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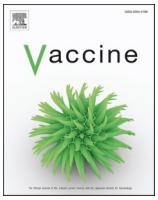

Short communication

A second-generation recombinant BCG strain combines protection against murine tuberculosis with an enhanced safety profile in immunocompromised hosts

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ABSTRACT

Bacille Calmette-Guérin (BCG) remains the only licensed vaccine against tuberculosis (TB). While BCG protects against TB in children, its protection against pulmonary TB in adults is suboptimal, and the development of a better TB vaccine is a global health priority. Previously, we reported two recombinant BCG strains effective against murine TB with low virulence and lung pathology in immunocompromised mice and guinea pigs. We have recently combined these two recombinant BCG strains into one novel vaccine candidate (BCGΔBCG1419c::ESAT6-PE25SS) and evaluated its immunogenicity, efficacy and safety profile in mice. This new vaccine candidate is non-inferior to BCG in protection against TB, presents reduced pro-inflammatory immune responses and displays an enhanced safety profile.

1. Introduction

The development of a highly effective vaccine that can improve control of tuberculosis (TB), a disease that causes over 1.5 million deaths per year, is a global health priority. BCG is currently the only licensed vaccine against TB. However, BCG-induced protection wanes over time, has limited efficacy in adults, cannot be applied to individuals with co-morbid HIV infection [1,2], and is contraindicated in people with impaired immunity [3,4]. Therefore, there is an urgent need for the development of vaccine candidates that are safer and more effective than conventional BCG [5]. Recent pre-clinical studies published by our groups and others have shown that recombinant BCG (rBCG) strains can induce a more effective immune response with fewer side effects in immunocompromised mice than the original BCG strain [6–10].

The recombinant strain BCGΔBCG1419c is an attenuated BCG strain lacking a gene involved in biofilm formation, *BCG1419c* [11]. Immunological studies have indicated that BCGΔBCG1419c is more effective than conventional BCG at inducing an immune response in lymph nodes and that it conferred similar levels of protection against *Mycobacterium tuberculosis* (*Mtb*) replication in mice [9]. Moreover, mice and guinea-pigs immunized with BCGΔBCG1419c have been shown to exhibit a reduced inflammatory lung profile and other pathological signs compared to animals receiving conventional BCG after *Mtb* infection, and BCGΔBCG1419c is associated with low virulence in immunocompromised mice [9,12,13]. A second rBCG strain, BCG::ESAT6-PE25SS, secretes full-length ESAT-6 *Mtb* antigen through the BCG secretion system ESX-5, and can generate protective specific T cells against *Mtb* infection [7]. Additionally, safety studies have shown that vaccination with BCG::ESAT6-PE25SS is associated with low virulence in immunocompetent and immunocompromised mice [7]. We have now generated

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a second-generation vaccine candidate, called BCGΔBCG1419c::ESAT6-PE25SS, which combines the features of the aforementioned rBCG strains (BCGΔBCG1419c and BCG::ESAT6-PE25SS). We hypothesized that it could be an outstanding TB vaccine candidate in terms of protection and safety in immunocompromised mice.

2. Materials and methods

2.1. Bacterial strains and culture conditions

BCG strains Pasteur ATCC 35734 (referred to as BCG), BCG::ESAT6-PE25SS [7] (referred to as E6), BCGΔBCG1419c [14] (referred to as Δ19), BCGΔBCG1419c::ESAT6-PE25SS (referred to as Δ19::E6), and *Mtb* H37Rv were cultured in Middlebrook 7H9 (BD Difco) broth supplemented with 10 % oleic albumin dextrose catalase (OADC) growth supplement (USBiological Life Sciences), 0.05 % Tween80, 0.4 % glycerol and appropriate antibiotics at 37 °C with continuous shaking. Bacteria were cultured until log phase at an OD_{600} of 0.8–1, either used directly or harvested and stored at −80 °C for subsequent use. For lung and spleen bacterial counts, serial dilutions were plated onto Middlebrook 7H11 agar supplemented with 0.5 % glycerol, 10 % OADC, containing 25 μg/ml kanamycin for E6 and Δ19::E6. For *Mtb* culture, cycloheximide and penicillin were added to 7H11 agar plates to restrict the growth of non-specific bacteria and fungi. Agar plates were sealed and incubated aerobically for 5–8 weeks at 37 °C. Colonies were counted and the total colony forming units (CFU) per organ were calculated based on dilution factors and organ size.

2.2. Generation of BCGΔBCG1419c::ESAT6-PE25SS

The plasmid pMV306hsp::esxA-PE25SS used for the generation of BCG::ESAT6-PE25SS [7], was transformed into BCG Pasteur (ATCC 35734) and BCGΔBCG1419c [14] to generate kanamycin-resistant recombinant BCG::ESAT6-PE25SS (E6 in ATCC 35734 background) and BCGΔBCG1419c::ESAT6-PE25SS (Δ19::E6), respectively. Kanamycin-resistant colonies with the esxA-PE25SS construct were analyzed by PCR to confirm the presence or deletion of the *BCG1419c* gene, as explained elsewhere [14,15].

2.3. Mice

For the immunogenicity studies, specific pathogen–free (SPF), 6–8-week-old female B6 mice were obtained from Bioterio Morelos (Tlayacapan, Morelos, Mexico) to be used for experiments performed at Centro de Investigación y Asistencia en Tecnología y diseño del Estado de Jalisco (CIATEJ), A.C., Mexico. Mice were maintained in a Biosafety Level 2 Animal facility. The Internal Committee for the Care and Use of Laboratory Animals (CICUAL for its acronym in Spanish) from CIATEJ approved the experiments with project number 2022-005 A. The animal research adheres to the ARRIVE guidelines (https://arriveguidelines.org/arrive-guidelines). All experiments complied with the Mexican guidelines regarding ethical and safe handling of experimental animals and final disposal of materials such as: NOM-07- SEMARNAT-SSA1–2002, NOM-033-ZOO-1995, and NOM-062- ZOO-1999.

For the efficacy studies, 6–8-week-old female C57BL/6 (B6) mice were purchased from the Animal Resource Centre (ARC; Perth, Australia). For safety studies, *Rag2*$^{-/-}$*IL2rg*$^{-/-}$ mice were bred and maintained in-house. Prior to vaccination or challenge, all mice were acclimatized for at least one week and kept in biosafety level 2 or 3 facilities, under SPF conditions at the Australian Institute of Tropical Health and Medicine (AITHM), James Cook University, Australia. All experiments were conducted in accordance with the National Health and Medical Research Council (NHMRC) animal care guidelines and were approved by the animal ethics committee (A2837) of James Cook University, Australia.

2.4. Vaccinations and infections with BCG

For the immunogenicity studies, C57BL/6 mice were vaccinated with BCG, E6, Δ19 or Δ19::E6, subcutaneously (s.c.) with approximately 10^6 CFU of freshly cultured bacteria in 100 μl of sterile phosphate-buffered saline (PBS). For the vaccine efficacy experiments, C57BL/6 mice were immunized with approximately 10^5 CFU of thawed and washed bacteria in 100 μl of sterile PBS. To determine safety in immunocompromised hosts, *Rag2*$^{-/-}$*IL2rg*$^{-/-}$ mice were intravenously infected with 1×10^6 CFU of freshly cultured bacteria in 200 μl of sterile PBS. Infected immunocompromised mice were weighed, and their wellbeing was monitored weekly for 25 weeks. The disease indicators (i.e. signs of disease) assessed in this study included: reluctance to move, isolation, ruffled fur, hunched position, breathing difficulties, distended abdomen, soft feces, low food or water intake and head tilting (indicative of an ear infection). The wellbeing of each mouse was scored from 0 to 3, 0 being a symptom-free and healthy mouse. Mice losing 15 % weight or scoring 3 at any point were sacrificed as determined by our protocols for humane endpoint.

2.5. Mtb challenge

Mtb challenge was performed in a BSL3 laboratory 60 days after vaccination. Frozen *Mtb* aliquots were thawed, diluted to the appropriate concentration, and treated in an ultrasound water bath to disrupt bacterial clumps. Mice were infected with very low dose 10–20 CFU *Mtb* H37Rv using a Glas-Col inhalation exposure system. The initial infectious dose was determined by plating lung tissues collected from 5 mice one day after aerosol infection on 10 % OADC enriched 7H11 agar plates.

2.6. Sample collection

Lungs and spleens were aseptically collected 60 days post vaccination and processed for CFU determination or cell isolation and flow cytometry analysis for the immunogenicity studies. Sera were collected from immunized mice at different points post vaccination by bleeding mice from the tail vein. For the efficacy studies lungs and spleens were collected 45 days after *Mtb* challenge for bacterial burden determination and histopathology analysis.

2.7. Histopathology

Lung lobes from infected mice were fixed overnight with 10 % neutral buffer formalin, transferred to 70 % ethanol, and embedded in paraffin. Processed lungs were sectioned with a microtome into 5 μm slices, transferred to glass slides, dewaxed and stained with H&E. Stained slides were scanned with an Aperio LV1 (Leica) followed by analysis with QuPath v0.4.0, Image Scope (Leica) and ImageJ software (NIH). To calculate the percentage of lung damage, the total surface area was measured followed by measuring the areas affected by *Mtb*, as described previously [16].

2.8. Cell isolation from lungs and spleens for flow cytometry analysis

Lungs were mechanically disrupted to obtain single cell suspensions. Cell suspensions were incubated with erythrocyte lysis buffer, washed with PBS, and passed through a 70 μm cell strainer. Concentration of live cells per sample was determined by trypan blue dye exclusion and automated cell counting. Two million cells were cultured in the presence or absence of 10 μg/ml of bovine Purified Protein Derivative (PPD) (PRONABIVE, obtained from *Mycobacterium bovis* AN5) in RPMI media supplemented with 10 % FBS and antibiotic for 24 h at 37 °C and 5 % CO_2. Spleens were perfused with DMEM and the resulting cell suspension was incubated in erythrocyte lysis buffer. One million splenocytes were cultured in the presence or absence of 10 μg/ml of PPD in DMEM

media supplemented with 10 % FBS, 1 % non-essential amino acids, 1 % *w/v* sodium pyruvate and antibiotic for 24 h at 37 °C in a humidified chamber and 5 % CO_2. Prior to cell fixation and labeling, 5 μg/ml of brefeldin A was added to cultures and incubated for 6 h before harvesting.

2.9. Flow cytometry

After incubation, cell supernatants were collected and stored at −20 °C. Identification of T cells was performed by using the following antibodies and fluorochromes against CD3 (APC; clone 17A2), CD8 (FITC; clone 53–6.7), CD4 (PE; clone RM4-5), CD69 (PerCP/Cy5.5; clone H1.2F3), CD62L (PerCP/Cy5.5; clone MEL-14), CD103 (APC; clone QA17A24), CD44 (APC; clone IM7), IFN-γ (PerCP/Cy5.5; clone XMG1.2), IL-2 (PerCP/Cy5.5; clone JES6-5H4) and TNF-α (APC; clone MP6-XT22); all purchased from Biolegend. For cell staining, cells were first incubated with 0.5 μg of FC-purified anti-mouse CD16/CD32 (Cytek, 70–0161) at 4 °C for 20 min. After washing with 2 % BSA-PBS, cells were incubated with 0.2 μg of anti-mouse antibodies in 50 μL of total volume. After washing, cells were fixed and permeabilized with the eBioscience Intracellular Fixation and Permeabilization kit (Invitrogen, 88-8824-00). Staining of intracellular cytokines was performed by incubating permeabilized cells with the corresponding intracellular anti-mouse antibodies and finally washed and preserved in a 4 % solution of paraformaldehyde at 4 °C protected from light. Unstained cells, and cells stained with the single antibodies were used as controls for the compensation setup. Stained cells were acquired in a BD Accuri C6 flow cytometer; 50,000 and 100,000 cells from spleens and lungs were recorded, respectively. Effector memory T cells were considered as $CD44^+CD62L^-$, central memory T cells as $CD44^+CD62L^+$ and resident memory T cells as $CD69^+CD103^+$ cells.

2.10. Mouse antibody isotype detection

The sera obtained from the blood samples collected in the immunization experiments were used to determine the relative levels of mouse IgG2b using a mouse monoclonal antibody isotyping kit (Sigma, USA, ISO2). For this, 50 ng of bovine PPD were added to each well of Costar 96 well plates (Corning, USA, 3590) and incubated at 37 °C for 1 h. Plates were blocked with 3 % BSA-PBS for 1 h at 37 °C and 50 μl of mouse sera (1:25 in PBS) were added and incubated at 37 °C for an additional hour. Wells were washed 3 times PBST (PBS, 0.05 % Tween 20), 50 μl of an anti-mouse-isotype antibody diluted 1:1000 in PBS were added, and the plate was incubated at 37 °C for 1 h. After washing three times, the plate was incubated at 37 °C for 1 h with an HRP-conjugated anti-goat IgG antibody (Sigma, USA, 075KA773) at 1:5000 in 1 % BSA-PBS. After washing three times, 50 μL of 1-Step TMB Ultra TMB-ELISA (Thermo Scientific, 34028) were added. Five minutes after adding the substrate, 50 μl of sulfuric acid 0.5 M were added to stop the reaction, and the optical density (OD) at 450 nm was determined using a microplate spectrophotometer (xMark Biorad, USA).

2.11. Statistics

Statistical analysis was performed, and graphs were generated using Prism version 10.1.1 (GraphPad). Normal data were analyzed by one-way ANOVA with Tukey's multiple comparison post-test and non-normal data were compared by Kruskal-Wallis with Dunn's multiple comparison post-test; and survival data were compared by Log-rank (Mantel-Cox) test and multiple comparisons were considered by performing a Bonferroni correction, as outlined in the figure legends. $p < 0.05$ was considered significant, unless otherwise stated.

3. Results

To test whether the new rBCG strain BCGΔBCG1419c::ESAT6-PE25SS (Δ19::E6 from now on), or the parental strains (abbreviated to E6 and Δ19) were capable of protecting adult immunocompetent mice against aerosol challenge with *Mtb*, C57BL/6 mice were vaccinated and 60 days later infected with a very low dose of *Mtb* (Fig. 1a). Bacterial burden was determined 45 days post infection in the lung and spleen. Lung damage was determined as the percentage of the area of dense cell infiltration in H&*E*-stained lung sections. Results indicated that all BCG strains significantly reduced *Mtb* bacterial burden to a similar level in the lungs and spleens of infected mice and decreased the damaged lung area compared to unvaccinated mice (Fig. 1b, c).

Immunological parameters were evaluated to determine whether the strains induced different immune responses. For this study, the lungs and spleens from vaccinated mice were collected 60 days after vaccination and single cell suspensions were prepared and stimulated *ex-vivo* with purified protein derivative (PPD) antigen. Evaluation of surface markers by flow cytometry indicated that Δ19 induced more central memory T cells in the lung and conversely fewer central memory T cells in the spleen compared to conventional BCG and E6 (Fig. 1d). Interestingly, Δ19::E6 induced fewer $CD8^+$ effector memory T cells in the spleen compared to Δ19. Intracellular staining of type I cytokines indicated that the newly developed Δ19::E6 strain induced fewer i) lung $CD4^+$ TNF-α^+ compared to Δ19; and ii) CD4 and $CD8^+$ IFN-γ^+ cells compared to E6 and Δ19, respectively (Fig. 1e). Mice receiving the Δ19::E6 strain also presented reduced splenic $CD4^+$ and $CD8^+$ IL-2^+ cells compared to the parental strains Δ19 and E6 (Fig. 1f). Lastly, Δ19::E6 produced less IgG2b compared to parental strains E6 and BCG (Fig. 1g). Together, these data indicate that despite reduced expression of pro-inflammatory cytokines (usually indicative of effective BCG vaccination), Δ19::E6 performs similarly to the parental rBCG strains and conventional BCG in terms of protection against *Mtb* challenge.

Next, we sought to investigate the safety profile of Δ19::E6 in immunocompromised mice. For this, *Rag2*$^{-/-}$*IL2rg*$^{-/-}$ mice, deficient in T, B and NK cell responses, were infected intravenously with a high dose of the different rBCG strains or conventional BCG. Weight and symptoms were monitored for 25 weeks, and persistence of BCG bacterial burden was determined 30 days after infection in a second cohort of mice. Results revealed that immunocompromised mice injected with Δ19::E6 gained more weight over time compared to BCG (Fig. 2a, b). Survival analysis showed that all rBCG strains were safer than conventional BCG and that the percentage of mice free of symptoms was higher in animals infected with Δ19 and Δ19::E6 compared to those receiving conventional BCG (Fig. 2c, d). All BCG strains persisted to a similar level in the *Rag2*$^{-/-}$*IL2rg*$^{-/-}$ mice one month after infection; however, the lung bacterial burden in mice infected with Δ19::E6 was pronouncedly lower compared to the original infectious dose, and significantly lower in the spleen (Fig. 2e). BCG persistence was also evaluated in immunocompetent C57BL/6 mice 60 days after vaccination. All BCG strains persisted at very low levels compared to the original vaccination dose in the lungs and spleens of vaccinated mice, however the bacterial burden in the spleen was higher compared to the lung. The bacterial burden in the lungs of mice immunized with BCG, E6, Δ19 and Δ19::E6 was lower compared to the initial injection dose (Fig. 2f). The spleen bacterial burden of C57BL/6 mice immunized with E6 and Δ19::E6 was lower compared to the injection dose and those receiving conventional BCG. These analyses suggest that Δ19::E6 and the parental strain Δ19 are safer compared to conventional BCG in immunocompromised mice.

4. Discussion

Here, we report the generation and evaluation of a second-generation rBCG strain as a promising TB vaccine candidate. The incorporation of more than one feature in rBCG strains is a common strategy used to enhance the safety and efficacy profile of conventional BCG [17]. For several decades, TB vaccine development efforts have been focused on safer subunit vaccines, many of which showed limited efficacy compared to BCG. However, in the last two decades,

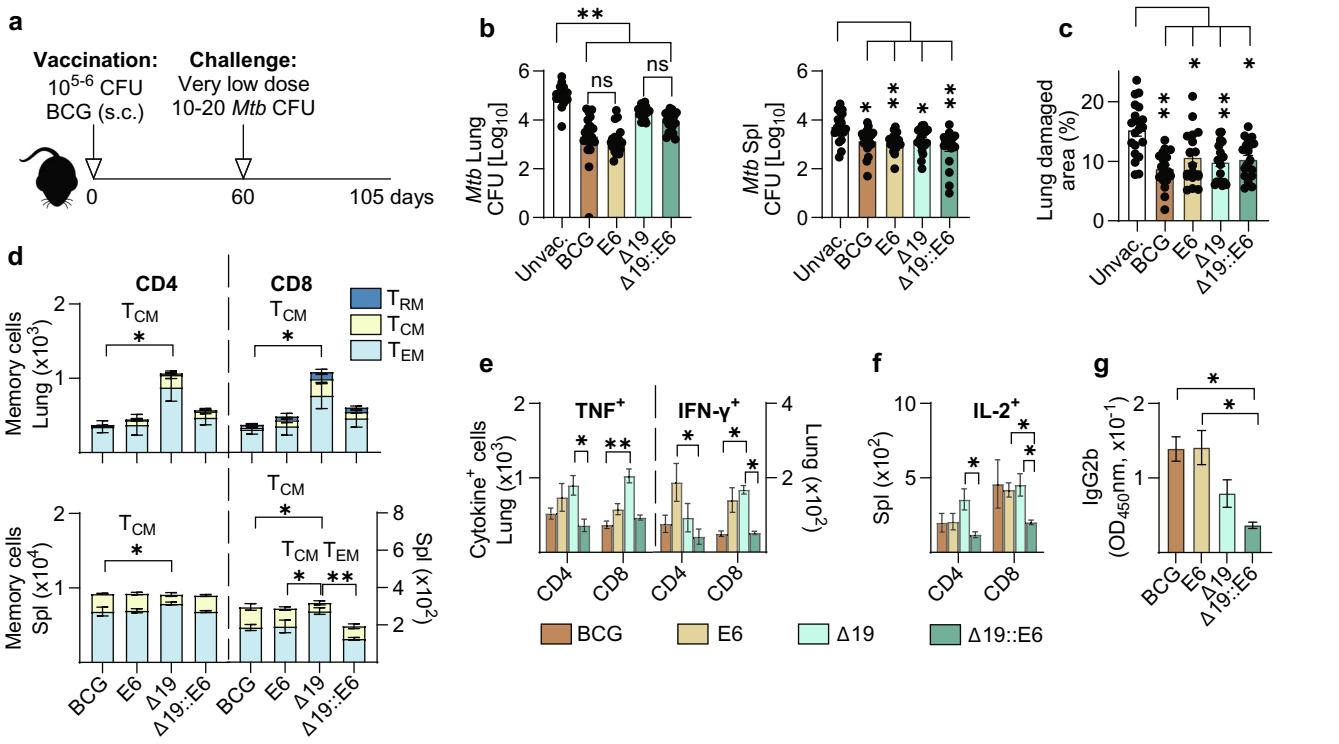

Fig. 1. Δ19::E6 confers TB protection with reduced pro-inflammatory immune responses. (a) C57BL/6 mice were vaccinated with 10^5 CFU (vaccine efficacy experiments) or 10^6 CFU (immunogenicity studies) of BCG Pasteur strain (BCG), BCGΔBCG1419c (Δ19), BCG::ESAT6-PE25SS (E6), or BCGΔBCG1419c::ESAT6-PE25SS (Δ19::E6) or left unvaccinated (Unvac.). Sixty days later, mice were either aerosol infected with 10–20 CFU of virulent *Mtb* H37Rv or spleens and lungs were collected for flow cytometry analysis. Sera were collected at different time points post-vaccination and antibody titers were determined. *Mtb* bacterial burden was determined 45 days post infection in lungs and spleens and lung damage was determined as the percentage of the area affected by *Mtb* in H&*E*-stained lung sections. (b) Bacterial burden in lung and spleen. (c) Histological analysis of the left lung lobe. (d) Analysis of memory T cell subpopulations in the lung and the spleen. (e-f) Intracellular staining analysis of bovine PPD-*ex vivo* stimulated $CD4^+$ and $CD8^+$ T cells in lung and spleen, at 60 days post-vaccination. (g) IgG2b antibody titers against bovine PPD at day 28 post-vaccination, expressed as OD450nm. Data from (b) and (c) ($n = 20$) were log transformed and compared by one-way ANOVA with Tukey's multiple comparison post-test. Number of cells in (d) to (f) are expressed as events per 100,000 cells per lung and 50,000 cells per spleen. Data from (d) to (g) ($n = 5$) were compared by Kruskal-Wallis with Dunn's multiple comparison post-test. All data show means ± SEM for individual mice. $^{**}p < 0.01$; $^{*}p < 0.05$.

mycobacterium-derived inactivated and live attenuated vaccines have shown encouraging results in pre- and clinical studies. For example, a rBCG (VPM1002), an attenuated *Mtb* strain (MTBVAC) and heat-killed non-tuberculous mycobacterium (*M. vaccae*) are currently in Phase III clinical trials [18]. The rBCG vaccine candidate VPM1002 (BCG*ΔureC::hly*) secretes listeriolysin, which enables antigen to escape from phagosomes, and is deficient in an urease C, which in turn modulates the pH for optimal listeriolysin performance [19]. Despite promising results shown by VPM1002 in clinical trials, a second-generation strain with enhanced pro-apoptotic profile, the BCG*ΔureC::hly ΔnuoG*, has been produced recently, with studies in mice showing increased efficacy and safety compared to the parental strain BCG*ΔureC::hly* [20]. This serves as an example of why it is important to invest and to generate a larger repertoire of 2nd- and 3rd-generation rBCG strains, ready to be fed into the pipeline of clinical studies to achieve the goal of finding an effective BCG replacement vaccine for TB, which hopefully will lead to TB elimination.

Our 2nd-generation vaccine candidate is deficient in the cyclic di-GMP phosphodiesterase-encoding gene *BCG1419c* and secretes full-length ESAT-6. As mentioned before, Δ19 exhibits an increased *in vitro* biofilm production compared to BCG [11,13]. Although it is still debatable whether biofilms are formed during TB disease *in vivo*, recent studies have reported the presence of biofilm-like structures in mice, macaques, and human samples [21]. Independently of the mechanism of action, vaccination studies in mice and guinea pigs with rBCG strain lacking the *BCG1419c* gene have shown that it protects against pulmonary and extrapulmonary TB. Furthermore, enhanced safety compared to BCG, and increased induction of memory $CD8^+$ T cell-associated immunogenicity in lungs and spleens of C57BL/6 mice were reported [9,10,12,22]. Findings reported here have shown that Δ19::E6 maintains a similar profile in terms of efficacy and safety compared to parental strain Δ19. Furthermore, ESAT-6 is a *Mtb* pore-forming toxin involved in mycobacterial cytosolic translocation within infected macrophages [23]. ESAT-6 expression not only induces potent specific T cell responses to ESAT-6 but ensures the availability of a broader antigenic repertoire in the cytosol of infected cells, which consequently can lead to the priming of antigen-specific $CD8^+$ T cells and the activation of cytosolic pattern recognition receptors. Conventional BCG lacks ESAT-6, which limits the antigen repertoire presented by BCG-infected cells. Therefore, rBCG strains expressing ESAT-6 or with alternative phagosomal escape properties are promising vaccine candidates which should be further explored. In summary, here we showed that Δ19::E6 is non-inferior to BCG and its parental strains (E6 and Δ19) at reducing pulmonary and disseminated *Mtb* infection in adult immunocompetent mice and that it exhibits a reduced pro-inflammatory T cell response in the lung and spleen. Importantly, several disease parameters indicate that Δ19::E6 is safer than BCG in immunocompromised mice, which may translate to improved vaccine safety in immunocompromised individuals, a feature that is absent from conventional BCG.

These findings warrant further comprehensive *in vivo* studies, including in models using more physiological ultra-low doses of *Mtb* challenge, latent TB re-activation and mice with other co-morbidities such as type 2 diabetes.

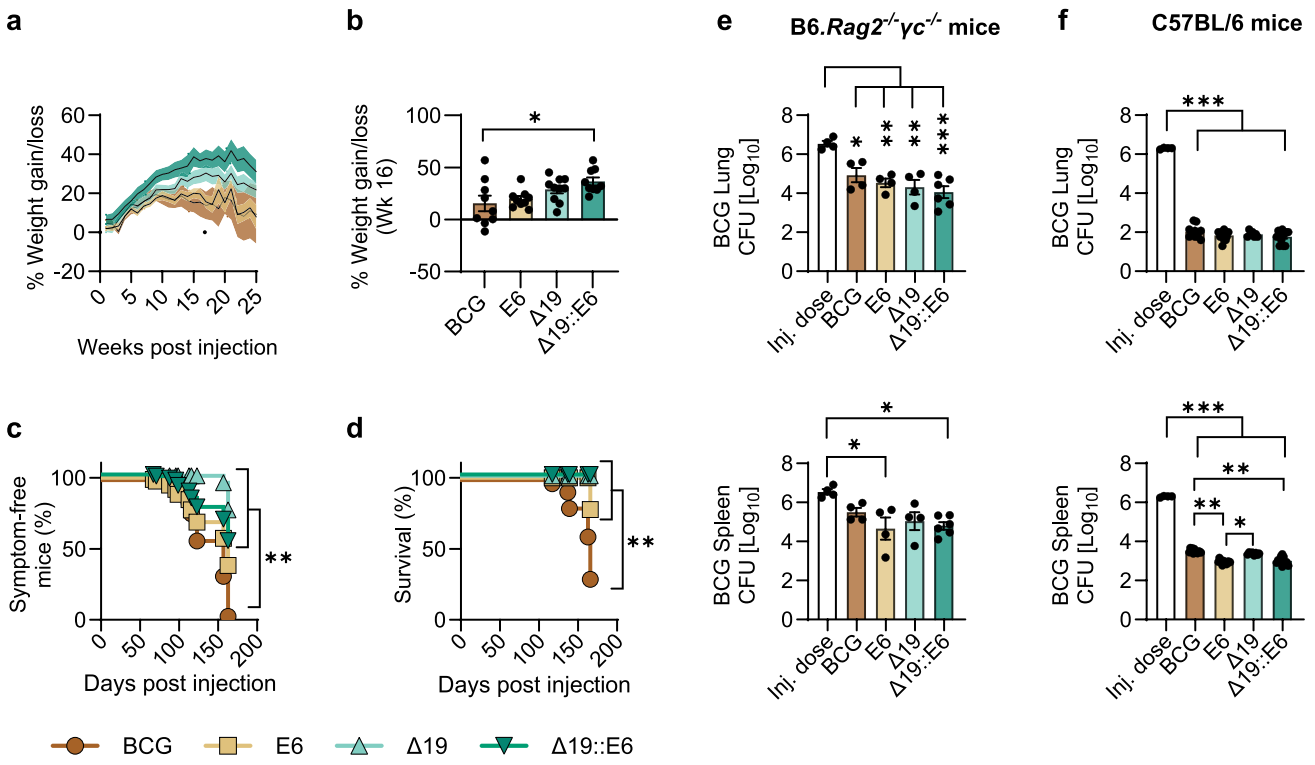

Fig. 2. Δ19::E6 is safer than BCG in immunocompromised mice. B6.*Rag2*$^{-/-}$*IL2rg*$^{-/-}$ mice were infected with 10^6 CFU of the different rBCG and conventional BCG strains intravenously. Weight and signs of disease were monitored over a period of 25 weeks and persistence (BCG bacterial burden) was determined 30 days after infection in a second cohort of mice. (a) Percentage of weight gain/loss over time compared to original weight on the day of infection. (b) Percentage of weight gain/loss at week 16, when all mice were still alive. Percentage of symptom-free (c) and alive mice over time (d). BCG bacterial burden in lung and spleen of immunocompromised (e) and immunocompetent B6 mice (f) 60 days post vaccination. Data in (b) (n = 9–10) were compared by Kruskal-Wallis with Dunn's multiple comparison post-test; data in (e) (n = 4–5) and (f) (n = 8–12) were log transformed and compared by one-way ANOVA with Tukey's multiple comparison post-test. Data in (c) and (d) (n = 9–10) were compared by Log-rank (Mantel-Cox) test and multiple comparisons were considered by performing a Bonferroni correction. All data displayed show means ± SEM for individual mice. $^{**}p < 0.01$; $^{*}p < 0.05$.

CRediT authorship contribution statement

Ana Maria Valencia-Hernandez: Writing – review & editing, Writing – original draft, Investigation, Funding acquisition. **Guangzu Zhao:** Investigation, Funding acquisition. **Socorro Miranda-Hernandez:** Investigation, Funding acquisition. **Cristian Alfredo Segura-Cerda:** Investigation. **Cesar Pedroza-Roldan:** Investigation. **Julia Seifert:** Investigation. **Michel de Jesus Aceves-Sanchez:** Investigation. **Mirna Burciaga-Flores:** Conceptualization. **Abel Gutierrez-Ortega:** Conceptualization. **Lidia del Pozo-Ramos:** Investigation. **Mario Alberto Flores-Valdez:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization. **Andreas Kupz:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Andreas Kupz reports financial support was provided by National Health and Medical Research Council. Ana Maria Valencia-Hernandez reports financial support and travel were provided by VALIDATE Network. Mario Alberto Flores Valdez reports financial support was provided by VALIDATE Network. Socorro Miranda-Hernandez reports financial support was provided by VALIDATE Network. Guangzu Zhao reports financial support was provided by VALIDATE Network. Andreas Kupz reports a relationship with Bill and Melinda Gates Foundation CTVD that includes: board membership, funding grants, and travel reimbursement. Andreas Kupz has a patent 'Recombinant strains of Mycobacterium bovis BCG' issued to James Cook University. Mario Alberto Flores Valdez has patent Patent number: #363576 issued to CIATEJ. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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