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Engineering a new vaccine platform for heterologous antigen delivery in live-attenuated *Mycobacterium tuberculosis*

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ABSTRACT

Live vaccines are attractive vehicles for antigen delivery as a strategy to immunize against heterologous pathogens. The live vaccine MTBVAC is based on rational attenuation of *Mycobacterium tuberculosis* with the objective of improving BCG protection against pulmonary tuberculosis. However, the development of recombinant mycobacteria as antigen-presenting microorganisms has been hindered due to their fastidious genetic manipulation. In this study, we used MTBVAC as a genetic platform to deliver diphtheria, tetanus, or pertussis toxoids, which are the immunogenic constituents of the DTP vaccine. When using nonoptimal genetic conditions, the expression of these immunogens was barely detectable. Accordingly, we pursued a rational, step-by-step optimization of the genetic components to achieve the expression and secretion of these toxoids. We explored variants of the L5 mycobacteriophage promoter to ensure balanced antigen expression and plasmid stability. Optimal signal sequences were identified by comparative proteomics of MTBVAC and its parental strain. It was determined that proteins secreted by the Twin Arginine Translocation pathway displayed higher secretion in MTBVAC, and the Ag85A secretion sequence was selected as the best candidate. Because the coding regions of diphtheria, tetanus, and pertussis toxoids significantly differ in G + C content relative to mycobacterial genes, their codon usage was optimized. We also placed a 3xFLAG epitope in frame with the C-terminus of these toxoids to facilitate protein detection. Altogether, these optimizations resulted in the secretion of DTP antigens by MTBVAC, as demonstrated by western blot and MRM-MS. Finally, we examined specific antibody responses in mice vaccinated with recombinant MTBVAC expressing DTP antigens.

1. Introduction

Vaccination is the public health intervention offering the best cost-to-benefit ratio. Millions of lives are saved each year through vaccination efforts; however, due to this silent benefit, the potential of vaccines is usually undervalued [1]. The advent of the COVID-19 disease in 2019 (COVID-19), a pandemic that has now surpassed 4 million deaths since December 2019 [2], has highlighted the clinical and social value of vaccines. The development of new vaccine platforms based on messenger RNA (mRNA) or adenoviral vectors has proven crucial to tackle the COVID-19 pandemic. These two vaccine strategies were initially developed decades earlier as proof-of-concept against cancer and other infectious diseases [3,4]. Exploration of classical and alternative vaccination strategies is essential to endow research with the technological arsenal to fight current, emerging and/or future infectious diseases.

Tuberculosis (TB) is an infectious disease mainly caused by the bacterium *Mycobacterium tuberculosis,* which killed 1.4 million people in 2019, predominantly in developing countries [5]. This high rate of mortality is reached despite the existence of the centenary vaccine "Bacille de Calmette et Guérin" (BCG). BCG, the only vaccine against TB currently in use, is a live vaccine consisting of the causative agent of TB in cattle, *Mycobacterium bovis*, which

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was randomly attenuated during laboratory subcultivation between 1907 and 1921 [6]. Even if BCG is efficacious in preventing severe forms of TB in children, it is less effective in preventing pulmonary TB in adults. Thus, several vaccine candidates are being developed to improve BCG. MTBVAC is a live attenuated vaccine designed to replace BCG that has completed phase IIa dose-finding trials in both adolescents and newborns in South Africa (NCT02933281 and NCT03536117) [7]. Unlike BCG, which was attenuated by random loss of chromosomal regions during subcultivation, MTBVAC has been rationally attenuated by deletion of the *phoP* and *fadD26* genes involved in virulence [8–11]. Furthermore, the MTBVAC parent strain (Mt103) is a human TB clinical isolate of *M. tuberculosis*, which differs from the *M. bovis* cow pathogen used to develop BCG [12]. Accordingly, MTBVAC maintains the whole T-cell epitope repertoire of the human pathogen, including>25% of these epitopes lost in BCG during subcultivation [13,14]. Altogether, cumulative preclinical and clinical studies position MTBVAC as a plausible new TB vaccine.

BCG has been historically proposed as a recombinant vaccine vehicle to express heterologous antigens because of its exceptional features [15]: it can persist for long periods inside macrophages, inducing long-lasting humoral and cellular immune responses; mycobacterial preparations exhibit intrinsic adjuvant properties, and they are indeed the basis of Freund's complete adjuvant; it is safe for newborns; and it is low-cost compared with subunit vaccines. In addition, the existing paradigm in which vaccines only protect against the target infection is being revisited [16] in light of recent findings demonstrating that BCG protects against unrelated pathogens [17–19], resulting in fewer hospitalizations in children [20] or reduced viral respiratory infections in the elderly [21]. Taking into account that MTBVAC maintains or even improves these properties of BCG [12,22], it would be of interest to evaluate the suitability of MTBVAC as a vaccine delivery platform for heterologous antigen expression.

In addition to the vaccine field, there is a need for new delivery systems, or probiotics, to compose live biotherapeutics: the existing options, such as *Escherichia coli*, Bacteroides, and lactic acid bacteria, are not able to stably colonize [23] or enter eukaryotic cells. MTBVAC could serve as an alternative scaffold in synthetic biology to deliver therapeutics inside host cells because it is capable of persisting inside macrophages and nonphagocytic cells [11,24]. However, synthetic biology toolkits in mycobacteria are far from reaching the potential of *E. coli*, partly because of the slow growth rate of *M. tuberculosis*, its poor DNA uptake, and its high rate of illegitimate recombination [25]. Despite these obstacles, a variety of genetic tools for mycobacteria have been developed, such as integrative and replicative plasmids, reporter systems, inducible and wide range strength promoters, selectable markers [26], recombineering [27], or even CRISPR-based [28,29] methodologies. However, to our knowledge, these methods have not been integrated into synthetic biology studies in *M. tuberculosis*. In this study, we used MTBVAC as an *M. tuberculosis* derivative to pave the way for synthetic biology in this fastidious pathogen.

In a first attempt to test the ability of MTBVAC to express heterologous antigens, we used the p2auxoHIVA plasmid, previously tested in BCG, to express and secrete human immunodeficiency virus (HIV) epitopes in lysine-auxotrophic MTBVAC. We achieved the expression of HIV epitopes in auxotrophic MTBVAC, which induced a specific immune response against those epitopes [30]. This proof-of-concept study supported the use of MTBVAC as an antigen delivery platform.

Regarding the heterologous antigens to be expressed, here, we used diphtheria, tetanus, and pertussis toxoids, which are the immunogenic constituents of the DTP vaccine. These immunogens were selected based on the observation that BCG or MTBVAC administered before the DTP vaccine was able to trigger Th1 immune responses against diphtheria, tetanus, and pertussis in mice. Immunization with DTP alone elicited a Th2 or a Th1/Th2 response. In addition, antibody responses against DTP antigens were also enhanced by previous immunization with BCG or MTBVAC in mice [31].

Genetic inactivation of diphtheria and pertussis toxins has been described without loss in their immunogenicity, while fragment C of the tetanus toxin is immunogenic with no associated toxicity. While genetic inactivation makes DTP toxoids ideal for recombinant expression in MTBVAC, clinical features could also be relevant. The DTP vaccine efficiently protects against tetanus and diphtheria, but protection against whooping cough caused by *Bordetella pertussis* remains less than optimal [32]. Recently, maternal immunization with pertussis vaccines and transplacental transfer of maternal antibodies have helped to diminish whooping cough cases in newborns, who are the most vulnerable population [33].

Altogether, based on this previous evidence, we hypothesize that constructing a recombinant MTBVAC expressing and secreting the DTP constituents would not only enhance immune responses against these immunogens but also pave the way to develop a combined vaccine against TB, diphtheria, tetanus, and pertussis. This latter possibility opens interesting avenues for live vaccines in terms of reducing vaccine production costs. The use of lyophilized microorganisms can avoid the need for cold chain distribution in developing countries or afford a combined, long-term immunization with a single shot.

2. Material and methods

2.1. Bacteria

The *Escherichia coli* HST08 (Stellar™; Clonetech) strain, used for cloning steps, was grown at 37 °C in Luria-Bertani (LB) broth or on agar plates supplemented with kanamycin (km; 20 μg/mL) when necessary.

M. tuberculosis MTBVAC (Universidad de Zaragoza) and its derived strains were grown at 37 °C in Middlebrook 7H9 broth supplemented with albumin-dextrose-catalase (ADC) 10% and with 0.05% (v/v) Tween-80 (7H9T-ADC). When necessary, MTBVAC was cultured on solid Middlebrook 7H10 plates supplemented with 0.5% glycerol and 10% ADC. When required, km was added at a concentration of 20 μg/mL. Bacterial suspensions for vaccination were prepared by diluting frozen PBS stocks previously quantified by plating serial dilutions.

2.2. Construction of MTBVAC-pJKB7, MTBVAC-pJKD6 and MTBVAC-pJKC1

pJKB7, pJKD6, and pJKC1 plasmids were previously generated [34]. Electrocompetent MTBVAC was prepared as follows: bacterial cultures at an optical density at 600 nm of 0.6–0.8 were supplemented with glycine at 0.2 M and incubated at 37 °C for 24 h. Then, bacteria were pelleted and washed twice with 0.05% Tween-80 and once with 10% glycerol-0.05% Tween-80 and finally suspended in 1/100 of the initial culture volume of 10% glycerol-0.05% Tween-80. Aliquots of 200–400 μL were electroporated with 0.5–1 μg of replicative pJKB7, pJKD6, and pJKC1 plasmids. Gap cuvettes (0.2 cm) (Bio-Rad) were used with a single pulse (2.5 kV, 25 μF, 1000 Ω) in a GenePulser XcellTM (Bio-Rad). Cells were recovered on 7H9T-ADC and incubated for 24 h at 37 °C to express antibiotic resistance genes. Then, serial decimal dilutions were plated on 7H10 plates containing the appropriate antibiotic. Colonies typically appeared in 3–4 weeks.

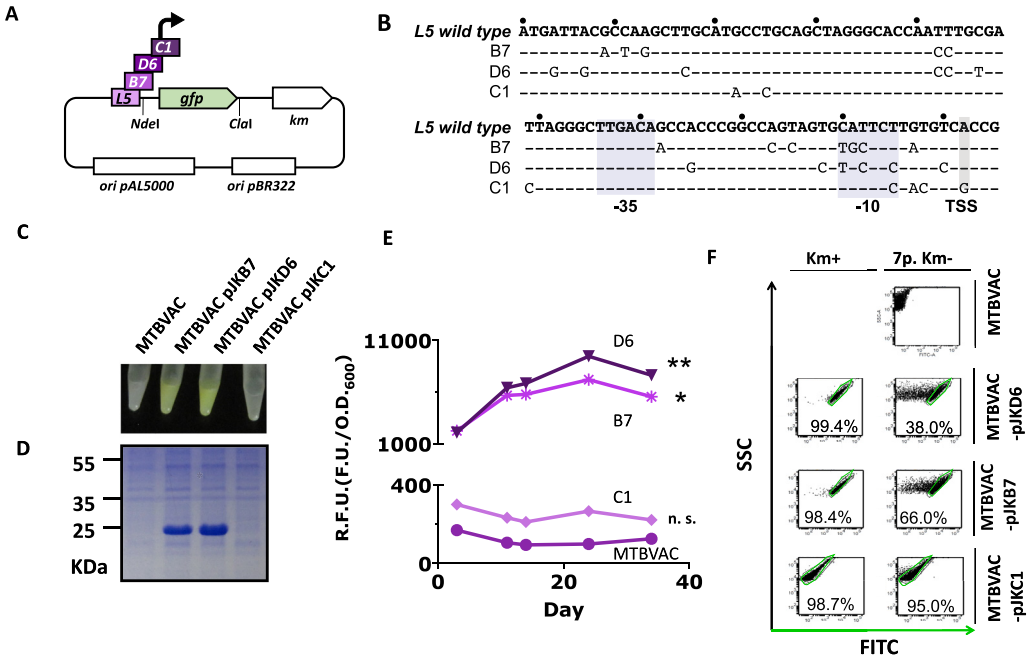

Fig. 1. Optimization of the L5 mycobacteriophage promoter for use in *M. tuberculosis*. A. Vector (pJK) for heterologous antigen expression in mycobacteria. The locations and directions of the *gfp* and kanamycin resistance (km) genes are indicated. This vector contains pAL5000 and pBR322 replicative origins for mycobacteria and *E. coli*, respectively. The L5 mycobacteriophage-derived promoters (C1, D6 and B7) are located upstream of *gfp*. *Nde*I and *Cla*I cloning sites are indicated. B. Partial sequences of the C1, D6 and B7 L5 mycobacteriophage promoters used in this study. The −35 and −10 boxes, as well as the transcription start site (TSS), are indicated. Note the different nucleotide polymorphisms spanning the promoter length in each variant. C. Liquid cultures of MTBVAC transformed with the plasmid depicted in panel A carrying C1, D6 and B7 promoters. Note the green colouration of the cultures resulting from GFP expression from the D6 and B7 promoters. D. Whole-cell fractions of cultures depicted in panel C stained with Coomassie blue dye. Note that the prominent band at 25 kDa is compatible with the molecular weight of the GFP protein. E. Relative fluorescence of MTBVAC strains carrying the *gfp* gene controlled by either the C1, D6 or B7 promoter. These promoters show different strengths ranging from high (D6) to medium (B7) and low (C1), which are maintained during the 35 days of the experiment. Statistical analysis was performed using the Friedman test followed by Dunn's multiple comparison test using MTBVAC as a control. Asterisks indicate the following p-values: *= 0.05 > p > 0.01; **=0.01 > p > 0.0005. F. Cytometry measurements of GFP activity in bacterial populations carrying the *gfp* gene under the control of C1, B7 and D6 promoters. The diagram indicates GFP measures in cultures with antibiotic (km +) pressure or cultures after seven passages without antibiotic (7p. km-). Note the gradual loss of plasmids with the D6 and B7 promoters in contrast to the stability of the plasmid bearing the C1 promoter. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.3. Construction of pJKD6ssXGFP plasmids

To generate the pJKD6ssXGFP plasmid (Fig. 2C), where X represents the different signal sequences (ss), DNA fragments containing the ss of ssag85A, ssag85B, ssag85C, ssblaC, ssrv0180c, ssrv0203, ssrv1987, and sscfp10 were amplified by PCR from MTBVAC genomic DNA. The oligonucleotides used are listed in Supplementary Table 1 as pJKD6ssX_fw and GFPssX_rv, except in the case of sscfp10, in which ss was cloned after the *gfp* gene in the *Bsr*GI cloning site. These PCR fragments were cloned in the pJKD6 plasmid previously digested with *NdeI* or *ClaI* by using an In-Fusion cloning kit (TaKaRa) system that allows ligase-independent cloning. *E. coli* Stellar was transformed with the reaction product, and proper recombination was checked by PCR and Sanger sequencing. MTBVAC was electroporated with pJKD6ssXGFP derivatives as previously described.

2.4. Construction of rMTBVACss-FC, rMTBVACssCRM and rMTBVACssS1 strains

The pJK-derived plasmids containing the signal sequence of Antigen 85A and the codon-optimized genes for FC, CRM197, or S1 were synthesized and subcloned into *Nde*I-*Cla*I sites by Genscript Company (Piscataway, USA). A schematic representation of the pJK derivatives is shown in Fig. 3A. MTBVAC was electroporated with pJK derivatives as previously described.

2.5. Construction of rMTBVAC-FC, rMTBVAC-CRM and rMTBVAC-S1 strains

To generate pJK plasmids without signal sequences (Fig. 3E), DNA fragments containing S1, FC, or CRM197 with optimized codons were amplified from pJKD6ssA-S1, pJKD6ssA-FC, or pJKD6ssA-CRM by PCR. These PCR fragments with exactly 15 bp identity to pJKD6 were inserted into pJKD6 previously digested with *Nde*I and *Cla*I. The correct construction checked by PCR and by Sanger sequencing was electroporated into MTBVAC.

2.6. Protein extraction

Intracellular protein extracts of mycobacteria were prepared from late log-phase cultures grown in 7H9T-ADC. Cells from 50 mL of culture were pelleted by centrifugation (4,000 g for 10 min at 4 °C). Mycobacterial cell pellets were washed twice with PBS to remove albumin and then resuspended in 1 mL of cold PBS. Mycobacterial suspensions were disrupted by sonication using the BioRuptor (Diagenode) for 15 min (30 s pulse at high power), allowing cooling in an ice-water bath for 30 s between pulses. The samples were centrifuged at 4000 g for 10 min at 4 °C, and the supernatant containing whole-cell protein extracts was filtered through a 0.22 µm-pore-size low protein-binding filter (Pall).

For extraction of secreted proteins, mycobacterial strains were cultured in 7H9T medium supplemented with dextrose, NaCl, and catalase in the absence of albumin. Cultures were grown until

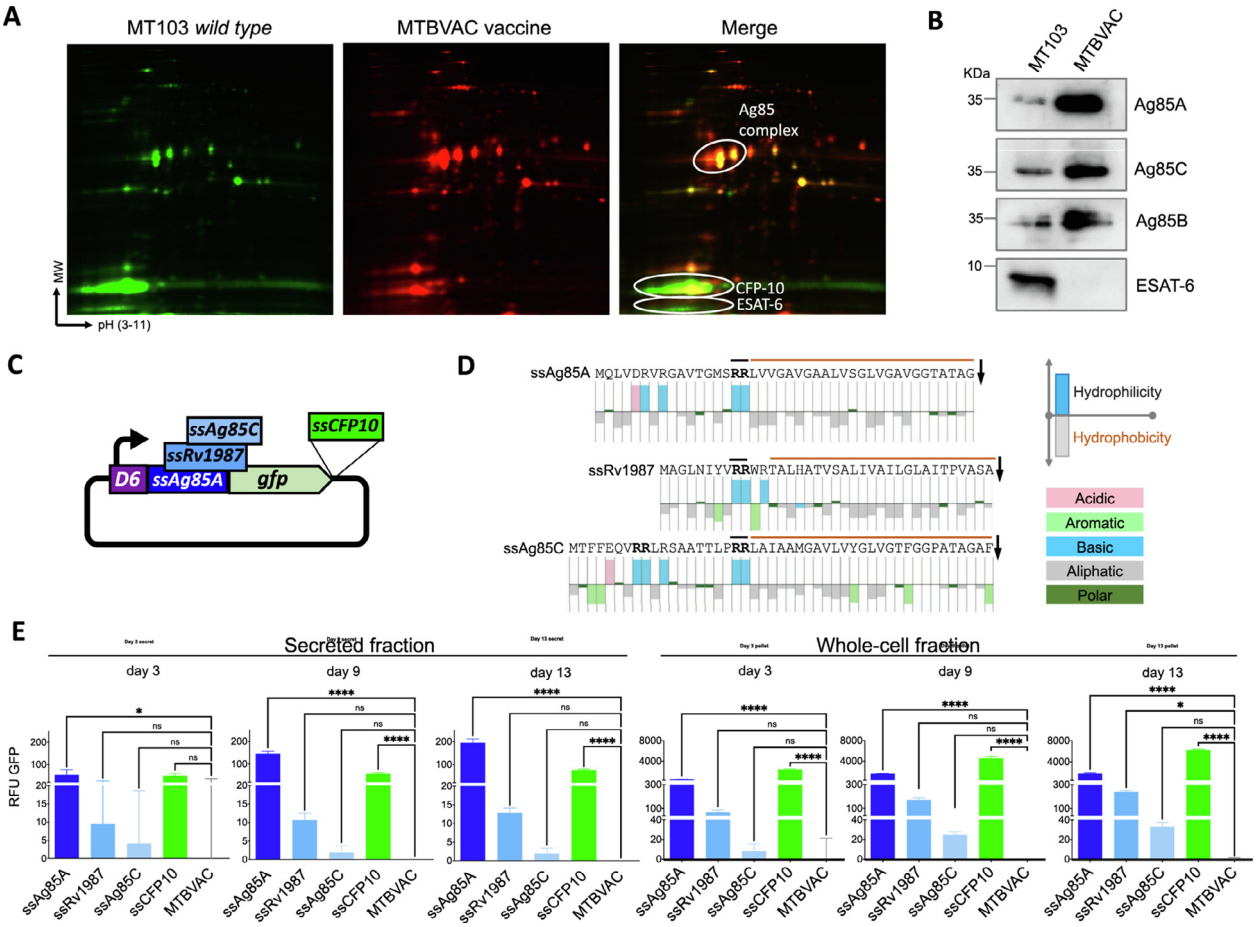

Fig. 2. Selection of an optimized signal sequence to secrete heterologous antigens in MTBVAC. A. Images from 2D-DiGE gels showing the secreted proteomes of the Mt103 strain (labelled in green), the MTBVAC vaccine (labelled in red) and the merged image. Note the predominant secretion of ESAT-6 and CFP-10 in Mt103 relative to MTBVAC. Additionally, note the increased secretion of the Ag85 complex in MTBVAC relative to Mt103. B. Western blot of Ag85A, 85B, 85C and ESAT-6 proteins in secreted fractions of Mt103 and MTBVAC. C. Construction of a reporter vector of protein secretion in mycobacteria. The plasmid in panel 1A was cut with *Nde*I, and the secretion signals of Ag85A, 85C and Rv1987 were introduced upstream of the coding sequence of the *gfp* gene. The secretion signal of CFP-10 was cloned into the *Cla*I site. D. Characteristics of the Ag85A, 85C and Rv1987 secretion signals. The hydrophilic profile of each residue and the RR motif of the TAT secretion pathway are indicated. The downside arrow indicates the putative processing site. E. Relative fluorescence unit (RFU) measurements of secreted and whole-cell fractions of MTBVAC transformed with plasmids indicated in panel C. Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparison test using MTBVAC as the control group. Asterisks indicate the following p-values: * 0.05 > p > 0.01; **** 0.0001 > p. Note that GFP was successfully secreted with each signal secretion tested, and the Ag85A secretion signal resulted in the best secretion efficacy. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the late log phase and then centrifuged (4000 g for 10 min at 4 °C). The supernatant was filtered through a 0.22 μm-pore-size low protein-binding filter (Pall). The extracellular proteins were precipitated for 1 h at 4 °C with 10% v/v trichloroacetic acid. Next, pellets were collected by centrifugation (4000 g, 1 h at 4 °C), washed with cold acetone, and resuspended in 200 μL of 150 mM Tris-HCl pH 8.8. In both cases, proteins were quantified using the QuantiPro BCA assay (Sigma-Aldrich) and stored at −80 °C.

2.7. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Ten micrograms of each protein extract was boiled for 5 min in the presence of 3 μL of 150 mM Tris/HCl pH 7.4, 3% SDS, 0.3 mM sodium molybdate, 30 mM sodium pyrophosphate, 30 mM NaF, 30% glycerol, 30% mercaptoethanol and 0.06% bromophenol blue. Then, proteins were separated by electrophoresis through a 5% stacking gel over a 10–15% resolving polyacrylamide gel containing 0.1% SDS in running buffer (25 mM Tris, 192 mM glycine, 3.4 mM SDS) at a constant amperage of 30 mA/gel. PageRuler Plus Prestained Protein Ladder (Thermo Scientific) was used as a molecular weight marker. The gels were stained with NOVEX Coomassie colloidal (INVITROGEN).

2.8. Western blot

Proteins contained in SDS-PAGE gels without Coomassie staining were transferred to PVDF membranes (preactivated with methanol) in transfer buffer (48 mM Tris/HCl pH 8.3, 39 mM glycine, 0.037% SDS, 20% methanol) for 1 h at 20 V with a semi-dry electrophoretic transfer cell (BioRad). The membrane was blocked with 5% skim powdered milk in wash buffer (0.9% (w/v) NaCl, 10 mM Tris/HCl pH 8.0, 0.1% Tween-20) for 1 h at room temperature with mild shaking. Thereafter, the membrane was incubated with primary antibody (Supplementary Table 3) in wash buffer with 5% skim powdered milk overnight at 4 °C. Three washes with wash buffer were performed before incubation with human serum adsorbed (HSA) secondary antibodies conjugated with horseradish peroxidase (HRP) in wash buffer with 5% skim powdered milk for 1 h at room temperature and mild shaking. Then, the membrane was washed again three times with wash buffer. Immunocomplex detection was performed by incubation with a chemiluminescent HRP substrate (ImmobilonTM western, Millipore) and subsequent exposure in a Proxima 2850 instrument (Isogen).

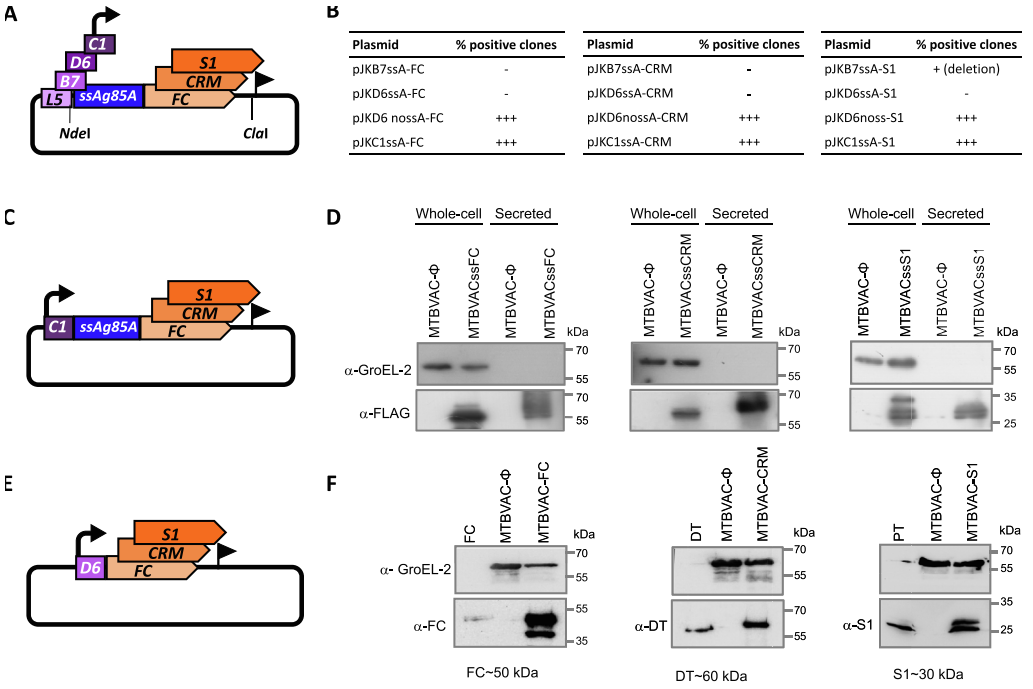

Fig. 3. Experimental validation of rMTBVAC strains expressing or secreting DTP antigens. A. Construction of plasmids for heterologous antigen secretion in MTBVAC. The DNA sequence for a protein consisting of the Ag85A secretion signal in fusion with each heterologous antigen (S1, FC or CRM197) and bearing a 3xFLAG epitope in the C-terminal end was introduced into the *Nde*I and *Cla*I sites under the control of C1, D6 and B7 promoters. B. The tables represent the percentage of positive clones resulting from every combination, where +++ indicates numerous colonies and – indicates no colonies. C. Schematic representation of the constructs selected from panel B to express and secrete DTP antigens by rMTBVAC. D. Western blots of whole-cell and secreted fractions of rMTBVAC transformed with S1, FC and CRM197 secretion plasmids. The GroEL2 protein was used either as a loading control in whole-cell samples or as a control in the absence of undesired lysis in secreted fractions, and wild-type MTBVAC served as the negative control. Each DTP antigen was detected using an anti-FLAG antibody, allowing the detection of the fusion proteins. E. Schematic representation of the constructs used to express DTP antigens in rMTBVAC, avoiding secretion toxicity. F. Western blots of whole-cell fractions of rMTBVAC carrying S1, FC or CRM197 expression plasmids. The GroEL2 protein was used as an internal standard; control DTP antigens were detected using specific antibodies against S1 (PT), FC and CRM197 (DT). The first lane in every panel corresponds to purified antigen protein. MTBVAC wild type serves as the negative control.

2.9. Multiple reaction Monitoring/Mass spectrometry (MRM/MS)

The MRM assay approach was applied for the measurement of specific peptides in complex mixtures. To detect S1, FC and CRM197 in the rMTBVAC protein extracts, MRM/MS reactions were performed by the Proteomics Facility of Servicios Científico Técnicos del CIBA (IACS-Universidad de Zaragoza). Cytosolic and secreted protein extracts from rMTBVACs were electrophoretically separated by SDS-PAGE. After staining with colloidal Coomassie (INVITROGEN), the gel bands were cut, and these bands included the adequate molecular weight of each antigen. For in-solution digestion, purified toxins were dried in a speed-vac (Thermo) and resuspended in denaturing buffer (6 M urea, 100 mM Tris-HCl pH 7.8). Cysteines were reduced with 200 mM DTT for 30 min at 37 °C and alkylated with 200 mM iodoacetamide for 30 min in the dark. Unreacted iodoacetamide was consumed by adding 20 µL of the reducing agent (200 mM DTT) for 30 min at room temperature. Samples were diluted with 25 mM ammonium bicarbonate to a final concentration of less than 1 M urea. Partial digestion was carried out with trypsin (Gold Trypsin, Promega) at a 1:50 ratio (enzyme/protein) for 3 h at 37 °C. The reaction was stopped by adding concentrated formic acid (Sigma). Protein bands from the rMTBVAC samples were excised at an adequate molecular weight from SDS-PAGE gels and washed with water, ammonium bicarbonate (25 mM NH_4HCO_3), and acetonitrile. Next, samples were reduced by incubation with dithiothreitol (10 mM) at 60 °C for 45 min and alkylated by incubation with iodoacetamide (50 mM) at room temperature for 30 min. Finally, proteins were trypsin digested overnight at 37 °C with an enzyme:protein ratio of 1:10 (Trypsin Gold, Promega). Digestion was stopped by the addition of 0.1% trifluoroacetic acid, and tryptic peptides were extracted sequentially with increasing concentrations of acetonitrile in water. Samples were dried in a speed-vac (Thermo) and reconstituted in 98% H2O, 2% acetonitrile, and 0.1% formic acid.

Protein identification was performed on a nano-LC system (Tempo MDLC, Eksigent, Dublin, CA, USA) coupled to a hybrid triple quadrupole/linear ion trap mass spectrometer (4000 QTRAP, Sciex, Foster City, CA). After precolumn desalting, tryptic digests (1 µg) were separated on a C18 column (Acclaim PepMap100, 75 µm id, 15 cm, 3 µm particle size, Thermo Scientific, USA) at a flow rate of 300 nL/min, with a 90 min linear gradient from 5 to 35% acetonitrile in 0.1% formic acid. The injection volume was 10 µL. The mass spectrometer was interfaced with a nanospray source equipped with an uncoated fused silica emitter tip (20 µm inner diameter, 10 µm tip, New Objective, Woburn, MA) and was operated in positive ion mode. MS source parameters were as follows: capillary voltage 2800 V, source temperature 150 °C, declustering potential (DP) 85 V, curtain, ion source gas (nitrogen) 20 psi, and collision gas (nitrogen) high. Analyses were performed using an information-dependent acquisition (IDA) method with the following steps: single enhanced mass spectra (EMS, 400–1400 *m*/*z*) from which the 8 most intense peaks were subjected to an enhanced product ion [EPI (MS/MS)] scan. Once the 8 most intense ions were subjected to downstream analysis, they were ignored for a period of 25 s.

2.10. MRM analysis

The weight and migration parameters of each peptide were stabilized to perform the MRM/MS technique with each purified

digested toxin. The purified toxins used were pertussis toxin (PT) from Calbiochem and fragment C of tetanus toxin kindly provided by Dra. Rosario Osta and Diphtheria toxin (DT) from Calbiochem. Optimal transitions were selected for each peptide with open-source Skyline software (MacCoss Lab Software, Seattle, WA, version 2.6.0.6851). Briefly, 3 to 5 transitions were selected for each peptide based on the intensity of y-fragment ions in the MS/MS spectra that were obtained in the LC-MS/MS analysis described above. MRM analyses were conducted on the nano-LC system coupled to the 4000QTRAP mass spectrometer with the same chromatographic and source parameter settings described above. MRM transitions for each peptide were recorded with a dwell time of 40 ms. Collision energies were automatically computed using the embedded rolling collision energy equations of the Skyline software. The mass spectrometer was instructed to switch from MRM to enhanced product ion (EPI) scanning mode when an individual MRM transition signal exceeded 1000 counts. Data were analysed by submitting the MS/MS data to a MASCOT server (version 2.3) (Matrix Science Ltd., London, UK). The data were searched against the SwissProt *M. tuberculosis* sequence database supplemented with the sequences of PT, FC, and DT toxins. The identification parameters were as follows: maximum missed cleavages 1, fixed modifications carbamidomethyl (cysteines), peptide mass tolerance 0.5 Da, fragment mass tolerance 0.3 Da.

2.11. 2D-DiGE (Differential in gel Electrophoresis)

Four independent cultures of Mt103 and MTBVAC were grown in 7H9 0.05% Tween 80 supplemented with 0.2% dextrose and 0.085% NaCl. After 4 weeks of incubation at 37 °C, cultures were pelleted by centrifugation at 4000 g for 10 min. The supernatant containing secreted proteins was incubated with 10% trichloroacetic acid (TCA) for one hour at 4 °C and then centrifuged for 90 min at 4000 g at 4 °C. Pelleted proteins were incubated with cold acetone for 2 h at −20 °C and centrifuged for 30 min at 4000 g and 4 °C, and the resulting pellet was air-dried for 15 min on ice. The pelleted fraction of protein was resuspended in 200 µL DiGE labelling buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris, Milli-Q water). Protein sample pH was checked and adjusted to 9.5 with KOH 1 N, quantified with the RC DC Protein Assay Kit (BioRad), and protein integrity and absence of albumin contamination were checked by SDS-PAGE and Coomassie staining. Secreted proteins were analysed through fluorescence two-dimensional difference gel electrophoresis (2D-DiGE). Briefly, Mt103 and MTBVAC samples were labelled with N-hydroxysuccinimidyl (NHS) ester derivatives of the cyanine dyes Cy3 and Cy5, respectively, and a pooled internal standard of equal amounts of Mt103 and MTBVAC samples was labelled with Cy2. These dyes provide different fluorescence wavelengths for detection, allowing two or more differentially labelled samples to be combined before being loaded onto an electrophoresis gel. The three samples were separated in the first dimension using isoelectric focusing (IPG strips pH 3–7) and in the second dimension using standard SDS-polyacrylamide gel electrophoresis (PAGE). Four different 2D-DiGE gels were prepared to quantify differential protein secretion in the four biological replicates of Mt103 and MTBVAC. For quantitative protein analysis, 2D-DiGE gels were exposed to different wavelengths, allowing specific detection of cyanine dyes. The abundance of each protein spot in a biological sample was measured as a ratio to its corresponding spot in the internal standard, allowing a direct quantitative comparison within each gel and normalizing abundance values for every protein in each sample. A total of 315 proteins showing significant differences between Mt103 and MTBVAC were picked from 2D-PAGE gels and analysed by MALDI-ToF, resulting in accurate identification of 24 proteins (Supplementary Table 1).

2.12. Ethics

Mice were maintained in the regulated "Centro de Investigaciones Biomédicas de Aragón" (CIBA, Zaragoza, Spain) facilities with reference number ES 50 297 0012 011. The procedures were carried out under Project Licence PI50/14 approved by the Ethics Committee for Animal Experiments from the University of Zaragoza. The care and use of animals were performed according to the Spanish Policy for Animal Protection RD53/2013, which meets the European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purposes.

2.13. Animal studies

In all cases, 5- to 7-week-old female BALB/c mice were purchased from Javier Labs. Groups of 5 female BALB/c mice were immunized subcutaneously with a single dose of 100 µL containing 10^6 CFU of the corresponding recombinant MTBVAC expressing the genetically inactivated toxins S1, CRM197, or FC. Saline (phosphate-buffered saline) inoculation was used as a control (Fig. 4). Blood was collected from immunized mice eight weeks after immunization from heart punch in euthanized mice. Mice were euthanized by CO_2 exposure for 10 min.

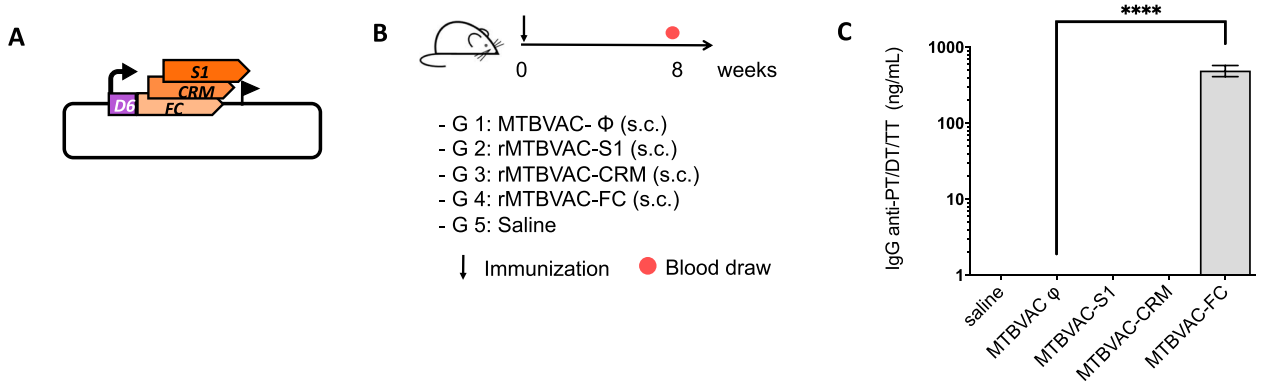

Fig. 4. Immunization of mice with rMTBVAC expressing the FC tetanus antigen results in a specific anti-FC antibody response. A. Schematic representation of the constructs selected for *in vivo* immunization that express DTP antigens by rMTBVAC, avoiding secretion toxicity. B. Experimental design of *in vivo* experiment. Groups of five BALB/c female mice were immunized subcutaneously with 10^6 CFU of the corresponding rMTBVAC expressing but not secreting S1, FC or CRM197, with MTBVAC wild type or saline as controls. Eight weeks later, the mice were euthanized, and blood was extracted for anti-S1, FC or CRM197 IgG evaluation. C. Specific IgG production in mice immunized with rMTBVAC indicated in panel B carrying the constructs specified in panel A. The bar and error bars indicate the mean and standard deviation of antibody titers from five vaccinated animals per vaccine group. Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparison test using MTBVAC as the control group. Asterisks indicate **** 0.0001 > p-value.

2.14. Flow cytometry

For cytometry studies of rMTBVAC-pJK expressing GFP, the bacteria were fixed with 4% paraformaldehyde. A Gallios flow cytometer (Beckman) was used to determine the single-cell fluorescence of gated bacterial cells based on the median fluorescein isothiocyanate signal (FITC; with 488-nm excitation and 500- to 560-nm emission filters).

2.15. Serum antibody measure ELISA

For determination of the specific anti-antigen IgG levels in serum, MaxiSorp ELISA plates (NUNC) were coated with 1 μg/mL of PT, TT or DT as previously mentioned, or with the standard curve samples for calculating the IgG concentration (mouse reference serum, Bethyl Technologies). The plates were incubated overnight at 4 °C. After a washing step with PBS-Tween 20 0.05% (v/v) buffer (washing buffer), plates were blocked with incubation buffer (bovine serum albumin 1% (w/v) in washing buffer) for 1 h at 37 °C. Then, plates were incubated with 50 μL of proper serum dilution for 90 min at 37 °C. After three washes, plates were incubated for 1 h at 37 °C with goat anti-mouse IgG diluted 1:10,000 (KPL). After another three washes, plates were incubated for 1 h at 37 °C with HRP-conjugated rabbit anti-goat IgG diluted 1:10,000 (KPL). Finally, an enzyme-substrate reaction was developed using 3,3′,5,5′- tetramethylbenzidine (Sigma) as the substrate, and the reaction was stopped with 0.1 N H_2SO_4. Optical density was measured at 450 nm.

3. Results

3.1. Classic genetic strategies are not suitable to express diphtheria, tetanus, or pertussis toxoids in BCG or MTBVAC

As a first step, we envisaged a modular construction consisting of each toxoid fused at the N-terminus to a signal secretion sequence and controlled by a mycobacterial promoter (Supplementary Fig. 1A). As signal sequences, we selected antigen (Ag) 85A (*fbpA*/Rv3804c) or Ag85B (*fbpB*/Rv1886c), which are well-known secreted substrates in *M. tuberculosis*. As promoters, we selected those from Ag85A, Ag85B, or Hsp60 (*groEL2*/Rv0440) genes. The genetically inactivated sequences of pertussis (S1), tetanus (FC), and diphtheria (CRM197) toxins were cloned in frame with each secretion sequence, and these sequences were placed under the control of the different promoters. We generated 12 different modular constructs that were subsequently cloned in the pMV361 integrative plasmid (Supplementary Fig. 1A). The resulting plasmids were checked by specific PCR (Supplementary Fig. 1B) and Sanger sequencing and subsequently introduced into MTBVAC or BCG by electroporation. In total, 12 recombinant (rMTBVAC) and 12 recombinant BCG (rBCG) strains were constructed.

The amounts of the S1, FC, and CRM197 mRNAs measured by qRT-PCR revealed marginal expression levels compared to the *sigA* endogenous gene (Supplementary Fig. 2A). Corroborating this finding, western blots using specific antibodies raised against each antigen showed undetectable antigen expression in most recombinant strains, except weak S1 expression in 2 rBCG strains (Supplementary Fig. 2B). This result is unexpected considering that the *sigA*, *fbpA*, *fbpB* and *groEL2* genes driven from their own promoters showed robust mRNA expression levels in our previous RNA-seq experiments [35]. Accordingly, we can hypothesize mRNA instability in these constructs and decide to design another step-by-step rationale strategy to optimize each genetic part of the constructs.

3.2. Promoters derived from the L5 mycobacteriophage allow tuning of expression levels in M. tuberculosis

In a previous study, three different promoters derived from the L5 mycobacteriophage were tested in BCG and *Mycobacterium smegmatis* [34]. These promoters were placed upstream of the enhanced green fluorescent protein (e-*gfp*) gene reporter in a replicative shuttle plasmid containing the pAL5000 and pBR322 replication origins from mycobacteria and *E. coli*, respectively (Fig. 1A). The different promoters showed nucleotide polymorphisms spanning the regulatory region and affecting the −10 box and the transcription start site (TSS) (Fig. 1B). As a result, these promoters demonstrated different strengths in BCG and *M. smegmatis*, ranging from high (B7), medium (D6) or low (C1) promoter strengths, based on measures of GFP activity [34]. However, since these promoters had not been tested in *M. tuberculosis*, we were prompted to study their promoter activities in MTBVAC. Cultures of transformed MTBVAC readily developed a green colour that was visible by the naked eye, indicative of the high GFP expression in the B7 and D6 transformants (Fig. 1C). This result was confirmed by Coomassie staining of the total protein fraction of the MTBVAC transformants, showing a prominent band in the range of 25 kDa compatible with the molecular weight of the GFP protein (27 kDa) (Fig. 1D). Next, we followed GFP activity for 35 days in liquid cultures and observed that the D6 promoter showed higher activity, followed by the B7 promoter, in MTBVAC (*M. tuberculosis*). These results contrast with those results obtained in BCG and *M. smegmatis*. The C1 promoter displayed lower activity in MTBVAC, BCG, and *M. smegmatis* (Fig. 1E). Finally, we decided to investigate plasmid stability in the absence of antibiotic pressure. rMTBVACs transformed with the different plasmids were subcultured 7 times in the absence of kanamycin. We observed that 38%, 66% and 95% of MTBVAC cells containing the D6, B7 and C1 promoters, respectively, maintained GFP fluorescence after 7 passages (Fig. 1F). This result indicates that plasmid loss is proportional to promoter strength. We hypothesize that the high GFP synthesis driven by D6 and, to a lesser extent, B7 promoters might represent a metabolic burden for MTBVAC. Altogether, characterization of promoter strengths and plasmid stability are useful data to select the best plasmid for every experiment. We should remember that the rMTBVAC strains constructed in this study are intended for vaccination experiments in animal models, where antibiotic pressure does not exist. Accordingly, to ensure long-term plasmid stability *in vivo*, the C1 promoter would be the preferred choice compared to the B7 and D6 promoters.

3.3. Secretion signals of twin arginine Translocation substrates allow efficient protein export in MTBVAC

To detect proteins differentially exported by MTBVAC compared with the parental *M. tuberculosis* Mt103 strain, we performed a proteomic comparison of the secreted protein fractions from these strains. The results from 2D differential gel electrophoresis (DiGE) revealed spots of differentially secreted proteins in *M. tuberculosis* or MTBVAC (Fig. 2A). Those spots showing robust differential secretion in four biological replicates were selected for protein identification by MALDI-TOF. The most prominent, low molecular weight bands with higher secretion in *M. tuberculosis* compared to MTBVAC corresponded to ESAT-6 and CFP-10 (Fig. 2A), corroborating our previous findings [11,36–38]. Among the major proteins secreted by MTBVAC, most putatively corresponded to proteins secreted by the Twin Arginine Translocation (TAT) pathway (Supplementary Table 2). Specifically, the most noticeable bands secreted in MTBVAC were proteins from the Ag85 complex (Ag85A, Ag85B, and Ag85C), which are also TAT-dependent substrates (Fig. 2A). To confirm the results from 2D-DiGE, we analysed

the abundance of the Ag 85 complex and ESAT-6 in *M. tuberculosis* Mt103 and MTBVAC by western blot. The results showed a consistently higher secretion of Ag85 proteins and absence of ESAT-6 in MTBVAC (Fig. 2B). Once those proteins showing higher secretion in MTBVAC were characterized, we decided to select an optimal signal sequence that allows heterologous protein secretion. We aimed to place signal sequences from Ag85A, Ag85B, Ag85C, BlaC, Rv0203 and Rv1987 proteins upstream of the *gfp* gene, and these constructs were introduced under the control of the D6 promoter (Fig. 2C). Of the attempted constructs, we successfully obtained plasmids carrying the Ag85A, Ag85C and Rv1987 signal sequences, which were characterized in terms of the presence of the twin-arginine motif and their putative cleavage sites (Fig. 2D). As a secreted protein internal control, we decided to use the CFP-10 signal sequence, which was previously demonstrated to be efficiently secreted in MTBVAC [38,39]. It is important to note that the CFP-10 signal sequence was placed as a C-terminal fusion, unlike TAT signal sequences, which were placed as N-terminal fusions, to maintain the signal sequence locations of the original proteins. Next, we analysed the secretion efficacy of the GFP protein fused to the abovementioned signal sequences. We followed fluorescence in the secreted and whole-cell fractions after 3, 9, and 13 days in liquid culture. After examination of the normalized fluorescence data, we concluded that Ag85A, Ag85C, and Rv1987 signal sequences allow proper secretion of GFP and that this secretion increased with time (Fig. 2E). The Ag85A secretion signal showed the best secretion efficacy, even higher than CFP-10 (Fig. 2E), which is prominently secreted by MTBVAC. In addition, of the three secretion signals tested, only Ag85A showed significant differences in either production or secretion of the reporter GFP protein.

3.4. Optimized coding genes of DTP toxoids placed under the control of the Ag85A secretion signal allow heterologous protein secretion in MTBVAC

In a final step, we aimed to design a modular construct to achieve our objective of heterologous protein secretion in MTBVAC. We used the previously characterized C1, B7 or D6 promoters to ensure different expression levels (Fig. 1E). As a signal sequence, we selected Ag85A, which showed the best secretion efficacy (Fig. 2E). The S1, FC and CRM197 coding regions were placed in fusion with the Ag85A signal peptide in their N-terminal ends and to a 3xFLAG epitope in their C-terminal ends (Fig. 3A). This latter peptide allowed detection of the fusion protein by western blot. It is important to note that S1, FC, and CRM197 toxoids were derived from *B. pertussis*, *Clostridium tetani,* and *Corynebacterium diphtheriae,* respectively, and that the G + C content of these bacteria (67%, 28% and 53%, respectively) differed from that of *M. tuberculosis* (65%). Accordingly, the coding sequences of the toxoids were optimized for codon usage in *M. tuberculosis* (Supplementary Fig. 3A). After attempting to introduce these codon-optimized sequences under the control of promoters with different strengths into MTBVAC, we obtained the highest number of transformants with the C1 promoter. These transformants also tested positive by PCR (Fig. 3B). The transformants resulting from the D6 and B7 promoters were usually derived from small colonies and ultimately resulted in false-positive clones (Fig. 3B). Altogether, we continued with the molecular characterization of rMTBVAC expressing the DTP toxoids under the C1 promoter. We aimed to detect the specific production of DTP antigens by using specific S1, FC and CRM197 antibodies. Since we were not able to detect these proteins using the specific antibodies mentioned above (Supplementary Fig. 4), with slight exception for FC, we used anti-FLAG antibodies to specifically detect each toxoid fused with the 3xFLAG epitope (Fig. 3D). Our results demonstrated the production of recombinant proteins in the whole-cell and secreted fractions of rMTBVAC strains, indicative of proper secretion of these heterologous proteins into the culture supernatant (Fig. 3D). The specificity of the antibody was confirmed by the lack of these bands in wild-type MTBVAC. To further confirm the secretion of S1, FC, and CRM197 toxoids in the rMTBVAC strains, we analysed secreted fractions by multiple reaction monitoring (MRM), a highly specific and sensitive label-free proteomic technique for identifying and quantifying targeted proteins. The results demonstrated the secretion of heterologous toxoids after the detection of a subset of specific peptides from the S1, FC, and CRM197 proteins (Supplementary Fig. 5). Difficulties in obtaining positive clones derived from the D6 and B7 promoters (Fig. 3B) might indicate putative toxicity of TAT secretion sequences placed under the control of a strong promoter, probably by saturating the TAT machinery with high molecular weight substrates carrying a TAT secretion signal. To prove this hypothesis, we cloned the S1, FC and CRM197 codon-optimized genes without a signal sequence under the control of the strong D6 promoter (Fig. 3E). Remarkably, we obtained a high number of transformants with this construct, which resulted in positive clones after PCR verification (Fig. 3B). Additionally, these positive clones showed efficient production of S1, FC, and CRM197 antigens in rMTBVAC as detected by specific antibodies (Fig. 3F).

3.5. Immunization of mice with rMTBVAC expressing or secreting the FC antigen elicits a specific antibody response against tetanus toxoid

Once obtained the rMTBVAC strains which expressed, and/or secreted our heterologous antigens, our goal was to demonstrate that vaccination with these strains resulted in specific immune responses. Unlike immunity against TB, which mainly relies on cellular-mediated immune responses, protective responses against DTP antigens are proportional to the levels of seroprotective antibodies triggered after vaccination. Thus, we selectively focused on the quantification of anti-DTP antibodies in the serum of vaccinated animals. Mice were immunized by the subcutaneous route with wild-type MTBVAC as a control or rMTBVAC secreting S1, FC or CRM197 toxoids whose expression was controlled by the C1 promoter. Eight weeks after immunization, blood was collected from the immunized mice to detect specific antibodies in the serum. Using these constructs, we did not detect anti-S1, anti-FC or anti-CRM197 responses, probably due to low antigen secretion levels in rMTBVAC. Then, we decided to immunize mice with rMTBVAC-producing but not rMTBVAC-secreting S1, CRM197, and FC toxoids from the D6 promoter. We previously verified that these rMTBVAC strains expressed high amounts of DTP antigens without producing toxicity (Fig. 3F). Our results confirmed a specific anti-FC response in mice immunized with the recombinant vaccine expressing the FC tetanus fragment (Fig. 4), which correlates with robust expression of the FC antigen in the corresponding rMTBVAC strain. Altogether, we have demonstrated that recombinant vaccines based on MTBVAC are able to elicit dose–response antibody responses against heterologous antigens after a single dose, and our results pave the way for future optimization of this vaccine platform.

4. Discussion

4.1. Suitability of live TB vaccines as genetic platforms to express heterologous proteins

Vaccination, antibiotics, clean water, and sanitation are among the most effective public health interventions that have prevented, or even eradicated, infectious diseases that once killed millions of people. Accordingly, the prevention of current, emerging, or future infectious diseases through vaccination has the potential to make

an enormous contribution to the health of modern societies [1]. Today, the World Health Organization lists 25 vaccine-preventable diseases [40], and many others are in development. Some of these vaccines are administered in combinations to confer immunity against diverse pathogens with a single shot. Accordingly, vaccinology benefits not only from the investigation of new vaccine candidates but also in developing new vaccine combinations, or even in designing recombinant live vaccines able to express the immunogenic constituents from other vaccines. Although some bacteria have been proposed as vaccine vehicles, it is important to note that none of these bacteria are currently used in vaccination policies, with the notable exception of BCG [41].

BCG is a centenary vaccine highly efficacious in preventing severe TB in infants. However, since BCG has limitations in preventing TB in adults, new vaccines are being investigated. Most of the TB vaccines in the pipeline are based on subunits or inactivated vaccine candidates, and live TB vaccines are limited to BCG revaccination, rBCG, or the attenuated MTBVAC vaccine [42]. MTBVAC presents some advantages relative to BCG: it is based on the human pathogen *M. tuberculosis* [38]; it maintains all epitopes present in *M. tuberculosis* [13], including those lost in BCG; it secretes higher amounts of immunodominant antigens compared to BCG; and it is produced in uniform clinical lots, avoiding the risk of the emergence of "daughter strains" as happened with BCG. Furthermore, recent animal studies have demonstrated that MTBVAC confers a significant improvement, compared to BCG, in protecting rhesus macaques against an aerosol challenge with *M. tuberculosis* [43] and that MTBVAC protects mice against the most widely distributed *M. tuberculosis* lineages in the human population [38]. These advantages are endorsed by the successful results in phase I and IIa clinical trials [7,44].

We have previously demonstrated that MTBVAC persists for several weeks in the lymph nodes of vaccinated mice [11], and consequently, we hypothesize that MTBVAC-based immunization could stimulate host immunity during a long-term period. In addition, MTBVAC maintains or even improves the beneficial nonspecific effects documented for BCG [12]. Taken together, these observations highlight the potential of MTBVAC as a vaccine platform to synthesize unrelated antigens to afford combined protection against TB and unrelated diseases.

4.2. The rationale to express DTP antigens in MTBVAC

In this study, we sought to construct rMTBVAC expressing and secreting DTP antigens. This rationale is based on our previous findings demonstrating that vaccination of mice with BCG or MTBVAC before vaccination with DTP results in higher antibody and Th1 responses than vaccination with DTP alone. Furthermore, exploration of human epidemiological data showed that pertussis incidence was 10-fold lower in countries that use DTP and BCG than in countries that use only DTP, supporting the hypothesis of a nonspecific immune enhancement of BCG favouring the DTP immune response [31]. Additionally, it is estimated that prevention of diphtheria, tetanus, and pertussis largely benefits from the 85% coverage of the different DTP vaccines available, and immunization with BCG against TB reached >90% vaccine coverage in 2019 [45]. Because BCG and DTP have similar vaccination coverages, a combined TB and DTP vaccine would offer benefits in terms of lower production, distribution, and administration costs.

The period between birth and the first DTP immunization is usually two months, and it is critical for newborns: they are not protected against diphtheria, tetanus, or whooping cough, and this is when most pertussis cases occur [46]. Since vaccines against TB are administered at birth, vaccination with rMTBVAC expressing DTP antigens could help to cover this window of susceptibility. Moreover, DTP vaccination requires at least 3 doses to achieve adequate protection. Since MTBVAC persists for long periods in vaccinated mice [11], the production of recombinant DTP antigens by rMTBVAC would result in a constant presentation of these antigens to the immune system and might reduce the number of DTP doses required for maximum protection.

Although previous works have attempted to construct rBCG vaccines expressing either pertussis or diphtheria antigens [47–49], to our knowledge, this is the first study to demonstrate the feasibility of expressing and secreting the three DTP antigens in a live TB vaccine. In addition, since we have used the *M. tuberculosis* derivative MTBVAC, this work aims to establish the basis for synthetic biology in the *Mycobacterium* genus.

4.3. Challenges and perspectives for heterologous protein expression in Mycobacterium

Even if the arsenal of mycobacterial genetic tools is continuously growing, it does not necessarily reach the potential and versatility of genetic manipulation in other model organisms, such as *Escherichia coli*. In addition, the construction of recombinant mycobacteria is hampered due to the fastidious growth and pathogenicity of these microorganisms. Indeed, the use of classic molecular genetic tools in BCG or MTBVAC resulted in negligible expression of S1, FC and CRM197 (Supplementary Fig. 1). Remarkably, we obtained these negative results after a combination of three different promoters, two signal secretion sequences, and three genes coding heterologous proteins (Supplementary Fig. 1). The fact that DTP and other antigens have been efficiently expressed in BCG using comparable tools [48–51] reinforces the notion that heterologous expression in *M. tuberculosis* is not trivial.

After optimization of each genetic part of the construction following a rational process, we succeeded in the expression and secretion of the three DTP antigens in the *M. tuberculosis* derivative MTBVAC. Our results indicated conclusions that could be of interest for future synthetic biology approaches in *M. tuberculosis*. First, the promoter strength was inversely proportional to vector stability in the absence of selective pressure (Fig. 1D,E,F). This is a key observation for infection or vaccination experiments in animal models, where selective pressure for plasmid maintenance is usually absent. Second, signal secretion sequences from the Ag85A and CFP-10 proteins placed at the N- and C-terminal ends, respectively, are suitable candidates for protein secretion in *M. tuberculosis* (Fig. 2E). Remarkably, the signal peptides of Ag85A and CFP-10 proteins are recognized by two different secretion systems, namely, TAT and ESX-1, respectively [52,53], which provides versatility to achieve protein secretion in *M. tuberculosis*. Third, higher secretion of TAT substrates in MTBVAC is probably related to the *phoP* gene mutation in this vaccine. A previous transcriptomic comparison of *M. tuberculosis* and its *phoP* mutant demonstrated that a noncoding RNA, *mcr7*, is the more regulated RNA from the PhoP regulon. Mcr7 post-transcriptionally regulates translation of the *tatC* gene, which is a constituent of the TAT machinery [34]. Consequently, TAT-exported proteins show higher secretion in *M. tuberculosis phoP* mutants [38]. Fourth, the use of a strong promoter is incompatible with secretion signals of the TAT secretion pathway. We hypothesize that the high expression of high molecular weight proteins bearing a TAT secretion signal might result in saturation of the TAT machinery. It is plausible that the efficiencies of transcription, translation, and secretion processes should be tightly orchestrated through an appropriate combination of promoters, ribosome binding sites, and signal peptides, as documented in other bacteria [54]. Fifth, considering the 65% G + C content in mycobacteria, codon optimization is recommended for the heterologous expression of proteins from non-mycobacterial species. Sixth, a 3xFLAG epitope placed in the C-terminal end of our constructs resulted in a versa-

tile approach to detect proteins from different origins. This approach might be even more useful to detect synthetic proteins or in cases where there are no commercial antibodies available. Seventh, high levels of antigen production are necessary to induce an antibody response *in vivo*. In rBCG, this has also been shown for pneumococcal antigens, and secretion/exportation increased the antibody response [55]. Eighth, antigens expressed in rMTBVAC can induce an antibody response even if they are not secreted *in vitro*. This has also been observed with rBCG-DTP antigens, where both CRM197 and FC (cell wall and cytosolic localization) induced specific antibody formation [49,50]. Altogether, we have paved the way to design an engineered TB vaccine platform for the expression of unrelated proteins.

We should bear in mind that the efficacy of genetic tools does not necessarily correlate across mycobacterial species. As an example, the L5 mycobacteriophage promoters used in this study show different strengths depending on the genetic backgrounds [34]. Other evidence comes from the observation that during the construction of rBCG-DTP vaccines, the exportation of the antigens to the cell wall was achieved for S1 and CRM197 but not for FC. In these studies, exportation was intended through use of the β-lactamase promoter and signal sequence, indicating that the secretion efficacy of this signal sequence depends on the antigen [48–50]. In summary, since most genetic tools have been developed either for fast-growing *M. smegmatis* or *M. bovis*-derived BCG, endeavors should be devoted to adapt these tools to the *M. tuberculosis* pathogen.

4.4. Future prospects for live TB vaccines

Vaccinology is an evolving science. The first attempts of Edward Jenner and Louis Pasteur to develop attenuated smallpox and rabies vaccines were soon followed by the discovery that immunization with toxoids or inactivated microorganisms protected against diphtheria, tetanus or whooping cough. Recently, the development of cell culture and recombinant DNA technologies has allowed the design of vaccines against a variety of bacteria and viruses [56]. The 21st century has projected novel technologies such as reverse vaccinology, which has resulted in vaccines against type B meningitis, or the recent advent of adenoviral vectors or mRNA vaccines [57].

In this study, we propose a complementary approach through the use of attenuated vaccines as bacterial factories to produce the immunogenic constituents of other proof-of-concept vaccines. This approach is limited to live attenuated vaccines such as the typhoid vaccine, the TB vaccine BCG, or BCG-replacement strategies such as MTBVAC. The development of live vaccine factories opens interesting perspectives in terms of multivalent immunization, persistent presentation of the antigen to the immune system, or its preparation as lyophilized formulations. In addition to health benefits, the combination of multiple immunizations in a single vaccine allows lower costs in terms of production, distribution and personnel required for administration. We should also remember that live TB vaccines confer a plethora of beneficial, nonspecific effects [58] such as protecting against unrelated pathogens [18,22] through priming trained immunity [59] or enhancing the immunity of unrelated vaccines [31]. Taken together, these heterologous effects of live TB vaccines together with the potential to construct recombinant derivatives open exciting perspectives for next-generation vaccine strategies.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. Carlos Martín and Jesús Gonzalo-Asensio are authors of the patents entitled "Tuberculosis Vaccine" and "Compositions for use as a prophylactic agent to those at risk of infection of tuberculosis, or as secondary agents for treating infected tuberculosis patients". Both patents protect the MTBVAC vaccine.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.csbj.2021.07.035.

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