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Immunogencity of antigens from *Mycobacterium tuberculosis* self-assembled as particulate vaccines

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ABSTRACT

Traditional approaches to vaccine development have failed to identify better vaccines to replace or supplement BCG for the control of tuberculosis (TB). Subunit vaccines offer a safer and more reproducible alternative for the prevention of diseases. In this study, the immunogenicity of bacterially derived polyester beads displaying three different Rv antigens of *Mycobacterium tuberculosis* was evaluated. Polyester beads displaying the antigens Rv1626, Rv2032, Rv1789, respectively, were produced in an endotoxin-free *Escherichia coli* strain. Beads were formulated with the adjuvant DDA and subcutaneously administered to C57BL/6 mice. Cytokine responses were evaluated by CBA and antibody responses by ELISA. Specificity of the IgG response was assessed by immunoblotting cell lysates of the vaccine production strains using sera from the vaccinated mice. Mice vaccinated with beads displaying Rv1626 had significantly greater IgG1 responses compared to mice vaccinated with Rv1789 beads and greater IgG2 responses than the group vaccinated with Rv2032 beads ($p < 0.05$). Immunoblotting of antisera from these mice indicated the antibody responses were Rv1626 antigen-specific and there was no detectable immune response to the polyester component of the vaccine. Overall, this study suggested that selected TB antigens derived from reverse vaccinology approaches can be displayed on polyester beads to produce antigen-specific immune responses potentially relevant to the prevention of TB.

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1. Introduction

It is estimated that in 2014, 9.6 million people developed TB and 1.5 million died from the disease, making it the leading cause of death from a single infectious disease worldwide (WHO, 2015). BCG, the only vaccine available against TB is insufficient for global control (Andersen and Doherty, 2005). Subunit vaccines, administered with selected adjuvant and delivery systems designed to improve immune responses represent a promising alternative to replace or supplement BCG.

Polyhydroxyalkanoates, a group of various biopolyesters comprised of natural 3-hydroxy fatty acids, are deposited as intracellular inclusions produced in bacteria as an energy and carbon storage material (Ma et al., 2013; Rehm, 2010). PHB is the most extensive and well-characterized member of the polyhydroxyalkanoates. PHB can be produced heterologously by introducing the genes that encode for the enzymes PhaA, PhaB, and PhaC (Peoples and Sinskey, 1989; Schubert et al., 1988) and then providing the appropriate conditions for bacterial cultivation (Chen et al., 2014). Due to their properties of biodegradability, biocompatibility and ease of production from renewable resources, there is significant interest in applications of biopolyesters as functional biobeads (Parlane et al., 2016) On the bead surface it is possible to display the desired antigens, fused to PhaC, which is covalently anchored to the PHB core of the beads (Parlane et al., 2009). This approach has enabled overproduction of desired proteins on the carrier bead, while the beads themselves have shown adjuvant properties such as enhancing the cell-mediated immune responses to the antigens (Parlane et al., 2012). This also allowed co-delivery of antigens and adjuvant as a single particle to APCs of the immune system. In addition, less antigen was required when compared to soluble antigens, which suggested this system promoted efficient antigen delivery to APCs.

Reverse vaccinology is an alternative to the traditional way of identifying vaccine candidates. The process uses the genomic

Abbreviations: TB, tuberculosis; DDA, dimethyldioctadecylammonium bromide; CBA, cytometry bead array; BCG, Bacillus Calmette-Guerin; PHB, Poly(3-hydroxybutyrate); PhaA, β–ketothiolase; PhaB, acetoacetyl-CoA reductase; PhaC, PHB synthase; APCs, antigen presenting cells; OD, optical density; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; PPDB, bovine purified protein derivative; LPS, lipopolysaccharide; SEM, scanning electron microscopy.

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sequence of the pathogen, allowing access (*in silico)* to all the proteins that the pathogen may express *in vivo.* Potential vaccine candidates are identified using bioinformatic tools including the identification of structural motifs and immunological epitopes (Rappuoli, 2000). In the case of *M. tuberculosis,* 271 antigens were experimentally confirmed as being immunogenic, but only 35% of these antigens have been tested in animal models so far (Bertholet et al., 2014). Derrick et al. in 2013 tested 16 of these antigens using challenge of mice with *M. tuberculosis*. Six of these antigens, Rv1626, Rv1735, Rv1789, Rv2032, Rv2220, and Rv3478 significantly reduced the colony forming units in the lungs of experimentally infected animals (Derrick et al., 2013). Previous studies showed that the display of various antigens on bacterial polyester beads enhanced their immunogenicity and stimulated a Th1 and Th2 immune response (Parlane et al., 2012, 2009). Hence, in this study we investigated whether the display of these novel Rv antigens on polyester beads produced inside engineered *E. coli* will also be immunogenic.

This paper describes the design and construction of plasmids containing rv genes to engineer and produce antigen displaying beads in *Clearcoli,* an endotoxin free strain of *E. coli*. Novel polyester beads displaying the antigens Rv1626, Rv2032 or Rv1789 were produced and their immunogenicity evaluated in C57BL/6 mice.

2. Materials and methods

2.1. Generation of plasmids for production of beads displaying Rv antigens

Plasmids and oligonucleotides used in this study are described in Table 1. Genes encoding Rv antigens were synthesized by GenScript (USA). General molecular cloning procedures (Promega, 2004) were used to generate new plasmids and their sequences were confirmed by DNA sequencing.

2.2. Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table 1. *E. coli* XL-1Blue was used for general cloning steps and was grown in Luria-Bertani (Acumedia, USA) broth, supplemented with ampicillin (100 μg/mL) at 37 °C. *Clearcoli* was used for beads production and media were supplemented with D-glucose 0.2% (w/v) (Merck, Germany) and NaCl (5 g/L) (Ajax Finechem, Australia). Cultures were grown in flasks in a shaking incubator at 37 °C at 200 rpm and the OD was monitored. Cultures were induced by addition of IPTG (1 mM) (Goldbio, USA) when an OD of 0.5 was reached and the incubation was continued at 25 °C and 200 rpm for 48 h.

2.3. Bead isolation

Cells were harvested and resuspended in lysis buffer (50 mM Tris, pH 11, 10 mM EDTA, 0.08% (w/v) SDS) (Herbert et al., 2012) prior to disruption by using a microfluidizer (Microfluidics M-110P, USA). Beads were recovered after centrifugation of the whole cell lysate at 6000 × *g* during 30 min at 4 °C. Beads were washed twice with lysis buffer, treated with 70% (v/v) ethanol to kill residual bacteria and stored in PBS buffer (10 mM Na_2HPO_4, 137 mM NaCl).

2.4. Bead characterization

Proteins attached to the beads were separated by using 8% SDS-PAGE and stained with Coomassie Blue. The amount of fusion protein was calculated with Image Lab software version 3.0 using a standard curve of increasing BSA concentrations. Using BSA as the standard, the software estimates the amount of proteins run per lane on the gel and assigns a quantity to each protein band. This is used to determine the ratio: ng of fusion protein/mg of beads. (Hay et al., 2014). A Western Blot anti-PhaC protein was performed using a rabbit anti-PhaC antibody (Genscript, USA) and a goat anti-rabit IgG HRP conjugate (Abcam,UK). Beads were visualised by scanning electron microscopy (SEM).

2.5. Vaccine preparation and mice immunization

DDA was added to PBS at a concentration of 1.25 mg/mL and heated at 80 °C for 10 min with constant stirring until the formation of micelles. The emulsion was cooled to room temperature and added to the beads. Each vaccine dose contained 250 mg DDA. The particle size of the vaccine formulations was measured using a Mastersizer (Malvern Mastersizer 3000, UK). Wild-type (Wt) beads (5 μg of PhaC), Rv1626 beads (5 μg of Rv1626), Rv2032 beads (5 μg of Rv2032) or Rv1789 beads (1.5 μg Rv1789) were used for vaccination, respectively. Female C57BL/6 mice were obtained from the animal breeding facility of AgResearch, Ruakura, Hamilton, New Zealand. Two hundred μL of the vaccine preparations were injected subcutaneously three times at 2 weeks intervals (6 mice per group). BCG was administered only once at a dose of 10^6 CFU. One group was administered DDA only as a negative control. All animal experiments were approved by the AgResearch Grasslands Animal Ethics Committee (Palmerston North, New Zealand).

2.6. Preparation of mice samples and immunological assays

Three weeks after the last immunization, mice were sedated, bled, euthanased and a single cell suspension of splenocytes obtained as described by Parlane et al. in 2009. Mice splenocytes were stimulated with PPDB (5 μg/mL) (Thermofisher,USA) and cultured for 3 days in 10% CO_2. Culture supernatant was removed and frozen at −20 °C until assayed. Cytokine release was measured using a BD CBA Mouse Th1/Th2/Th17 cytokine kit (BD Biosciences, USA) adapted to Falcon V bottom polypropylene plates. Samples were acquired with a BD FACS Verse flow cytometer (BD Biosciences, USA) and data analysed with FCAP array software (BD Biosciences, USA).

IgG responses in sera were analysed by ELISA. Microlon high-binding plates (Greiner Bio-One, Germany) were incubated overnight at 4 °C with 5 μg of antigen (beads) or 10 μg/mL of PPDB as a positive control in 0.05 M Carbonate/Bicarbonate buffer. Serial dilutions of sera from individual mice were added to the plates and goat anti-mouse IgG1 or IgG2c HRP-conjugate antibodies (ICLLab, USA) were used for detection of IgG1 and IgG2c antibodies, respectively. To investigate the specificity of the IgG response, pooled mice sera were diluted 1500-fold and used for immunoblotting against all bead proteins, total host cell proteins or purified soluble proteins. An anti-mouse IgG HRP-conjugate (abcam, United Kingdom) was used for detection of bound IgG antibodies.

2.7. Statistical analysis

Dunn's test of multiple comparisons following a significant Kruskal-Wallis test was performed to analyze significant differences between the groups mean values from the CBA and ELISA assays using the program Minitab17. Statistical significance was considered at $p < 0.05$.

3. Results

3.1. Construction of plasmids containing mycobacterial Rv genes and their ability to mediate polyester bead production in clearcoli

Different combinations of the rv genes fused to 5′ and/or 3′ ends of the *phaC* gene were generated and the resultant plas-

Table 1
Strains, plasmids and oligonucleotides used.

Strains, plasmids or oligonucleotides	Genotype or description	Source or reference
E. coli strains		
XL1-Blue	*endA1 gyrA96(nalR) thi-1 recA1 relA1 lac glnV44 F'[:Tn10 proAB+ lacIq Δ(lacZ)M15] hsdR17(rK- mK+)*	Stratagene
Clearcoli BL21 (DE3)	F– *ompT hsdSB (rB- mB-) gal dcm lon* λ(DE3 [*lacI lac*UV5-T7 gene 1 *ind1 sam7 nin5*]) *msbA148 ΔgutQΔkdsD ΔlpxL ΔlpxMΔpagP ΔlpxP ΔeptA*	Lucigen
Plasmids	Contains hybrid rv genes used for subcloning steps	GenScript
pUC57-rv1626-rv3478-rv2220	Contains hybrid rv genes used for subcloning steps	
pUC57-rv1789-rv2032-rv1785		
pPOLY-C-phaC	Amp^r. pET-14b derivative containing *phaC* from *Ralstonia eutropha* and *StuI, XhoI, XmaI* and *BamHI* sites downstream of *phaC*.	Hay et al. (2014)
pMCS69	Cm^r. pBBR1MCS derivative containing genes *phaA* and *phaB* from *R. eutropha*	Amara and Rehm (2003)
pPOLY-C-phaC-rv1626	pPOLY-C-phaC derivative containing *rv1626* inserted into *XhoI/BamHI* sites.	This study
pPOLY-C-phaC-rv2032	pPOLY-C-phaC derivative containing *rv2032* inserted into *XhoI/BamHI* sites.	This study
pPOLY-C-phaC-rv1789	pPOLY-C-phaC derivative containing *rv1789* inserted into *XhoI/BamHI* sites.	This study
pPOLY-C-rv1626	pPOLY-C-phaC derivative containing *rv1626- rv3478* hybrid gene inserted into	This study
−rv3478-phaC-rv2220	*XbaI/SpeI* sites and *-rv2220* inserted into *XhoI/BamHI* sites.	This study
pPOLY-C-phaC-rv2220	pPOLY-C-phaC derivative containing *rv2220* inserted into *XhoI/BamHI* sites.	This study
pPOLY-C-phaC-rv1735	pPOLY-C-phaC derivative containing *rv1735*inserted into *XhoI/BamHI* sites.	This study
pPOLY-C-6His-rv1626	pPOLY-C derivative containing *rv1626* and a 6xHis tag upstream.	
Oligonucleotides		
1626-XhoI	5′-GCTCTCGAGATGTACACAGGACCTACGACG-3′	This study
1626-BamHI	5′-CCGGATCCTGTATCTTTTGGTGTACCAAGCGTT-3′	This study
2220-XhoI	5′-GCTCTCGAGATGACGGAAAAAACGCCAGAT-3′	This study
2220-BamHI	5′-GCTGCTGGATCCCACATCATAATAAAGAGCAAATTC-3′	This study
3478-BamHI	5′-GCTGGATCCTCCAGCAGCAGGCGTTCTAGG −3′	This study
1789-XhoI	5′-GCTCTCGAGGATTTTGGTGCTCTTCCT −3′	This study
2032-BamHI	5′-GCTGGATCCTCCAGCAGCAGGCGTTCTAGG-3′	This study
1789-BamHI	5′-GCTGGATCCTCCAGCAAAAGGAGGTCGAGC-3′	This study
2032-XhoI	5′-GCTCTCGAGCCAGATACGATGGTG-3′	This study
2032-BamHI	5′-GCTGGATCCTCGATGATCTTTAGCTCTAAC-3′	This study
1735-XhoI	5′-GCTCTCGAGGGTGCTACGGCAATTACA-3′	This study
1735-BamHI	5′-GCTGGATCCCATTGGTTGGTTACGACGAAA-3′	This study
His1626-SpeI	5′-GCTACTAGTATGCATCATCACCATCACCACTACACAGGACCTACGACGGAT-3′	This study
1626-Xhostop	5′-GCTCTCGAGTTATGTATCTTTTGGTGTACCAAG-3′	This study
		This study

Table 2
Concentration of proteins in beads.

Protein	Amount of PhaC-Rv antigen fusion/wet beads (ng protein/mg beads)	Amount of Rv antigen/wet beads (ng protein/mg beads)	Molecular weight (kDa)
PhaC	695	–	64
PhaC-Rv1626	1210	393	88.7
PhaC-Rv2032	1183	409	102.4
PhaC-Rv1789	367	131	104.4

mids were transformed into the bead production strain *Clearcoli*. Plasmids pPOLY-C-rv1626-rv3478-phaC-rv2220, pPOLY-C-phaC-rv2220, pPOLY-C-phaC-rv1735, pPOLY-C-phaC-rv1789, pPOLY-C-phaC-rv1626 and pPOLY-C-phaC-rv2032 each mediated PHA bead production. However only plasmids pPOLY-C-phaC-rv1626, pPOLY-C-phaC-rv2032, pPOLY-C-phaC-rv1789 and pPOLY-C-phaC (Wt beads) mediated production of beads displaying the fusion protein as the dominant protein. The dominant bands observed corresponded to the expected molecular weights of the respective PhaC-fusion proteins (Table 2). In addition, minor protein bands were observed and these were likely to be bacterial host cell proteins that co-purified with the beads and were not removed in the cleaning process (Fig. 1A). An immunoblot using anti-PhaC antibodies (Fig. 1B) further confirmed the stability and presence of the respective full-length fusion proteins. As Wt beads did not have antigen protein fused to PhaC, they were used as a positive control for the production of beads and as a negative control for immunising mice.

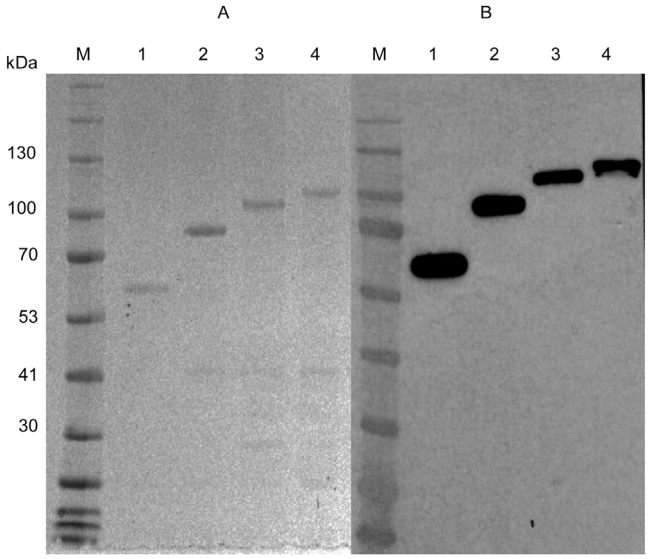

Fig. 1. A, SDS-PAGE and B, Western Blot analysis of proteins attached to various polyester beads isolated from Clear coli. Lane 1, molecular weight standard; lane 2, wild-type beads (PhaC: 64 kDa); lane 3, beads displaying Rv1626 (PhaC-Rv1626: 88.7 kDa); lane 4, beads displaying Rv2032 (PhaC-Rv2032: 102.4 kDa); lane 5, beads displaying Rv1789 (PhaC-Rv1789: 104.4 kDa).

3.2. Bead properties

The amount of fusion protein displayed on the various beads was calculated by densitometry using a BSA standard curve. This

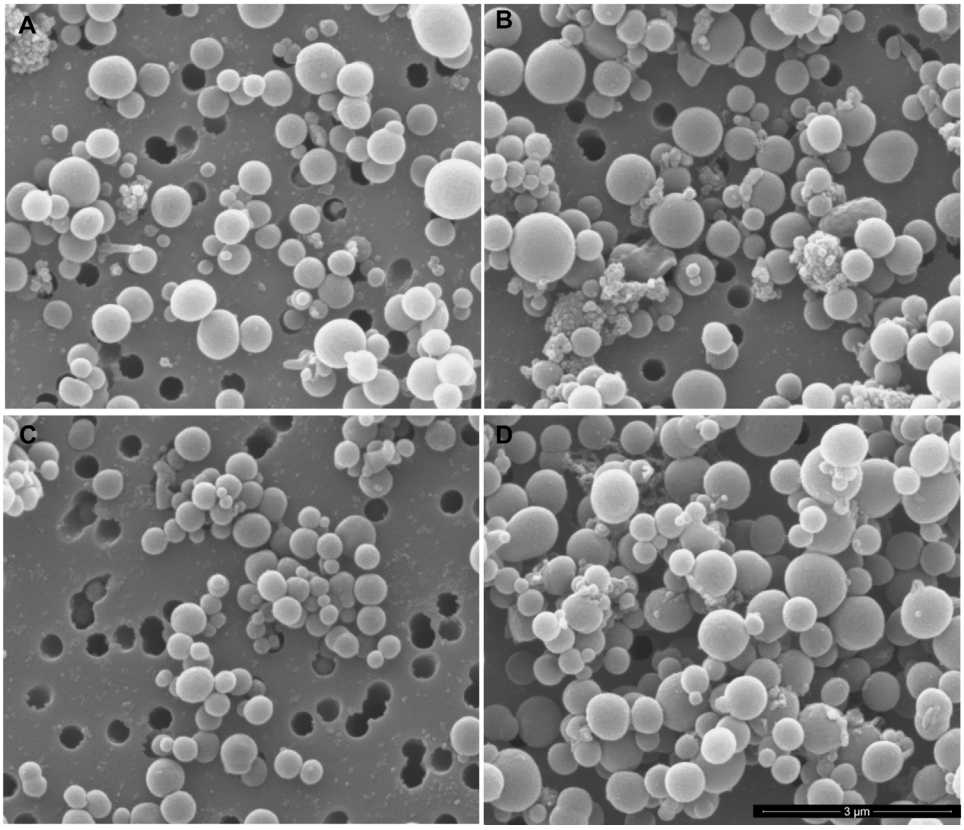

Fig. 2. SEM of various PHA beads. A, Wt beads, B, Rv1626 beads, C, Rv2032 beads and D, Rv1789 beads.

Table 3
Size distribution of beads in vaccine formulations (μm) as measured my laser scattering. Dx represents the particle diameter corresponding to X% cumulative particle size distribution.

	D_{10}	D_{50}	D_{90}
Wt beads	3.42	8.74	65.9
Rv1626 beads	3.82	9.52	33.7
Rv2032 beads	4.18	13.7	89.0
Rv1789 beads	3.78	27.8	186.0

was used to deduce the amount of fusion protein and Rv antigen per bead mass for each type of bead (Table 2). SEM images (Fig. 2) showed that, except for Rv2032 beads which appeared smaller than the other beads, bead size distribution (~500–750 nm) and surface morphology was similar for all other beads. Measurements of the particle size distribution of the formulations used for injection (i.e. after formulation with adjuvant) (Table 3) demonstrated that the beads aggregated in solution as the particle size increased after addition of adjuvant.

3.3. Vaccine formulation and mice immunization

Derrick et al. in 2013 used 5 μg of soluble recombinant protein for vaccination. The ratio of Rv antigen to bead was sufficiently high for mice to be immunized with a dose of beads containing 5 μg of Rv antigen in the case of beads displaying Rv1626 and Rv2032. However, due to the lower level of expression of Rv1789 antigen on beads, mice could only be immunized with 1.5 μg Rv1789. Control mice each received a dose of Wt beads containing 5 μg of PhaC.

No adverse effects due to immunization were observed and animal weights gradually increased during the time course of the trial with no significant differences between the groups. Small lumps (~2 mm) were observed in mice vaccinated with beads but not with BCG or DDA alone, suggesting these lumps were due to an immune response to the beads.

3.4. Cytokine responses

All splenocytes were stimulated with PPDB and following incubation, IFNγ, IL-10, IL-17A, Il-2, IL-4, IL-6 and TNFα levels were measured in the culture supernantants (Fig. 3). There was no detectable IL-4 released.TNFα responses were significantly higher in all groups immunized with beads compared to the group given DDA alone. IFNγ production was significantly higher for the BCG-vaccinated group than for groups immunized with Wt beads, Rv2032 beads and DDA.

3.5. Antibody responses

IgG1 and IgG2c responses were evaluated by ELISA (Fig. 4) using microtiter plates coated either with beads or PPDB and using sera from immunized mice. PPDB contains a wide range of immunodominant mycobacterial proteins (Cho et al., 2012), and was used to determine antibody response in BCG-vaccinated mice as well as the mice vaccinated with beads. The results showed strong IgG1 and IgG2 responses to a Rv1626 bead preparation in mice vaccinated with the Rv1626 beads. The IgG1 responses were significantly higher ($p < 0.05$) than IgG1 responses produced by mice vaccinated with Rv1789 beads and IgG2c responses were higher than those elicited by mice vaccinated with Rv2032 beads ($p < 0.05$). The response to wild-type beads was similar in all groups and there were no differences in responses to the various antigen beads in this group. Minimal antibody responses to PPDB were measured in groups vaccinated with Wt, Rv1789 beads, BCG and DDA. Although not significantly different, the responses to PPDB by mice

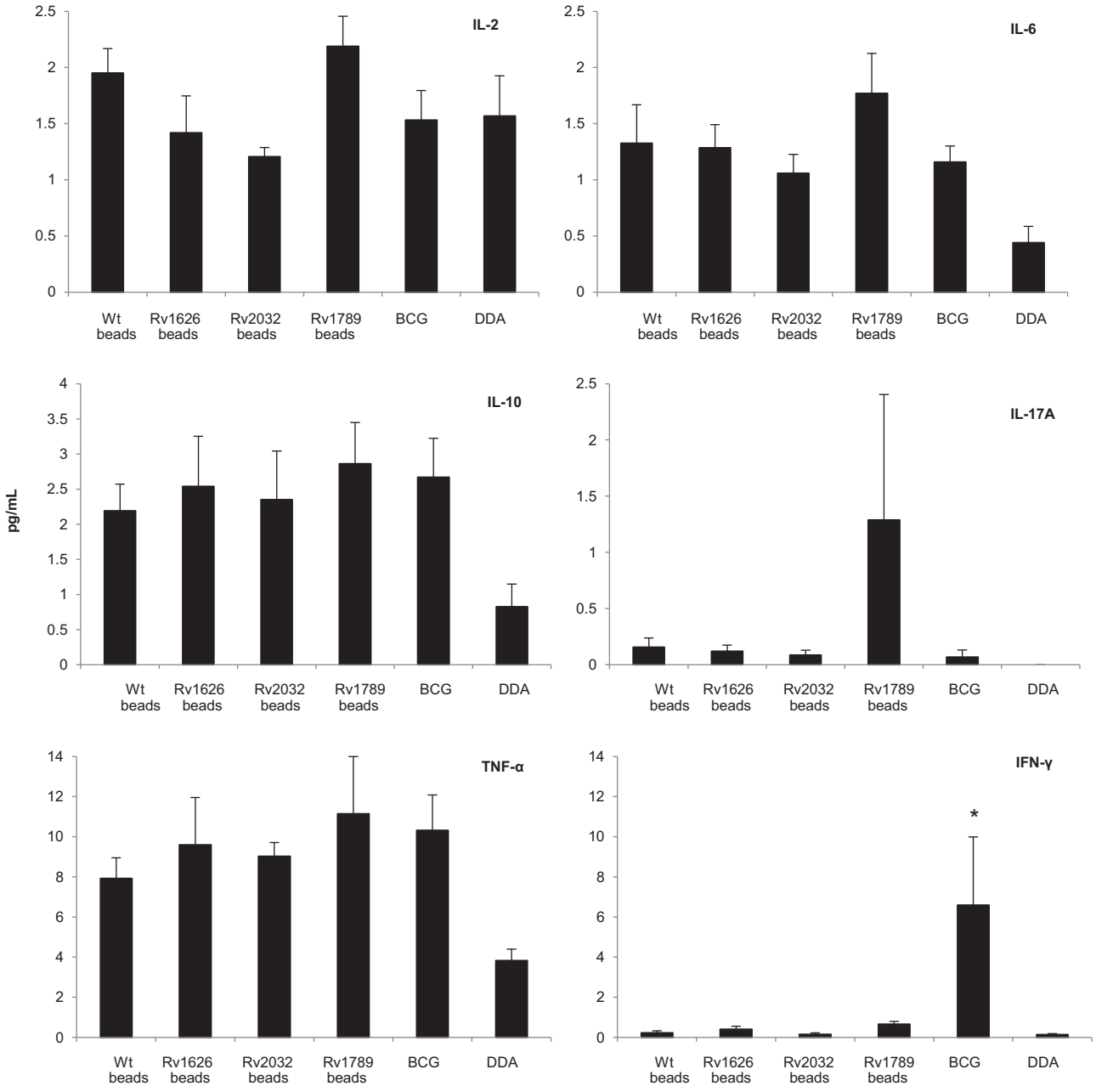

Fig. 3. Cytokine responses of mice splenocytes upon stimulation with PPDB and analysed by cytometric bead array. Each data point represents the mean ± the standard error of the mean. Wt beads, beads negative control (beads without antigen); Rv number bead: beads displaying reverse vaccinology antigens; BCG (Bacillus Calmette–Guérin); DDA (dimethyldioctadecylammonium bromide) adjuvant used for vaccine formulation and as a negative control. *, significantly higher than wild type beads ($p < 0.05$).

vaccinated with Rv1626 and Rv2032 were higher than responses induced by Wt beads.

3.6. Specificity of IgG response

In order to confirm which proteins on the beads generated the immune response, an immunoblot was performed. Each of the beads used for immunising mice was tested for reactivity against sera produced from mice immunized with the various vaccines. Sera from mice immunized with wild-type beads, Rv2032 or Rv1789 beads recognized no proteins. However, sera from mice vaccinated with Rv1626 beads specifically recognized a protein band corresponding to the expected molecular weight of the fusion protein PhaC-Rv1626 (Fig. 5). Further immunoblot analysis with sera from mice immunized with Rv1626 beads showed a very specific IgG response as only Rv1626 was recognized in whole cell lysates of *Clearcoli* bacteria producing Rv1626 beads and no antigen was detected in the cell lysate of *Clearcoli* harbouring only the vector as the negative control (Fig. 6). In these immunoblots, purified soluble Rv1626 served as a positive control.

4. Discussion

Reverse vaccinology approaches which combine genomics/proteomics with vaccinology has enabled the successful development of a broadly protective protein-based vaccine against the serogroup B *Neisseria menigitidis* (Donati and Rappuoli, 2013). Potential vaccine candidates from *M. tuberculosis* have been identified by reverse vaccinology. When tested as soluble proteins, these antigens have been shown to stimulate protective immunity

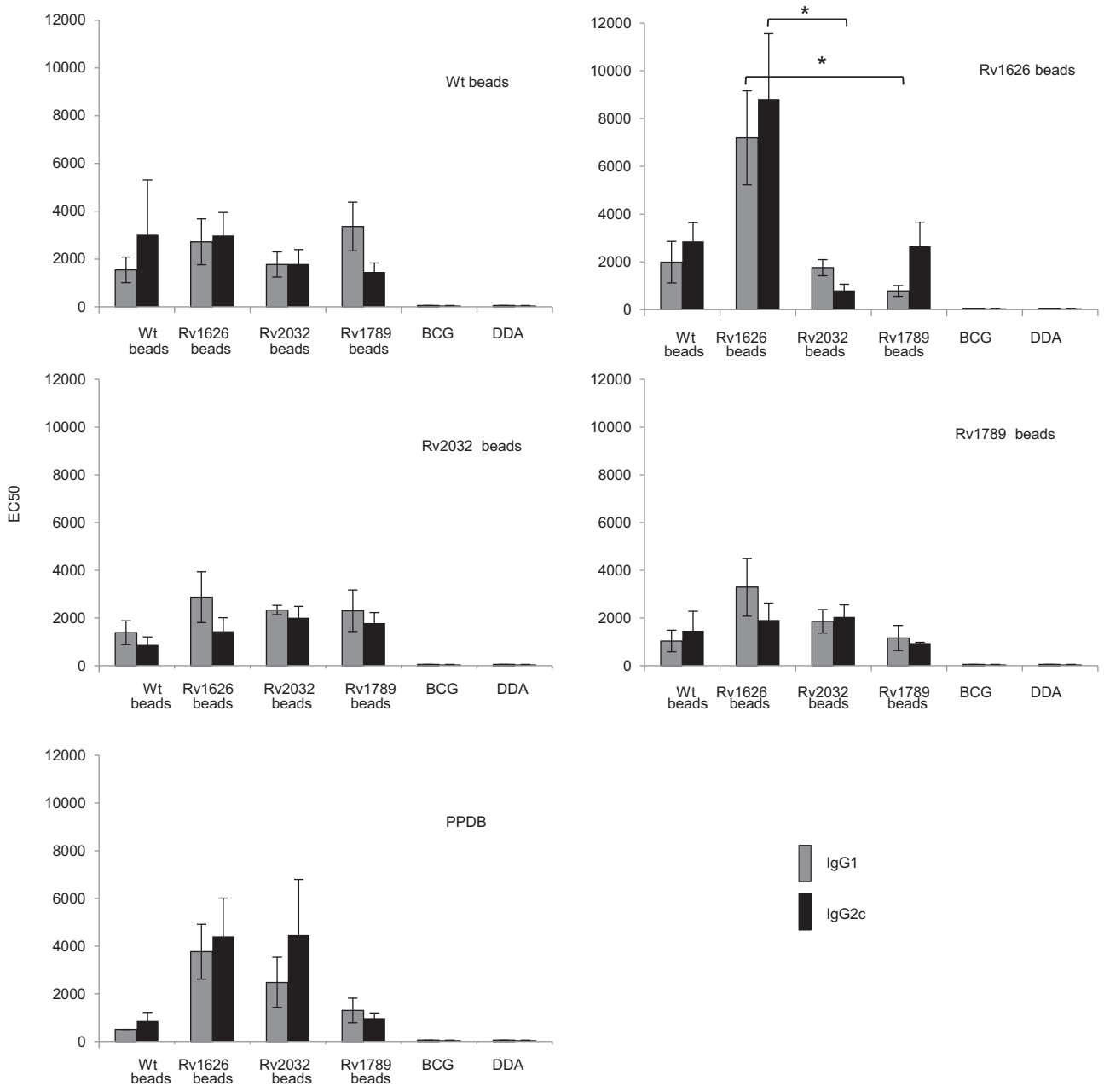

Fig. 4. IgG1 and IgG2c titers expressed in EC50 values in response to various beads and PPDB analysed by ELISA for each immunized group. Each data point represents the mean ± the standard error of the mean. Wt beads, beads negative control (beads without antigen); Rv number bead: beads displaying reverse vaccinology antigens; BCG (Bacillus Calmette–Guérin); DDA (dimethyldioctadecylammonium bromide) adjuvant used for vaccine formulation and as a negative control. *, significantly greater than other group vaccinated with beads ($p < 0.05$).

(Derrick et al., 2013). In the current study, beads were engineered to display three of these mycobacterial antigens, Rv1626, Rv1789 and Rv2032.

The various Rv antigens were translationally fused to different termini of PhaC in order to determine which fusion protein orientation was permissive i.e. produced a fusion protein which can be stably overproduced while mediating efficient bead assembly in *E. coli*. Although in some cases beads were produced, the fusion protein was not detectable by SDS-PAGE analysis of the beads. Hence, a different fusion strategy was required to increase the level of antigen produced on the surface of the beads. For these preliminary investigations, it was determined that the most efficient design for each of the three Rv antigens was a C-terminal fusion with PhaC (Fig. 1). Respective isolated beads showed the antigen-PhaC fusion protein stably and abundantly produced at the bead surface (Fig. 1).

Another important consideration for bead vaccines is the amount of antigen protein that can be administered practically, due to the viscosity of bead suspensions. Hence, a high ratio of antigen-PhaC fusion to bead mass is preferred, as this provides the option of immunising animals with higher doses of antigen and a lower bead content. In this study, it was possible to produce beads overproducing sufficient levels of Rv1626 and Rv2032 to administer the same dose (5 μg) as tested previously for the Rv1626 and Rv2032 proteins alone (Derrick et al., 2013). However, in the case of Rv1789, production of antigen fusion was only sufficient to allow administration of the equivalent of 1.5 μg of Rv1789 (Table 2). If higher levels of antigen production could be achieved for these beads,

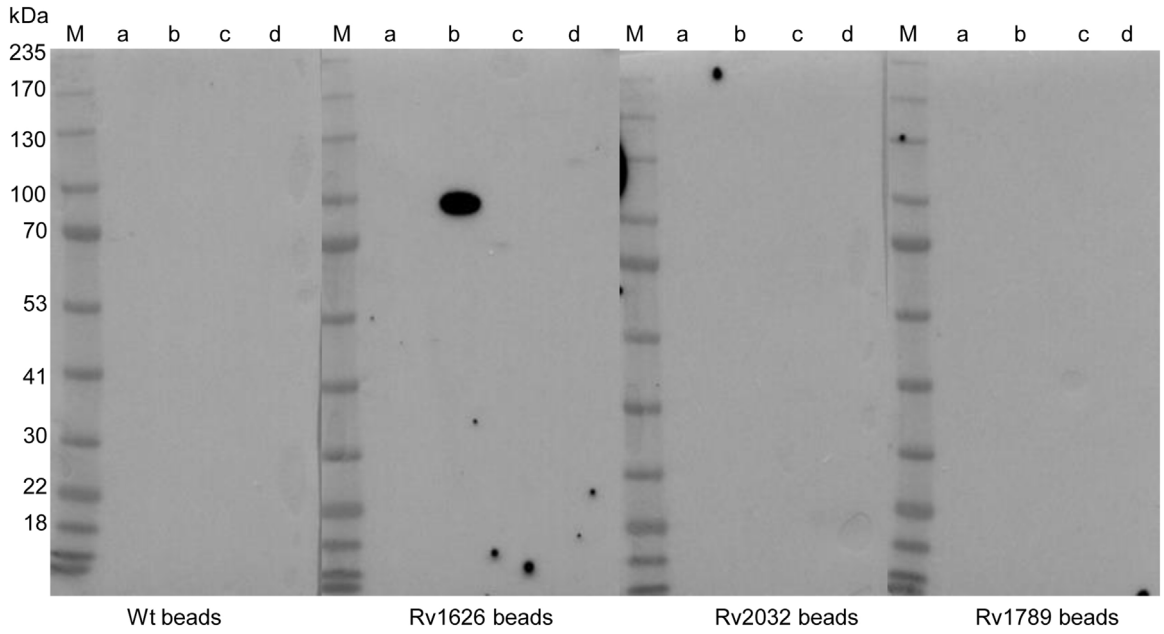

Fig. 5. Immunoblot of beads associated proteins using pooled sera from mice immunized with the various beads. Beads used for immunization were indicated below the blot. Lanes a-d represent the protein profiles of the various beads as follows: Lane M, molecular weight standard, lane a, Wt beads (negative control without antigen); lane b, beads displaying Rv1626; lane c, beads displaying Rv2032 and lane d, beads displaying Rv1789.

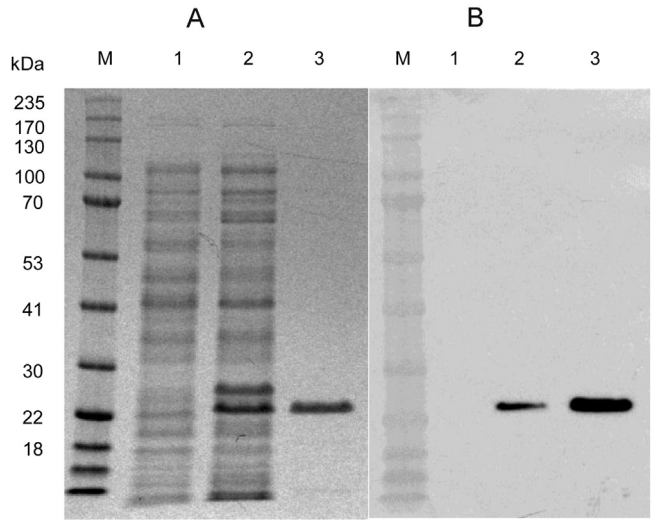

Fig. 6. Specific recognition of the Rv1626 antigen by sera of mice immunized with beads displaying Rv1626. A, SDS-PAGE; B, immunoblot. Lane 1, negative control (lysate of Clear coli cells containing plasmid pPoly-C); lane 2, lysate of Clear coli cells producing recombinant Rv1626; Lane 3, purified recombinant Rv1626. Pooled sera from mice immunized with beads displaying Rv1626 were used for the immunoblot.

immunization of mice with an increased antigen dose may have generated stronger immune responses. *Clearcoli*, is a novel strain derived from the commonly used BL21(DE3) strain. This host contains a genetically modified LPS which does not trigger an endotoxic response in human cells (Woodard et al., 2012). LPS is considered a major pathogenic element in Gram-negative septicaemia in humans (Hurley, 1995) and it is usually removed from biotechnological preparations (Magalhães et al., 2007). *Clearcoli* is an expression system that allows production of virtually endotoxin-free recombinant proteins (Woodard et al., 2012). All the beads used for immunization were produced by engineered Clearcoli and exhibited showed particle sizes of <1 μm. However, after formulation, the various beads formed aggregates of different sizes, which might be due to the interaction of the different proteins on the surface of the beads with each other and with the adjuvant. The variation between beads in the extent of aggregation may account for the different immune responses they elicited. When comparing polystyrene beads conjugated with ovalbumin of 20, 40, 100, 500, 1000 and 2000 nm, beads of 40 nm induced the strongest IFN-γ and IgG responses (Fifis et al., 2004). However, when comparing poly-lactic acid particles of 600 to 26,000 nm, only the 4000 nm particles were able to induce antibody production (Nakaoka et al., 1996). In a more recent study using the same particles, a particle size of 2000–8000 nm induced a greater IgG response than particles of <20,000, 10,000–70,000 and 50,000–150,000 nm (Katare et al., 2005). Hence, it might be considered that Wt beads and Rv1626 beads exhibited a more suitable size range to induce stronger immune responses than the larger Rv2032 and Rv1789 beads (Fig. 2, Table 3). However, what happens after injection and how the morphology or size of the vaccines changes *in vivo* is unknown.

To assess the mode of immune response induced by the various polyester beads, splenocytes of vaccinate mice were stimulated with PPDB and their cytokine profiles were analysed. While BCG mediated a Th1-type immune response as indicated by IFN-γ and TNFα production, both Wt and antigen displaying beads induced a strong TNFα response but did not elicit induction of other cytokines (Fig. 3). Hence, the cytokine profile analysis suggests the beads did not induce a strong Th1 and Th17-type immune response as was previously observed for beads displaying the TB antigens ESAT6 and Ag85A and beads displaying the Core antigen of HCV (Parlane et al., 2011, 2012, 2009). While tuberculin is known to contain Rv1626 (Cho et al., 2012), stronger cytokine responses may have been observed by using the specific antigens rather than PPDB.

The production of IFN-γ post-vaccination has been linked with the efficacy of the vaccine in humans (Black et al., 2002). Although IFN-γ, IL-2 and TNFα produced by $CD4^+$ cells have been used to predict the capability of TB vaccines to induce protective immunity, recent vaccine candidates that generated considerable levels of multifunctional $CD4^+$ producing these cytokines were unsuccessful to confer enhanced protection (Bhatt et al., 2015).

The immunological properties of other *M. tuberculosis* Rv antigens such as e.g. Rv0577 (Byun et al., 2012), and Rv3425 (Xu et al.,

2015) had been previously shown to induce a Th-1 like immune response. Rv0978c and Rv0754 induced maturation and activation of dendritic cells (Bansal et al., 2010) and synthetic peptides from Rv1733c improved the control of already-established infection after *M. tuberculosis* challenge (Coppola et al., 2015).

In contrast to the minimal IFN-γ responses induced by the beads, immunization of mice with the various beads, including the Wt beads, induced antibody responses (Fig. 4). The Rv1626 beads induced an antigen-specific IgG2c response significantly greater than responses in the groups vaccinated with Rv2032 beads, BCG or DDA alone and a greater IgG1 response than mice vaccinated with RV1789 beads, BCG or DDA alone. IgG levels were low for mice vaccinated with BCG, and were similar to levels produced in mice vaccinated with DDA adjuvant alone. The subclass of IgG induced after immunization is associated with the type of immune response. IgG1 production is induced by Th2-type cytokines and IgG2 by Th1-type cytokines (Petrushina et al., 2003). The IgG response to Rv1626 beads was mixed, as indicated by induction of both Th1 and Th2 responses.

Vaccination with soluble Rv1626 and Rv2032 had been shown to reduce the bacterial burden when compared with non vaccinated controls from 0.3–0.5 $\log_{10}$ in a challenge with *M. tuberculosis* and Rv1789 reduced it by more than 0.5 $\log_{10}$ (Derrick et al., 2013). Rv1789 is a member of the PPE protein family, which play an important role in *M. tuberculosis* virulence (Fishbein et al., 2015). However, in this study, Rv1626 beads showed the strongest immunogenicity, which could infer that Rv1626 could be more protective antigen when this antigen is displayed on the surface of beads and used as vaccine. It should be noted that the stronger immunogenicity of the Rv1626 beads could also be due to the more suitable size range of these beads to induce an immune response as aforementioned.

The mice produced IgG responses to wild-type bead constituents and the sera from these showed a degree of antibody reactivity to the various antigen-displaying beads. This may be due to common impurities derived from the production host. These components present in bead preparations may also have triggered the TNFα responses observed in all the immunized groups of mice. Further assessment of the specificity of sera obtained from the immunized groups by immunoblotting against the various bead proteins clearly indicated that antibodies induced by the Rv1626 beads were specific for Rv1626 (Fig. 5). Since the other PhaC fusion proteins were not detected, PhaC, which anchors the antigen to the beads, does not seem to be immunogenic. Immunoblotting using sera from mice vaccinated with Rv1626 beads against whole cell lysates of the respective production host, recombinant *Clearcoli* either or not producing Rv1626 confirmed that beads only induced a specific response to Rv1626 and no other potential host cell derived components were immunogenic i.e. did not induce any detectable immune response (Fig. 6).

The Th1/Th2 paradigm assumes that *M. tuberculosis*, as an intracellular pathogen, induces cellular immune responses (Muraille et al., 2014), and the classic vaccinology approach assumes that the natural immune response to infection is sufficient for protection against a future infection. Nevertheless, for all licensed vaccines with the exception of BCG and herpes zoster, antibodies mediate protection (Plotkin, 2010). *M. tuberculosis* induces a humoral immune response to a wide variety of antigens despite being an intracellular pathogen (Steingart et al., 2009) and numerous studies have shown that antibodies modify the course of experimental mycobacterial infection in mice to the benefit of the host (Acosta et al., 2013). In the context of TB infection, instead of mimicking natural immunity, vaccine induced protection might require “uncommon” or “unnatural” immunity (Nunes-Alves et al., 2014). Utilisation of the bead antigen display strategy could facilitate screening and identification of vaccine candidates and include components that trigger an immune response different to the response naturally induced by the pathogen. These alternative responses may be important for protective immunity.

In conclusion, the mycobacterial antigens, Rv1626, Rv1789 and Rv2032 identified previously from a reverse vaccinology approach were successfully displayed on polyester beads. Immunisation of mice with these beads confirmed Rv1626 as an immunogenic antigen mediating an antigen-specific antibody response. Polyester beads are an efficient antigen delivery systems mediating specific immune responses to the delivered antigen. The potential of the Rv1626 antigen to induce protective immunity to tuberculosis will be investigated in future studies.

Author contributions

Performed the majority of the experiments: PRR, Designed the research study: PRR, NAP, DNW, BHAR. Contributed reagents/materials/analysis tools and assisted in post-mortem analysis: NAP. Drafted the manuscript: PRR, BHAR. All authors read and approved the final manuscript.

Conflict of interest statement

BHAR is shareholder and CSO of PolyBatics Ltd that commercialises the polyester bead technology.

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References

Acosta, A., Lopez, Y., Nor, N.M., Pando, R.H., Alvarez, N., Sarmiento, M.E., Glatman-Freedman, A., 2013. The role of antibodies in the defense against tuberculosis. In: Mahboub BH, V.M. (Ed.), Tuberculosis—Current Issues in Diagnosis and Management.

Amara, A.A., Rehm, B.H.A., 2003. Replacement of the catalytic nucleophile cysteine-296 by serine in class II polyhydroxyalkanoate synthase from *Pseudomonas aeruginosa*-mediated synthesis of a new polyester: identification of catalytic residues. Biochem. J. 374, 413–421.

Andersen, P., Doherty, M., 2005. The success and failure of BCG—implications for a novel tuberculosis vaccine. Nat. Rev. Microbiol. 3, 656–662.

Bansal, K., Elluru, S.R., Narayana, Y., Chaturvedi, R., Patil, S.A., Kaveri, S.V., Bayry, J., Balaji, K.N., 2010. PE_PGRS antigens of *Mycobacterium tuberculosis* induce maturation and activation of human dendritic cells. J. Immunol. 184, 3495–3504.

Bertholet, S., Reed, S., Rappuoli, R., 2014. Reverse vaccinology applied to TB. In: Acosta, A., Nor, N.M., Sarmiento, M.E. (Eds.), The Art & Science of Tuberculosis Vaccine Development. , 2nd ed.

Bhatt, K., Verma, S., Ellner, J.J., Salgame, P., 2015. Quest for correlates of protection against tuberculosis. Clin. Vaccine Immunol. 22, 258–266.

Black, G.F., Weir, R.E., Floyd, S., Bliss, L., Warndorff, D.K., Crampin, A.C., Ngwira, B., Sichali, L., Nazareth, B., Blackwell, J.M., Branson, K., Chaguluka, S.D., Donovan, L., Jarman, E., King, E., Fine, P.E.M., Dockrell, H.M., 2002. BCG-induced increase in interferon-gamma response to mycobacterial antigens and efficacy of BCG vaccination in Malawi and the UK: two randomised controlled studies. Lancet 359, 1393–1401.

Byun, E.H., Kim, W.S., Kim, J.S., Jung, I.D., Park, Y.M., Kim, H.J., Cho, S.N., Shin, S.J., 2012. *Mycobacterium tuberculosis* Rv0577, a novel TLR2 agonist, induces maturation of dendritic cells and drives Th1 immune response. FASEB J. 26, 2695–2711.

Chen, S., Parlane, N.A., Lee, J., Wedlock, D.N., Buddle, B.M., Rehm, B.H.A., 2014. New skin test for detection of bovine tuberculosis on the basis of antigen-displaying polyester inclusions produced by recombinant escherichia coli. Appl. Environ. Microbiol. 80, 2526–2535.

Cho, Y.S., Dobos, K.M., Prenni, J., Yang, H., Hess, A., Rosenkrands, I., Andersen, P., Ryoo, S.W., Bai, G.-H., Brennan, M.J., Izzo, A., Bielefeldt-Ohmann, H., Belisle, J.T., 2012. Deciphering the proteome of the in vivo diagnostic reagent purified protein derivative from *Mycobacterium tuberculosis*. Proteomics 12, 979–991.

Coppola, M., van den Eeden, S.J., Wilson, L., Franken, K.L., Ottenhoff, T.H., Geluk, A., 2015. Synthetic long peptide derived from *Mycobacterium tuberculosis* latency antigen rv1733c protects against tuberculosis. Clin. Vaccine Immunol. 22, 1060–1069.

Derrick, S.C., Yabe, I.M., Yang, A., Kolibab, K., Hollingsworth, B., Kurtz, S.L., Morris, S., 2013. Immunogenicity and protective efficacy of novel *Mycobacterium tuberculosis antigens*. Vaccine 31, 4641–4646.

Donati, C., Rappuoli, R., 2013. Reverse vaccinology in the 21 st century: improvements over the original design. Ann. N. Y. Acad. Sci. 1285, 115–132.

Fifis, T., Gamvrellis, A., Crimeen-Irwin, B., Pietersz, G.A., Li, J., Mottram, P.L., McKenzie, I.F.C., Plebanski, M., 2004. Size-dependent immunogenicity: therapeutic and protective properties of nano-vaccines against tumors. J. Immunol. 173, 3148–3154.

Fishbein, S., van Wyk, N., Warren, R.M., Sampson, S.L., 2015. Phylogeny to function: PE/PPE protein evolution and impact on *Mycobacterium tuberculosis* pathogenicity. Mol. Microbiol. 96, 901–916.

Hay, I.D., Hooks, D.O., Rehm, B.H.A., 2014. Use of bacterial polyhydroxyalkanoates in protein display technologies. In: Springer Protocols Handbooks. Humana Press, Totowa, NJ, pp. 1–16.

Herbert, A.B., Rehm, B.H.A., Saravolac, E.G., Thompson, T., 2012. Compositions for separation methods. WO2013190453 A2.

Hurley, J.C., 1995. Endotoxemia: methods of detection and clinical correlates. Clin. Microbiol. Rev 8, 268–292.

Katare, Y.K., Muthukumaran, T., Panda, A.K., 2005. Influence of particle size, antigen load, dose and additional adjuvant on the immune response from antigen loaded PLA microparticles. Int. J. Pharm. 301, 149–160.

Ma, H.K., Liu, M.M., Li, S.Y., Wu, Q., Chen, J.C., Chen, G.Q., 2013. Application of polyhydroxyalkanoate (PHA) synthesis regulatory protein PhaR as a bio-surfactant and bactericidal agent. J. Biotechnol. 166, 34–41.

Magalhães, P.O., Lopes, A.M., Mazzola, P.G., Rangel-Yagui, C., Penna, T.C.V., Pessoa, A., 2007. Methods of endotoxin removal from biological preparations: a review. J Pharm. Pharm. Sci. 10, 388–404.

Muraille, E., Leo, O., Moser, M., 2014. TH1/TH2 paradigm extended: macrophage polarization as an unappreciated pathogen-driven escape mechanism? Front. Immunol. 5, 603.

Nakaoka, R., Inoue, Y., Tabata, Y., Ikada, Y., 1996. Size effect on the antibody production induced by biodegradable microspheres containing antigen. Vaccine 14, 1251–1256.

Nunes-Alves, C., Booty, M.G., Carpenter, S.M., Jayaraman, P., Rothchild, A.C., Behar, S.M., 2014. In search of a new paradigm for protective immunity to TB. Nat. Rev. Microbiol. 12, 289–299.

Parlane, N.A., Wedlock, D.N., Buddle, B.M., Rehm, B.H., 2009. Bacterial polyester inclusions engineered to display vaccine candidate antigens for use as a novel class of safe and efficient vaccine delivery agents. Appl. Environ. Microbiol. 75, 7739–7744.

Parlane, N.A., Grage, K., Lee, J.W., Buddle, B.M., Denis, M., Rehm, B.H.A., 2011. Production of a particulate hepatitis C vaccine candidate by an engineered *Lactococcus lactis* strain. Appl. Environ. Microbiol. 77, 8516–8522.

Parlane, N.A., Grage, K., Mifune, J., Basaraba, R.J., Wedlock, D.N., Rehm, B.H., Buddle, B.M., 2012. Vaccines displaying mycobacterial proteins on biopolyester beads stimulate cellular immunity and induce protection against tuberculosis. Clin. Vaccine Immunol. 19, 37–44.

Parlane, N.A., Gupta, S.K., Rubio-Reyes, P., Chen, S., Gonzalez-Miro, M., Wedlock, D.N., Rehm, B.H.A., 2016. Self-assembled protein-coated polyhydroxyalkanoate beads: properties and biomedical applications. ACS Biomater. Sci. Eng., http://dx.doi.org/10.1021/acsbiomaterials.6b00355, in press.

Peoples, O.P., Sinskey, A.J., 1989. Poly-beta-hydroxybutyrate (PHB) biosynthesis in Alcaligenes eutrophus H16. Identification and characterization of the PHB polymerase gene (phbC). J. Biol. Chem. 264, 15298–15303.

Petrushina, I., Tran, M., Sadzikava, N., Ghochikyan, A., Vasilevko, V., Agadjanyan, M.G., Cribbs, D.H., 2003. Importance of IgG2c isotype in the immune response to β-amyloid in amyloid precursor protein/transgenic mice. Neurosci. Lett 338, 5–8.

Plotkin, S.A., 2010. Correlates of protection induced by vaccination. Clin. Vaccine Immunol. 17, 1055–1065.

Promega, 2004 Subcloning notebook in: Promega (Ed.), 1–68.

Rappuoli, R., 2000. Reverse vaccinology. Curr. Opin. Microbiol. 3, 445–450.

Rehm, B.H.A., 2010. Bacterial polymers: biosynthesis, modifications and applications. Nat. Rev. Micro 8, 578–592.

Schubert, P., Steinbüchel, A., Schlegel, H.G., 1988. Cloning of the Alcaligenes eutrophus genes for synthesis of poly-beta-hydroxybutyric acid (PHB) and synthesis of PHB in *Escherichia coli*. J. Bacteriol. 170, 5837–5847.

Steingart, K.R., Dendukuri, N., Henry, M., Schiller, I., Nahid, P., Hopewell, P.C., Ramsay, A., Pai, M., Laal, S., 2009. Performance of purified antigens for serodiagnosis of pulmonary tuberculosis: a meta-analysis. Clin. Vaccine Immunol. 16, 260–276.

WHO, 2015. Global TB Report, 19 ed.

Woodard, R.W., Meredith, T.C., Aggarwal, P., 2012. Viable non-toxic gram-negative bacteria. US 8303964 B2.

Xu, Y., Yang, E., Huang, Q., Ni, W., Kong, C., Liu, G., Li, G., Su, H., Wang, H., 2015. PPE57 induces activation of macrophages and drives Th1-type immune responses through TLR2. J. Mol. Med. 93, 645–662.