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Immunogenicity and Protection Induced by a *Mycobacterium tuberculosis sigE* Mutant in a BALB/c Mouse Model of Progressive Pulmonary Tuberculosis[▽]

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Tuberculosis is still one of the main challenges to human global health, leading to about two million deaths every year. One of the reasons for its success is the lack of efficacy of the widely used vaccine *Mycobacterium bovis* BCG. In this article, we analyze the potential use of an attenuated mutant of *Mycobacterium tuberculosis* H37Rv lacking the sigma factor σ^E as a live vaccine. We have demonstrated that BALB/c mice infected by the intratracheal route with this mutant strain showed significantly higher survival rates and less tissue damage than animals infected with the parental or complemented mutant strain. Although animals infected with the *sigE* mutant had low bacillary loads, their lungs showed significantly higher production of the protective factors gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), inducible nitric oxide synthase (iNOS), and β-defensins than those of animals infected with the parental or complemented mutant strain. Moreover, we demonstrate that the *sigE* mutant, when inoculated subcutaneously, was more attenuated than BCG in immunodeficient nude mice, thus representing a good candidate for a novel attenuated live vaccine strain. Finally, when we used the *sigE* mutant as a subcutaneous vaccine, it was able to induce a higher level of protection than did BCG with both H37Rv and a highly virulent strain of *M. tuberculosis* (Beijing code 9501000). Taken together, our findings suggest that the *sigE* mutant is a very promising strain for the development of a new vaccine against tuberculosis.

Tuberculosis (TB) is still one of the leading causes of mortality throughout the world (10, 30). The HIV/AIDS pandemic, the deterioration in public health systems in developing countries, and the emergence of multidrug-resistant (MDR) forms of TB are important factors contributing to the high toll imposed by this disease on the human population. Prophylactic vaccination with the attenuated strain of *Mycobacterium bovis* bacille Calmette-Guérin (BCG) is used in most countries. BCG vaccination, even if effective against severe forms of childhood tuberculosis, has a limited efficacy against adult pulmonary disease, the most transmissible form of the infection (11). Hence, new rationally constructed vaccine candidates are required.

Mycobacterium tuberculosis is a remarkable pathogen capable of adapting to and surviving various harsh conditions encountered during infection. Such adaptation is due mostly to a complex transcriptional regulatory network able to modulate the expression of its complex genome (37).

Sigma factors bind to the RNA polymerase holoenzyme, providing its specificity for particular promoters, and play a key role in the regulation of gene expression and adaptation to stress in prokaryotes (5). The *M. tuberculosis* genome encodes 13 sigma factors, 10 of which belong to the extracytoplasmic function (ECF) subclass (also referred to as group four) (33). Among the mycobacterial sigma factors, σ^E (belonging to the ECF subclass) is probably the best characterized. It is subject to very complex regulation (8) and was clearly shown to be deeply involved in virulence. A mutant in which its structural gene (*sigE*) was disrupted was not only sensitive to various surface-disrupting stresses, such as the detergent sodium dodecyl sulfate (SDS), the cationic peptide polymyxin, and the antibiotic vancomycin (12, 26, 31), but was also unable to grow in resting macrophages and dendritic cells, was more sensitive to killing from activated macrophages, and was severely attenuated in mice (3, 14, 27, 29). The σ^E transcriptome was analyzed by DNA microarrays following SDS-induced surface stress and during macrophage infection (13, 29): interestingly, σ^E was found to regulate genes involved in mycolic acid biosynthesis and fatty acid degradation, as well as genes involved in membrane protein quality control and membrane stabilization. Taken together, these data suggest that σ^E is responsible for controlling surface stability and composition following exposure to damaging environmental conditions.

Finally, recent *in vitro* studies comparing the transcriptional responses of human and murine macrophages (13) and human dendritic cells (14) infected with wild-type *M. tuberculosis* strain H37Rv and the *sigE* mutant revealed that components of the σ^E regulon modulate the innate immune system, so that in the *sigE* mutant-infected cells, there was an upregulation of proteins of the acute-phase response, Toll-like receptors 1 and 2, proinflammatory cytokines, chemokines, and prostaglandins. Thus, the *sigE* mutant strain stimulates the host immune system during macrophage infection, suggesting that this strain could be an efficient live attenuated vaccine strain.

Rationally attenuated, live replicating mutants of *M. tuberculosis* are potential vaccine candidates (16). The advantage of using attenuated *M. tuberculosis* strains is that they produce a large number of protective antigens, including those that are

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TABLE 1. Primers used for quantitative RT-PCR determinations

Product of target gene	Primer sequence	
	Forward (5′–3′)	Reverse (5′–3′)
G3PDH	CATTGTGGAAGGGCTCATGA	GGAAGGCCATGCCAGTGAGC
TNF-α	TGTGGCTTCGACCTCTACCTC	GCCGAGAAAGGCTGCTTG
IFN-γ	GGTGACATGAAAATCCTGCAG	CCTCAAACTTGGCAATACTCATGA
IL-4	CGTCCTCACAGCAACGGAGA	GCAGCTTATCGATGAATCCAGG
IL-10	AAAGGCACTGCACGACATAGC	TGCGGAGAACGTGGAAAAAC
βD3	TCTGTTTGCATTTCTCCTGGTG	TAAACTTCCAACAGCTGGAGTGG
βD4	TCTGTTTGCATTTCTCCTGGTG	TTTGCTAAAAGCTGCAGGTGG

absent from BCG (4). Thus, vaccination with live attenuated *M. tuberculosis* can induce a stronger and longer immune stimulation, conferring higher levels of protection against TB than BCG (16). In the first part of this article, we describe the survival, lung bacillary loads, histopathology, and cytokine profile expression in BALB/c mice after intratracheal infection with the *M. tuberculosis sigE* mutant, its parental strain, H37Rv, and the *sigE* mutant complemented strain. In the second part, we analyze the potential of the *sigE* mutant as a vaccine. After corroborating its attenuation when administered by the subcutaneous route and comparing its immunogenicity to that of BCG before challenge, we tested its protective efficacy after challenge with either *M. tuberculosis* H37Rv or one clinical isolate (Beijing strain 9501000) previously shown to be strikingly hypervirulent in BALB/c mice (24). These experiments showed that the *sigE* mutant is able to confer a significantly better protection against challenge with virulent *M. tuberculosis* than BCG.

MATERIALS AND METHODS

Ethics statement. Animal studies were approved by the Institutional Ethics Committee of the National Institute of Medical Sciences and Nutrition Salvador Zubirán in accordance with the guidelines of the Mexican national regulations on Animal Care and Experimentation, NOM 062-ZOO-1999.

Growth of bacterial strains. ST28, a *sigE* mutant, and its complemented derivative, ST29, were obtained from *M. tuberculosis* H37Rv as previously described (29). The BCG strain used was *M. bovis* BCG Phipps. This BCG substrain was the most protective of 10 strains tested in our BALB/c model of progressive pulmonary tuberculosis (6). The Beijing strain 9501000 was donated by D. van Soolingen (RIVM, Netherlands). Strains were grown in Middlebrook 7H9 medium (Difco Laboratories) supplemented with oleic acid, albumin, catalase, and dextrose (OADC) (Difco Laboratories). After 1 month of culture, mycobacteria were harvested, adjusted to 2.5×10^5 bacteria in 100 μl phosphate-buffered saline (PBS), aliquoted, and maintained at −70°C until used. Before use, bacterial aliquots were thawed and their viability was checked.

Experimental model of progressive pulmonary tuberculosis in BALB/c mice. Virulence (as determined by survival, lung pathology, and bacterial load) and the immune response induced by each isolate were evaluated in 8- to 10-week-old male BALB/c mice as previously described (15). Bacilli were grown in liquid culture medium 7H9 and monitored by densitometry. As soon as the culture reached mid-log phase, the bacilli were harvested and suspended in PBS containing 0.05% Tween 80 by shaking for 10 min with glass beads. The suspension was centrifuged for 1 min at $350 \times g$ to remove large clumps of bacilli. Then, a preliminary bacterial count was achieved by smearing the supernatant at a known ratio of volume to area and counting 10 random fields after Ziehl-Neelsen staining. The suspension was finally diluted to 2.5×10^5 bacteria in 100 μl of PBS and aliquoted at −70°C. Before use, bacteria were recounted and viability was checked as described previously (22). To induce progressive pulmonary tuberculosis, mice were anesthetized with sevoflurane and inoculated intratracheally with 2.5×10^5 CFU of *M. tuberculosis* H37Rv, the *sigE* mutant, or the *sigE* complemented strain suspended in 100 μl PBS (15). After animal infection, the remnant of the bacterial inoculum was plated to confirm the number of CFU administrated to the animals.

Infected mice were kept in a vertical position until the effect of anesthesia passed. Animals were maintained in groups of five in cages fitted with microisolators connected to negative pressure. Twenty mice from each group were left undisturbed to record survival from day 8 up to day 120 after infection. Six animals from each group were sacrificed by exsanguinations at 1, 3, 7, 14, 21, 28, 60, and 120 days after infection. One lung lobe, right or left, was perfused with 10% formaldehyde, dissolved in PBS, and prepared for histopathological studies. The other lobe was snap-frozen in liquid nitrogen and then stored at −70°C for microbiological and immunological analysis (15). All procedures were performed in a laminar flow cabinet in a biosafety level III facility.

Preparation of lung tissue for histology and automated morphometry. One lobe of the lung was fixed by intratracheal perfusion with 10% formaldehyde for 24 h and then sectioned through the hilus and embedded in paraffin. Sections 5 μm thick were stained with hematoxylin-eosin for the histological-morphometric analysis. The percentage of the pulmonary area affected by pneumonia was determined using an automated image analyzer (Q Win Leica; Leica, Milton Keynes, United Kingdom) (15).

Determination of CFU in infected lungs. Right or left lungs from four mice at each time point, in two separate experiments, were used for colony counting. Lungs were homogenized with a Polytron homogenizer (Kinematica, Luzern, Switzerland) in sterile 50-ml tubes containing 3 ml of isotonic saline. Four dilutions of each homogenate were spread onto duplicate plates containing Bacto Middlebrook 7H10 agar (Difco Labs, Detroit, MI) enriched with OADC. Plates were incubated for 21 days prior to determination of CFU (15).

Real-time PCR analysis of cytokines in lung homogenate. Left or right lung lobes from three different mice per group in two different experiments were used to isolate mRNA using the RNeasy minikit (Qiagen), according to the recommendations of the manufacturer. Quality and quantity of RNA were evaluated through spectrophotometry (260/280) and on agarose gels. Reverse transcription of the mRNA was performed using 5 μg RNA, oligo(dT), and the Omniscript kit (Qiagen, Inc.). Real-time PCR was carried out using the 7500 real time PCR system (Applied Biosystems) and Quantitect SYBR green Mastermix kit (Qiagen). Standard curves of quantified and diluted PCR product, as well as negative controls, were included in each PCR run. Specific primers for genes encoding glyceraldehyde-3-phosphate dehydrogenase (G3PDH), tumor necrosis factor alpha (TNF-α), gamma interferon (IFN-γ), interleukin 4 (IL-4), IL-10, β-defensin 3 (βD3), and β-defensin 4 (βD4) were designed using the program Primer Express (Applied Biosystems) (Table 1).

Cycling conditions used were as follows: initial denaturation at 95°C for 15 min, followed by 40 cycles at 95°C for 20 s, 60°C for 20 s, and 72°C for 34 s. Quantities of the specific mRNA in the sample were measured according to the corresponding gene-specific standard. The mRNA encoding G3PDH was used as an internal invariant control to normalize the expression of the cytokine-expressing genes. Data were shown as copies of cytokine-specific mRNA/10^6 copies of G3PDH-specific mRNA (1).

Comparison of immunogenicity with BCG- and *sigE* mutant-vaccinated mice before challenge. To study bacterial growth and ability to disseminate, we determined CFU in different organs after subcutaneous vaccination (1).

Groups of four BALB/c mice were vaccinated by inoculating the best protective dose of live bacilli (8,000 cells, determined as described above; data not shown) subcutaneously at the base of the tail. After animal vaccination, aliquots of the bacterial inoculum were plated to confirm the number of CFU administrated to the animals.

Animals were killed at 15, 30, and 60 days postvaccination. The inguinal lymph nodes, spleen, lungs, and subcutaneous tissue at the site of vaccination (base of the tail) were immediately dissected and homogenized for determination of

bacillary loads by CFU quantification, following the same procedure described above.

Another group of four vaccinated BALB/c mice per time point was used to determine immunogenicity by comparing the production of IFN-γ by cell suspensions from inguinal lymph nodes, spleen, and lungs after stimulation with mycobacterial culture filtrate antigens (CFA) or the immunodominant recombinant antigens ESAT-6, Hsp65, and Ag85 (1). After mice were killed, spleen, inguinal lymph nodes, and lungs were immediately removed, placed in 2 ml of RPMI medium containing 0.5 mg/ml collagenase type 2 (Worthington, NJ), and incubated for 1 h at 37°C. Samples were then passed through a 70-μm cell sieve, crushed with a syringe plunger, and rinsed with the medium. Cells were centrifuged at 1,500 rpm for 5 min, and the supernatant was removed; red cells were eliminated with a lysis buffer. After washing, cells were resuspended in RPMI medium supplemented with 2 mM L-glutamine, 100 U of penicillin per ml, 1 μg of streptomycin per ml (Sigma), and 10% fetal calf serum. Cultures for cytokine production (10^6 cells in 1 ml of culture medium) were performed in flat-bottomed 24-well plates without and with mycobacterial antigens (CFA, ESAT-6, Hsp65, and Ag85). After 3 days of antigenic stimulation, cells were centrifuged and the supernatant was used for IFN-γ quantification through a commercial enzyme-linked immunosorbent assay (ELISA) test kit (Pharmingen, San Diego, CA). Preliminary dose-response curve experiments showed that the best antigen concentration was 5 μg during 3 days of culture stimulation (data not shown).

Infection of nude mice. Groups of 20 nude mice were vaccinated subcutaneously at the base of the tail with one dose of 8,000 live *sigE* mutant or BCG bacilli. After animal infection, the remnant of the bacterial inoculum was plated to confirm the number of CFU administrated to the animals (1).

Evaluation of protection against *M. tuberculosis* H37Rv and a high-virulence Beijing strain in BALB/c mice vaccinated with the *sigE* mutant or BCG. Two separate experiments were performed, using 10 mice for each of four experimental groups. Animals were vaccinated by inoculating the best protective dose of live bacilli (8,000 cells; data not shown) subcutaneously at the base of the tail. At 60 days postvaccination, the first group of 10 mice was challenged through the intratracheal route with 2.5×10^5 CFU of *M. tuberculosis* H37Rv, while the second group with the same number of animals was challenged by the same route and dose with the highly virulent Beijing strain code 9501000. The third and fourth groups corresponded to control animals, which were not vaccinated and were intratracheally infected with the same dose of either H37Rv or the Beijing strain. Three mice per group were euthanized, and their lung homogenates were used to determine the infecting dose which resulted in around 2.1×10^5 to 2.3 CFU (not shown). After 2 and 4 months postchallenge, levels of protection were determined by the quantification of CFU in lung homogenates, following the same procedure described above, and by automated morphometry, measuring the lung surface affected by pneumonia. Ten more animals per group were left untouched, and deaths were recorded to construct survival curves.

Statistical analysis. Statistical analysis for survival curves was performed using Kaplan-Meier plots and log rank tests. Student's *t* test was used to determine the statistical significance of CFU, histopathology, and cytokine expression; a *P* value of <0.05 was considered significant.

RESULTS

Characterization of *sigE* mutant pathogenicity after intratracheal administration. In order to characterize the attenuation of the *sigE* mutant in our model, groups of BALB/c mice (70 per group) were infected intratracheally with 2.5×10^5 CFU of H37Rv, the *sigE* mutant, or its complemented strain. All the animals infected with the *sigE* mutant survived after 4 months of infection. In contrast, mice inoculated with the complemented or parental strain started to die at 3 weeks postinfection, and all had died by 8 weeks (Fig. 1A). These survival rates correlated well with the CFU in lung homogenates. During the first and second weeks of infection, similar numbers of CFU were detected in the three groups, whereas after days 21 and 28 postinfection, significantly lower bacterial loads were found for mice infected with the *sigE* mutant than were detected for animals infected with the parental or complemented strain (Fig. 1B). At days 60 and 120, animals infected with the mutant strain still showed a low bacterial burden, while the other animals were dead.

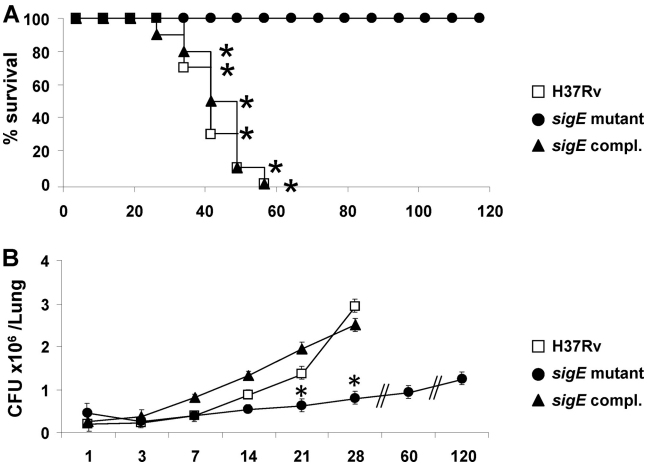

FIG. 1. Pathogenicity of the *sigE* mutant after intratracheal inoculation. (A) Survival of BALB/c mice (20 mice per strain) infected by intratracheal injection of *M. tuberculosis* H37Rv, the *sigE* mutant, and the complemented strain. (B) Lung bacterial burden in mice infected with *M. tuberculosis* H37Rv, the *sigE* mutant, and the complemented mutant strain. Mice were sacrificed after 1, 3, 7, 14, 21, 28, 60 and 120 days postinfection. Asterisks represent statistical significance compared to results for *sigE* mutant-infected mice.

The histopathological analysis showed inflammatory infiltrate predominantly constituted by lymphocytes and activated macrophages (large cells with a compact cytoplasm and nucleus with apparent nucleoli) in the alveolar-capillary interstitium and around small blood vessels and bronchial walls after 1 and 2 weeks of the infection with either of the strains, with levels being higher in animals infected with the *sigE* mutant, which showed well-formed granulomas after 7 days of infection, while the mice infected with the parental or complemented strain showed granulomas after 2 weeks of infection. After 28 days postinfection, only H37Rv induced a significant pneumonia, involving about 30% of the lung surface. In contrast, in mice infected with the *sigE* mutant, well-formed granulomas and comparable pneumonia appeared only after 60 and 120 days postinfection, when the animals infected with H37Rv or the complemented strain were all dead (data not shown).

Evaluation of cytokine, iNOS, and β-defensin expression during infection. The amounts of mRNA specific for various cytokines, iNOS, and β-defensins 3 and 4 were evaluated during the infection with the three different strains. Although the lungs of mice infected with the *sigE* mutant showed significant lower bacillary loads and inflammation than those of animals infected with the parental or complemented strain, they showed a significantly higher and constant expression of genes encoding IFN-γ, TNF-α, and β-defensin 3 (Fig. 2), as well as a progressive induction of iNOS expression in late time points (Fig. 2). Also, expression of IL-10 was higher in *sigE* mutant-infected mice but only from 21 and 28 days after the infection (Fig. 2). The only cytokine shown to be expressed at a significantly lower level in the *sigE* mutant-infected animals was IL-4 (Fig. 2).

Comparison of *sigE* mutant and BCG attenuation in mice. In order to compare the virulence of the *sigE* mutant to that of BCG, groups of BALB/c mice (12 per group) were inoculated

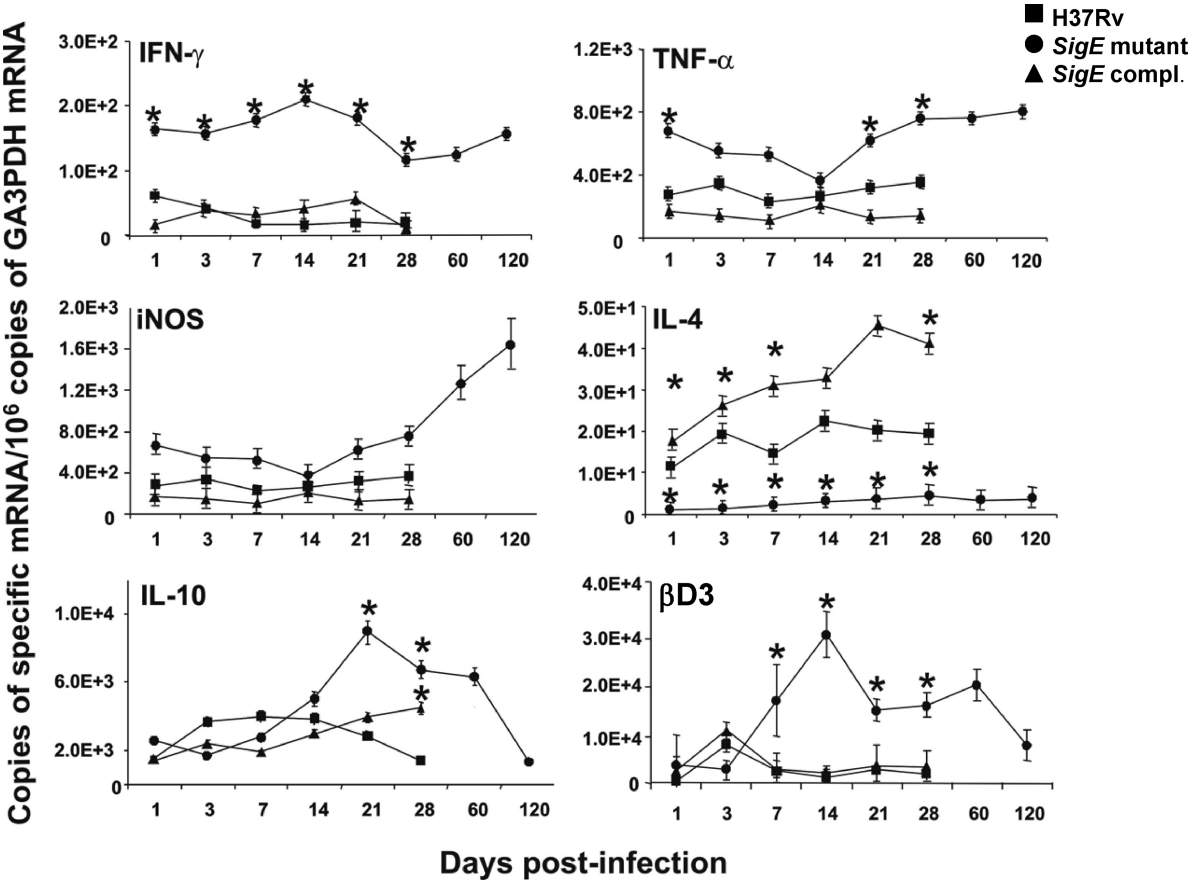

FIG. 2. Quantitative expression of mRNA was determined by real-time PCR in lungs from mice infected with the *sigE* mutant, H37Rv, or the complemented strain. Data are expressed as means and standard deviations for four different animals at each time point. Asterisks represent statistical significance ($P < 0.05$) compared with results for H37Rv-infected mice. No data at day 120 postinfection are presented for H37Rv-infected and complemented mutant strain-infected mice because no surviving animals were available in these experiments.

subcutaneously with 8,000 CFU of either of these two bacterial strains. Two weeks after inoculation, animals which received the *sigE* mutant showed a significant, 2-fold-higher bacterial load at the inoculation site and in the lungs. However, at days 30 and 60 postvaccination, the two groups of vaccinated animals showed similar bacillary loads in the inoculation site, inguinal lymph nodes, spleen, and lungs (Fig. 3), suggesting that the *sigE* mutant is not more virulent than BCG in mice. To further investigate the virulence potential of the *sigE* mutant strain compared to that of BCG, we compared the survival rates of nude mice (20 per group) inoculated subcutaneously with 8,000 CFU of either of the two bacterial strains. Results, shown in Fig. 4, show that even if no significative difference in the 50% survival time point was found between the two groups, at the end of the experiment there was a significant difference in survival between the two groups, suggesting that the *sigE* mutant is more attenuated than BCG in these immunodeficient animals.

Comparison of *sigE* mutant and BCG immunogenicity following vaccination. In order to compare the efficiency of cellular immunity activation induced by the *sigE* mutant or BCG vaccination, we quantified by ELISA the IFN-γ production in cell suspensions collected from the spleen, lung, and inguinal lymph nodes collected at different time points after vaccination and stimulated with mycobacterial antigens. Figure 5 shows that spleen and lung cells from animals vaccinated with the *sigE* mutant stimulated with culture filtrate antigens (CFA) or with the other recombinant antigens produced significantly higher levels of IFN-γ than those of BCG-vaccinated mice at day 60 postvaccination. Since BCG lacks the ESAT-6 structural gene, animals vaccinated with this strain did not produce or secrete any significant amount of IFN-γ after stimulation with this antigen.

Comparative protection against *M. tuberculosis* H37Rv or Beijing 9501000 in BALB/c mice vaccinated with the *sigE* mutant or BCG. In order to compare the levels of protection induced by BCG and the *sigE* mutant, groups of BALB/c mice (40 per group for 2 separate experiments) were vaccinated subcutaneously in the base of the tail with 8,000 CFU of either strain. At 60 days postvaccination, mice were challenged intratracheally with 2.5×10^5 CFU of *M. tuberculosis* H37Rv. Ten mice were euthanized at 60 or 120 days postchallenge. Levels of protection were determined by survival rates, quantification of CFU recovered from the lungs, and the extension of tissue damage, evaluating the percentage of the lung surface affected by pneumonia at both time points.

After 4 months postchallenge, 98% of the mice vaccinated with the *sigE* mutant were still alive, while 20% of BCG-

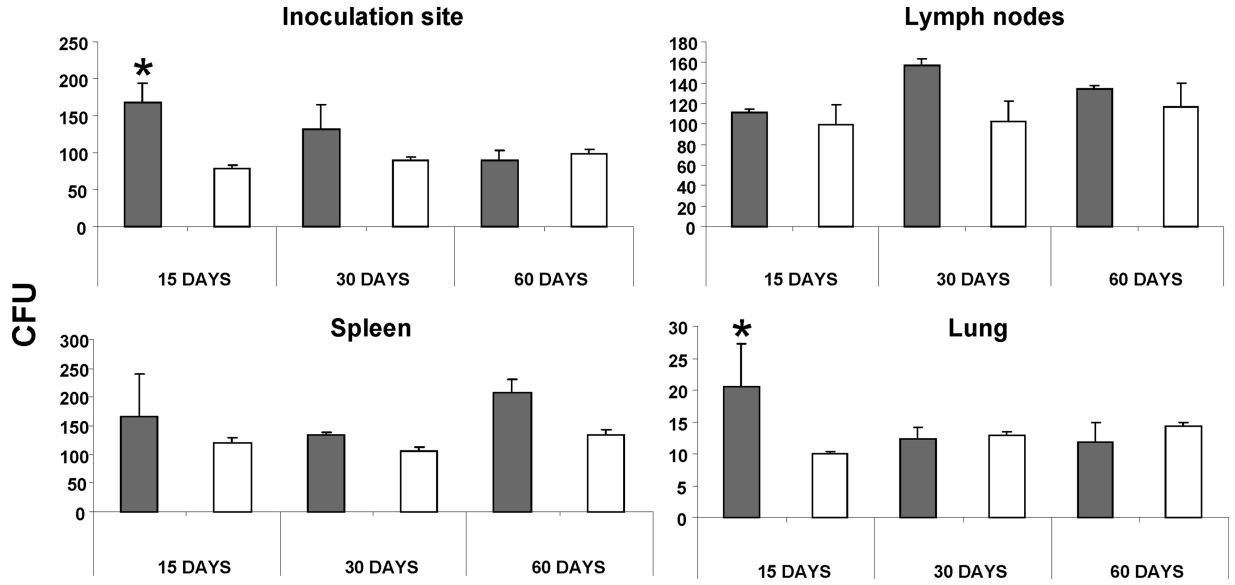

FIG. 3. Bacillary loads at the site of vaccination (subcutaneous tissue base of the tail), inguinal lymph nodes, spleen, and lungs from BALB/c mice inoculated 8,000 CFU of BCG (white bars) or the *sigE* mutant (gray bars) at different time points before the challenge. Bars represent the means and standard deviations for four different animals at each time point in two separate experiments. Asterisks represent statistical significance ($P < 0.05$) among the indicated groups.

vaccinated mice had died. All nonvaccinated controls died after 11 weeks of infection (Fig. 6A, left panel). These results correlated well with lung bacillary loads and histopathology, showing significantly fewer CFU and less pneumonia for mice vaccinated with the *sigE* mutant than for BCG-vaccinated or control nonvaccinated animals. In particular, *sigE* mutant-vaccinated mice showed a reduction in lung CFU of 1.1 and 1.0 $\log_{10}$ with respect to BCG-vaccinated mice at 60 and 120 days postinfection and a reduction of 1.4 $\log_{10}$ with respect with nonvaccinated controls at day 60 postinfection (Fig. 6B and C, left panel).

In a second vaccination experiment, animals vaccinated following the same protocol were challenged with the highly virulent *M. tuberculosis* strain Beijing 9501000. Nonvaccinated animals started to die after 4 weeks from the challenge, and after 6 weeks all were dead. Mice vaccinated with BCG showed a 30% survival rate after 4 months postchallenge, whereas animals vaccinated with the *sigE* mutant exhibited a significantly higher survival rate of 80% (Fig. 6A, right panel). These results were in agreement with lung CFU determinations (Fig. 6B, right panel): after 60 days from infection, mice vaccinated with the *sigE* mutant had a lung bacillary load 0.72 $\log_{10}$ lower than those vaccinated with BCG, while after 120 days the reduction was of 0.79 $\log_{10}$ (Fig. 6B, right panel). After 60 days from the challenge, *sigE* mutant-vaccinated animals showed a higher percentage of lung surface affected by pneumonia than BCG-vaccinated mice, suggesting a more rapid and higher expression of proinflammatory cytokines, such as IFN-γ and TNF-α, in *sigE* mutant-vaccinated animals, which could correlate with better protection. However, after 120 days from the challenge, this difference disappeared (Fig. 6C, right panel).

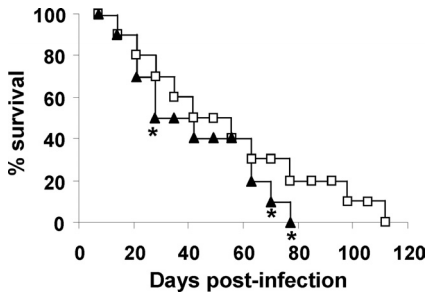

FIG. 4. Survival of nude mice (20 mice per strain) vaccinated by the subcutaneous route in the base of the tail (8,000 CFU) with BCG (black triangles) or the *sigE* mutant (white squares). Asterisks represent statistical significance ($P < 0.05$).

DISCUSSION

During infection, bacteria confront different environments, determined by the site in which the pathogen resides and the level of activation of the host immune response. To survive and grow, the pathogen must be able to adapt to these different milieus. Most bacterial adaptive mechanisms are based on the regulation of gene expression, which consequently plays a very important role in bacterial pathogenesis (37). Examples of this regulation are the two-component regulatory systems, such as PhoP-PhoQ (38), and σ factors (28).

σ^E, a member of the ECF subclass of sigma factors, is induced after exposure to different stress conditions, such as heat shock, SDS-mediated cell surface stress, vancomycin, oxidative stress, and alkaline pH, and during growth in human macrophages (33). Its regulon includes several genes involved in stress response and surface biology, including activities such as mycolic acid biosynthesis, fatty acid degradation, membrane protein quality control, and membrane stabilization.(33). In a previous study, we demonstrated that the *sigE* mutant was attenuated in immunodeficient SCID and immunocompetent

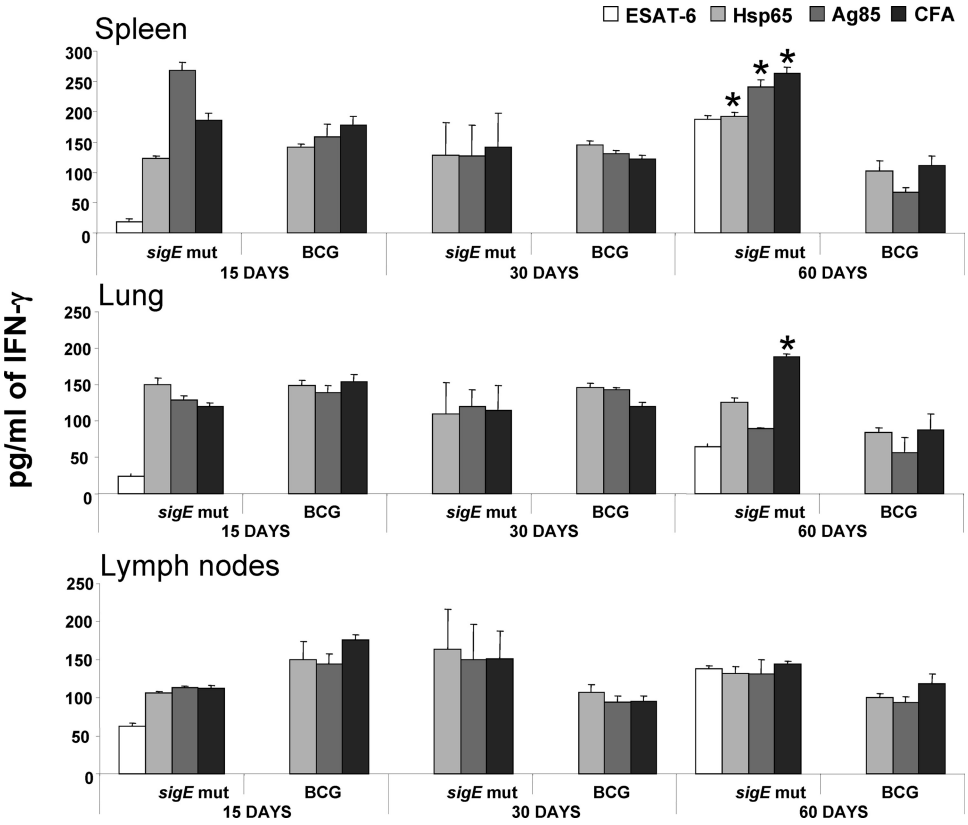

FIG. 5. IFN-γ quantification by ELISA. IFN-γ was quantified in cell suspension supernatants from inguinal lymph nodes, lungs, and spleen of BCG or *sigE* mutant-vaccinated mice after stimulation with culture filtrate mycobacterial antigens (CFA) and the immunodominant recombinant antigens ESAT-6, Hsp65, and Ag85. Bars represent the means and standard deviations for four different animals at each time point. Asterisks represent statistical significance ($P < 0.05$).

BALB/c mice after intravenous infection (27). The aims of the present study were to further characterize its pathogenicity and immunogenicity in BALB/c mice after infection by the intratracheal route and then to evaluate the potential of this mutant as an attenuated vaccine. Our BALB/c mouse model of progressive pulmonary tuberculosis is suitable for determining the virulence and immune response induced by mutant mycobacteria, since it is based on respiratory infection, which is the usual infection route in humans. Moreover, in this model the rate of bacterial multiplication in the lungs correlates well with the extent of tissue damage (pneumonia) and mortality, and the infection is successfully controlled as long as a strong Th1-cell response is sustained (18–20), in agreement with previous evidence on the protective role of Th1-cell cytokines against mycobacterial infection (7).

Our results confirmed that the *sigE* mutant is highly attenuated, permitting complete survival of the infected animals after 4 months of infection, with significantly lower bacillary loads and less tissue damage than for animals infected with the parental and complemented strains. Despite the observation that lungs of mice infected with the *sigE* mutant had a lower bacterial burden and inflammation, they exhibited significantly higher expression of IFN-γ and TNF-α than the lungs of mice infected with the parental or complemented strain, suggesting that the *sigE* mutant elicits a stronger immune response. Moreover, the fact that increased levels of IFN-γ and TNF-α were already detectable after 1 day of infection underscores the ability of the *sigE* mutant to very rapidly induce the secretion of these cytokines. These results are in agreement with recent *in vitro* observations of macrophages infected with the same mutant (13). These studies showed that in comparison with resting macrophages infected with the parental strain H37Rv, *sigE* mutant-infected cells exhibited higher expression of the transcriptional factor T-bet and, in consequence, more IFN-γ production. Moreover, IFN-γ-activated macrophages infected *in vitro* with the mutant strain induced high expression of TNF-α (13), which could explain the reason for the high induction of iNOS expression that we detected in the *sigE* mutant-infected lungs. Interestingly, during the late stage of infection, the lungs of mice infected with the *sigE* mutant showed higher expression of IL-10, an anti-inflammatory cytokine that may limit migration of lymphocytes and reduce tissue damage but that under certain conditions can also exert stimulatory effects on CD4 and CD8 T cells, leading to increased IFN-γ production (23, 25). This finding is in perfect agreement with the high production of IL-10 that we previously observed in human dendritic cells infected *in vitro* with the *sigE* mutant (14).

Another interesting observation was the increased expression of β-defensins 3 and 4 in the lungs of mice infected with the *sigE* mutant. These molecules are cationic natural antimicrobial peptides that can kill microbes, and some of them have

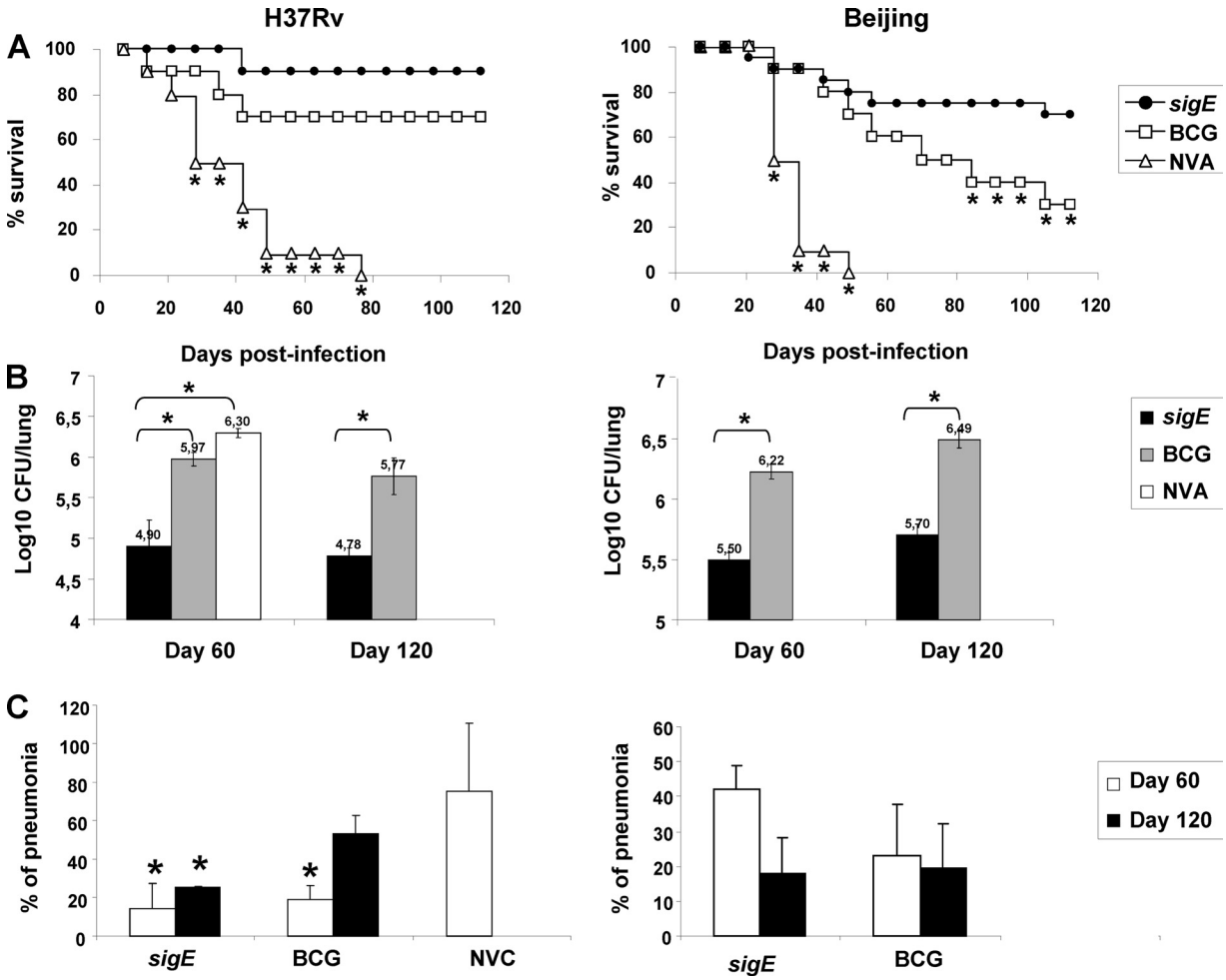

FIG. 6. Survival, lung bacillary loads, and histopathology after intratracheal challenge with H37Rv (left panel) or Beijing 9501000 (right panel) in BALB/c mice vaccinated with the *sigE* mutant or BCG and in control nonvaccinated animals (NVA). (A) Survival of the different groups of BALB/c mice (20 mice per strain) challenged with the indicated strains. (B) Lung bacillary loads in the different groups of BALB/c mice challenged with the indicated strains after 60 and 120 days from challenge. (C) Percentage of lung surface affected by pneumonia, determined by automated morphometry. The results are expressed as the means ± standard deviations of results for four mice. Asterisks represent statistical significance ($P < 0.005$) between the represented groups. No data at 2 and 4 months postchallenge are presented for the control nonvaccinated animals challenged with the Beijing strain and at 4 months postchallenge for the control nonvaccinated animals challenged with H37Rv, since no surviving animals were available.

chemotactic activities on immune cells (9). We have previously shown, in this animal model of tuberculosis, a rapid and high expression of β-defensins 3 and 4 during the phase of efficient control of bacillary replication (32). This finding was in perfect agreement with the observation that macrophages infected *in vitro* with the *sigE* mutant upregulate genes encoding Toll-like receptors 1 and 2 and β-defensins (13). Finally, we showed that mice infected with the *sigE* mutant produced significatively less IL-4 than those infected with the wild-type parental strain. This is of extreme interest, since in our model of infection, induction of IL-4 production correlates with exacerbation of the disease and failure of the immune system to control bacterial replication (17, 36). Thus, the predominant Th1 response plus the high expression of β-defensins in mice infected with the *sigE* mutant could be the basis of its attenuation, allowing 100% survival in association with very low CFU numbers and tissue damage. It is worth noting that *sigE*-complemented strain-infected animals produced IL-4 at levels higher than those produced by animals infected with H37Rv; this might be explained by preliminary unpublished data suggesting that in the complemented strain, the level of *sigE* expression is higher than that in the wild-type strain.

These observations justify the hypothesis that the *sigE* mutant could have strong potential as a novel attenuated vaccine, since the response to its infection fits well into the proposition that the aim of a "classical" vaccine is to mimic natural infection as closely as possible, inducing a strong immune protective response without causing extensive disease (39). In addition, the lack of a Th2 response in the presence of a strong Th1 response is considered one of the essential characteristics for a new antitubercular vaccine (34–36). Moreover, the *sigE* mutant can be considered a good vaccine candidate since it is highly attenuated in SCID mice (27) and at the latest time points produces a significantly lower mortality than BCG in nude

mice. This is of particular importance, since one of the problems of BCG is that it can cause disease in immunocompromised patients, so a new attenuated vaccine strain should be more attenuated than BCG in this kind of patient. Finally, another promising observation was that after vaccination and before challenge, spleen and lung cell suspensions stimulated with mycobacterial antigens from mice vaccinated with the *sigE* mutant were more efficient in the production of IFN-γ than those from animals vaccinated with BCG. Taken together, these observations suggest that the *sigE* mutant is safer and more immunogenic than BCG.

Beside the downregulation of the genes in the σ^E regulon, some of which are involved in surface biology, σ^E absence has a pleiotropic effect on the bacterial surface, as demonstrated by the transcriptional profile of the *sigE* mutant after *in vitro* macrophage infection, showing the induction of genes related to cell wall structure and protein secretion (13). Thus, the *sigE* mutant might have cell envelope defects resulting in both its attenuation and its high immunogenicity. We are currently comparing the composition of the cell envelope and secretome of the *sigE* mutant and wild-type parental strain, H37Rv, in order to identify differences that could be the basis of their different pathogenic and immunogenic behaviors.

Several mycobacterial mutants have already been demonstrated to have good potential as new efficient vaccines (reviewed in reference 10), and three of them have been analyzed using the model of infection used in this work: (i) a mutant lacking *phoP*, which was able to induce protection similar to that of BCG (1); (ii) a mutant lacking *fadD26* (lacking the cell wall lipid complex phthiocerol dimycocerosate), which conferred 70% survival after 4 months of challenge with the highly virulent strain Beijing 9501000 but showed only a partial attenuation (21); (iii) a mutant lacking the mammalian cell entry gene 2 (*mce2*), which was severely attenuated and induced a 72% survival after 4 months of challenge with the highly virulent strain Beijing 9501000 (2). We show here that the *sigE* mutant is as attenuated as the *mce2* mutant but induced better protection, allowing 80% mouse survival after 4 months of challenge with strain Beijing 9501000. Interestingly, mice infected with the *mce2* mutant, in contrast to those infected with the *sigE* mutant, produced significatively less IFN-γ than those infected with the wild-type strain and did not produce more TNF-α. Thus, the *sigE* mutant is until now the best vaccine candidate tested in this experimental mouse model.

In our experiments, the level of BCG-induced protection was lower than that usually provided by this vaccine. This could be explained by the fact that the mice used in this study received Mexico City autoclaved, but not filtered, tap water, known to contain high loads of environmental mycobacteria. Preexposure to these environmental mycobacteria probably resulted in a basal level of immunization against mycobacterial antigens, which has been hypothesized to cause poor responsiveness to BCG vaccination (34, 36). Further experiments in other animal models in which the protective potential of BCG is higher than that obtainable in our model are needed to confirm the superiority of the protective potential of the *sigE* mutant with respect to that of BCG.

We conclude that these results encourage further studies of the *sigE* mutant as a potential vaccine strain; for this purpose, the construction of a double mutant in order to create a more attenuated and highly immunogenic mutant or the overexpression of protective antigens in this strain could represent valuable strategies for further development.

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