Relief from Zmp1-Mediated Arrest of Phagosome Maturation Is Associated with Facilitated Presentation and Enhanced Immunogenicity of Mycobacterial Antigens

Pål Johansen,1 Antonia Fettelschoss,1 Beat Amstutz,2 Petra Selchow,2 Ying Waechterle-Men,1 Peter Keller,2 Vojo Deretic,3 Leonhard Held,4 Thomas M. Küng,1 Erik C. Böttger,2,5 and Peter Sander2,5*

Department of Dermatology, University Hospital Zurich, Zurich, Switzerland1; Institute of Medical Microbiology, University of Zurich, Zurich, Switzerland2; Department of Molecular Genetics and Microbiology, University of New Mexico Health Sciences Center, Albuquerque, New Mexico3; Division of Biostatistics, Institute for Social and Preventive Medicine, University of Zurich, Zurich, Switzerland4; and Nationales Zentrum für Mykobakterien, Gloriastrasse 30/32, 8006 Zurich, Switzerland5

Received 27 December 2010/Returned for modification 3 February 2011/Accepted 28 March 2011

Pathogenic mycobacteria escape host innate immune responses by blocking phagosome-lysosome fusion. Avoiding lysosomal delivery may also be involved in the capacity of mycobacteria to evade major histocompatibility complex (MHC) class I- or II-dependent T-cell responses. In this study, we used a genetic mutant of Mycobacterium bovis BCG that is unable to escape lysosomal transfer and show that presentation of mycobacterial antigens is affected by the site of intracellular residence. Compared to infection with wild-type BCG, infection of murine bone marrow-derived dendritic cells with a mycobacterial mutant deficient in zinc metalloprotease 1 (Zmp1) resulted in increased presentation of MHC class II-restricted antigens, as assessed by activation of mycobacterial Ag85A-specific T-cell hybridomas. The zmp1 deletion mutant was more immunogenic in vivo, as measured by delayed-type hypersensitivity (DTH), antigen-specific lymphocyte proliferation, and the frequency of antigen-specific gamma interferon (IFN-γ)-producing lymphocytes of both CD4 and CD8 subsets. In conclusion, our results suggest that phagosome maturation and lysosomal delivery of BCG facilitate mycobacterial antigen presentation and enhance immunogenicity.

Mycobacterium tuberculosis is a pathogen with one of the highest death tolls, and its morbid success is in part ascribed to the bacterium’s ability to manipulate host defense mechanisms by evading or controlling host immune responses. One hallmark of M. tuberculosis infection is that the bacterium resists lysosomal delivery following phagocytosis (30). Phagolysosomes are equipped with the machinery to generate peptide-MHC class II complexes (39), and inhibition of phagosome-lysosome fusion is one proposed mechanism by which M. tuberculosis may escape efficient MHC class II antigen presentation (25). Arrest of phagosome maturation may also affect cross-presentation of mycobacterial peptides via the putative phagosome-to-cytosol MHC class I antigen presentation pathway (21) or via MHC class I presentation that occurs by fusion and fission of phagosomes with endoplasmic reticulum-derived vesicles containing newly synthesized MHC I molecules (27).

BCG is a live attenuated vaccine derived from Mycobacterium bovis. More than four billion doses of the vaccine have been administered worldwide since its introduction in 1921. Although relatively safe and inexpensive, the efficacy of BCG to protect against adult lung tuberculosis is highly variable (7). The reasons for the poor efficacy of BCG in protection against tuberculosis are poorly understood. One explanation builds on the observation that BCG has lost important genes during the laboratory attenuation process (2). As a consequence, much work has been done to improve, with varying success, the efficacy of BCG by introducing additional copies of existing genes (14) or by reintroducing some of the genes that were lost during the in vitro attenuation process (26).

It is widely assumed that tuberculosis disease will protect, at least partially, against subsequent reinfection (1). However, the failure of natural disease to protect against reinfection (36) indicates that immunity evoked by natural infection is limited, partially explaining the relative ineffectiveness of vaccination with BCG (24). The limited postinfection immunity may also indicate that M. tuberculosis actively escapes immune surveillance (12, 25, 28). We have previously reported that a putative mycobacterial zinc metalloprotease, Zmp1, plays an important role in disease pathogenesis by interfering with inflammasome activation and phagosome maturation (23), two central pathways of pathogen defense. In the present study, we hypothesized that the limited efficacy of BCG reflects the natural course of tuberculosis infection and the organism’s ability to subvert the immune system. Consequently, rather than attempting to improve BCG efficacy by introducing additional genes, we followed the idea that further genetic deletions might increase BCG immunogenicity.

Processing and presentation of mycobacterial antigens are impaired in mycobacterial infections (30), and it is important to determine whether this is connected with phagosome maturation. Pursuing the hypothesis that the mechanism through which a vaccine is processed in antigen-presenting cells affects its efficacy, we speculated that zmp1 deletion might alter BCG immunogenicity. Using an M. bovis BCG mutant lacking the zmp1 gene, we assessed the possible influence of Zmp1 on
presentation of mycobacterial antigens by professional anti-
gen-presenting cells and the induction of CD4- and CD8-posi-
tive T-cell responses to mycobacterial antigens in vivo. By
various measures of immune reactivity, we show that Zmp1
deletion increased the immunogenicity of BCG both in vitro
and in vivo.

MATERIALS AND METHODS

Mice. Female BALB/c (H-2b) or C57BL/6 (H-2b) mice were obtained from Harlan (Horst, Netherlands). Female SCID (C57-17/Prkdc(scid)) mice were obtained from the Jackson Laboratory (Bar Harbor, ME). All mice were ob-
tained and kept under specific-pathogen-free (SPF) conditions in facilities at the University Hospital Zurich and were used at 6 to 10 weeks of age. The experi-
ments were reviewed by the local ethical committee and were performed accord-
ing to Swiss experimental and ethical guidelines.

Preparation and cultivation of bacteria. Mycobacterial strains were grown and propagated according to standard microbiological techniques. The con-
struction of the BCGzmp1 knockout mutant has been described previously (23). BCGzmp1 was complemented by transformation with the zmp1-contain-
ing plasmid, PMV361-hyg-zmp1, and the complemented BCG strain is hence-
forth referred to as BCGcompl. Zmp1 expression in the complemented mutant is under the control of the zmp1 promoter.

Western blot analyses using rabbit anti-Ag85 antisum (gift from Colorado State University) showed comparable expression levels for Ag85 in all three strains: BCG, BCGzmp1, BCGcompl (data not shown).

Confocal imaging. Bone marrow stem cells were isolated from mice femurs and differentiated for 7 days in Dulbecco's modified Eagle's medium supple-
mented with 10% fetal calf serum (FCS), 10% L-cell conditioned medium, and penicillin/streptomycin on petri dishes. The cells were then mounted on 0.7-mm
glass coverslips in 24-well plates at 1.5 × 10^5 cells per well and infected with BCG as described previously (29). Colocalization studies were done as described previ-
ously (29), and the coverslip contents were left unidentified before analysis.

Antigen presentation studies in dendritic cells. Dendritic cells (DCs) were prepared from C57BL/6 and BALB/c mice as described previously (17). Briefly, femurs were aseptically harvested and bone marrow cells were cultured in RPMI 1640 medium supplemented with 10% FCS, glutamine, sodium pyruvate, peni-
cillin, and streptomycin in the presence of 10% supernatant from granulocyte-
macrophage colony-stimulating factor (GM-CSF)-secreting X-63 cells. The X-63
cell line was transfected and kindly provided by A. Rolink (University of Basel).
After 6 to 7 days, 1 × 10^5 DCs per well were plated in 96-well flat-bottom culture plates and infected with BCGwt, BCGzmp1, or BCGcompl at different multiplic-
ties of infection (MOIs) (10, 1, 0.1, 0.01, or 0.001). Fluorescence-activated cell sorter (FACS) analyses of DCs infected with Alexa Fluor 488-labeled mycobacteria demonstrated comparable mean fluorescence levels for BCGwt, BCGzmp1, and BCGcompl, and fluorescence microcopy of macrophages showed similar infec-
tion rates for all three strains (data not shown). Within 1 h, 1 × 10^6 De10 (H-2b) or 2E5 (H-2b, H-2a) T-cell hybridoma cells specific for the M. tuberculosis Ag85A peptides comprising amino acids 241 to 260 [Ag85A(241–260)] and amino acids 101 to 120 [Ag85A(101–120)], respectively, were added to each well. The anti-
genants were purchased from EMK (Tübingen, Germany), and the hybridomas were kindly provided by C. Leclerc (Institut Pasteur, Paris). After incubation at 37°C
for 20 h, supernatants were collected and frozen for later analysis of interleukin-2 (IL-2) by standard enzyme-linked immunosorbent assay (ELISA) (R&D Sys-
tems, Abingdon, United Kingdom).

Analysis of delayed-type hypersensitivity. C57BL/6 mice were immunized with different doses of BCGwt or BCGzmp1 in 100 μl phosphate-buffered saline (PBS) by subcutaneous injections in the neck region. After 3 weeks, the mice were challenged by injection of 50 μl of a 5-μg/ml solution of tuberculin purified protein derivative (PPD) (SSI, Copenhagen, Denmark) in saline, into the plantar
side of the hind right footpad. Injection of saline alone was used as a negative control. One to 4 days later, a delayed-type hypersensitivity (DTH) reaction was analyzed by measuring the swelling of the footpad in comparison to the prechall-
enge length of the footpad. The measurements were done using a spring-
loaded digital micrometer from Mitutoyo (Kawasaki, Japan).

Immunogenicity testing. C57BL/6 mice were immunized by subcutaneous delivery of 10^6 CFU BCG on days 0 and 21. On day 28, the mice were euthanized, and spleens harvested. The spleens were homogenized, the erythro-
cytes were lysed in a hypotonic buffer, and triplicates of 2 × 10^6 splenocytes were restimulated in vitro with 5 or 1 μg/ml PPD for determination of proliferation and cytokine secretion. After 3 days of cultivation, supernatants were collected
and frozen for later analysis of IFN-γ secretion by ELISA (R&D Systems). Parallel cultures were pulsed with 3H-labeled thymidine at 1 μCi per well for an additional 1 h for analysis of proliferation by β-scintillation.

IFN-γ-producing cells were analyzed by enzyme-linked immunospot (ELISPOT) assay (Diaclone, Besançon, France) by restimulating 2 × 10^5 splenocytes from immunized mice with or without antigen for 16 h in multiscreen 96-well Millipore polyvinylidene difluoride (PVDF) plates (Fisher Scientific AG, Wohlen, Swit-
zerland) prewetted with 1 μg/ml anti-IFN-γ. The ELISPOT plates were then washed and developed according to the manufacturer’s protocol. The spots were analyzed on an AID EliSpot reader system from Autoimmun Diagnos-
tika (Strassberg, Germany), and the results were expressed as spots per 2 × 10^5 splenocytes.

IFN-γ-producing splenocytes were analyzed by intracellular staining and flow cytometry. Triplicates of 2 × 10^5 splenocytes were restimulated in 24-well plates at 37°C for 1 h with 5 μg/ml PPD, the last 4 h also with 2.5 μg/ml brefeldin A (Sigma-Aldrich, Buchs, Switzerland). The cells were washed, fixed in protein-free PBS/paraformaldehyde (1%) on ice for 10 min, washed, and then permeabilized in PBS/NI-40 (0.1%) on ice for 3 min. After washing, the cells were resuspended in PBS/NI-40 (2%), incubated on ice for 5 min with anti-CD16/CD32 for Fc-
receptor blocking, and then stained with anti-CD4 (fluorescein isothiocyanate [FITC]), anti-CD8 (PerCP-Cy5), anti-CD44 (phycoerythrin [PE]), and anti-
IFN-γ (allophycocyanin [APC]) antibodies for 40 min. After washing, the cells were acquired using a FACS Canto (BD Biosciences, San Jose, CA). All anti-
bodies were purchased from BD Pharmingen (Basel, Switzerland) or from eBio-
science (Bender MedSystems, Vienna, Austria). The analysis was done using the FlowJo 8.5.2 software from Tree Star, Inc. (Ashland, OR), and the frequency of IFN-γ-producing cells was defined by gating on CD44-positive CD4 or CD8 lymphocytes.

Persistence of BCG in immunocompetent mice. The effect of Zmp1 on the growth of BCG in immunocompetent mice was tested after tail vein injection of
10^6 CFU of BCGwt or BCGzmp1 in C57BL/6 mice (n = 40). At different time
points thereafter, 5 to 7 animals from each group were euthanized, and the lungs, spleen, and frontal lobe of each animal were harvested aseptically in 5 ml PBS. The tissues were homogenized using a Polytron PT 3000 from Kinematica (Littau, Switzerland), incubated for 1 h in 0.5% quillaja bark saponin (Sigma-
Aldrich), serially diluted, and plated on Middlebrook 7H10 plates for the deter-
mination of the numbers of BCG bacteria. The colonies were enumerated after 3 weeks of incubation at 37°C.

Pathogenicity of BCG in SCID mice. To test whether the deletion of Zmp1 in BCG affects its pathogenicity in immunodeficient mice, the survival of SCID mice upon inoculation with BCGwt and BCGzmp1 was assessed. The two BCG strains were administered by tail vein injections at 10^7, 10^8, and 10^9 CFU in groups of six SCID mice. Body weight and survival were assessed over a period of 6 months. The animals were euthanized when they met the preestablished endpoint (20% weight loss compared to the weight at the day of inoculation). Statistical analysis. For the analysis of dependent observations, a random-
effects analysis of variance (ANOVA) was performed, with the BCG strain, immunization dose, and type of antigen used for restimulation as fixed factors
and the individual mouse as a random effect. Prior to ANOVA, the data were square root transformed to meet the equal variance assumption. Nonparametric and independent data were analyzed by the two-sided Mann-Whitney U test for two groups or by the Kruskal-Wallis test for three groups or more. For analysis of the survival data, mean survival time and hazard ratios (with 95% confidence intervals) were computed. Kaplan-Meier survival curves were compared with the use of the Cox proportional-hazards models, and statistical significance was tested by log rank test. Analyses were done using the statistical software R (30) or GraphPad Prism. The significance level was set at 5%.

RESULTS

Zmp1-dependent phagosome maturation arrest. At early
stages, the course of M. tuberculosis infection in macrophages is characterized by the bacterially induced arrest of phago-
somal maturation. This allows the bacterium to replicate in vacuoles that retain a high pH, show limited hydrolytic activity, and interact poorly with antigen presentation pathways (30). BCGwt arrests phagosome maturation at the stage of an early
endosome. However, deletion of zmp1 suspends the ability of the bacteria to arrest phagosome maturation, and BCGzmp1 colocal-

Clin. Vaccine Immunol. 908 JOHANSEN ET AL.
in vivo antigen presentation of BCG. Zmp1 interferes with innate immune responses (23), which prompted us to investigate whether the deletion of Zmp1 affects mycobacterial antigen presentation in professional antigen-presenting cells. Bone marrow DCs were prepared and infected at different MOIs with BCG wt, BCGzmp1, or BCGcompl. The infected DCs were incubated with MHC class II-restricted T-cell hybridomas DE10 and 2E5, respectively, and the secretion of IL-2 from the hybridoma cells was measured as a marker of antigen-stimulated T-cell activation. Presentation of Ag85A antigens was found to be more efficient in cells infected with the wild type or the complemented strains in cells infected with the Zmp1-deficient BCG strain than in cells infected with the wild type or the complemented strains (Fig. 2). Increased antigen presentation in BCGzmp1-infected cells was independent of MHC restriction, as it was observed in DCs derived from both H-2b C57BL/6 (Fig. 2A) (P = 0.04) and H-2d BALB/c (Fig. 2B) (P < 0.0001) mice.

Immunogenicity of the BCG wild type and Zmp1 mutants. To analyze the role of Zmp1 in the immunogenicity of BCG, mice were immunized with titrated doses of wild-type and Zmp1-deficient strains. Subsequently, we measured T-cell responses by assessing DTH to PPD injections into footpads. Different thresholds for the two strains were observed. While inoculation of 10⁵ CFU BCG wt was required to produce notable and specific DTH to PPD, 10³ CFU was sufficient to induce an equivalent response with BCGzmp1 (Fig. 3A). BCGzmp1 induced a significantly stronger DTH response than did BCGwt at 10⁵ CFU (P = 0.0004), while the difference at 10⁴ CFU (P = 0.08) was of borderline significance (Fig. 3A). The peak of footpad swelling was observed with inoculations of between 10⁴ and 10⁵ CFU (data not shown). Data from a second independent experiment were available, and the analysis of the combined data (n = 78) verified a significantly stronger DTH response at both 10⁴ (P < 0.0001) and 10⁵ (P = 0.04) CFU. When the kinetics of the DTH response were analyzed for inocula of 10⁴ CFU BCG, the results revealed that BCGzmp1 produced stronger and faster DTH responses than BCGwt, the maximum swelling being reached 48 h earlier for BCGzmp1 than for BCGwt (Fig. 3B).

The increased immunogenicity of the Zmp1-deficient and lysosome-targeting BCG strain was associated with increased antigen-specific T-cell proliferation and cytokine secretion of splenocytes from immunized mice. Figure 4A illustrates antigen-specific T-cell proliferation after inoculation of 10⁵ CFU BCG and for splenocytes restimulated with PPD. First, while 1 μg/ml was sufficient for restimulation and proliferation of splenocytes from mice immunized with BCGzmp1, a 5-fold higher concentration was required for splenocytes harvested from mice immunized with BCGwt or the rescued, complemented mutant BCG. In addition, the degree of proliferation was significantly increased following BCGzmp1 immunization (P = 0.02 by ANOVA). The assessment of secreted IFN-γ by ELISA revealed increased cytokine amounts following immunization with BCGzmp1 compared to BCGwt (P < 0.0001).
In additional experiments, mice were immunized as described above, but the splenocytes were stimulated with mycobacterial Ag85A or TB10.3 antigens (not shown). A two-way random-effects ANOVA revealed that splenocytes from BCG zmp1-deleted mutant than in mice immunized with BCG wt. The results with untreated controls, BCG primed T cells for production (CD4, P = 0.004; CD8, P = 0.0006; Mann-Whitney test). Splenocytes from mice immunized with BCG wt contained significantly higher frequencies of IFN-γ-producing CD4 (P = 0.004) and CD8 (P = 0.008) T cells than did splenocytes from mice immunized with BCG wt or BCG zmp1, while the two latter strains induced comparable numbers of IFN-γ positive cells.

In additional experiments, mice were immunized as described above, but the splenocytes were stimulated with mycobacterial Ag85A or TB10.3 antigens (not shown). A two-way random-effects ANOVA revealed that splenocytes from BCG zmp1-immunized mice had a stronger proliferation capacity (P = 0.007) and produced more IFN-γ (P = 0.02) than splenocytes from BCG wt-immunized mice. Here, a three-way random-effects ANOVA applied to all data available (n = 360 observations from 30 mice) revealed that stronger immune reactions were observed in mice immunized with the zmp1-deleted mutant than in mice immunized with BCG wt (P < 0.0001), independent of the in vivo BCG dose or the type of antigen used for restimulation in vitro.

**BCG persistence and growth in wild-type and SCID mice.** To test if deletion of zmp1 and increased phagosome maturation is associated with differences in dissemination and persistence of BCG, wild-type mice were infected with 10⁶ CFU BCG wt or BCG zmp1 intravenously. General signs of disease and the bacterial load of various organs were monitored over a period of 85 days. No overt signs of clinical disease were observed during the experiment. Systemic infection caused a high mycobacterial load in the spleen and liver (Fig. 5). The titers slowly declined, but mycobacteria were still detectable in livers and in spleens nearly 3 months postinfection. The bacterial load in the lungs was approximately 1% of the load in the liver 1 day postinfection and declined to undetectable levels at the end of the study (not shown). No difference in dissemination or persistence was observed between BCG wt and BCG zmp1.

We also addressed the possible unexpected adverse effects of zmp1 deletion by infecting SCID mice and monitoring their survival. The animals received 10⁶, 10⁷, or 10⁸ CFU of either BCG zmp1 or the parental BCG wt strain by tail vein injection, and their survival was monitored. The criterion for termination was a weight loss of 20%, as illustrated in Fig. 6A for an inoculum of 10⁶ CFU BCG. The mice started to lose weight after 5 to 6 weeks following infection and had to be euthanized at approximately 9 to 12 weeks postinfection, independent of Zmp1 expression (Fig. 6A). The Kaplan-Meier survival curves for all inoculum doses revealed dose dependency but strain independency with mean survival times of 9 and 16.5 days (10⁶ CFU), 42.5 and 44 days (10⁷ CFU), and 78.5 and 80.5 days (10⁸ CFU) for BCG wt and BCG zmp1, respectively (Fig. 6B). Statistical analysis comprising dose pairwise comparisons of mice infected with BCG zmp1 or BCG wt using log rank and hazard
The present study shows that a genetic deletion of BCG may evoke improved antigen presentation and immunogenicity. Our approach is based on two assumptions. First, *M. tuberculosis* has evolved to subvert antimycobacterial immune responses by arresting the maturation of phagosomes (30). As a result, phagosome-lysosome fusion is blocked and efficient MHC class II-mediated antigen presentation is impaired. Second, BCG has, at least partially, retained these properties. We characterized a BCG mutant that is deficient in the gene encoding zinc metalloprotease 1 (Zmp1), a protein which previously has been shown to inhibit phagosome maturation by preventing inflammasome activation and caspase-dependent IL-1β production (23). Using mycobacterial Ag85A-specific T-cell hybridomas, we found that infection of murine DCs with Zmp1-deficient *M. bovis* BCG significantly enhanced antigen presentation compared to that of wild-type BCG. This was observed for both I-Ab- and I-Eα-restricted MHC class II pathways of presentation, as similar data were obtained with C57BL/6 (H-2b) and BALB/c (H-2d) mice, respectively. Increased antigen presentation was accompanied by enhanced BCG immunogenicity: (i) the DTH reaction upon immunization with BCG/zmp1 was obtained with 1/10 of the dose required for BCG/wt, (ii) the induction of antigen-specific splenocyte proliferation was increased, (iii) the frequency of antigen-specific IFN-γ-producing CD4 and CD8 T cells was higher, and (iv) the amount of IFN-γ secreted was heightened following immunization with BCG/zmp1. To directly demonstrate that the enhanced immunogenicity of the zmp1 deletion mutant is due to inactivation of the structural zmp1 gene, we complemented the zmp-deficient mutant with wild-type zmp1. Complementation with zmp1 abrogated phagosome maturation (23), and the mutants increased immunogenicity.

Despite extensive use of BCG, the correlates of protection are still ill defined (19). While activated macrophages are critical for disease containment (6), both hypo- and hyperactivation have been suggested to contribute to disease progression (42). The finding that zmp1 deletion did not affect survival of BCG-infected immunodeficient SCID mice nor BCG growth and dissemination in immunocompetent mice indicates that the enhanced immunogenicity of BCG/zmp1 does not come at the cost of decreased persistency or heightened pathology. The exact mechanism of mycobacterium-induced phagosome maturation arrest is still unclear. The bacteria are taken up by macrophages or DCs and are internalized into phagosomes that fuse with early and late endosomes (4, 31, 33). Phagosome maturation from early phagosomes to phagolysosomes comprises a series of fusion, fission, and trafficking events, which depend on a complex network of Rab GTPases and phospholipids that control endocytic processes (3, 32, 34, 35). Besides its direct antibacterial effects, the lysosome and its acidic environment are vital for antigen presentation through degradation and unfolding of bacterial proteins and the substitution of the invariant chain that allows binding to MHC class II molecules (30). In addition to Zmp1, several other proteins and lipids reportedly inhibit phagosome maturation and lysosomal delivery of pathogenic mycobacteria. Cell wall lipids are associated with the blocking of phagosome-lysosome fusion, e.g., lipoarabinomannan (8, 13, 37), sulfolipids (9), and trehalose dimycolate (15), and the bacterial phosphatase SapM (38), the...
nucleoside diphosphate kinase Ndk (34), and the serine/threonine protein kinase G (PknG) (5, 40) have all been suggested to affect the maturation of phagosomes.

Our results are at variance with recent observations that the arrest of phagosome maturation, as mediated by deletion of the serine/threonine protein kinase G (PknG) (5, 40), does not affect the presentation of BCG antigens (22). While we cannot directly explain this discrepancy, subtle, yet unknown differences in phagosome maturation may be responsible for functional differences between the Zmp1- and the PknG-deficient mutants. Support for our conclusion that phagosome maturation does play a role in presentation and immunogenicity of mycobacterial antigens is provided by recent data from studies in which rapamycin was used to pharmacologically enhance trafficking of BCG to lysosomes by means of autophagy. Upon treatment with rapamycin, mycobacteria colocalized with the late endosomal markers Rab7 and CD63, and this result correlated with enhanced antigen presentation (16). In addition, Ag85A-deficient virulent Mycobacterium tuberculosis bacteria reside in a phagosomal compartment enriched for the acidicotropic dye Lysotracker and LAMP-1, suggesting enhanced fusion with late endosomes, and this is correlated with improved T-cell priming in vitro (18). Together with our results, these studies indicate that phagosome maturation and antigen presentation are interconnected and that the immunogenicity of BCG is affected by triggering phagosome maturation and lysosomal delivery of mycobacterial antigens.

In summary, this study demonstrates that deletion mutagenesis of BCG may not only facilitate phagosome maturation but also increase antigen presentation and immunogenicity of BCG. These findings may lead to a better understanding of the molecular mechanisms involved in tuberculosis-associated subversion of the host’s immune system. The results also suggest that deletion of genes that suppress antigen presentation and immunogenicity is a promising approach in the rational design of new vaccines against M. tuberculosis.

ACKNOWLEDGMENTS

This project was supported in part by the University of Zurich, the European Union (EU-FP7 New TBVac, project no. 241745), the Swiss National Science Foundation (31003A-120326), and the Niedersächsischer Verein zur Bekämpfung der Tuberkulose, Lungen- und Bronchialerkrankungen e.V. P.J., T.M.K. (3100AO-122221), and Y.W.-M. (3200BO-118202) were supported by the Swiss National Science Foundation. The authors disclose no conflict of interest.

We thank Nicole Graf for assistance with statistical analysis, Deepa Mohanan for FACS sorting of infected dendritic cells, and Claude Chavrier, P., R. G. Parton, H. P. Hauri, K. Simons, and M. Zerial.

REFERENCES

1. Barnes, P. F., and M. D. Cave. 2003. Molecular epidemiology of tuberculosis. N. Engl. J. Med. 349:1149–1156.
2. Behr, M. A., and P. M. Small. 1997. Has BCG attenuated to impotence? Nature 389:133–134.
3. Chavrier, P., R. G. Parton, H. P. Hauri, K. Simons, and M. Zerial. 1999. Localization of low molecular weight GTP binding proteins to exocytic and endocytic compartments. Cell 98:317–329.
4. Clemens, D. L., and M. A. Horwitz. 1996. The Mycobacterium tuberculosis phagosome interacts with early endosomes and is accessible to exogenously administered transferrin. J. Exp. Med. 184:1349–1355.
5. Cowley, S., et al. 2004. The Mycobacterium tuberculosis protein serine/threonine kinase PknG is linked to cellular glutamate/glutamine levels and is important for growth in vivo. Mol. Microbiol. 52:1691–1702.
6. Dorboi, A., S. T. Reece, and S. H. Kaufmann. 2011. For better or for worse: the immune response against Mycobacterium tuberculosis balances pathology and protection. Immunol. Rev. 240:235–251.
7. Fine, P. E. 1995. Variation in protection by BCG: implications of and for heterologous immunity. Lancet 346:1339–1345.
8. Fratti, R. A., J. Chua, I. Vergne, and V. Deretic. 2003. Mycobacterium tuberculosis glycosylated phospholipidylinositol causes phagosome maturation arrest. Proc. Natl. Acad. Sci. U. S. A. 100:5437–5442.
9. Grobe, L. F., et al. 2005. Increased vaccine efficacy against tuberculosis of recombinant Mycobacterium bovis bacille Calmette-Guérin mutants that secrete listeriolysin. J. Clin. Invest. 115:2472–2479.
10. Hess, J., et al. 2000. Secretion of different listeriolysin cognates by recombinant attenuated Salmonella Typhimurium: superior efficacy of haemolytic non-haemolytic constructs after oral vaccination. Microbes Infect. 2:1799–1806.
11. Hmama, Z., R. Gabathuler, W. A. Jeffries, G. de Jong, and N. E. Reiner. 1998. Enhancement of HLA-DR expression by mononuclear phagocytes in infected mice with Mycobacterium leprae, Mycobacterium marinum and M. tuberculosis. J. Immunol. 161:8882–4893.
12. Hmama, Z., et al. 2004. Quantitative analysis of phagolysosome fusion in intact cells: inhibition by mycobacterial lipoxaraminomannan and rescue by an alpha1-2,3-diacylglucosaminyl lipid A. J. Pathol. 204:118–126.
13. Horwitz, M. A., G. Harth, B. J. Dillon, and S. Maslesa-Galic. 2000. Recombinant bacillus Calmette-Guérin (BCG) vaccines expressing the Mycobacterium tuberculosis 30-kDa secretory protein induce greater protective immunity against tuberculosis than conventional BCG vaccines in a highly susceptible animal model. Proc. Natl. Acad. Sci. U. S. A. 97:13853–13858.
14. Indrigo, J. R., R. L. Hunter, Jr., and J. K. Actor. 2003. Cord factor trehalose 6,6-dimycoclate (TDM) mediates trafficking events during mycobacterial infection of murine macrophages. Microbiology 149:2049–2059.
15. Jagannath, C., et al. 2009. Autophagy enhances the efficacy of BCG vaccine by increasing peptide presentation in mouse dendritic cells. Nat. Med. 15:767–770.
16. Johansen, P., et al. 2003. Anti-mycobacterial immunity induced by a single injection of M. leprae Hsp65-encoding plasmid DNA in biodegradable microparticles. Immunol. Lett. 90:81–85.
17. Katti, M. K., et al. 2008. A DeltaPA mutant derived from Mycobacterium tuberculosis H37Rv has an enhanced susceptibility to intracellular antimicrobial oxidative mechanisms, undergoes limited phagosome maturation and activates macrophages and dendritic cells. Cell. Microbiol. 10:1266–1303.
18. Kaufmann, S. H. 2010. Future vaccination strategies against tuberculosis: an overview outside the box. Immunol. Rev. 237:43–57.
19. Kaufmann, S. H., G. Hussey, and P. H. Lambert. 2010. New vaccines for tuberculosis. Lancet 375:2110–2119.
20. Kovacs-cvics-Bankowski, M., and K. L. Rock. 1995. A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules. Science 267:243–246.
21. Majless, L., et al. 2007. Inhibition of phagosome maturation by mycobacteria does not interfere with presentation of mycobacterial antigens by MHC molecules. J. Immunol. 179:1825–1833.
22. Master, S. S., et al. 2008. Mycobacterium tuberculosis prevents inflammasome activation. Cell Host Microbe 3:224–232.
23. Mollenkopf, H. J., M. Kursar, and S. H. Kaufmann. 2004. Immune response to posterior tuberculosis in mice: Mycobacterium tuberculosis and Mycobacterium bovis bacille Calmette-Guérin induce equal protection. J. Infect. Dis. 190:588–597.
24. Pancol, P., A. Mirza, N. Bhardwaj, and R. M. Steinman. 1993. Sequestration from immune CD4+ T cells of mycobacteria growing in human macrophages. Science 260:984–986.
25. Pym, A. S., et al. 2003. Recombinant BCG exporting ESAT-6 confers enhanced protection against tuberculosis. Nat. Med. 9:533–539.
26. Raghavan, M., N. Del Cid, S. M. Rizvi, and L. R. Peters. 2008. MHC class I-restricted listeriolysin. J. Clin. Invest. 119:100–111.
27. Ramachandra, L. E., N.oss, W. H. Boom, and C. V. Hardin. 2001. Processing of Mycobacterium tuberculosis antigen 85B involves intraphagosomal formation of peptide-major histocompatibility complex II complexes and is inhibited by live bacilli that decrease phagosome maturation. J. Exp. Med. 194:1421–1432.
28. Rampini, S. K., et al. 2008. LspA inactivation in Mycobacterium tuberculosis results in attenuation without affecting phagosome maturation activation. Microbiology 154:2991–3001.
29. Rohde, K., R. M. Yates, G. E. Purdy, and D. G. Russell. 2007. Mycobacterium tuberculosis and the environment within the phagosome. Immunol. Rev. 219:54–75.
30. Russell, D. G. 2003. Phagosomes, fatty acids and tuberculosis. Nat. Cell Biol. 6:775–778.
31. Smith, A. C., et al. 2007. A network of Rab GTPases controls phagosome...
maturation and is modulated by Salmonella enterica serovar Typhimurium. J. Cell Biol. 176:263–268.
33. Sturgill-Koszycki, S., U. E. Schaible, and D. G. Russell. 1996. Mycobacterium-containing phagosomes are accessible to early endosomes and reflect a transitional state in normal phagosome biogenesis. EMBO J. 15:6960–6968.
34. Sun, J., et al. 2010. Mycobacterial nucleoside diphosphate kinase blocks phagosome maturation in murine RAW 264.7 macrophages. PLoS One 5:e8769.
35. Sun, R., et al. 2009. Novel recombinant BCG expressing perfringolysin O and the over-expression of key immunodominant antigens: pre-clinical characterisation, safety and protection against challenge with Mycobacterium tuberculosis. Vaccine 27:4412–4423.
36. van Rie, A., et al. 1999. Exogenous reinfection as a cause of recurrent tuberculosis after curative treatment. N. Engl. J. Med. 341:1174–1179.
37. Vergne, I., J. Chua, and V. Deretic. 2003. Tuberculosis toxin blocking phagosome maturation inhibits a novel Ca2+/calmodulin-PI3K hVPS34 cascade. J. Exp. Med. 198:653–659.
38. Vergne, I., et al. 2005. Mechanism of phagolysosome biogenesis block by viable Mycobacterium tuberculosis. Proc. Natl. Acad. Sci. U. S. A. 102:4033–4038.
39. Vyas, J. M., A. G. Van der Veen, and H. L. Ploegh. 2008. The known unknowns of antigen processing and presentation. Nat. Rev. Immunol. 8:607–618.
40. Wallburger, A., et al. 2004. Protein kinase G from pathogenic mycobacteria promotes survival within macrophages. Science 304:1800–1804.
41. WHO. 2007. Global tuberculosis control: surveillance, planning, financing. WHO, Geneva, Switzerland.
42. Yeremeev, V. V., et al. 2000. The 19-kD antigen and protective immunity in a murine model of tuberculosis. Clin. Exp. Immunol. 120:274–279.