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Parenteral adenoviral boost enhances BCG induced protection, but not long term survival in a murine model of bovine TB

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ABSTRACT

Boosting BCG using heterologous prime-boost represents a promising strategy for improved tuberculosis (TB) vaccines, and adenovirus (Ad) delivery is established as an efficacious boosting vehicle. Although studies demonstrate that intranasal administration of Ad boost to BCG offers optimal protection, this is not currently possible in cattle. Using Ad vaccine expressing the mycobacterial antigen TB10.4 (BCG/Ad-TB10.4), we demonstrate, parenteral boost of BCG immunised mice to induce specific $CD8^+$ IFN-γ producing T cells via synergistic priming of new epitopes. This induces significant improvement in pulmonary protection against *Mycobacterium bovis* over that provided by BCG when assessed in a standard 4 week challenge model. However, in a stringent, year-long survival study, BCG/Ad-TB10.4 did not improve outcome over BCG, which we suggest may be due to the lack of additional memory cells ($IL\text{-}2^+$) induced by boosting. These data indicate BCG-prime/parenteral-Ad-TB10.4-boost to be a promising candidate, but also highlight the need for further understanding of the mechanisms of T cell priming and associated memory using Ad delivery systems. That we were able to generate significant improvement in pulmonary protection above BCG with parenteral, rather than mucosal administration of boost vaccine is critical; suggesting that the generation of effective mucosal immunity is possible, without the risks and challenges of mucosal administration, but that further work to specifically enhance sustained protective immunity is required.

1. Introduction

Tuberculosis (TB) caused by infection with *Mycobacterium 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]. Despite this inconsistent performance, BCG remains one of the most widely used human vaccines in the world and due to its partial efficacy and proven safety record, is unlikely to be withdrawn.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 towards improving the efficacy of BCG by a number of approaches; prominent among which is boosting BCG with heterologous vaccines [4,5].

It is clear that optimal protection against TB requires CD4 T cells, as well as the effector cytokines IFN-γ and TNF-α [reviewed in 6]. However, as other studies demonstrate, CD4 T cell derived IFN-γ is not an exclusive component of vaccine-mediated immunity [7] 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 [6].

A number of viral heterologous boost vehicles for tuberculosis vaccines have been evaluated [reviewed in 8], with modified Vaccinia Ankara (MVA) and Adenovirus (Ad) recently progressing to clinical trials [9,10].

We previously reported the efficacy of an ESAT-6 protein family member, RV3019c as a subunit vaccine against bovine TB [11]. Another member of this family, RV0288 (TB10.4) [12], has been found to be highly immuno-dominant in BCG immunised, *M. tuberculosis* [13] and *M. bovis* infected mice [14,15] and human TB patients [12]. As this antigen is expressed by BCG, *M. tuberculosis* and *M. bovis*, it may therefore represent an ideal candidate as a boost vaccine following BCG immunisation. Indeed, studies have reported the efficacy of multivalent adenoviral vaccines incorporating TB10.4 against both *M. tuberculosis* [16–19] and *M. bovis*

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[20]. Although murine studies predominantly demonstrate intranasal/respiratory delivery to be optimal, given the One Health approach of our research, and that respiratory vaccination remains a technical challenge in bovines, we wished to evaluate the potential of Ad expressing TB10.4 as an injectable BCG boost in a murine model of immunity against *M. bovis*.

We first established the efficacy of TB10.4 protein vaccination against *M. bovis*, then expressed TB10.4 in type 5 Ad (Ad-TB10.4). We subsequently parentally boosted BCG induced immunity with Ad-TB10.4. BCG/Ad-TB10.4 prime-boost increased the frequency of $CD8^+$ IFN-γ^+ T cells via recognition of additional epitopes, but not the frequency of multifunctional $CD4^+$ or $CD8^+$ T cells. Significantly, systemic boosting increased pulmonary protection against an *M. bovis* challenge. Despite this encouraging increase in protection, long term survival was unchanged, which we suggest may be due to the lack of additional memory cell (IL-2^+) responses. These data indicate BCG-prime/parenteral-Ad-TB10.4-boost to be a promising candidate for future development, but also highlight the need for further understanding of the mechanisms by which vaccination induces more effective and sustained protective immunity against TB.

2. Materials and methods

2.1. Ethics

All animal work was carried out in accordance with the UK Animal (Scientific Procedures) Act 1986; under appropriate licences. The study protocol was approved by the APHA Animal Use Ethics Committee (UK PCD number 70/6905).

2.2. 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 Advisory Committee on Dangerous Pathogens (ACDP) Containment Level 3 (equivalent to BSL3) facilities at APHA, according to the Code of Practice for the Housing and Care of Animals Bred, Supplied or Used for Scientific Purposes [21]. All animals were randomly assigned to treatment groups, and group housed (6 or 8 mice per cage, as specified below), with water and food *ad libitum* and provided with maximum environmental enrichment (e.g. toys, nesting and seeds) as was possible under biological containment. For immunological analyses, provision of normally distributed data required minimum sample size $n = 6$ (Kolmogorov and Smirnov test). For enumeration of bacterial load, $n = 8$ is the minimum required to detect a 0.5 log reduction, at 95% power, assuming a standard deviation of 0.25 log10 based on previous laboratory data. Similar rationale was used for use of $n = 8$ in 'survival' analyses groups.

After challenge with *M. bovis*, all mice were weighed twice weekly and assessed for clinical signs of tuberculosis daily. Clinical signs of tuberculosis in mice manifest as: weight loss, hunching, piloerection, unresponsiveness to stimuli and difficulty breathing. Animals were scored daily for any of these clinical criteria using an in-house scoring system approved by the APHA Named Veterinary Surgeon (NVS) and UK Home Office Animal Inspectorate, and specified in the relevant licences. Animals were euthanized at a pre-determined humane endpoint based on these clinical criteria.

2.3. Mycobacteria, mycobacterial enumeration and antigens

The 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 [14]. Recombinant mycobacterial protein TB10.4 (Proteix sro, Prague, Czech Republic) was used for immunisation and stimulation as described [14]. Additionally, peptides mapping TB10.4 were used for ELISPOT stimulation (Pepscan, Lelystad, The Netherlands). 13 of the possible 14 peptides (16mers, overlapping by 10 aa; no peptide was available for P7) mapping the entire protein sequence were used individually, or as a pool.

Mycobacteria were enumerated in aseptically removed spleen and lungs from animals after euthanasia. Organs were homogenised, serially diluted and plated out onto modified Middlebrook 7H11 agar medium as previously described [22]. Bacterial colonies were enumerated four weeks later following incubation at 37 °C. CFU data were log transformed ($Y = \log[Y]$) and expressed as $\log_{10}$/organ.

2.4. Adenovirus-TB10.4 construction

The mycobacterial gene TB10.4 was amplified from *M. tuberculosis* H37Rv genomic DNA using primers: Rv0288 BamF (GCGGATCCATGTCGCAAATCATGTACAAC, BamHI site) and Rv0288 XBAR (ATTATCTAGACTAGCCGCCGCCCCATTTGGCGGCTTC, XBAI site). The amplification conditions were: 94 °C for 15 s, followed by 30 cycles of 15 s at 94 °C, hybridization and extension at 68 °C for 3 min, then a final extension for 3 min at 68 °C. PCR products and adenoviral shuttle vector pVQ Ad5CMV K were digested with *Bam*HI and *XBaI*, ligated following standard protocols, and transformed into *E. coli* DH5α. Sequencing was used to confirm the correct sequence and orientation of the cloned fragments. Subsequent cloning of pVQ Ad5CMV-RV0288 into pVQ HuAd5 backbone and amplification was performed by Viraquest Inc. (North Liberty, Iowa), as described [23].

2.5. Immunisation and challenge

2.5.1. Protein vaccinations

The protein sub-unit vaccination/challenge schedule is summarized in Fig. 1A. Mice ($n = 8$) were immunised via the subcutaneous route (s.c.) three times (two weeks apart) with 100 μl containing 10 μg of TB10.4 (RV0288) protein emulsified in MPL/DDA adjuvant consisting of 25 μg detoxified Lipid A (MPL) (Avanti polar Lipids, Alabaster, Alabama), dissolved in 0.2% triethylamine. This was mixed by multiple syringing with 250 μg of dimethyldioctadecyl ammonium bromide (DDA) micelles (created by heating to 80 °C, E. Agger SSI, *pers. comm.*) (both Sigma, Poole, UK). Control mice were immunised with MPL-DDA adjuvant alone. A separate group of mice were immunised with a single injection of 2×10^5 Colony Forming Units (CFU) of BCG intradermally (i.d.) in the base of the tail. Four weeks following final sub-unit immunisation, mice were challenged via intravenous route (i.v.) which is less stringent than intranasal challenge [24], with 1000 CFU of *M. bovis*. Four weeks later they were euthanized, and lungs and spleens removed for bacterial enumeration.

2.5.2. Ad-TB10.4 dose response

Mice ($n = 3$), were immunised with 100 μl containing: 5×10^6, 5×10^7 or 5×10^8 Plaque Forming Units (PFU) of Ad-TB10.4 or Ad-empty, (i.d.) in the base of the tail. Twelve days post-immunisation (p.i.) they were euthanized and spleen cells prepared. Specific $CD4^+$ T cell responses were assessed using intracellular staining (ICS) as previously described [14], following stimulation with 2 μg/ml TB10.4 protein.

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