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

Leander Grode,1 Peter Seiler,1 Sven Baumann,1 Jürgen Hess,1 Volker Brinkmann,1 Ali Nasser Eddine,1 Peggy Mann,1 Christian Goossman,1 Silke Bandermann,1 Debbie Smith,2 Gregory J. Bancroft,2 Jean-Marc Reyrat,3 Dick van Sooijen,4 Bärbel Raupach,1 and Stefan H.E. Kaufmann1

1Max Planck Institute for Infection Biology, Berlin, Germany. 2Immunology Unit, Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, United Kingdom. 3INSERM, Faculté de Médecine Necker-Enfants Malades, Paris, France. 4National Mycobacteria Reference Laboratory, National Institute of Public Health and the Environment, Bilthoven, The Netherlands.

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 (bly+ 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

Nonstandard abbreviations used: BCG, Mycobacterium bovis bacille Calmette-Guérin; Hly, listeriolysin; rBCG, recombinant BCG; bly+ rBCG, Hly-secreting rBCG; ∆ureC bly+ rBCG, ureC–deficient bly+ rBCG; ureC, urease C.

Conflict of interest: L. Grode, S.H.E. Kaufmann, and J. Hess are the inventors of the rBCG strains, and they have exclusively licensed them through the Max Planck Society to Vakzine Projekt Management GmbH (VPM, Hannover, Germany). S.H.E. Kaufmann reports having received research grant support from VPM, and L. Grode is now an employee of VPM.

Citation for this article: J. Clin. Invest. 115:2472-2479 (2005). doi:10.1172/JCI24617.
milieu for Hly activity, a urease C-deficient (ureC-deficient) Hly-secreting BCG–vaccine strain was constructed (15). Mycobacterial ureC plays a role in pH neutralization of the phagosome and thus inhibits maturation of the phagolysosome (14, 16). Here, we demonstrate that Hly-secreting recombinant BCG (hly+ rBCG) and ureC-deficient hly– rBCG (ΔureC hly+ rBCG) mutant strains induce more efficacious protection against M. tuberculosis than does the parental BCG strain in a low-to-medium dose aerosol-infection mouse model.

**Results**

**Generation and characterization of ΔureC hly+ rBCG.** In order to generate a live vaccine that provides improved protection against M. tuberculosis, we equipped BCG with the membrane-perforating Hly of L. monocytogenes (hly+ rBCG) (Figure 1A). To improve the pH milieu for Hly activity, a ΔureC hly+ rBCG strain that lacked ureC activity was constructed. As predicted, hemolytic activities of supernatants from hly+ rBCG and ΔureC hly+ rBCG cultures against sheep red blood cells were considerably more intense than those seen with parental BCG (Figure 1B). To show whether absence of ureC allowed phagosomal acidification, the pH of mycobacterial phagosomes was determined by ratiometric analysis of dye-labeled phagosomes containing parental or ΔureC hly+ rBCG organisms. Macrophages were infected with DIDS-stained BCG strains. Phase contrast with fluorescence overlay is shown in Figure 1, C and E, while parts D and F show the same cells with pH pseudocolor. As expected, parental BCG faced an almost neutral pH (Figure 1, C and D) whereas Hly produced by ΔureC hly+ rBCG faced an acidic milieu between pH 4.5 and pH 5.5 (Figure 1, E and F). Similar effects were observed with parental BCG or ΔureC hly+ rBCG cova-

![Figure 1](https://www.jci.org)  
**Figure 1**  
Hemolytic activity 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 DIDS (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 DIDS and its high affinity to the hydrophobic cell wall. Scale bar: 20 μm.

**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 parental BCG considerably reduced M. tuberculosis load in the lungs of aerosol-challenged mice as compared with naïve controls (Figure 2A). In the early (day 30) to intermediate (day 90) phase of infection, the course of M. tuberculosis H37Rv infection did not significantly 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 magnitude 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 naïve 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-
ment, vaccination with ∆ureC \textit{Hly}+ rBCG reduced CFU in the lung by more than 100-fold.

\textbf{In vivo safety of \textit{Hly}+ rBCG and \textit{ ureC 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 10⁶ CFU (Figure 4A). At this high dose, SCID mice vaccinated with \textit{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 \textit{ ureC 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 \textit{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 \textit{Hly}+ rBCG and \textit{ ureC 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).

\textit{Antigen translocation and apoptosis of host cells infected with \textit{Hly}+ rBCG}. We wanted to gain deeper insight into the biological mechanisms responsible for superior vaccine efficacy of \textit{ ureC Hly}+ rBCG. Two mechanisms can be envisaged, both depending on the capacity of \textit{Hly} to perforate the phagosomal membrane. These mechanisms are not mutually exclusive. First, perforation of the membrane surrounding the mycobacterial phagosome facilitates translocation and subsequent MHC I loading of mycobacterial antigens (11, 17). Second, membrane perforation promotes leakage of phagolysosomal proteases such as cathepsins into the cytosol, which activate
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.

Figure 4
High safety profile of hly+ rBCG and ΔureC hly+ rBCG vaccines. (A) SCID immune-deficient mutant mice were vaccinated i.v. with 0.5 – 1 × 10⁶ CFU of hly+ rBCG (diamonds), ΔureC hly+ rBCG (triangles), and parental BCG (squares) to determine survival rate. P = 0.001 for hly+ rBCG compared with parental BCG, and P < 0.001 for ΔureC hly+ rBCG compared with parental BCG. (B) RAG1−/− immunodeficient mutant mice were vaccinated i.v. with 0.5 – 1 × 10⁶ CFU of hly+ rBCG (diamonds), ΔureC hly+ rBCG (triangles), and parental BCG (squares) to determine bacterial load in lung and spleen. Differences were not statistically significant (P = 0.7 for lung and P = 0.5 for spleen; Mann-Whitney U test). Shown is 1 representative experiment of each of 2 similar experiments.

Figure 5
Antigen presence in the cytosol. (A–D) Bone marrow macrophages infected for 24 hours with ΔureC hly+ rBCG (A and B) and parental BCG (C and D). The images are projections of 2 confocal planes from specimens stained for BCG (green), F-actin (red), and DNA (blue) (overlay in A and C). Material stained with an antibody raised against BCG is dispersed throughout the cytoplasm in cells infected with ΔureC hly+ rBCG while BCG material is exclusively located in bacteria within cells infected with parental BCG. Scale bar in A–D: 10 μm. (E and F) Fine structural analysis of mycobacterial localization in bone marrow macrophages 24 hours after infection with ΔureC hly+ rBCG (E) and parental BCG (F). Panel E shows strong staining of a bacterium in a phagosome; additional staining for mycobacterial material was found in the cytoplasm (indicated by an arrow). However, cytoplasmic staining was greatly reduced in cells infected with parental BCG (F). Quantification of the density of immunogold signal in the cytoplasm of infected cells: parental BCG, 3.5 ± 2.3 gold particles/μm²; ΔureC hly+ rBCG, 7.0 ± 3.7 gold particles/μm²; P = 0.022 (Mann-Whitney U test). Scale bar in E and F: 1 μm.

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 \textit{M. tuberculosis} strains, the laboratory strain H37Rv and a clinical isolate of the Beijing/W genotype family, revealed that \textit{hly} rBCG and, to a greater extent, \textit{∆ureC hly} rBCG vaccine strains were significantly more efficacious in inducing protective immunity against tuberculosis than parental BCG.

The \textit{∆ureC} rBCG strain used in our experiments was originally engineered by allelic exchange (15). \textit{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 \textit{ureC} has been suggested as promoting \textit{M. tuberculosis} H37Rv trafficking to the macrophage cell surface (22). Hly is a major virulence factor of \textit{L. monocytogenes}. By perforating the phagosomal membrane, it facilitates egression of \textit{L. monocytogenes} into the cytosol. The acidic pH optimum of Hly restricts its activity in the maturing phagosome. However, BCG arrests phagosomal maturation at an early stage and prohibits pH acidification. This obstacle of maturation arrest could be overcome, at least partially, by the removal of \textit{ureC}. In the absence of \textit{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 \textit{ΔureC hly} rBCG was more active than that secreted by \textit{hly} rBCG because of an optimized phagosomal pH milieu for Hly.

BCG vaccination protects newborns against conventional \textit{M. tuberculosis} isolates (1, 7, 23, 24) but has apparently lost vaccine efficacy against newly emerging isolates, notably of the \textit{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 \textit{M. tuberculosis} Beijing/W in BCG-vaccinated mice. In contrast, \textit{ΔureC hly} rBCG not only induced more potent protection against the laboratory strain \textit{M. tuberculosis} H37Rv but also showed greater protection against \textit{M. tuberculosis} Beijing/W.

With respect to the mechanisms underlying improved protection by \textit{hly} rBCG, 2 explanations, which are not mutually exclusive, are conceivable. First, the \textit{hly} 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 \textit{ΔureC hly} 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 \textit{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 \textit{ΔureC hly} rBCG induces apoptosis via a different mechanism than does \textit{M. tuberculosis}. Hly from \textit{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 \textit{L. monocytogenes} or by \textit{ΔureC hly} 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 \textit{hly} 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 phagosomally sequestered tubercle bacillus (9). Recent experiments demonstrated that vesicles containing mycobacterial antigens derived from macrophages infected with BCG are capable of adoptively transferring protection against \textit{M. tuberculosis} to mice (our unpublished observations).
In sum, we assume that improved vaccine efficacy of \( \Delta \text{ureC hly} \) rBCG as compared with \( \text{hly} \) 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 \( \Delta \text{ureC hly} \) rBCG.

Concerning in vivo safety of the \( \Delta \text{ureC hly} \) rBCG and \( \text{hly} \) 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 \( \Delta \text{ureC hly} \) rBCG was highly attenuated in SCID mice, \( \Delta \text{ureC rBCG} \) has already shown a slight decrease in multiplication 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 \( \Delta \text{ureC hly} \) 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 \( \Delta \text{ureC hly} \) 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 transferred into Dubos broth base 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—\( \text{M. bovis} \) Beijing/W (RIVM No. 17919, 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 mastocytes cells and murine macrophage-like cells J774A.1, both of H-2\( ^{d} \) haplotype, 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 RPMI medium.

**In vitro hemolysis assay.** One ml of a 50% sheep erythrocyte solution in Alsever buffer (Fiebig-Nährstofftechnik) was incubated with each 2 × 10\(^{6} \) CFU of \( \text{hly} \) rBCG, \( \Delta \text{ureC hly} \) rBCG, and parental BCG as well as with \( \text{L. monocytogenes} \) 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 DIF (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 DMIRB microscope equipped with a Leica I FLUOTAR 40x/1.00 — 0.50 oil, a Nikon DYM 1200 digital camera, and a 100 W HBO lamp (Osram). 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 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 × 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-μM mersensin and 10-μM 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 \( \Delta \text{ureC hly} \) 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 Axiovert 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 minufuge (Heraeus) at 3,600 g 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

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 gesundheitliche 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′-AAGGGGCA-CAGGTATGAAAG-3′; and reverse-primer: 5′-GATTTACCTCCGCT-GTTTCT-3′. PCR products were analyzed by agarose gel electrophoresis.
Vaccination and challenge of mice. Mice were immunized i.v. with 10^6 CFU of BCG strains. Immunization via the i.v. route has been shown to result in the same level of protection against \textit{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 \textit{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 placing the complete lungs and spleens of mice onto Middlebrook 7H11/ampicillin plates (Becton Dickinson).

Statistical analyses. Statistical analyses were performed using Mann-Whitney \textit{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 $P < 0.05$.

Acknowledgments

The authors thank Beatrix Fauler for immunostainings of ultra-thin cryosections and Silke Bandermann for excellent technical assistance. The BCG Pasteur 1173P2 strain was kindly provided by B. Gicquel. We thank D. Russell for providing unpublished data on the impact of ureC on pH neutralization. The paper received financial support from European Union FPS (QLK2-1999-01093) and FPG (LSHP-CT-2003-503367) as well as from the German Science Foundation Priority Programme Novel Vaccination Strategies (SPP 1089, KA 573/4-2).

Received for publication January 31, 2005, and accepted in revised form June 7, 2005.

Address correspondence to: Stefan H.E. Kaufmann, Max Planck Institute for Infection Biology, Department of Immunology, Schumannstrasse 21/22, D-10117 Berlin, Germany. Phone: 49-30-28460-500 or 49-30-28460-506; Fax: 49-30-28460-501; E-mail: Kaufmann@mpib-berlin.mpg.de.

Leander Grode’s present address is: Vakzine-Projekt Management GmbH, Braunschweig, Germany.

Peter Seiler’s present address is: Bayer HealthCare AG, Wuppertal, Germany.

Jürgen Hess’s present address is: responsiv GmbH, Erlangen, Germany.

Leander Grode, Peter Seiler, and Sven Baumann contributed equally to this work.