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BCG *Δzmp1* vaccine induces enhanced antigen specific immune responses in cattle

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ABSTRACT

Mycobacterium bovis (*M. bovis*) causes major economy and public health problem in numerous countries. In Great Britain, despite the use of a test-and-slaughter strategy, the incidence of bovine tuberculosis (bTB) in cattle has steadily risen in recent years. One strategy being considered to reduce the burden of bTB in cattle is the development of an efficient vaccine. The only current potentially available vaccine against tuberculosis, live attenuated *M. bovis* bacille Calmette–Guérin (BCG), has demonstrated variable efficacy in both humans and cattle and the development of improved vaccination strategies for cattle is a research priority. In this study we assessed the immunogenicity in cattle of two recombinant BCG strains, namely BCG Pasteur *Δzmp1::aph* and BCG Danish *Δzmp1*. By applying a recently defined predictive immune-correlate of protection (T cell memory responses measured by cultured ELISPOT), we have compared these two recombinant BCG with wild-type BCG Danish SSI. Our results demonstrated that both strains induced superior T cell memory responses compared to wild-type BCG. These data provide support for the prioritisation of testing BCG Danish *Δzmp1* in vaccination/*M. bovis* challenge studies to determine its protective efficacy.

1. Introduction

Mycobacterium bovis (*M. bovis*), a member of the *Mycobacterium tuberculosis* complex, is the causative agent of bovine tuberculosis (bTB) which causes major economy and public health problem in numerous countries [1]. In Great Britain, despite the use of a test-and-slaughter strategy, the incidence of bTB in cattle has steadily risen in recent years. One strategy being considered to reduce the burden of bTB in cattle is the development of an efficient vaccine. The only current licensed vaccine against tuberculosis, live attenuated *M. bovis* bacille Calmette–Guérin (BCG), has demonstrated variable efficacy in both humans and cattle [2,3]. However, in recent years there have been several advances in cattle vaccine development [4,5], with the most promising approaches being prime/boost strategies combining BCG and DNA, protein or virally-vectored vaccines. As such, the development of cattle vaccines has closely mirrored those of human TB vaccine development programmes. Improved vaccines based on recombinant BCG with mutations enhancing BCG immunogenicity are also being developed and two such vaccines are the subject of the present study [4,5].

One of the proposed mechanisms involved in the success of tuberculosis infection is the ability of pathogenic mycobacteria to inhibit phagosome–lysosome fusion [6]. One potential consequence of this is that *M. tuberculosis* antigens are not efficiently presented by MHC class I [7,8] and MHC class II antigens [9,10]. It has been previously reported that mycobacterial Zmp1 inhibits phagolysosome maturation by preventing inflamasome activation [11]. Zmp1 encodes a zinc metalloprotease with structural similarity to human proteases Neprilysin and endothelin converting enzyme [12]. Its activity optimum is at a slightly acidic pH [13]. A recombinant BCG vaccine based on BCG Pasteur lacking *zmp1* gene (BCG Pasteur *Δzmp1::aph*) has been shown to have increased immunogenicity in mice both *in vitro* and *in vivo*. This vaccine strain contained an antibiotic resistance selection marker, and to overcome this limitation, an unmarked BCG lacking the *zmp1* (BCG Danish *Δzmp1*) gene was constructed. This construct was based also on BCG Danish to reflect the use of BCG Danish SSI currently as the gold standard vaccine strain both in human and cattle TB vaccine development.

Building upon the synergy between cattle and human TB vaccine programmes, in the current study we have assessed two potential bTB cattle vaccines based on BCG Denmark, designated BCG

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Danish *Δzmp1* and BCG Pasteur *Δzmp1::aph*. We investigated the ability of these vaccines to induce cell mediated immunity in cattle with a view to assessing their suitability for future efficacy studies.

2. Materials and methods

2.1. Ethics

All animals were housed at the Animal Health and Veterinary Laboratories Agency (AHVLA) at the time of blood sampling and procedures were conducted within the limits of a United Kingdom Home Office Licence under the Animal (Scientific Procedures) Act 1986, which was approved by the local ethical review committee.

2.2. Animals and vaccination schedule

Male Holstein–Friesian calves (approximately 6 months old at the beginning of the study) were recruited from tuberculosis-free GB farms. Prior to inclusion of these animals into the experiments, all cattle tested negative in the whole-blood gamma interferon (IFN-γ) assay using the BOVIGAM bovine IFN-γ ELISA kit (Prionics, Switzerland), applying the standard GB interpretation criteria of this test (reactivity to purified protein derivative of *M. bovis* (PPD-B)—purified protein derivative of *M. avium* (PPD-A) < 0.1 $OD_{450\,nm}$). Although, IFN-γ responses in 15 and 11 cattle out of 20 for PPD-A and PPD-B, respectively, were >0.1 $OD_{450\,nm}$, all PPD-B minus PPD-A IFN-γ responses for these animals were <0.1 $OD_{450\,nm}$ as stated above. All animals were housed in an appropriate biosafety levels containment facilities and allowed access to food and water *ad libitum*.

The vaccines BCG WT, BCG Danish *Δzmp1* or BCG Pasteur *Δzmp1::aph* (see Section 2.3 for a description of their production) were administered to groups of 5 cattle via the subcutaneous route. All vaccines were prepared from frozen stocks 1 h prior to immunisation. Doses (given in 1 ml volumes) for all groups were confirmed on the basis of colony forming units (CFU) by culture; BCG WT, BCG Danish *Δzmp1* and BCG Pasteur *Δzmp1::aph* vaccinated animals received 3×10^6, 1.2×10^6 and 3.1×10^5 CFU, respectively.

2.3. Vaccines

BCG Danish wild-type (BCG WT), BCG Danish *Δzmp1* and BCG Pasteur *Δzmp1::aph* were provided by University of Zurich. The construction of the BCG Pasteur *Δzmp1::aph* knockout mutant has been described previously [11]. Briefly, BCG Pasteur *Δzmp1::aph* is *zmp* deletion mutant of a streptomycin resistant BCG Pasteur strain. Two consecutive *Nhe*I fragments within genomic *zmp1* were substituted by an *aph* cassette conferring kanamycin resistance by transformation with an rpsL-based suicide vector [14]. The strain has been described previously. Generation of BCG Danish *Δzmp1*: Recombinant DNA techniques were initially done in *Escherichia coli* and applied to clone a vector for generation of the unmarked BCG *zmp1* deletion mutant. Antibiotics were used at the following concentrations: Ampicillin 50 μg/ml, kanamycin 50 μg/ml for *E. coli*; kanamycin 50 μg/ml, hygromycin 25 μg/ml for BCG. Sucrose for counter selection was applied at 2% (vol/vol). Briefly, plasmid ptrpA-1-rpsL_zmp [11] was digested with *Nhe*I and two consecutive *Nhe*I fragments were deleted and substituted by "triple translation stop insert" to result in plasmid ptrpA_rpsL-zmp3xstop. The *Not*I-fragment carrying the inactivated *zmp* allele was subsequently cloned into *E. coli* plasmid pGEM7_sacB_aph_hyg to result in plasmid pzmp_sacB_aph_hyg. Plasmid pzmp_sacB_aph_hyg was subsequently transformed into electrocompetent BCG Danish 1331 (vaccine lot) and selected on Middlebrook 7H10-oleic acid dextrose albumin (OADC) with appropriate antibiotics. BCG was made competent as previously described [15]. Integration of suicide vector and deletion of *zmp1* from the genome of BCG after sucrose counter selection was tested by Southern blot analyses using *Hind*III digested genomic DNA and a *zmp1* upstream probe. Removal of vector backbone was phenotypically confirmed by antibiotic susceptibility testing (KanS, HygS). BCG strains were grown in Middlebrook 7H9 supplemented with OADC (10% v/v) until **OD600** = 0.4 to 0.6. Bacterial aliquots were frozen and stored at −80 °C. Serial dilutions of thawed aliquots were plated on 7H10 OADC agar plates and the number of colony forming units was determined after 3–4 weeks of incubation at 37 °C.

2.4. Antigens

Purified protein derivatives PPD-A and PPD-B prepared from *M. avium* and *M. bovis*, respectively, were obtained from Prionics, (Schlieren, Switzerland) and used in culture and cellular immunoassays at a dilution of 1:100. *M. tuberculosis* recombinant protein Ag85A was provided by Lionex Diagnostics and Therapeutics GmbH (Braunschweig, Germany) and used at 5 μg/ml in cellular immunoassays. Pokeweed Mitogen (PWM; Sigma-Aldrich, Poole, UK) was included as a positive control at a final concentration of 5 μg/ml, while tissue culture medium (RPMI 1640 [Life Technologies, Poole, UK] supplemented with 5% Foetal Bovine Serum [Sigma], non-essential amino acids [Sigma], 5×10^{-5} M 2-mercaptoethanol, 100 U/ml penicillin and 100 mg/ml streptomycin sulphate [Gibco, UK]) was used as a negative control.

2.5. IFN-γ enzyme-linked immunosorbent assay (ELISA)

Measurement of antigen stimulated IFN-γ in whole blood cultures was determined by culturing duplicate 250 μl aliquots of whole blood in the presence of PPD-A, PPD-B, Ag85A or in RPMI1640 media alone. Plasma supernatants were harvested following 20 h incubation at 37 °C/5% CO_2 and tested for IFN-γ using the BOVIGAM bovine IFN-γ ELISA kit (Prionics, Switzerland). IFN-γ responses were evaluated at pre-vaccination (week 0) and post vaccination weeks 2, 4, 8 and 10. For reporting, IFN-γ optical density values at 450 nm (OD 450 nm) were transformed into units of pg/ml by generating a standard curve using recombinant bovine IFN-γ (Endogen, USA).

2.6. Short-term T-cell lines for cultured ELISPOT and intracellular cytokine staining assay

Peripheral blood mononuclear cells (PBMC) were isolated from heparinised blood by Histopaque-1077 (Sigma-Aldrich) gradient centrifugation and cells were resuspended at 1×10^6/ml in tissue culture medium. Short-term bovine T-cell lines were generated as previously described [16]. In brief, PBMC at 2×10^6 cells/ml (1 ml aliquot) were stimulated with PPD-B in a 24 well plate and incubated for 14 days at 37 °C/5% CO_2. During these 14 days of incubation, recombinant human IL-2 (Sigma, UK) at 10 U/ml was added to the PBMC cultures at days 3 and 7. At days 10 and 12, half of the supernatant from PBMC cultures was removed and replaced by fresh culture media. On day 14, PBMC cultures were washed twice with HBSS solution (Life Technologies, UK) by centrifugation (300 g for 5 min), viable cells were counted and re-suspended at required cell titres for cultured ELISPOT and intracellular cytokine staining (ICS) analysis.

2.6.1. IFN-γ cultured ELISPOT assay [16]

On day 14 of the PBMC cultures, fresh PBMC were used as antigen-presenting cells (APCs) and added to required wells at