

# Pulmonary *Mycobacterium bovis* BCG Vaccination Confers Dose-Dependent Superior Protection Compared to That of Subcutaneous Vaccination

Nacho Aguilo,<sup>a,b</sup> Ana Maria Toledo,<sup>c</sup> Eva Maria Lopez-Roman,<sup>c</sup> Esther Perez-Herran,<sup>c</sup> Eamonn Gormley,<sup>d</sup> Joaquin Rullas-Trincado,<sup>c</sup> Iñigo Angulo-Barturen,<sup>c</sup> Carlos Martin<sup>a,b,e</sup>

Grupo de Genética de Micobacterias, Departamento de Microbiología, Medicina Preventiva y Salud Pública, Faculty of Medicine, Universidad de Zaragoza, Zaragoza, Spain<sup>a</sup>; CIBER Enfermedades Respiratorias, Instituto de Salud Carlos III, Madrid, Spain<sup>b</sup>; Diseases of the Developing World, GlaxoSmithKline, Tres Cantos, Madrid, Spain<sup>c</sup>; School of Veterinary Medicine, University College Dublin (UCD), Dublin, Ireland<sup>d</sup>; Servicio de Microbiología, Hospital Universitario Miguel Servet, IIS Aragón, Zaragoza, Spain<sup>e</sup>

**Worldwide, the *Mycobacterium bovis* BCG vaccine is one of the most widely used vaccines. However, it appears to be ineffective in preventing pulmonary tuberculosis. Here, we show that pulmonary BCG vaccination of mice with a broad dose range provides superior protection against *Mycobacterium tuberculosis* challenge compared to that of subcutaneous vaccination.**

Vaccine delivery through the natural route of infection has been pursued as a strategy to improve protective efficacy. In the case of tuberculosis (TB), a pulmonary disease, *Mycobacterium bovis* BCG is the only vaccine available and is administered by the intradermal route globally. Even though BCG is reported to confer protection against severe disseminated forms of TB disease, the level of protection conferred against pulmonary TB is variable, limited, and short-lived (1). As a result, alternative vaccine candidates and strategies are being explored (2). Recently, a novel candidate vaccine based on the viral vector MVA85A, which is designed to work as a BCG prime-MVA85A boost strategy, was found to have no statistically significant impact on vaccine efficacy in a clinical trial involving BCG-vaccinated infants (3). A failure to generate long-lasting immunological memory in the lungs has been proposed as a possible cause for the poor levels of protective immunity induced by BCG vaccination (4). The particular vaccine delivery route may also influence the levels of immunity, and there are preclinical data suggesting that aerogenic delivery of BCG (5–7) or other TB vaccine candidates (8, 9) can improve vaccine efficacy compared to that of intradermal or subcutaneous (s.c.) delivery methods. Other studies have demonstrated that s.c. BCG vaccination protects uniformly against experimental challenge when delivered across a wide range of doses, both in murine and guinea pig models (10). However, relatively few studies have been conducted comparing different doses and delivery routes of live BCG (11).

Based on the hypothesis that BCG delivered parenterally induces weak immune responses in the lungs and leads to poor protection, we compared the protective efficacy conferred by intratracheal (i.t.) and s.c. BCG strain Danish 1331 vaccination of mice against a low-dose (100 CFU) *Mycobacterium tuberculosis* strain H37Rv i.t. challenge. A broad range of doses of the BCG vaccine (from 10<sup>1</sup> to 10<sup>7</sup> CFU) was tested to study the dose-response effect for each route of administration. The s.c. route was selected, as it is routinely used in vaccine studies using a mouse model, and the i.t. route was used because it is the only respiratory route that allows for the accurate delivery of defined vaccine doses in the range used here. The data obtained from s.c. vaccination confirmed a lack of association between vaccine dose and protection, with approximately a 1-log reduction in *M. tuberculosis*

growth in the lungs in all groups of mice vaccinated with BCG subcutaneously, compared to that of nonvaccinated controls, apart from the lowest vaccine dose group (10<sup>1</sup> CFU) (Fig. 1A). In contrast, a strong dose-response effect was observed in mice vaccinated by the i.t. route. The 10<sup>7</sup> BCG CFU vaccination by the i.t. route reduced the pulmonary bacterial counts up to 3 logs compared with the nonvaccinated group (Fig. 1B). In previous studies that have shown no differences in terms of protection between the s.c. and aerosol routes of BCG vaccination, the BCG dose used was 10<sup>3</sup> CFU (12). Consistent with this, we also failed to see a statistically significant difference between the s.c. and i.t. delivery routes using 10<sup>3</sup> CFU of BCG (Fig. 1A).

Previous studies in mice have also shown that aerosol vaccination with the fast-growing mycobacterial strain *Mycobacterium* resulted in higher specific immune responses in the spleen compared to that with s.c. vaccination (9). BCG aerosol vaccination of guinea pigs has also shown similar results (13). In this study, we used flow cytometry with lung and spleen samples to measure the proportion of gamma interferon (IFN- $\gamma$ )-producing CD4<sup>+</sup> T cells induced by the s.c. and i.t. vaccination routes after *ex vivo* overnight stimulation with tuberculin purified protein derivative (PPD) (10  $\mu$ g) and antigen 85A (Ag85A) or Ag85B (2  $\mu$ g). Intratracheal BCG vaccination resulted in higher immune responses in the spleen at all vaccine doses (Fig. 2A), suggesting that this route induces a more effective systemic immunity than that of the s.c. route. The data obtained in lungs also indicate a higher specific immunity in mice vaccinated i.t. with all BCG doses with each of the three antigens. Compared with the i.t. route, the response in the lungs induced by s.c. vaccination was very low at the three BCG doses tested (Fig. 2B). We noted a lower percentage of IFN-

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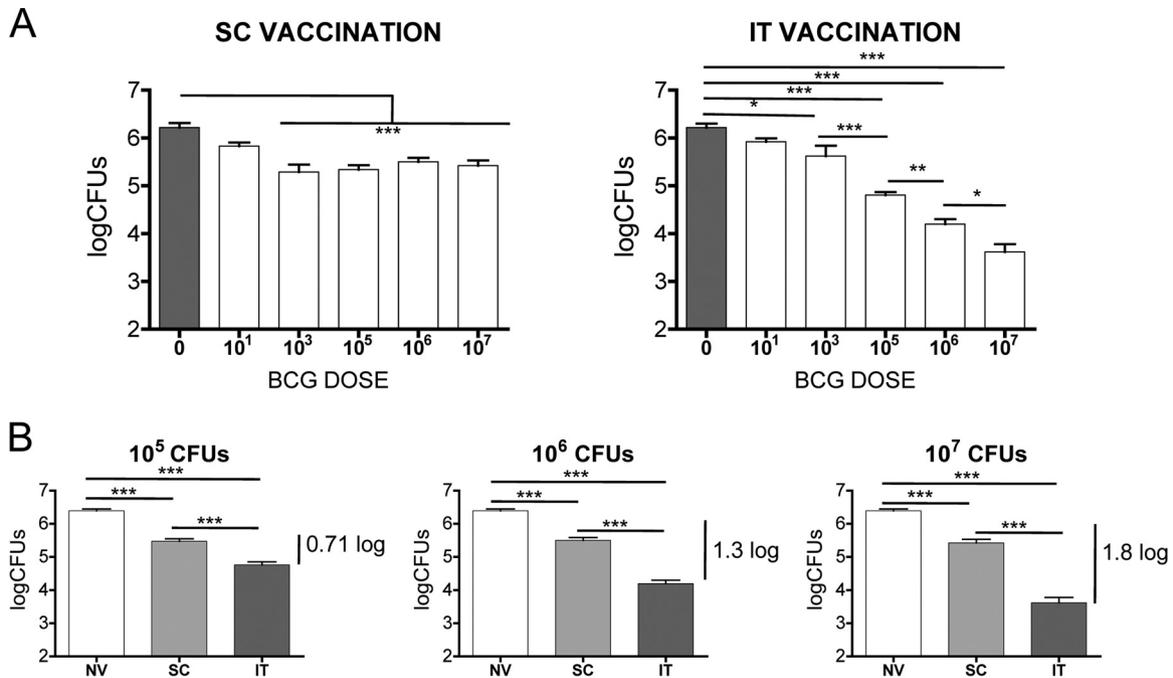
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Address correspondence to Carlos Martin, carlos@unizar.es.

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**FIG 1** Protection in the lung comparing subcutaneous and intratracheal BCG vaccination. Groups of five to eight C57/BL6 mice were nonvaccinated (NV) or vaccinated once via the s.c. or i.t. route with BCG Danish 1331 (SSI Denmark) vaccine using the doses indicated in the figure. Two months postvaccination, the mice were challenged i.t. with low-dose (100 CFU) *M. tuberculosis* H37Rv challenge, and 1 month later, the bacterial burden in the lungs was determined. (A) Data compared by vaccine dose. (B) Data compared by route of administration. Data are represented as mean  $\pm$  SD. Statistical analysis was carried out using one-way analysis of variance (ANOVA) and Bonferroni's posttest. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

$\gamma$ -producing cells in the lungs from mice inoculated i.t. with the highest BCG dose ( $10^7$  CFU) than mice vaccinated with  $10^5$  CFU BCG, despite the higher level of protection observed in the high-BCG-dose group. However, the immune responses were only measured at a single time point. A time course study of Th1- and Th2-type cytokine responses would be required to investigate whether there is a BCG dose-responsive kinetic profile associated with the level of protection (14).

Our results demonstrate that the immune protection generated by s.c. BCG delivery appears to occur independently of the range of BCG doses tested. A possible explanation is that delivery of BCG by the s.c. route results in less efficient migration of bacilli to draining lymph nodes than does the i.t. route, with which the bacilli will rapidly encounter alveolar macrophages and other primed antigen-presenting cells. This may impact the strength of the induced immune responses. Another study comparing intranasal and s.c. routes of vaccination in mice demonstrated a dose-dependent response in the levels of protection generated by both routes, albeit over a narrower range of delivered doses (11). The reasons for the discrepancies in the results of these studies are unclear, though it is noted that the earlier study used the BCG Pasteur strain and measured vaccine efficacy in the BALB/c mouse strain model, whereas the current study was conducted with the BCG Danish strain using C57/BL6 mice. Elsewhere, it has been shown that BALB/c mice are relatively susceptible to primary pulmonary BCG infection compared with C57BL/6 mice (14).

Consistent with our results, studies have also demonstrated that i.t. BCG vaccination of mice induces a higher accumulation of IFN- $\gamma$ -producing cells in the lungs (15, 16) than in those vaccinated by the s.c. route, which is associated with better protection.

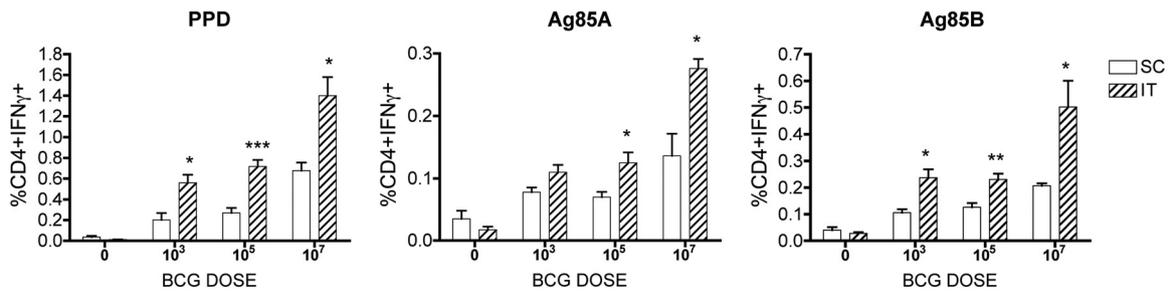
In our study, even though a clear dose-response protection effect was measured by the i.t. BCG vaccination at 4 weeks postchallenge, we acknowledge that this effect might be an artifact of the time interval between the vaccination and challenge, as well as the duration of challenge. As described by Gruppo and Orme (10), the dose-response effects observed with a short BCG vaccination-to-challenge interval are not evident when the postvaccination time to challenge is extended.

In our study, we used a broader range of inoculum doses than that in the study conducted by Tree et al. (11). Although i.t. vaccination is not clinically applicable for practical reasons, it is useful in experimental animal models, as it allows for a more accurate delivery of defined doses than with intranasal or aerosol vaccination.

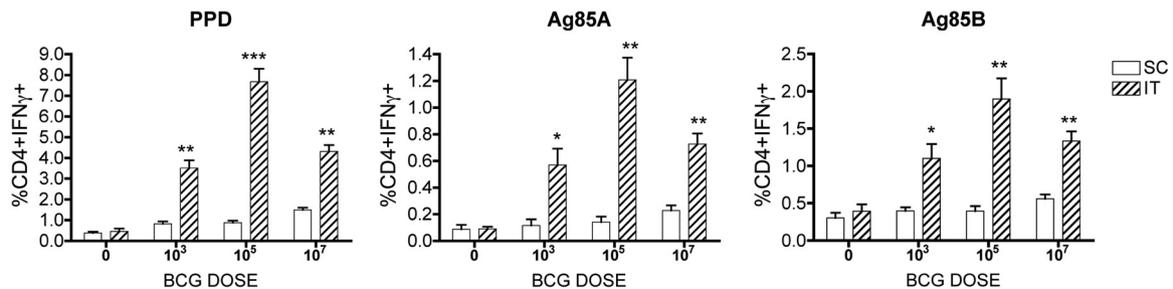
Studying the safety of the pulmonary route for vaccination was beyond the scope of this study and requires further knowledge on dose response and dissemination from the initial site of inoculum deposition. However, safety studies of aerosolized BCG (either  $3 \times 10^6$  or  $3 \times 10^7$  CFU per treatment) have been conducted in patients with metastatic lung cancer (17). These patients exhibited a transient and characteristic symptom complex beginning with chills 4 to 8 h posttreatment. However, all patients were symptom free within 24 to 36 h after treatment. This syndrome did not occur in other patients who received intradermal or oral BCG only. The study concluded that the aerosol route of administration of BCG was feasible and well tolerated in patients. For BCG delivery by the pulmonary route, the most effective dose might be the highest possible that generates protective immunity while being balanced to minimize the risk of severe adverse reactions.

Historically, the rationale for parenteral BCG administration

## A. SPLEEN



## B. LUNG



**FIG 2** Immunogenicity conferred by BCG vaccination. Groups of five C57/BL6 mice were nonvaccinated (NV) or vaccinated once via the s.c. or i.t. route with BCG Danish 1331 vaccine using the doses indicated in the figure. Two months postvaccination, cellular suspensions of spleen (A) and lungs (B) were obtained and stimulated overnight with tuberculin PPD (10  $\mu$ g), Ag85A (2  $\mu$ g), or Ag85B (2  $\mu$ g). IFN- $\gamma$ -producing CD4<sup>+</sup> cells were analyzed by flow cytometry. Data are represented as mean  $\pm$  SD. Statistical analysis was done using an unpaired two-tailed *t* test. \*, *P* < 0.05; \*\*, *P* < 0.01; \*\*\*, *P* < 0.001.

has been that vaccinated subjects showed rapid and consistent positive conversion for the tuberculin skin test (TST) (18). According to WHO guidelines, the TST is not a useful measure of BCG vaccine potency, as TST positive conversion is largely irrelevant from the perspective of vaccine protective efficacy (19). In addition, preclinical data demonstrate that guinea pigs vaccinated with BCG by the aerosol route show positive sensitivity to PPD inoculation, and pulmonary vaccination with 10<sup>3</sup> CFU of BCG has been shown to induce elevated local cellular immune responses and higher levels of protective immunity than that with intradermal vaccination (13). Pulmonary vaccination poses several advantages over other vaccination routes for tuberculosis, because as the natural route of vaccination for the respiratory route of infection, it may enhance local and systemic immunity.

Although the search for new and more efficient TB vaccines continues, the emerging information suggests that the development and testing of novel vaccines should include clinical trials to study the efficacy of BCG and new vaccine candidates in the context of different aerogenic routes of delivery. Understanding all of the factors that influence and determine protective immunity might expedite the time required to license next-generation vaccines.

All animal studies were ethically reviewed and carried out in accordance with European Directive 2010/63/EU and the Glaxo-SmithKline (GSK) Policy on the Care, Welfare, and Treatment of Animals.

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