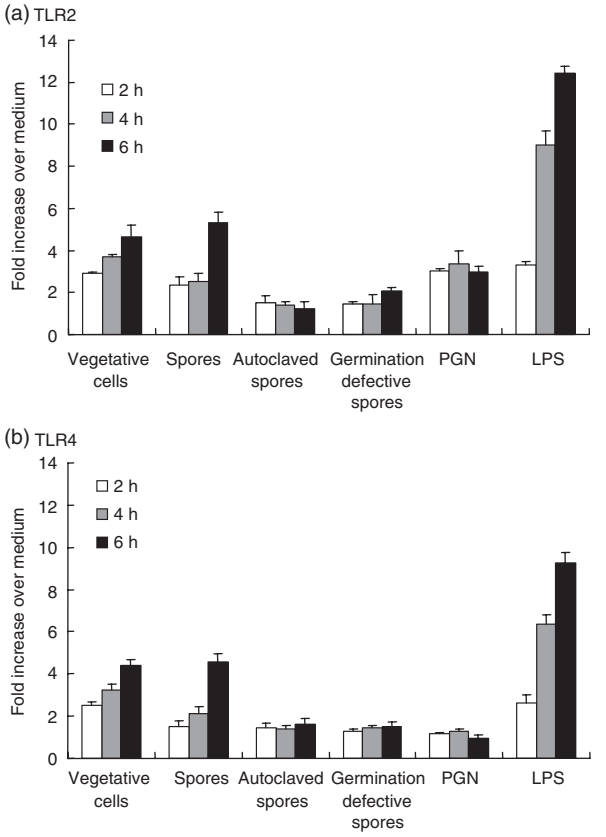

Fig. 4. TLR mRNA expression in the murine, macrophage-like, RAW264.7 cell line. Macrophage monolayers were stimulated with PY79 vegetative cells, PY79 spore, autoclaved PY79 spores, germination defective spores, peptidoglycan and lipopolysaccharide for 2 h. At the indicated time point, mRNA was isolated and TLR2 (a) and TLR4 (b) amplified and quantified by real time PCR. The β-actin gene was used as a control housekeeping gene to compare relative levels of expression. Results showed the fold increase over medium only. Asterisks show statistically significant differences compared with the naïve group at $P < 0.05$ using Mann–Whitney *U*-test.

using spores suspended in cell growth medium revealed that after 2 h incubation there was a noticeable increase in CFU, which could only occur if spores had germinated and then initiated cell growth and multiplication. These results demonstrate that the progressive increase in TLR induction could reflect cell growth and replication and, in the case of spores, spore germination. Therefore, to determine whether spores themselves could induce expression of either TLR2 or TLR4 gene expression we repeated the experiment, but this time using PY79 spores that had been autoclaved and with spores (strain SC2376, and congenic to strain PY79) that carried the *gerD-cwlD* mutation. The *gerD-cwlD* allele confers a severe germination defect to intact spores, drastically reducing germination to 0.0015% compared with the isogenic PY79 strain (Duc *et al.*, 2003a). Both controls failed to

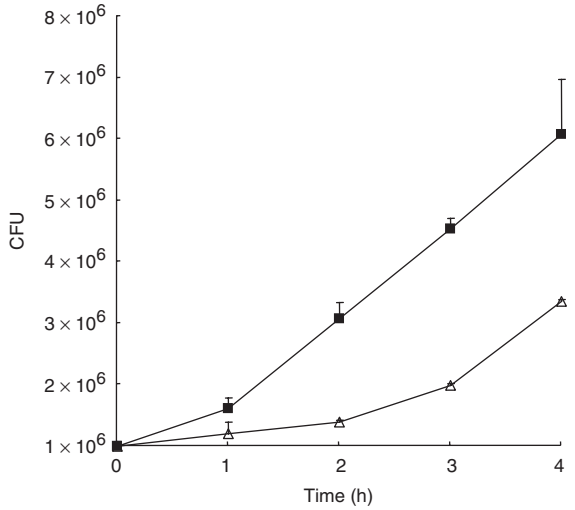

Fig. 5. Growth of *Bacillus subtilis* in complete DMEM medium. Spores (△) or vegetative cells (■) of *B. subtilis* strain PY79 were suspended to *c.* 1×10^6 CFU mL^{-1} in complete culture medium supplemented with HEPES and seeded (3 mL) in six-well cell-culture plates. Plates were incubated at 37 °C in 5% CO_2 and at designated time points, 0.5 mL of medium was collected and serial dilutions were plated on DSM agar to determine the total viable counts.

show any evidence that spores could induce expression of TLR2 or TLR4 (Fig. 4).

Discussion

Members of the *Bacillus* genus are normally considered to be soil organisms but there is growing evidence that while they may be found in the soil in the spore-form their true habitat is that of an animal gut and shedding in the faeces leads to their abundance in the soil (Tam *et al.*, 2006). In any event spores, whether ingested with food, or as commensals, are likely to interact with the GALT and there are a multitude of commercial probiotic products containing *Bacillus* spores for human and animal use (Sanders *et al.*, 2003; Hong *et al.*, 2005). The purpose of this study was to evaluate the immune response of mice dosed with suspensions of spores of three species of *Bacillus, B. subtilis, B. licheniformis* and *B. flexus* obtained from the human gut. Although it is possible that for different species the dose of spores may affect the resulting response, our approach was to choose one fixed dose and compare responses. In order to do this localized responses in the Peyer's patches and in the small intestine were assessed. *Bacillus subtilis* and *B. licheniformis* isolates were chosen because they are in current use as probiotics (Hong *et al.*, 2005) and in the case of one commercial product, BioPlus 2B, they are used in combination for poultry and livestock. We also used this study to determine if there were significant differences between field gut isolates and a laboratory, or domesticated, strain, in this case *B. subtilis* strain PY79. All isolates were able to survive within the murine gut, although clear differences were observed with the laboratory strain of *B. subtilis* showing the shortest retention time, and with the exception of one, HU68, all natural isolates persisted in the GIT for between 7 and 14 days longer. Considering that the time required for a spore to transit the mouse GIT is as little as 3 h (Hoa *et al.*, 2001) we consider this evidence that all spore-forming isolates are able to survive and persist within the GIT.

To survive within the GIT we believe that spores must germinate and establish residency, most likely achieving this by virtue of the vegetative cell that can form biofilms, a feature prevalent in natural isolates of *Bacillus* but not in laboratory isolates (Branda *et al.*, 2001; Faille *et al.*, 2002). In other work we have shown that HU58 does form biofilms (Tam *et al.*, 2006) and in this work we have found that HU14, HU53 both form biofilms while HU68 did not (results not shown). These findings appear to support a link between biofilm formation and gut residency although it should be emphasized that no evidence for *Bacillus* biofilm formation has been demonstrated in the animal GIT. Biofilm formation may enhance the ability of *Bacillus* communities to sporulate, since spore formation has been shown to occur *in vivo* in the murine GIT (Tam *et al.*, 2006) and specialized fruiting bodies within biofilms enable localized centers of sporulation (Branda *et al.*, 2001).

Direct counting of spores in organs of mice that had been given a single, oral, dose of spores (1×10^9) has shown that *B. subtilis* can enter the Peyer's patches, MLN and spleen (Duc *et al.*, 2003b). In a separate study, M cells, which line the follicle-associated epithelium, have been shown to transport *B. subtilis* from the lumen into the Peyer's patches (Rhee *et al.*, 2004). Peyer's patches are rich in antigen-presenting cells (APCs; macrophages and dendritic cells) and B and T lymphocytes. Proliferation of cells in the follicles of the Peyer's patches is a good indication, then, of antigen stimulation (David *et al.*, 2003). B lymphocytes are found in abundance in germinal centers of the Peyer's patches and interact with APCs leading to antibody production. Using a histological approach we found that with the exception of HU14, administration of a single dose of *Bacillus* spores produced a clear increase in cell mitosis, coupled with increased number of tingible body macrophages (suggestive of increased lymphocyte differentiation). Importantly, there was no increase in germinal center size, indicating that cell proliferation and lymphocyte proliferation was balanced and excessive tissue expansion must be limited (David *et al.*, 2003). Activation of proliferative responses in the mouse has been demonstrated using a number of probiotics including *B. subtilis* var. *natto* (Ichikawa *et al.*, 1999; Samanya & Yamauchi, 2002), the strain used in the Japanese fermented food known as Natto. T lymphocyte proliferation in the Peyer's patches was shown by the

positive staining of the CD3 marker and APCs by staining of the F4/80 marker (Hume *et al.*, 1984), although, interestingly, TNF-α was not detected. Although in this work we did not examine direct evidence for B cell proliferation we assume this based on recent studies that have examined proliferative responses in mice dosed with recombinant spores of *B. subtilis* (D'Arienzo *et al.*, 2006). In addition, it has been shown that antibody responses specific to orally administered spores are generated and include spore-specific IgG but also secretory IgA with a distinct Th1 bias (Duc *et al.*, 2003b). Additionally, IgG and sIgA specific to germinated spores has also been measured, demonstrating that spores must germinate, either in the GIT lumen or following uptake and phagocytosis by APCs (Duc *et al.*, 2003a, 2004b). The production of antibodies to orally administered spores shows that spores are immunogenic and cannot, when used at the doses studied here, be considered a food. The doses that have been used in this study, as well as in other studies using *B. subtilis* spores, are unlikely to be encountered naturally. However, when used as a probiotic, regular doses of 1×10^7–2×10^9 spores per dose are used in humans, and while still not comparable to those used in the mouse model here, it is likely that continued ingestion of spores would stimulate the GALT. This is consistent with our analysis of cytokines, which shows that *in vivo* a number of cytokines are produced in the spleen. This includes the proinflammatory cytokines TNF-α and IL-6 and most probably are a result of interaction of *Bacillus* with APCs. Interestingly, despite differences in the persistence in the GIT no substantial differences in cytokine levels between species could readily be observed. Possibly, a more detailed analysis of the temporal profiles of cytokines, coupled with a more extensive analysis of different cytokines, might reveal differences.

A seminal finding here is that *B. subtilis* is likely to interact with the TLR proteins; an interaction of importance in the innate immune response when establishing first contact between potential 'pathogen' and the phagocyte (Akira *et al.*, 2001; Kaisho & Akira, 2001; Armant & Fenton, 2002). These receptor proteins recognize unique signals or patterns of particular molecules present on microorganisms and transmit this information to the cytoplasm of the phagocyte, which in turn leads to expression of cytokines. Our work shows clearly that vegetative cells of *B. subtilis* upregulate expression of TLR2 and TLR4. TLR2 is known to recognize molecular components of Gram-positive bacteria including lipoproteins and peptidoglycan (Takeuchi *et al.*, 1999; Kaisho & Akira, 2001) and TLR4 is stimulated by lipotechoic acids of Gram-positive bacteria (Kaisho & Akira, 2001). Therefore, we can account for how vegetative cells interact with both TLRs and indeed this is consistent with one other study using a *Bacillus* species, namely *Bacillus anthracis*, that was shown to interact with macrophages via TLR2 (Hughes *et al.*, 2005) and TLR4 (Hsu *et al.*, 2004; Park *et al.*, 2004). In this study, the existence of additional TLRs was proposed and our work here shows that spores unable to germinate interact with neither TLR2 nor TLR4. Since nongerminating spores are known to elicit humoral immune responses when administered orally, we would predict the existence of at least one known, or unknown, TLR which interacts with spores.

In conclusion, in this study we have shown that *Bacillus* spores are able to stimulate proliferation of cells within the GALT and promote a potent immune response. This completes much of our understanding on the fate of ingested spores and shows that they should not be considered simply as a food. Following uptake by M cells, they can interact with lymphocytes and APCs in the Peyer's patches to stimulate humoral and cellular responses.

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