Variable Virulence and Efficacy of BCG Vaccine Strains in Mice and Correlation With Genome Polymorphisms

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Bacille Calmette–Guérin (BCG), an attenuated strain of Mycobacterium bovis, is the only vaccine available for tuberculosis (TB) control. However, BCG is not an ideal vaccine and has two major limitations: BCG exhibits highly variable effectiveness against the development of TB both in pediatric and adult populations and can cause disseminated BCG disease in immunocompromised individuals. BCG comprises a number of substrains that are genetically distinct. Whether and how these genetic differences affect BCG efficacy remains largely unknown. In this study, we performed comparative analyses of the virulence and efficacy of 13 BCG strains, representing different genetic lineages, in SCID and BALB/c mice. Our results show that BCG strains of the DU2 group IV (BCG-Phipps, BCG-Frappier, BCG-Pasteur, and BCG-Tice) exhibit the highest levels of virulence, and BCG strains of the DU2 group II (BCG-Sweden, BCG-Birkhaug) are among the least virulent group. These distinct levels of virulence may be explained by strain-specific duplications and deletions of genomic DNA. There appears to be a general trend that more virulent BCG strains are also more effective in protection against Mycobacterium tuberculosis challenge. Our findings have important implications for current BCG vaccine programs and for future TB vaccine development.

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INTRODUCTION

Tuberculosis (TB) remains one of the world’s deadliest infectious diseases, causing 1.5 million deaths and 8–10 million new infections annually. Bacille Calmette–Guérin (BCG) is currently the only vaccine used for immunoprophylaxis of TB. BCG was included in the WHO Expanded Program on Immunization in 1974 and more than 4 billion people have been immunized with BCG. Over 90% of children worldwide are vaccinated with BCG and >120 million doses of BCG are administered annually, making it the world’s most widely used vaccine. Clinical studies have confirmed that BCG protects against disseminated TB including meningitis and miliary TB in children.1,2 However, in nonendemic countries, BCG vaccination is not performed due to the variable effectiveness (ranging from 0 to 80%) in preventing pulmonary TB in adults and the relatively low incidence of disease in these regions.3 Another issue concerning BCG is its safety. Although BCG is generally considered very safe, there is a substantially higher risk of disseminated BCG disease in children with primary immune deficiencies or HIV infection, which apparently outweighs the potential benefit of TB prevention.4 As a result, the current WHO policy recommends that children who are known to be HIV infected, even if asymptomatic, should not be immunized with BCG.4

BCG was derived from a virulent strain of Mycobacterium bovis through in vitro attenuation (230 times passaging) from 1908 to 1921. Beginning in 1924, BCG was distributed to multiple countries worldwide, leading to its diversification into a number of genetically distinct substrains.5 Comparative genome analyses using a variety of techniques (including subtractive hybridization, spotted oligonucleotide arrays, microarray based resequencing, and whole genome sequencing) have uncovered numerous large sequence polymorphisms including deletions and duplications, as well as single nucleotide polymorphisms among BCG strains.6–14 Based on these studies, BCG strains can be separated into several different groups. For example, a tandem duplication-2 (DU2) occurs in all BCG strains examined so far, but DU2 exists in four different forms (Table 1). Consequently, BCG strains are divided into four major groups based on the DU2 forms: group I (BCG-Russia, BCG-Japan, and BCG-Moreau), II (BCG-Sweden and BCG-Birkhaug), III (BCG-Danish, BCG-Prague, BCG- Glaxo, and BCG-China), and IV (BCG-Phipps, BCG-Tice, BCG-Frappier, and BCG-Pasteur) (Table 1).6–10 The genetic clustering of BCG strains is generally consistent with the historical records of BCG dissemination. For example, BCG strains of DU2 groups I and II were disseminated before 1927 (the early strains) and the strains
of groups III and IV were distributed after 1927 (the late strains). Groups III and IV strains also exhibit a deletion in the Region of Difference-2 (RD2) (Table 1).7

It is unequivocal that BCG has evolved over time, but whether this matters in terms of BCG effectiveness against TB is being debated.15,16 Reviews of clinical trials led to a conclusion that the variation in BCG strains is not a significant determinant of overall efficacy.17,18 However, these analyses were based on very limited data available from human studies.17,18 Considering the vast number of publications on BCG, clinical and animal studies directly comparing the effectiveness of different BCG strains have been remarkably scarce (reviewed in ref. 19). Given the recent delineation of genetic differences between BCG strains, a head-to-head comparison of BCG strains of genetic diversity is of great interest. In this study, we compared the virulence and efficacy of 13 BCG strains, representing all genetic lineages, in SCID and BALB/c mice, respectively.

RESULTS

Evaluation of virulence of BCG strains in SCID mice

SCID mice, which lack T- and B lymphocytes and are highly immunocompromised, are the reference model for the safety test of live vaccines including recombinant BCG and attenuated M. tb strains in preclinical studies, consented among TB vaccine researchers and regulatory bodies.20,21 The safety of a live vaccine is inferred from its virulence in SCID mice, which is reflected in the ability of the vaccine to replicate in the animal and to cause mortality. To directly compare the virulence of BCG strains, we performed SCID mice infection of 13 BCG strains and monitored the survival of animals over 18 weeks.

Strikingly, there were significant differences in the survival curves of SCID mice infected with different groups of BCG strains (P < 0.0001, log-rank test; Figure 1a). The majority of SCID mice infected with BCG-Phipps, BCG-Frappier, BCG-Pasteur, and BCG-Tice were 48, 53.5, 55, and 58 days, respectively. In contrast, all SCID mice infected with BCG-Japan, BCG-Birkhaug, BCG-Sweden, BCG-Glaxo, and BCG-Prague survived during the 18-week experiment. The survival rate of SCID mice infected with BCG-Japan, BCG-Birkhaug, BCG-Sweden, BCG-Glaxo, and BCG-Prague were 93.75, 100, 100, 100, and 100%, respectively, which were the same as the phosphate-buffered saline (PBS) control group (87.5%). The virulence levels of the other BCG strains were in between these two groups. The median survival time of SCID mice infected with BCG-China, BCG-Moreau, BCG-Russia, and BCG-Danish were 83, 94.5, 99.5, and 114 days, respectively (Figure 1a). Accordingly, the 13 BCG strains fall into five groups based on their virulence levels: BCG-Phipps, BCG-Pasteur, BCG-Frappier, BCG-Tice > BCG-China > BCG-Russia, BCG-Moreau > BCG-Danish > BCG-Glaxo, BCG-Prague, BCG-Japan, BCG-Sweden, and BCG-Birkhaug. The differences were statistically significant between groups, but not between members of the same group (log-rank analysis).

There was a general parallel between the SCID mice survival curves and the ability of individual BCG strains to replicate in the animals. At week 4 postinfection, BCG-Phipps, BCG-Frappier, BCG-Pasteur, and BCG-Tice exhibited the highest counts in the lungs of individual SCID mice, reaching 7.35 log10, 7.34 log10, 6.76 log10, and 7.11 log10 colony-forming units (CFUs), respectively, which were on average 2.5 log10 higher than their counts at the week 1 timepoint (4.11 log10, 4.92 log10, 4.25 log10, and 5.04 log10 CFUs for BCG-Phipps, BCG-Frappier, BCG-Pasteur, and BCG-Tice, respectively) (Figure 1b). BCG-Japan, BCG-Birkhaug, BCG-Prague, and BCG-Glaxo showed little growth in the lungs of SCID mice during the same period, with an average of 4.3 log10 CFUs at week 4 postinfection, which was essentially the same as the average counts (4.1 log10 CFUs) at the week 1 time point. BCG-Russia, BCG-Moreau, BCG-China, BCG-Danish, and BCG-Sweden showed intermediate levels of replication, ranging from 1.2 to 2.1 log10 CFUs higher in the lungs of SCID mice.
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at week 4 than at week 1 postinfection (Figure 1b). A similar trend was observed for BCG counts in the spleen of SCID mice (Figure 1c).

In a separate experiment, three BCG strains (BCG-Pasteur, BCG-Russia, BCG-Japan) representing different virulence levels were selected to repeat the SCID mice infection experiment. Consistent with the results above, all SCID mice infected with BCG-Japan survived the 18-week experiment, while mice infected with BCG-Pasteur and BCG-Russia had a median survival time of 42 and 84 days, respectively.

Evaluation of efficacy of BCG strains in BALB/c mice

Immunocompetent inbred mice (BALB/c and C57BL/6) are widely used for TB vaccine studies because of the low cost and the availability of immunological reagents. To compare the protective efficacy of BCG strains, we used a low-dose (100 CFUs of M. tb H37Rv), aerosol infection mouse (BALB/c) model to mimic the natural M. tb infection in humans. As classically demonstrated, the M. tb infection of BALB/c and C57BL/6 mice by aerosol challenge is followed by two phases. The first is the progressive phase in which M. tb grows essentially uninhibited for the first 3–4 weeks, resulting in 10^6–10^7 CFUs in lungs. The progressive phase ends with the inhibition of further M. tb growth by adaptive immunity. This is followed by a stationary phase, in which the bacterial burden remains largely unchanged for 9–12 months before the mice succumb to TB.

At week 4 post-M. tb challenge, the nonvaccinated group of BALB/c mice had a mean M. tb burden of 7.27 log_{10} CFUs in the lungs, and there was a significant difference between the mean M. tb burden in the lungs of animal groups vaccinated with various BCG strains and the PBS control group (P < 0.0001, one-way analysis of variance (ANOVA)) (Figure 2a; Table 2). For multiple pair-wise comparisons, one-way ANOVA and Tukey's post hoc tests were performed. It was found that mice vaccinated with BCG-Phipps, BCG-Frappier, BCG-Pasteur, BCG-Tice, BCG-Danish, BCG-Prague, BCG-Russia, and BCG-Moreau had significantly lower M. tb burdens than the nonvaccinated PBS group (Figure 2a), ranging from 0.58 log_{10} (BCG-Moreau group) to 1.03 log_{10} CFUs (BCG-Phipps group) lower (Table 2). Mice vaccinated with BCG-China, BCG-Danish, BCG-Russia, and BCG-Moreau had 0.25–0.52 log_{10} lower M. tb counts than the PBS group but the differences were not statistically significant (Table 2). In a comparison between the BCG-vaccinated groups, mice vaccinated with BCG-Phipps had significantly lower M. tb counts in the lungs than animals vaccinated with BCG-Birkhaug (ΔCFU = 0.78 log_{10}, P < 0.0001), BCG-Sweden

Figure 1 Differential virulence of BCG strains in SCID mice. (a) Survival curves of SCID mice infected with 13 different BCG strains. The survival curves were plotted using the Kaplan–Meier method and differences between curves were analyzed using the log-rank test. BCG strains belonging to the same DU2 groups are highlighted in the same color. (b, c) CFUs in the lungs (b) and spleen (c) of SCID mice at 1 and 4 weeks after infection with 13 different BCG strains. The first bar of each strain is the data from week 1 and the second bar is the data from week 4. Data were plotted as mean ± SD (n = 4). BCG, Bacille Calmette–Guérin; CFU, colony-forming unit; PBS, phosphate-buffered saline.
M. tuberculosis counts than the PBS group, although the differences were not statistically significant. The *M. tuberculosis* burden in animals vaccinated with the remaining *BCG* strains, except *BCG*-China, were lower than the PBS control group, ranging from 0.34 log$_{10}$ (BCG-Japan) to 0.58 log$_{10}$ CFUs (BCG-Russia), but these differences were not statistically significant (one-way ANOVA and Tukey’s post hoc test).

At week 9 postchallenge, the infection has entered into the stationary phase and the *M. tuberculosis* burden in the PBS group was stabilized at 6.77 log$_{10}$ and 6.07 log$_{10}$ CFUs in the lungs and spleen, respectively (Table 2). The differences in *M. tuberculosis* burden between the *BCG*-vaccinated groups and the PBS group diminished. *BCG*-vaccinated animals had on average 6.65 log$_{10}$ and 5.89 log$_{10}$ CFUs in the lungs and spleen, respectively, and none of the *BCG*-vaccinated animal groups were significantly different than the PBS control group (Table 2).

To determine if the results above were reproducible, two *BCG* strains of each genetic lineage (DU2 groups I–IV) were selected and the experiment was performed at a different laboratory under similar conditions (i.e., s.c. vaccination with *BCG* strains followed by aerosol infection of *M. tuberculosis*). The *M. tuberculosis* burden in the lungs of animals at week 4 postinfection were determined and analyzed. Consistently, mice vaccinated with *BCG*-Phipps, *BCG*-Tice, *BCG*-Danish, *BCG*-Russia, and *BCG*-Moreau had significantly lower *M. tuberculosis* burden in the lungs, ranging from 0.54 to 0.83 log$_{10}$ CFUs lower than the PBS group (P < 0.05, one-way ANOVA and Tukey’s post hoc test; Figure 2c). No significant differences were found between the bacterial burden in mice vaccinated with *BCG*-Sweden, *BCG*-Birkhaug, and *BCG*-Glaxo compared to the PBS group.

The protective efficacy of *BCG* strains appeared to correlate with their genetic clustering based on tandem duplications. We replotted the lung *M. tuberculosis* burdens of Figure 2a by combining data of *BCG* strains of the same DU2 group (Figure 3a). The mean *M. tuberculosis* burden of animals vaccinated with groups I–IV *BCG* strains were 6.70 log$_{10}$, 6.98 log$_{10}$, 6.63 log$_{10}$, and 6.47 log$_{10}$ CFUs in the lungs, respectively. One-way ANOVA and Bonferroni’s multiple-comparison test showed that strains of group IV (*BCG*-Phipps, *BCG*-Frappier, *BCG*-Pasteur, and *BCG*-Tice) and group III (*BCG*-China, *BCG*-Danish, *BCG*-Prague, and *BCG*-Glaxo) afforded significantly better protection than strains of group II (*BCG*-Sweden and *BCG*-Birkhaug) in vaccinated mice.

The most protective *BCG* group, DU2 group IV, also had the highest level of virulence in SCID mice, and there appeared to be a correlation between virulence and efficacy. In Figure 3b, we replotted the data of Figure 2a by dividing the *BCG* strains into three groups based on the virulence level (Figure 1a), the most virulent group (*BCG*-Phipps, *BCG*-Frappier, *BCG*-Pasteur, and *BCG*-Tice), the intermediate virulent group (*BCG*-China, *BCG*-Russia,
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BCG-Moreau, and BCG-Danish), and the least virulent group (BCG-Japan, BCG-Glaxo, BCG-Prague, BCG-Sweden, and BCG-Birkhaug). The mean \( M. \text{tb} \) burden for these three groups were 6.47 log\(_{10}\), 6.64 log\(_{10}\), and 6.81 log\(_{10}\) CFUs in the lungs, respectively, and the difference between the most virulent group and the least virulent group was statistically significant (\( P < 0.0001 \), one-way ANOVA and Bonferroni’s test).

DISCUSSION

In this study, we performed a head-to-head comparison of the virulence and efficacy of 13 BCG strains representing different genetic lineages in murine models. Previously, there had been over a dozen studies comparing the effectiveness of different BCG strains using several animal models including mice, guinea pigs, hamsters, and bank vole (reviewed in ref. 19). In the majority of these studies, the number of BCG strains included for comparative analyses were limited, and only three studies compared ≥10 strains of BCG.\(^19\) Differences in the animal model and study designs make it difficult to compare results from different studies. As such, no consistent conclusion can be drawn from them.\(^19\) Early studies in the 1970s by Ladefoged and coworkers compared 11 BCG strains in bank voles and 12 BCG strains in guinea pigs.\(^26,27\) However, in the bank vole study, the 11 BCG strains were compared in six separate experiments, each involving 5 strains, and BCG-Danish was

Table 2 CFUs in lungs and spleens after \( M. \text{tb} \) challenge in mice immunized with 13 different BCG strains

| Animal groups | Log
| CFU/lung (mean ± SD) | Log
| CFU/spleen (mean ± SD) |
|---------------|--------------------|--------------------|
|               | Week 4             | Week 9             | Week 4             | Week 9             |
| PBS           | 7.27 ± 0.10        | 6.77 ± 0.14        | 5.56 ± 0.49        | 6.07 ± 0.11        |
| Phipps        | 6.23 ± 0.16        | 6.52 ± 0.31        | 4.88 ± 0.42        | 5.91 ± 0.45        |
| Frappier      | 6.43 ± 0.22        | 6.31 ± 0.43        | 4.90 ± 0.28        | 5.85 ± 0.55        |
| Pasteur       | 6.55 ± 0.29        | 6.63 ± 0.07        | 4.74 ± 0.28        | 5.75 ± 0.31        |
| Tice          | 6.65 ± 0.17        | 6.71 ± 0.28        | 4.76 ± 0.34        | 5.95 ± 0.25        |
| Russia        | 6.64 ± 0.18        | 6.25 ± 0.46        | 4.98 ± 0.17        | 5.64 ± 0.17        |
| Moreau        | 6.69 ± 0.48        | 6.81 ± 0.22        | 4.94 ± 0.38        | 6.22 ± 0.19        |
| Japan         | 6.77 ± 0.34        | 6.83 ± 0.08        | 5.22 ± 0.17        | 5.83 ± 0.10        |
| China         | 6.75 ± 0.13        | 6.85 ± 0.41        | 5.58 ± 0.46        | 5.92 ± 0.43        |
| Danish        | 6.48 ± 0.17        | 6.45 ± 0.32        | 4.84 ± 0.31        | 5.60 ± 0.20        |
| Prague        | 6.51 ± 0.27        | 6.48 ± 0.33        | 4.85 ± 0.22        | 5.81 ± 0.25        |
| Glaxo         | 6.79 ± 0.23        | 6.75 ± 0.18        | 5.03 ± 0.10        | 6.22 ± 0.28        |
| Sweden        | 6.95 ± 0.21        | 7.16 ± 0.60        | 5.02 ± 0.37        | 6.06 ± 0.42        |
| Birkhaug      | 7.01 ± 0.14        | 6.74 ± 0.25        | 5.08 ± 0.22        | 5.83 ± 0.34        |

BCG, Bacille Calmette–Guérin; CFU, colony-forming unit.
the only strain common in all experiments, which limits the comparison of the results. In the guinea pig study, the *M. tb* strain used in the challenge was inadvertently attenuated and the data on protective efficacy was unavailable. A more recent study by Castillo-Rodal et al. compared the effectiveness of 10 BCG strains in BALB/c mice. However, 6 of the 10 BCG strains (BCG-Phipps, BCG-Tice, BCG-Pasteur, BCG-Frappier, BCG-Connaught, and BCG-Mexico) included in this study are from the DU2 group IV, while only 1 strain each from group I (BCG-Moreau) and group III (BCG-Danish) and 2 strains from group II (BCG-Sweden and BCG-Birkhaug) were included. Therefore, the BCG strains chosen for this study are not equally represented. Recognizing the limitations of the previous studies, we performed comparative studies of 13 BCG strains in the same experiment, which has the advantage of removing variability between experiments. The BCG strains chosen for our experiments are also well characterized genetically (e.g., the availability of genome sequences), and these BCG strains represent all genetic lineages identified so far, including three, two, four, and four strains from DU2 groups I–IV, respectively. In addition, we also compared the virulence of these 13 BCG strains in SCID mice, which, to the best of our knowledge, has never been performed previously. One limitation of the present study is that laboratory strains instead of commercial BCG vaccines were used for experiments. However, analyses of commercial BCG vaccines are complicated by the observation that commercial preparations could have mixed BCG strains.

Our results show that BCG strains exhibit variable virulence and efficacy. The segregation of the virulence of BCG strains generally coincides with their genetic clustering based on genome tandem duplications (Figure 1). The most virulent strains (BCG-Phipps, BCG-Pasteur, BCG-Frappier, and BCG-Tice) all belong to the DU2 group IV (Figure 1), and the two strains of the DU2 group II (BCG-Sweden and BCG-Birkhaug) are among the least virulent group. Tandem duplications are a major mechanism of BCG adaptation to in vitro growth conditions, thus it is not surprising that gene amplification via tandem duplication has a major influence on BCG properties. Comparison of the DU2 groups reveals that groups III and IV contain a 15765-bp (3,590,902–3,606,667) duplication that does not occur in groups I and II (Table 1). This duplication includes two regulatory genes: *sigH* and *whiB1*. *SigH* plays a critical role in the oxidative stress responses in *M. tb* and *WhiB1* is a nitric oxide-responsive transcription factor. Elevated expression of *sigH* and *whiB1* in BCG strains of groups III and IV may enhance their replication, thereby exhibiting higher virulence in SCID mice. BCG-Glaxo is less virulent than BCG-China and BCG-Danish of the same group (group III) likely because BCG-Glaxo is naturally deficient in the production of phthiocerol dimycolates and phenolic glycolipids. Phthiocerol dimycolates and phenolic glycolipids are multiple methyl-branched fatty acid-containing lipids in the mycobacterial cell wall and their critical roles in virulence have been demonstrated in multiple pathogenic mycobacteria including *M. tb*, *M. bovis*, and *M. marinum*. The low virulence of BCG-Prague may be related to the *phoP* mutation in this strain. *PhoP* is a response regulator of the PhoP-PhoR two-component system. *PhoPR* controls the synthesis and export of multiple virulence factors in *M. tb* including EsxA, an effector of the type VII secretion system ESX-1, and lipids of polyacyltrehalose and sulfolipid families, and therefore is critical for *M. tb* virulence. However, since BCG has lost the RD1 region encoding the ESX-1, in addition to the impaired *phoPR* regulation system in *M. bovis* and BCG due to single nucleotide polymorphisms in this locus, the extent to which the *phoP* mutation contributes to the attenuation of BCG-Prague remains unknown. BCG-Japan has a lower level of virulence than BCG-Russia and BCG-Moreau in group I strains presumably because it is deficient in the production of phthiocerol dimycolates/phenolic glycolipids and triacylglycerols. For BCG-Sweden and BCG-Birkhaug of group II, the deletion of *whiB3*, a reductive stress regulator, and *trcR*, a response regulator of the *trcR-trcS* two-component system, may account for their low virulence. Strain-specific single nucleotide polymorphisms revealed by whole genome sequencing may also account for the differential virulence of individual BCG strains. For example, BCG-Russia and BCG-Moreau of group I are more virulent than BCG-Danish but less virulent than BCG-China of the same group (group III). Future studies are required to test these hypotheses.

There appears to be a general trend that more virulent BCG strains are also more protective. BCG strains of the most virulent group (BCG-Phipps, BCG-Pasteur, BCG-Frappier, BCG-Tice) demonstrated better protection than BCG-Sweden and BCG-Birkhaug of group II. Among the group I strains, BCG-Japan is the least virulent and also less protective than BCG-Russia and BCG-Moreau. Consistent with this notion, a previous study found that recombinant BCG strains complemented with the RD1 region exhibited increased virulence in SCID mice but also better protection in C57BL/6 mice and guinea pigs. The correlation is less straightforward when comparing strains in group III (BCG-Danish, BCG-China, BCG-Praque, and BCG-Glaxo). For example, BCG-Prague is quite attenuated but well protecting, suggesting that multiple factors are involved. Current strategies to develop the next generation of TB vaccines include the construction of recombinant BCG. Candidates that have entered clinical trials include rBCG30,51 and VPM1002 (rBCG::Δ*phoP*) which in the preclinical animal studies (mice or guinea pigs), consistently reduced the *M. tb* burden by 0.5–1.0 log₁₀ CFUs compared to the corresponding parental BCG strains. Notably, we found that mice vaccinated with BCG-Phipps and BCG-Frappier had 0.5–0.8 log₁₀ fewer *M. tb* than those vaccinated with BCG-Birkhaug, BCG-Sweden, BCG-Japan, or BCG-Glaxo (Figure 2a), highlighting the importance of selecting specific BCG strain(s) for the construction of recombinant BCG.

Currently, all BCG strains are considered “equal” in clinical use. The most widely used BCG vaccines include both early (BCG-Japan, BCG-Moreau, BCG-Russia) and late BCG (BCG-Pasteur, BCG-Danish, BCG-Connaught, distributed after 1927) strains. Reviews of clinical trial data found no evidence that efficacy was directly comparing different BCG strains. The conclusion drawn was based on comparisons between different clinical trials, which is compounded by multiple factors including differences in trial method and the population of the study. A randomized trial study comparing two BCG strains in 300,000 infants in Hong Kong found...
that a more virulent strain, BCG-Pasteur, administered at a lower dosage, provided a significantly greater (40%) protection against childhood forms of TB than a less virulent strain, BCG-Glaxo.56 However, in a retrospective analysis of cohorts in Kazakhstan, vaccination of neonates by one of the least virulent strains, BCG-Japan, reduced the risk of TB by 69%, by 43% after BCG-Serbia vaccination, and only by 22% after BCG-Russia.57 While it is difficult to compare results between clinical trial studies, and between animal and human studies, these results suggest there are significant differences in effectiveness against TB between BCG strains. Multiple studies have also demonstrated that BCG exhibits strain-dependent variations in immune responses in humans.58 Taken together, these pieces of evidence call for a clinical trial study directly comparing the effectiveness of different BCG strains. Based on the findings of our study, we suggest that the trial should include BCG strains from a diverse genetic background—that is, representatives of each of the four major groups (DU2 group I-IV). The outcome of such a clinical trial may not only identify the most effective BCG strain(s) for current clinical use, but also uncover genetic factors that influence the vaccine effectiveness, which will be useful for the development of the next generation of TB vaccines.

MATERIALS AND METHODS

**Bacterial strains and culture conditions.** All BCG strains included in this study have been previously described.48 BCG strains and M. tb H37Rv were grown at 37 °C in Middlebrook 7H9 broth (Difco) supplemented with 0.2% glycerol, 10% albumin–dextrose–catalase (ADC; BD BBL, Shanghai, China), and 0.05% Tween-80 or on Middlebrook 7H11 agar (Difco) supplemented with 0.5% glycerol and 10% oleic acid–albumin–dextrose–catalase (OADC; BD BBL).

**Analysis of BCG virulence in SCID mice.** All of the animal procedures were approved by the local animal care committees. Female SCID mice were purchased from Beijing HFK Bioscience and the mice were matched aged (7–8 weeks) within each experiment. Mice (27 per group) were infected i.v. via the tail vein with 10^7 CFU of the different BCG strains in 0.1 ml PBS/0.01% Tween-80. At day 1 postinfection, two mice from each group were sacrificed and the lungs and spleens were harvested, homogenized in PBS, and plated on 7H11 agar to enumerate bacterial burden. This was performed to confirm the actual infection dosage. To analyze the replication of BCG strains in SCID mice, four mice from each group at week 1 and week 4 postinfection were sacrificed and the CFUs of BCG in lungs and spleen were determined. The survival of the remaining mice was monitored over 18 weeks.

**Protection against M. tb challenge.** Groups of 12 female BALB/c mice were vaccinated s.c. on the scruff of the neck with 10^7 CFU of the different BCG strains in 0.1 ml PBS/0.01% Tween-80 or PBS/0.01% Tween-80 alone as a control. At 8 weeks postvaccination, mice were aerochallenged with 100 CFU of M. tb H37Rv using a GlasCol nebulizer. Mice were euthanized at 4 and 9 weeks postchallenge (five mice per group per time point) to harvest the lungs and spleen, which were then homogenized and plated on 7H11 agar to enumerate the burden of M. tb. Plates were incubated at 37 °C and counted over 2.5–3 weeks. Actual infection dose was confirmed by homogenizing whole lungs of two mice at day 1 postinfection and plating on 7H11 to enumerate M. tb.

**Statistical analysis.** SCID mice survival was plotted using the Kaplan–Meier method and differences between curves were analyzed using the log-rank test. One-way ANOVA with Tukey’s multiple comparisons were performed for M. tb burdens (log_−transformed CFU data) when there are more than six groups. One-way ANOVA and Bonferroni’s multiple comparisons were performed for six or fewer groups.
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