IAI Journals.ASM.org

Mucosal Vaccination against Tuberculosis Using Inert Bioparticles

Rajko Reljic,[a] Laura Sibley,[b] Jen-Min Huang,[b] Ilaria Pepponi,[a] Andreas Hoppe,[c] Huynh A. Hong,[b] Simon M. Cutting[b]

Infection and Immunity Research Centre, St. George's University of London, London, United Kingdom[a]; School of Biological Sciences, Royal Holloway University of London, Egham, United Kingdom[b]; Department of Computing & Information Systems, Kingston University, Kingston upon Thames, United Kingdom[c]

Needle-free, mucosal immunization is a highly desirable strategy for vaccination against many pathogens, especially those entering through the respiratory mucosa, such as *Mycobacterium tuberculosis*. Unfortunately, mucosal vaccination against tuberculosis (TB) is impeded by a lack of suitable adjuvants and/or delivery platforms that could induce a protective immune response in humans. Here, we report on a novel biotechnological approach for mucosal vaccination against TB that overcomes some of the current limitations. This is achieved by coating protective TB antigens onto the surface of inert bacterial spores, which are then delivered to the respiratory tract. Our data showed that mice immunized nasally with coated spores developed humoral and cellular immune responses and multifunctional T cells and, most importantly, presented significantly reduced bacterial loads in their lungs and spleens following pathogenic challenge. We conclude that this new vaccine delivery platform merits further development as a mucosal vaccine for TB and possibly also other respiratory pathogens.

A third of the world's population is estimated to be infected with *Mycobacterium tuberculosis*, the causative pathogen of tuberculosis (TB). Although the incidence of TB has been slowly decreasing from year to year, this disease still accounts for over a million deaths per annum, mostly in the developing world. TB, however, is also beginning to have an impact on the more developed nations, for example, because of the increased levels of people movement and international travel, which only serve to disseminate TB. Controlling TB relies on a number of strategies, including vaccination, drug therapy, and improved living standards. However, only improved living standards have been shown to have any real impact, and both the current TB drugs and vaccination strategies carry a number of inherent flaws. With drug therapy, multiresistant strains of *M. tuberculosis* and, more recently, extensively drug-resistant (XDR) *M. tuberculosis* variants have emerged, showing that drug development may, at most, produce only a short-term impact. The current vaccine against TB, *M. bovis* BCG, is generally considered unsatisfactory because of its variable efficacy (in different parts of the world) and the short-lived protection that it confers, especially against the reactivated pulmonary form of the disease. For these reasons, BCG is no longer routinely used in some countries.

Bearing in mind these concerns, a better vaccine is now a priority. It should be noted that the results of the first-ever efficacy trial (phase 2b) of a new TB vaccine (MVA85A) that could boost the efficacy of BCG was concluded in early 2013 but with disappointing results (1). Although other trials are planned or are in progress for at least nine other TB vaccines, it is clear that the tasks ahead will be challenging. There are some important issues regarding the design of TB vaccines that might now be reconsidered, for example, the general assumption that cellular responses are of paramount importance (2–4), the emphasis being placed on specific *M. tuberculosis* antigens, and finally, the choice of mucosal vaccination as a potentially better route for administration. Bearing in mind both the considerable effort and cost of developing a new TB vaccine and the poor outcome of the recent MVA85A clinical trial, perhaps now is the time to consider alternative and preferably low-cost strategies that could be affordably implemented in developing countries, which bear the brunt of the TB epidemic.

One such approach, which is evaluated in this paper, is the use of bacterial spores as a delivery vehicle. Bacterial spores of the species *Bacillus subtilis* are approximately 1 μm in length and have been shown to have utility at delivering antigens to the mucosa and generating protective immune responses (5). Of particular interest is recent work where killed bacterial spores were loaded with protein antigens or, indeed, whole virions and used to confer protection in animal models of infection (5). In the case of influenza virus, killed spores adsorbed with inactivated H5N1 were able to fully protect mice against challenge with live H5N2 when administered by the intranasal (i.n.) route (6). A defining feature of the use of spores as a vaccine delivery system is their ability to generate mucosal immune responses, i.e., secretory IgA (sIgA), coupled with their intrinsic adjuvant properties (5). For TB, in which the primary portal of entry is via the upper respiratory tract, there is possibly a case for immunizing by this route, where not only cellular immune responses but possibly also humoral immune responses might contribute to protection (7–9).

In this study, we set out to determine whether killed bacterial spores have potential as a TB vaccine delivery system and, if so, whether they could be developed further. Using two *M. tuberculosis* antigens, MPT64 and Acr-Ag85B (a hybrid protein consisting of alpha-crystallin [Acr] and antigen 85B [Ag85B]), adsorbed onto the surface of killed spores, we show evidence that is compelling. That is, in four independent experiments, spores could confer a significant, greater than 1-log-unit reduction in *M. tuberculosis* counts in infected mice. We emphasize at the outset that we

Received 25 June 2013 **Returned for modification** 29 July 2013
Accepted 12 August 2013
Published ahead of print 19 August 2013
Editor: A. Camilli
Address correspondence to Simon M. Cutting, s.cutting@rhul.ac.uk.
R.R. and L.S. contributed equally to this article.
Supplemental material for this article may be found at http://dx.doi.org/10.1128/IAI.00786-13.

doi:10.1128/IAI.00786-13

are not here defining a specific TB vaccine but, rather, demonstrating that spores can be developed as a vaccine vehicle that is simple in design and realistic, offering simplicity in production, efficacy, and utility.

MATERIALS AND METHODS

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), United Kingdom, 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 anesthesia, using isoflurane, and all efforts were made to minimize suffering.

Strains. HU58 is a nondomesticated strain of *B. subtilis* isolated from the human gastrointestinal (GI) tract (10). DS127 (*cotC-gfp*) is a *B. subtilis* strain that expresses green fluorescent protein (GFP) on the spore surface and has been described elsewhere (11). *M. tuberculosis* H37Rv was used for challenge experiments and was grown in Difco 7H9 medium supplemented with albumin-dextrose-catalase Middlebrook enrichment.

Production of *B. subtilis* spores. Strains were routinely grown in Difco sporulation medium (DSM), and spores were prepared in large quantities using growth on solid DSM (12). Spores were washed and purified before use, and aliquots were stored at −20°C until use. For preparation of killed HU58 ($HU58^K$) spores, they were autoclaved (121°C, 15 lb/in^2, 30 min) before use.

Detection of green fluorescence. Approximately 1×10^8 DS127 spores were added to a coverslip coated with 0.01% poly-L-lysine, incubated at room temperature (RT) for 45 min, and washed seven times with phosphate-buffered saline (PBS). The coverslip was mounted onto a microscope slide and observed using a fluorescein isothiocyanate band-pass filter (excitation, 475 to 490 nm) on a Nikon Eclipse Ti fluorescence microscope.

***In vivo* dissemination studies.** Studies were performed using C57BL/6 mice (female; age, 6 to 8 weeks; Charles River, United Kingdom). Mice were sedated using isoflurane, and DS127 spores (2×10^9 spores/dose) were administered i.n. (30 μl). After 2, 4, 6, 24, and 48 h, mice were euthanized using CO_2 and lungs were fixed in 10% neutral buffered formalin. Histological processing and hematoxylin-eosin (H&E) staining were performed by TUPI Ltd. (United Kingdom).

***In vitro* studies using RAW267.4 macrophages.** RAW267.4 macrophages were seeded and grown on coverslips coated with 0.01% poly-L-lysine and placed in a 24-well plate for 2 days at 37°C in 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), penicillin, streptomycin, and L-glutamine. Cells were then infected with approximately 1×10^6 DS127 spores (multiplicity of infection = 1) and incubated at 37°C in 5% CO_2 for 30 min. Supernatants were removed, and cells were washed with PBS and then fixed with 4% formaldehyde. 4′,6-Diamidino-2-phenylindole (DAPI) was used to stain the macrophage nuclei. Coverslips were mounted onto slides and analyzed using an EVOS fluorescence microscope (magnification, ×100) using the blue and green laser blocks.

Recombinant proteins. The *M. tuberculosis* MPT64 antigen was produced in *Escherichia coli* BL21(DE3) from a pET28b expression vector (Novagen) that carried the *mpt64* gene fused to a C-terminal polyhistidine tag. High levels of expression of the 25-kDa recombinant MPT64 (rMPT64) polypeptide were obtained upon induction with isopropyl-β-D-thiogalactopyranoside (IPTG), and rMPT64 was purified by nickel affinity column chromatography. Eluted rMPT64 protein was checked for integrity by SDS-PAGE, and the concentration was determined using a Bio-Rad DC protein assay kit. The Acr, Ag85B, and Acr-Ag85B fusion proteins were subcloned and expressed in *E. coli* BL21 as previously described (13). These proteins were expressed with a His tag, purified from bacterial lysates by nickel affinity chromatography, refolded, and freed from endotoxin by polymyxin B chromatography.

Production of polyclonal antibodies (PAbs). C57BL/6 mice were immunized by intraperitoneal injection three times with 2 μg of rMPT64 or recombinant Acr-Ag85B (rAcr-Ag85B) over a period of 6 weeks. After a further 2 to 8 weeks, mice were culled and samples of heart blood were taken. Serum was separated from clotted blood by centrifugation (9,000 × *g*, 20 min). Antiserum was purified using protein G Spin-Trap columns (GE Healthcare).

Adsorption of proteins onto spores. Suspensions containing 2×10^9 spores were centrifuged, and pellets were suspended in 0.2 ml of 0.01 M PBS at pH 4, 7, or 10. Purified proteins (10 μg) were added to the spore suspension, and the binding mixture was incubated for 30 min at RT. Spores were centrifuged (1 min), and the pellet was washed two times with PBS (at the same pH as that in the binding mixture). Adsorption was evaluated by (i) extraction and solubilization of adsorbed proteins, (ii) confocal imaging of spores, and (iii) measurement of the zeta potential (see Fig. S1 in the supplemental material). Spore coat protein extractions were done as described previously (5) and required suspending the spore pellet in 100 μl of spore coat extraction buffer, incubation at 68°C for 1 h to solubilize spore coat-adsorbed proteins, and 12% SDS-polyacrylamide gel electrophoresis, followed by detection of bound protein by Western blotting using rMPT64 or rAcr-Ag85B PAbs (1:2,000 dilution). For microscopy, spores were washed with PBS, aliquots of 20 μl were placed onto coverslips coated with 0.01 M poly-L-lysine (Sigma, United Kingdom), and the coverslips were incubated for 5 min. Excess liquid was removed, and the spores were left to dry. Spores were washed with PBS, blocked with 2% bovine serum albumin (Sigma, United Kingdom) for 15 min, and then washed nine times with PBS. Primary antibody (MPT64 or Acr-Ag85B PAbs) was added (1:500), and the mixture was incubated at RT for 45 min. The coverslips were washed three times with PBS, and then anti-mouse IgG-tetramethyl rhodamine isocyanate (1:200) was added and the coverslips were incubated at RT for 45 min. The coverslips were washed three times with PBS, mounted onto slides, and examined using a Nikon Eclipse fluorescence microscope and a Cy3 laser (excitation, 530 to 560 nm) with a 100-ms exposure.

Immunizations. Groups ($n = 9$) of mice (C57BL/6; female; age, 6 to 8 weeks; Charles River, United Kingdom) were used to evaluate the efficacy and immunogenicity of spores adsorbed with rMPT64 or rAcr-Ag85B. The two dosing regimens used were (i) three i.n. doses of $HU58^K$ spores adsorbed with protein antigens (rMPT64 or rAcr-Ag85B) and (ii) a parenteral (subcutaneous [s.c.]) injection of BCG (5×10^5 CFU of BCG Pasteur), followed by two i.n. doses of $HU58^K$ spores adsorbed with recombinant proteins (rMPT64 or rAcr-Ag85B). The dosing schedule is shown in Fig. 3. Control groups included animals (i) dosed once (s.c.) with BCG, (ii) dosed i.n. with $HU58^K$ spores only, (iii) dosed i.n. with rMPT64 or rAcr-Ag85B, and (iv) dosed three times i.n. with PBS. In all cases, the number of $HU58^K$ spores used per dose was 2×10^9. For i.n. dosing, mice were anesthetized using isoflurane and a volume of 30 μl. For rMPT64 and rAcr-Ag85B, 10 μg of protein was used. For adsorbed spores, 10 μg of recombinant protein was adsorbed to spores at pH 4.0. The adsorption experiments were done 60 min prior to immunization. At 6 weeks after the final immunization, two mice from each group were culled for immunogenicity studies and seven mice from each group were challenged with *M. tuberculosis*.

***M. tuberculosis* challenge and enumeration of bacteria in organs.** Mice were lightly anesthetized (isoflurane) and challenged i.n. with 5×10^5 CFU of *M. tuberculosis* H37Rv in a total volume of 50 μl. After 4 weeks, mice were culled, lungs and spleen were homogenized (using a Stomacher), and serial dilutions were plated onto 7H11 agar (Difco, BD). Plates were incubated at 37°C for 28 days, after which the numbers of CFU were counted.

IFN-γ enzyme-linked immunosorbent assay (ELISA). Spleen cells (5×10^5) were seeded in 96-well cell culture plates in complete medium. Cells were stimulated with 10 μg/ml of rMPT64, rAcr, or rAg85B, 1 μg/ml

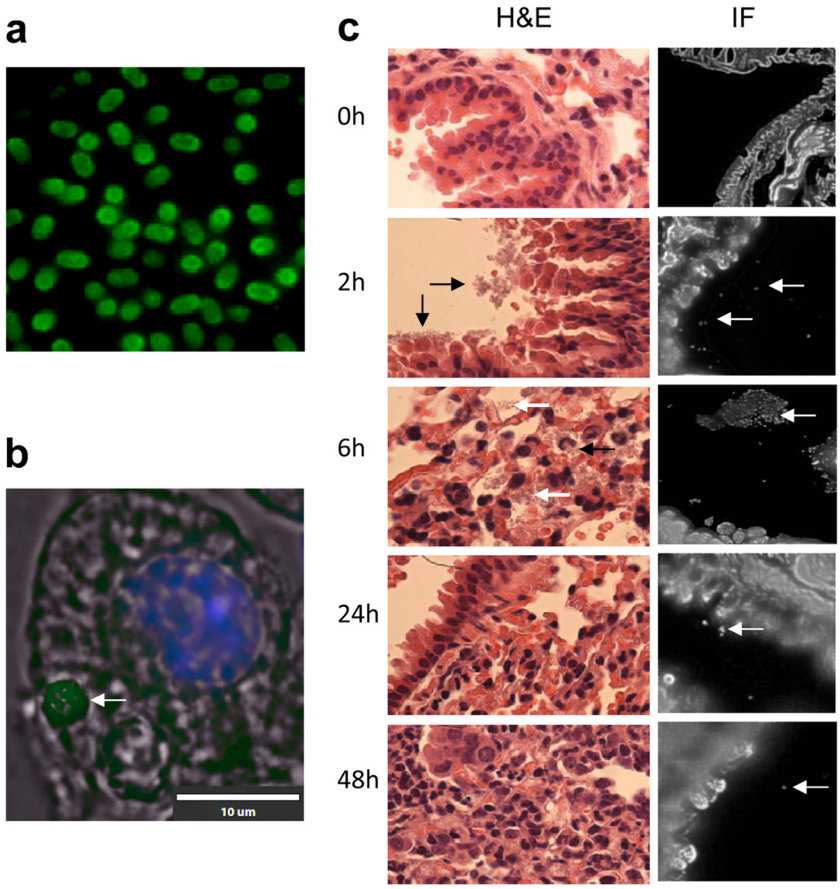

FIG 1 Dissemination of spores *in vivo* and *in vitro*. (a) Representative images of DS127 spores (length, ~1 μm) expressing GFP. (b) A representative RAW264.7 macrophage following 30 min of incubation of a monolayer with DS127 spores. An accumulation of spores (~10 to 20) contained within an endosome is shown (arrow), and the nucleus is DAPI stained. Imaging was performed using an EVOS fluorescence microscope. (c) Mice were dosed i.n. with DS127 spores expressing GFP. Lung tissues were taken from mice after 2, 6, 24, and 48 h and examined by H&E staining and immunofluorescence (IF) imaging. The 0-h sample was taken from naive animals. Arrows, clumps or individual spores. Magnification, ×100.

concanavalin A as a positive control, or medium only at 37°C in 5% CO_2. Supernatants were harvested from cultures after 48 h of incubation. Microtiter plates (96-well Maxisorb plates; Nunc) were coated with anti-mouse gamma interferon (IFN-γ) monoclonal antibody (BD Biosciences) in 0.1 M carbonate-bicarbonate buffer overnight at 4°C. Free binding sites were blocked with 10% FCS in PBS at RT for 1 h. Culture supernatants were tested at RT for 2 h, and IFN-γ was detected with a biotinylated anti-mouse IFN-γ–streptavidin–horseradish peroxidase conjugate (BD Biosciences) at RT for 1 h. Recombinant mouse IFN-γ (BD Biosciences) was used as a standard.

IFN-γ ELISPOT assay. An IFN-γ enzyme-linked immunosorbent spot (ELISPOT) assay was carried out using a kit (Mabtech, Sweden) as described in detail in the Methods in the supplemental material.

Polyfunctional T cell analysis. Polyfunctional T cell analysis was performed using the strategies described elsewhere (14), as described in detail in the Methods in the supplemental material.

Antibody ELISA. Serum was prepared from blood samples from the tail and lung lavage fluid specimens recovered from euthanized mice as described elsewhere (15). Antigen-specific (IgG, IgG1, IgG2, and IgA) responses were determined using indirect ELISA. Plates were coated with 2 μg/ml of rMPT64, rAcr, or rAg85B, blocked with 5% dried milk in PBS, pH 7.2, incubated with serially diluted serum, and finally, probed with specific secondary antibody-enzyme conjugates (all from Sigma). Endpoint titers were calculated as the dilutions producing the same optical density as a 1/100 (IgG) or a 1/4 (IgA) dilution of a preimmune serum sample.

Statistical analysis. The results for *M. tuberculosis* enumeration in organs were analyzed by analysis of variance (ANOVA) and Tukey's multiple-comparison test, using GraphPad software. The differences were considered significant when the *P* value was less than 0.05. The Student *t* test was used to compare the results between groups, with a *P* value of <0.05 considered significant.

RESULTS

Fate and dissemination of spores following intranasal administration. To investigate the fate and dissemination of *B. subtilis* spores administered by the intranasal (i.n.) route, we made use of spores engineered to display green fluorescent protein (GFP) on their surface by fusion of GFP to the spore coat protein CotC (11) (Fig. 1a). We first examined the interaction of DS127 spores with macrophages *in vitro*. Thirty minutes after addition of DS127 spores to growing RAW267.4 macrophages, spores were shown to be readily taken up into the phagosome. This can be seen in Fig. 1b, where clusters of between 10 and 20 spores can be visualized within a phagosome. The results show that macrophages are able to phagocytize spores (8) and so theoretically could act as antigen-presenting cells (APCs) for display of antigens carried on the surface of spores.

Next, we examined *in vivo* the fate and dissemination of DS127 spores administered as a single i.n. dose in mice. At 2, 6, 24, and 48

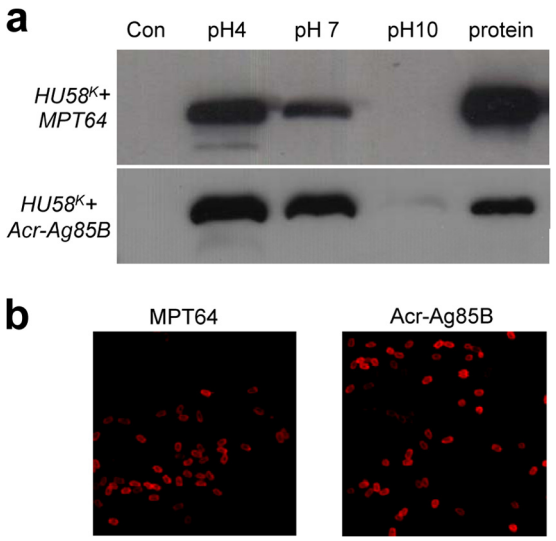

FIG 2 Adsorption of TB antigens on the spore surface. Purified suspensions of $HU58^K$ spores in PBS buffers at different pHs were mixed with purified recombinant protein (2 μg) for 1 h at RT. Spores were centrifuged and washed two times, and adsorbed proteins were extracted. One-tenth of the extracted sample was used for detection by Western blotting (a). The result for purified rMPT64 (25-kDa) or rAcr-Ag85B (50-kDa) protein (0.1 μg each) is shown for comparison. The control lane (Con) shows the corresponding blot from spores taken through the entire procedure without protein. (b) Detection of adsorbed proteins (at pH 4.0) using confocal microscopy.

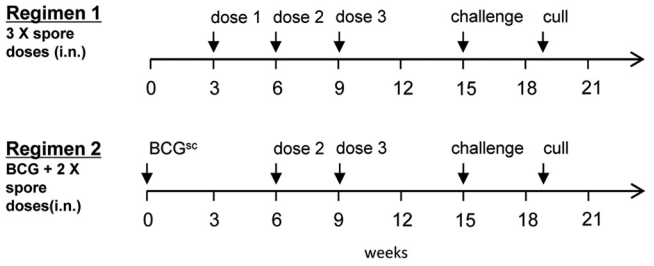

FIG 3 Dosing regimens. Two dosing regimens were conducted in parallel. Animal groups receiving regimen 1 received three i.n. doses of PBS buffer (naive group), $HU58^K$, rMPT64, rAcr-Ag85B, and spores adsorbed with rMPT64 [$HU58^K$(MPT)] or rAcr-85B [$HU58^K$(AA)]. For regimen 2, one group received a single s.c. dose of BCG and then further groups received additional i.n. doses of $HU58^K$, rMPT64, $HU58^K$(MPT), or $HU58^K$(AA). At day 99, all animals were challenged with *M. tuberculosis*. This protocol was repeated in its entirety three times.

h postdosing, animals were culled and sections of lungs were examined using hematoxylin-eosin (H&E) staining and immunofluorescence (IF) microscopy (Fig. 1c). At 2 h, spores could be seen to be clumping together in the bronchioles and at the epithelial barriers. At 6 h, the spores appeared to have infiltrated the tissue and were seen within the alveoli. Phagocytosis of spores was inferred by the change in morphology of some lung cells around the spores. After 24 h, very few spores were seen in the tissue, indicating that the phagocytes had cleared them. This is in accordance with the findings of previous studies showing that particles ranging in size from 1 to 5 μm can be taken up by macrophages or M cells in the respiratory tract, whereas particles in the nanometer size range can be endocytosed by epithelial cells (16). In addition, the size of the particles was shown to affect deposition within the lungs. Particles that lie in the size range of 1 to 3 μm are ideal for delivery to alveolar and respiratory bronchioles, whereas 80% of particles smaller than 1 μm are not deposited at all because of low inertia (17). Our experiments demonstrated that at least a proportion of the spores administered i.n. can reach the alveoli and are not retained in the upper respiratory tract.

Design and evaluation of an inert carrier for TB antigens. Two candidate *M. tuberculosis* antigens, MPT64 and a fusion protein, Acr-Ag85B, were chosen as vaccine candidates. MPT64 is a 25-kDa secreted and highly immunogenic protein whose structural gene is absent in many BCG strains due to attenuation (18) and as a vaccine candidate has been shown to confer partial protection in mouse models (19). Acr-Ag85B (molecular mass, 50 kDa) is a fusion protein made of the Acr (HspX; 16-kDa) antigen, which is preferentially recognized by latently infected individuals (20), and Ag85B, which is one of the most protective known immunodominant *M. tuberculosis* antigens (21) and is expressed in BCG.

As a vaccine, autoclaved killed spores of *B. subtilis* strain HU58, referred to as $HU58^K$, were used as an inert delivery vehicle by adsorbing either rMPT64 or rAcr-Ag85B on the spore surface. To optimize binding, we first measured the adsorption of both recombinant proteins to $HU58^K$ spores using different buffers. PBS was found to be the most suitable, and Fig. 2a shows the binding at different pHs. At pH 7.0, $\sim 1.5 \times 10^{-4}$ pg of rMPT64 and $\sim 1.2 \times 10^{-3}$ pg of rAcr-Ag85B bound per spore. At pH 4.0, $\sim 3.5 \times 10^{-4}$ pg of rMPT64 and $\sim 1.75 \times 10^{-3}$ pg of rAcr-Ag85B bound per spore. pH 4.0 was therefore the most appropriate buffer for adsorption, as it is below the pIs of rMPT64 (pI 4.7) and rAcr-Ag85B (pI 5.7) and results in 10 times more adsorption of rAcr-Ag85B than rMPT64. Confocal imaging using polyclonal antibodies specific to MPT64 and Acr-Ag85B confirmed the surface display of both antigens on $HU58^K$ spores (Fig. 2b). We also measured the zeta potential (or surface charge) of spores (see Fig. S1 in the supplemental material) as an indicator of surface adsorption, which showed that at pH 4.0 and pH 7.0, $HU58^K$ spores were noticeably less negatively charged when adsorbed with either rMPT64 or rAcr-Ag85B.

Evaluation of spore TB vaccines. Groups of mice were immunized with $HU58^K$ spores adsorbed with either rMPT64 or rAcr-Ag85B using two regimens run in parallel. The first consisted of a three-dose i.n. dosing regimen, and the second consisted of a BCG parenteral dose followed by two i.n. doses of adsorbed spores (Fig. 3). In each case, the estimated amount of recombinant protein delivered nasally per dose was 2.4 μg for rMPT64 and 6 μg for rAcr-Ag85B (see Fig. S2 in the supplemental material). The dosing regimens included control groups receiving recombinant proteins or $HU58^K$ alone as well as a naive group receiving PBS buffer. For both dosing regimens, the single BCG parenteral dose served as a positive control. Following the last immunization, animals were challenged i.n. with *M. tuberculosis* and the bacterial load in spleens and lungs was determined. The outcome of the first study, exemplified in Fig. 4, showed that compared to the *M. tuberculosis* counts for the naive group (receiving only three doses of PBS buffer), $HU58^K$ spores adsorbed with either rMPT64 or rAcr-Ag85B produced significant reductions ($P < 0.01$ and $P < 0.001$, respectively) of about 1 log unit in the counts of *M. tuberculosis* found in the lungs. These reductions were similar to those found in animals dosed with BCG. The use of three doses of $HU58^K$ spores on their own or rMPT64 or rAcr-Ag85B proteins without

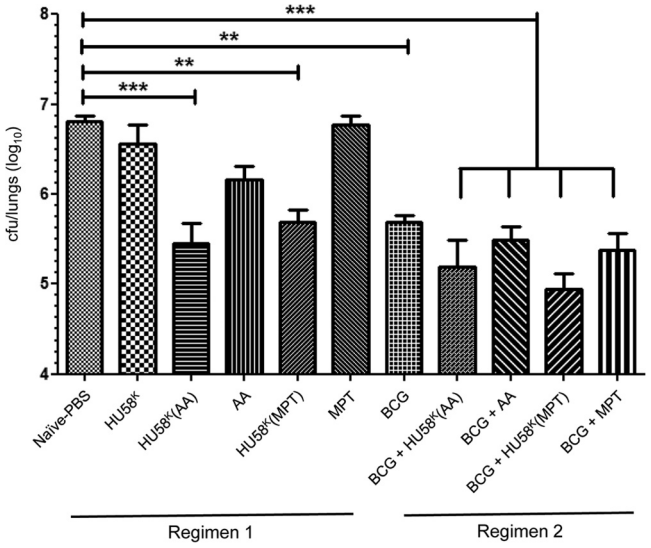

FIG 4 Bacterial load in the lungs of immunized mice. Mice ($n = 7$) were immunized i.n. with three doses of spores of HU58^K alone, rMPT64 (MPT) protein, rAcr-Ag85B (AA) protein, or HU58^K adsorbed with either rMPT64 [HU58^K(MPT)] or rAcr-Ag85B [HU58^K(AA)]. A negative-control group received three doses of PBS. In a parallel experiment, mice were given a subcutaneous injection of BCG vaccine, followed by two i.n. doses of HU58^K adsorbed with either rMPT64 or rAcr-Ag85B or the rMPT64 or rAcr-Ag85B protein alone. The positive-control group received a single s.c. dose of BCG. Means and SEMs are shown. Data were analyzed using a one-way ANOVA, followed by Tukey's multiple-comparison test. ***, $P < 0.001$; **, $P < 0.01$.

spores did not result in statistically significant reductions in lung *M. tuberculosis* counts, although rAcr-Ag85B induced a small reduction in the count compared to the count for the PBS control. In animals dosed first with BCG and then with two i.n. doses of HU58^K spores adsorbed with recombinant proteins, further small reductions in lung counts were observed, but these were not significantly different from those achieved with BCG alone.

This experiment was performed four times in total, and cumulative data from representative groups are shown in Fig. 5. Our data show that, similar to BCG, HU58^K adsorbed with rMPT64 or rAcr-Ag85B produced reductions in *M. tuberculosis* counts in both lungs and spleens that were significantly lower than those in naive animals. Two i.n. doses of antigen-adsorbed HU58^K spores following an initial BCG injection did not result in a further reduction of the infection in the lungs, though a difference was observed ($P < 0.05$) in the spleens with both rMPT64 and rAcr-Ag85B. When the results of the four separate trials are taken together, it is apparent that killed HU58 spores adsorbed with recombinant antigens can elicit a significant reduction in *M. tuberculosis* infection in the lungs equivalent to that obtained with the use of a single dose of BCG. Moreover, when used as a two-dose supplement to BCG, a further reduction in bacterial load in the spleens can be achieved, suggesting that with further optimization of the boost protocol, this vaccination strategy could also be applied as a boost or a supplement to BCG.

Cellular and humoral responses. IFN-γ is considered one of the most important components of the protective immune response against TB infection. Splenocytes from animals dosed with the spore vaccines using regimen 1 were examined for IFN-γ production using stimulation with rMPT64 and rAcr-Ag85B and both an ELISA and an ELISPOT assay (Fig. 6). Control treatments of groups including animals dosed with the recombinant proteins failed to elicit any significant levels of IFN-γ. In contrast, vaccine-dosed groups showed significant levels ($P < 0.05$) of IFN-γ production upon stimulation with rMPT64 (Fig. 6a) or with rAcr or rAg85B (Fig. 6b). ELISPOT assay IFN-γ data for the number of spot-forming units corroborated the ELISA data for both rMPT64- and rAcr-Ag85B-stimulated cells, and these responses were greater than those for cells derived from animals given one

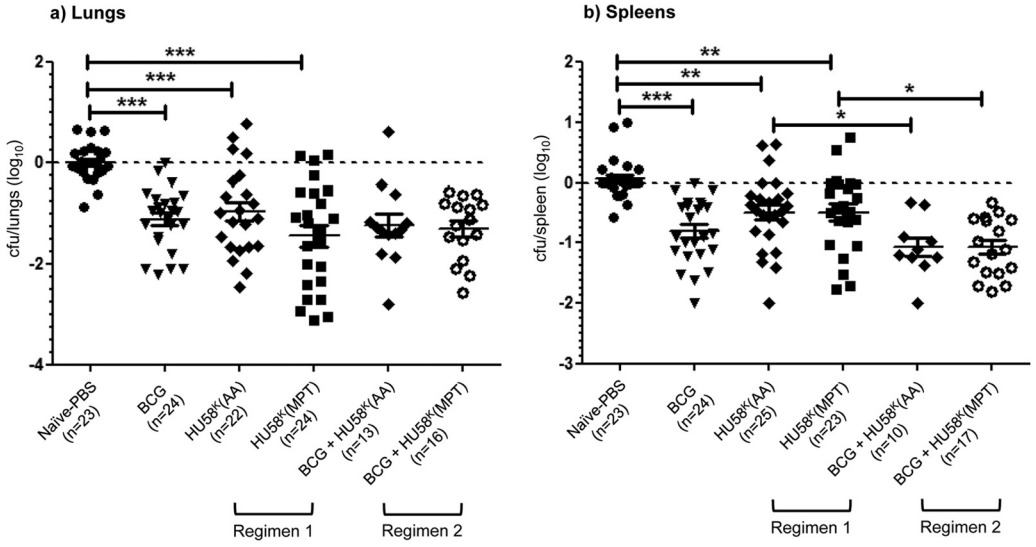

FIG 5 Cumulative data for bacterial loads from four experimental studies. Bacterial loads in the lungs (a) or spleens (b) of mice from combined experiments are shown as the median $\log_{10}$ numbers of CFU and SEMs. Data are combined from four experiments, except for the BCG prime data (regimen 2), for which data are from three experiments. All data are normalized against those for the internal PBS controls for each experiment (i.e., the $\log_{10}$ differences from the mean number of CFU for PBS in that experiment are shown). Horizontal lines and error bars, means and SEMs of the accumulative data, respectively; dotted lines, point of normalization; *n*, number of animals at the end of the experiments (some animals were lost due to premature death or tissue contamination). Data were analyzed using a one-way ANOVA, followed by Tukey's multiple-comparison test. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

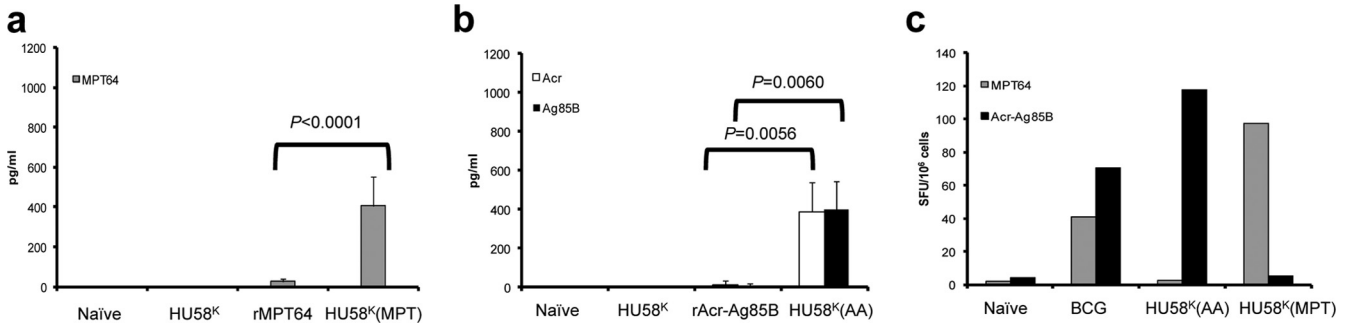

FIG 6 IFN-γ responses. IFN-γ was determined from splenocytes stimulated using an ELISA or ELISPOT assay. (a and b) ELISA detection of IFN-γ from splenocytes stimulated with rMPT64 (a) or with rAcr or rAg85B (b). (c) ELISPOT assay determination of the number of spot-forming units (SFU) in splenocytes stimulated with rMPT64 or rAcr-Ag85B. Spleens were taken from mice ($n = 2$) immunized using dosing regimen 1. The BCG group received one dose of BCG.

dose of BCG. Interestingly, some IFN-γ-producing cells could be detected in BCG-immunized animals upon rMPT64 stimulation, which was unexpected, as this antigen is absent from the Pasteur strain of BCG used here (18).

Although humoral responses are generally not considered primary correlates of protection in TB, we appraised the levels of IgG in blood for each antigen. As shown in Fig. S3 in the supplemental material, the IgG levels were consistently the highest for animals immunized with antigen-coated spores compared to the levels for animals immunized with either antigens or spores alone. We also determined the serum levels of the two IgG isotypes (IgG1 and IgG2a) indicative of a T helper (the Th2 versus Th1 type) immune response (see Fig. S3 in the supplemental material). In animals dosed using regimen 1, anti-MPT64 IgG1 and Ig2a were clearly produced in response to the vaccine consisting of spores loaded with rMPT64 [HU58^K(MPT)], with IgG1 being produced prior to IgG2a. In animals dosed with spores loaded with rAcr-Ag85B [HU58^K(AA)], significant levels of anti-Ag85B IgG1 and IgG2a were evident, with IgG1 induction also being shown to precede IgG2a induction. Lower titers of anti-Acr were observed, with significant levels of only IgG1 being observed. Extrapolation of the ratio of IgG1 (Th2 specific) to IgG2a (Th1 specific) (see Fig. S4 in the supplemental material) revealed that for MPT64- and Acr-Ag85B-specific responses, the ratio declined during the course of the immunization, indicating a shift from a Th2-dominated IgG1 response to a balanced distribution of responses of the IgG1 and IgG2a isotypes.

Mucosal IgA (sIgA) in lung lavage fluid samples also revealed some evidence of local immunity (see Fig. S5 in the supplemental material). That is, an IgA response to MPT64 that was higher when the antigen was adsorbed onto spores than when it was administered on its own was detected ($P < 0.05$), and a similar trend was also observed for anti-Ag85B, though the difference was not statistically significant.

Polyfunctional T cell responses. Analysis of intracellular cytokine staining in splenocyte cultures stimulated *in vitro* with either rMPT64 or rAcr-Ag85B revealed that both vaccine formulations induced antigen-specific CD4$^+$ and CD8$^+$ T cells (Fig. 7 and 8). Thus, rMPT64 predominantly induced single cytokine-expressing CD4$^+$ cells (mostly interleukin-2 [IL-2] and some IFN-γ), while a small proportion of cells simultaneously produced IFN-γ and tumor necrosis factor alpha (TNF-α) (Fig. 8a). rAcr-Ag85B induced a greater proportion (than rMPT64) of double-cytokine-producing cells (IFN-γ plus TNF-α or IFN-γ plus IL-2), while the remaining cells produced IL-2 (Fig. 8c). Both vaccine formulations predominantly induced IL-2-producing CD8$^+$ T cells (Fig. 8b and d), and a proportion of MPT64-specific cells also produced IFN-γ, while a very small proportion of Acr-Ag85B-specific cells expressed IFN-γ and TNF-α simultaneously. Neither antigen formulated with the spores induced significant proportions of triple-cytokine-producing T cells.

DISCUSSION

An improved vaccine against TB should ideally be easy to administer to encourage and to facilitate its use in low-income countries, where the vaccine is the most needed. Since TB is the most prevalent in regions with a high incidence of HIV infection, a needle-free vaccine would be particularly desirable. In principle, immunization with subunit vaccines is an attractive immunization strategy, but the drawback of this approach has been finding suitable adjuvants, due to the many safety constraints and difficulties inherent to adjuvant development. Here, to address that issue, we tested a novel biotechnological approach based on mucosal delivery of TB antigens adsorbed on inert *B. subtilis* spores. Bacterial spores have a number of physicochemical properties that make them particularly amenable for mucosal vaccine delivery. They are naturally transmitted via the mucosal route (i.e., respiratory or oral), they are resistant to the immediate effects of the innate immune system, they can overcome the various anatomical and physiological barriers in the mucosa, and importantly, they can be made to display foreign proteins on their surface. Critically, *B. subtilis* spores have a very good safety record as dietary supplements (probiotics) in both humans and animals (22), therefore facilitating their potential use in vaccine delivery.

We reasoned that the interaction with the respiratory mucosa achieved by the delivery of the mycobacterial antigens displayed on the surface of bacterial spores would mimic, in part, the interaction of the respiratory mucosa with the *M. tuberculosis* pathogen itself and induce the most appropriate immune responses. We tested this novel approach for mucosal vaccine delivery in the mouse model of *M. tuberculosis* infection and demonstrated that it can effectively induce both humoral and cellular immune responses and confer a significant level of protection against the infection.

To demonstrate the feasibility of this novel vaccine delivery platform, we first showed that nasally administered spores can

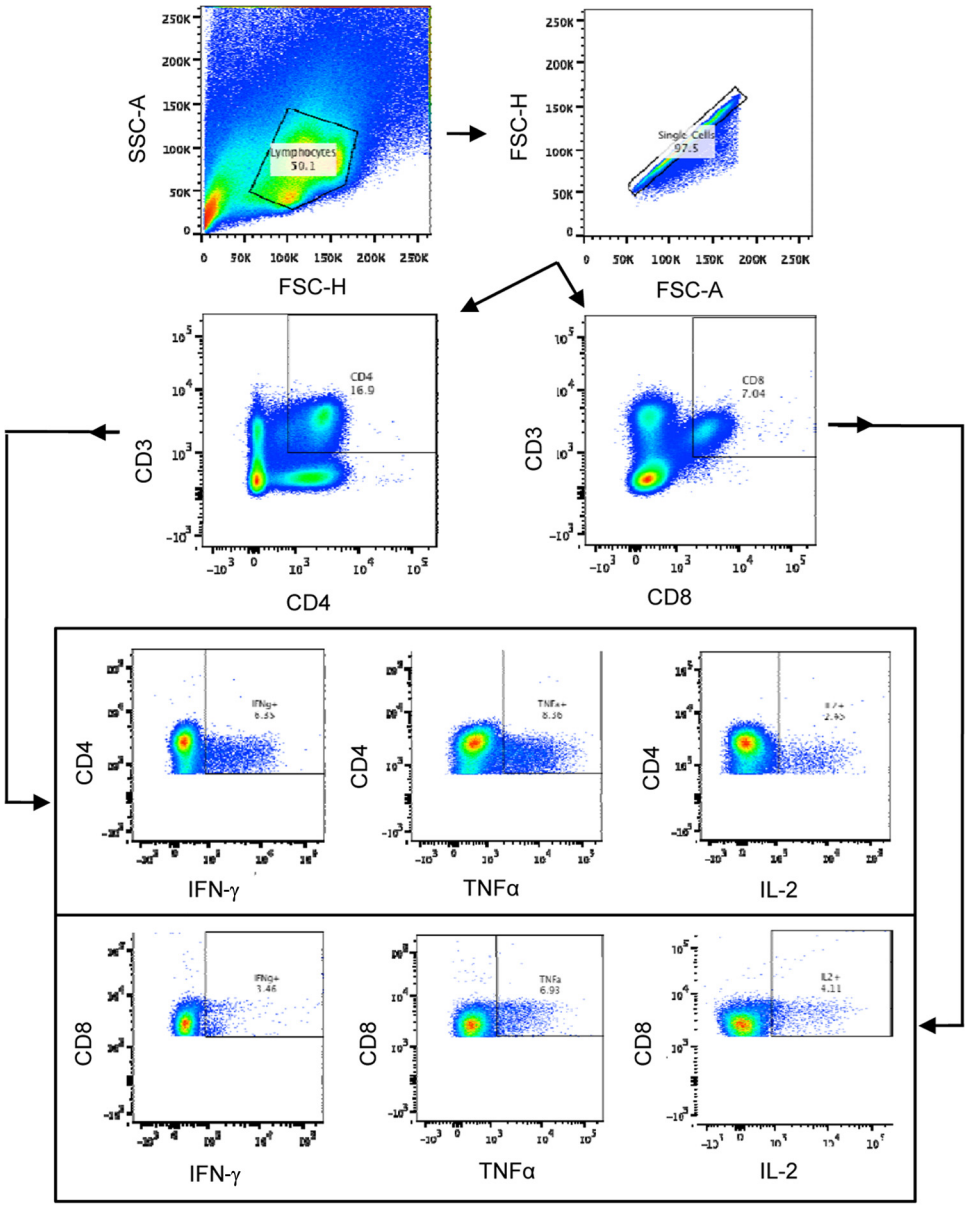

FIG 7 Gating strategy for polyfunctional T cell analysis. Representative FlowJo scatter plots of a naive mouse splenocyte sample are presented to show the gating strategy. Samples were first gated by forward scatter (FSC-A)/side scatter (SSC-A) to select lymphocytes and were then gated by FSC-H/FSC-A to gate for single cells. This population was then gated for $CD3^+$ $CD4^+$ or $CD3^+$ $CD8^+$ staining. These two populations were then analyzed for IFN-γ, IL-2, and TNF-α by staining.

enter the lungs. Elsewhere it has been shown that anesthesia prior to i.n. dosing facilitates entry of the vaccine formulation into the lungs, and indeed, when using doses greater than 20 μl, like those used here, it would be expected that a considerable proportion (50%) of the vaccine reaches the lungs (23, 24). The remainder interacts with the nasal passages, with a significant proportion (~30%) entering the gastrointestinal (GI) tract (24). Spores are approximately 1 μm in length (defining them as microparticles) and hydrophobic and carry a net negative charge (5). The nasal-associated lymphoid tissue (NALT) contains specialized M cells, B and T lymphocyte follicles, macrophages, and dendritic cells (25). While soluble antigens can penetrate the nasal mucosa, microparticulate antigens are taken up by M cells into the NALT (26). It is clear that our results show that cellular and humoral responses can be achieved and these responses are greater than those achieved with the delivery of antigen alone. Moreover, spores loaded with *M. tuberculosis* antigens could reprogram a Th2-biased (i.e., IgG1) response to a more balanced Th2/Th1 profile with both IgG1 and IgG2a antigen-specific responses. Clearly, a balanced immune response consisting of both cellular and humoral immunity is preferable to either a Th2-biased response, which, in the case of TB, would lack cellular immunity, or a Th1-biased response, which would carry the risk of autoimmunity.

Spores clearly enhance or augment immune responses, and these responses are consistent with those found in other studies showing that bacterial spores carry adjuvant properties (5, 27).

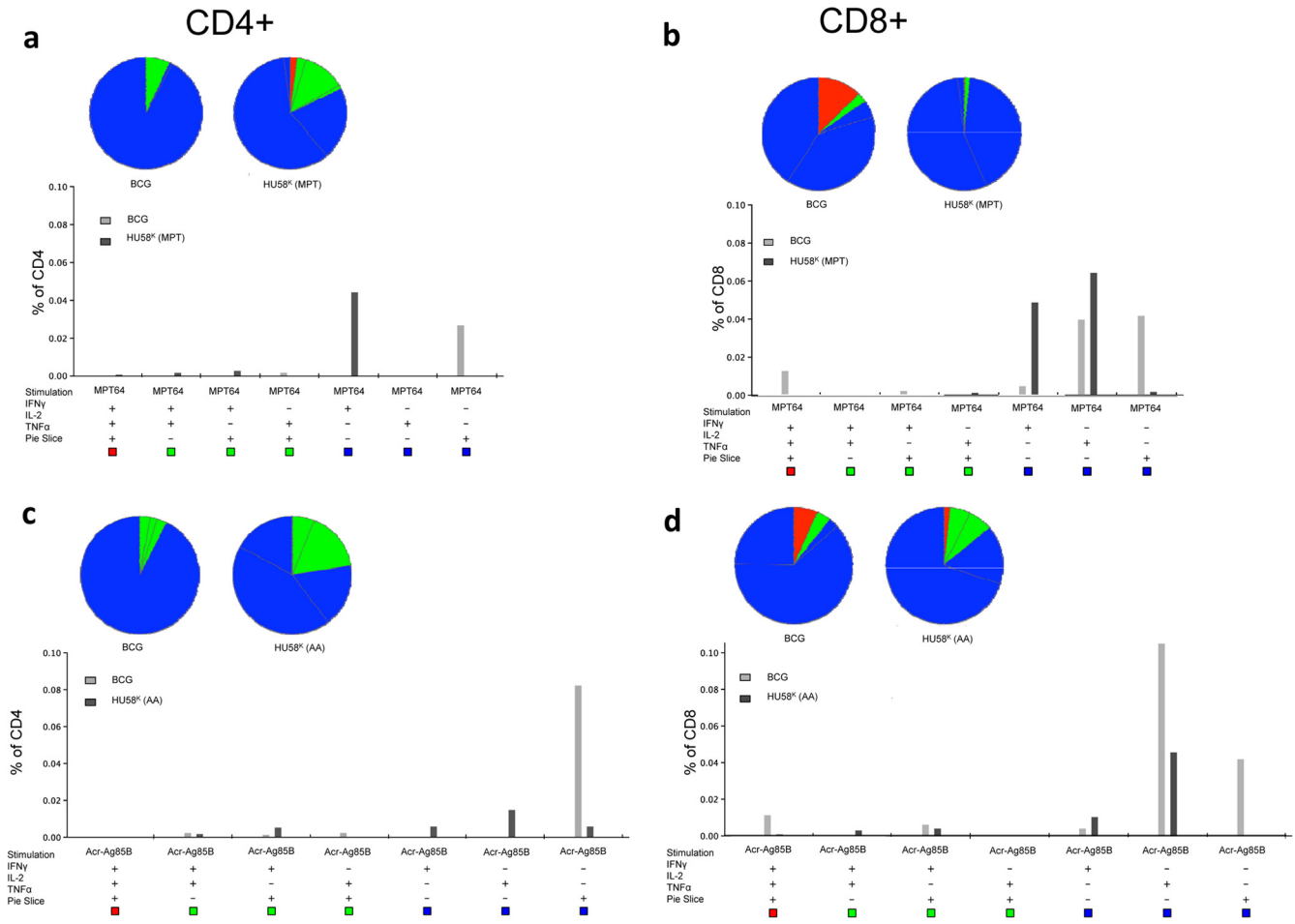

FIG 8 Polyfunctional T cell analysis. The frequencies of antigen-specific cytokine-producing $CD4^+$ and $CD8^+$ splenocytes (collected at day 78) induced after vaccination are shown. The groups included in the analysis were dosed with BCG only (one s.c. dose) and three i.n. doses of either $HU58^K$(MPT) or $HU58^K$(AA). (a and b) Splenocytes stimulated with rMPT64 (MPT); (c and d) splenocytes stimulated with rAcr-Ag85B (AA). Cells producing single cytokines predominate in both spore groups, with IL-2-producing cells being present at the highest proportion. However, some double-cytokine-producing and a small proportion of triple-cytokine-producing functional $CD4^+$ cells were induced in $HU58^K$(MPT64)-vaccinated mice, and some double- and triple-cytokine-producing functional $CD8^+$ cells were induced by $HU58^K$(Acr-Ag85B)-vaccinated mice.

Presumably, spores facilitate entry of the *M. tuberculosis* antigens into the major histocompatibility complex class I and II presentation pathways, and their fate parallels the fate of other microparticulate carriers, such as immune-stimulating complexes (ISCOMs), virus-like particles (VLPs), and biodegradable microspheres (28–31). In other work, we have shown that spores can prime the innate immune system by (i) promoting maturation of dendritic cells, (ii) recruiting NK cells into the lungs, and (iii) activating the NK-κB pathway, most probably through interaction of specific spore ligands with one or more Toll-like receptors (6). We predict, then, that these responses may also play a role in protection against *M. tuberculosis*, for which the importance of innate immunity is now becoming apparent (32). For some diseases, such as HIV infection and TB, it is now being considered that a full range of adaptive immune responses is required, including $CD4^+$ and $CD8^+$ T cell effectors at mucosal and systemic sites (33).

Spores as a mucosal vaccine delivery system appear to fulfill many of these requirements, offering a number of advantages over the other delivery systems. They are safe and easily produced and stored, amenable for formulation with any given protein, easily administered without the need for a needle, and biodegradable *in vivo*; have intrinsic adjuvant properties; and most importantly, are capable of inducing a range of immune responses both locally and systemically. Thus, we showed that antigen-specific sIgA can be readily detected in the lung lavage fluid, and also, high IgG titers could be detected in the sera of immunized animals; moreover, the systemic immune response induced by mucosal vaccination with antigen-coated spores was evidenced by T cell proliferation, IFN-γ secretion, and the recruitment of multifunctional T cells in the spleen. In contrast, intranasal delivery of either live (27) or killed (34) spores fails to generate a significant humoral or cellular response directed against the spore itself but generates a response only against the coadministered antigen. Regarding heat stability, extensive studies have shown that genetically modified spores of *B. subtilis* expressing a heterologous antigen can retain almost complete efficacy as vaccines for up to 12 months when stored at ambient temperature (35). For adsorption to spores, however, the long-term stability of adsorbed antigens is unlikely to apply and may require further process development.

The level of protection against *M. tuberculosis* infection conferred by this immunization approach is significant and is comparable to that conferred by the BCG vaccine, with the difference being that the immune response is directed toward an antigenic subunit rather than the whole organism. That makes it possible to further manipulate this vaccine strategy and test a range of antigens or, indeed, their combinations. We chose, in this proof-of-principle study, to test the spore approach with a TB antigen absent from BCG (MPT64) and a fusion protein made of two antigens present in both *M. tuberculosis* and BCG (Acr-Ag85B). Interestingly, in the case of the latter, the spore approach appeared to be more effective in the homologous than in the heterologous BCG prime/subunit boost vaccination approach. It may be that the boosting capacity of the new vaccine candidate was limited by an insufficient time span between the BCG prime and vaccine boost or, indeed, by the particular choice of antigens in our study. In a study conducted by Rouanet et al., heterologous prime/boost immunization with BCG followed by subcutaneous administration of the heparin-binding hemagglutinin adhesion (HBHA) subunit vaccine in the presence of a pro-Th1 adjuvant had no significant effect on BCG-conferred protection when boosting at 3 weeks or 12 weeks after the BCG prime (36). Lengthening the boosting time, however, to 32 weeks had a much stronger effect, as evidenced by a large reduction in lung and spleen bacterial loads (36). A similar significant boosting effect was also observed with the Ag85B-ESAT6/LTK63 vaccine candidate, when it was administered 8 months after BCG (37), and *M. tuberculosis* 72F/AS02A, when it was administered 16 weeks after BCG (38); both of these boosting times are significantly longer than the boosting time used in our study. Alternatively, other antigens would perhaps have been more suited for boosting the waning BCG immunity, and this should be tested in future studies.

However, our data provide what we believe is compelling evidence for the efficacy of inert bacterial spores as a mucosal vaccine delivery platform that can be utilized for inducing protective immune responses against TB and possibly also other mucosal pathogens. Our evidence shows that a mucosal subunit TB vaccine that is at least as effective as the current TB vaccine (BCG) is a realistic prospect, with a significant scope for further refinement and improvement. It is hoped that such novel vaccination approaches will ultimately lead to the development of a more efficacious vaccination strategy for TB in the foreseeable future.

ACKNOWLEDGMENTS

This work was funded by the SWAN Alliance (South-West Alliance Network, of Royal Holloway University of London, St. Georges University of London, and Kingston University), an EU grant (Innovac, LSH-2005-036871) to S.M.C., and the Dunhill Medical Trust (to R.R.).

We have no conflict of interest to declare.

REFERENCES

1. **Tameris MD, Hatherill M, Landry BS, Scriba TJ, Snowden MA, Lockhart S, Shea JE, McClain JB, Hussey GD, Hanekom WA, Mahomed H, McShane H, Team MATS.** 2013. Safety and efficacy of MVA85A, a new tuberculosis vaccine, in infants previously vaccinated with BCG: a randomised, placebo-controlled phase 2b trial. Lancet **381:**1021–1028.
2. **Agger EM, Andersen P.** 2002. A novel TB vaccine; towards a strategy based on our understanding of BCG failure. Vaccine **21:**7–14.
3. **Checkley AM, McShane H.** 2011. Tuberculosis vaccines: progress and challenges. Trends Pharm. Sci. **32:**601–606.
4. **Scanga CA, Flynn JL.** 2010. Mycobacterial infections and the inflammatory seesaw. Cell Host Microbe **7:**177–179.
5. **Huang JM, Hong HA, Van Tong H, Hoang TH, Brisson A, Cutting SM.** 2010. Mucosal delivery of antigens using adsorption to bacterial spores. Vaccine **28:**1021–1030.
6. **Song M, Hong HA, Huang JM, Colenutt C, Khang DD, Nguyen TV, Park SM, Shim BS, Song HH, Cheon IS, Jang JE, Choi JA, Choi YK, Stadler K, Cutting SM.** 2012. Killed *Bacillus subtilis* spores as a mucosal adjuvant for an H5N1 vaccine. Vaccine **30:**3266–3277.
7. **Hamasur B, Haile M, Pawlowski A, Schroder U, Kallenius G, Svenson SB.** 2004. A mycobacterial lipoarabinomannan specific monoclonal antibody and its F(ab′) fragment prolong survival of mice infected with *Mycobacterium tuberculosis*. Clin. Exp. Immunol. **138:**30–38.
8. **Glatman-Freedman A.** 2006. The role of antibody-mediated immunity in defense against *Mycobacterium tuberculosis*: advances toward a novel vaccine strategy. Tuberculosis (Edinb.) **86:**191–197.
9. **Kallenius G, Pawlowski A, Brandtzaeg P, Svenson S.** 2007. Should a new tuberculosis vaccine be administered intranasally? Tuberculosis (Edinb.) **87:**257–266.
10. **Hong HA, Khaneja R, Tam NM, Cazzato A, Tan S, Urdaci M, Brisson A, Gasbarrini A, Barnes I, Cutting SM.** 2009. *Bacillus subtilis* isolated from the human gastrointestinal tract. Res. Microbiol. **160:**134–143.
11. **Isticato R, Di Mase DS, Mauriello EM, De Felice M, Ricca E.** 2007. Amino terminal fusion of heterologous proteins to CotC increases display efficiencies in the *Bacillus subtilis* spore system. Biotechniques **42:**151–152, 154, 156.
12. **Nicholson WL, Setlow P.** 1990. Sporulation, germination and outgrowth, p 391–450. *In* Harwood CR, Cutting SM (ed), Molecular biological methods for *Bacillus*. John Wiley & Sons Ltd., Chichester, United Kingdom.
13. **Pepponi I, Stylianou E, van Dolleweerd C, Diogo GR, Paul MJ, Drake PM, Ma JK, Reljic R.** 2013. Immune-complex mimics as a molecular platform for adjuvant-free vaccine delivery. PLoS One **8:**e60855. doi:10.1371/journal.pone.0060855.
14. **Sharpe SA, McShane H, Dennis MJ, Basaraba RJ, Gleeson F, Hall G, McIntyre A, Gooch K, Clark S, Beveridge NE, Nuth E, White A, Marriott A, Dowall S, Hill AV, Williams A, Marsh PD.** 2010. Establishment of an aerosol challenge model of tuberculosis in rhesus macaques and an evaluation of endpoints for vaccine testing. Clin. Vaccine Immunol. **17:**1170–1182.
15. **Yu LH, Cutting SM.** 2009. The effect of anti-spore antibody responses on the use of spores for vaccine delivery. Vaccine **27:**4576–4584.
16. **Chadwick S, Kriegel C, Amiji M.** 2010. Nanotechnology solutions for mucosal immunization. Adv. Drug Deliv. Rev. **62:**394–407.
17. **Yang W, Peters JI, Williams RO, III.** 2008. Inhaled nanoparticles—a current review. Int. J. Pharm. **356:**239–247.
18. **Behr MA, Small PM.** 1999. A historical and molecular phylogeny of BCG strains. Vaccine **17:**915–922.
19. **Delogu G, Howard A, Collins FM, Morris SL.** 2000. DNA vaccination against tuberculosis: expression of a ubiquitin-conjugated tuberculosis protein enhances antimycobacterial immunity. Infect. Immun. **68:**3097–3102.
20. **Roupie V, Romano M, Zhang L, Korf H, Lin MY, Franken KL, Ottenhoff TH, Klein MR, Huygen K.** 2007. Immunogenicity of eight dormancy regulon-encoded proteins of *Mycobacterium tuberculosis* in DNA-vaccinated and tuberculosis-infected mice. Infect. Immun. **75:**941–949.
21. **Olsen AW, Williams A, Okkels LM, Hatch G, Andersen P.** 2004. Protective effect of a tuberculosis subunit vaccine based on a fusion of antigen 85B and ESAT-6 in the aerosol guinea pig model. Infect. Immun. **72:**6148–6150.
22. **Hong HA, Duc le H, Cutting SM.** 2005. The use of bacterial spore formers as probiotics. FEMS Microbiol. Rev. **29:**813–835.
23. **Vajdy M, O'Hagan DT.** 2001. Microparticles for intranasal immunization. Adv. Drug Deliv. Rev. **51:**127–141.
24. **Eyles JE, Williamson ED, Alpar HO.** 1999. Immunological responses to nasal delivery of free and encapsulated tetanus toxoid: studies on the effect of vehicle volume. Int. J. Pharm. **189:**75–79.
25. **Kuper CF, Koornstra PJ, Hameleers DM, Biewenga J, Spit BJ, Duijvestijn AM, van Breda Vriesman PJ, Sminia T.** 1992. The role of nasopharyngeal lymphoid tissue. Immunol. Today **13:**219–224.
26. **Behrens I, Pena AI, Alonso MJ, Kissel T.** 2002. Comparative uptake studies of bioadhesive and non-bioadhesive nanoparticles in human intestinal cell lines and rats: the effect of mucus on particle adsorption and transport. Pharm. Res. **19:**1185–1193.
27. **Barnes AG, Cerovic V, Hobson PS, Klavinskis LS.** 2007. *Bacillus subtilis* spores: a novel microparticle adjuvant which can instruct a balanced Th1

and Th2 immune response to specific antigen. Eur. J. Immunol. **37:**1538–1547.
28. **Peter K, Men Y, Pantaleo G, Gander B, Corradin G.** 2001. Induction of a cytotoxic T-cell response to HIV-1 proteins with short synthetic peptides and human compatible adjuvants. Vaccine **19:**4121–4129.
29. **Yan M, Peng J, Jabbar IA, Liu X, Filgueira L, Frazer IH, Thomas R.** 2004. Despite differences between dendritic cells and Langerhans cells in the mechanism of papillomavirus-like particle antigen uptake, both cells cross-prime T cells. Virology **324:**297–310.
30. **Sanders MT, Brown LE, Deliyannis G, Pearse MJ.** 2005. ISCOM-based vaccines: the second decade. Immunol. Cell Biol. **83:**119–128.
31. **Raychaudhuri S, Rock KL.** 1998. Fully mobilizing host defense: building better vaccines. Nat. Biotechnol. **16:**1025–1031.
32. **Korbel DS, Schneider BE, Schaible UE.** 2008. Innate immunity in tuberculosis: myths and truth. Microbes Infect. **10:**995–1004.
33. **Pulendran B, Ahmed R.** 2006. Translating innate immunity into immunological memory: implications for vaccine development. Cell **124:**849–863.
34. **Song JM, Hossain J, Yoo DG, Lipatov AS, Davis CT, Quan FS, Chen LM, Hogan RJ, Donis RO, Compans RW, Kang SM.** 2010. Protective immunity against H5N1 influenza virus by a single dose vaccination with virus-like particles. Virology **405:**165–175.
35. **Lee S, Belitsky BR, Brown DW, Brinker JP, Kerstein KO, Herrmann JE, Keusch GT, Sonenshein AL, Tzipori S.** 2010. Efficacy, heat stability and safety of intranasally administered Bacillus subtilis spore or vegetative cell vaccines expressing tetanus toxin fragment C. Vaccine **28:**6658–6665.
36. **Rouanet C, Debrie AS, Lecher S, Locht C.** 2009. Subcutaneous boosting with heparin binding haemagglutinin increases BCG-induced protection against tuberculosis. Microbes Infect. **11:**995–1001.
37. **Dietrich J, Andersen C, Rappuoli R, Doherty TM, Jensen CG, Andersen P.** 2006. Mucosal administration of Ag85B-ESAT-6 protects against infection with *Mycobacterium tuberculosis* and boosts prior bacillus Calmette-Guerin immunity. J. Immunol. **177:**6353–6360.
38. **Reed SG, Coler RN, Dalemans W, Tan EV, DeLa Cruz EC, Basaraba RJ, Orme IM, Skeiky YA, Alderson MR, Cowgill KD, Prieels JP, Abalos RM, Dubois MC, Cohen J, Mettens P, Lobet Y.** 2009. Defined tuberculosis vaccine, Mtb72F/AS02A, evidence of protection in cynomolgus monkeys. Proc. Natl. Acad. Sci. U. S. A. **106:**2301–2306.