otic chromatin-associated factors. These two domains, SET and SWIB, have not been identified outside eukaryotes. Most remarkably, a diverged copy of the SWIB domain was found fused to the COOH-terminus of the chlamydial topoisomerase I. It appears that SWIB and SET domains, together with the Swi/Snf2 family of helicases (CT555 and CT708), may participate in the chromatin condensation-decondensation that is characteristic of the chlamydial developmental cycle (*34*). Moreover, these findings suggest that there are mechanistic similarities between these processes in chlamydiae and eukaryotic chromatin dynamics.

Most of the "eukaryotic" proteins of chlamydia do not appear to be specifically related to their animal homologs. Unexpectedly, they tend to group with plant proteins in phylogenetic analyses (Fig. 3). A clue as to their previous niche may be suggested by the observation of *Chlamydia*-like endosymbionts of *Acanthamoebae* (*35*). The phylogenetic position of *Acanthamoebae* is near the original branching point of the eukaryotes, and grouping with plants cannot be ruled out (*36*). It is possible that the evolution of chlamydiae as intracellular parasites started with an opportunistic interaction with amoebal hosts, and the protochlamydiae became amoebal parasites or symbionts for a period long enough to acquire the "plant-like" genes, whose origin may be amoebal. Thus, the emergence of host-dependent protochlamydiae may have started in single-cell amoebic hosts before moving to multicellular invertebrate hosts, perhaps then diverging into the major chlamydial genera prior to adapting to vertebrate hosts (*37*).

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39. E. V. Koonin, L. Aravind, R. L. Tatusov, data not shown.

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Attenuation of Virulence by Disruption of the *Mycobacterium tuberculosis erp* Gene

François-Xavier Berthet,* Micheline Lagranderie, Pierre Gounon, Christine Laurent-Winter, Danielle Ensergueix, Pierre Chavarot, Françoise Thouron, Eddie Maranghi, Vladimir Pelicic,† Denis Portnoï, Gilles Marchal, Brigitte Gicquel‡

The virulence of the mycobacteria that cause tuberculosis depends on their ability to multiply in mammalian hosts. Disruption of the bacterial *erp* gene, which encodes the exported repetitive protein, impaired multiplication of *M. tuberculosis* and *M. bovis* Bacille Calmette-Guérin in cultured macrophages and mice. Reintroduction of *erp* into the mutants restored their ability to multiply. These results indicate that *erp* contributes to the virulence of *M. tuberculosis*.

Mycobacterium tuberculosis, *M. bovis*, and *M. africanum* cause tuberculosis, an infectious disease killing more than 3 million people per year worldwide (*1*). These bacteria constitute the *M. tuberculosis* complex (MTC), which also includes the tuberculosis vaccine strain *M. bovis* Bacille Calmette-Guérin (BCG) and the murine pathogen *M. microti*. Members of the MTC multiply within phagocytic cells in a specialized vacuolar compartment called the phagosome (*2*). Phagosomes containing mycobacteria do not acidify (*3*), and they escape fusion with lysosomes (*4*). Remodeling of the phagosome architecture by these pathogenic bacteria is thought to be critical for their intracellular multiplication

and survival (*5*, *6*).

Mycobacterial proteins that are exported intracellularly are likely to participate in phagosome remodeling. Using a genetic approach to identify such exported proteins, we recently identified the *M. tuberculosis erp* gene (*7*). It encodes the exported repetitive protein (ERP), a protein previously identified in *M. bovis* as the PGLTS secreted antigen (*8*). Although no function has been ascribed to ERP, sequences homologous to *erp* are found exclusively in mycobacteria causing tuberculosis (*7*, *8*) and leprosy (*9*).

To characterize *M. tuberculosis* ERP, we overproduced it in *Escherichia coli* as a fusion protein containing six histidines at the COOH terminus (ERP-6His) (*10*). ERP-6His formed cytoplasmic inclusion bodies and was purified by nickel-affinity chromatography under denaturing conditions (*11*). Renatured soluble ERP-6His (36 kD) was used to immunize rabbits, and high-titer anti-ERP polyclonal sera detected immunoreactive bands both in cell-associated fractions (36 kD) and trichloroacetic acid–precipitated culture filtrates (36 and 34 kD) of BCG and *M. tuberculosis* (Fig. 1A).

To determine the subcellular localization of ERP, we performed immunocytochemical analyses of *M. bovis* BCG bacilli using rabbit antisera to ERP and a gold-labeled anti-rabbit conjugate (*12*, *13*). Transmission electron microscopy revealed intense surface labeling at the periphery of the bacilli (127 ± 30 gold grains per bacterium), which indicates that ERP is a surface-exposed molecule (Fig. 1B). We next investigated whether ERP was produced during intracellular multiplication of *M. tuberculosis* within cultured macrophages. J774 murine macrophages were infected with a clinical isolate of *M. tuberculosis* and processed for immunoelectron microscopy (*12*, *14*). Specific labeling of the mycobacterial cell wall and the phagosomal lumen was observed with sera from ERP-immunized rabbits (Fig. 1F) but not with anti-ERP serum previously adsorbed against purified ERP-6His (Fig. 1E). Furthermore, small vesicles near the phagosomes also contained the ERP label. These findings demonstrate that ERP is produced in *M. tuberculosis* phagosomes, and they suggest that it may traffic intracellularly.

We then examined whether ERP is essential for intracellular growth of the mycobacteria. A targeted null mutation was introduced at the *erp* locus in *M. tuberculosis* H37Rv and in the model tuberculosis vaccine strain *M. bovis* BCG. A sucrose-counterselectable suicide vector (pJQ200) was used to deliver a mutated allele of *erp* (*erp::aph*) into the chromosome of *M. bovis* BCG (*15*, *16*). The corresponding *M. tuberculosis* mutant strain was constructed with the *ts-sacB* technology (*17*). Mutant strains resulting from allelic exchange (Fig. 2A) were referred to as BCG *erp::aph* and H37Rv *erp::aph*. By using a mycobacteriophage MS6-derived integrative vector pIPX70, we reintroduced a single copy of *erp* at the *attB* site in BCG *erp::aph* and H37Rv *erp::aph* (Fig. 2A). The genotype of these strains was analyzed by Southern (DNA) blotting with an *erp*-specific DNA probe. This analysis revealed that there was an insertion of 1.3 kb in the restriction fragment carrying *erp* and that an additional copy of *erp* was present in the chromosome of complemented strains (Fig. 2B). Analysis of cell-associated fractions and

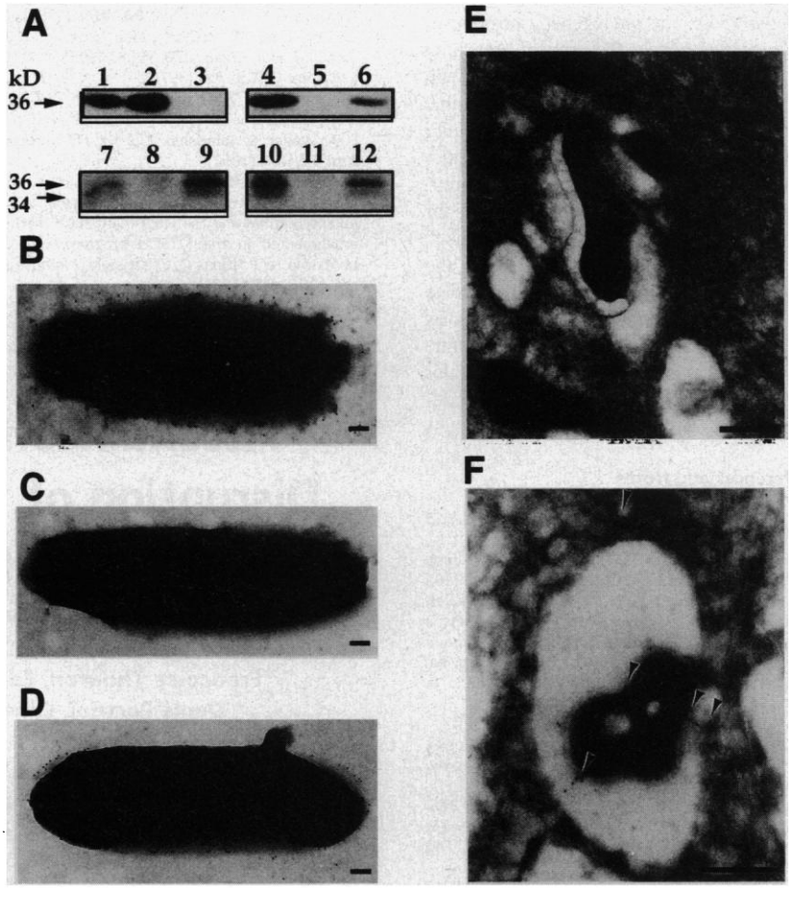


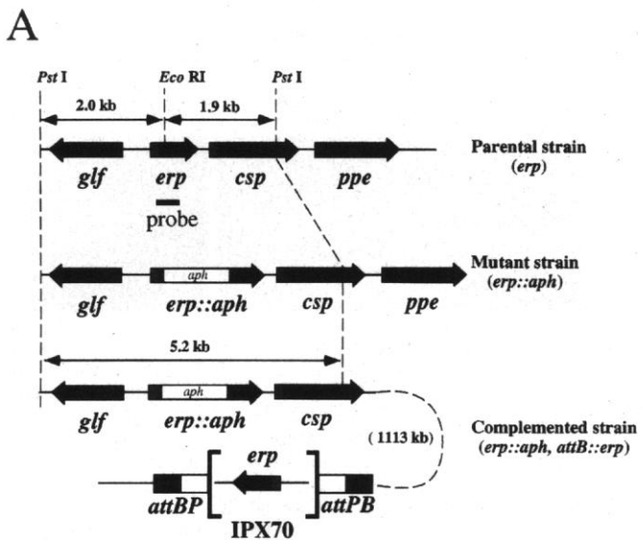
Fig. 1. Expression, immunodetection, and localization of ERP. (**A**) Immunodetection of ERP in cell-associated (lanes 1 through 6) or concentrated supernatant (lanes 7 through 12) fractions of *M. bovis* BCG (lanes 1 through 3 and 7 through 9) and *M. tuberculosis* (lanes 4 through 6 and 10 through 12). Protein extracts were prepared from parental (lanes 1, 4, 7, and 10), mutant (lanes 3, 5, 8, and 11), and complemented (lanes 2, 6, 9, and 12) strains. (**B** through **D**) Surface analysis of parental (B), mutant (C), or complemented (D) *M. bovis* BCG cells processed for immunogold labeling with anti-ERP serum. (**E** and **F**) Immundetection of ERP in *M. tuberculosis*–infected, J774 murine macrophage sections incubated with anti-ERP serum previously adsorbed against purified ERP-6HIS (E) or anti-ERP sera (F); arrowheads in (F) show ERP labeling of an intraphagosomal mycobacterium (bottom) and a small cellular vesicle (top). Scale bars in (B) through (F), 200 nm.

F.-X. Berthet, D. Ensergueix, V. Pelicic, D. Portnoï, Unité de Génétique Mycobactérienne, Institut Pasteur, 25 rue du Docteur Roux, 75724 Paris Cédex 15, France. M. Lagranderie and F. Thouron, Laboratoire du BCG, Institut Pasteur, 25 rue du Docteur Roux, 75724 Paris Cédex 15, France. P. Gounon, Station Centrale de Microscopie Electronique, Institut Pasteur, 25 rue du Docteur Roux, 75724 Paris Cédex 15, France. C. Laurent-Winter, Laboratoire d'Electrophorèse Bidimensionnelle, Institut Pasteur, 25 rue du Docteur Roux, 75724 Paris Cédex 15, France. P. Chavarot, E. Maranghi, G. Marchal, Unité de Physiopathologie de l'Infection, Institut Pasteur, 25 rue du Docteur Roux, 75724 Paris Cédex 15, France. B. Gicquel, Unité de Génétique Mycobactérienne, Institut Pasteur, 25 rue du Docteur Roux, 75724 Paris Cédex 15, France, and Laboratoire du Bacille Calmette Guérin (BCG), Institut Pasteur, 25 rue du Docteur Roux, 75724 Paris Cédex 15, France.

*Present address: SmithKline Beecham Biologicals, Laboratory of Molecular Bacteriology, Rue de l'Institut 89, B-1330 Rixensart, Belgium.

†Present address: Department of Biological Sciences, University of Padova, Via Trieste 75, 35121 Padova, Italy.

‡To whom correspondence should be addressed. E-mail: bgicquel@pasteur.fr



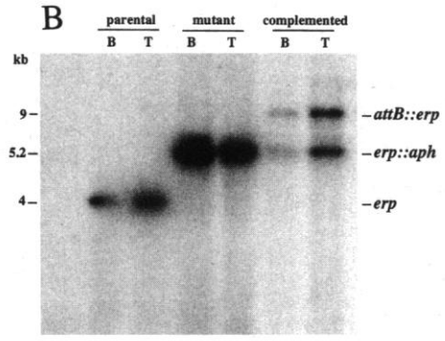


Fig. 2. Disruption of the *erp* gene. (**A**) Chromosomal organization of the *erp* gene in the parental, mutant, and complemented strains of *M. bovis* BCG and *M tuberculosis*. Gene symbols are as follows: *glf* (UDP galactomutase), *csp* (secreted protein), *ppe* (repetitive protein of the PPE family), *aph* (aminoglycoside phosphotransferase), and *attB* (bacterial chromosome attachment site for the Ms6 mycobacteriophage). (**B**) Southern blot analysis of chromosomal DNA from the parental, mutant, and complemented strains of *M. bovis* BCG (B) and *M. tuberculosis* (T). The *erp* internal probe used for hybridization is depicted in (A).

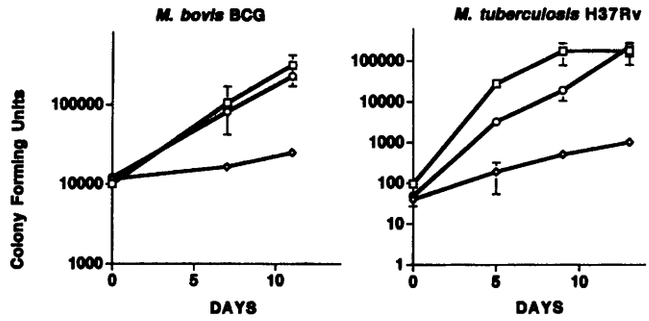


Fig. 3. Effect of the *erp::aph* mutation on the intracellular multiplication of *M. bovis* BCG and *M. tuberculosis*. CFU counts in bone marrow–derived murine macrophages infected with the parental (squares), mutant (diamonds), or complemented (circles) strains are shown. Results are expressed as means and standard deviations of four different measurements.

concentrated supernatants (Fig. 1A) from BCG *erp::aph* and H37Rv *erp::aph* cultures indicated that disruption of *erp* abolished the production of ERP. This was confirmed by the disappearance of immunogold labeling (32 ± 10 gold grains per bacterium) on *M. bovis* BCG *erp::aph* bacilli (Fig. 1C). In contrast, integration of *erp* at the Ms6 *attB* site of *erp::aph* mutant strains restored the production of ERP, both at the cell surface (Fig. 1, A and D) (112 ± 57 gold grains per bacterium) and in the culture medium (Fig. 1A) of *M. tuberculosis* and *M. bovis* BCG. The parental, mutant, and complemented strains of BCG and H37Rv showed similar colony morphology, doubling time, and growth characteristics in Middelbrook 7H9/ADC broth or minimal Sauton medium. Taken together, these data show that *erp* is not essential for the growth of BCG and H37Rv under axenic conditions.

To determine whether BCG *erp::aph* and H37Rv *erp::aph* grow within phagocytes, we compared the multiplication capacity of mutant and parental strains in cultured bone marrow–derived macrophages (*18*). Analysis of colony-forming units (CFUs) indicated that multiplication of *erp::aph* mutants was impaired within murine macrophages, whereas parental and complemented strains grew normally (Fig. 3). Moreover, the H37RV *erp::aph* strain showed reduced cytopathic effects as compared to the parental or complemented strains. To determine whether the *erp::aph* mutation also affected multiplication within the host, we analyzed the persistence of BCG *erp::aph* and H37Rv *erp::aph* in mice. BALB/c mice were injected intravenously with 10^6 viable units of parental, *erp::aph* mutant, and *erp*-complemented strains, and bacterial infection was monitored by counting CFUs over a 56-day period (*19*). We analyzed the lungs, liver, and spleen, which are the three organs known to contain most of the mycobacterial load after intravenous inoculation. The BCG *erp::aph* mutant was rapidly cleared from the lungs of infected animals, whereas the parental and complemented strains colonized and survived within this organ (Fig. 4A). In contrast, the H37Rv *erp::aph* mutant survived but multiplied very slowly in the lungs as compared to the parental and complemented strains (Fig. 4A). The lungs are the primary site of infection in tuberculosis. Multiplication of *erp::aph* mutants was also greatly impaired in the spleen (Fig. 4B) and the liver. In addition, whereas parental BCG showed a "spreading" colony morphology after animal passaging, the mutant no longer spread and exhibited retarded growth (by 1 week as compared to the parental strain) (Fig. 4C). The significance of this observation is unknown, but a loss of the spreading phenotype has been correlated with the lowest levels of residual virulence among BCG substrains (*20*). The non-spreading phenotype we observed was lost after restreaking of the mutant on 7H11 plates, and reintroduction of *erp* restored the parental phenotype.

We have shown that the *M. tuberculosis* *erp* gene encodes a surface-exposed protein produced during phagosomal growth and that this protein is required for multiplication of the pathogen within the host cell. Thus, *erp* may be a good candidate for the rational attenuation of *M. tuberculosis* and the construction of new live-attenuated vaccines against tuberculosis. Indeed, the multiplication characteristics of H37Rv *erp::aph* in vivo are comparable to that observed for the tuberculosis vaccine strain *M. bovis* BCG. Sensitization of guinea pigs with mycobacterial purified protein derivative 1 month after immunization with BCG *erp::aph* resulted in a delayed-type hypersensitivity response comparable to that induced by the parental strain. This observation suggests that BCG *erp::aph* persists long enough to stimulate antimycobacterial immunity. Although the function of ERP is still unclear, this work opens new avenues for studying the pathogenetic mechanisms of the mycobacteria causing tuberculosis.

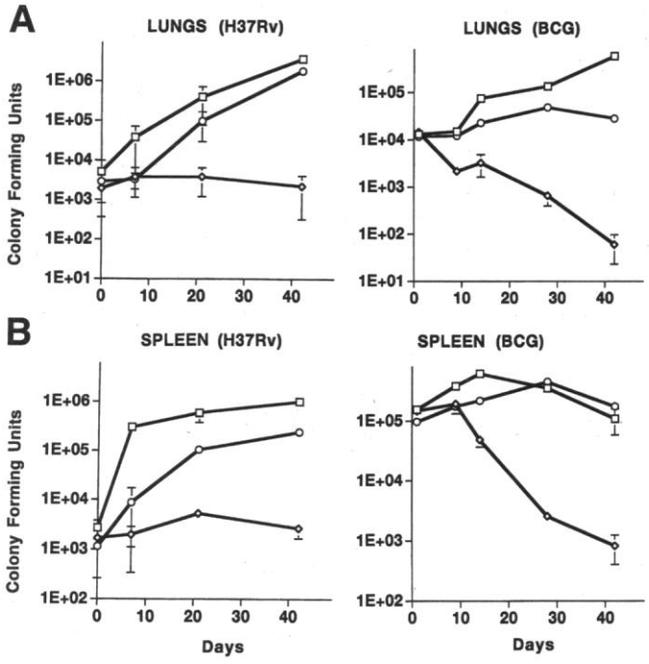


Fig. 4. Effect of the *erp::aph* mutation on the survival and multiplication of *M. bovis* BCG and *M. tuberculosis* in the lungs (**A**) and the spleen (**B**) of BALB/c mice. CFU counts are shown for the parental (squares), mutant (diamonds), and complemented (circles) strains. The results for each time point are the means and standard deviations of CFU counting performed on five mice. The *y* axis reflects the number of viable bacteria recovered from a specific organ. (**C**) Morphologic differences between parental (*erp*), mutant (*erp::aph*), and complemented (*erp::aph, attB::erp*) BCG colonies recovered from the liver of infected mice at day 7. Bacteria were grown for 20 days (parental and complemented strains) or 27 days (mutant strain) on 7H11 plates, and the resultant colonies were photographed at the same scale (scale bar, 1 cm).

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11. Liquid cultures of *E. coli* strain M15 [pHis233] were grown in Luria-Bertani broth, induced with isopropyl-β-D-thiogalactoside, and processed for protein purification under denaturing conditions using nickel-nitrilotriacetic acid agarose resin (QIAGEN). Eluted ERP-6His was dialyzed twice for 12 hours against phosphate-buffered saline (PBS) and stored frozen at −20°C. Two rabbits (strain New Zealand) were injected with 100 μg of protein and then injected every 15 days with 150, 200, and 250 μg of ERP-6His emulsified in incomplete Freund's adjuvant. Hyperimmune anti-ERP serum was obtained by bleeding animals 6 weeks after immunization.
12. Cells were fixed with 1% paraformaldehyde in 0.1 M phosphate buffer, washed in the same buffer, and then applied to Formvar–carbon-coated nickel grids that had previously been made hydrophylic by glow discharge. Grids were then processed for immunocytochemistry (*16*), rinsed with distilled water, and negatively stained with 1% ammonium molybdate in water.
13. Grids were treated sequentially with drops of the following reagents: NH_4Cl (50 mM) in PBS for 10 min, bovine serum albumin (BSA) (1% w/v) in PBS for 5 min, antiserum to ERP diluted 1/100 in PBS-BSA for 60 min, and PBS-BSA (three washes, 2 to 5 min each). Samples were then labeled with goat anti-rabbit immunoglobulin G (H and L chains); conjugated to gold grains (5 or 10 nm in size; British Biocell International, UK); diluted 1/20 in PBS plus 0.1% skin fish gelatin (Sigma) for 30 to 45 min; and washed in PBS (one wash, 1 min) and distilled water (three washes, 1 min each). Finally, samples were fixed with 1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 2 min.
14. Bacteria or infected macrophages (multiplicity of infection = 1) were fixed with 2% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer. Cells were harvested and embedded in 10% gelatin. Pelleted cells were incubated from 2 to 14 hours in 1.8 M sucrose and 15% polyvinyl pyrolidone (molecular weight, 10,000). Small blocks were mounted on stubs, frozen in liquid nitrogen, and sectioned at −120°C with a cryoultramicrotome (Reickert fetal calf serum). Thin sections were recovered in a drop of 2.3 M sucrose and transferred to Formvar–carbon-coated nickel grids. Grids were then processed for immunocytochemistry (*16*), rinsed with distilled water, and embedded in methylcellulose containing 0.3% uranyl acetate.
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16. A 3.9-kb DNA fragment encompassing the full-length *erp* gene was excised from pIPX412 by Pst I digestion and cloned into the corresponding site of pACYC177. The resulting plasmid (pPB1) was linearized by Eco RI, which cuts at a unique site within *erp*. In parallel, an *aph* cassette conferring resistance to kanamycin was excised by Pst I digestion from the plasmid pUC-4K. Both pPB1 and the *aph* fragment were treated with T4 DNA polymerase (Boehringer Mannheim) to create blunt ends and were ligated to produce pPB2. A 5.2-kb DNA fragment containing *erp::aph* was excised from pPB2 by Pst I digestion and cloned into the nonreplicative plasmid vector pJQ200, resulting in the vector pPB3. Five micrograms of pPB3 was introduced by electroporation into *M. bovis* BCG, which was subsequently plated onto 7H11 Middelbrook plates supplemented with kanamycin (20 μg/ml). Colonies were screened by PCR, with oligonucleotides flanking the Eco RI site used for insertion of *aph* and by replica-spotting on plates containing kanamycin (20 μg/ml) and 2% sucrose. One clone that contained an insertion of 1.3 kb and that was no longer sensitive to sucrose was analyzed by Southern blotting.
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18. Mice were maintained according to the Institut Pasteur guidelines for laboratory animal husbandry. Bone marrow–derived macrophages were isolated from the femurs of 7-week-old female BALB/c mice. Cells were seeded at 5×10^5 cells per well into 8-well Labtek TM (Nunc, Denmark) culture chambers and were cultivated for 7 days as described (*5*). Differentiated macrophages were infected with 10^5 viable units of BCG. Macrophages were lysed with the Cell Culture Lysis Reagent (Promega, Madison). Bacteria were diluted in Middelbrook 7H9 culture medium and spread on 7H11 plates for CFU analysis.
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