Cell-Mediated Immunity Induced by Recombinant Mycobacterium bovis Bacille Calmette-Guérin Strains Against an Intracellular Bacterial Pathogen: Importance of Antigen Secretion or Membrane-Targeted Antigen Display as Lipoprotein for Vaccine Efficacy

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Cell-Mediated Immunity Induced by Recombinant Mycobacterium bovis Bacille Calmette-Guérin Strains Against an Intracellular Bacterial Pathogen: Importance of Antigen Secretion or Membrane-Targeted Antigen Display as Lipoprotein for Vaccine Efficacy

Leander Grode,* Mischo Kursar,* Joachim Fensterle,† Stefan H. E. Kaufmann,* and Jürgen Hess‡

Live recombinant vaccines expressing defined pathogen-derived Ags represent powerful candidates for future vaccination strategies. In this study, we report on the differential induction of protective cell-mediated immunity elicited by different recombinant Mycobacterium bovis Bacille Calmette-Guérin (BCG) strains displaying p60 Ag of Listeria monocytogenes in secreted, cytosolic, or membrane-attached form for T cell recognition. Anti-listerial protection evoked by the membrane-linked p60 lipoprotein of rBCG Mp60 and that of the p60 derivative secreted by rBCG Sp60-40 were nearly equal, whereas cytosolic p60 displayed by rBCG Np60 failed to protect mice from listeriosis. In vivo depletion of CD4 or CD8 T cell subpopulations in rBCG Mp60-vaccinated mice before listerial challenge revealed interactions of both T cell subsets in anti-liserial protection. In rBCG Sp60-40-vaccinated animals, CD4 T cells predominantly contributed to anti-listerial control as shown by the failure of anti-CD8 mAb treatment to impair the outcome of listeriosis in rBCG Sp60-40-vaccinated mice after L. monocytogenes challenge. Hence, differential Ag display by rBCG influences cell-mediated immunity, which in turn may impact vaccine efficacy due to the different requirements of CD4 or CD8 T cells for pathogen elimination. The Journal of Immunology, 2002, 168: 1869–1876.

Mycobacterium bovis bacille Calmette-Guérin (BCG), an attenuated M. bovis strain, offers great potential as a live recombinant carrier delivering foreign protein Ags to be recognized by the immune system. Indeed, BCG is currently the most common human vaccine that has already been used to vaccinate more than three billion people against tuberculosis (TB) (1). Numerous viral, bacterial, and parasitic Ags have been successfully expressed in BCG and were shown to stimulate humoral and cell-mediated immune responses against respective pathogens (2–4). Moreover, several reports have emphasized the importance of differential Ag display by BCG in the context of Ab-mediated protective immunity (5–7). Display of relevant Ags (OspA, PspA, and MSP1) as secreted proteins or membrane-associated lipoproteins by rBCG is central for protection against Streptococcus pneumoniae, Borrelia burgdorferi, or Plasmodium yoelii, respectively (5–7). In infectious disease models with emphasis on CD4 and CD8 T cell-controlled immunity, it remains to be determined whether Ag display by rBCG influences vaccine efficacy due to the lack of protection studies (4, 8–10).

In contrast, for attenuated recombinant Salmonella typhimurium vaccines, several reports underline the importance of Ag secretion to elicit protective T cell-mediated immune responses against lymphocytic choriomeningitis virus or Listeria monocytogenes infection in mice (11–14). Both Ag carriers, S. typhimurium and BCG, reside within phagocytic vacuoles inside host cells, thereby sharing several similarities (15). In general, Ags delivered into the phagolysosome are preferentially processed via the MHC class II pathway and, as a consequence, stimulate CD4 T cells predominantly (16). In contrast, Ags with cytosolic localization are processed along the MHC class I pathway to induce CD8 T cells (17). Therefore, intraphagosomal bacteria such as attenuated S. typhi-murium or BCG preferentially activate CD4 T cells, whereas bacteria capable of leaving the vacuolar compartment to enter the host cell cytosol additionally induce CD8 T cells (4, 10, 16). One member of this latter group of microorganisms is represented by the Gram-positive rod-like pathogen L. monocytogenes, which causes listeriosis in mice and man (16). Due to the intracytosolic localization of L. monocytogenes within host cells, the listerial protein p60, which is secreted in large amounts into the bacterial environment, represents a prominent source for MHC class I and II epitopes (18–20). Due to its bifunctional properties during bacterial septation and host cell entry by L. monocytogenes, the biological role of p60 remains controversial (21, 22). CD8 T cells of H-2Kd haplotype and specific for the epitopes p60449–457 are capable of adaptively transferring protection to naive BALB/c mice against subsequent challenge-infection with L. monocytogenes (23, 24). Although p60449–457 represents the predominant epitope among listerial MHC class I-associated peptides with up to 10,000 epitopes per cell after 6 h of in vitro infection.
(25), it elicited a subdominant T cell response in vivo compared with that induced by the p60 \textsuperscript{217–225} epitope (25). Recombinant \textit{S. typhimurium} secreting p60 fusion proteins via the type-1-secretion machinery induced cell-mediated immunity against murine listeriosis, whereas recombinant \textit{S. typhimurium} blocked in their capability to secrete this p60 hybrid Ag failed to evoke anti-listerial protection, underlining the highly immunogenic potential of secreted Ags displayed by viable delivery devices (11). This indicates that p60 is a valuable tool for the evaluation of Ag display by rBCG in mice. Therefore, experiments with rBCG strains expressing this immunodominant p60 Ag in different bacterial compartments were performed to quantitatively analyze the contribution of Ag display for protective immune responses. In this study, we report on the superior vaccine efficacy of p60 targeted as lipoprotein derivative to the mycobacterial membrane surface against listeriosis. Furthermore, we show that anti-listerial protection depended on interactions between CD8 and CD4 T cells with Th1-reporters.

Materials and Methods

**Mice**

BALB/c mice were kept under specific pathogen-free conditions in our facilities at the “Bundesgesundheitsamt für Veterinärmedizin und Verbraucherschutz” (Berlin, Germany) and were fed autoclaved food and water ad libitum. In a given experiment, mice were age and sex matched.

**Bacteria and cells**

BCG strain Danish 1331 (Statens Serum Institut, Copenhagen, Denmark) was cultured in Dubos broth base (Difco, Detroit, MI) supplemented with Dubos medium albumin (Difco) at 37°C. A mid-logarithmic culture was aliquoted and stored at −70°C until use. \textit{L. monocytogenes} EG5 5y 1/2a and M3 strains were grown in brain heart infusion broth (Difco) at 37°C with aeration (26). Plasmid pSK-5 was a generous gift of Drs. I. Gentschev and W. Goebel (University of Würzburg, Germany; Ref. 27). The mycobacteria-\textit{Escherichia coli} shuttle vectors pAT261, p2619s, and pRB26 were kindly provided by MedImmune (Gaithersburg, MD; Ref. 28). P815 mastocytoma cells were obtained from the American Type Culture Collection (Manassas, VA) and were cultured in RPMI 1640 (Life Technologies, Karlsruhe, Germany) supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 U/ml), and 2-ME. This medium is referred to as RPMI 10 medium.

DNA manipulation and sequencing

Extrachromosomal pAT261, pRB26, and p2619s expression plasmids were used for compartmentalized p60 expression by different rBCG strains as described previously (5, 6, 28). The 1-kb \textit{PstI}–\textit{SalI} fragment (original \textit{PstI}–\textit{SalI} position 942; Ref. 27) of a pUC18–p60 construct harboring a PCR-derivediap gene of \textit{L. monocytogenes} EG5 (used oligonucleotides for PCR: forward-p60-TACC CGGGATC CGACGAAGCCTGAGTGTCGA and reverse-p60-ATCC CGGCGTAC CGTTATAGCGACGCAGAA) was applied for constructing pRB26:p60. The complete \textit{BamHI}–\textit{SalI} DNA fragment of pRB26:p60 coding for the C-terminal 319 aa of p60 was introduced into the following plasmids: p2619s and pAT261. The correct DNA sequence of the resulting constructs p2619s:p60, pRB26:p60, or pAT261:p60 at the sites of fragment insertion was determined using the following oligonucleotides 2619-GGTTAGAAGCCTGAGTACA, RB26: CGATGCTAGTATCCAGTG, and AT261-CGGCGATCATGAGTTCA, respectively (Sequiserve, Vaterstetten, Germany).

**Characterization of rBCG strains**

The plasmids pRB26:p60, pAT261:p60, or p2619s:p60 were introduced into BCG strain Danish 1331 by a standard electroporation protocol (5, 6, 28) and then r-colonies of rBCG strains Np60, Sp60-40, or Mp60 were selected on Middlebrook 7H10 agar supplemented with kanamycin (15 μg/ml). Kanamycin-resistant colonies were grown to mid-logarithmic phase in Dubos liquid medium (Difco) containing 10% Dubos medium albumin (Difco) and 15 μg/ml kanamycin for 3 wk. The bacteria of each rBCG strain normalized to a CFU of 5 × 10^6 microorganisms were harvested by centrifugation at 10,000 × g for 10 min. The supernatant referred to now on as the somatic fraction was then stored at −20°C. The pellet was resuspended with PBS/0.05% (v/v) Tween 20, and was again ribulized at the speed of 6.5 for 45 s and was centrifuged at 10,000 × g for 10 min. The supernatant referred to now on as the membrane fraction was then stored at −20°C. The protein content of each preparation was adjusted to 2 mg/ml total protein. According to the manufacturer’s instructions, 20 mg was separated on 10% SDS-PAGE (Bio-Rad, Richmond, CA). The separated proteins were electrophoretically transferred to a Hybond ECL nitrocellulose membrane (Amersham, Little Chalfont, U.K.) which was blocked overnight with PBS containing 1% BSA. The membrane was washed in PBS-Tween 0.05%, and was subsequently incubated with appropriate rabbit Abs specific for p60 diluted in PBS for 2 h. After washing, the membrane was stained with HRP-coupled anti-rabbit IgG (diluted 1/20,000; BD Pharmingen, San Diego, CA) for 1 h. The signal development on x-ray film (XOMAT-AR; Eastman Kodak, Rochester, NY) was performed with ECL kit (Amersham) for 30 s up to 1 min (29).

**Vaccination of mice with rBCG strains**

One group of BALB/c mice was vaccinated i.v. with 10^5 microorganisms of rBCG strains or BCG control and was sacrificed for CFU analysis 15, 30, and 60 days postvaccination. At indicated time points postvaccination, lung and spleen of these mice were removed and homogenized with a lab blender (Seward Medical, London, U.K.). rBCG microorganisms were enumerated by plating serial 10-fold dilutions in PBS on Middlebrook agar plates (Difco) supplemented with 10% oleic acid albumin dextrose catalase enrichment (Difco) and 15 μg/ml kanamycin. After 3–4 wk of incubation at 37°C, mycobacterial colonies were counted. The other group of rBCG-vaccinated mice were challenged-infected i.v. with 10^4 L. monocytogenes EGD bacteria (10 × LD\textsubscript{50}) on day 120 postvaccination, and survival was monitored for up to 10 days postchallenge. On day 10 postchallenge, listerial CFU in spleens of rBCG Mp60- or Sp60-40-vaccinated BALB/c survivors were determined by plating serially diluted organ homogenates on PALCAM-L. monocytogenes-selective agar (Merck, Darmstadt, Germany).

**Generation of tetrameric PE-conjugated H2-K\textsuperscript{d}-p60\textsuperscript{217–225} peptide complexes**

Modified forms of the full-length cDNA of the H2-K\textsuperscript{d} H chain and human β2-microglobulin were kindly provided by E. G. Pamer. Tetrameric H2-K\textsuperscript{d} peptide complexes (p60\textsuperscript{217–225}) were generated following the protocol described previously (30, 31).

**Staining and flow cytometry analysis**

For flow cytometry analysis, 1 × 10^6 cells per reaction were added to a 1.5-ml Eppendorf tube. The cells were incubated for 15 min at 4°C with 10 μg/ml Fc block (BD Pharmingen), 10 μg/ml ChromPure Rat IgG (Dia-nova, Hamburg, Germany), and 50 μg/ml unconjugated streptavidin (Molecular Probes, Eugene, OR) in 120 μl of PBS (pH 7.45) containing 0.5% BSA and 0.01% sodium azide. After incubation, the cells were triple stained for 60 min at 4°C with 3 μg/ml Cy-5-conjugated anti-CD8α mAb.
in pRB26:p60. The rBCG construct carrying pRB26:p60 was
inserted into the vectors pAT261 and p2619s, which were termed now pAT261:p60 and p2619s:p60,
respectively (Table I). The corresponding rBCG strains harboring
these plasmids were called Sp60-40 and Mp60. As outlined in Fig.
1, the plasmids pAT261:p60 and p2619s:p60 directed p60 expres-
sion via respective signal peptides to the supernatant or to the
membrane surface of the rBCG strains, Sp60-40 and Mp60. To
characterize p60 expression by the different rBCG strains Np60,
Mp60, or Sp60-40, appropriate supernatants, mycobacterial lysates,
and membrane preparations of mid-logarithmic-grown cultures
were prepared as described in Materials and Methods. In membrane
fractions, the p60 hybrid Ag was only detected in Mp60-specific protein preparations at 43 kDa (Fig. 2). Moreover, as expected,
only the cytoplasmic Ag samples of rBCG strains Np60 and Sp60-40 could be stained with anti-p60 Ab (Fig. 2).

Impact of differential p60 Ag display by rBCG on the speci-
cific immune response

The frequencies of epitope-specific CD8 T cells recognizing
p60217–225 peptide induced by a single i.v. immunization with rBCG strains were determined on days 12, 30, and 60 postvacci-
nation. To verify the importance of membrane-anchored p60 lo-
calization for the strength of the CD8 T cell response, direct ex
vivo analysis of p60217–225-specific CD8 T cell frequencies were

Results

Expression and localization of p60 from L. monocytogenes in
rBCG

To find out the most immunogenic mode of Ag display by rBCG
for the induction of protective cell-mediated immunity against mu-
rine listeriosis, several rBCG strains were constructed with differen-
tial p60 localization. The E. coli- mycobacteria shuttle vectors
pRB26, pAT261, and p2619s were used to generate rBCG strains
expressing p60 of L. monocytogenes in different mycobacterial
compartments (5, 6, 32). A PCR-derived 1-kb Pmr-SalII DNA frag-
ment coding for the C-terminal 319 amino acids of p60 (position
166–484; Ref. 27) was inserted into the plasmid pRB26, resulting in
pRB26:p60. The rBCG construct carrying pRB26:p60 was
 termed Np60 (Table I). Subsequently, the BamHI–SalII DNA frag-
ment of pRB26:p60 was introduced into the vectors pAT261 and
p2619s, which were termed now pAT261:p60 and p2619s:p60,
respectively (Table I). The corresponding rBCG strains harboring
these plasmids were called Sp60-40 and Mp60. As outlined in Fig.
1, the plasmids pAT261:p60 and p2619s:p60 directed p60 expres-
sion via respective signal peptides to the supernatant or to the

FIGURE 2. Localization of p60 fusion proteins in rBCG strains. The
rBCG lysates and membrane samples were prepared by centrifugation
and phase partitioning as described in Materials and Methods. Samples of each
rBCG strain were normalized to a CFU of 5 × 10^5 microorganisms and
were subjected to SDS-PAGE, electroblotted to nitrocellulose, and immu-
nostained with rabbit anti-p60 Ab. The control of each fraction is flanked
on each site for cytosol of BCG wild-type (left) and membrane BCG wild-
type (right).
performed by means of FACS staining with labeled H-2Kd-
p60\textsuperscript{217–225} tetrameric complexes. In four independent experi-
mental kinetics, the highest CD8 T cell frequencies for the p60\textsuperscript{217–225} epitope were always measured on day 30 postvaccination (Fig. 3). At this time point, the proportion of p60\textsuperscript{217–225} tetramer-positive CD8 T lymphocytes ranged from 1.1 to 1.5, which is similar to data obtained in \textit{L. monocytogenes} infection experiments at day 7 postinfection (Fig. 3) (25).

Correlation between the immune responses elicited by different rBCG strains expressing p60 and protection against murine listeriosis

To correlate immune effector mechanisms elicited by these rBCG strains with the respective vaccine efficacy, BALB/c mice were immunized i.v. with \textsuperscript{10\textsuperscript{6}} CFU of Np60 (\(\square\)), Mp60 (\(\blacktriangle\)), Sp60-40 (\(\blacktriangleleft\)), or the parental BCG vector. All control mice succumbed to this high listerial challenge inoculum. Vaccination with Mp60 displaying p60 as a membrane-anchored lipoprotein derivative rendered mice fully protected, and Sp60-40 induced 80% protection (Fig. 4). The two survival curves of Mp60- or Sp60-40-immunized mice were not significantly different as assessed by log rank statistics. The rBCG strain Np60 and the BCG vector strain failed to protect mice from lethal listeriosis in a statistically significant way (Fig. 4). The CFU in spleens of survivors vaccinated with rBCG Mp60 or Sp60-40 were determined at day 10 postchallenge. Interestingly, at this day, Mp60-immunized animals showed a significantly lower listerial load in spleens than mice vaccinated with rBCG Sp60-40 (Fig. 4; \(p < 0.05\), unpaired \textit{t} test).

To determine the contribution of CD4 and CD8 T cells to anti-
listerial protection induced by Mp60 or Sp60-40 vaccination, BALB/c mice were depleted from respective T cell subpopulations by anti-
CD4 or anti-CD8 mAb treatment 3 days before and 2 days postchal-
lenge infection with \textsuperscript{5}LD\textsubscript{50} of \textit{L. monocytogenes}. These treatments
caused \(\approx 98\%\) depletion of the respective T cell subset before listerial
challenge as assessed by cyto-
fluorometry. To underline the effect of
the T cell depletion on anti-listerial immunity, a control group of
BALB/c mice was included that was immunized with a sublethal dose of \textit{L. monocytogenes} and was challenged similarly to the rBCG-vac-
cinated animals. Anti-CD4 mAb treatment led to the death of nearly
all Mp60-, Sp60-40-, or BCG control-vaccinated mice by day 10 post-
challenge (Fig. 5 and Table II). In contrast, animals previously im-
uminized with \textit{L. monocytogenes} survived the lethal listerial infection
after CD4 T cell depletion, suggesting that CD8 T cells were sufficient

**FIGURE 3.** Direct ex vivo TCR staining of p60\textsuperscript{217–225}-specific T cells. \textit{A,} Kinetics of p60\textsuperscript{217–225}-specific CD8 T cell frequencies from three spleens of BALB/c mice per indicated time point were stained with Cy-5-conjugated anti-CD8α mAb, FITC-conjugated anti-CD62L mAb, and PE-conjugated p60\textsuperscript{217–225} tetramers as described in Materials and Methods. The animals were vaccinated i.v. with \textsuperscript{10\textsuperscript{6}} CFU of rBCG strains or the parental BCG vector. \textit{B,} Measurement of frequencies of CD8 T cells recognizing p60\textsuperscript{217–225} by tetramer staining in three independent experiments at day 30 postvaccination. BALB/c mice were immunized i.v. with \textsuperscript{10\textsuperscript{6}} CFU of Mp60 (\(\blacktriangle\)), BCG (\(\blacktriangle\)), or \textsuperscript{10\textsuperscript{3}} CFU of \textit{L. monocytogenes} (\(\bullet\)). Splenocytes of each experimental group were collected from three nonvaccinated or vaccinated mice.

**FIGURE 4.** Vaccine efficacy of different rBCG strains against murine listeriosis. \textit{A,} Survival of five BALB/c mice per group vaccinated i.v. with the rBCG strains Np60 (\(\triangle\)), Mp60 (\(\blacktriangle\)), Sp60-40 (\(\bullet\)), or the parental BCG (\(\square\)) after lethal \textit{L. monocytogenes} challenge (10 \(\times\) LD\textsubscript{50}). The survival differences between Mp60- versus Sp60-40-vaccinated groups are not statistically significant (log rank test). \textit{B,} \textit{L. monocytogenes}-specific CFU in spleens of Mp60- (\(\blacktriangle\)) and Sp60-40-vaccinated (\(\bullet\)) survivors at day 10 after listerial challenge. The median of CFU results from four or five animals per group is presented. CFU differences between rBCG Mp60- and Sp60-40-treated mice are statistically significant (\(p < 0.05\), unpaired \textit{t} test). The same experiment was repeated twice with similar results.
induced protective p60-specific CD4 T cells for listerial control, whereas membrane-anchored p60 lipoprotein displayed by rBCG Mp60 stimulated both p60-specific CD8 T and CD4 T lymphocytes to keep L. monocytogenes infection in check.

**Discussion**

This report describes rBCG strains expressing the immunodominant Ag p60 of *L. monocytogenes* in different bacterial compartments, and correlates induction of cell-mediated immune responses with protection. We found that derivatives of p60 directed to distinct compartments (cytosolic, membrane, and supernatant) were detectable in respective fractions of different rBCG strains; that the persistence of the different rBCG strains was not significantly influenced by p60 expression in vivo; that the p60(217–225) epitope-specific CD8 T cell response of rBCG Mp60 peaked on day 30 postvaccination as assessed by tetramer staining; that at day 10 postrchallenge, anti-listerial protection induced by rBCG Mp60 was superior to that of Sp60-40 as measured by CFU analysis on day 10; and that in vivo depletion of CD4 and CD8 T cell subpopulations in rBCG Mp60-vaccinated mice before listerial challenge revealed interactions of both T cell subsets in vaccine-mediated protection. In the case of rBCG Sp60-40, CD4 T cells predominantly contributed to anti-listerial control.

The C-terminal part of listerial p60 used for the construction of the rBCG strains carries three immunodominant T cell epitopes of H-2^d^ haplotype (19, 20, 24, 27). Besides the two H-2K^d^-specific CD8 T cell epitopes p60(217–225) and p60(449–457) Ag p60 also carries an immunodominant p60(301–312) epitope for H-2^A^ restricted CD4 T cells (19, 20, 24). Altogether, p60 of *L. monocytogenes* represents a versatile model Ag for analyzing CD4/CD8 T cell responses on the clonal level. Experimental infection with *L. monocytogenes* represents a well-established infectious disease model in which both CD4 and CD8 T cells contribute to cell-mediated protection. Hence, it offers an ideal system for the evaluation of rBCG strains in terms of cell-mediated immunity (16). It is known that live BCG microorganisms induce nonspecific antibacterial activity in mice, which is mediated mainly by inflammatory cytokines and nitric oxide that is protective against a lethal *L. monocytogenes* challenge within the first week after BCG vaccination (33). For this reason, we selected a vaccination schedule for the evaluation of anti-listerial protection by rBCG:p60 with vaccination-challenge intervals of 120 days.

To our knowledge, this is the first report in which the mycobacteria-*E. coli* shuttle vectors pRB26, p2619s, and pAT261 with extrachromosomal replication modes were compared for the evaluation of cell-mediated immunity induced by different rBCG strains due to the genetic consistency of used plasmid constructs. Although we are aware that p60 expression in distinct compartments of these rBCG strains was not analyzed quantitatively, the consistent usage of the pAL5000 replicon and the hsp60 promoter by the corresponding vectors allow the immunological evaluation of different rBCG strains in terms of Ag compartmentalization (3). Moreover, we believe that in vitro quantification of rAg expressed by rBCG would not reflect the actual quantity of rAg available in vivo (34). Our report describes the different qualities of cell-mediated immunity demonstrated especially by the rBCG strains Sp60-40 and Mp60, which may not only be due to different amounts of Ag available for immune recognition because Ag secretion by rBCG may result in increased Ag abundance rather than a membrane-anchored acylated Ag expression (34).

As already outlined by Köhler et al. (27), the high positive charges of the p60 fusion proteins of rBCG Np60, Mp60, or Sp60-40 can explain the considerable discrepancies between the experimentally determined sizes and the calculated molecular

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**FIGURE 5.** In vivo depletion of distinct T cell subsets in rBCG-vaccinated animals before and after lethal *L. monocytogenes* challenge. A. Iso- type-specific mAb-treated BALB/c animals immunized with BCG (■), Mp60 (▲), Sp60-40 (▼), or *L. monocytogenes* (●). B. Anti-CD4 mAb-treated mice vaccinated with rBCG (■), Mp60 (▲), Sp60-40 (▼), or *L. monocytogenes* (●). C. Anti-CD8 mAb-treated mice vaccinated with rBCG (■), Mp60 (▲), Sp60-40 (▼), or *L. monocytogenes* (●). BALB/c mice were vaccinated i.v. with 10^6 microbes of the rBCG Mp60 and Sp60-40 or the parental BCG strain for 120 days and were then cleared from remaining mycobacteria by chemotherapy. Five days later, in vivo depletion of distinct CD4 or CD8 T cell subsets was performed 3 days before and 2 days after challenge infection with 5 × 10^5* L. monocytogenes* as described in Materials and Methods. The same experiment was repeated twice with similar results. The differences of survival data between each group (Sp60-40, Mp60, BCG, or *L. monocytogenes*) and the BCG control group were statistically significant (p < 0.05; log rank test and Kaplan Meier statistics).
2 days after challenge infection with 5 10^5 H9253 mycobacteria by chemotherapy. Five days later, in vivo depletion of CD4 or CD8 T cell subsets from CD4 T cells, as previously described for listeriolysin of speciation of mice could be possibly described by the following two p60-specific and -protective CD4 T cells by rBCG Mp60 immunized animals. The apparently lower stimulation of cell priming was more potent in rBCG Sp60-40-vaccinated than in evidenced by CD4 T cell depletion, it could be suggested that CD4 T cells in protection of rBCG Sp60-40-vaccinated mice was confirmed by depletion of immune CD8 T cells before L. monocytogenes challenge. These animals survived lethal listeriosis, suggesting a dominant contribution of CD4 T cells to protective immunity without CD8 T cell requirement. Consistent with this finding, p60_217–225 tetramer-positive CD8 T cells were not significantly induced in rBCG Sp60-40-vaccinated mice up to 60 days postinfection. This finding is in line with anti-listerial protection of naive BALB/c mice adoptively conferred by IFN-γ-secreting Th1-biased CD4 T lymphocytes specific for the p60_301–312 peptide (20). Recently, several subdominant p60 epitopes were identified by novel ex vivo ELISPOT analysis of separated T cell populations from L. monocytogenes-infected BALB/c mice, which could additionally contribute to anti-listerial protection induced by rBCG Sp60-40 (39).

Our report describes the impact of membrane-anchored acylated Ag display on anti-listerial immunity induced by rBCG Mp60. Mice vaccinated with rBCG Mp60 and depleted of immune CD4 or CD8 T cells before challenge were not protected against L. monocytogenes infection. In contrast to rBCG Sp60-40-mediated protection, only a combination of CD4 and CD8 T cells improved the outcome of listeriosis after rBCG Mp60 vaccination. As evidenced by CD4 T cell depletion, it could be suggested that CD4 T cell priming was more potent in rBCG Sp60-40-vaccinated than in Mp60-immunized animals. The apparently lower stimulation of p60-specific and -protective CD4 T cells by rBCG Mp60 immunization of mice could be possibly described by the following two explanations: 1) loss of p60-derived epitopes from MHC class II-loading compartments lead to the impaired stimulation of p60-specific CD4 T cells, as previously described for listeriolysin of L. monocytogenes (40), due to subcellular trafficking of the acylated p60 fusion protein within APC; or 2) involvement of acylated 19 kDa in inhibition of Toll-like receptor 2-dependent down-regulation of MHC class II Ag processing by macrophages (41). We favor the first notion for the failure of CD4 T cell induction by rBCG Mp60 vaccination because only the coding region for the 19-kDa signal peptide and not the whole open reading frame of the 19-kDa protein was used for the generation of the rBCG Mp60 strain. The question remains open whether the lipid tail of the 19-kDa protein alone could inhibit MHC class II processing of macrophages (41). In three independent experiments, we observed similar frequencies of p60_217–225 tetramer-positive CD8 T cells in mice after infection with L. monocytogenes or rBCG Mp60 at days 15, 30, or 60.

In general, the mechanisms by which lipoprotein derivatives of rBCG evoke such a broad spectrum of immune responses are still to be elucidated. Note that immunization of mice with peptides conjugated to a palmitoyl chain stimulated increased CD4 and CD8 T cell responses without adjuvants in a wide range of MHC class I and II haplotypes (42). It is also well known that the expression of foreign Ag with the signal sequence of the 19-kDa lipoprotein of M. tuberculosis improves the induction of humoral immunity in mice. This is consistent with a B cell-activating capacity of these lipid residues (5, 6). Recently, the potential immunostimulatory effects of the 19-kDa lipoprotein were also shown for the generation of T cell responses as indicated by markedly increased IL-12 production via Toll-like receptor engagement in human macrophages (43). The acylated 19-kDa Ag of M. tuberculosis expressed by the nonpathogenic host strain Mycobacterium vaccae resulted in the induction of strong Th1-biased immune responses to the 19-kDa Ag (44). However, challenge experiments of immunized mice with M. tuberculosis did not provide evidence for protection by the 19-kDa protein, and the presence of the rAg 19 kDa abrogated the limited protection conferred by the M. vaccae vector strain (44). Yet, other experiments identified the 19- and 38-kDa lipoproteins of M. tuberculosis as prominent sources for MHC class I-restricted epitopes in mice and humans and, hence, support the notion of immunological relevance of these Ags (45–47). Furthermore, a recent study revealed that peptides derived from the 19-kDa protein or 19-kDa fusion protein have direct access to the MHC class I presentation pathway via TAP-independent mechanisms (48). Electron microscopy and subcellular fractionation of BCG-infected bone marrow-derived macrophages suggested that the acylated and membrane-linked 19 kDa protein is exported from the mycobacteria-containing phagosome during the first hour after phagocytosis of microorganisms (48). Subcellular trafficking of 19-kDa protein derivatives expressed by different rM. vaccae strains depended on acylation and was independent from glycosylation of the secreted 19-kDa lipoprotein (48). A causal link between this lipid component attached to the amino-terminal cysteine residue of fusion proteins carrying the 19-kDa-derived signal peptide and elevated MHC class I presentation of a 19-kDa Ag fusion with the influenza virus

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**Table II. Survival after L. monocytogenes infection**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Survival (%) 10 Days Postinfection*</th>
<th>Untreated</th>
<th>CD4 depleted</th>
<th>CD8 depleted</th>
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<td></td>
<td>I</td>
<td>II</td>
<td>III</td>
<td>I</td>
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<tr>
<td>Sp60-40</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Mp60</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>20</td>
</tr>
<tr>
<td>BCG</td>
<td>20</td>
<td>20</td>
<td>15</td>
<td>20</td>
</tr>
<tr>
<td>L.m.</td>
<td>20</td>
<td>20</td>
<td>15</td>
<td>100</td>
</tr>
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</table>

* BALB/c mice were vaccinated i.v. with 10^7 microorganisms of the rBCG Mp60 and Sp60-40 or the parental BCG strain for 120 days and then cleared from remaining mycobacteria by chemotherapy. Five days later, in vivo depletion of CD4 or CD8 T cell subsets from five animals of each vaccinated group was performed 3 days before and 2 days after challenge infection with 5 × 10^6 L. monocytogenes. Subsequently, the survival of these challenged mice was monitored up to 10 days. The differences of survival between each group (Sp60-40, Mp60, BCG or L. monocytogenes (L.m.)) and the BCG-control group were statistically significant (p < 0.05; log rank test and Kaplan Meier statistics).
nucleoprotein (NP) was demonstrated by the failure of a nonacylated 19-kDa-NP mutant to stimulate NP-specific CTL after infection of bone marrow-derived macrophages with \textit{M. vaccae} expressing the appropriate 19-kDa-NP hybrid construct (48). In summary, only acylation of a mycobacterial surface-attached and intracellularly secreted 19-kDa Ag fusions promoted induction of Ag MHC class I-restricted CTL via intracellular trafficking of these lipoproteins in bone marrow-derived macrophages and dendritic cells (48).

In general, due to the limited knowledge about immunodominant mycobacterial Ags and the lack of highly efficacious adjuvants for humans, which could in combination induce protective cell-mediated immunity against TB, improvement of BCG remains the best choice for rational design of TB vaccines (49). Our study provides new insights into the importance of targeting Ags unique for the proteome of \textit{M. tuberculosis} to highly immunostimulatory compartments of BCG. We learned from our study that Ag compartmentalization by rBCG apparently influenced distinct cellular branches of cell-mediated immunity. Many groups have now demonstrated the importance of anti-mycobacterial CD8 T cells besides the well-known participation of CD4 T cells in TB control (50–54). For achieving protective cell-mediated immunity with emphasis on CD4 and CD8 T lymphocytes against TB, we consider Ag display as membrane-anchored lipoprotein, as well as secreted protein, to be the combined and most immunodominant mode for mycobacterial Ag delivery by improved BCG vaccines.

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References


