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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widely used human vaccines in the world and due to its partial efficacy and proven safety record, is unlikely to be withdrawn.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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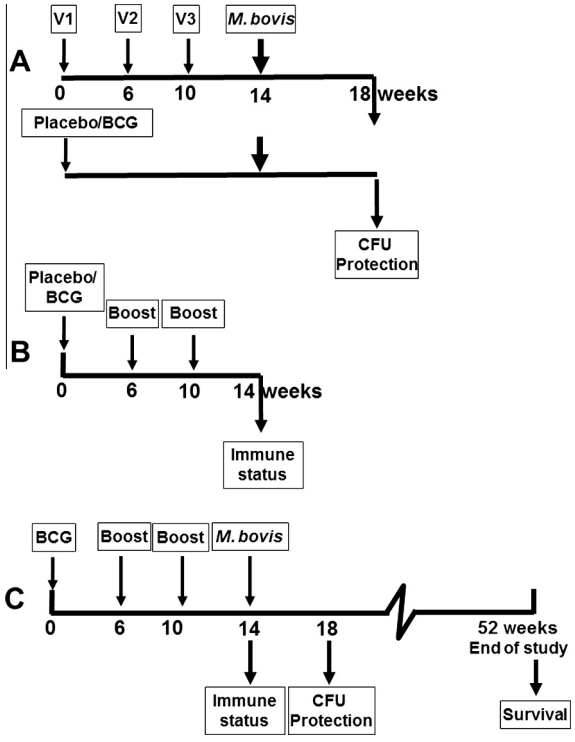

Fig. 1. Vaccination regimen schedules. Mice were immunised with BCG, TB10.4/adjuvant or adjuvant alone and bacterial burden assessed 4 weeks after intravenous challenge with 1000 CFU of *M. bovis* (A). BCG immunised or naïve mice were boosted i.d. with two doses of either Ad-TB10.4 or Ad-empty and the immune status was evaluated prior to challenge (B). Bacterial burden and survival rate in BCG Prime-Boost immunised mice were assessed after intranasal challenge with 300 CFU of *M. bovis* (C).

2.5.3. BCG-prime boost experiments

The prime-boost vaccination/challenge schedule is summarized in Fig. 1B and C. There were six separate treatment groups ($n = 22$/group) of mice: Placebo; BCG; BCG/Ad-TB10.4; BCG/Ad-empty; Ad-TB10.4 and Ad-empty. Groups of mice were immunised with a single injection (50 μl) containing 2×10^5 CFU of BCG, i.d. in the base of the tail. Six weeks later groups of placebo or BCG immunised mice were boosted twice four weeks apart, in the same location with 5×10^7 PFU (50 μl) of Ad-TB10.4 or Ad-empty. Four weeks later, six mice per group were euthanized for immunological analyses, and all remaining mice were challenged via the intranasal route (i.n.) with 300 CFU *M. bovis* as previously described [24]. Bacterial loads in spleen and lungs in eight mice per group were enumerated four weeks after challenge as previously described above, and for survival data, eight mice per group were monitored for a further 318 days post challenge, or until clinical manifestations of TB necessitated euthanisation at a humane endpoint according to a pre-determined clinical scoring system.

2.6. Cell isolation and stimulation

Following euthanasia, spleens were aseptically removed and cells prepared as previously described [14]. Following washing (300 g/8 mins), 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.

2.7. IFN-γ ELISPOT

Cells were incubated with TB10.4 protein, a pool of TB10.4 peptides, or the individual peptides as stated and the frequency of antigen-specific IFN-γ secretors (expressed as Spot Forming Units or SFU) detected by ELISPOT (Mabtech, Sweden), as previously described [14].

2.8. Cytokine production

Cells (5×10^6/ml) were cultured in the presence of antigen (TB10.4 protein) 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.

2.9. Flow cytometry

For intracellular staining (ICS), cells were stimulated with antigen (TB10.4 protein) and anti-CD28 (BD Biosciences, Oxford, UK) as previously described [14]. 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, CD3-FITC, CD27-PE and LIVE/DEAD® Fixable Yellow Dead Cell Stain ('YeViD', Life Technologies, Paisley, UK). Subsequently, cells were washed, fixed/permeabilized and stained by ICS with IFN-γ-APC (BD Bioscience), IL-2-PE-Cy7 and TNF-α-FITC as previously described [14]. For MHC class II-peptide tetramer staining, spleen cells were enriched for CD4 T cells (>90% purity) using the Dynal Mouse CD4 Negative Isolation Kit (Life Technologies) as per manufacturer's instructions. Cells were then stained (45 min/37 °C/5% CO_2) in culture media with TB10.4 peptide: MHCII I-$A^{(d)}$ (SSTHEANTMAMMARDT) tetramer-complex, labeled with APC; or I-$A^{(d)}$ negative control (PVSKMRMATPLLMQA) tetramer-APC (both provided by NIH Tetramer Core Facility, Atlanta, Georgia). Following washing, they were stained (15 min/4 °C) in staining buffer with CD4-Brilliant Violet (BV) 711, CD44-BV785, CD62L-BV605, CD25-BV421, CD19-Alexa Fluor (AF) 700, TER119-AF700, CD8-APC-H7, CD16/32-AF700, CD3-FITC, CD45RB-PE, and YeViD, washed and fixed with Cytofix (BD Bioscience). All antibody conjugates were purchased from eBioscience (Hatfield, UK) except where stated. Data were acquired using a SORP LSR Fortessa (BD Bioscience) (utilizing a 532 nm laser for PE and PE-Cy7) and analysed on Flowjo v.10.0.7 (Tree Star, USA) software. All analyses were gated on a minimum of 100,000 live lymphocytes.

2.10. Statistical analysis

Non-survival data were analysed by 1-way ANOVA with Bonferroni's *post hoc* test, using GraphPad Prism 5 software (GraphPad, USA). Prior to analysis, mycobacterial counts were $\log_{10}$ transformed and ELISA/ELISPOT data were normalised by subtracting the unstimulated from the antigen-stimulated culture values. Survival data was analysed with the Mantel-Cox Log Rank test. Differences with a $\rho < 0.05$ (or $\rho = 0.06$ for survival data) were considered significant and denoted with $*$, $\rho < 0.01$ with $**$, $\rho < 0.001$ with $***$ and $\rho < 0.0001$ with $****$.

3. Results

3.1. TB10.4 induces significant protection against virulent mycobacterial challenge as a protein subunit vaccine

As an immuno-dominant antigen in BCG immunised mice [14,15], we investigated the potential of TB10.4 as a protective vaccine antigen for bovine TB, with a view for use in a BCG

prime-boost approach. Mice ($n = 8$) were immunised three times with TB10.4 emulsified in adjuvant or with a single dose of BCG. Mice were challenged 1 month after final immunisation and lung bacterial load measured 4 weeks later. Intravenous (i.v.) challenge, which is less stringent than intranasal challenge [24], and our preferred model for evaluating non-BCG component vaccines, was employed in this study. As shown in Fig. 2, TB10.4 induced significant protection (0.64 Log_{10}, $\rho < 0.05$ *vs* adjuvant control) in the lungs, as did BCG (1.43 Log_{10}, $\rho < 0.001$ *vs* adjuvant control). In the spleen protection induced by TB10.4 was less, but still significant (0.30 Log_{10}, $\rho < 0.05$), compared to BCG (1.8 Log_{10}, $\rho < 0.001$ *vs* adjuvant control). These data clearly indicate the potential for TB10.4 as a protective antigen for *M. bovis* challenge.

3.2. Optimisation of Ad-TB10.4 dose

Having demonstrated the potential of TB10.4 as a protective sub-unit vaccine, we cloned TB10.4 into pVQ Ad5 vector to produce Ad-TB10.4 vaccine as described in Material & Methods. The optimum dose of Ad-TB10.4 for subsequent use in prime-boost experiments was determined by dose-titration. Mice ($n = 3$) were immunised once with 5×10^6, 5×10^7 or 5×10^8 PFU of Ad-TB10.4 or 5×10^8 PFU Ad-Empty control and immune responses assessed 12 days later. ICS analyses (Fig. S2) revealed that whilst 5×10^6 and 5×10^8 PFU induced significant frequencies of $CD4^+$ IFN-γ^+ cells ($\rho < 0.01$ and $\rho < 0.05$, respectively) compared to the control vector, 5×10^7 PFU induced the highest frequency, although not quite significant ($\rho = 0.07$). No $CD8^+$ response was observed (data not shown). Thus, 5×10^7 PFU was chosen as the dose for further experiments.

3.3. Ad-TB10.4 boosts both IFN-γ^+ cell frequency, and breadth of epitope recognition following BCG immunisation

To assess the boosting effect of Ad-TB10.4, BCG immunised or naïve mice ($n = 6$) were boosted six weeks after vaccination with two doses of either Ad-TB10.4 or Ad-empty, as illustrated in Fig. 2A. In order to ensure maximum effect, the common strategy [reviewed in 24] of two boosts were used. To establish immune status prior to challenge, one month after final vaccination, spleen cells from individual mice in each vaccine regimen were subjected to IFN-γ ELISPOT assay (Fig. 3). As shown in Fig. 3A, BCG induced higher responses than the PBS control immunisation (425 *vs* 49 SFU), as did BCG/Ad-empty (583 *vs* 49 SFU). Interestingly, Ad-TB10.4 alone, induced a higher response than BCG (1342 SFU, $\rho < 0.001$), indicating the ability of adenovirus delivery to initiate a strong IFN-γ response. Strikingly, Ad-TB10.4 boost synergised with BCG, inducing significantly higher responses (2171 SFU) than any other regimen ($\rho < 0.001$ *vs* all groups) and 50% higher than Ad-TB10.4 alone.

In addition, we examined responses of pooled spleen samples from the BCG, BCG/Ad-TB10.4, BCG/Ad-empty and Ad-TB10.4 groups to 13 individual overlapping peptides mapping the protein sequence of TB10.4, or a peptide pool. As shown in Fig. 3B, BCG induced a response to one epitope only (P13-14), whilst the use of Ad-TB10.4 alone induced the recognition of an additional epitope(s) contained within peptides 3–5 and not that of P13. The combination of BCG and Ad-TB10.4 did not induce responses to any epitopes other than the individual vaccine components themselves, but boosted responses to peptides 3–5 (but not P13-14) by 50%. BCG/Ad-empty only induced a response to the P13-14 epitope.

The observation that Ad-TB10.4 boosted BCG induced responses by increasing the breadth of epitope recognition, was further confirmed when we examined the frequency of CD4 T cells from individual mice in each vaccine regimen specific to the single dominant TB10.4 P13 peptide using an MHC class II tetramer–peptide complex (Fig. 3C). These data show that BCG/Ad-TB10.4 failed to increase the frequency of tetramer$^+$ (i.e. P13-specific) $CD27^-$ $CD4^+$ T cells above that induced by BCG alone, indicating the increased IFN-γ responses in boosted animals were not due to stimulation of P13-specific CD4 T cells.

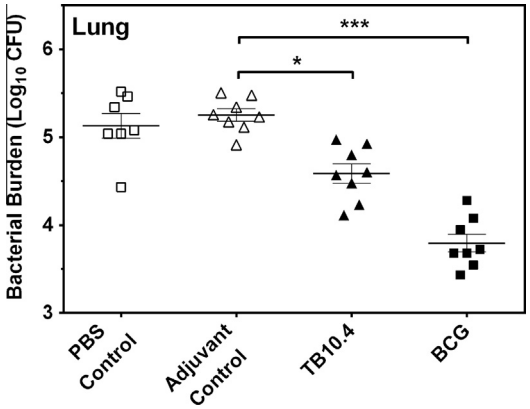

Fig. 2. TB10.4 induces significant protection against virulent mycobacterial challenge as a protein subunit vaccine. Mice ($n = 8$) were immunised once i.d. with BCG, or three times s.c. with the indicated vaccine. Spleen and Lungs from individual mice were removed 4 weeks following challenge and bacteria enumerated. Data represent the mean bacterial burden per organ ± SE. $^*\rho < 0.05$, $^{***}\rho < 0.001$ *vs* adjuvant controls; ANOVA with Bonferroni's *post hoc* test. Data are representative of one of two independent experiments.

3.4. Adenovirus vector immuno-modulates BCG induced $CD4^+$ T cell responses

To further analyse the immune response initiated by the different vaccination regimes, individual spleen cell cultures from each regimen were cultured with TB10.4 protein and three day supernatants assayed by chemiluminescent ELISA. As shown in Fig. 4, BCG initiated a TB10.4-specific recall response to all cytokines measured, although none were significantly higher than in naïve control mice ($\rho < 0.001$). Surprisingly, and in contrast to the IFN-γ ELISPOT results, BCG/Ad-TB10.4 failed to boost any of these responses and Ad-TB10.4 alone failed to initiate a substantial recall response.

In contrast, BCG/Ad-empty increased TB10.4 recall responses: inducing 100% more IL-4, IL-6 ($\rho < 0.05$) and IL-12 ($\rho < 0.001$); 300% more IFN-γ and IL-10 and a striking 800% more IL-17 ($\rho < 0.001$) than BCG alone. These data indicated a strong immuno-modulatory effect of the Ad-empty boost despite not being co-administered with BCG.

3.5. Ad-TB10.4 significantly boosts antigen-specific $CD8^+$ but not $CD4^+$ T cells

The capacity of Ad-TB10.4 to boost both CD8 as well as CD4 T cell responses was interrogated by ICS and flow cytometric analysis (gating strategy Fig. S1). As described in Fig. 5A, and previously reported [14,15], BCG induced a significant ($\rho < 0.0001$ *vs* control) frequency of IFN-γ^+/IL-2^+/TNF-α^+ multifunctional CD4 T cells. The frequency of these cells was not boosted by Ad-TB10.4. Contrastingly, BCG immunisation induced a poor antigen-specific CD8 T cell response, but Ad-TB10.4 boost significantly increased the frequency of both IFN-γ^+ TNF-α^+ bi-functional and of IFN-γ^+ monofunctional $CD8^+$ T cells ($\rho < 0.001$ vs BCG) by 100% and 500%, respectively (Fig. 5B). As observed in the initial titration, no $CD8^+$ T cell response to Ad-TB10.4 in the absence of BCG was observed

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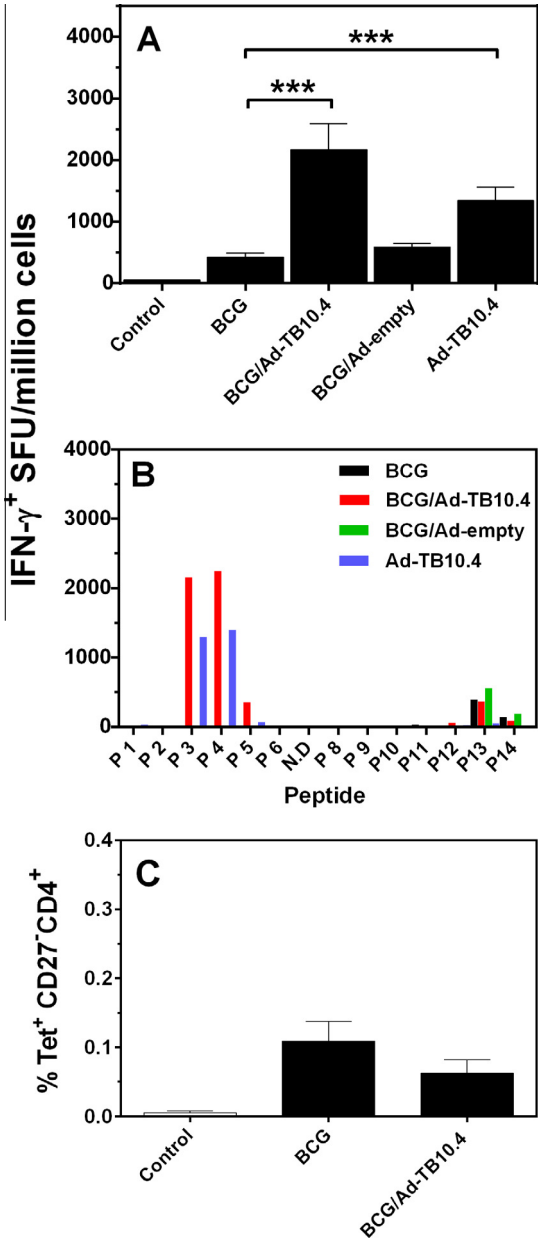

Fig. 3. Ad-TB10.4 boosts IFN-γ^+ cell frequency and breadth of epitope recognition. BCG immunised or naïve mice ($n = 6$) were boosted six weeks after vaccination with two doses of either Ad-TB10.4 or Ad-empty, one month apart. Four weeks after final vaccination, individual mouse spleen cells from each vaccine regimen were subjected to IFN-γ ELISPOT assay after stimulation with the BCG-derived recombinant protein TB10.4 (A). Bars represent the mean ± SEM. $^{***}\rho < 0.001$ *vs* BCG group, ANOVA with Bonferroni's *post hoc* test. Pooled spleen cells from each vaccine regimen were stimulated with TB10.4 derived peptides to map the response to individual peptides (B). Bars represent represent mean response of each vaccine group to individual peptides mapping entire protein sequence. The frequency of CD4 T cells, from individual mice specific to the single dominant TB10.4 P13 peptide was examined using an MHC class II tetramer–peptide complex specific for this peptide (C). Bars represent the mean ± SEM % frequency of tetramer$^+$ cells within the live CD4$^+$ CD27$^-$ T cell population minus the result of the non-specific negative control tetramer. Data are representative of one of two independent experiments.

(Fig. 5B). It is of interest to note that the CD8$^+$ populations induced by BCG/Ad-TB10.4 did not include IFN-γ^+/IL-2$^+$/TNF-α^+ multifunctional cells, or indeed any IL-2 producing cells. It was not possible in this study to extensively phenotype these cells, so whether they represent a similar T_{EM} population as the BCG-specific CD4 T cells previously characterised [14,15] remains unclear.

3.6. Systemically administered Ad-TB10.4 boosting of BCG induces significantly higher protection against pulmonary tuberculosis

In order to assess the protective efficacy of these vaccine regimens, one month after the last vaccination all remaining mice were challenged intranasally with ~300 CFU of *M. bovis*. Bacterial burden in spleen and lungs were assessed one month later in individual mice ($n = 8$), as illustrated in Fig. 2B. As shown in Fig. 6, this challenge resulted in a substantial *M. bovis* burden of: 4.45 Log_{10} in the spleen and 6.31 Log_{10} CFU in the lungs of naïve control mice. BCG induced protection of 1.55 Log_{10} in the spleen ($\rho < 0.001$ *vs* controls) and 1.65 Log_{10} in the lungs ($\rho < 0.001$ *vs* controls).

BCG boosted by Ad-TB10.4 vaccination was sufficient to increase this protection by a further 0.66 Log_{10} in spleen ($\rho = 0.06$ *vs* BCG), and 0.61 Log_{10} in the lungs ($\rho < 0.01$ *vs* BCG). BCG/Ad-empty induced equivalent protection to BCG alone, whilst Ad-TB10.4 alone failed to induce significant protection.

3.7. Ad-TB10.4 boosting of BCG does not enhance long-term protection

In order to assess the long term protection induced by vaccination, all remaining mice ($n = 7/8$) were kept for a further 318 days post-challenge, or until clinical manifestations of TB necessitated euthanisation according to a clinical scoring system. Survival, therefore, was counted as time to humane endpoint. Because of technical issues, these mice were housed at Specific Animal Pathogen Order (SAPO, HSE, UK) Level 4, which precluded any sampling of these animals for histology, bacterial counts or immune status. As shown in Fig. 7, BCG increased survival with a Median Survival Time (MST) of 285 days *vs* naïve controls MST = 188 days ($\rho = 0.06$). Despite significantly reducing bacterial burden at 4 weeks post-challenge (as shown in Fig. 7), BCG/Ad-TB10.4 vaccination did not further increase survival (MST = 286 days).

4. Discussion

Given the unlikelihood of BCG being removed from use in the intermediate future, heterologous prime-boost based on prior BCG immunisation is the most likely scenario for improved TB vaccines [5]. This has established an intensive effort to evaluate potential heterologous boost vaccines, with over 45 candidates tested to date [reviewed in 25]. Here we report our in-house development and efficacy testing of a BCG boost vaccine (parenteral Ad-TB10.4) for bovine TB which under One Health principles is relevant for *M. tuberculosis* vaccine development.

Much controversy exists regarding the suitability of the most tested (and therefore safe) HuAd5 serotype used here and many other studies, for use in an eventual target populations with potential pre-existing immunity against the vector. We chose Ad5 for its exceptional safety record, [reviewed in 26] and certainly, there would be no issue of cattle having prior exposure. For potential human use, there are now strong incontrovertible data that despite any pre-existing immunity, Ad5 is still safe and provides an effective delivery vehicle [10,27].

The ability of boost vaccines to increase the breadth of CD4 T cell epitope recognition has been documented in HIV vaccines using DNA/Ad [28], and bovine TB vaccines using BCG/MVA prime-boost [29]. Also, Billeskov et al. [30], reported that protein immunisation primed recognition of CD4 T cell epitopes which are subdominant following BCG immunisation or *M. tuberculosis* infection.

The increased epitope recognition was detected by ELISPOT, which does not discern between IFN-γ produced by CD4 and CD8 T cells. That BCG/Ad-TB10.4 was demonstrated by ICS to induce CD8$^+$, but not enhance CD4$^+$ T cell responses compared to BCG alone, suggests additional epitopes were CD8$^+$ restricted, further

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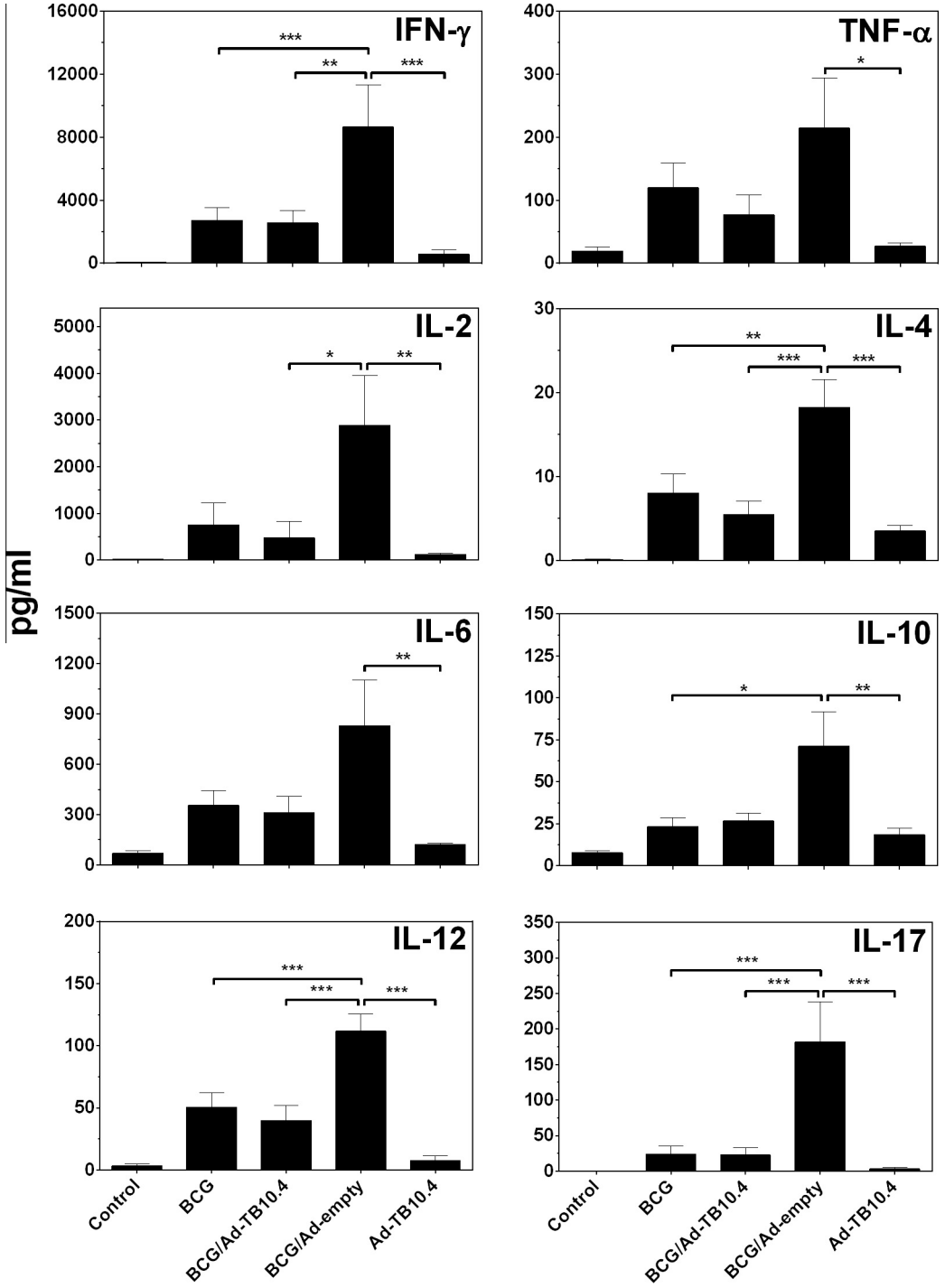

Fig. 4. Adenovirus vector immuno-modulates BCG induced recall responses. BCG immunised or naïve mice ($n = 6$) were boosted six weeks after vaccination with two doses of either Ad-TB10.4 or Ad-empty, one month apart. Four weeks after final vaccination, spleen cells of individual mice from each vaccine regimen were cultured with the BCG-derived recombinant protein TB10.4, and three day supernatants assayed by chemiluminescent ELISA. Bars represent the mean ± SEM. $^{*}\rho < 0.05$, $^{**}\rho < 0.01$, $^{***}\rho < 0.001$, ANOVA with Bonferroni's *post hoc* test.

supported by the lack of boosting observed in $CD4^{+}$ $tetramer^{+}$ cells. As protein immunisation increases CD4 T cell epitope recognition [30], perhaps a combination of protein and Ad boosting of BCG may offer the best strategy for the broadest recognition of T cell epitopes in future. A recent study [31] demonstrated the promise of this in a TB vaccine model using an adjuvanted protein subunit prime-Ad boost, but not in the context of prior BCG immunisation.

Whilst we still do not fully understand the contribution of individual TB10.4 epitopes to protection, the human response to BCG is highly restricted [12] and thus the Ad-TB10.4 induction of a broader T cell repertoire to this one protective antigen could still offer a significant opportunity to improve upon BCG, especially due to its synergistic effect as a BCG boost.

We have previously described that BCG induces a broad multi-polarised T cell recall cytokine response (stimulated with a defined cocktail of antigens) [32], and here we demonstrate a similar response stimulated by TB10.4 in the cell culture supernatant assay. This is a consistent observation both in our model and previous reports [31,32] and may indicate such a broad response is

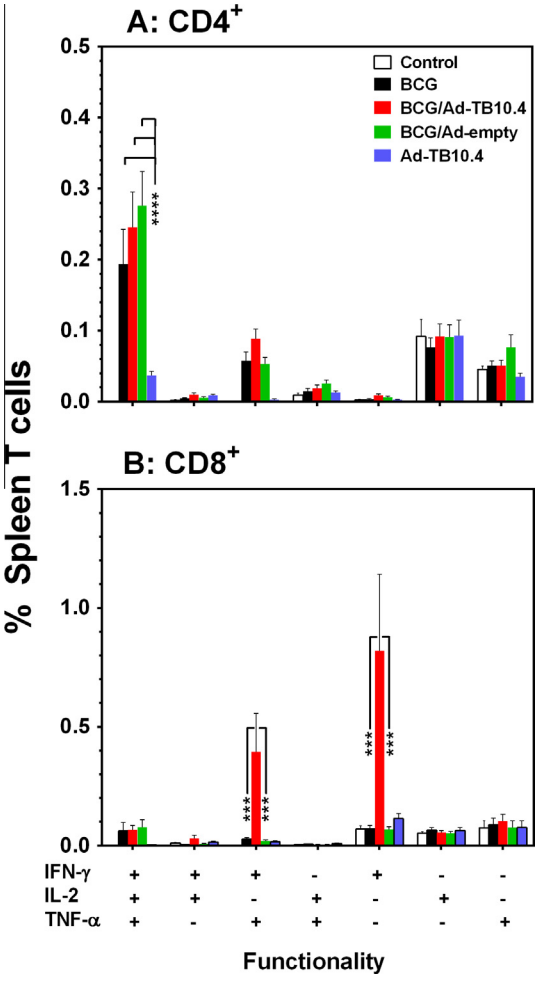

Fig. 5. Ad-TB10.4 significantly boosts antigen-specific $CD8^+$ but not $CD4^+$ T cells. BCG immunised or naïve mice ($n = 6$) were boosted six weeks after vaccination with two doses of either Ad-TB10.4 or Ad-empty, one month apart. Four weeks after final vaccination, spleen cells of individual mice from each vaccine regimen were stimulated with the BCG-derived recombinant protein TB10.4, stained by intracellular cytokine staining (ICS) and interrogated by flow cytometry. Graphs represent the percentage of live $CD4^+$ (A) and $CD8^+$ (B) lymphocytes detected in the analysis, as determined by the inclusion of a LIVE/DEAD fixable yellow dead cell stain. Plots were gated as described in Supplementary Fig. S1 and analysed for all combinations of simultaneous IFN-γ, IL-2 and TNF-α production. Bars represent the mean ± SEM. $^{**}p < 0.01$; ANOVA with Bonferroni's *post hoc* test. Data are representative of one of two independent experiments.

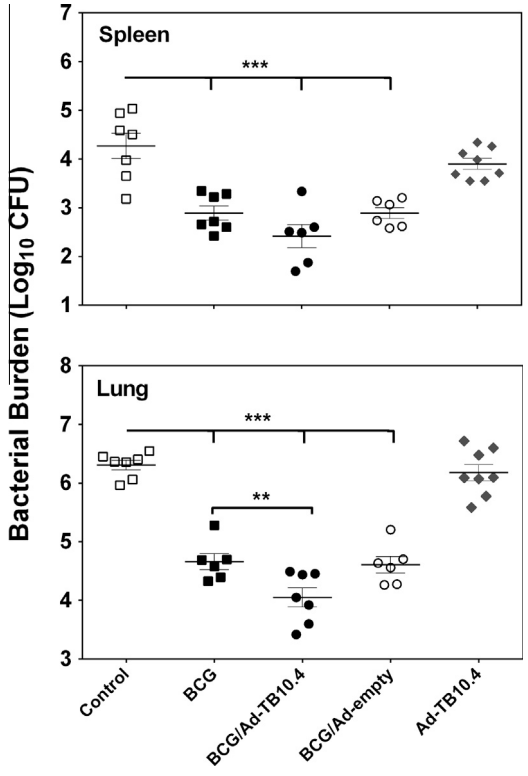

Fig. 6. Systemically administered Ad-TB10.4 boosting of BCG induces significantly higher protection against pulmonary tuberculosis. BCG immunised or naïve mice ($n = 6$–8) were boosted i.d. six weeks after vaccination with two doses of either Ad-TB10.4 or Ad-empty, one month apart. Spleen and Lungs from individual mice were removed 4 weeks following challenge and bacteria enumerated. Data represent the mean bacterial burden per organ ± SE. $^{*}\rho < 0.05$; $^{**}\rho < 0.005$; $^{***}\rho < 0.0005$; *vs* control or BCG; ANOVA with Bonferroni's *post hoc* test.

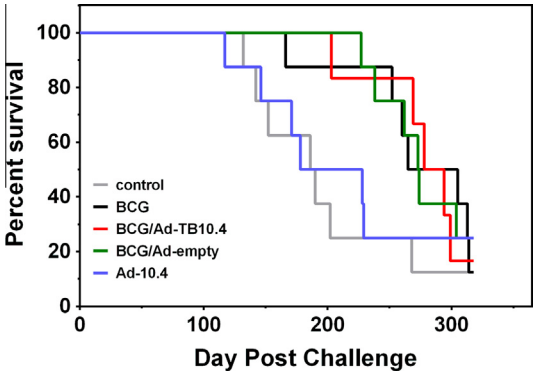

Fig. 7. Ad-TB10.4 boosting of BCG fails to enhance long-term protection. Mice ($n = 7$/8) were infected with 300 CFU of *M. bovis* i.n. and kept until clinical manifestations of TB necessitated euthanisation according to a clinical scoring system. Survival was counted as time to humane endpoint, or until the end of experiment at 318 days post-challenge. Survival data was analysed with the Mantel-Cox Log Rank test; $^{*}\rho = 0.06$.

optimal for BCG-induced protection. The reduced antigen-specific IFN-γ production in the culture assay, was surprising given the significant IFN-γ ELISPOT responses observed. This may reflect different parameters measured (secretor frequency *vs* magnitude; effector *vs* memory), and that ELISPOT is more sensitive [33]; detecting immediate/short-term IFN-γ release, accumulatively. The disparity between IFN-γ detection by ELISPOT and ICS, may be due to: (i) the fact that peptides were used for the IFN-γ-ELISPOT stimulation, whilst ICS used protein; therefore, insufficient antigen-processing may occur to present the specific epitopes revealed in the epitope mapping, or (ii) assay sensitivity.

The immunomodulatory effect of the Ad-empty vector administration on the TB10.4-specific BCG response was also surprising, but is likely due to a non-specific adjuvant effect. Analysis of total virus particles in batches of Ad-TB10.4 & Ad-empty revealed no differences, and therefore does not explain this differential effect. It is known that Ad vectors induce innate responses [34], and data demonstrate BCG to persist long after vaccination [35–37]. Combined with previous reports [38–40], we propose the Ad vector is adjuvanting persistent BCG, enhancing specific immune responses. The exact mechanisms of this adjuvant effect are not yet known but are the subject of current studies. These responses together with a lack of additional protection in this group, supports the increasingly established hypothesis that increasing the magnitude of responses alone is not beneficial [41,42].

In light of this vector adjuvant effect on CD4 T cell responses, it was equally intriguing that Ad-TB10.4 failed to do the same. Elucidating the mechanisms of this observation was beyond the scope

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of this study, but we propose two potential explanations. First, the antigen-specific CD8 T cells identified here may dominate the response to Ad-TB10.4, thus down-regulating CD4 T cell responses. Alternatively, peripheral negative selection may occur [43]. The initial Ad-TB10.4 boost may have primed very high affinity TB10.4-specific CD4 T cells, which undergo apoptosis upon subsequent boost, by peripheral repertoire tuning.

The increase in pulmonary protection over BCG achieved by BCG/Ad-TB10.4 immunisation compares to other non-mucosal prime-boost vaccine regimes performed in mice using a similar vaccination-challenge interval (reviewed in [25]). This is however, to our knowledge, the first demonstration of efficacy using a systemic Adenoviral boost in mice.

The reason for our study demonstrating efficacy with non-respiratory adenoviral boost where others have failed is at this point unclear, although a recent study in non-human primates (NHP) has shown equivalent efficacy between respiratory and parenteral Ad-Ag85A boost of BCG [44]. There are several potential factors which may explain this discrepancy with many published studies. Importantly, our study challenged with *M. bovis* which although an order of magnitude more virulent than *M. tuberculosis* in mice (Hogarth, P.J., *unpublished data*, [45]) is the parent strain of BCG & thus more closely related than *M. tuberculosis* [46]. Our boost vaccine (Ad-TB10.4) has not previously been tested using either i.d. route or multiple boost. It is also one of few boost vaccines to show improvement upon BCG which is not RV3804c (Ag85A) [25] specific; thus, there is potential for improved efficacy if used in a multivalent/combined vaccine formulation with Ag85A and other protective antigens.

That improved protection did not translate into enhanced survival was disappointing, although a common feature of many studies [25]. We propose that the absence of IL-2 producing $CD8^+$ cells may indicate the lack of memory phenotype [47]. In this case, additional protection may be limited to the failure of short-term protective responses to persist. We are currently investigating alternate immunisation regimes to boost immune memory and thus longer term protection.

This study indicates BCG/Ad-TB10.4 prime-boost to be a promising candidate, but also highlights the need for further understanding of the mechanisms of T cell priming and associated memory using adenovirus delivery systems. The fact that we were able to generate significant improvement over BCG with a parenteral, rather than mucosal administered boost is of great promise, demonstrating effective mucosal immunity is possible without the risks and challenges of mucosal administration.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2016.06.032.

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