

Recombinant *Bacillus subtilis* spores expressing MPT64 evaluated as a vaccine against tuberculosis in the murine model

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Abstract

Recombinant *Bacillus subtilis* spores expressing a TB antigen, MPT64, were tested for their ability to protect mice against tuberculosis challenge. A chimeric gene consisting of the spore coat gene *cotB* fused to *mpt64* was constructed, and expression of a stable CotB-MPT64 hybrid protein of the spore coat verified. Spores were evaluated as a live vaccine and also formaldehyde inactivated. Mice were given three doses of spores or alternatively used in a prime-boost regimen with BCG. The results showed that inactivated recombinant spores were able to reduce the bacterial burden in the lungs of mice to comparable levels to that of BCG. In the prime-boost regimen, both live and inactivated spores showed a reduction in bacterial load in comparison with BCG. ELISPOT and polyfunctional T-cell analysis were performed to examine cellular responses and showed that antigen-specific secretion of Th1 cytokines was stimulated after immunisation with inactive recombinant spores and BCG. In summary, recombinant spores can elicit Th1 responses, which are important for protection against TB disease.

Introduction

The use of recombinant bacterial spores has been shown to offer promise as a mucosal and heat-stable vaccine delivery system. Spores of *Bacillus* are quiescent cell forms, currently used worldwide as probiotics (Hong *et al.*, 2005). Their robustness and safety are the main reasons they have been considered for vaccine delivery. Spores of *Bacillus subtilis* have been used as both recombinant vaccines and as nonrecombinant vaccines. In the former, spores expressing heterologous antigens on the spore coat have conferred protection observed in animal models dosed mucosally. Relevant examples include tetanus, anthrax and *Clostridium difficile* (Duc *et al.*, 2003, 2007; Permpoonpattana *et al.*, 2011). In the nonrecombinant approach, antigens are adsorbed onto the spore surface by a combination of covalent and hydrophobic bonding (Huang *et al.*, 2010a). Interestingly, this approach has been shown to work well using killed spores

of *B. subtilis* adsorbed with influenza H1N1 virions as well as the MPT64 and Acr-Ag85B antigens of *Mycobacterium tuberculosis* (Song *et al.*, 2012; Reljic *et al.*, 2013). In both cases, nasal administration of adsorbed spores conferred measurable levels of protection in murine models of infection (Song *et al.*, 2012; Reljic *et al.*, 2013).

The BCG vaccine is effective in protecting against the infant form of tuberculosis, but has a limited and variable effect in adults against the pulmonary form of the disease, making the development of an improved vaccine critical in reducing the 8–10 million new tuberculosis infections per year. An improved vaccine against tuberculosis should preferentially be economic and easy to administer to encourage and facilitate its use in low-income countries, where the vaccine is most needed.

The aim of this study was to assess the potential use of recombinant *B. subtilis* spores expressing the MPT64 antigen as a vaccine against tuberculosis. Two vaccination approaches and two dosing strategies were evaluated.

Both live recombinant spores and formaldehyde-inactivated recombinant spores were used as vaccines where the latter has the advantage of addressing ethical issues relating to the deliberate release of GMOs. Two dosing regimens were also evaluated, dosing alone or in a prime-boost regimen with BCG. Many novel TB vaccines in current development are using BCG as a prime (McShane *et al.*, 2005), partly to build on the protection it does confer and also, since many populations are already vaccinated with BCG.

Materials and methods

Construction and evaluation of recombinant *B. subtilis* strains that express MPT64

Recombinant strains of *B. subtilis* strain HU58 referred to as HU58-MPT64 and HU58B-MPT64, expressed the *M. tuberculosis* MPT64 protein fused to the C-terminus of the spore coat protein CotB (Donovan *et al.*, 1987). HU58 is a natural isolate of *B. subtilis* from the human gastro-intestinal tract (Hong *et al.*, 2009a). The first stage in the construction of HU58-MPT64 was the synthesis of the *cotB* gene and a region 900-bp downstream (including the *ywrJ* gene) in the plasmid pCotVac-precursor (by Geneart GmbH). This gene cassette was based on the published genome sequence of *B. subtilis* 168 (Genbank accession number AL009126) with in frame EcoRI, BamHI, SacII and XbaI sites, a stop codon and NotI and MluI sites replacing 318 bp of the *cotB* C-terminus. The *dif-cat-dif* cassette was amplified from pTOPO.bac-DifCAT (Bloor & Cranenburgh, 2006) by PCR using primers DifCAT2-for (5'-CACGCGGCCGCTAATACCGGTGGT GACCACTTC-3') and DifCAT2-rev (5'-GACACGCGTC TGCAGAACTCGCCCTTTTG-3'), cut with NotI and MluI and ligated to the pCotVac-precursor cut with the same enzymes to create the plasmid pCotVac.

The *mpt64* gene was codon optimised for *B. subtilis*, synthesised and supplied in plasmid pGA4-Bs.mpt64 (by Geneart GmbH), then excised using EcoRI and BamHI, then ligated into pCotVac cut with the same enzymes to create pCotVac-mpt64 (Supporting Information, Figs S1–S3). The CotVac-mpt64 cistron comprises the 205 codons of the mature *mpt64* fused to codon 275 of *cotB*, separated by two codons from the EcoRI site. This was then linearised using DraIII, transformed into *B. subtilis* HU58 and selected on LB agar containing 20- $\mu\text{g mL}^{-1}$ chloramphenicol, where it integrated into the *cotB* locus by a double cross-over recombination, replacing wild-type *cotB* with *cotB-mpt64*. This integrant strain was then subcultured in LB broth in the absence of antibiotics, where the *cat* gene was excised by the Xer-cise mechanism utilising endogenous RipX and CodV

recombinases (Bloor & Cranenburgh, 2006) to generate strain HU58-MPT64.

To improve the amount of MPT64 displayed in the spore coat, the integration plasmid pAX01 was used, chosen as it contained homology to a suitable integration site in the *lacA* locus (*Bacillus* Genetic Stock Center; (Hartl *et al.*, 2001) The *cotB* gene and its promoter were amplified by PCR from *B. subtilis* 168 chromosomal DNA using primers *cotB*-for (5'-CGGATCCGGATGTGATCT GCGAGTAT-3') and *cotB*-rev (5'-CGAATTCCTTTCAC GATTCTCCTCCT-3'), cut with EcoRI-BamHI and cloned into pAX01 cut with the same enzymes.

Production and purification of spores

HU58 and HU58B-MPT64 strains were grown on solid DSM (Difco Sporulation Media) (Nicholson & Setlow, 1990) agar for 48 h at 37 °C and then harvested and purified as described elsewhere (Nicholson & Setlow, 1990).

Inactivation of spores using formaldehyde

Spores at 2×10^9 were incubated with 0%, 1% or 4% formaldehyde [37% w/v (Sigma)] for 24 or 48 h at RT or 37 °C. After each incubation, the spores were washed twice with PBS (0.01 M, pH 7.4) and plated out onto DSM agar plates and incubated overnight at 37 °C to assess the effect of formaldehyde on spore inactivation. The optimum conditions required for inactivation were 4% formaldehyde 37 °C for 24 h, and these were used to inactivate spores prior to dosing the mice in the study. The levels of formaldehyde were measured in the supernatants after the wash steps using the formaldehyde test kit (HACH).

rMPT64 protein

The *M. tuberculosis* MPT64 antigen was produced as described previously (Reljic *et al.*, 2013) and detailed in the Supporting Information, Data S1.

Antibodies

Polyclonal antibodies were raised in C57BL/6 female mice immunised by the intraperitoneal (i.p.) route with 2 μg of purified rMPT64 protein on days 1, 14 and 28. Serum was collected from terminal heart bleeds.

Western blot

Spore coats were extracted from 1×10^9 spores using an SDS-DTT extraction buffer as described elsewhere (Huang

et al., 2010a) and run alongside rMPT64 protein on a 12.5% SDS-PAGE gel. The gel was then transferred to a nitrocellulose membrane, blocked with 5% skimmed milk powder followed by probing with anti-MPT64 or anti-CotB antibodies (both 1 : 2000 both in-house). Antibody bound to protein was detected using anti-mouse-IgG-HRP (1 : 4000), and the membrane developed using chemiluminescence reagents (Amersham ECL, GE Healthcare) and captured onto film (GE Healthcare).

Confocal microscopy

Live HU58B-MPT64 spores were applied to microscope cover slips (VWR) that had been treated with 0.01% (w/v) poly-L-lysine (Sigma). The procedure is as outlined by Duc *et al.* (Duc *et al.*, 2004) and detailed in the Data S1.

Ethics statement

This study was carried out in strict accordance with the recommendations in the Animals (Scientific Procedures) Act 1986, issued by the Home Office (HO), UK, specifically, HO project licenses 70/6625 and 70/7490 for R.R. and 70/7025 for S.M.C. The protocols were approved by the Ethics Committees of the St. George's University of London and Royal Holloway University of London, prior to obtaining Home Office animal project license approval. All inoculations were performed, while the animals were under light anaesthesia, using isoflurane, and all efforts were made to minimise suffering.

Immunisations

For experiment one, groups of six C57BL/six mice (female, aged 6 weeks old) were immunised. The BCG only group was immunised subcutaneously with 5×10^5 BCG Pasteur on day 1. The mice receiving HU58 spores were administered 2×10^9 HU58 spores (30 μ L) intranasally using a pipette on days 15, 36 and 57. For the prime-boost group, mice were primed subcutaneously with 5×10^5 BCG Pasteur on day 1 and received the boosters on day 36 and 57 with 2×10^9 live HU58B-MPT64 spores delivered intranasally (i.n.) (30 μ L). Control groups include groups receiving PBS (30 μ L) by the intranasal route. In experiment two, groups of ten C57BL/6 mice were used. The BCG group and PBS control groups were treated as in experiment one. The inactive spores group was immunised i.n. with 2×10^9 inactive HU58-MPT64 spores (30 μ L) on days 21, 42 and 63. The prime-boost group was immunised subcutaneously with 5×10^5 BCG Pasteur on day 1, followed by i.n. dosing with 2×10^9 inactive HU58-MPT64 spores (30 μ L) on days 42 and 63.

MTB challenge and bacterial enumeration in organs

From experiment one, $n = 5$ and, from experiment two, $n = 8$ mice per group were used for challenge with MTB and the others were used for immunological assays. Mice were lightly anaesthetised (isoflurane) and challenged i.n. with 5×10^5 CFU MTB H37Rv (50 μ L). After 4 weeks, mice were culled, lungs and spleen homogenised (using a Stomacher) and serial dilutions plated onto 7H11 agar (Difco, BD), plates were incubated at 37 °C for 28 days after which the colony-forming units (CFU) were counted. Those mice that did not appear to be infected (i.e. no MTB detected in the lungs or spleen) were not kept in the analysis.

Statistics

The results for the MTB enumeration in organs were analysed by Mann–Whitney *U*-test, using the GraphPad software. The differences were considered significant when the *P* value was < 0.05 .

Ex-vivo IFN γ Spleen ELISPOT

Two weeks after completion of the immunisations, one mouse from experiment one and two mice from experiment two from each group were used for immunological assays. IFN γ ELISPOT was carried out using a kit (Mabtech, Sweden) and is described fully in the Data S1.

Polyfunctional T-cell analysis

Two weeks after completion of the immunisations, two mice from experiment two from each group were used for immunological assays. Polyfunctional T-cell analysis was carried out as described elsewhere (Sharpe *et al.*, 2010) and described in more detail in the Data S1.

Results and discussion

Recombinant spores expressing *M. tuberculosis* MPT64

MPT64 is a 25 kDa secreted and highly immunogenic protein whose encoding gene, due to attenuation, is absent in many BCG strains (Behr & Small, 1999). In this work, we adopted a recombinant approach constructing a GMO with MPT64 expressed on the spore surface. The *mpt64* gene, codon optimised, was fused to the *cotB* gene of *B. subtilis* HU58, a recently described isolate obtained from the human GI tract (Hong *et al.*, 2009b). *cotB* was chosen as a fusion partner since, firstly, its gene product,

CotB, (43 kDa), is a major component of the outer spore coat layer (Henriques & Moran, 2007) and secondly, CotB has successfully been used for surface display of a number of antigens including segments of *Clostridium tetani* tetanus toxin, *Escherichia coli* labile toxin, *Clostridium perfringens* alpha toxin and *C. difficile* toxin A (Isticato *et al.*, 2001; Mauriello *et al.*, 2004; Hoang *et al.*, 2008; Permpoonpattana *et al.*, 2011).

Using plasmid pCotVac-mpt64 (Data S1), the amount of MPT64 antigen expressed as a 53.7 kDa CotB-MPT64

chimera on the spore coat was estimated by immune dot blotting as 2×10^{-6} pg per spore (Fig. 2a), which was regarded as insufficient for a successful immunisation. Therefore, following the example of Isticato *et al.* (Isticato *et al.*, 2001), a complete wild-type copy of *cotB* was reinserted into the chromosome. The resulting plasmid, pAX01-cotB (Data S1), was linearised using PvuI and transformed into HU58-MPT64, selecting the integrant on $1 \mu\text{g mL}^{-1}$ erythromycin/ $25 \mu\text{g mL}^{-1}$ lincomycin to generate HU58B-MPT64. In this strain, two copies of *cotB* exist: *cotB* fused to MPT64 in the wild-type *cotB* locus, and a full-length copy of *cotB* at the *lacA* locus (Fig. 1). Western blotting revealed a significant increase (100-times) in expression of MPT64 on the spore corresponding to $c. 3.8 \times 10^{-4}$ pg per spore (Fig. 2a).

Expression of MPT64 on the surface of HU58B-MPT64 spores was verified using confocal imaging (Fig. 2b and c). This finding is similar to the work of Isticato *et al.* (Isticato *et al.*, 2001) where a fusion of TTFC to the C-terminus of CotB (strain RH103) failed to allow any expression unless in the presence of an additional copy of *cotB*. The higher levels of expression are believed to result from the stable assembly of Cot monomers in the spore coat layer, and a process that fails to occur when the Cot is modified yet can occur if at least some unadulterated Cot monomers are present (Isticato *et al.*, 2001).

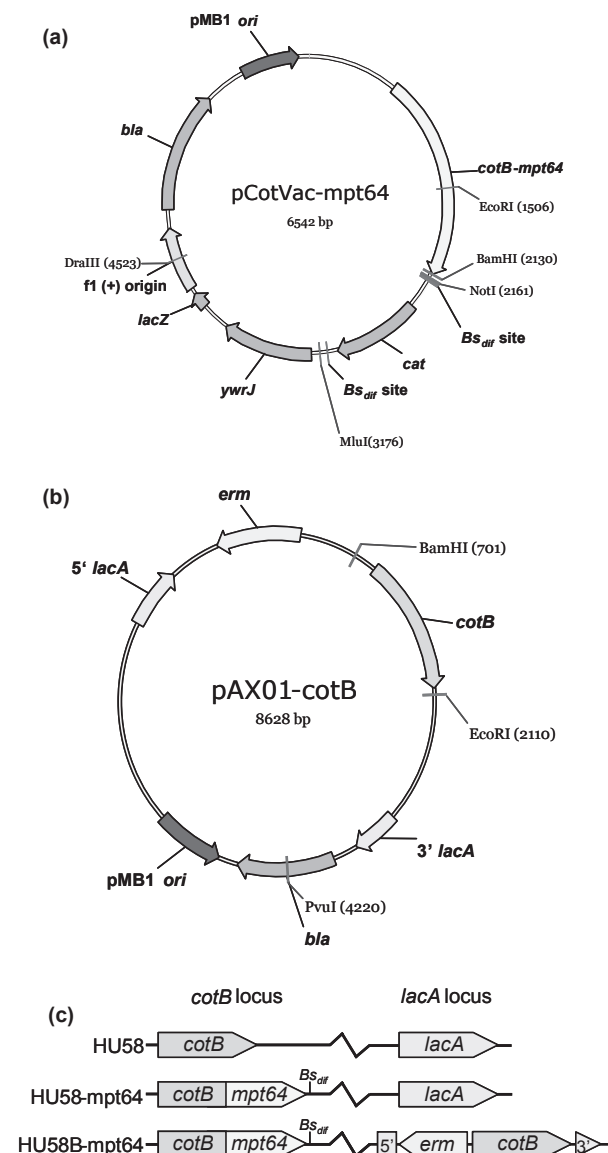


Fig. 1. Recombinant expression of MPT64 on *Bacillus subtilis*. The chromosomal loci of these strains are shown with the chloramphenicol resistance gene *cat* having been excised in the *cotB* locus by Xer-cise to leave a single *BS_{diff}* site, while the erythromycin resistance gene *erm* is retained in the *lacA* locus.

Inactivation of spores using formaldehyde

The use of GMOs is potentially a regulatory issue that needs to be addressed especially for bacterial spores that could possibly be released to the environment. As part of an ongoing study to address methods for inactivating spores, we evaluated the use of formaldehyde because this agent is used routinely in vaccine preparation. For example, Polio (Salk & Salk, 1984), Hepatitis A (Pellegrini *et al.*, 1993), toxic shock syndrome (Gampfer *et al.*, 2002) and foot-and-mouth disease (Twomey *et al.*, 1995) are all inactivated using formaldehyde. HU58B-MPT64 spores were inactivated using 1% or 4% formaldehyde, for 24 or 48 h at RT or 37 °C to elucidate the optimum conditions for inactivating spores. The results showed that 4% formaldehyde substantially reduced spore viability at both RT and 37 °C (Fig. 3a). There was also some evidence that after prolonged incubation (> 48 h), formaldehyde was less effective and possibly the formaldehyde degraded over time. Formaldehyde is toxic and carcinogenic, and therefore, it is important to remove it before vaccination. After washing the spores, the supernatant was tested for formaldehyde content using the formaldehyde test kit and it was found that the levels of formaldehyde were reduced to < 0.05% after two washes (Fig. 3b) which was significantly less than the threshold of 0.2% permitted in

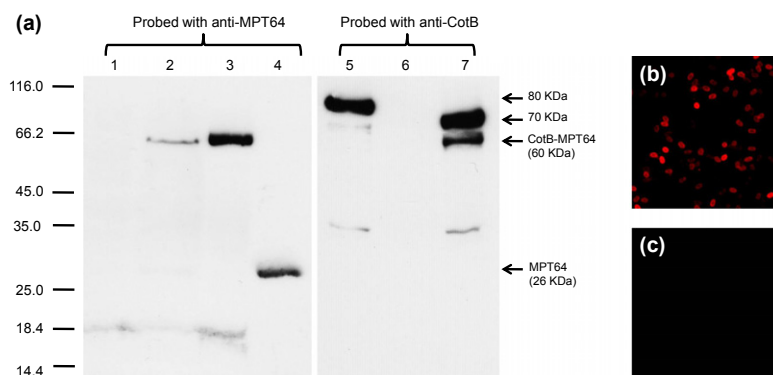


Fig. 2. Expression of MPT64. Expression of MPT64 was validated using a number of immunotechniques. (a) shows proteins probed with anti-MPT64 (lanes 1–4) and anti-CotB antibodies (lanes 5–7). Lane 1, HU58 spore coat protein extracts; lane 2, HU58-MPT64 spore coat proteins; lane 3, HU58B-MPT64 spore coat proteins; lane 4, MPT64 protein (0.1 μ g); lane 5, HU58 coat proteins; lane 5, HU58-MPT64 coat proteins; and lane 7, HU58B-MPT64 coat proteins. Spore coat proteins were extracted from 10^9 spores and fractionated on 12.5% SDS-PAGE as described elsewhere (Nicholson & Setlow, 1990). Confocal imaging was used to confirm surface expression of MPT64 (b and c). HU58B-MPT64 spores were labelled with mouse anti-MPT64 serum followed by the anti-mouse IgG-TRITC conjugate (b); HU58 control spores are shown in (c). Images were taken using a Nikon Eclipse fluorescence microscope equipped with a BioRad Radiance 2100 laser scanning system. The image size was $16 \times 16 \mu\text{m}$.

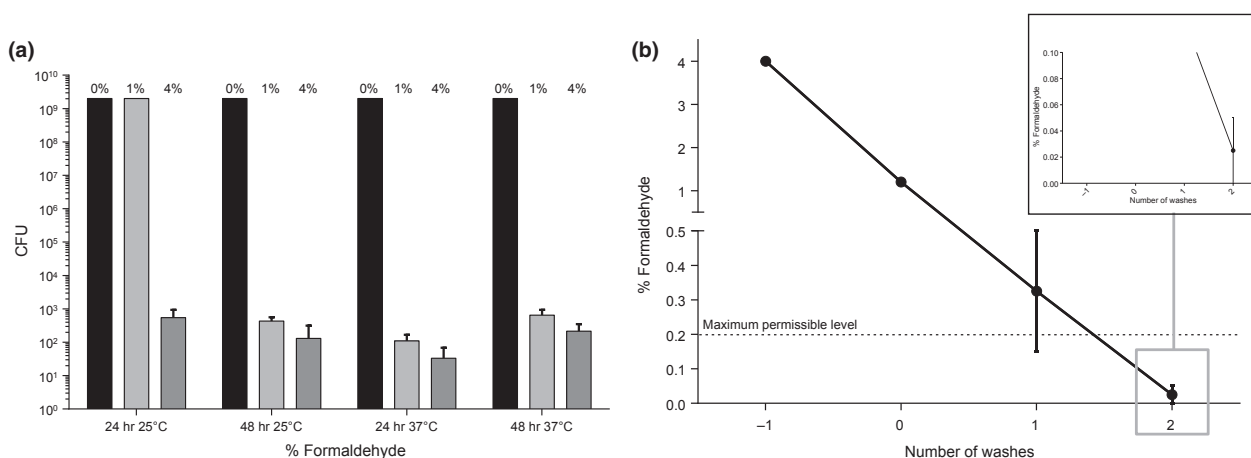


Fig. 3. Inactivation of recombinant HU58B-MPT64 spores with formaldehyde. (a) CFU of surviving HU58B-MPT64 after incubation of spore suspensions with 0%, 1% or 4% formaldehyde at either ambient temperature or 37 °C. (b) percentage of residual formaldehyde left in samples of treated spores after wash steps with PBS as determined using the HACH test. The maximum permissible level is that defined by the European Pharmacopoeia 2014 (EDQM, 2014).

vaccine formulations (EDQM, 2014). We emphasise that these studies are ongoing and currently, with other spore vaccines under development, we have achieved greater than nine-logs of spore inactivation using formaldehyde (using a different method to be reported elsewhere).

Cellular immune responses

IFN γ is a Th1 cytokine that has been shown to be important in protection against TB disease, as knockout mice lacking IFN γ are far more susceptible to TB (Cooper

et al., 1993). IFN γ ELISPOT is a commonly used assay used to assess immune responses to TB vaccinations and was used here to assess the IFN γ production in splenocytes in immunised animals. Low levels of IFN γ production were observed in animals dosed with HU58 spores and inactivated recombinant spores. Both live and inactive spores in the prime-boost regimen with BCG showed IFN γ production higher than BCG alone (Fig. 4).

IFN γ production is certainly important for TB protection, but it has been shown not to correlate with protection, that is, high levels of IFN γ do not correlate with

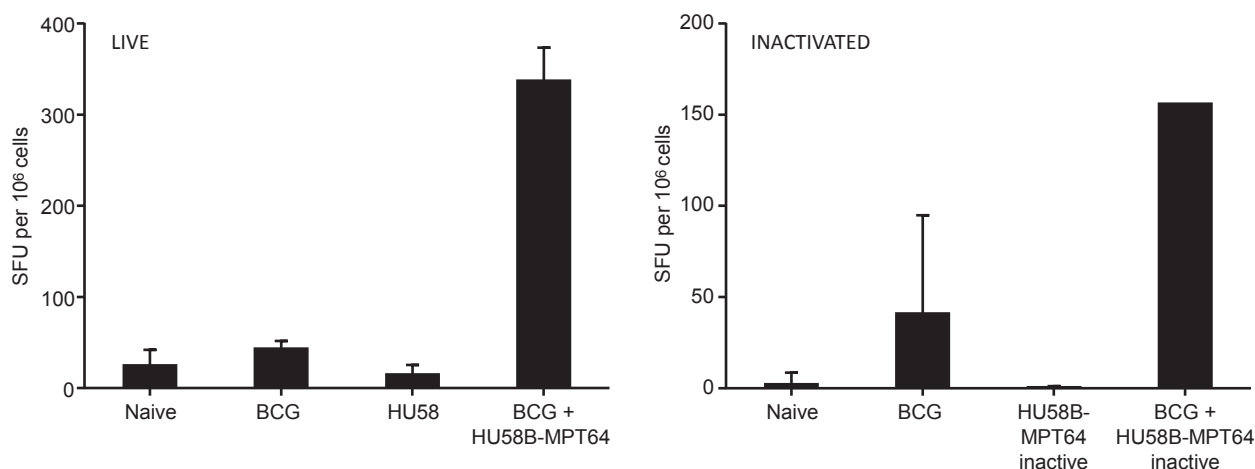


Fig. 4. IFN γ responses. ELISPOT assay determination of the number of spot-forming units (SFU) in splenocytes stimulated with rMPT64 protein in two representative mice taken from each group 2 weeks after the last immunisation. Those mice from prime-boost groups show the highest number of cells secreting IFN γ .

disease prevention although IFN γ production is certainly required. There is a lack of biomarkers and correlates of protection for TB, but one assay that is being actively investigated is polyfunctional T-cell analysis that can simultaneously produce more than one cytokine. CD4⁺ and CD8⁺ cells producing combinations of IFN γ , IL-2 and TNF α have been implicated in improved protection against viruses including HIV. For example, in patients with HIV, those patients with cells producing three cytokines were better able at controlling infection, carried a lower viral load and were better able to maintain antiviral CD4⁺ cells (Kannanganat *et al.*, 2007). Cells producing all three cytokines are considered to indicate a more robust immune response as well as a higher level of cytokine production (Kannanganat *et al.*, 2007) and are deemed important in protective immunity to viral infections and intracellular pathogens, most notably TB. MVA85A, one of the lead candidate TB vaccines, has been shown to induce multifunctional T-cells in humans and NHPs in several trials (McShane *et al.*, 2005; Beveridge *et al.*, 2007; Sharpe *et al.*, 2010; Odotola *et al.*, 2012; Rowland *et al.*, 2012; White *et al.*, 2013).

In our study presented here, cytokine production was assessed in CD4⁺ and CD8⁺ cells in the BCG, inactive HU58B-MPT64 and BCG + inactivated HU58B-MPT64 vaccination groups (Fig. 5). The results for CD4⁺ cytokine production showed undetectable levels of cytokine production in the BCG group, which may be because rMPT64 protein was used for stimulation and MPT64 is not functionally present in this strain of BCG, and may therefore be a poor stimulator (Fig. 5a). The HU58B-MPT64 group showed production of double-positive cells as well as IFN γ ⁺ single cytokine-producing cells. In the BCG + inactivated HU58B-MPT64 group, the cytokine-producing

populations had two sets of double-positive cells and IFN γ ⁺ and IL2⁺ single-positive cells. The CD8⁺ cells in the BCG vaccination group show induction of triple-functional cells, double-positive cells and IL-2⁺ and TNF α ⁺ single cells (Fig. 5b). The inactive HU58B-MPT64 CD8⁺ cell populations had two sets of double-positive cells and IFN γ ⁺ and TNF α ⁺ cells. The inactive recombinant spores used in the BCG prime-boost regimen were able to stimulate production of triple-functional cells, IFN γ ⁺ IL-2⁺ and IL-2⁺ TNF α ⁺ double-positive cells and all three cytokines separately (Fig. 5a and b). The spores used in the BCG prime-boost regimen showed the widest variety of cytokine-producing cells.

Evaluation of recombinant spores as a TB vaccine

Live HU58B-MPT64 spores were tested in a BCG prime-boost regimen (Fig. 6a and b), and inactivated spores were tested alone as well as in a BCG prime-boost regimen (Fig. 6c and d). Following immunisation, animals were challenged with *M. tuberculosis*. HU58 spores did not show any significant protection in comparison with PBS. The inactivated HU58B-MPT64 spores did show a significant reduction in bacterial load in comparison with PBS in the lungs, which was comparable with BCG (Fig. 6c), which demonstrates the ability of spores to carry antigens and be able to provide specific protection against infection. There was a reduction in CFU in the spleen in comparison with PBS, although this was not equivalent to BCG. The results seen were variable, and some appeared to be better protected than others, which could be an artefact of the intranasal dosing, which could be improved by aerosolising the formulation in future.

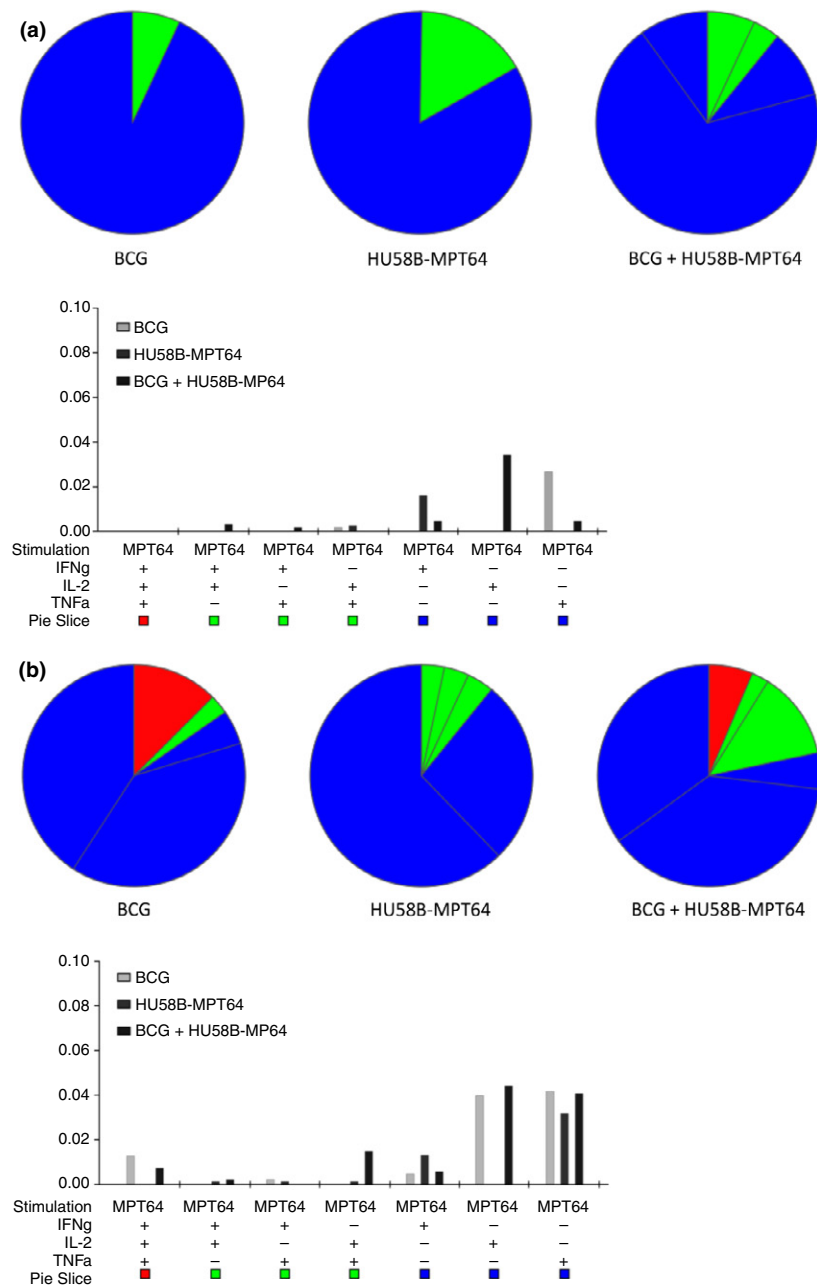


Fig. 5. Polyfunctional T-cell analysis. Frequency of antigen-specific cytokine-producing CD4+ and CD8+ splenocytes induced after vaccination. Groups included in analysis were BCG only, inactive HU58B-MPT64 and BCG with inactive HU58B-MPT64 boost. Results were from two representative mice taken from each group 2 weeks after the last immunisation. (a) CD4+ data; (b) CD8+ data. Recombinant spores predominantly have single cytokine-producing cells producing IFN γ , and TNF α from CD8+ cells. With BCG, there is more IL-2 produced. BCG and BCG-supplemented samples both induce triple-functional CD8+ cells.

The results, however, are promising, and further work could be performed to improve the protection observed.

In both prime-boost regimens with live and inactive HU58B-MPT64 spores, the bacterial loads were lower in comparison with the HU58 and inactive HU58B-MPT64 spore groups. The BCG + live HU58B-MPT64 spores showed a reduction in *M. tuberculosis* CFU in the lungs that was significantly lower than PBS (Fig. 6a). There was also a reduction in the spleen CFU in comparison with BCG, which suggested that dissemination of disease was decreased with the addition of the live recombinant

spores (Fig. 6b). In the prime-boost regimen with inactive spores, the bacterial load in the lungs was lower than BCG immunised animals (Fig. 6d). The bacterial load in the spleen after immunisation with BCG + inactive HU58B-MPT64 was significantly different in comparison with PBS, but not significantly better than BCG alone.

The amount of variety in the types of cytokine-producing cells induced in the polyfunctional T-cells appear to correlate with bacterial burden. The inactive HU58B-MPT64 group produced the least variety of cell types producing cytokine in both CD4+ and CD8+ cell types, and

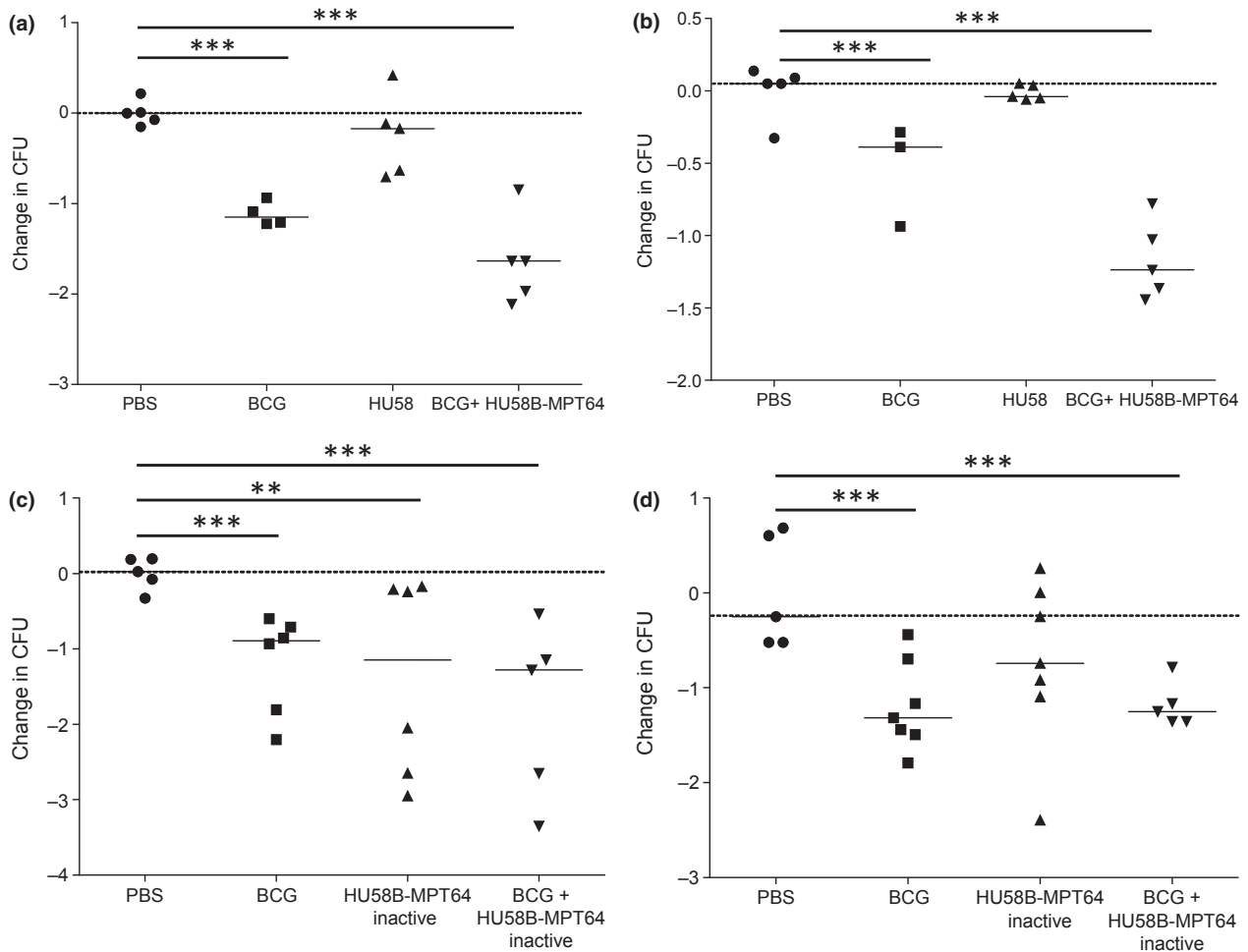


Fig. 6. Colony-forming units (CFU) of *Mycobacterium tuberculosis* in the lungs and spleen of mice 4 weeks after challenge. (a) CFU data from the lungs from mice dosed i.n. with either HU58 spores, BCG or BCG followed by live HU58B-MPT64 spores; (b) CFU data from the spleens of mice dosed with either HU58 spores, BCG or BCG followed by live HU58B-MPT64 spores; (c), CFU data from the lungs of mice dosed with either BCG, formaldehyde-inactivated HU58B-MPT64 or BCG followed by formaldehyde-inactivated HU58B-MPT64; and (d) CFU data from the spleen of mice dosed with either BCG, formaldehyde-inactivated HU58B-MPT64 or BCG followed by formaldehyde-inactivated HU58B-MPT64. In each case, comparisons were made to PBS control groups. Medians are shown, and mice that were not infected were removed from analysis. Data analysed by the Mann–Whitney *U*-test. **, $P = 0.01$, ***, $P = 0.001$.

the bacterial burden was higher than in the prime-boost group. This group showed a reduction in bacterial load in comparison with PBS, so the Th1 response and the presence of double-positive and single-positive cells were able to activate an immune response able to control disease to some degree. However, the BCG + inactive HU58B-MPT64 group, which had a high IFN γ ELISPOT response and a wide range of cytokine-producing cells, including triple-functional CD8 $^{+}$ cells showed the best reduction in bacterial burden in the lungs. BCG also induced triple-functional CD8 $^{+}$ cells, so it appears that these cells could correlate with a reduction in bacterial load. However, the results from the booster group are not

significantly different to BCG and improving the spore inactivation could perhaps reduce the variation in the data and prove more conclusively that inactive spores can improve levels of protection. MPT64 is not present in BCG, but in this study, we have seen an additional benefit to including spores expressing MPT64 in the booster regimen. In previous work, it has been demonstrated that by adding antigens such as MPT64, which are RD2 antigens from *Mycobacterium tuberculosis*, the protection has been improved because RD2 antigens are important in protective efficacy against TB (Wang *et al.*, 2011). The polyfunctional T-cell data also showed that spores induced different cytokine-producing T-cells in comparison with

BCG alone, and so rather than a boosting effect perhaps, we are seeing a supplementation to the vaccine that improves the range of cytokine-producing cells and protection. MPT64 has been tested previously in bacterial vaccines formats including in a *Salmonella* vector (Huang *et al.*, 2010b) and *E. coli* vector (Sali *et al.*, 2014). Both of these expression systems demonstrated the induction of specific immune responses against MPT64 and reduced infection and confirms the utility of recombinant bacterial vectors as vaccine adjuvants.

In conclusion, we have shown that recombinant spores, live and formaldehyde inactivated, are able to provide protection with regard to bacterial load in the lungs alone in a prime-boost regimen using BCG and that active and inactive recombinant spores can activate Th1 immune responses in the IFN γ ELISPOT and polyfunctional T-cell analysis. Interestingly, inactivated spores alone were able to provide comparable protection to BCG alone, demonstrating that they are immunogenic and able to stimulate relevant immune responses. *Bacillus subtilis* spores could provide an excellent vaccine adjuvant for TB because they are relatively straightforward to produce, generally considered safe for human use (and in current use as a probiotic) and can be transported at ambient temperatures which also keeps costs lower. The other clear advantage of using spores as a mucosal vaccine is that there is no requirement for needles, making it easier and safer to deliver. Together these attributes make spores an attractive adjuvant for TB because it is a disease that primarily affects population in developing countries, so making an affordable and easily transportable vaccine is crucial. Our approach reported here complements a previous study where we used heat-killed spores as a carrier for *M. tuberculosis* antigens (including MPT64) (Reljic *et al.*, 2013). Together, bacterial spores as a carrier for *M. tuberculosis* antigens whether as GMOs or non-GMOs offer potential for a simple and low-cost vaccination approach to TB.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. cotB sequence alignments.

Fig. S2. Recombinant expression of MPT64 on *B. subtilis*.

Fig. S3. ELISA detection of MPT64 on spores.

Data S1. Methods.