Increased vaccine efficacy against tuberculosis of recombinant Mycobacterium bovis bacille Calmette-Guérin mutants that secrete listeriolysin

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The tuberculosis vaccine Mycobacterium bovis bacille Calmette-Guérin (BCG) was equipped with the membrane-perforating listeriolysin (Hly) of Listeria monocytogenes, which was shown to improve protection against Mycobacterium tuberculosis. Following aerosol challenge, the Hly-secreting recombinant BCG (hly+ rBCG) vaccine was shown to protect significantly better against aerosol infection with M. tuberculosis than did the parental BCG strain. The isogenic, urease C–deficient bly+ rBCG (∆ureC bly+ rBCG) vaccine, providing an intraphagosomal pH closer to the acidic pH optimum for Hly activity, exhibited still higher vaccine efficacy than parental BCG. ∆ureC bly+ rBCG also induced profound protection against a member of the M. tuberculosis Beijing/W genotype family while parental BCG failed to do so consistently. Hly not only promoted antigen translocation into the cytoplasm but also apoptosis of infected macrophages. We concluded that superior vaccine efficacy of ∆ureC bly+ rBCG as compared with parental BCG is primarily based on improved cross-priming, which causes enhanced T cell–mediated immunity.

Introduction

Mycobacterium bovis bacille Calmette-Guérin (BCG) represents the most widely used viable vaccine for tuberculosis, with over 3 billion doses administered (1). General agreement exists that BCG can protect against, or at least ameliorate, severe forms of systemic tuberculosis in children, particularly meningitis. However, BCG’s protective value against adult pulmonary tuberculosis ranges from little to none. Although approximately 95% of all tuberculosis cases occur in the developing world, several countries of eastern Europe, including Russia, have witnessed increasing incidences of tuberculosis in recent years, with an estimated 9 million new tuberculosis cases globally in 2002 (2). Worldwide estimated mortality ranges from 1.6 to 2.2 million lives per year, depending on whether the high proportion of multidrug-resistant strains, which are rapidly expanding globally (6, 7). In light of increasing incidences of multidrug-resistant M. tuberculosis strains, the urgent need for a more efficacious tuberculosis vaccine is no longer restricted to the developing world.

The preferred intraphagosomal location of M. tuberculosis and BCG in professional APCs — mostly macrophages and DCs — dictates trafficking of their antigens through the MHC II pathway, which results in preferential stimulation of CD4 T cells (8). Yet, MHC I–restricted CD8 T cells are known to contribute to immunity to M. tuberculosis (8). Recent evidence suggests that M. tuberculosis induces apoptosis in infected cells, which in turn results in the formation of vesicles carrying mycobacterial antigens that can be taken up by bystander APC (9). This process, termed cross-priming, not only promotes MHC I antigen processing and CD8 T cell stimulation but also bypasses immunosuppressive mechanisms imposed on the infected cell (10). BCG only induces weak apoptosis and CD8 T cell stimulation (9). To improve access of mycobacterial antigens to the MHC I pathway, which would result in better CD8 T cell stimulation, we constructed an rBCG strain that secretes listeriolysin (Hly) of Listeria monocytogenes (11). The Hly enables L. monocytogenes to escape from the phagosomes of infected host cells (12). Hly has an acidic pH optimum of greater than 5.8 (13) while BCG modifies the phagosomal pH to remain neutral (14). To improve the pH...
load in (hly, we equipped BCG with the membrane-perforating 6 than load in the lungs of C B 43x53 milieu between pH 4.5 and pH 5.5 (Figure 1, E and F). Similar and D) whereas Hly produced by 43x64 Macrophages were infected with DID-stained BCG strains. Phase 43x141 ureC allowed phagosomal acidification, the pH of mycobacterial 43x152 seen with parental BCG (Figure 1B). To show whether absence of sheep red blood cells were considerably more intense than those 43x174 supernatants from 43x207 Hly of 43x218 tuberculosis 43x229 eradicate a live vaccine that provides improved protection against 43x240 Generation and characterization of 43x251 Results 43x273 in infection mouse model. 43x284 does the parental BCG strain in a low-to-medium dose aerosol-challenged mice as compared with naive controls (Figure 4). Shown is 1 out of 3 representative experiments. (B) Hemolysis 43x405 Superior vaccine efficacy of hly rBCG and ΔureC hly rBCG against M. tuberculosis laboratory strain H37Rv and clinical isolate of the Beijing/W genotype family. BALB/c mice were vaccinated (one single i.p. injection of 10⁶ CFU) with hly rBCG, ΔureC hly rBCG, or parental BCG and aerosol challenged 120 days after vaccination with M. tuberculosis H37Rv microorganisms. At this time point, BCG had been completely eradicated in vaccinated mice (verified by CFU analysis, data not shown). Vaccination with hly rBCG and paren- tal BCG considerably reduced M. tuberculosis load in the lungs of aerosol-challenged mice as compared with naive controls (Figure 2A). In the early (day 30) to intermediate (day 90) phase of infec- tion, the course of M. tuberculosis H37Rv infection did not signifi- cantly differ between the 2 vaccinated groups. However, at late stages (day 150) of infection, the M. tuberculosis H37Rv loads in the lung were significantly (P = 0.016) reduced by 1 order of magni- tude in hly rBCG–vaccinated mice as compared with mice in the parental BCG group.

Although both ΔureC hly rBCG and parental BCG protected against tuberculosis, protection evoked by ΔureC hly rBCG was substantially more successful than that evoked by parental BCG (Figure 2, B and C). This was true for infection with medium doses (200 CFU; Figure 2C) and low doses (30 CFU; Figure 2B) of M. tuberculosis. In the experiment shown in Figure 2B, protection led to an almost 1,000-fold reduction of the M. tuberculosis load in lungs as compared with naive mice at day 200 after challenge. Also, at earlier time points, enhanced protection was seen with ΔureC hly rBCG as compared with parental BCG (Figure 2B).

An equally striking improvement was noted after challenge with the clinical genotype strain M. tuberculosis Beijing/W (Figure 3, A and B). The ΔureC hly rBCG strain significantly (P < 0.001 at day 200) protected against the Beijing/W genotype strain in both lung and spleen over the entire 200-day period of observation (P < 0.05). Compared with vaccination with parental BCG, which virtually failed to protect against M. tuberculosis Beijing/W in this experi-

Figure 1
Hemolytic activity of and phagosomal acidification by ΔureC hly rBCG. (A) RT-PCR analysis of BCG-strains for Hly-secretion. Secretion of Hly was analyzed by RT-PCR using RNA from parental BCG (lane 1), rBCG ΔureC (lane 2), hly rBCG (lane 3), and ΔureC hly rBCG (lane 4). Shown is 1 out of 3 representative experiments. (B) Hemolysis by hly rBCG and ΔureC hly rBCG but not parental BCG. Sheep red blood cells were incubated with hly rBCG (open triangles), ΔureC hly rBCG (closed triangles), parental BCG (closed squares), or L. monocytogenes (open diamonds), or remained untreated (open circles). At indicated time points, aliquots were taken and release of hemoglobin was determined by optical absorption as measurement for lysis of red blood cells. Shown is 1 representative experiment of 4. (C–F) Primary murine macrophages were infected with parental BCG (C and D) or ΔureC hly rBCG (E and F) for 2.5 hours. Images on the left show BCG stained with DID (signal shown in green). Images on the right show a pseudocolor representation of the fluorescence ratio in the blue and green channels with the Lyso Sensor Yellow/Blue dye. Images are merged with a black-and-white phase contrast image. Note that not all bacteria in each batch were stained due to the poor solubility of DID and its high affinity to the hydrophobic cell wall. Scale bar: 20 μm.
ment, vaccination with ΔureC hly+ rBCG reduced CFU in the lung by more than 100-fold.

In vivo safety of hly+ rBCG and ΔureC hly+ rBCG. So that we could analyze the safety of the new rBCG vaccine strains in vivo at high doses, SCID mice were vaccinated i.v. with approximately 10^8 CFU (Figure 4A). At this high dose, SCID mice vaccinated with hly+ rBCG survived significantly longer (mean survival time, 65 days; P = 0.001) than did SCID mice vaccinated with parental BCG (mean survival time, < 25 days). The ΔureC hly+ rBCG–vaccinated mice survived even longer (mean survival time, 80 days; P < 0.001) than mice vaccinated with parental BCG. Thus, in the SCID mouse model, virulence of the rBCG strains was substantially reduced as compared with parental BCG. In further support of the high safety of hly+ rBCG vaccine candidates, CFUs in spleen and lung did not differ among BALB/c wild-type mice vaccinated with the 3 different BCG strains, and all 3 strains were cleared by a similar kinetics (data not shown). Similarly, CFU in spleen and lung of RAG1–/– mutant mice after vaccination with hly+ rBCG and ΔureC hly+ rBCG did not significantly differ from CFU of mice vaccinated with parental BCG (Figure 4B). Consistent with these findings, histological analysis of vaccinated mice did not reveal any alterations in gross pathology in lung, spleen, and liver of either immunocompetent BALB/c mice or RAG1–/– mutant mice (data not shown).

Figure 2
Greater protection against M. tuberculosis aerosol challenge by vaccination with hly+ rBCG and ΔureC hly+ rBCG. (A) BALB/c mice were vaccinated with hly+ rBCG (triangles) or parental BCG (circles) or left naive (squares). At day 120 after vaccination, mice were aerosol challenged with 200 CFU of M. tuberculosis H37Rv. P = 0.016 (Mann-Whitney U test) for hly+ rBCG compared with parental BCG at day 150 after infection (p.i.). Shown is 1 representative experiment of 2. The mean of results from 7 mice is shown (± SEM). (B and C) BALB/c mice were vaccinated with ΔureC hly+ rBCG (diamonds) or parental BCG (circles) or left naive (squares). At day 120 after vaccination, mice were aerosol challenged with 30 CFU (B) and 200 CFU (C) of M. tuberculosis H37Rv. M. tuberculosis load in lung was determined at the indicated time points. Shown are 2 representative experiments. *P = 0.0012 and **P = 0.001 (Mann-Whitney U test) for ΔureC hly+ rBCG compared with parental BCG at day 90 p.i. The mean of results for 7 mice is shown (± SEM).

Figure 3
Greater protection against M. tuberculosis Beijing/W by vaccination with ΔureC hly+ rBCG. BALB/c mice were vaccinated with ΔureC hly+ rBCG (diamonds) or parental BCG (circles) or left naive (squares). At day 120 after vaccination, mice were aerosol challenged with 200 CFU of M. tuberculosis Beijing/W. M. tuberculosis load in lung was determined at the indicated time points. Upper panel: lung, P < 0.001 (Mann-Whitney U test) for ΔureC hly+ rBCG compared with parental BCG at day 200 p.i.; and P < 0.05 (Friedman test) for the whole observation period. Lower panel: spleen, P < 0.001 (Mann-Whitney U test) for ΔureC hly+ rBCG compared with parental BCG at day 200 p.i. Shown is 1 representative experiment of 2. The mean of results for 7 mice is shown (± SEM).
caspase cascade-inducing apoptotic cell death (11, 18–20). Apoptosis in turn results in cross-priming of mycobacterial antigens (9). Due to the immunosuppressive activities of mycobacteria (10), cross-priming would not only promote MHC I, but would also promote MHC II–restricted T cell stimulation. As shown in Figure 5, A–E, mycobacterial antigens were detected in the cytoplasm of macrophages infected with ΔureC hly+ rBCG; the antigens remained more concentrated in the phagosome of cells infected with parental BCG. Release of BCG from the phagosome of ΔureC hly+ rBCG–infected macrophages is indicated by arrows in Figure 5E. When we analyzed the density of the immunogold signal in the cytoplasm of infected cells, we found that ΔureC hly+ rBCG–infected macrophages had significantly (P = 0.026) more mycobacterial antigen in the cytoplasm than did macrophages infected with parental BCG (7.0 ± 3.7 gold particles/μm² for ΔureC hly+ rBCG vs. 3.5 ± 2.3 particles/μm² for parental BCG). Noninfected macrophages showed negligible background staining of 0.14 ± 0.07 particles/μm². Moreover, ΔureC hly+ rBCG induced profound apoptosis in both murine (P < 0.001) and human macrophages (P < 0.05) (Figure 6, A and B, respectively). In contrast, parental BCG marginally increased apoptosis above background levels. The hly+ rBCG–induced macrophage apoptosis was further increased by the absence of the ureC gene in ΔureC hly+ rBCG, presumably due to the optimized pH for Hly activity. These results were supported by TUNEL staining of infected macrophages (Figure 6, C–E). We concluded that cytosolic MHC I loading and, to a greater extent, cross-priming contribute to improved vaccine efficacy of hly+ rBCG.

Discussion
The present report describes superior protection against tuberculosis by vaccination of mice with hly+ rBCG of L. monocytogenes as
compared with vaccination with parental BCG. Murine aerosol-challenge models with 2 different M. tuberculosis strains, the laboratory strain H37Rv and a clinical isolate of the Beijing/W genotype family, revealed that ∆ureC bly+ rBCG and, to a greater extent, ∆ureC bly rBCG vaccine strains were significantly more efficacious in inducing protective immunity against tuberculosis than parental BCG.

The ∆ureC rBCG strain used in our experiments was originally engineered by allelic exchange (15). ureC is involved in the maintenance of neutral intraphagosomal pH within BCG-containing vacuoles (21). Hence, lack of this enzyme allows phagosomal acidification, thereby creating an optimal pH milieu for cytolytic functions of Hly. In addition, absence of ureC has been suggested as promoting MHC II trafficking to the macrophage cell surface (22). Hly is a major virulence factor of L. monocytogenes. By perforating the phagosomal membrane, it facilitates egress of L. monocytogenes into the cytosol. The acidic pH optimum of Hly restricts its activity in the maturing phagosome. However, BCG arrests phagosome maturation at an early stage and prohibits pH acidification. This obstacle of maturation arrest could be overcome, at least partially, by the removal of ureC. In the absence of ureC, the phagosomal pH of infected macrophages first decreases, and full activity of Hly is achieved only at optimal (acidic) pH. It is possible that the pH gradient subsequently breaks down due to proton influx through Hly-mediated pores. We concluded that Hly secreted by ∆ureC bly+ rBCG was more active than that secreted by bly+ rBCG because of an optimized phagosomal pH milieu for Hly.

BCG vaccination protects newborns against conventional M. tuberculosis isolates (1, 7, 23, 24) but has apparently lost vaccine efficacy against newly emerging isolates, notably of the M. tuberculosis Beijing/W genotype family (3, 24–28). In experimental animal models, BCG confers only weak protection against the clinical isolate Beijing/W (29). Consistent with this observation, we found low to absent protection against M. tuberculosis Beijing/W in BCG-vaccinated mice. In contrast, ∆ureC bly+ rBCG not only induced more potent protection against the laboratory strain M. tuberculosis H37Rv but also showed greater protection against M. tuberculosis Beijing/W.

With respect to the mechanisms underlying improved protection by bly+ rBCG, 2 explanations, which are not mutually exclusive, are conceivable. First, the bly+ rBCG vaccine strains may have enhanced cytosolic antigen loading of MHC I molecules in infected APCs to prime CD8+ T cells (17). Consistent with this notion, we detected a higher abundance of mycobacterial antigens in the cytoplasm of macrophages infected with ∆ureC bly+ rBCG as compared with those infected with parental BCG. Second, perforation of the phagosomal membrane by Hly may promote cross-priming by induction of apoptosis within the infected macrophage (9). It remains to be clarified whether M. tuberculosis preferentially induces or inhibits apoptosis (9, 30, 31), and it is likely that the outcome of apoptosis regulation depends on specific conditions. Additionally, we assume that ∆ureC bly+ rBCG induces apoptosis via a different mechanism than does M. tuberculosis. Hly from L. monocytogenes has been shown to induce apoptotic cell death in different cell types (19, 20, 32). It is likely that membrane perforation by Hly secreted by L. monocytogenes or by ∆ureC bly+ rBCG facilitates delivery of phagolysosomal proteases into the cytosol (32), where cathepsins have been shown to induce apoptosis via the mitochondrial pathway (18). Although first experiments with a macrophage cell line did not reveal cytolytic activity of bly+ rBCG by lactate dehydrogenase–release assay (33), we found significant induction of apoptotic cell death by measuring DNA-fragmentation of primary macrophages. Apoptosis of infected cells promotes cross-priming of mycobacterial antigens via uptake of apoptotic vesicles by DCs and therefore improves cell-mediated immune responses (9). Transfer of antigens from infected cells to potent APCs such as DCs may represent an alternative pathway involving infection-mediated apoptosis of host cells, by which CD8+ T cells recognize antigens of the phagosome-secluded tubercle bacillus (9). Recent experiments demonstrated that vesicles containing mycobacterial antigens derived from macrophages infected with BCG are capable of adoptively transferring protection against M. tuberculosis to mice (our unpublished observations).
In sum, we have improved vaccine efficacy of ΔureC hly+ rBCG as compared with hly+ rBCG and parental BCG is best explained by efficient perforation of phagosomal membranes by Hly. Hly meets an acidic pH optimum in the phagosome due to absent ureC activity. Perforation promotes antigen translocation into the cytoplasm and facilitates cross-priming through increased apoptosis. We consider the latter mechanism of major importance for augmented vaccine efficacy of ΔureC hly+ rBCG.

Concerning in vivo safety of the ΔureC hly+ rBCG and hly+ rBCG vaccines, we observed even lower virulence as compared with that of parental BCG in immunodeficient SCID mice, which is most likely due to reduced intracellular persistence of both rBCG strains in infected host cells (34, 35). Consistent with our finding that ΔureC hly+ rBCG was highly attenuated in SCID mice, ΔureC rBCG has already shown a slight decrease in multiplicity and persistence in the lung of vaccinated mice compared with parental BCG (36). This is in agreement with the observation that ureC-deficient BCG is significantly attenuated in its capacity to block MHC class II expression (22). Both for safety and for vaccine efficacy reasons, we selected ΔureC hly+ rBCG for good manufacturing practices (GMP) production and clinical trials after licensing to Vakzine Projekt Management GmbH (Hannover, Germany).

A strategy to improve antigen display by BCG has previously been reported (37). Increased vaccine efficacy of the 2 rBCG strains is based on different mechanisms — elevated antigen secretion by the rBCG strain of Horwitz et al. (37) and altered intracellular behavior of the rBCG strain described here. A combination of both strains, therefore, may be considered for replacing conventional BCG for vaccination of newborns. Recently, several subunit vaccine candidates have been described, which can be used to boost immune responses induced by BCG prime (38–42). It is tempting to assume that a combination of priming with improved BCG such as ΔureC hly+ rBCG and boosting with the most efficacious subunit vaccine would provide more powerful intervention measures against one of the major threats among infectious diseases, tuberculosis.

**Methods**

**Mycobacterial strains and cell lines.** The BCG strains Danish 1331 (Statens Serum Institut, Copenhagen, Denmark) and BCG Pasteur 1173P2 (originally provided by B. Gicquel, Institut Pasteur, Paris, France) were cultured in Dubos broth base (Difco; BD Diagnostics) supplemented with Dubos medium albumin (Difco; BD Diagnostics) at 37°C. A midlogarithmic culture was aliquoted and stored at –70°C until use. The mycobacteria –Escherichia coli shuttle vector pMV306 was used to integrate the hly gene into the mycobacterial chromosome of BCG Danish 1331, which resulted in the respective Hly recombinant strain, hly+ rBCG. ΔureC hly+ rBCG was electrocompetent M. boris BCG Bacterium ΔureCaph (15) was transformed using the chromosomal integrative shuttle vector pMV306hly+Hly, and hygromycin-resistant clones were selected. Plasmid pMV306hly+Hly was obtained by inserting the hygromycin resistance gene from pMV206-hyg into pMV306-Hly (35) to replace the kanamycin-resistance marker. M. tuberculosis H37Rv (originally obtained from J.K. Seydel, Forschungsinstitut Borstel, Borstel, Germany) and M. tuberculosis Beijing/W (RIVM No. 17,919, country of isolation: Mongolia) were passaged in mice and grown in Dubos broth base supplemented with 10% Dubos medium albumin (Difco; BD Diagnostics), then stored in aliquots at –70°C. P815 mastocytoma cells and murine macrophage-like cells J774A.1, both of H-2b haplootype, were obtained from ATCC and cultured in RPMI 1640 (Life Technologies Inc.) supplemented with 10% FCS, penicillin (100 IU/ml), streptomycin (100 IU/ml), and 2-mercaptoethanol. This medium is referred to as RP10 medium.

All animal experiments were conducted with the approval of the Landesamt für Arbeitsschutz, Gesundheitsschutz und technische Sicherheit (Berlin, Germany). BALB/c mice were bred at the central animal facilities of the Max Planck Institute for Infection Biology at the Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinärmedizin (Berlin, Germany). Animals were kept under specific pathogen-free conditions and fed autoclaved food and water ad libitum.

**RT-PCR.** Total RNA from BCG was isolated using TRIzol RNA isolation kit (Gibco BRL; Invitrogen Corp.). The quality, purity, and concentration of the RNA was determined by Bioanalyzer (Agilent). RNA (1 μg) was used for RT-PCR using Access Quick RT-PCR System (Promega). PCR was carried out for 30 cycles (94°C for 1 minute; 57°C for 1 minute; 72°C for 1.5 minutes) with the following primers (MWG Biotech): forward-primer: 5′-AAAGGGGCA-CAGGATGAAAG-3′; reverse-primer: 5′-GATTTCATCCGCGT-GTTTCT-3′. PCR products were analyzed by agarose gel electrophoresis.

**In vitro hemolysis assay.** One ml of a 50% sheep erythrocytes solution in Alsever buffer (Fiebig-Nährstofftechnik) was incubated with each 2 x 10^6 CFU of hly+ rBCG, ΔureC hly+ rBCG, and parental BCG as well as with L. monocytogenes as control at 37°C in a water bath under constant shaking. Every 60 minutes, the absorption at 410 nm was measured in order to determine the amount of free hemoglobin.

**Phagosomal pH measurements.** Bone marrow macrophages or RAW 264.7 murine macrophage cells were grown on glass slides at 37°C and infected with the different BCG strains for various time periods as indicated in the figure legends. To identify lysosomes that contained bacteria, the BCG were stained with DIT (Vibram Cell Labeling Kit; Invitrogen Corp.). LysoSensor Yellow/Blue (Invitrogen Corp.) was added to the medium at a final concentration of 15 μM. This dye is spontaneously taken up by the cells into lysosomes and phagosomes. Fluorescence microscopic experiments were performed with a Leica DM RB microscope equipped with a Leica IL Fluotar 40x/1.00 — 0.50 oil, a Nikon DYM 1200 digital camera, and a 100 W HBO lamp (Osmar). For LysoSensor green fluorescence detection, the filter set was composed of exciter 380/13 nm, dichroic 400 nm, and emitter 560/80 nm, while for blue fluorescence the combination was exciter 360/40 nm, dichroic 400 nm, and emitter 360/40 nm. DID fluorescence was detected with a regular filter set for dark red dyes. For ratiometric analysis, micrographs of dye-labeled phagosomes containing bacteria were recorded. Digital images were processed using ImageJ software version 1.33a (NIH; http://rsb.info.nih.gov/ij). The green and blue channels were extracted from the RGB (red, green, blue) images, and the fluorescence intensity over lysosomes was measured. The ratio of blue to green fluorescence of the lysosomes was calculated according to the following formula: intensity (resulting image) = 25 x intensity (blue)/intensity (green). The resulting images were converted to pseudocolor to indicate pH values. To obtain a pH calibration curve, cells were stained with LysoSensor Yellow/Blue coupled to dextran (Invitrogen Corp.), treated with 10-mM monensin and 10-mM nigericin, and equilibrated for 2 minutes with calibrated PBS buffer solutions (pH from 4.0 to 7.5) prior to image acquisition. In another set of experiments, parental BCG and ΔureC hly+ rBCG were covalently coupled to the pH-sensitive dye Cypher 5 (Amersham Biosciences). This dye emits dark red fluorescence that becomes more intense upon acidification. Cypher 5–coupled bacteria were used to infect murine bone marrow macrophages, and changes in fluorescence intensity over 60 minutes after infection were recorded on a Zeiss Axiovirt 200M microscope equipped with a Cy5 filter set on a Hamamatsu Orca digital camera.

**Determination of apoptosis.** Apoptotic cell death was determined according to the method of Nicoletti et al. (43). Briefly, cells were harvested and centrifuged in a minifuge (Heraeus) at 3,600 for 5 minutes, washed once with PBS, and subsequently resuspended in a buffer containing 0.1% (w/v) sodium citrate, 0.1% (v/v) Triton X-100, and 50 μg/ml propidium
Munsiff, S.S., et al. 2003. Persistence of a highly resistant strain of tuberculosis in New York City. After incubation at 4°C in the dark for at least 16 hours, apoptotic nuclei were quantified by FACScan (BD). For detecting apoptotic nuclei by the TUNEL method, bone marrow macrophages were seeded on glass coverslips, infected with the various BCG strains for 8 and 24 hours, and fixed. The TUNEL assay kit (In Situ Cell Death Detection Kit; Roche Diagnostics Corp.) was employed following the instructions of the manufacturer. The signal of the FITC-labeled nucleotides was intensified using an anti-FITC antibody coupled to Alexa Fluor 488 (Invitrogen Corp.). Nuclei were counterstained with Bisbenzimide (Hoechst 33258), and the percentage of TUNEL-positive cells was determined using ImageJ software version 1.33a.

Confocal microscopy. Bone marrow macrophages grown on coverslips were infected with BCG strains for the indicated periods, fixed in 4% paraformaldehyde, permeibilized in 1% Triton X-100 in PBS, blocked, and stained with an antibody raised against BCG. To indicate localization in the cells, F-actin was stained with phallolidin and DNA with Draq5 (Biostatus Ltd.). Samples were embedded in Mowiol (Sigma-Aldrich) and analyzed with a Leica TCS-SP confocal microscope.

Cryoelectron microscopy. Bone marrow macrophages were infected with BCG strains. Infected cultures were fixed with 4% PFA/PBS and harvested using a rubber policeman. Suspended cells were transferred to a centrifuge tube and pelleted at 1000 g for 2 minutes. The supernatant was discarded and the cells were carefully suspended in 30 μl of melted low melting point agarose (2% PBS; Fluka). Droplets of the suspension were placed on Parafilm (American National Can Company) in a humidified chamber and left to gel on ice. After gelling, droplets were cut to cubes with an edge length of about 1 mm. The cubes were gradually infiltrated in a mixture of 1.6 M saccharose/25% (w/v) polyvinylpyrrolidone in PBS. Infiltrated cubes were mounted on aluminium stubs, snap frozen in liquid nitrogen, and transferred to an RMC ultramicrotome equipped with a cryo chamber. The specimens were trimmed using a diamond trimming device and then cut to ultrathin cryosections. The sections were collected on Formvar-coated nickel grids (Plano) and incubated on blocking buffer (1% BSA, 5% NGS, 10% cold water fish gelatin in PBS). The anti-BCG rabbit serum was diluted to 1:1,000 in the same buffer and reacted with the specimens for 1 hour at 37°C followed by addition of a goat anti-rabbit serum conjugated to 12 nm gold colloids. As controls, noninfected macrophages with anti-BCG rabbit serum was diluted to ultrathin cryosections. The sections were collected on Formvar-coated nickel grids (Plano) and incubated on blocking buffer (1% BSA, 5% NGS, 10% cold water fish gelatin in PBS). The anti-BCG rabbit serum was diluted to 1:1,000 in the same buffer and reacted with the specimens for 1 hour at 37°C followed by addition of a goat anti-rabbit serum conjugated to 12 nm gold colloids. As controls, noninfected macrophages with anti-BCG rabbit serum and nonimmune rabbit serum were used. Sections were analyzed in a Leo 906E transmission electron microscope (Leo/Zeiss). Statistical analysis on cytoplasmic staining intensity was performed using ImageJ software version 1.33a. Twenty random images (magnification, x12,930) of murine bone marrow macrophages infected with the BCG strains or not infected were analyzed for immunogold localization not linked to phagosomes. Staining intensity was expressed as gold particles per μm².

Vaccination and challenge of mice. Mice were immunized i.v. with 10⁶ CFU of BCG strains. Immunization via the i.v. route has been shown to result in the same level of protection against M. tuberculosis challenge as immunization via the s.c. route (44). At 120 days after vaccination, animals were aerosol challenged with 100–200 CFU of M. tuberculosis H37Rv or Beijing/W per lung using an aerosol chamber (Glas-Col) as described (42). The inoculum was confirmed at day 1 after infection by plating the complete lungs and spleens of mice onto Middlebrook 7H11/ampicillin plates (Becton Dickinson).

Statistical analyses. Statistical analyses were performed using Mann-Whitney U test (CFU assays), Friedman test (CFU time courses), log-rank test (survival assays), and 1-way ANOVA for repeated measurements (apoptosis assays). Differences were considered significant at P < 0.05.

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