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BOVINE TUBERCULOSIS

Boosting BCG with inert spores improves immunogenicity and induces specific IL-17 responses in a murine model of bovine tuberculosis

M. Carmen Garcia-Pelayo [a, 1], Daryan A. Kaveh [a, *, 1], Laura Sibly [b, 2], Paul R. Webb [a], Naomi C. Bull [a], Simon M. Cutting [b], Philip J. Hogarth [a]

[a] Vaccine Immunology Team, Department of Bacteriology, Animal & Plant Health Agency (APHA), Addlestone, Surrey KT15 3NB, UK
[b] School of Biological Sciences, Royal Holloway, University of London, Egham, Surrey TW20 0EX, UK

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SUMMARY

Tuberculosis (TB) remains a global pandemic, in both animals and man, and novel vaccines are urgently required. Heterologous prime-boost of BCG represents a promising strategy for improved TB vaccines, with respiratory delivery the most efficacious to date. Such an approach may be an ideal vaccination strategy against bovine TB (bTB), but respiratory vaccination presents a technical challenge in cattle. Inert bacterial spores represent an attractive vaccine vehicle. Therefore we evaluated whether parenterally administered spores are efficacious when used as a BCG boost in a murine model of immunity against *Mycobacterium bovis*.

Here we report the use of heat-killed, TB10.4 adsorbed, *Bacillus subtilis* spores delivered via subcutaneous injection to boost immunity primed by BCG. We demonstrate that this approach improves the immunogenicity of BCG. Interestingly, this associated with substantial boosting of IL-17 responses; considered to be important in protective immunity against TB. These data demonstrate that parenteral delivery of spores represents a promising vaccine vehicle for boosting BCG, and identifies potential for optimisation for use as a vaccine for bovine TB.

1. Introduction

Tuberculosis (TB) caused by infection with *Mycobacterium tuberculosis* (*M. tuberculosis*) or *Mycobacterium bovis* remains one of the most important infectious diseases of man and animals, respectively; inflicting a huge cost in both health, welfare and financial terms [1]. At present the only available vaccine against TB is *M. bovis* bacille Calmette–Guérin (BCG), which demonstrates variable efficacy in humans and cattle [2,3]. In humans in particular, BCG induces some protection against childhood disseminated TB and tuberculous meningitis, but poor protection against pulmonary TB in adolescents and adults [4]. Despite this inconsistent performance, BCG remains the most widely used human vaccine in the world and due to its partial efficacy and proven safety record, is unlikely to be withdrawn. Hence, a great deal of research effort is targeted toward improving the efficacy of BCG by a number of approaches; prominent among which is boosting BCG with heterologous vaccines [5,6]. Although respiratory delivery appears to provide the most efficacious boost (reviewed in [7]), this may not be practical for a cattle vaccination strategy against bovine TB (bTB).

Optimal protection against TB requires CD4 T cells, as well as the effector cytokines IFN-γ and TNF-α (reviewed in Ref. [8]). However, studies have demonstrated, CD4 T cell derived IFN-γ is not an exclusive component of vaccine-mediated immunity [9] and identification of other critical components of protection remains elusive. The role of CD8 T cells in protection against TB is somewhat less clear and as yet poorly defined [8].

A number of heterologous boost vehicles for tuberculosis vaccines have been evaluated and (reviewed in [10]). Inert bioparticles, such as *Bacillus subtilis* spores, are effective delivery vehicles with immunomodulatory properties [11,12], capable of inducing protective immunity against disease [13,14], including showing

* Corresponding author. Tel.: +44 (0) 2080 269564; fax: +44 1932 357260.
E-mail address: daryan.kaveh@apha.gsi.gov.uk (D.A. Kaveh).
[1] These authors contributed equally to this work.
[2] Present address: Microbiological Services Division, Public Health England, Porton Down, Salisbury SP4 0JG, UK.

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promise via respiratory vaccination against TB [15]. These data, combined with the desirable properties of cost and scalability [16], suggest *B. subtilis* spores may represent an ideal vehicle candidate for boosting BCG in a vaccination strategy against bTB. As respiratory vaccination, however, remains a technical challenge in bovine species, we wished to evaluate the potential of such spores as an injectable BCG boost in a murine model of immunity against *M. bovis*.

We chose to evaluate an ESAT-6 family protein, RV0288 (TB10.4) [17] as a model antigen to adsorb onto heat-killed (HK) *B. subtilis* spores as a subunit BCG boost vaccine against bTB. TB10.4 is highly immuno-dominant in BCG immunised [18], and *M. bovis* infected mice [19,20], and cattle [21]. As TB10.4 is expressed by both BCG and *M. bovis*, it may therefore represent an ideal candidate in a BCG boost strategy.

Here we report the use of inert, TB10.4 adsorbed, *B. subtilis* spores delivered via subcutaneous injection (s.c) to boost immunity previously primed by BCG, in a murine *M. bovis* challenge model. We demonstrate that this approach induced significant immunological boosting, most notably of TB10.4-specific IL-17, which is considered to be important in protection against TB [22].

2. Materials & methods

2.1. Animals

Female BALB/c mice were obtained from SPF facilities at Charles River UK Ltd. and used at 8 weeks of age. All animals were housed in appropriate BSL3/SAPO 4 containment facilities at APHA. After challenge with *M. bovis*, all mice were weighed twice weekly and assessed for clinical signs of tuberculosis daily.

2.2. Strains and antigens

HU58 is a non-domesticated strain of *B. subtilis* isolated from the human gastrointestinal (GI) tract [23]. The BCG vaccination strain was the human vaccine *M. bovis* BCG Danish 1331 prepared as per manufacturer's instructions (SSI, Copenhagen, Denmark). *M. bovis* strain AF2122/97 was used for all challenge experiments as described [19]. Recombinant mycobacterial protein TB10.4 (Proteix sro., Prague, Czech Republic) was used for stimulation as described previously [19].

2.3. Antisera

Polyclonal sera reactive to TB10.4 was obtained from previous experiments. Briefly, mice were immunised three times at two week intervals with 10 μg TB10.4 emulsified in MPL/DDA adjuvant. Pooled serum was prepared from terminal cardiac bleeds taken two weeks after final immunisation.

2.4. Production of B. subtilis spores

Strains were routinely grown in Difco sporulation medium (DSM, Difco, UK), and spores were prepared in large quantities using growth on solid DSM [24]. Spores were washed and purified before use, and aliquots were stored at −20 °C until use. For preparation of HK HU58 spores, they were autoclaved (121 °C, 15 lb/in^2, 30 min) before use.

2.5. 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, and the pellet was washed twice with PBS (at the same pH as that in the binding mixture). Adsorption was evaluated by extraction and solubilisation of adsorbed proteins. Spore coat protein extractions were done as described previously [25] by suspending the spore pellet in spore coat extraction buffer, incubation at 68 °C for 1 h, and gel electrophoresis, followed by detection of bound protein by Western blotting using TB10.4 reactive sera.

2.6. Immunisation, mycobacterial enumeration and challenge

There were six separate treatment groups (n = 22) of mice: Placebo; BCG; BCG/Spore-TB10.4; BCG/Spore-empty; Spore-TB10.4 and Spore-empty. Groups of mice were immunised with a single intradermal (i.d.) injection (50 μl) containing 2×10^5 CFU of BCG or PBS placebo, in the base of the tail. Six weeks later groups of placebo or BCG immunised mice were boosted twice subcutaneously (s.c. 50 μl) with 10^{10} TB10.4 adsorbed (1.5 μg TB10.4/dose) *B. subtilis* spores (Spore-TB10.4) or control spores (Spore-empty), four weeks apart. Four weeks later, six mice per group were euthanised for immunological analyses, and all remaining mice were challenged via intranasal route (i.n.) with ~300 CFU *M. bovis* as described previously [26]. Four weeks later, 6–8 mice/group were euthanised, lungs and spleens removed, homogenised, serially diluted and plated out onto modified Middlebrook 7H11 agar medium [27]. Bacterial colonies were enumerated four weeks later following incubation at 37 °C. For survival data, remaining mice (n = 8) were monitored for a further 318 days post-challenge, or until clinical manifestations of TB necessitated euthanasia at a humane endpoint according to a clinical scoring system.

2.7. Cell isolation and stimulation

Following euthanasia, spleens were aseptically removed and cells prepared as previously described [19]. Following washing (300 g/8 min), all cells were re-suspended at 5×10^6/ml for assays. Cells were cultured with specific antigen as stated, with each individual protein or peptide antigen at a final concentration of 2 μg/ml for all assays.

2.8. IFN-γ ELISPOT

Cells were incubated with TB10.4 protein, and the frequency of antigen-specific IFN-γ secretors detected by ELISPOT (Mabtech, Stockholm, Sweden), as previously described [19].

2.9. Cytokine production

Cells (5×10^6/ml) were cultured in the presence of antigen for 72 h (37 °C/5% CO_2) prior to harvest of supernatant. Production of IFN-γ, TNF-α, IL-2, IL-4, IL-6, IL-10, IL-12, and IL-17 was measured by multiplex chemi-luminescent ELISA using the Meso Scale Discovery platform (MSD®, Rockville, Maryland) according to manufacturer's instructions.

2.10. Flow cytometry

For intracellular cytokine staining (ICS), cells were stimulated with TB10.4 antigen and anti-CD28 (BD Biosciences, Oxford, UK) as previously described [19]. They were surface stained with pre-titrated antibodies: CD4–Brilliant Violet (BV) 711, CD44–BV785, CD62L–BV605, CD25-BV421, CD19-Alexa Fluor (AF) 700, TER119-AF700 (all Biolegend, London, UK), CD8-APC-H7 (BD Bioscience), CD16/32-AF700, CD27-PE and LIVE/DEAD® Fixable Yellow Dead