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Raw starch microparticles as BCG adjuvant: Their efficacy depends on the virulence of the infection strains

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ABSTRACT

The persistence of tuberculosis (TB) as one of the top 10 causes of death worldwide, the growing incidence of multidrug-resistant tuberculosis and the controversial efficacy of the Bacille Calmette-Guérin (BCG) vaccine drives the development of new generation multistage vaccines against this disease that can boost BCG-primed immunity. The use of polymeric microparticles for this purpose increases due to their advantages, especially their good safety levels and intrinsic immunostimulant properties. We recently explored and demonstrated the reinforcing and adjuvant potential of starch microparticles (SMPs) that administered intranasally to BCG-primed BALB/c mice, alone or in combination with a recombinant antigen, increased survival rates and induced a reduction of bacterial load in the lungs of mice infected with tuberculosis. Here, we tested the effect of SMPs added to the BCG vaccine as adjuvant to the whole-cell vaccine and investigated their contribution to the improvement of the protective efficacy of subcutaneous vaccination in mice challenged with virulent strains of *Mycobacterium tuberculosis*. As expected, our results were dependent on the infection strains, showing that virulence is a crucial factor that affects the adjuvant activity of SMPs. Our results also confirm the adjuvant activity of this carbohydrate and its usefulness in diverse vaccination strategies not only for mucosal but also for parenteral administration.

1. Introduction

A safe, effective and affordable TB vaccine would represent a major advancement in the control of the disease. The highly variable protection conferred by the Bacilli Calmette Guérin (BCG) vaccine against pulmonary TB in adults, in addition to other factors, such as the exposure to saprophytic mycobacteria, the presence of other diseases such as diabetes and HIV coinfection, as well as the emergence of multidrug resistant strains of *M. tuberculosis*, are perhaps the most important reasons why it has been so difficult to control and eradicate the disease. In 2016, 1.3 million people died, and nearly 10 million people contracted the disease, with 490,000 cases of multidrug-resistant TB (MDR-TB) [1–3]. For this reason, developing a strategy that boosts the BCG vaccination and prevents active TB in adolescents and adults is a main goal that has directed research efforts. Several TB subunit vaccine (viral vectored or fusion protein-based vaccines) candidates have entered human clinical trials and have shown different levels of efficacy [4]. However, the challenge of the regulatory implications and safety concerns continues, in particular because even approved adjuvants can be implicated in postvaccination adverse reactions which are grouped as a new syndrome named ASIA (Autoimmune/inflammatory Syndrome Induced by Adjuvants) [5], and adjuvants used in new TB vaccines in the clinical phase of evaluation are not approved for human use. Such is the case of IC31®, CAF01 and the Adjuvant System AS01, which are three vaccine adjuvants that have demonstrated the ability to induce cellular immune responses and that comprise a two-component approach that combines particulate systems (i.e., liposomes) and appropriate immunomodulators (i.e., antibacterial peptides, synthetic oligodeoxynucleotides, saponins) [6–10].

As safer alternative candidates for the adjuvant and delivery of anti-TB vaccines, the use of polymeric microparticles, especially

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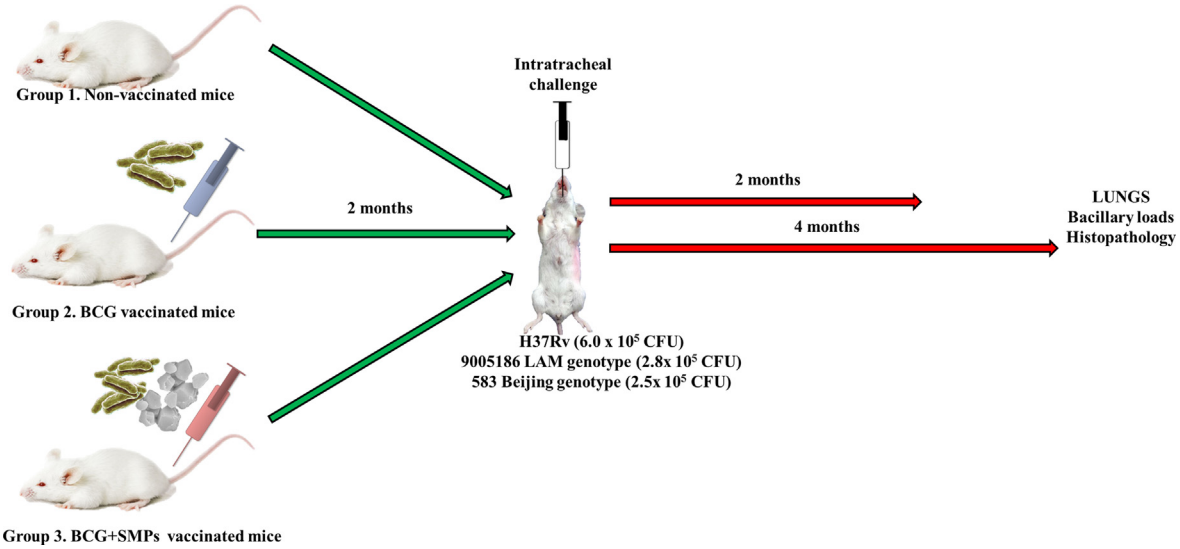

Fig. 1. Experimental strategy. Eighteen mice were randomly assigned to each group, of which six mice were left untouched for recording deaths after infection and construction of survival curves. Independent experiments were performed for each strain evaluated (54 total mice per strain per experiment).

polysaccharide polymers, has gained more attention [11–17]. These microparticles show greater stability and low toxicity and are biodegradable and biocompatible. These microparticles also have better cellular uptake by antigen presenting cells (APCs) and show immunostimulant properties, *in vitro* and *in vivo*, that can induce both protective and cell-mediated (CD4 and CD8) immune responses in animal models. Particularly, poly(lactide-co-glycolide) (PLGA) [11,12], chitosan and its derivatives [13,14], inulin [15,16]and starch [17]have been proposed as adjuvant and/or delivery systems in TB vaccination strategies, especially to enhance the protection against infection in mice acting as booster vaccines to improve BCG-primed immunity. Previously, we evaluated the reinforcing and adjuvant potential of raw starch microparticles (SMPs) in BCG-primed BALB/c mice. The intranasal administration of these unmodified particles, alone or in combination with a recombinant antigen, one month after BCG vaccination induced a significant reduction of bacterial loads in the lungs of mice, even more than in mice that only received BCG. Moreover, the microparticles did not contribute to the progress of pneumonia, which diminishes the safety concerns related to the administration of SMPs [17]. The questions that subsequently arose are whether the SMPs are able to reinforce the BCG vaccine and whether this carbohydrate of daily use can be an adjuvant for that complex vaccine with so many antigens. In this work, we report the results observed when administering BCG with SMPs in a murine model of tuberculosis challenged with *M. tuberculosis* (Mtb) strains of different levels of virulence.

2. Materials and methods

2.1. Ethics statement

This study was approved by the Institutional Animal Research Committee of the National Institute of Medical Science and Nutrition Salvador Zubirán (NIMSNSZ) (PAT-1834–16/19–1) in accordance with the guidelines of the Mexican national regulations on Animal Care and Experimentation, NOM 062-ZOO-1999.

2.2. Mice vaccination

Male BALB/c mice (6–8 weeks old; weighing 20–22 g) obtained from the Animal Facilities of the NIMSNSZ were used for subcutaneous (*s.c*) vaccination with BCG strain Phipps [18] combined with the SMPs. Groups of 6 mice were housed in cages (Allentown MicroVent units) fitted with microisolators (Allentown Air Flow System) connected to negative pressure. Conditions of temperature, humidity and light/dark cycles were maintained under control, and animals received food and water *ad libitum*. The BCG strain Phipps was harvested in the logarithmic phase ($O.D._{600} \cong 0.6$) and washed three times with phosphate buffer saline supplemented with 0.05% Tween 80 (PBS-5 T) (Sigma-Aldrich, St. Louis, MO, USA) to avoid bacterial aggregates. The morphology of the bacilli was corroborated by Ziehl-Neelsen staining, and CFU were titrated by plaque culture in Bacto Middlebrook 7H10 agar (Difco Labs, Detroit, MI) enriched with oleic acid, albumin, dextrose and catalase (OADC) (Difco Labs, Detroit, MI). Then, 8×10^3 CFU and 250 μg of the SMPs obtained from a prewashed raw starch suspension (100 mg/mL) were suspended in 100 μL of isotonic saline solution (ISS), mixed and inoculated *s.c.* at the base of the tail. No local adverse reactions were observed after injection of BCG + SMPs.

SMPs have an average size of 2.5 μm, with particles of angular and polygonal shapes. As determined by a proximate chemical analysis they are 97.6% carbohydrates (as nitrogen-free extract); 0.17% crude protein; 1.77% lipids (as ethereal extract) and 0.43% ashes in dry base.

2.3. Mice infection

Two months after *s.c.* vaccination, the mice were infected intratracheally (*i.t*) with one of 3 different strains of Mtb. The moderate virulent H37Rv strain (6×10^5 CFU) [19], the very high virulent Beijing strain (5 8 3) from South Africa (2.5×10^5 CFU) [20], and the hypervirulent isolate from the Latin American-Mediterranean (LAM) genotype 9,005,186 (2.8×10^5 CFU) [21]. The experimental strategy is shown in Fig. 1. Bacteria were harvested in the logarithmic phase ($O.D._{600} \cong 0.6$) washed three times with PBS-5T, and their microscopic morphology was corroborated by Ziehl-Neelsen staining. CFU titrated by plaque culture were suspended in a volume of 100 μL PBS. Sevoflurane-anesthetized animals were inoculated *i.t* and housed in groups of six in a microisolator system with constant flow and air filtration ensuring no exchange of air between cages or the external environment. The infection process was performed in a laminar flow cabinet in a Biosafety Level III facility. Two independent experiments for each strain were performed.

2.4. Sampling

Two and four months after infection, mice from each group were anesthetized with an intraperitoneal (*i.p.*) injection of sodium pentobarbital (210 mg/kg) and euthanized by exsanguination. The lungs were aseptically removed. The lungs were fixed by intratracheal perfusion with ethanol for histopathological analysis and kept at room temperature, while the lungs for CFU quantification were kept at −70 °C until analyzed. The sampling was also performed in a laminar flow cabinet in a Biosafety Level III facility.

2.5. CFU testing and histological analyses

Quantification of CFU in lung homogenates was performed to determine the levels of protection of BCG and BCG + SMPs vaccination. In sterile conditions, the right lungs from at least three mice per group were homogenized in 1 mL of PBS-5 T three to four times (4.5 m/s, 20 s) with a FastPrep®-24 homogenizer (M.P. Biomedicals). Four or five dilutions of each homogenate were dropped in duplicate in Bacto Middlebrook 7H10 agar + OADC (Difco Labs, Detroit, MI) plates and incubated for 21 days at 37 °C and 5% CO_2 to count the colonies and determine the total number of CFU per lung.

Fixed lungs were sectioned through the hilus and embedded in paraffin for histological-morphometric analysis. Tissue blocks stored at room temperature were cut into 4 μm thick sections using a microtome and stained with hematoxylin-eosin (H/E). The percentage of the pulmonary area (μm^2) affected by pneumonia was determined using an automated image analyzer Leica Qwin (Leica Microsystems Imaging Solutions ltd., Cambridge, UK, 25X).

2.6. Statistical analysis

Statistical analysis for survival curves was performed using Kaplan-Meier plots and log rank tests. CFU data and histology were analyzed using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, California, USA. Multigroup comparisons were performed by analysis of variance (ANOVA) with Tukey's posttest. The results are expressed as the mean ± SD. Differences were considered significant at $P < 0.05$.

3. Results

The efficacy of SMPs as adjuvant of the whole-cell BCG vaccine was evaluated by comparing the levels of protection conferred by BCG without SMPs in the control group. Two, four or six months after challenging with *M. tuberculosis* strains of different virulence, the level of protection was determined by comparing the survival between groups and assessing the bacillary load and the tissue damage (pneumonia) in the lungs.

3.1. Protection after challenge with M. Tuberculosis H37Rv

M. tuberculosis H37Rv, which is a moderately virulent strain, killed the nonvaccinated mice 90 days after infection, while BCG vaccinated mice survived until day 120, although they started to die at 95 days. Furthermore, animals that received BCG combined with SMPs as adjuvant were still alive 180 days after infection when the experiment stopped. The contribution of the SMPs to BCG vaccination regarding the survival of animals was statistically significant ($P < 0.001$) (Fig. 2A). In agreement with these results, the bacterial load was also significantly lower in these animals at

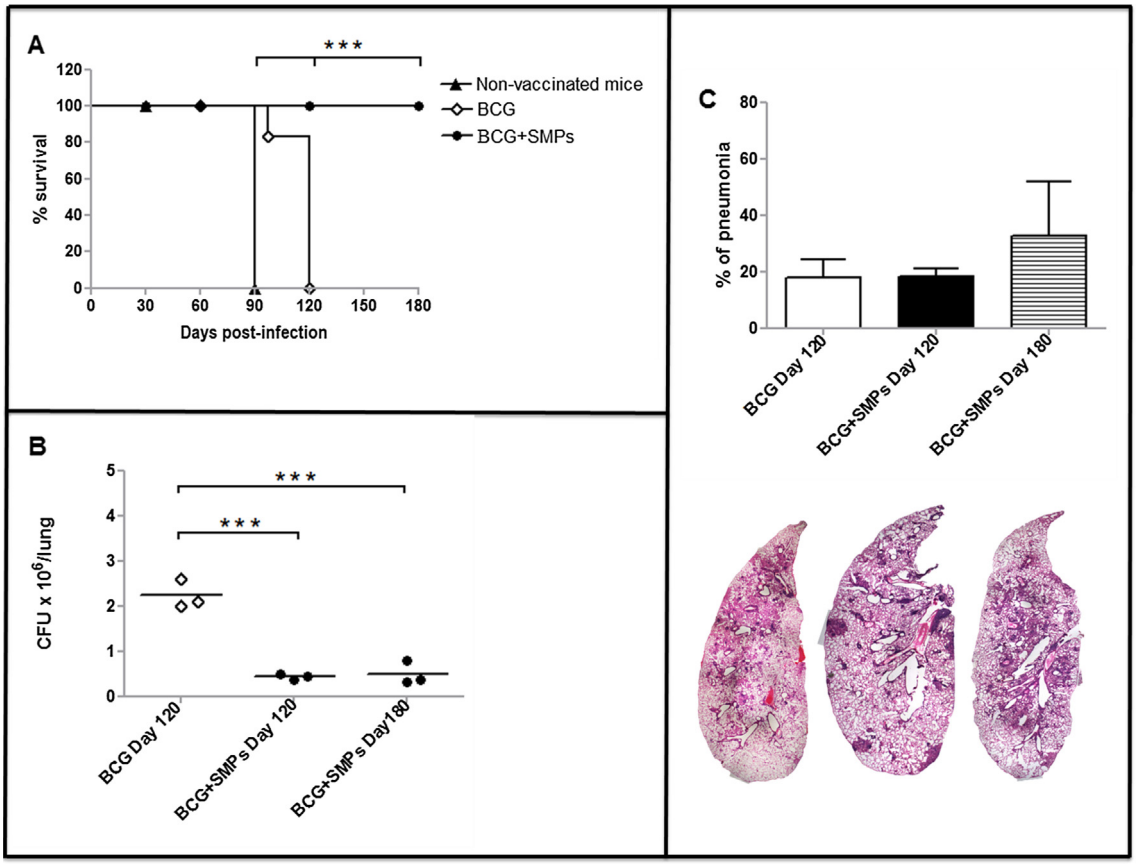

Fig. 2. Protection against *M. tuberculosis* H37Rv. (A) Survival. (B) Lung bacillary loads. (C) Percentage of lung surface affected by pneumonia and representative micrographs (H/E staining). The results are expressed as the mean ± SD (***$P < 0.001$).

four months and remained constant until six months postinfection (Fig. 2B). Concerning the tissue damage, at 120 days, the lungs of mice vaccinated with BCG and BCG + SMPs had the same percentage of the lung area affected by pneumonia. At 180 days, the pneumonia increased in the animals treated with BCG-SMPs; however, as there were no survivors of the mice only treated with BCG, it was not possible to establish any comparison. However, no significant differences were observed among the groups (Fig. 2C).

3.2. Protection after challenge with M. Tuberculosis 9,005,186 LAM and 583 Beijing

Highly virulent strains were also used to determine the protective efficacy of vaccination with BCG alone or with BCG + SMPs. *M. tuberculosis* 9,005,186 of the LAM genotype killed the nonvaccinated mice between 27 and 41 days ($P < 0.001$ compared with the vaccinated groups). In the group vaccinated with BCG, 60% of mice were still alive by day 84; however, the group only survived until day 97. No differences were observed among the survival rates of the vaccinated groups. Animals vaccinated with BCG + SMPs started to die at day 84 and survived until day 97 (Fig. 3A). Bacillary loads, determined two months after infection, were practically the same for both groups (Fig. 3B); the same was observed for tissue damage (Fig. 3C).

However, mice infected with *M. tuberculosis* 583 Beijing reacted differently. Nonvaccinated mice died between 41 and 51 days, while mice vaccinated with BCG lived for 90 days. In contrast, more than 90% of the mice that received BCG + SMPs were still alive by day 120, succumbing to the disease by day 158, which indicates that although BCG doubled the life expectancy when exposed to the *M. tuberculosis* 583 Beijing infection, BCG + SMPs tripled it (Fig. 4A). The bacterial load determined four months after infection in the latter group was lower than that in animals that only received BCG ($P < 0.05$) (Fig. 4B), although this had no impact on the progress of pneumonia for both vaccinated groups (Fig. 4C).

4. Discussion

In this study, we present the effect of starch microparticles as adjuvant to BCG vaccination against three strains of TB: *M. tuberculosis* H37Rv, which is a laboratory strain, and two clinical isolates (Beijing 583 and LAM 9005186); while most studies only show the effect on H37Rv [16], very few studies show results against clinical isolates [14]. In addition, it should be noted that a natural microdroplet infection usually contains a few bacilli, and, in this work, the mice were infected with 10^5 bacteria; thus, the effect of the microparticles as a boost or direct adjuvant is very important.

In previous works, we have provided evidence for the efficacy of SMPs not only as carrier for mucosal administration of antigens (Patent 347183) [22] but also as potential vaccine adjuvant that can be used alone or in combination with recombinant proteins to reinforce the BCG vaccination (patent pending Mx/a/2016/005434). The very desirable advantages of these microparticles, such as biocompatibility, biodegradability, the correct size to be engulfed by alveolar macrophages [23,24], as well as their immunostimulant properties that decrease bacterial loads and provide efficient protection against TB infection in mice [12], are important for the development of vaccines against infectious

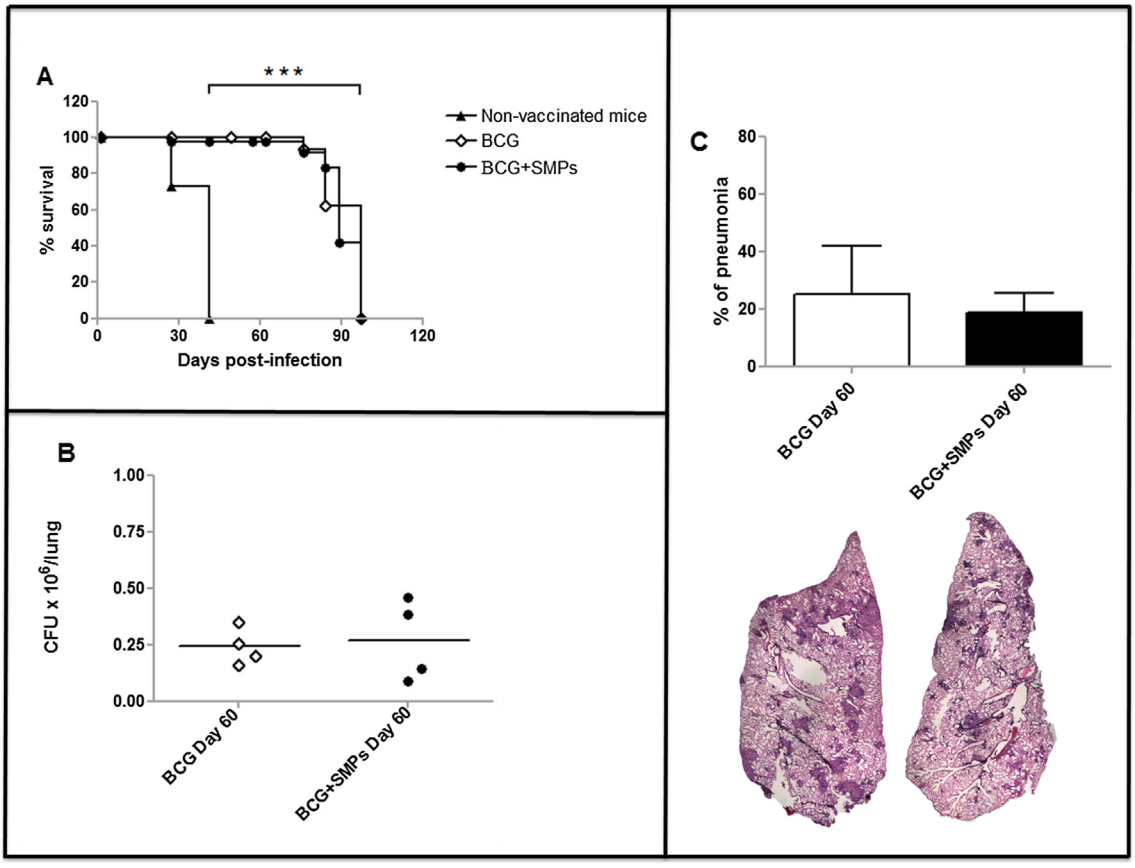

Fig. 3. Protection against *M. tuberculosis* 9,005,186 LAM. (A) Survival. (B) Lung bacillary loads. (C) Percentage of lung surface affected by pneumonia and representative micrographs (H/E staining). The results are expressed as the mean ± SD (***$P < 0.0001$).

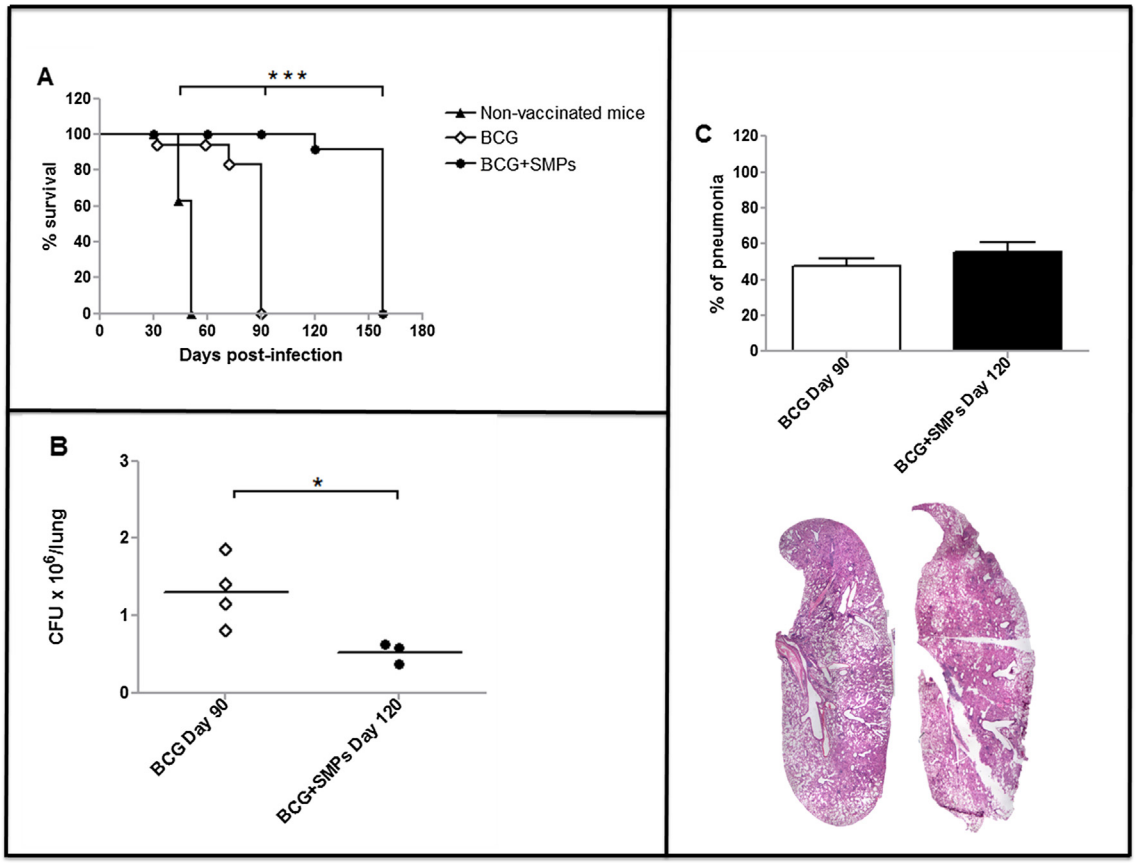

Fig. 4. Protection against *M. tuberculosis* 583 Beijing. (A) Survival. (B) Lung bacillary loads. (C) Percentage of lung surface affected by pneumonia and representative micrographs (H/E staining). The results are expressed as the mean ± SD (*$P < 0.05$).

diseases and particularly for new generations of TB vaccines or adjuvants that can boost BCG-primed immunity [25,26].

Factors such as human and mycobacterial genetics, exposure to environmental mycobacteria and socioeconomic and nutritional status, among other factors, affect the efficacy of this vaccine to confer sterilizing immunity to TB [27,28]; consequently, finding an appropriate substitute has been very difficult. Additionally, despite the variable protection conferred with BCG, the components of its wall are essential for an adequate stimulation of the immune system that contributes to the protection [27]. Accordingly, finding adjuvants that can increase the immunity and memory T cells activated with BCG is important, and microparticles appear to be a good alternative derived from the fact they can also interact with cells of the innate immune system, such as macrophages, can induce their activation [8,25] and can even influence the distribution and persistence of BCG postvaccination as well as modify the frequencies of CD4 + and CD8 + T cells expressing IFN-γ and IFN- γ/TNF-α in mice [29].

Here, animals vaccinated with BCG + SMPs did not die from infection with *M. tuberculosis* H37Rv and at least tripled their survival against infection with *M. tuberculosis* 583 Beijing. In both experiments, a significant decrease in the bacillary load in the lung was observed, which is very interesting because some experiments suggest that the BCG-induced immunological defense may protect against most *M. tuberculosis* strains but not against *M. tuberculosis* Beijing isolates [30]. It is even suggested that BCG vaccination may favor the selection of Beijing strains that resist BCG-induced immunity [31], which, in turn, is very dangerous for the population because some studies suggest that these strains may have a particular propensity for acquiring drug resistance [32]. Our results indicate a contribution of SMPs to the protective response of BCG against the Beijing strain or at least a contribution counteracting the pathology induced by these bacteria, which have been proven to drive the immune response towards nonprotective mechanisms due to a rapid but inefficient activation of macrophages favoring massive tissue damage and early mortality [30]. We can understand this effect from the microparticles if we consider microparticles as inductors of phagocytosis and the phagocytosis as a prerequisite for the activation of macrophages with the consequent induction of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-12; or on the other hand, the induction of respiratory burst and reactive nitrogen intermediates that subsequently favor the cell-mediated immune response and an adequate elimination of the bacilli. For example, it has been reported that macrophages of mouse or human origin that are infected with Mtb and exposed to polymeric microparticles induced macrophage activation and bactericidal profiles; thus, stimulating the phagocytes is a good alternative that the authors recommended to increase the bacilli elimination [11].

By contrast, we did not observe additional effects of adding microparticles to BCG in the case of mice infected with the 9,005,186 LAM strain. This result is contrary to that obtained in our previous study where the subcutaneous vaccination with BCG was boosted with the nasal administration of SMPs. In that case, the mice infected with 9,005,186 LAM and not with 583 Beijing were protected better [17]. These differences can be explained in terms of the route and scheme of administration of the vaccine and microparticles. Cells participating in the recognition, uptake, processing and presentation of BCG and in the recognition and uptake of SMPs are different depending on the location. If they are resident epidermal, nasal mucosal or alveolar macrophages, and their different polymorphisms in the pattern-recognition

receptors (PRR) may be an important factor that contributes to the induced response as well as contributes to the susceptibility to TB or to the effectiveness of BCG [33]. As observed for other particulate vaccine-adjuvants, the effect of the SMPs can also be the result of their interaction with these cells depending on the route of administration [34] and, of course, can be affected by the immune response induced by the strain of infection. The wide variation in the immune response to infection with different strains, especially in terms of pro-inflammatory cytokines production [35] is also the factor within the Mtb complex that explains the variable protection of BCG against hypervirulent isolates [24]; therefore, the immune response dependent on administration route becomes one of the most important factors that must be considered for the rational design of new vaccines and vaccine-adjuvants for tuberculosis. Especially in particulate adjuvants with intrinsic properties of size, charge and shape that also determine their interaction with the immune system and determine their safety and tolerability. Chitosan and its derivatives, for example, have shown important qualities as a system of administration/adjuvant for anti-tuberculosis vaccines, however some authors have observed some limitations to be solved in chitosan nanoparticles, such as the reduction of the positive charge in the surface in order to reduce their toxicity without affecting immune responses, and also the size (<100 nm) in order to diminish the risk of evasion of the reticuloendothelial system [13].

Although the general mechanism of action of SMPs and their adjuvant potential with other immunostimulant or antigenic molecules is not known, we want to emphasize that, as far as we know, there are no reports of adjuvants made of natural polymers that demonstrate the immunostimulating properties that SMPs have shown. Other reported nano or microparticulated systems have obtained a similar response to the SMPs, increasing the protective efficacy of BCG vaccine against infection with hypervirulent *M. tuberculosis* strains, when they act in combination with immune activators such as CpG oligonucleotides, monophosphoryl lipid A (MPL) or polyinosinic: polycytidylic acid (poly I: C) [13,14,16].

5. Conclusions

In the present study, we used SMPs simultaneously as adjuvant of the BCG vaccine administered by *s.c.* route, which implies the first time we used these particles via a parenteral route and accompanied by a whole-cell vaccine. We observed an improvement in the protective efficacy of the BCG vaccine with the added particles in the animals infected with *M. tuberculosis* H37Rv and 583 Beijing but not with 9,005,186 LAM. These observations imply that the level of virulence of infection strains is a crucial factor that affects the adjuvant activity of SMPs and is one of the most important factors affecting the immune response to hypervirulent strains and the protection conferred by BCG. Although the effects and response generated must be revised further, our results confirm the suitability and potential of this carbohydrate adjuvant to combine in diverse vaccination strategies not only for mucosal but also for parenteral administration.

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Conflict of interest statement

There is no conflict of interest among authors.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.04.027.

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