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The BCGΔBCG1419c strain, which produces more pellicle *in vitro*, improves control of chronic tuberculosis *in vivo*

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ABSTRACT

Mycobacterium tuberculosis (Mtb) has been a threat to humans since ancient times, and it is the main causative agent of tuberculosis (TB). Until today, the only licensed vaccine against Mtb is the live attenuated *M. bovis* Bacillus Calmette-Guérin (BCG), which has variable levels of protection against the pulmonary form of infection. The quest for a new vaccine is a priority given the rise of multidrug-resistant Mtb around the world, as well as the tremendous burden imposed by latent TB. The objective of this study was to evaluate the immunogenicity and capacity of protection of a modified BCG strain (BCGΔBCG1419c) lacking the c-di-GMP phosphodiesterase gene *BCG1419c*, in diverse mice models. In a previous report, we have shown that BCGΔBCG1419c was capable of increasing biofilm production and after intravenous infection of immunocompetent mice; this strain persisted longer in lungs than parental BCG Pasteur. This led us to hypothesize that BCGΔBCG1419c might therefore possess some advantage as vaccine candidate. Our results in this report indicate that compared to conventional BCG, vaccination with BCGΔBCG1419c induced a better activation of specific T-lymphocytes population, was equally effective in preventing weight loss despite being used at lower dose, reduced tissue damage (pneumonic scores), increased local IFNγ$^+$ T cells, and diminished bacterial burden in lungs of BALB/c mice infected intratracheally with high dose Mtb H37Rv to induce progressive TB. Moreover, vaccination with BCGΔBCG1419c improved resistance to reactivation after immunosuppression induced by corticosterone in a murine model of chronic infection similar to latent TB. Furthermore, despite showing increased persistence in immunocompetent mice, BCGΔBCG1419c was as attenuated as parental BCG in nude mice. To our knowledge, this is the first demonstration that a modified BCG vaccine candidate with increased pellicle/biofilm production has the capacity to protect against Mtb challenge in chronic and reactivation models of infection.

1. Introduction

Mycobacterium tuberculosis (Mtb) is the etiological agent of tuberculosis (TB), which causes 1.5 million deaths annually in the world. The World Health Organization estimates that Mtb latently infects around two billion people worldwide, despite a 90% global coverage vaccination [1]. Bacillus Calmette-Guérin (BCG) is the only vaccine approved for use in humans. BCG is somewhat ineffective in providing protection against pulmonary and latent TB [2]. Meta-analyses showed that protection conferred by BCG is highly variable and the related mechanisms are diverse and not completely elucidated [3].

Several efforts have been made to increase the efficacy of BCG, mostly centered on the stimulation of T helper 1 (Th1) immune responses [4], as in murine models of infection (the most widely used), production of IFN-γ is the hallmark associated with bacterial load reduction in lungs [3]. rBCGΔ*ureC::hly* expresses listeriolysin

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O to disrupt the phagosome membrane and enhance antigen presentation [5]. A rBCG strain (AERAS-422) expressing both perfringolysin O and Rv3407 (preferentially recognized by T-cells from latent TB infected people) [6] showed enhanced immunogenicity and protection against Mtb challenge in mice, but was disregarded during a clinical trial phase I because of side effects [6,7].

Biofilm formation in diverse bacteria has been associated with enhanced survival under environmental stress, including challenge with antibiotics [8]. Furthermore, biofilm-grown microorganisms or products derived from them are able to induce a protective effect against infection. In mycobacteria, usefulness of revisiting this strategy has been recently suggested [9]. In BCG, the *BCG1419c* gene encodes for a protein with phosphodiesterase activity that is involved in degradation of the second messenger bis-(3′-5′)-cyclic dimeric GMP (c-di-GMP), a molecule linked to biofilm formation, differentiation and virulence of bacteria [10]. Deletion of the *BCG1419c* gene in *M. bovis* BCG Pasteur (BCGΔBCG1419c) increases its capacity for biofilm production, tolerance to nitrosative stress *in vitro* and increases its persistence in lungs and spleen of immunocompetent BALB/c mice, when administered intravenously, in comparison with parental BCG strain [11]. With this information in mind, we decided to determine the attenuation (safety), immunogenic and protective capacity of BCGΔBCG1419c in diverse murine models of TB infection. Our results show that this vaccine candidate is able to improve the control of chronic infection better that parental BCG.

2. Materials and methods

2.1. Bacterial strains and plasmids

BCG Pasteur 1173P2 (referred to as BCG Pasteur) was used as reference strain. Its isogenic derivative BCGΔBCG1419c has been described previously [11]. For challenge experiments in mice, the *M. tuberculosis* H37Rv strain was used.

2.2. Ethical statement

Local animal ethics committee approved all experiments, which were performed following Mexican guidelines regarding ethical and safe handling of experimental animals such as: NOM-07-SEMARNAT-SSA1-2002, NOM-033-ZOO-1995, and NOM-062-ZOO-1999.

2.3. Mycobacterial growth conditions

Mycobacteria were grown in Middlebrook 7H9 broth supplemented with 0.2% glycerol, 10% OADC, and 0.05% Tween 80 (Sigma-Aldrich, USA). Cultures were maintained in 75 cm^2 vented cap tissue culture flask at 37 °C, 100 rpm, in an orbital shaker for immunization experiments where challenge was followed, or at 37 °C, 5% CO_2, static, when response to PPD was assessed. After incubation, cells were prepared by washing with 7H9 medium, serially diluted and plated onto 7H10 agar supplemented with 0.5% glycerol and 10% OADC. Plates were incubated for 3 weeks and Colony Forming Units (CFUs) were determined. After one month of culture, Mtb H37Rv were harvested, washed one time with 7H9 and serially diluted to adjust to 2.5×10^5 bacteria in 100 μL, samples were aliquoted at −70 °C.

2.4. Comparison of immunogenicity with BCGΔBCG1419c and BCG Pasteur-vaccinated mice before challenge

Pathogen-free, female, 8–9 weeks old BALB/c mice were obtained from Harlan Laboratories (Mexico). Mice were maintained in cages with food and water *ad libitum*. Two groups of mice (n = 4) were immunized subcutaneously in the base of the tail with 1×10^6 Colony Forming Units (CFUs) of BCG strains suspended in 100 μL of saline solution. After priming, mice were maintained for 8 weeks; afterwards, they were euthanized by cervical dislocation. The spleens were removed in aseptic conditions and transferred to Petri dishes with 1 mL of DMEM (Dulbeccós Modified Eaglés Medium) (Invitrogen, USA). Spleens were perfused with 3 mL of DMEM, and splenocytes were recovered by 10 min centrifugation at 2000 rpm at room temperature (RT). Then, 3 mL of erythrocyte lysis buffer were added and incubated for 5 min at RT. Next, we added 5 mL of DMEM followed by centrifugation for 10 min at 2000 rpm at RT. Cells were suspended in 5 mL of DMEM supplemented with 10% FBS, 1% Non-essential amino acids, 1% sodium pyruvate, 1% penicillin-streptomycin, and 1% 2-mercaptoethanol. Cellular viability was checked using trypan blue dye. One million splenocytes were cultured in a 48-well plate with supplemented DMEM in the presence or absence of 10 μg Purified Protein Derivative (PPD) and incubated 48 h at 37 °C and 5% CO_2. Splenocytes were recovered after centrifugation at 2000 rpm for 10 min at RT. Cells were suspended in 100 μL cytometry buffer (PBS 1X and 1% FBS) containing anti-CD3 APC, anti-CD4 PE, anti-CD8 FITC fluorochrome-conjugated monoclonal antibodies (Biolegend, USA) and incubated for 30 min at 4 °C in the dark. After labeling, cells were suspended in 500 μL of cytometry buffer, then centrifuged at 2000 rpm for 10 min at RT. CytoFix/CytoPerm kit (BDBiosciences, USA) was used for permeabilization and labeling of the splenocytes with anti-IFN-γ PE/Cy7 (Biolegend, USA) according to manufacturer's instructions. After final washing and centrifugation, pellets of cells were suspended in 300 μL of PBS 1X and stored at 4 °C until acquisition in a Guava Via count cytometer (Merck Millipore, USA). At least 10,000 events were collected. Data were analyzed in the Incyte software (Merck Millipore, USA). Experiments were performed two times.

2.5. Infection of nude mice to determine safety of BCG vaccine candidate

Groups of 10 nu/nu mice were infected subcutaneously at the base of the tail with one dose of 8000 live BCG strains, and aliquots of the bacterial inoculum were plated to confirm the number of CFU administrated to the animals. Animals were left with water and food *ad libitum*, monitored for signs of disease, and comparative survival was determined.

2.6. Evaluation of protection against M. tuberculosis H37Rv in a model of progressive disease in BALB/c mice vaccinated with BCGΔBCG1419c or BCG Pasteur

The progressive infection model and assessment of changes due to *M. tuberculosis* H37Rv infection have been described previously, and is characterized by high pulmonary bacilli loads, widespread tissue damage (pneumonia) and high mortality after two months of infection [12]. In this work, two independent experiments were performed using 10 mice for each experimental group. Mice were vaccinated by inoculating BCG strains (8000 bacilli) subcutaneously at the base of the tail (except for the experiments were weight lost was determined, where 2500 CFU of BCGΔBCG1419c or 8000 CFU of parental BCG were used); the control group only received sterile saline. 2.5×10^5 CFU of *M. tuberculosis* H37Rv were administered intratracheally (i.t.) to each mouse.

Mice were weighted every two weeks, and after 4 and 6 months post-challenge, infected animals were euthanized. Animals were maintained in groups of five in cages fitted with microisolators

connected to negative pressure. All practices were performed in a biological safety cabinet in a biosafety level 3 facility.

2.7. Evaluation of protection against M. tuberculosis H37Rv reactivation in a model of chronic infection similar to latent tuberculosis in B6D2F1 mice vaccinated with BCGBΔCG1419c or BCG Pasteur

The chronic infection model used in this work has been described previously [22]. Briefly, two groups of 5–8-week-old female mice B6D2F1 (C57BL/6J × DBA/2J) were infected i.t. with 1000 viable Mtb H37Rv suspended in 100 μL of isotonic sterile endotoxin-free saline solution. After 5 months of infection, groups of five mice were vaccinated with BCG strains or saline solution in the control group. Two months later, corticosterone was administered in drinking water (3 mg/L) for 1 and 2 months in order to induce reactivation. Mice were euthanized under terminal anesthesia; the lungs were obtained and used to determine bacillary burdens and pneumonia by CFU quantification and automated histomorphometry, as described below.

2.8. Determination of pulmonary bacilli loads and tissue damage

The right lung was obtained from five mice at each time point, in two separate experiments. Immediately after the mouse euthanasia, the right lung was frozen by immersion in liquid nitrogen and kept at −70 °C until use. Lung homogenates were obtained with Polytron (Kinematica, Luzern, Switzerland) in isotonic saline, and four dilutions of each homogenate were spread onto duplicate plates containing Middlebrook 7H10 agar (Difco Labs, Detroit MI, USA) enriched with 10% OADC. Plates were incubated for 21 days to determine colony forming units (CFU) [12].

The left lung was fixed by intratracheal perfusion with 10% formaldehyde for 24 h, then sectioned through the hilus and embedded in paraffin. Sections, 5 μm thick, were stained with hematoxylin-eosin. The percentage of the pulmonary area affected by pneumonia was determined using an automated image analyzer (Q Win Leica, Milton Keynes) [13].

The percentages of lymphocytes or macrophages (the latter labeled for F4/80) that produced IFN-γ were determined in the pneumonic areas by immunohistochemistry and automated morphometry. The same paraffin embedded tissue used for conventional histology was used for immunohistochemistry. Sections 5 μm thick were deparaffinised, the endogenous peroxidase quenched and incubated with rabbit antibodies anti-IFNγ diluted 1/250 in PBS (Santa Cruz Biotechnology, Santa Cruz, CA), followed by incubation with goat anti-rabbit IgG labeled with peroxidase. After extensive washings with PBS, sections were stained with hematoxylin, mounted and examined. The number of IFNγ immunostained lymphocytes and macrophages were determined in 10 randomly chosen pneumonic areas in each lung by automated morphometry. Then, the percentage of IFNγ positive cells was obtained in each mouse comparing BCGΔBCG1419c and BCG Pasteur after challenge in both experimental TB models.

2.9. Statistical analysis

Data were expressed as mean ± error standard deviations of the mean. A two tailed-Mann-Whitney *T* test and a one-way ANOVA with a Tukey's multiple comparison *post hoc* test were performed using Graph Pad prism version 5.0 for OS X. The Log-rank test was used to determine differences in survival times.

3. Results

3.1. Evaluation of BCGΔBCG1419c virulence in athymic mice

We determined the safety of the BCGΔBCG1419c strain by infecting groups of nu/nu mice subcutaneously. A follow up was performed for 350 days and we found that the median survival time of both BCG Pasteur and BCGΔBCG1419c was 303 days, in contrast to BCG Phipps where the median survival time was 112 days (Fig. 1, p values 0.004 and <0.0001 in Phipps vs Pasteur, and Phipps vs BCGΔBCG1419c, respectively). Thus, BCGΔBCG1419c maintain its attenuated profile similarly to its parental strain, despite being able to persist at higher bacillary burden in immunocompetent mice [11].

3.2. Vaccination with Mycobacterium bovis Pasteur BCGΔBCG1419c increases the levels of activated T lymphocytes in mice

$CD4^+$ T and $CD8^+$ T lymphocytes obtained from BCGΔBCG1419c or BCG Pasteur vaccinated mice showed similar increases in total number of T cells in response to PPD (Fig. 2A-B). Intracellular expression of IFN-γ allowed us to determine that $CD4^+$ T and $CD8^+$ T lymphocytes obtained from the vaccinated group with BCGΔBCG1419c showed higher expression of IFN-γ in response to PPD in comparison to the BCG Pasteur vaccinated group (Fig. 2C-D). Thus, vaccination of mice with BCGΔBCG1419c increases the frequency of PPD specific-IFN-γ secreting T cells.

3.3. Vaccination with Mycobacterium bovis Pasteur BCGΔBCG1419c prevents weight loss, reduces bacterial loads and tissue damage in lungs of immunocompetent mice

Weight loss is a characteristic feature of human active TB and is also used as a humanitarian criterion to proceed to euthanasia of infected animals. All groups of mice showed a similar kinetics of gain and loss of weight until week 22, when the non-vaccinated group, decreased at least 2 g with respect to week 2. At week 24 and 28 the control group reduced significantly (Fig. 3, $p = 0.013$ and $p = 0.045$, respectively) its weight in comparison with BCG Pasteur and BCGΔBCG1419c vaccinated groups, which did not differ between them despite BCGΔBCG1419c-vaccinated mice received less bacteria. As there was no apparent reversion after 28 weeks post-infection, this was the last time point assessed because of ethical and humanitarian reasons [14,15].

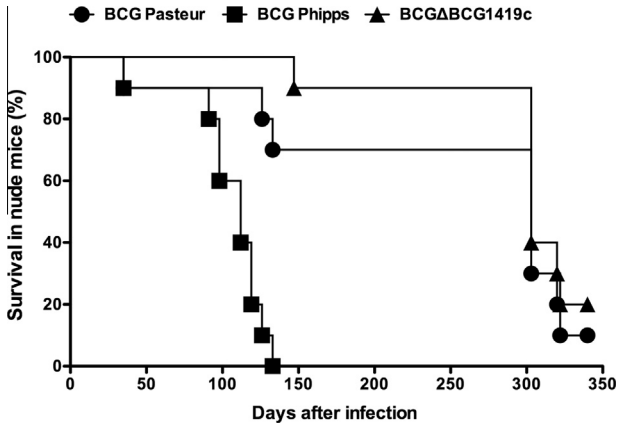

Fig. 1. Virulence of BCG strains in nude mice. Groups of nude mice were infected with BCGΔBCG1419c, BCG Pasteur, and BCG Phipps and maintained for 350 days. Median survivals were as follow; BCG Pasteur and BCGΔBCG1419c were 303 days and BCG Phipps 112 days. BCGΔBCG1419c vs BCG Phipps $p < 0.0001$ and BCG Pasteur vs BCG Phipps $p = 0.004$.

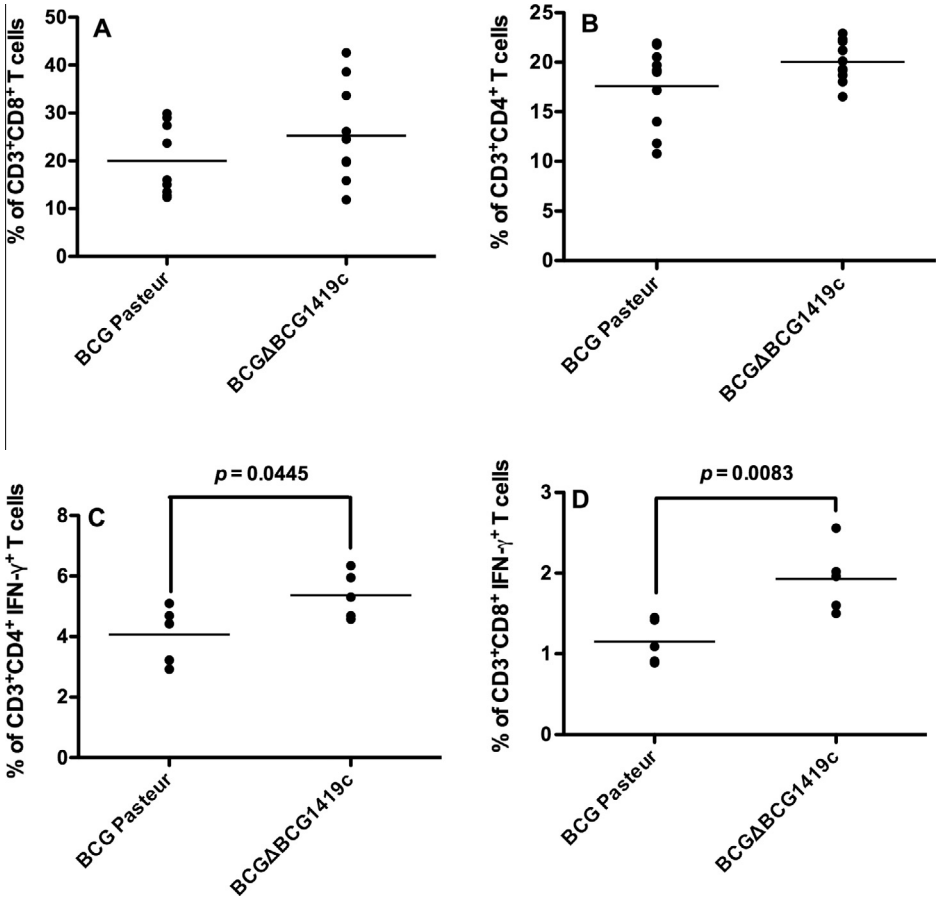

Fig. 2. Cellular immune response determination in BALB/c mice after vaccination with BCG strains. Groups of mice were vaccinated with BCGΔBCG1419c and BCG Pasteur and after 2 months were sacrificed. Splenocytes where stimulated with PPD for 48 h and analyzed by flow cytometry. (A) Percentage of $CD8^+$ T lymphocytes and in (B) percentage of $CD4^+$ T lymphocytes after stimulation. In (C) percentage of activated $CD4^+$ T-lymphocytes secreting IFN-γ and in (D) percentage of activated $CD8^+$ T-lymphocytes secreting IFN-γ.

Regarding lung bacillary burdens, in comparison with the control group, in average 0.65 and 0.55 $\log_{10}$ less bacteria were found in BCG and BCGΔBCG1419c-vaccinated mice respectively four months post-infection (Fig. 4A), dropping by 0.7 $\log_{10}$ ($p = 0.022$) and 0.9 $\log_{10}$ ($p = 0.001$) six months post-infection (Fig. 4E). Here, the difference between BCG Pasteur and BCGΔBCG1419c vaccinated groups was close to be statistically significant ($p = 0.060$).

In the model of progressive TB we used, a reduction in pneumonic area is a significant indicator of vaccine protection. At four months post-infection, compared to control mice, a 5% decrease in pneumonia was observed in BCG vaccinated mice, while a 20% reduction was seen in mice vaccinated with BCGΔBCG1419c (Fig. 4B, I-K). The 15% difference observed between BCGΔBCG1419c- and BCG-vaccinated mice was significant ($p = 0.048$), although it was not maintained after 6-month of infection (Fig. 4F).

On the other hand, after 4 months of infection, mice vaccinated with BCGΔBCG1419c showed significantly higher percentage of IFN-γ^+ T cells (mean 80%) than mice vaccinated with BCG (mean 58%, $p < 0.0014$) (Fig. 4C), with similar numbers at 6 months after challenge (Fig. 4G). BCGΔBCG1419c-vaccinated mice showed in average 6% less activated macrophages than mice immunized with parental BCG (Fig. 4D, $p = 0.017$) at 4 months post-infection. In both groups of mice, the percentages of activated macrophages were lower in comparison with saline control (BCGΔBCG1419c vs SS, $p = 0.0001$ and BCG Pasteur vs SS, $p = 0019$). At six-months post-infection, BCGΔBCG1419c-vaccinated animals maintained a lower proportion of activated macrophages in comparison to BCG-vaccinated mice. A 15% reduction in activated macrophages was seen in saline group at six-months post-infection in comparison with the experiment at fourth month post-infection (Fig. 4H). Together these results indicate that vaccination with BCGΔBCG1419c is more effective than using parental BCG in protecting mice from weight loss, bacterial loads and tissue damage in late phase of progressive pulmonary TB.

3.4. Vaccination with Mycobacterium bovis Pasteur BCGΔBCG1419c improves resistance to reactivation of Mycobacterium tuberculosis after mice immunosuppression

Next, we decided to determine the capacity of BCG strains to protect against reactivation from chronic infection post-immunosuppression with corticosterone. One-month post-immunosuppression, in average, 0.2 $\log_{10}$ less bacterial load was observed in the group vaccinated with BCG Pasteur in comparison with non-vaccinated animals. Interestingly, an almost 1-$\log_{10}$ reduction of bacterial load was observed in the group of mice vaccinated with BCGΔBCG1419c, which was significant (Fig. 5A, $p = 0.032$) in comparison with controls. After two months of immunosuppression (Fig. 5E), all mice showed bacterial loads increased by around 0.5 $\log_{10}$ in comparison with mice immunosuppressed for one month, where CFUs found in all BCG vaccinated animals (regardless of the strain) being lower in comparison with

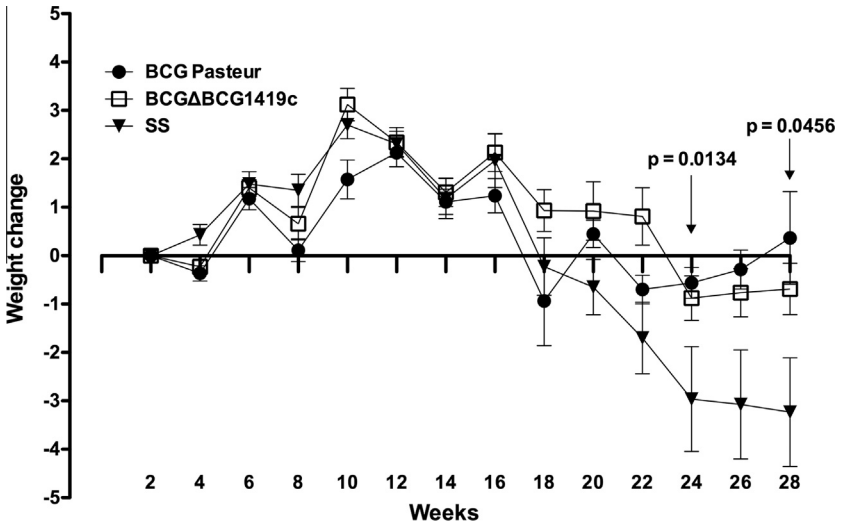

Fig. 3. Weight change in vaccinated mice after H37Rv challenge. Groups of BALB/c mice where vaccinated with BCGΔBCG1419c and BCG Pasteur, or received sterile saline (SS) as control, and challenged with *M. tuberculosis* H37Rv 60 days post-vaccination. Weight change was determined every two weeks for 6 months. Data represented as mean ± SEM.

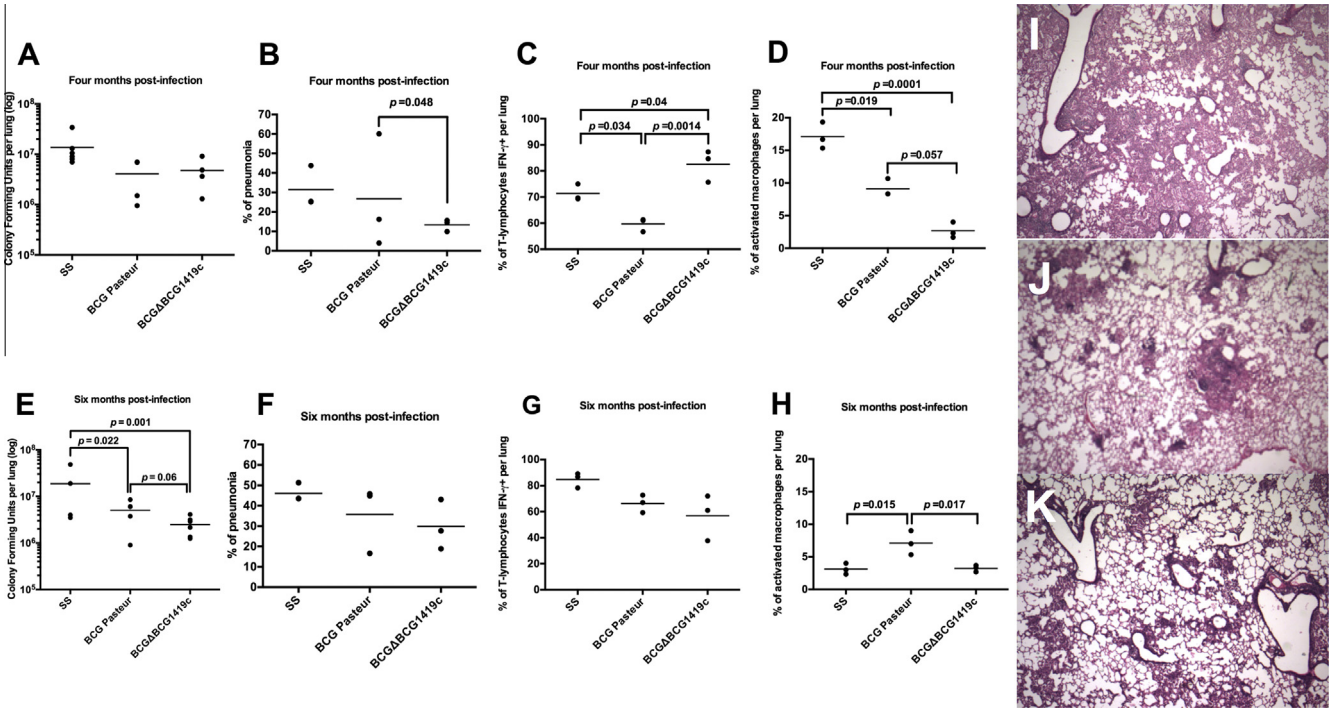

Fig. 4. Bacterial loads, pneumonia and immune cells present in lungs of vaccinated mice after *M. tuberculosis* H37Rv challenge. Groups of BALB/c mice where vaccinated with BCGΔBCG1419c and BCG Pasteur, or received sterile saline (SS) as control, and challenged with *M. tuberculosis* H37Rv 60 days post-vaccination. Experiment at four month post-challenge in (A) bacterial load, (B) percentage of pneumonic areas, (C) percentage of IFN-γ secreting lymphocytes and (D) percentage of activated macrophages in lungs. Experiment at six month post-challenge in (E) bacterial load, (F) percentage of pneumonic areas, (G) percentage of IFN-γ secreting lymphocytes and (H) Percentage of activated macrophages in lungs. Representative histopathology sections of pneumonic areas of (I) control, (J) BCG Pasteur, and (K) BCGΔBCG1419c immunized mice.

saline control (BCG vs saline, $p = 0.003$ and BCGΔBCG1419c vs saline, $p = 0.015$).

After one month of immunosuppression, we observed a 3% reduction in pneumonia of animals vaccinated with BCG, whereas a statistically significant 30% reduction ($p = 0.027$) was found for BCGΔBCG1419c-vaccinated mice compared to controls (Fig. 5B, I-K). The difference between BCGs-vaccinated groups was not significant ($p = 0.342$). After two months of immunosuppression (Fig. 5F), no difference was observed between groups.

IFNγ$^+$ lymphocytes numbers were also similar between all vaccinated groups (57–62%) (Fig. 5C and G), and after 2 months of corticosteroid treatment, they were significantly higher than those in control group ($p = 0.042$). After one-month of immunosuppression, BCGΔBCG1419c vaccinated mice showed 20% of activated macrophages in pneumonic areas, in comparison with only 3% in BCG vaccinated animals ($p = 0.026$) (Fig. D). After two-months of immunosuppression, all groups showed similar levels of activated macrophages.

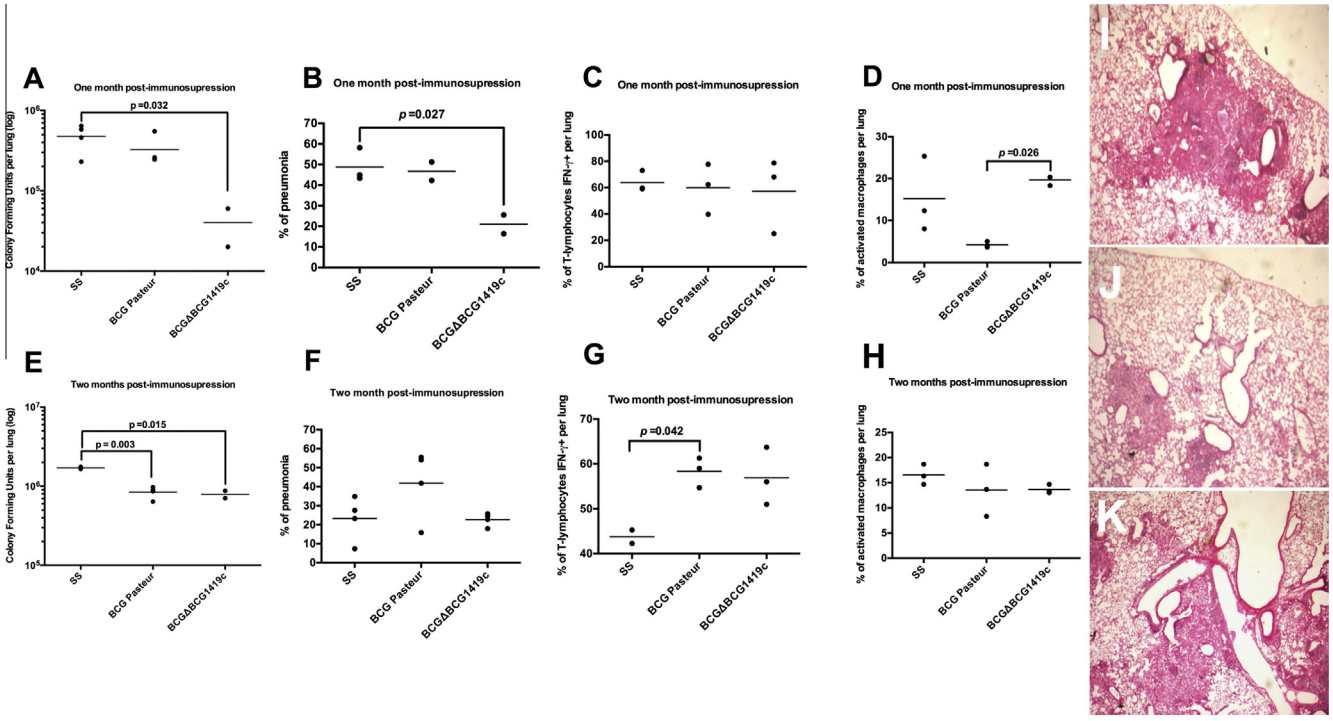

Fig. 5. Protection against *M. tuberculosis* H37Rv reactivation. Groups of B6D2F1 (C57/6J × DBA/2J) F1 were intratracheally infected with *M. tuberculosis* H37Rv. After 5 months were vaccinated with BCGΔBCG1419c and BCG Pasteur and saline solution as a control. 60-days later were administered with corticosterone daily for one month and two months. Experiment at one month of immunosuppression. In (A) bacterial load, (B) percentage of pneumonic areas, (C) percentage of IFN-γ secreting lymphocytes and (D) percentage of activated macrophages in lungs. Experiment at two month of immunosuppression. In (E) bacterial load, (F) percentage of pneumonic areas, (G) percentage of IFN-γ secreting lymphocytes and (H) percentage of activated macrophages in lungs. Representative histopathology sections of pneumonic areas of (I) control, (J) BCG Pasteur, and (K) BCGΔBCG1419c immunized mice.

4. Discussion

Considering no vaccine candidate aiming to control latent TB has been approved yet for application to humans, we decided to evaluate the BCGΔBCG1419c strain regarding its safety, immunogenicity, and protective efficacy in diverse mice models. In athymic nu/nu mice (Fig. 1), the median survival times for BCG Pasteur- and BCGΔBCG1419c-infected animals were identical (303 days), indicating that lacking of *BCG1419c* gene does not modify its virulence although it showed a better capacity to survive in immunocompetent mice [11]. In contrast, infection with BCG Phipps induced rapid death (median survival of 112 days); a result close to the virulence observed for this BCG strain in nude mice in another study [16].

Mice immunized with BCGΔBCG1419c increased the proportion of specific $CD4^+$ T and $CD8^+$ T-lymphocytes in response to PPD in comparison with the group vaccinated with the BCG parental strain, including induced production of IFN-γ by $CD4^+$ T and $CD8^+$ T-lymphocytes (Fig. 2). It has been shown that control of chronic infection in mice requires T cells [17]. In fact, depletion of $CD4^+$ T and/or $CD8^+$ T lymphocytes allowed Mtb reactivation in mice [18]. $CD4^+$ T lymphocytes have been proposed to be relevant during the early phase of infection [19] and, on the other hand, $CD8^+$ T lymphocytes would be more associated to control of latent TB infection by reducing the numbers of remaining infected macrophages [20]. Thus, it could be that BCGΔBCG1419c is abler to induce enhanced immune response than parental BCG Pasteur, at least to some extent by promoting a higher activation of $CD4^+$ Th-1 and $CD8^+$ T-cells in response to PPD.

To date, no vaccine candidate has proven to induce "sterilizing" immunity. Therefore, reduction of pulmonary bacterial loads in animal models is a surrogate marker of protection. After four months post-challenge with Mtb H37Rv, BCGΔBCG1419c vaccinated mice showed a reduction of bacterial load in lungs (Fig. 4A). This result was in agreement with prevention of weight loss (Fig. 3) and significant reduction of tissue damage (Fig. 4B, K), as well as the significantly higher percentage of IFN-γ^+ T lymphocytes (Fig. 4C) in the pulmonary inflammatory infiltrates, with a significantly lower percentage of IFN-γ^+ macrophages (Fig. 4D), which altogether indicate a better protection conferred by BCGΔBCG1419c than its parental strain during progressive disease. This might have resulted in the diminished Mtb bacillary burdens observed at 6-month post-challenge, which was close to statistical significance ($p = 0.06$) when compared to BCG Pasteur vaccinated group and statistically different from the control group (Fig. 4E).

Changes in numbers and types of immune cells surrounding and forming the granuloma are likely associated with balancing the immune response, in order to control infection [21]. Our vaccinated groups show features that are congruent with what is proposed to occur in an active granuloma [21] because of the high bacterial burden with classically activated macrophages and Th1 cells present (Fig. 4). It is worth noting that control of progressive infection seems to rely more in IFN-γ^+ T lymphocytes than in activated macrophages (Figs. 4C-D), whereas for the reactivation model occurs the opposite (Figs. 5C-D). Determining, in the future, additional contribution of local immune cells known to participate in control of TB (alternatively activated macrophages, neutrophils, Th17 cells, $CD8^+$ T cells, T reg cells as well as B lymphocytes) would better explain how improved control of infection conferred by BCGΔBCG1419c is attained.

Immunosuppression induced by several conditions, such as HIV infection, diabetes or malnutrition increases the risk of latent TB reactivation [22]. Using a model of reactivation from chronic infection upon corticosteroid administration, we found that BCGΔBCG1419c-vaccinated mice were significantly more efficient to control Mtb reactivation than BCG Pasteur (with a 1 $\log_{10}$ drop

in CFU) (Fig. 5). The best control was observed after 1 moth of immunosuppression, with significantly lower bacillary burden, less percentage of pneumonia and higher percentage of activated macrophages (Fig. 5A-B-D). A 1 $\log_{10}$ drop in CFU was obtained with the subunit vaccine antigen Rv1759c using the same murine model [23]. Therapeutic use of a subunit vaccine candidate comprising Ag85B-ESAT6-Rv2260c, after challenge with Mtb, showed a reduction of bacillary burden in a range from 0.8 to 1.6-$\log_{10}$ [24].

We are aware that caveats of the model we used are both the ethical and practical concerns of uncontrolled pro-inflammatory responses in human beings with latent tuberculosis infection (LTBI) that could receive a boost with complete BCG, although this was not the primary intended use of BCGΔBCG1419c, should this vaccine candidate demonstrate effective results in other models. We rather aimed in this study to show this strain improves protection in models resembling chronic/latent infection, in the mean time we determine specific differences that may lead to subunitary vaccine candidates or we develop and test a different model, more similar to natural history of latent TB. Nevertheless, such boosting with BCG could be useful as it has just been shown that in South African volunteers, it boosted memory NKT-like and NK cells, with preclearance of Mtb by isoniazid preventive treatment of LTBI people, having little effect on immune response elicited by revaccination [25]. We acknowledge that BCG have mostly been assessed for its capacity to protect against active TB, although it might also protect against *M. tuberculosis* infection and latent TB reactivation, by induction of innate immune memory [26] or other yet to be described mechanisms.

Improved control of TB in mice after BCGΔBCG1419c immunization could be the result of many factors, for instance, differential expression of antigenic components, a factor already shown to occur even among BCG substrains [27]. Alternatively, c-di-GMP by itself may play an important role in modifying response to BCGΔBCG1419c, given it is bound by stimulator of interferon genes (STING) [28], stimulates expression of MHC II molecules in dendritic cells, increases secretion of IL-12, IFN-γ, IL-8 and several chemokine receptors such as CXCR4, CCR, and CCR7 [29].

BCGΔBCG1419c produces more pellicle (a type of biofilm) than parental BCG [11]. Biofilm surfaces contain lipopolysaccharides, DNA, proteins, and sugar-derived molecules that may affect innate and adaptive immune cells responses. Mtb biofilms produced upon thiol-reductive stress [30] changed the transcription of some genes compared to planktonic cells, some of them documented to be immunogenic: 9 PE_PEGRS [31,32] genes upregulated and 2 downregulated; and genes required for mycolic acids synthesis and modification being downregulated (*acpM*, *kasB*, *mmaA4*, *accD6*) [33–36]. We hypothesize that some changes occurring to BCGΔBCG1419c compared to wild type BCG might be conserved during its passage within the host. Thus, BCGΔBCG1419c could be more immunogenic and show higher protection than BCG because it differentially expresses some immunogenic proteins, maintains higher levels of c-di-GMP and/or modifies molecules associated with biofilm formation, and experiments ongoing should shed light on these matters.

In summary, our results suggest that vaccination with BCGΔBCG1419c might be a useful strategy for latent/chronic/persistent infection with Mtb, and further suggest that local control of progressive disease differs with respect to cell types needed to reduce damages associated to reactivation from chronic infection. Additional research is needed to determine the mechanisms that results in improved control of infection in these models after administration of BCGΔBCG1419c, including the use of additional animal models as well as different infection and immunization routes, to confirm or disregard the inclusion of this strain into the TB vaccine candidates pipeline.

Conflict of interest

M.A.F.V., C.P.R., R.H.P., M.J.A.S have filed for patent about BCGΔBCG1419c as vaccine candidate against tuberculosis.

Author contributions

Conceived and designed the experiments: CPR, RHP, MAFV. Performed the experiments: CPR, CG, JBP, DME, MJAS, BMC. Analyzed the data: CPR, BMC, RHP, MAFV. Wrote the paper: CPR, MAFV.

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