









SHORT REPORT



Time to face the proofs: the BCG Moreau vaccine promotes superior inflammatory cytokine profile *in vitro* when compared with Russia, Pasteur, and Danish strains

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ABSTRACT

Tuberculosis (TB) has been a major public health problem worldwide, and the Bacillus Calmette–Guérin (BCG) vaccine is the only available vaccine against this disease. The BCG vaccine is no longer a single organism; it consists of diverse strains. The early-shared strains of the BCG vaccine are stronger immunostimulators than the late-shared strains. In this study, we have employed a simple *in vitro* human model to broadly evaluate the differences among four widely used BCG vaccines during the characterization of strain-specific host immune responses. In general, the BCG Moreau vaccine generated a higher inflammatory cytokine profile and lower TGF- β levels compared with the Russia, Pasteur, and Danish strains in the context of early sensitization with TB; however, no changes were observed in the IL-23 levels between infected and noninfected cultures. Unsurprisingly, the BCG vaccines provided different features, and the variances among those strains may influence the activation of infected host cells, which ultimately leads to distinct protective efficacy to tackle TB.

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1. Introduction

Along with the recent coronavirus disease 2019 (COVID-19) pandemic, tuberculosis (TB) has also been a major public health problem worldwide. Since 2003, TB has remained as the leading cause of mortality due to an infectious pathogen, namely *Mycobacterium tuberculosis*, but this scenario is about to change shortly because by the time this text was written, COVID-19 deaths toll ranked close on (https://www.reddit.com/r/Coronavirus_COVID_19/comments/hgj86d/average_disease_deaths_per_day_worldwide_jun_2020/). However, COVID-19 is an epidemic, but TB has been endemic since the human modern history.



In 2019, an estimated 10 million people (88% adults, 56% male, and 8.2% people living with human immunodeficiency virus (HIV)) fell ill with TB. In addition, there has been an estimated 1.2 million TB deaths among HIV-negative people and 208,000 deaths among those who are HIV positive. The death toll from this infectious disease is still the highest among other diseases; thus, efforts to combat it must be accelerated.¹

TB is the classic model of cellular immunity, but both cell-mediated immunity (CMI) and humoral immune responses are implicated in protection against the disease.² Owing to the considerable complexity of TB and the highly heterogeneous nature of its progression, it has been difficult to demarcate different cytokines, chemokines, and types of effector T cells into protective *versus* destructive immune responses. Moreover, there is still

a significant level of unawareness of the quantitative and qualitative relationships between T cell responses and their magnitude.

The live and attenuated Bacillus Calmette–Guérin (BCG) vaccine, originally derived from a serial passage of a virulent strain of *M. bovis*, has been the only available vaccine against TB since its introduction in 1921. It is most effective in preventing pediatric TB, miliary TB, and tuberculous meningitis.³ However, it has a limited effect in preventing pulmonary TB, which occurs more frequently in adults.^{4,5} The protective efficacy of the present BCG vaccine against pulmonary TB ranges from 0%–85% in different clinical trials worldwide.⁶ There have been several explanations for its variable efficacy, such as the hypothesis that prior infection with environmental mycobacteria in adults influences the subsequent efficacy of the BCG vaccine and the absence of antigens that are protective against *M. tuberculosis*.^{6–9} The essence of such a hypothesis is that prior infection with some *Mycobacterium* species enhances the effect of BCG, whereas other species interfere.¹⁰ In fact, guinea pigs vaccinated with BCG were found to have been protected against *M. tuberculosis* as early as 15 days post-infection. Cytokine analysis by qRT-PCR also revealed an increase in proinflammatory cytokine, indicating Th1-biased immune responses in BCG-vaccinated animals.¹¹

The BCG vaccine is no longer a single organism; it consists of genotypically and phenotypically different strains (reviewed by¹²). Evolutionarily, the BCG vaccine

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was divided into “early-shared strains” (Group I) and more attenuated “late-shared strains” (Groups II to IV), with Group I strains being more effective than the others.^{13,14} Brazil has used BCG Moreau vaccine (Group I) to immunize neonates from 1930 to 2018, and some important data have been collected.³ In 2012, this strain was listed as a WHO BCG Reference Reagent in an international collaborative study approved by the WHO Expert Committee on Biological Standardization.¹⁵

There are substantial variations in the immunogenicity and specific immune responses of several BCG vaccine strains.^{16,17} Subsequent to the immunological characterization of most BCG vaccines, the early-shared strains (Moreau, Russia, Japan, Sweden, and Birkhaug) exhibited stronger induction activities of nitric oxide, IL-1 β , IL-6, IL-8, IL-12, and TNF- α from A549 and THP-1 human cell lines in the presence of IFN- γ than did the late-shared strains (Danish, Pasteur, and others).¹⁸ Thus, the early-shared strains appeared to be more potent immunostimulants than the late-shared strains. This could be attributed partially to several long fatty acids found in the cell wall of the BCG vaccine.¹⁸ In addition, another study has demonstrated that *in vivo* immune responses to the BCG vaccine differ by strain, which may account for the variable outcomes of BCG immunization.¹⁹ A given BCG strain may multiply and persist longer in organs after vaccination and this may be followed by different antigen priming and stimulation of T cell responses.¹⁶ Remarkably, there is a lack of induction of CD8 + T cells following immunization with the BCG Japan. In addition, BCG Prague and BCG Japan were unable to protect mice against challenge whereas Glaxo, Pasteur, or Russia BCG strains eliminated mycobacteria very efficiently.¹⁶ Also, BCG Danish immunized infants showed the highest IFN- γ , IL-13 and IL-10 levels. Conversely, clinical score differences, such as mortality were not significant.¹⁷ These differences may play a role in BCG vaccination efficiency. In contrast, meta-analyses of human studies have also demonstrated that the effects of different BCG strains are comparable.^{20,21}

To validate the hypothesis that the BCG Moreau vaccine is more effective than the other strains in terms of the induction of proinflammatory profile, we tested four widely used BCG vaccines in an *in vitro* human model in this study: BCG Moreau and BCG Russia (Group I), BCG Danish (Group III), and BCG Pasteur (Group IV) strains. Although BCG Russia may be much less effective than BCG Danish and BCG Japan (Group I),²² since 2018, Brazil has been using BCG made from a seed strain of BCG Russia.²³ However, little is known about BCG Russia protective properties or its immune response in comparison with the other BCG strains, particularly the BCG Moreau vaccine. Although BCG Moreau induces a strong delayed-type hypersensitivity reaction in skin tests, relatively few *in vitro* studies have investigated the origin of this protective response (reviewed by¹²).

2. Subjects, materials, and methods

The specimens tested in this study were collected from healthy adult donors (HD; $n = 27$) at a public blood bank (anonymous donations from individuals aged ≥ 18 years). Some samples were also used in a previous study to test BCG *in vitro* T cell

immune responses.²⁴ Since 1967, Brazil has had a policy of providing universal BCG vaccination soon after birth. Although we acknowledge that maternal BCG scar rather than just a history of receipt of BCG is likely to modify an infant’s response to BCG,²⁵ and hence the importance of the BCG, as well as other mycobacteria exposure in a given population, we were unable to determine the BCG status of our cohort (or even to perform additional tests) due to the blood bank depository guidelines. The IOC-Fiocruz (protocol # 35775014.0.0000.5248) institutional review board approved the study procedures.

PBMCs were separated within no more than 24 h (average of 5 h) of obtaining blood specimens from all the study participants and cultured as described elsewhere.²⁴ *In vitro* infections of freshly isolated PBMCs with the BCG Moreau (at a single dose, individual batches of sealed glass vials containing liquid suspension with approximately 1×10^7 viable bacilli), Pasteur, Danish, or Russia strains (both at a single dose, individual batches of vials containing frozen suspensions with approximately 1×10^7 viable bacilli each) were performed at a multiplicity of infection (MOI) of 2:1 (bacilli/host cell). No difference was observed between the aforementioned frozen and liquid lots, or MOI.²⁴ The respective master batches were used once for each infection and then subsequently discarded. The BCG Pasteur, Danish, and Russia ampoules were thawed shortly before the infection of cells. Cultured PBMCs (1×10^6 cells each) in RPMI medium (Sigma Immunochemicals, USA) supplemented with 10% human AB serum were stored at 37°C in a humidified 5% CO₂ atmosphere in individual 12 \times 75 mm sterile polystyrene tubes (Falcon, Corning Inc., USA) for 48 h. Subsequently, the supernatants were stored at -70°C for further use in cytokine detection assays.

Except for the BCG Russia, analysis of the concentrations of human TGF- β , IL-1 β , IL-6, IL-17, IL-23, and IFN- γ was included as part of our previous enzyme-linked immunosorbent assay (ELISA) and multiarray cytokine detection and induced by three BCG vaccine strains.²⁴ Cell-free supernatants were thawed once and subsequently assayed to determine the concentrations of human IL-1 β (lower detection limit (DL): 0.4 pg/mL and upper DL: 2,877 pg/mL), IL-6 (lower DL: 3.1 pg/mL and upper DL: 2,539 ng/mL), IL-17 (lower DL: 1.2 pg/mL and upper DL: 6,631 pg/mL), and IFN- γ (lower DL: 0.7 pg/mL and upper DL: 11,377 pg/mL) using the Bio-Plex protein multiarray system kit, and Luminex-based technology was adopted (Bio-Rad, USA). Correspondingly, we performed ELISA on human IL-23 (lower DL: 125 pg/mL and upper DL: 8,000 pg/mL) and TGF- β (lower DL: 31.2 pg/mL and upper DL: 2,000 pg/mL) using commercial kits (R&D Systems, USA) according to the manufacturer’s instructions.

The cytokine production values are reported as median and interquartile range. The normality of the distribution of the variables was tested using the Shapiro–Wilk tests and Q–Q plots. Between-group comparisons were performed using the Kruskal–Wallis test followed by Dunn’s post hoc test. In addition, Spearman’s rank correlation coefficient was adopted to analyze the correlation between the cytokine level and BCG strain using GraphPad InStat 8.0 (GraphPad Software Inc., La Jolla, USA). $P < .05$ was considered statistically significant.

3. Results

Better characterization of strain-specific host immune responses via comparison among the four widely used BCG strains (Moreau, Pasteur, Danish, and Russia) may facilitate in the development of rational approaches to improve the BCG vaccine. To achieve this, we conducted a merely cross-sectional, single-center cohort study. After appropriate sampling and *in vitro* stimulations, we simultaneously measured six cytokines from HD by multiparametric Luminex for IL-1 β , IL-6, IL-17, and IFN- γ and ELISA for IL-23 and TGF- β . A lower level of TGF- β was detected only in BCG Moreau-infected culture compared with the matched medium rest background (baseline) immune responses ($p < .03$; **Figure 1A**). Conversely, higher levels of IL-1 β and IL-6 were detected exclusively in BCG Moreau-infected culture compared with baseline ($p < .001$; **Figure 1B,C**, respectively). Similarly, the IL-17 levels of BCG Moreau-, Pasteur-, and Danish-infected cultures were found to be higher compared with baseline ($p < .001$), as well as compared with the level of BCG Russia-infected culture ($p < .006$; **Figure 1D**). Finally, the IFN- γ levels of BCG Moreau- and Danish-infected cultures were found to be higher compared with baseline ($p < .03$), as well as compared with the level of BCG Russia-infected culture ($p < .001$; **Figure 1E**). No differences were observed in the IL-23 levels between infected and noninfected cultures (data not shown).

According to a very recent study, the networks of cytokine correlations are quite complicated both in patient and in healthy control groups, and correlation analysis among the cytokines may explain the essential differences between them.²⁶ Thus, Spearman correlations of changes of four cytokines from all infected conditions demonstrated a positive correlation between IFN- γ and both the IL-1 β (data below) and IL-17 levels (**Table 1**), only when relating to BCG Russia. Also, all infected conditions demonstrated a positive correlation between IFN- γ and the IL-17 levels as well as IL-6 and both IFN- γ and IL-17 levels, except for the IL-17 levels using the Russia strain (**Table 1**). Finally, the IL-1 β levels exhibited a distinct pattern when compared with IL-6 ($\rho = 0.79$, $p < .01$; $\rho = 0.91$, $p < .001$; and $\rho = 0.51$, $p = ns$), IFN- γ ($\rho = 0.84$, $p < .003$; $\rho = 0.87$, $p < .002$; and $\rho = 1.00$, $p < .001$), and IL-17 levels ($\rho = 0.59$, $p = ns$; $\rho = 0.75$, $p < .01$; and $\rho = 0.98$, $p < .001$) for BCG Danish, Pasteur, and Russia, respectively, but not for BCG Moreau. The four BCG strains were not found to be correlated with TGF- β and IL-23 levels (data not shown).

4. Discussion

The success of *M. tuberculosis*, as one of the dreaded human pathogens, lies in its ability to utilize different defense mechanisms in response to the varied environmental challenges during

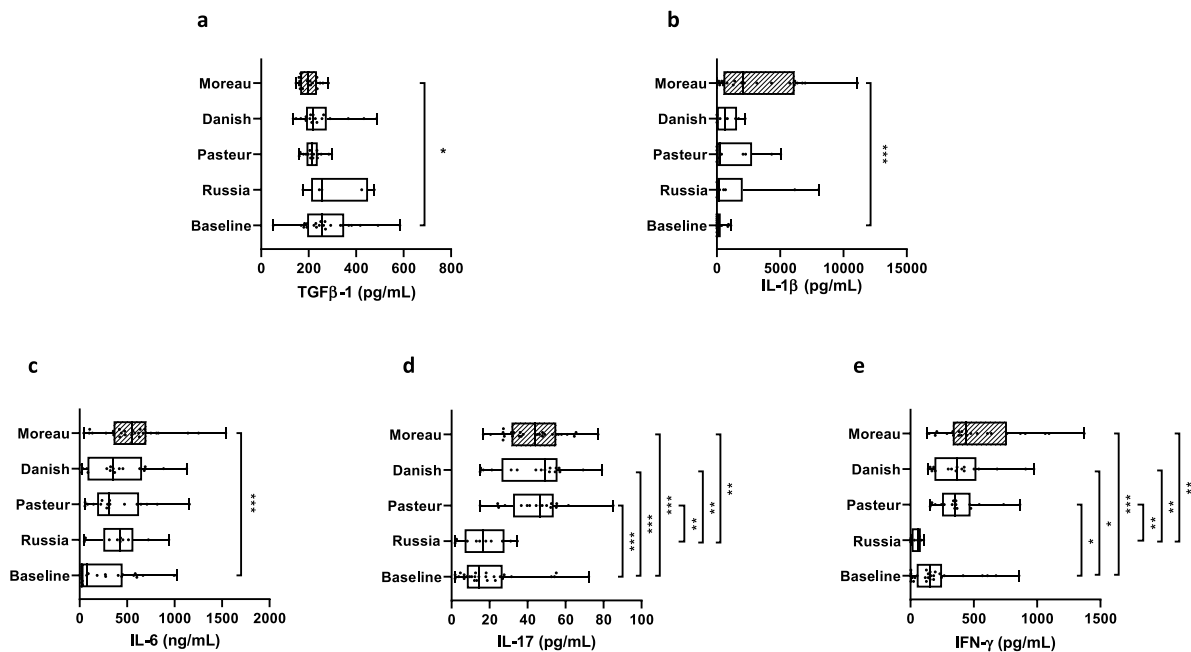


Figure 1. (A) TGF- β , (B) IL-1 β , (C) IL-6, (D) IL-17 and (E) IFN- γ levels (pg/mL, except C ng/mL) in healthy donors ($n = 27$) representing the baseline, uninfected cells, and the BCG Moreau (hatched), Pasteur, Danish, and Russia strains in 48 h *in vitro* infection of human mononuclear cells. Box and whiskers plot denote the median and the interquartile range cytokine values in each condition. * $p \leq .05$; *** $p \leq .0005$ compared with baseline, and ** $p \leq .005$ compared with BCG Russia, according to Dunn's paired test.

Table 1. Spearman's correlation analysis of associations between cytokine levels using the four different BCG strains.

Cytokines		BCG Danish		BCG Moreau		BCG Pasteur		BCG Russia	
		rho	p value	rho	p value	rho	P value	rho	p value
IL-6 vs.	IL-17	0.95	<0.001 ^a	0.45	0.018 ^a	0.92	<0.001 ^a	0.58	0.080
	IFN-y	0.97	<0.001 ^a	0.46	0.027 ^a	0.90	<0.001 ^a	0.73	0.045 ^a
IL-17 vs.	IFN-y	0.94	<0.001 ^a	0.55	0.005 ^a	0.90	<0.001 ^a	1.00	<0.001 ^a

the course of its intracellular infection, latency, and reactivation cycle.²⁷ This highlights the ability of *M. tuberculosis* for differential expression of genes required for its survival in the myriad of environmental conditions it faces during the course of infection.²⁸

To tackle TB, the BCG immunization policies are widely employed in countries burdened with high incidence of TB. Many countries have also continued the universal BCG vaccination despite the low incidence of TB. However, in other countries, BCG vaccination has been replaced by intensified case detection and supervised early treatment with contact tracing, whereas some chose to continue BCG vaccination but adopted a selective approach to be targeted only for the high-risk group.²⁹

As mentioned elsewhere, it is not surprising that diverse BCG strains could provide different adaptive features and that the variances between those strains may influence the activation of infected host cells.³⁰ Our present data has expanded and confirmed the results of a previous study²⁴ and validated that the BCG Moreau vaccine is the most potent immunostimulant among the four strains *in vitro* tested herein as it demonstrated the highest level of proinflammatory cytokines and reduced the TGF- β levels. Earlier, it has been shown that the protective effect of bacterial immunostimulants, like OM-85BV (a preparation of lysates of eight bacterial pathogens), is ascribed to the improvement of nonspecific immunity, as well general activation of the CMI.³¹ However, excessive inflammatory cytokine production can lead to tissue damage.³² Conversely, local antiseptic, anti-inflammatory and mucolytic products are substantially less frequently used by OM-85BV treated patients.³¹ In fact, although our knowledge of the BCG vaccine remains nascent, this field deserves more attention and deeper exploration. On the one hand, our study has found that there might be a recall and a specific immune response in adults with regard to the BCG Moreau vaccine strain, at least for three major monokines, i.e., IL-1 β , IL-6, and TGF- β . Since the HC subjects had been previously immunized with BCG-Moreau as infants, it was not unexpected that this strain would also induce the largest *in vitro* cytokine responses after stimulation with the same vaccine. A study has pointed out a strain-specific immune response at *in vitro* level with regard to two BCG vaccine strains, although different from the four strains used in the present study.³⁰ On the other hand, in our study IL-17 and IFN- γ were broadly induced by the BCG vaccine from three distinct groups: I, III, and IV. Additionally, this study found no correlation when IL-1 β levels were compared with IL-17 (for BCG Danish) and IL-6 (for BCG Russia). Surprisingly, the BCG Russia vaccine did not induce any cytokine tested herein, albeit this strain is classified as the closest to BCG Moreau.¹³

Previously, BCG Russia has been shown to be less immunogenic than other BCG strains, albeit it is currently the most widely administered BCG strain worldwide.²² BCG Russia induced lower frequencies of mycobacteria-specific polyfunctional CD4+ and cytotoxic CD8 + T cells, lower Th1 cytokine secretion and smaller skin test reactions when compared with other BCG strains in a small research clinical trial in Australia.³³ In another setting, the BCG Russia immune response of African infant vaccinees corroborated with the poor polyfunctional scores related to BCG Danish and tended

to accumulate in a CD4 + T cell naïve-like state.³⁴ Thus, the data suggested that BCG Russia did not enhance innate immunity in the same way as other BCG vaccines and possibly deviates the immune system away from an anti-inflammatory IL-10 immune response.

Our study also found a positive association involving IFN- γ , IL-17 and IL-6 levels in almost all BCG-infected conditions, although the IL-1 β levels exhibited a distinct pattern when compared with those cytokines. In fact, reports describing the effect of IFN- γ on IL-1 β production are conflicting.³⁵ It has been recognized that IL-1 β produced by macrophages and dendritic cells has a pro-inflammatory profile comparable to IFN- γ .³⁶ Two studies have found that IL-1 synergized with IL-6³⁷ and also IL-23³⁸ to maintain cytokine expression in effector Th17 cells. Typically, IL-1 β , TGF- β and IL-23 are essential driving forces of cell differentiation from Th0 to Th17 profile, resulting in increased IL-17 production.³⁹ In another setting, IFN- γ potentiated IL-1 β release from human cells.³⁵ Although still debated, an improved pro-inflammatory milieu has been related to the BCG vaccine, and ascribed as one of the main protection mechanisms provided by BCG against TB infection.^{24,39}

The WHO published the last BCG report²⁹ in 2018 and recommended that a single dose of the BCG vaccine be given to all infants at birth in countries where TB is highly endemic or where there is a high risk of exposure to TB, whereas countries with low incidence of TB may limit BCG vaccination to selected high-risk groups. Accordingly, the International Union Against Tuberculosis and Lung Diseases (IUATLD)⁴⁰ had set criteria to help countries in defining low endemicity and to consider the discontinuation of BCG vaccination or changing of its policy from a universal to a targeted approach. As the incidence of TB continues to decline in most developed countries,¹ selective BCG vaccination strategies in high-risk populations are increasingly being considered as an alternative to universal vaccination,^{41–44} particularly among countries that satisfy the IUATLD criteria for discontinuation.⁴⁰

There is general evidence that vaccines have important heterologous, off-target effects on children in low-income countries.⁴⁵ The hypothesis states that, until a different vaccine is administered, live attenuated vaccines induce a beneficial nonspecific immunity. In randomized clinical trials, the BCG Danish vaccine nearly halves all-cause mortality in neonates by dropping deaths from infections other than TB, and this multifaceted effect is stronger if the mother has had the BCG vaccine and much stronger after the child's second or subsequent dose of the BCG vaccine.^{45–47} These heterologous protections are mediated, at least in part, by epigenetic effects on innate immune function.⁴⁵ A recent study related to the agonistic effect, leading to a heterologous and rapid protection of the BCG vaccine, pointed out that BCG administration reduces human neonatal sepsis, suggesting that this defense mechanism requires the increase of G-CSF provided by the BCG vaccine.⁴⁸ More recently, though, there has been a debate about the nonspecific beneficial effects of the BCG vaccine on viral infections: In the strategy of vaccine repositioning, could BCG afford protection against COVID-19?⁴⁹ Consequently, clinical trials addressing this question are

underway (<https://mvec.mcri.edu.au/clinical-trial-of-bcg-vaccine-against-covid-19-brace/>). Thus, at present, the WHO does not recommend BCG vaccination for the prevention of COVID-19.

The development of an improved TB vaccine, which can act as an efficient prophylactic vaccine to boost the immune system of BCG-vaccinated individuals, is urgently needed. Currently, the improvement of BCG is still considered as one of the best choices for the rational design of a new antituberculosis vaccine to recall the primed CMI. Reportedly, a BCG-based delivery system is attributed with greater potency of live bacterial vectors in stimulating adaptive immune responses than peptides, proteins, or DNA vaccines; the ability to mimic a natural infection and to induce innate immune responses via toll-like receptors; and providing an inflammatory milieu for antigen capture by dendritic cells.^{50,51} Thus, the BCG vaccine itself is a vehicle with inherent adjuvant properties, expressing different genes in a live-attenuated vaccine format.⁵²⁻⁵⁴

The most important limitation of the current study was the small sample size. There were other limitations of this study. First, the difficulty in determining the significant differences *in vitro* among the different BCG vaccine strains after immunization in humans, since many of the strains used in vaccine manufacture contain more than one genotype.⁵⁵ More evidence on the efficacy, effectiveness, and adverse effects of the BCG vaccine strain is needed. Second, the strain specificity topic, since it is necessary to test our hypothesis using alternative cohort of matched individuals already vaccinated with any nonrelated BCG Moreau vaccine. Third, although unable to explore better in this study, we appreciate the use of pre-exposure markers to mycobacteria, such as the IGRA, in future studies, thus allowing relating the stimulus provided by the BCG vaccine to any eventual recall immune response to mycobacteria.⁵⁶

Taken together, the BCG Moreau vaccine generates an *in vitro* higher inflammatory cytokine profile and lower TGF- β levels compared with the Russia, Pasteur, and Danish strains in the context of early sensitization with mycobacteria (i.e., healthy adults vaccinated with BCG Moreau when they were young). It will be important to find a long-term association between adequate early vaccination with specific BCG strains, subsequent skin test reaction kinetics, and survival. Considering that this centennial vaccine has been safely used worldwide, we have obtained a significant amount of information about it. It would be remiss to not remark that astonishing progress has been made in TB vaccine research in response to this disease. Conclusively, this study demonstrates how harnessing the BCG vaccine field has led to new insights into its protective efficacy to tackle TB.

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Disclosure statement


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