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Mycobacterium bovis BCG as a Delivery System for the *dtb* Gene Antigen from Diphtheria Toxin

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Abstract

Diphtheria is a fulminant bacterial disease caused by toxigenic strains of *Corynebacterium diphtheriae* whose local and systemic manifestations are due to the action of the diphtheria toxin (DT). The vaccine which is used to prevent diphtheria worldwide is a toxoid obtained by detoxifying DT. Although associated with high efficacy in the prevention of disease, the current anti-diphtheria vaccine, one of the components of DTP (diphtheria, tetanus and pertussis triple vaccine), may present post vaccination effects such as toxicity and reactogenicity resulting from the presence of contaminants in the vaccine that originated during the process of production and/or detoxification. Therefore, strategies to develop a less toxic and at the same time economically viable vaccine alternatives are needed to improve existing vaccines in use worldwide. In this study, the Moreau substrain of BCG which is used in Brazil as a live vaccine against human tuberculosis was genetically modified to carry and express the gene encoding for the diphtheria toxin fragment B (DTB). As such, the DNA sequence encoding the *dtb* gene was cloned into the pUS977 shuttle vector for cytoplasmic expression and successfully introduced into BCG cells by electroporation. Mice immunized with recombinant BCG expressing DTB showed seroconversion with the detection of specific antibodies against DTB. Also, rBCGs stably expressing DTB persisted up to 60 days in the absence of selective pressure in mice and cell viability did not change significantly during the period tested. Finally, immune sera from BALB/c mice vaccinated with rBCGpUS977dtb_{PW8} were preliminarily tested for their capacity of neutralizing the diphtheria toxin in the Vero Cells assay.

Keywords

Recombinant BCG, Diphtheria Toxin *dtb* Gene, Park Williams 8 (PW8), *Corynebacterium diphtheriae*, $rDTB_{PW8}$, pUS977 Vector

1. Introduction

Corynebacterium diphtheriae, one of 59 described species in the *Corynebacterium* genus, causes the highly contagious infection diphtheria in humans [1] [2] [3]. *C. diphtheriae* produces diphtheria toxin (DT) and a pseudomembrane adherent to the tonsils, pharynx, and/or nose. The local and systemic manifestations of diphtheria are mainly related to the action of DT, which is the most studied virulence factor of this species [4] [5] [6] [7]. DT is a polypeptide of 535 amino acids with a molecular weight of approximately 58.3 kDa, which is proteolytically cleaved after secretion into two fragments with distinct activities. The aminoterminal fragment A (DTA) is responsible for toxicity and the nontoxic carboxyl fragment (DTB) with 341 amino acids is responsible for adherence and internalization of the DT [8] [9].

Mycobacterium bovis Bacillus Calmette-Guerin (BCG) is used as a vaccine for the control of tuberculosis, is currently the most widely used vaccine in the world and has been given to more than three billion people since 1921 [10] [11]. For this reason, BCG is among the best live vector candidates for delivery of protective antigens *in vivo,* especially in developing countries where the cost of vaccines is a major issue. BCG is able to elicit potent Th1-mediated immune responses and requires no additional adjuvant components in its formulation to evoke protective immunity, as demonstrated in several animal models of infectious diseases [12] [13] [14]. Additionally, several other advantages have been associated with the potential use of BCG as an antigen-presenting system such as the ability to induce a long lasting type 1 helper T cell (Th1) immune response and CD8+T-cell triggering with just one dose. BCG can also be given at birth, is one of the most thermostable vaccines to date and can induce mucosal immunity by oral or nasal administration [15] [16]. Up to this date, recombinant BCG (rBCG) vaccines expressing a variety of parasite, bacterial, or viral antigens have been shown to induce protective immune responses in murine and primate challenge models [12] [14] [17] [18] [19] [20] [21]. The major purpose of our study is the development of a safer, less reactogenic and cheaper diphtheria vaccine prototype based on the use of BCG, the vaccine against tuberculosis, as a live delivery vector. In this report we present data on the construction of a new rBCG strain prototype vaccine against diphtheria based on the Brazilian Moreau vaccine substrain of *M. bovis* BCG, expressing the *C. diphtheriae* gene dtb_{PW8} by means of the non-integrative plasmid vector pUS977. Analysis of *dtb* expression by BCG, structural stability of the pUS977dtb_{PW8} after vaccination, ability to induce a specific humoral response against the diphtheria toxoid and a prelimi-

nary evaluation of the neutralization power of sera from mice immunized with the rBCGpUS977*dtb*$_{PW8}$ prototype was carried out.

2. Materials and Methods

2.1. Bacterial Strains, Plasmid, and Culture Conditions

The *C. diphtheriae* strain Park Williams 8 (ATCC 13812) was obtained from the Institute Butantan (São Paulo, Brazil) as a source of genomic DNA. The DTP vaccine was obtained from Bio-Manguinhos/FIOCRUZ and the plasmid vector pUS977 from Dr. M.A. Medeiros (Institute of Bio-Manguinhos/FIOCRUZ, Rio de Janeiro, RJ, Brazil). *C. diphtheriae* was grown in liquid Brain Heart Infusion (Difco, Detroit, MI, USA). *Escherichia coli* strain DH5α, grown either on solid or in liquid Luria-Bertani medium, was used for amplification of plasmids throughout the study. The Moreau substrain of *M. bovis* BCG used in vaccine production was obtained from the Fundação Ataulfo de Paiva (Rio de Janeiro, Brazil) and was typically grown either in Middlebrook 7H9 broth (Difco) supplemented with 10% albumin-dextrose-catalase (ADC), 0.2% glycerol, and 0.05% Tween 80, or in Middlebrook 7H10 agar (Difco) supplemented with ADC. When necessary, kanamycin was added to the mycobacteria media at a concentration of 25 μg/mL for selection of recombinant bacteria.

2.2. Construction of the pUS977 Expression Vector pUS977*dtb*$_{pw8}$

A DNA fragment representing the entire *dtb* gene subunit was amplified from *C. diphtheriae* genomic DNA using the primers *dtb* forward A2633B07 5' CGT CTA GAA GGT AGC TCA TTG TC 3' and *dtb* A2633B08 reverse 5' GCT CTA GAC CCC ACT ACC TTT C 3', each containing a *Xba*I restriction site. The resulting 0.965 kb fragment was digested with *Xba*I and cloned in frame in pUS977, previously cut with the same enzyme, to create the construct pUS977*dtb*$_{PW8.}$ This construct was amplified in *E. coli* DH5α and used to transform BCG by electroporation using standard methods [22]. Transformants were later selected by their resistance to kanamycin.

2.3. Western Blotting and Localization of Heterologous Proteins in rBCG

After transformation, recombinant BCG strains were selected on 7H10 Middlebrook medium containing kanamycin 25 μg/mL. Several colonies were tested for their plasmid content by the electroduction method described by [23] and for expression of DTB by Western blotting. For Western blotting analysis, one or more kanamycin-resistant BCG transformants were grown individually in 5 mL 7H9 Middlebrook liquid cultures supplemented with kanamycin. After growth at 37˚C, 2.0 mL cultures were harvested at mid-log phase by centrifugation, resuspended in lysis buffer (10 mM Tris pH 8.0; 1 mM EDTA; 2 mM PMSF (phenyl methyl sulfonyl fluoride) with 50 mg of glass beads (0.1 mm diameter) and then mixed three times (one minute each time) and left to rest on ice. Then

the mycobacterial lysate was centrifuged and the resulting supernatant and pellet were separated and resuspended in a sample buffer 1:1 (100 mM Tris-HCL, 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol). Samples were subsequently boiled for 10 minutes and then loaded onto a 12% SDS-PAGE. After separation, proteins in the SDS-PAGE gel were electrotransferred onto a 0.45 μm nitrocellulose membrane (Bio-Rad, Hercules, CA, USA), which was incubated overnight in 3% nonfat dry milk in phosphate-buffered saline (PBS) containing 0.1% Tween 20 (vol/vol) (Sigma, St Louis, MO, USA). On the following day, the membrane was incubated with a 1:1000 dilution of anti-diphtheria toxoid polyclonal primary antibodies produced in-house. After washing out the primary antibody, the membrane was incubated with alkaline phosphatase-conjugated anti-mouse IgG (Sigma) diluted in PBS-T as the secondary antibody. Antibody binding was detected with NBT and BCIP (nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt) (Sigma) as a substrate.

2.4. Stability and Persistence of rBCG pUS977dtb_{PW8} in Human Monocytes (THP-1)

Cultures of human monocytes (THP-1) (1×10^5) were produced in 24-well plates using RPMI 1640 supplemented with 2 mM L—glutamine, 10 mM HEPES, and 10% fetal calf serum (FCS) as a growth medium. THP-1 monolayers were infected with equivalent levels of BCG or rBCG strains at a multiplicity of infection (MOI) of approximately 10 bacterial cells per monocyte. Bacteria necessary for infection was prepared by dilution of log phase cultures to the desired concentration based on direct enumeration by light microscopy. The actual number of viable BCG and rBCG strains used in each infection experiment was verified retrospectively by plating serial dilutions of the culture on Middlebrook 7H10 agar (Difco) with incubation for up to 4 weeks. Samples (4 wells per culture) were collected at 4 h, 24 h, 48 h, 5, 8, 9 and 12 days post-infection exposure. After that, infected THP-1 cells were lysed with 0.1% Tween 80 in distilled water (25 μL/well), serial diluted, and plated onto plain 7H10 agar (BCG control) or onto the same medium with kanamycin (rBCG). The levels of growth recovered at 4 h were used to calculate the relative infective capabilities of the rBCG in comparison to control BCG. The values obtained for colony forming units (CFU) recovered at other time points were used to calculate the degree of intracellular persistence of the rBCG. The functional stability of rBCG was assessed by comparing the CFU values obtained in media with and without kanamycin according to a previously reported protocol [24].

2.5. Immunization Procedures

Immunization experiments with 4 - 6 week-old male BALB/c mice were conducted in compliance with The Ethical Principles in Animal Experimentation established by the Brazilian College of Animal Experimentation and approved by

the Fundação Oswaldo Cruz-Animal Use Ethical Committee CEUA (P0163-03). Briefly, to evaluate the immune response against the rBCG strain, groups of 5 mice (BCG, BCGpUS977, rBCG pUS977*dtb*$_{PW8}$ and DTP), were immunized intraperitoneally (i.p.) with 10^6 CFU/0.1 mL in phosphate buffered saline (PBS) containing 0.05% Tween 80 (vol/vol) (Sigma, St Louis, MO) (PBS-T) at day 0 and boosted 9 weeks later. As a control, each animal in a group of 5 received 50 μL of the conventional DPT vaccine. All animals were bled at different time points after priming at the retro-orbital plexus and sera obtained from blood at each point were pooled and later analyzed by enzyme-linked immunosorbent assays (ELISA), which was performed for detection and quantitation of anti-rDTB$_{PW8}$ mouse antibodies. Sera were collected 4 weeks after immunization, pooled, and tested by ELISA for the presence of antibodies against DTB.

2.6. Evaluation of the Structural Stability of the pUS977*dtb*$_{PW8}$ Construct from rBCGs Recuperated from Mice

After immunization, recombinant BCGs were recovered out of the spleens of vaccinated mice for evaluation of the structural stability of the pUS977*dtb*$_{PW8}$ construct. Thus, constructs were then directly transferred from spleen reisolated rBCGs to *E. coli* DH5α by electroduction [23] and subsequently recuperated, purified and digested with restriction endonucleases (*Xba*I or *Kpn*I). The presence (or absence) of structural changes in the construct was evaluated by comparing it with the banding pattern generated by the plasmid preparation used to produce the original transformant, through electrophoresis in 1% agarose gel.

2.7. Analysis of the Humoral Immune Response Induced by rBCG pUS977*dtb*$_{PW8}$

Serum antibody responses were quantified by ELISA. Briefly, Maxisorp 96-well plates (Nunc International, Rochester, NY) were coated with the diphtheria toxoid (100 μL; 0.05 μg/mL in 0.2 M carbonate buffer/0.2 M bicarbonate pH 9.6; 4˚C overnight), then washed five times with PBS-T, blocked with 4% nonfat dry milk in PBS, and finally incubated with serial dilutions of mouse sera in PBS. After 1 hour at 37˚C, the plates were washed as described above and incubated with HRP-conjugated goat anti-mouse IgG (1:4,000) (Southern Biotechnology Associates, Inc.) in PBS at 37˚C for 1 hour. After another round of washings, antibodies were visualized by adding TMB substrate (100 μL; 10 mg/mL 3, 3', 5, 5' tetramethylbenzidine in citrate phosphate buffer, containing 0.01% hydrogen peroxide. After adding the substrate, the plates were sealed and incubated in the dark at room temperature for 10 min, after which the reaction was stopped by adding 50 μL of a 20% sulphuric acid solution, and the optical density (OD) of the yellow-orange color developed was measured at 450 - 492 nm in a spectrophotometer (Biorad). Absorbance values were plotted against serum dilutions.

2.8. Vero Cell Method Potency Test

The *in vitro* Vero cell (CCL-81) method chosen for the purposes of this study is

based on the protocol described by the WHO [25] and was used for titration of the diphtheria antitoxin. Briefly, sera in twofold dilutions in 96-well plates were incubated in the presence of 0.01 Lf/mL of DT (Sigma), which is usually neutralized by 0.031 IU/mL of standard anti-DT per mL in cell medium RPMI (Gibco), without bovine serum for an hour at room temperature in the dark. Vero cells in suspension (2.5×10^5 cells/mL) were added to each well of the plates containing medium 199 with Earl's salt's (Gibco) with 4.4% sodium bicarbonate, 4 mg/mL gentamicin and 5% fetal bovine serum. Plates were incubated for 96 h at 37˚C in a 5% CO_2 incubator and 90% relative humidity. The cells were later fixed with 10% formaldehyde for an hour and stained with 1% crystal violet for thirty minutes. After that, plates were washed in water to eliminate the excess stain. After the plates had dried completely, the Vero cells were examined visually on the microscope by observing the holes with approximately 50% of color (hole 50% of cells stained) multiplied by the reference serum titer [26] [27]. The titer of neutralizing antibodies in the pooled sera was calculated by multiplying the inverse of its endpoint dilution with that of the standard antitoxin, expressed in IU/ml. Each pool of sera was analyzed in duplicate, and controls for DT and standard anti-DT were included in all experiments.

3. Results

The pUS977*dtb*$_{PW8}$ expression vector was created on top of the mycobacterial pUS977 plasmid developed by Medeiros *et al.* (30) through the ligation of the 1051 bp diphtheria toxin *dtb* DNA amplified by PCR as described above, to the pUS977 *Xba*I restriction site. The **Figure 1(a)** shows the architecture of the pUS977*dtb*$_{PW8}$ construct which is essentially a small plasmid construct bearing origins of replication of *E. coli* and mycobacteria, a multiclonal restriction site, a kanamycin resistance gene as the selective marker and having as a major feature the *dtb* gene expression driven by the mycobacteria promoter pAN. The rBCG

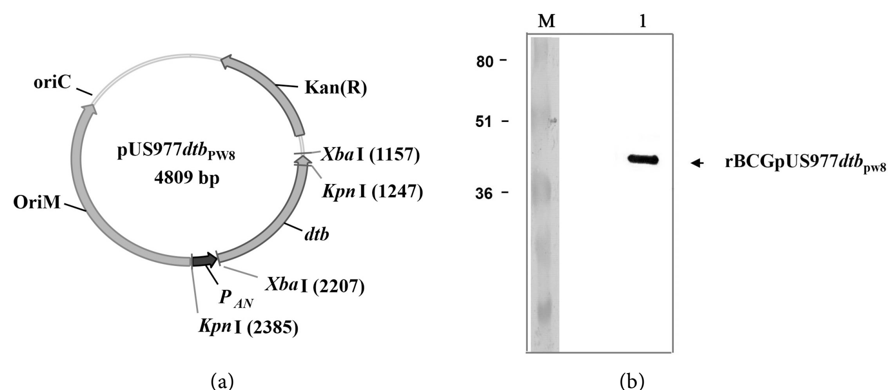

Figure 1. Architecture of the plasmid vector pUS977*dtb*$_{PW8}$ carrying the cassette for expression of the *dtb* gene in BCG (a). Western blot analyses of the BCGpUS977*dtb*$_{PW8}$ lysate separated by SDS-PAGE, with anti-diphtheria toxoid polyclonal antibodies. Lane M: molecular weight markers (Broad Range: 80, 51 and 36 kDa); lane 1: BCGpUS977*dtb*$_{PW8}$ lysate (b).

strain resulting from transformation with the construct pUS977*dtb*$_{PW8}$ was evaluated in terms of its capacity to express rDTB$_{pw8}$ *in vitro* as shown in **Figure 1(b)**. As expected, the lysate of rBCGs carrying the pUS977*dtb*$_{PW8}$ separated by SDS-PAGE revealed an immunoreactive band corresponding to an approximately 45-kDa protein, as recognized by anti-diphtheria toxoid polyclonal antibodies. Results published elsewhere (37) of electrophoretic separations of PCR products from rBCGpUS977*dtb*$_{PW8}$ cells reisolated from spleens of mice after vaccination with the rBCG prototype, revealed that rBCGs recovered from the spleens of vaccinated mice carried not only the construct but also the functional *dtb* expression cassette even sixty days after vaccination. In fact, analysis of the structural stability of rBCG constructs recovered from the spleens of mice showed no alteration in the structure of the plasmid, as essayed through the digestion of plasmids rescued from *E. coli* with either endonuclease *Xba*I or *Kpn*I. The structural stability of the recombinant plasmid introduced into the BCG should reflect the functional stability of the prototype vaccine with the expression of the target protein for several generations. Also very important is the evidence that the same rBCGs recovered from the spleen of BALB/c mice 60 days after vaccination, when subjected to six consecutive sub-cultures, showed no difference in the ability to express the DTB, thus demonstrating the persistence of functional stability of the rBCG transformed with pUS977*dtb*$_{PW8}$ construct. The size of the protein expressed by the rescued constructs was exactly as expected, and it was also recognized by anti-diphtheria toxoid polyclonal antibodies in western blottings. The infectivity/persistence study carried out in THP-1 human monocytes was done in two independent experiments (8 and 12 days, respectively), in the presence or absence of kanamycin [24]. The rBCGpUS977 *dtb*$_{pw8}$ retained full infectivity when compared to non-modified BCG and was able to persist in THP-1 cells up to the maximum time limit tested (12 days) with no plasmid loss in the absence of kanamycin (**Figure 2**). Additionally, viability counts of both rBCGpUS977*dtb*$_{pw8}$ and non-modified BCG originated from infected THP-1 cells were similar at all time points in both experiments, regardless of the presence of kanamycin, which denotes the stability of the rBCGpUS977*dtb*$_{pw8}$ inside a human cell as well, even in the absence of selective pressure. Similarly, the genetic integrity of the pUS977*dtb*$_{pw8}$ construct was confirmed later by PCR analyses of plasmids electroeluted to *E.coli* from rBCGs recovered from THP-1 cells.

In humans, BCG and DPT vaccines are administered by different routes, intradermally for BCG and intramuscularly for DPT. In this study, we chose to use the intraperitoneal route for both rBCG and DPT so that we could compare the response induced by the rBCG vaccine prototype and DPT in the same immunization context. We thus verified that BALB/c mice vaccinated with the rBCGpUS977*dtb*$_{pw8}$ were able to mount a specific immune response against the diphtheria toxoid. Sera collected from immunized mice and controls after up to 20 weeks revealed that the anti-DT immune response peaked 4 weeks after the

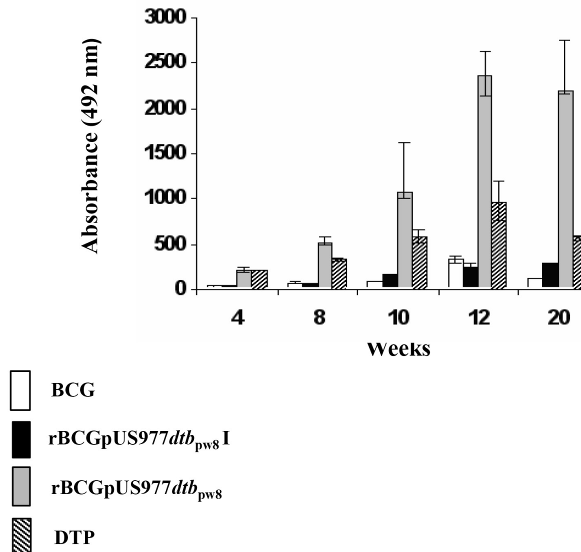

Figure 2. Humoral response induced in BALB/c mice after i.p. vaccination with BCGpUS977*dtb*$_{PW8}$. Four groups of 5 male mice each (4 to 6 weeks old) were immunized with 10^6 CFU mL of either BCG (non transformed), inactivated BCGpUS977*dtb*$_{PW8}$ (BCGpUS977*dtb*$_{PW8}$I), BCGpUS977*dtb*$_{PW8}$ or the DTP vaccine (positive control). The graphic above displays antibodies against the diphtheria toxoid in pooled sera collected from each group of mice at different time points as quantified by ELISA (A_{492nm}) and up to 20 weeks after immunization.

booster with high titer of IgG anti-fragment B antibodies and was two-fold higher when compared to the response induced by the classic cellular DTP vaccine (**Figure 3**). Sera from vaccinated mice were preliminarily tested for evidences of neutralization capacity against DT. So, pooled sera obtained from mice 60 days after immunization with rBCGpUS977*dtb*$_{PW8,}$ were able to neutralize the cytotoxic effects of DT at dilution 1:4 (equivalent to the titer of 0,062 IU/mL of the reference anti-diphtheria serum, SAD) using the WHO protocol for the Vero Cell assay (**Figure 4**). These results, although important, need to be confirmed by the rabbit neutralization assay.

4. Discussion and Conclusions

Since the first transformation of mycobacteria with foreign DNA, the recombinant BCG technology has been used to evaluate the expression of foreign protective antigens in BCG for the development of new and improved BCG vaccines [13] [14] [15] [16] [24] [28]-[36]. Although the expression of DT antigens in different bacterial species has been previously done [8] [37]-[41], in BCG the expressed DT antigens came from the mutant CRM 197 [27] and the diphtheria toxin *dtb* gene of the PW8 vaccine strain [42]. It is well known that DTB is crucial for the adherence of diphtheria toxin to target cell receptors, a fundamental step in the internalization of the DT toxic fragment A. Thus, if we are able to induce the production of antibodies against DTB with an rBCG engineered to do it so, it is expected that the antibodies generated will interfere with adherence

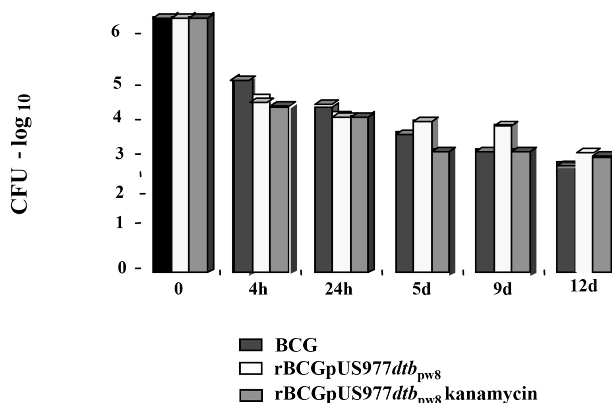

Figure 3. Analyzes of infectivity, persistence, and plasmid maintenance/integrity of BCGpUS977*dtb*$_{PW8}$ in human monocytes (THP-1) in the presence of kanamycin (25 μl per well) or not. The graphic displays the kinetics of the intracellular persistence of each strain up to 12 days. CFUs are shown in $\log_{10}$ units. Values recorded at 4 h, 24 h and 5, 9, 12 days represent the number of bacteria which had been internalized after 4 h of contact with cells.

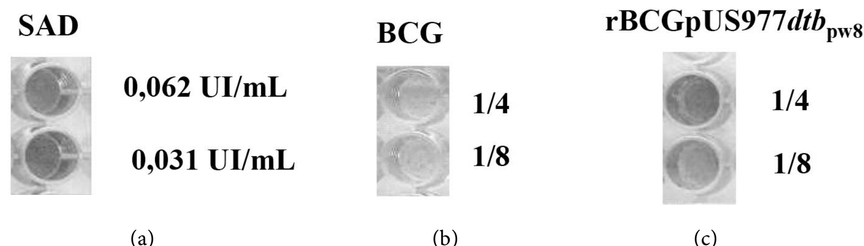

Figure 4. Neutralizing power of pooled sera from mice vaccinated with 10^6 CFU mL of BCGpUS977*dtb*$_{PW8}$ (c); Pooled sera from animals vaccinated with plain BCG were used as negative control (b); Anti-diphtheric serum (SAD) was used as positive control (a); Dilutions tested ranged from 1/4 and 1/8.

and the internalization process of DT and, consequently, will prevent the toxigenic effects of toxin produced by *C. diphtheriae* strains.

Despite the advances reached so far, a major limitation of the rBCG technology is the stability of the modified BCG [42]. In the present study, the promoter P_{AN} of *M. paratuberculosis* [43] was used with great success to drive the expression of DTB into the cytoplasm of the BCG cells. Data presented here showed that a 40 kDa DTB polypeptide was expressed, well tolerated by BCG and was also recognized by anti-diphtheria toxoid polyclonal antibodies. Moreover, the rBCGpUS977*dtb*$_{pw8}$ remained genetically stable and fully functional even after recovery from the spleens of BALB/c mice immunized with the same rBCGs expressing DTB, as demonstrated by the immunoblotting of anti-DT specific sera with rBCG lysates in the laboratory. The rBCGpUS977*dtb*$_{pw8}$ is definitely one of the most stable rBCGs ever constructed in our laboratory.

The ability of the rBCGpUS977*dtb*$_{pw8}$ to infect and persist in human monocyte cells (THP-1) in addition to its capacity of inducing a specific anti-DT immune response in BALB/c mice was demonstrated. The capability of the live modified

bacterial vector to enter and persist was observed in human monocyte (THP-1 lineage) for a given period of time in a fundamental part of the antigen presentation process, rBCGpUS977*dtb*$_{pw8}$. A previous study of our group using the same expression vector demonstrated the long term humoral response (up to 8 months) induced by mice vaccinated with an rBCG strain expressing the S1 subunit of pertussis toxin [30]. Our results showed that, contrary to DTP, the response to the rBCGpUS977*dtb*pw8 was strong, especially in the 12 - 20 week interval. Previous results with rBCG showed that the expression of CRM 197 was found to generate a neutralizing response [27]. In our study the rBCGpUS977*dtb*pw8 was also able to generate neutralizing antibodies, albeit at low sera dilutions. However, it is important to keep in mind that sera-neutralizing capabilities in mice, guinea pigs, rabbits and humans are not comparable and further studies will have to be done to assure the neutralizing quality of the response generated by the rBCGpUS977*dtb*pw8. Taken together, the results achieved so far reveal that the new strain rBCGpUS977*dtb*pw8 developed in our laboratory proved to be genetically stable up to 20 weeks after vaccination and was able to generate a specific humoral response against the target antigen DTB associated to the neutralization of the toxin produced by *C. diphtheria*. The rBCGpUS977*dtb*pw8 has a potential application as a vaccine prototype and may as well be useful in future studies of neutralizing immune responses and protection against *C. diphtheriae*.

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Conflict of Interest

We fully declare that no financial or other potential conflict of interest.

References

[1] Mattos-Guaraldi, A.L., Formiga, L.C.D., Camello, T.C.F., Pereira, G.A., Hirata Jr., R. and Halpern, M. (2001) *Corynebacterium diphtheriae* Threats in Cancer Patients. *Revista Argentina de Microbiologia*, **33**, 96-100.

[2] Hirata Jr., R., Pereira, G.A., Filardy, A.A., Gomes, D.L.R., Damasco, P.V., Rosa, A.C. P., Nagao, P.E., Pimenta, F.P. and Mattos-Guaraldi, A.L. (2008) Potential Pathogenic Role of Aggregative Adhering *Corynebacterium diphtheriae* of Different clonal Groups in Endocarditis. *Brazilian Journal of Medical and Biological Research*, **41**, 986-991. https://doi.org/10.1590/S0100-879X2008001100007

[3] Kimura, Y., Watanabe, Y., Suga, N., Suzuki, N., Maeda, K., Suzuki, K., Kitagawa, W., Miura, N., Morita, H. and Imai, H. (2011) Acute Peritonitis Due to *Corynebacterium ulcerans* in a Patient Receiving Continuous Ambulatory Peritoneal Dialysis: A Case Report and Literature Review. *Clinical and Experimental Nephrology*, **15**, 171-174. https://doi.org/10.1007/s10157-010-0346-4

[4] Pappenheimer Jr., A.M. (1993) The Story of a Toxic Protein, 1888-1992. *Protein Science*, **2**, 292-298. https://doi.org/10.1002/pro.5560020218

[5] Wang, J. and London, E. (2009) The Membrane Topography of the Diphtheria Toxin T Domain Linked to the Chain Reveals a Transient Transmembrane Hairpin and Potential Translocation Mechanisms. *Biochemistry*, **48**, 10446-10456. https://doi.org/10.1021/bi9014665

[6] Man, P., Montagner, C., Vitrac, H., Kavan, D., Pichard, S., Gillet, D., Forest, E. and Forge, V. (2010) Accessibility Changes within Diphtheria Toxin T Domain When in the Functional Molten Globule State, as Determined Using Hydrogen-Deuterium Exchange Measurements. *FEBS Journal*, **277**, 653-662. https://doi.org/10.1111/j.1742-4658.2009.07511.x

[7] Ladokhin, A.S. (2013). Ph-Triggered Conformational Switching along the Membrane Insertion Pathway of the Diphtheria Toxin T-Domain. *Toxins (Basel)*, **5**, 1362-1380. https://doi.org/10.3390/toxins5081362

[8] Lee, C.W., Halperin, S.A., Morris, A. and Lee, S.F. (2005) Expression of Diphtheria Toxin in *Streptococcus mutans* and Induction of Toxin Neutralizing Antisera. *Canadian Journal of Microbiology*, **51**, 841-846. https://doi.org/10.1139/w05-078

[9] Chellapandi, P., Sakthishree, S. and Bharathi, M. (2013) Phylogenetic Approach for Inferring the Origin and Functional Evolution of Bacterial ADP-Ribosylation Superfamily. *Protein and Peptide Letters*, **20**, 1054-1065. https://doi.org/10.2174/0929866511320090012

[10] Bloom, B.R. and Fine, P.E.M. (1994) The BCG Experience: Implications for Future Vaccines Against Tuberculosis. In: Bloom, B.R., Ed., *Tuberculosis: Pathogenesis, Protection, and Control*, ASM Press, Washington DC, 531-558. https://doi.org/10.1128/9781555818357.ch31

[11] Benévolo-de-Andrade, T.C., Monteiro-Maia, R., Cosgrove, C. and Castello-Branco, L.R. (2005) BCG Moreau Rio de Janeiro An Oral Vaccine against Tuberculosis Review. *Memórias do Instituto Oswaldo Cruz*, **100**, 459-465. https://doi.org/10.1590/S0074-02762005000500002

[12] Winter, N., Lagranderie, M., Gangloff, S., Leclerc, C., Gheorghiu, M. and Gicquel, B. (1995) Recombinant BCG Strains Expressing the SIVmac251 nef Gene Induce Proliferative and CTL Responses against nef Synthetic Peptides in Mice. *Vaccine*, **13**, 471-478.

[13] Chapman, R., Shephard, E., Stutz, H., Douglass, N., Sambandamurthy, V., Garcia, I., Ryffel, B., Jacobs, W. and Williamson, A.L. (2012) Priming with a Recombinant Pantothenate Auxotroph of *Mycobacterium bovis* BCG and Boosting with MVA Elicit HIV-1 Gag Specific CD8+ T Cells. *PLoS ONE*, **7**, e32769. https://doi.org/10.1371/journal.pone.0032769

[14] Grode, L., Ganoza, C.A., Brohm, C., Weiner, J., Eisele, B. and Kaufmann, S.H.E. (2013) Safety and Immunogenicity of the Recombinant BCG Vaccine VPM1002 in a Phase 1 Open-Label Randomized Clinical Trial. *Vaccine*, **31**, 1340-1348.

[15] Ohara, N. and Yamada, T. (2001) Recombinant BCG Vaccines. *Vaccine*, **19**, 4089-4098.

[16] Matsuo, K. and Yasutomi, Y. (2011) *Mycobacterium bovis* Bacille Calmette-Guerin as a Vaccine Vector for Global Infectious Disease Control. *Tuberculosis Research and Treatment*, **2011**, Article ID: 574591. https://doi.org/10.1155/2011/574591

[17] Yasutomi, Y., Koenig, S., Haun, S.S., Stover, C.K., Jackson, R.K. and Conard, P. (1993) Immunization with Recombinant BCG-SIV Elicits SIV-Specific Cytotoxic T

Lymphocytes in Rhesus Monkeys. *The Journal of Immunology*, **150**, 3101-3107.

[18] Langermann, S., Palaszynski, S.R., Burlein, J.E., Koenig, S., Hanson, M.S., Briles, D.E. and Stover, C.K. (1994) Protective Humoral Response against Pneumococcal Infection in Mice Elicited by Recombinant Bacilli Calmette-Guérin Vaccines Expressing Pneumococcal Surface Protein. *The Journal of Experimental Medicine*, **180**, 2277-2286. https://doi.org/10.1084/jem.180.6.2277

[19] Langermann, S., Palaszynski, S.R., Sadziene, A., Stover, C.K. and Koenig, S. (1994) Systemic and Mucosal Immunity Induced by BCG Vector Expressing an Outer-Surface Protein A of *Borrelia burgdorferi*. *Nature*, **372**, 552-555. https://doi.org/10.1038/372552a0

[20] Kaufmann, S.H.E. (2010) Foresight: Novel Tuberculosis Vaccination Strategies Based on Understanding the Immune Response. *Journal of Internal Medicine*, **267**, 337-353. https://doi.org/10.1111/j.1365-2796.2010.02216.x

[21] Da Cruz, F.W., McBride, A.J., Conceição, F.R., Dale, J.W., McFadden, J. and Dellagostin, O.A. (2001) Expression of the B-Cell and T-Cell Epitopes of the Rabies Virus Nucleoprotein in *Mycobacterium bovis* BCG and Induction of an Humoral Response in Mice. *Vaccine*, **20**, 731-736.

[22] Parish, T. and Stoker, N.G. (1998) Electroporation of Mycobacteria. Mycobacteria Protocols. *Method in Molecular Biology*, 120-144. https://doi.org/10.1385/0-89603-471-2:129

[23] Baulard, A., Jourdan, C., Mercenier, A. and Locht, C. (1992) Rapid Mycobacterial Plasmid Analysis by Electroduction between *Mycobacterium spp*. and *Escherichia coli*. *Nucleic Acids Research*, **20**, 4105. https://doi.org/10.1093/nar/20.15.4105

[24] Varaldo, P.B., Leite, L.C.C., Dias, W.O., Miyaji, E.M., Torres, F.I.G., Gebara, V.C., Armôa, G.R.G., Campos, A.S., Matos, D.C.S., Winter, N., Gicquel, B., Vilar, M.M., McFadden, J., Almeida, M.S., Tendler, M. and McIntosh, D. (2004) Recombinant *Mycobacterium bovis* BCG Expressing the Sm14 Antigen of *Schistosoma mansoni* Protects Mice from Cercarial Challenge. *Infection and Immunity*, **72**, 3336-3343. https://doi.org/10.1128/IAI.72.6.3336-3343.2004

[25] World Health Organization (WHO) (1993) Laboratory Methods for the Testing for Potency of Diphtheria (D), Tetanus (T), Pertussis (P) and Combined. 1-105.

[26] Miyamura, K., Nishio, S., Ito, A., Murata, R. and Kono, R. (1974) Micro Cell Culture Method for Determination of Diphtheria Toxin and Antitoxin Titres Used VERO Cells. I Studies on Factors Affecting the Toxin and Antitoxin Titration. *Journal of Biological Standardization*, **2**, 189-201.

[27] Miyaji, E.N., Mazzantini, R.P., Waldely, O.D., Nascimento, A.L.T.O. and Gupta, R.K. (2001) Induction of Neutralizing Antibodies against Diphtheria Toxin by Priming with Recombinant *Mycobacterium bovis* BCG Expressing CRM197, a Mutant Diphtheria Toxin. *Infection and Immunity*, **69**, 869-874. https://doi.org/10.1128/IAI.69.2.869-874.2001

[28] Jacobs, W.R., Tuckman, M. and Bloom, B.R. (1987) Introduction of Foreign DNA into Mycobacteria using a Shuttle Plasmid. *Nature*, **327**, 532-535. https://doi.org/10.1038/327532a0

[29] Medeiros, M.A., Delagostin, O.A., Armôa, G.R., Degrave, W.M., De Mendonça-Lima, L., Lopes, M.Q., Costa, J.F., Mcfadden, J. and McIntosh, D. (2002) Comparative Evaluation of *Mycobacterium vaccae* as a Surrogate Cloning Host for Use in the Study of Mycobacterial Genetics. *Microbiology*, **148**, 1999-2009. https://doi.org/10.1099/00221287-148-7-1999

[30] Medeiros, M.A., Armôa, G.R.G., McIntosh, D. and Delagostin, A.O. (2005) Diferen-

tial Humoral Immune Response Induced in Mice Immunized with Two Strains of Recombinant *Mycobacterium bovis* BCG Expressing the S1 Subunit of *Bordetella pertussis* Toxin. *Cannadian Journal Microbiology*, **51**, 1-6. https://doi.org/10.1139/w05-095

[31] Rezende, C.A.F., De Moraes, M.T.B., Matos, D.C.S., McIntosh, D. and Armôa, G.R.G. (2005) Humoral Response and Genetic Stability of Recombinant BCG Expressing Hepatitis B Surface Antigens. *Journal of Virological Methods*, **125**, 1-9.

[32] Michelon, A., Conceição, F. R., Binsfeld, P.C., da Cunha, C.W., Moreira, A.N., Argondizzo, A.P., McIntosh, D., Armôa, G.R., Campos, A.S., Faber, M., Mcfadden, J. and Dellagostin, O.A. (2006) Immunogenicity of *Mycobacterium bovis* BCG Expressing *Anaplasma marginale* MSP1 a Antigen. *Vaccine*, **11**, 6332-6339.

[33] Varaldo, P.B., Miyaji, E.N., Vilar, M.M., Campos, A.S.D., Dias, W.O., Armôa, G.R., Tendler, M., Leite, L.C. and McIntosh, D. (2006) Mycobacteria Codon Optimization of the Gene Encoding the Sm14 Antigen of *Schistossoma mansoni* in Recombinant *mycobacterium bovis* Bacille Calmette-Guerin Enhances Expression but not Protection against Cercarial Challenge in Mice. *FEMS Immunology and Medical Microbiology*, **48**, 132-139. https://doi.org/10.1111/j.1574-695X.2006.00133.x

[34] Santangelo, M.P., McIntosh, D., Bigi, F., Armôa, G.R., Campos, A.S.D., Ruybal, P., Dellagostin, O.A., McFadden, J., Mendum, T., Gicquel, B., Winter, N., Farber, M. and Cataldi, A. (2007) *Mycobacterium bovis* BCG as a Delivery System for the RAP-1 Antigen from *Babesia bovis*. *Vaccine*, **25**, 1104-1113.

[35] Nascimento, I.P., Dias, W.O., Quintilio, W., Christ, A.P., Moraes, J.F. and Vancetto M.D. (2008) Neonatal Immunization with a Single Dose of Recombinant BCG Expressing Subunit S1 from Pertussis Toxin Induces Complete Protection against *Bordetella pertussis* Intracerebral Challenge. *Microbes and Infection*, **10**, 198-202.

[36] Bastos, R.G., Borsuk, S., Seixas, F.K. and Dellagostin, O.A. (2009) Recombinant *Mycobacterium bovis* BCG. *Vaccine*, **27**, 6495-6503.

[37] Barbieri, J.T. and Collier, R.J. (1987) Expression of a Mutant, Full-Length Form of Diphtheria Toxin in *Escherichia coli*. *Infection and Immunity*, **55**, 1647-1651.

[38] Bishai, W.R., Miyanohara, A. and Murphy, J.R. (1987) Cloning and Expression in *Escherichia coli* of Three Fragments of Diphtheria Toxin Truncated within Fragment B. *Journal of Bacteriology*, **169**, 1554-1563. https://doi.org/10.1128/jb.169.4.1554-1563.1987

[39] Cabiaux, V., Phalipon, A., Wattiez, R., Falmagne, P., Ruysschaert, J.M. and Kaczorek, M. (1988) Expression of a Biologically Active Diphtheria Toxin Fragment B in *Escherichia coli*. *Molecular Microbiology*, **2**, 339-346. https://doi.org/10.1111/j.1365-2958.1988.tb00037.x

[40] Orr, N., Galen, J.E. and Levine, M.M. (1999) Expression and Immunogenicity of a Mutant Diphtheria Toxin Molecule, CRM197, and Its Fragments in *Salmonella typhi* Vaccine Strain CVD 908-htr A. *Infection and Immunity*, **67**, 4290-4294.

[41] Lee, C.W., Lee, S.F. and Halperin, S.A. (2004) Expression and Immunogenicity of a Recombinant Diphtheria Toxin Fragment A in *Streptococcus gordonii*. *Applied and Environmental Microbiology Application*, **70**, 4569-4574. https://doi.org/10.1128/AEM.70.8.4569-4574.2004

[42] Nascimento, D.V., Dellagostin, A.O., Hirata, J.R., Pereira, G.M.B., Mattos-Guaraldi, A.L. and Armôa, G.R.G. (2013) Plasmid Instability when the hsp60 Gene Promoter Is Used to Express the Protective Non-Toxic Fragment B of the Diphtheria Toxin in Recombinant BCG. *American Journal of Molecular Biology*, **3**, 81-86. https://doi.org/10.4236/ajmb.2013.32011

[43] Murray, A., Winter, N., Lagranderie, M., Hill, D.F., Rauzier, J., Timm, J., Leclerc, C., Moriarty, K.M., Gheorghiu, M. and Gicquel, B. (1992) Expression of *Escherichia coli β*-Galactosidase in *Mycobacterium bovis* BCG Using an Expression System from *Mycobacterium Paratuberculosis* which Induced Humoral and Cellular Immune Responses. *Molecular Microbiology*, **6**, 3331-3342.
https://doi.org/10.1111/j.1365-2958.1992.tb02201.x