LspA inactivation in *Mycobacterium tuberculosis* results in attenuation without affecting phagosome maturation arrest

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The success of *Mycobacterium tuberculosis* depends on its ability to survive within host macrophages. Here, *M. tuberculosis* avoids the acidic, hydrolytically competent environment of the phagolysosome by arresting phagosome maturation. Having shown previously that a *M. tuberculosis* mutant deficient in lipoprotein signal peptidase (LspA) is strongly attenuated *in vivo* in a mouse model of infection, we now studied putative mechanisms involved in attenuation of the *lspA::aph* mutant at a cellular level. In this work we investigated the ability of the mutant to interfere with two host defence mechanisms, i.e. Toll-like receptor (TLR)2-dependent immune response and phagosome maturation. While mycobacterial lipoproteins have been reported to trigger a TLR2 signalling pathway critical for innate immune responses, we found that growth control of the *lspA::aph* mutant was independent of TLR2. In addition, the *lspA::aph* mutant arrested phagosome maturation to an extent similar to that of the wild-type, as measured by lysosomal-associated membrane protein 1 (LAMP1) co-localization and intraphagosomal pH. These observations demonstrate severe attenuation even in the presence of arrested phagosome maturation, and point to a role for the early phagosome in growth restriction of the *M. tuberculosis* *lspA* mutant.

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INTRODUCTION

Mycobacterium tuberculosis is a major threat to human health, accounting for 1.6 million deaths and 8.8 million new infections in 2005 (World Health Organization fact sheet; <http://www.who.int/mediacentre/factsheets/fs104/en/>). The bacteria show a remarkable ability to survive and replicate within macrophages. A multitude of virulence factors contribute to the intracellular survival of pathogenic mycobacteria, such as the physical barrier of the mycobacterial cell wall and the ability of the bacilli to avoid activation of T cells by manipulation of the host immune

response (reviewed by Chua *et al.*, 2004; Russell, 2001). Among the most striking and prominent features of *M. tuberculosis* is its ability to arrest phagosome maturation, creating an intracellular niche with characteristics of early endosomes suitable for replication of the bacteria (Huyhn & Grinstein, 2007; Russell, 2001; Vergne *et al.*, 2004).

Lipoproteins are a class of secreted, lipidated proteins ubiquitously present in bacteria. Lipoproteins are involved in a variety of functions including cell wall synthesis, nutrient uptake, sensing and transmembrane signalling, and adhesion (Sutcliffe & Russell, 1995). Lipidation is thought to allow anchoring of these proteins to the cell surface. Lipoproteins are characterized by the presence of a consensus sequence that directs post-translational modifications. One step of the post-translational modifications depends on the lipoprotein signal peptidase (LspA), which cleaves off the signal peptide from immature prolipoproteins (Dev & Ray, 1984; Sander *et al.*, 2004; Sankaran & Wu, 1994). Mycobacterial lipoproteins have been found in both the plasma membrane and the cell wall fraction, with their correct localization depending on LspA (Mawuenyega *et al.*, 2005; Rezwan *et al.*, 2007a, b). A *M. tuberculosis*

Abbreviations: BMDM, bone marrow-derived macrophages; IFN, interferon; LAMP1, lysosomal-associated membrane protein 1; PIM, phosphatidylinositol mannoside; TLR, Toll-like receptor; TNF- α , tumour necrosis factor- α .

Four supplementary figures showing kinetics of *in vivo* growth of *M. tuberculosis* wild-type and mutants in spleen over a 108 day period, restoration of wild-type growth in IFN- γ -activated BMDM by complementation of *M. tuberculosis* *lspA*, growth of wild-type, mutant and complemented strains in Middlebrook 7H9-ADC, and TNF- α secretion by BMDM in response to infection with wild type and mutant strains, are available with the online version of this paper.

mutant deficient in *LspA* (*lspA::aph*) shows a very severe reduction in c.f.u. in lungs and complete absence of lung pathology in a mouse infection model (Sander *et al.*, 2004). The mechanisms that underlie attenuation remain to be elucidated; however, the phenotype of the *lspA::aph* mutant suggests strong interactions of lipoproteins with prominent host defence mechanisms.

Mycobacterial lipoproteins are recognized by the Toll-like receptor (TLR)2 (Brightbill *et al.*, 1999), which belongs to the family of pattern recognition receptors (Rock *et al.*, 1998). TLR2 is the core subunit of heterodimeric receptors that recognize lipidated cellular components: TLR2/1 binds tri-acylated mature lipoproteins, while TLR2/6 recognizes di-acyl moieties (Jin *et al.*, 2007). Pathogen recognition through TLR in principle serves three functions: sensing the presence and type of the pathogen, provoking an immediate anti-pathogen response and stimulating the development of a long-lasting adaptive response (Kanzler *et al.*, 2007). The response to TLR signalling is determined by factors specific to individual cell types and the nature of the ligand. It can include cell differentiation, proliferation or apoptosis, and secretion of cytokines, prominently interferons (IFNs) and tumour necrosis factor (TNF)- α (Kanzler *et al.*, 2007). TLR2 contributes to innate resistance against *M. tuberculosis* (Reiling *et al.*, 2002). *In vitro* investigations suggest that the triggering of TLR2 by *M. tuberculosis* may represent a double-edged sword: short-term signalling through TLR2 activates macrophages and initiates inflammation via TNF- α secretion that may help to control the acute infection (Underhill *et al.*, 1999); prolonged TLR2 signalling in macrophages results in downregulation of major histocompatibility complex (MHC)-II antigen processing, suppressing an acquired immune response (Gehring *et al.*, 2004; Noss *et al.*, 2001). The link between TLR2 triggering and phagosome maturation is controversial: Blander & Medzhitov (2004) reported that TLR stimulation promotes phagosome maturation, while Yates & Russell (2005) provided evidence that TLR2 or TLR4 stimulation does not affect phagosome acidification or phago-lysosomal fusion.

The phagocytic machinery of macrophages, i.e. phagocytosis and phagosome maturation, is a complex defence mechanism of the innate immune system. Following internalization, phagosomes mature to phago-lysosomes. The maturation process depends on vesicle fusion and fission. It involves acquisition, loss and modification of defined host proteins, and ultimately results in acidification of the phagosomal lumen to pH <5 and generates an environment in which microbes are exposed to lytic enzymes and reactive oxygen and nitrogen intermediates (Huynh & Grinstein, 2007; Schnappinger *et al.*, 2003; Vieira *et al.*, 2002). Pathogenic mycobacteria interfere with the phagosome maturation process (Armstrong & Hart, 1971; Clemens & Horwitz, 1995; Hasan *et al.*, 1997; Via *et al.*, 1997; Xu *et al.*, 1994). The mycobacterial phagosome retains the early endosomal GTPase rab5 (Clemens *et al.*, 2000; Kelley & Schorey, 2003; Via *et al.*, 1997) and access to

transferrin within the rapid recycling pathway (Clemens & Horwitz, 1996; Sturgill-Koszycki *et al.*, 1996). It does not acquire early endosomal antigen 1 (Fratti *et al.*, 2001), late endosomal/lysosomal markers [such as rab7, lysosomal-associated membrane protein 1 (LAMP1) and CD63] (Chua *et al.*, 2004; Russell, 2001) or vacuolar proton ATPase (Sturgill-Koszycki *et al.*, 1994), and it only acidifies mildly to about pH 6.4 (Oh & Straubinger, 1996; Russell *et al.*, 2005; Sturgill-Koszycki *et al.*, 1994). Although providing only few carbohydrates and little iron, the maintenance of *M. tuberculosis* in a compartment with early endosomal characteristics is thought to provide a favourable environment suitable for intracellular replication (Huynh & Grinstein, 2007; Russell, 2001; Schnappinger *et al.*, 2003; Vergne *et al.*, 2004). Upon activation with IFN- γ , macrophages restrict growth of intracellular mycobacteria; growth restriction is associated with phagosome maturation and further acidification (Hostetter *et al.*, 2002; MacMicking *et al.*, 2003; Schaible *et al.*, 1998; Via *et al.*, 1998). Phagosome maturation arrest is specific to live mycobacteria. Heat-killed mycobacteria undergo phagosome maturation and the intraphagosomal pH stabilizes at about 5.8 (Russell *et al.*, 2005). The mechanisms involved in phagosome maturation arrest are still elusive. Phagosome maturation arrest is in part dependent on lipid components of the mycobacterial cell wall, such as lipoarabinomannan, phosphatidylinositol mannosides (PIMs) and cord factor (Anes *et al.*, 2003; Fratti *et al.*, 2001, 2003; Indrigo *et al.*, 2003; Vergne *et al.*, 2003, 2004). Additional factors involved are the acid phosphatase SapM (Saleh & Belisle, 2000; Vergne *et al.*, 2005), the bacterial serine/threonine kinase PknG (Walburger *et al.*, 2004), and the zinc-metalloprotease Zmp1 (Master *et al.*, 2008).

The strong attenuation of the *M. tuberculosis lspA::aph* mutant (Sander *et al.*, 2004) prompted us to investigate the mechanism(s) involved in loss of virulence. We here investigated the ability of the mutant to interfere with two innate host defence mechanisms, i.e. TLR2 signalling and phagosome maturation.

METHODS

Bacterial strains and growth conditions. *M. tuberculosis* H37Rv #1424, a derivative of *M. tuberculosis* H37Rv, was used as wild-type strain in this study. The isogenic *lspA::aph* knockout mutant and *lspA::aph-lspA* complemented mutant have been described previously (Sander *et al.*, 2004). Strains were grown at 37 °C on Middlebrook 7H10 agar (Difco) supplemented with OADC (0.05 % oleic acid, 5 % BSA fraction V, 2 % glucose, 0.004 % beef catalase, 0.85 % NaCl; Difco) or in settling cultures at 37 °C in Middlebrook 7H9 broth (Difco) supplemented with OADC and 0.05 % Tween 80; antibiotics were added when appropriate (50 µg kanamycin ml⁻¹, 25 µg hygromycin ml⁻¹). For growth kinetics, strains at mid-exponential phase in roller bottles without antibiotics were diluted to OD₆₀₀ 0.01 in Middlebrook 7H9 supplemented with ADC [5 % albumin, 2 % dextrose, 0.0003 % catalase (beef)] and 0.05 % Tween 80, and OD₆₀₀ was monitored over 10–12 days. For infection experiments, strains were grown in 7H9 broth supplemented with

OADC and 0.05 % Tween 80 to mid-exponential phase in roller bottles without antibiotics. Heat-inactivated *M. tuberculosis* was used as a control. Heat-inactivation was performed at 85 °C for 30 min; inactivation was confirmed by plating on 7H10. All procedures were done under biosafety level III conditions.

Cells and growth conditions. Macrophages were derived from bone marrow of over eight-week-old specific-pathogen-free female BALB/c or C57BL/6 mice (both from Harlan) maintained in individual ventilated cages at Biologisches Zentrallabor (BZL), University of Zurich. Animal procedures were approved by the Kantonales Veterinäramt (Zurich). Bone marrow stem cells were differentiated at 37 °C in 5 % CO₂ for 7 days in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal calf serum (FCS) (Brunschwig), 10 % L-cell conditioned medium and 1 % penicillin/streptomycin (10 000 U ml⁻¹/10 000 µg ml⁻¹) (Gibco) on Petri dishes (Greiner). Viability controls using trypan blue exclusion revealed over 90 % viable cells. During infection assays, cells were maintained in DMEM supplemented with 10 % FCS, a medium which does not support mycobacterial multiplication (Bryk *et al.*, 2008). The mouse monocyte-macrophage cell line J774A.1 was also maintained in DMEM supplemented with 10 % FCS.

Mouse infection experiments. Female 6–8-week-old specific-pathogen-free C57BL/6 wild-type or TLR2^{-/-} mice back-crossed at least 10 times to a C57BL/6 background were maintained under biosafety level III conditions in individually ventilated cages. For infection experiments, *M. tuberculosis* cultures grown to mid-exponential phase were harvested, aliquoted and frozen at -80 °C. After thawing, viable cell counts were determined by plating serial dilutions on Middlebrook 7H10 agar supplemented with OADC. To ensure proper dispersion of *M. tuberculosis*, the thawed bacterial suspension was diluted appropriately and drawn through a non-pyrogenic needle (Microlance 3, BD) prior to every infection experiment. Mice were aerogenically infected by producing an aerosol of 5.5 ml of a solution containing 10⁶ c.f.u. ml⁻¹ *M. tuberculosis* in an aerosol generation device (Glas-Col) for 30 min, calibrated to seed ~1000 c.f.u. of the respective *M. tuberculosis* strain. The implanted inocula of the wild-type, mutant and complemented strains were confirmed by determination of c.f.u. in the lungs 1 day after infection. At each time point of analysis, lungs from five sacrificed animals were removed, weighed and homogenized. Serial dilutions of organ homogenates were plated on 7H10 agar supplemented with OADC, and c.f.u. were calculated as the arithmetical mean of duplicate determinations and geometric means of the groups at the indicated time points. All experiments performed were in accordance with the German Animal Protection Law and were approved by the Animal Research Ethics Board of the Ministry of Environment, Nature Protection and Agriculture (Kiel) and an institutional review board.

Intracellular growth experiments. Fully differentiated bone marrow-derived macrophages (BMDM) were seeded in 24-well plates (Nunc) at 1 × 10⁵ cells per well and maintained in DMEM supplemented with 10 % FCS for 72 h. For experiments with activated macrophages, 100 U ml⁻¹ recombinant murine IFN-γ (Lucerna Chem) was added to the medium when cells were seeded, and the IFN-γ level was maintained throughout the chase period. Bacterial suspensions were prepared using cultures grown in roller bottles without antibiotics. Macrophages were infected at 37 °C in 5 % CO₂ for 2 h at an m.o.i. of 0.5 bacteria per cell in triplicate. Extracellular bacteria were removed by washing each well three times with DMEM. The number of viable intracellular bacteria was determined at day 0, 1, 3, 5, 7 and 9 after infection by lysing adherent cells with ice-cold water and 0.04 % SDS. Lysates were plated in serial dilutions on 7H10 OADC and c.f.u. were counted after 3 weeks of incubation at 37 °C. Plating of the culture medium in preceding experiments had excluded significant extracellular bacterial growth (data not shown).

Measurement of TNF-α production. Supernatants from control and infected BMDM of BALB/c mice were harvested at 5, 24, 48 and 72 h post-infection, centrifuged at 4000 r.p.m. and aliquots were stored at -70 °C. Quantification of c.f.u. confirmed that the numbers of bacteria in the inocula were similar for the two strains (data not shown). TNF-α was detected by ELISA (Quantikine, R&D Systems) according to the manufacturer's instructions. Recombinant TNF-α (R&D Systems) was used to generate standard curves. Mock infection with culture medium served as a negative control. Each condition was measured in duplicate and in four independent experiments from distinct cell preparations.

Intracellular co-localization experiments. BMDM of BALB/c mice were cultured on 0.7 mm glass coverslips (Assistent) in 24-well plates at 1.5–2 × 10⁵ cells per well with or without 100 U ml⁻¹ recombinant murine IFN-γ. Washed mycobacteria were labelled with FITC (Sigma-Aldrich) by incubation at 37 °C for 30 min in PBS, pH 7.4, containing 10 mM Na₂CO₃ and 0.05 % Tween 80. Bacterial suspensions were pipetted 10 times through a 26-gauge needle to remove *M. tuberculosis* aggregates. Latex beads with a diameter of 2.07 µm (Bangs Laboratories) were labelled with FITC (Sigma-Aldrich) by incubation at 4 °C for 30 min with constant shaking at 700 r.p.m. in PBS, pH 8.3, containing 10 mM Na₂CO₃. Macrophages were infected at m.o.i. 50–100 in triplicate. Phagocytosis was synchronized by allowing the bacteria to adhere to host cells for 60 min at 4 °C before starting phagocytosis by washing each well, adding preheated (37 °C) culture medium and incubating at 37 °C in 5 % CO₂. After a chase of 5, 20, 60 or 1200 min, coverslips were fixed with 4 % paraformaldehyde in PBS, pH 7.4. Samples were incubated in PBS, pH 7.4, with 2 % FCS, 0.2 % BSA and 0.05 % saponin prior to staining with the primary antibody rat anti-LAMP1 (Santa Cruz) and secondary antibody goat anti-rat Alexa Fluor 568 (Molecular Probes). Coverslips were mounted in Mowiol (Calbiochem), blinded and sequentially analysed for co-localization with a Leica TCS SP2 confocal microscope (Mannheim).

Measurement of intraphagosomal pH. Intraphagosomal pH measurement was performed as described previously (Master *et al.*, 2008). Briefly, mycobacteria were labelled with 5(6)-carboxyfluorescein *N*-succinimidyl ester (CFSE) (Sigma-Aldrich) by incubation in PBS, pH 7.4, containing 0.05 % Tween 80 for 60 min and 5(6)-carboxyrhodamine 6G succinimidylester (CRSE) (Molecular Probes) for 30 min (added to the bacteria/CFSE suspension after 30 min) at 37 °C with shaking (400 r.p.m.) in the dark. Unreacted fluorophore was removed by washing with PBS, pH 7.4. Bacteria were added to triplicates of 2 × 10⁵ ml⁻¹ host cells (BMDM or cell line J774A.1) at m.o.i. 100–500 in 96-well plates. Bacteria were sedimented to the cells by spin inoculation (300 r.p.m. for 5 min at 4 °C) and incubated for 60 min at 4 °C for synchronization. Phagocytosis was initiated by washing each well, adding preheated culture medium and incubating at 37 °C in 5 % CO₂. After 5 and 150 min, cells were washed and maintained in PBS, pH 7.4, for recording with a spectrofluorimeter (Synergy HT, Bio-Tek). Intraphagosomal pH was calculated from CFSE/CRSE ratios using a calibration curve established from cells fed with double-labelled mycobacteria in PBS with defined pH (5.0–7.0) containing the ionophores nigericin (20 µM) (Fluka) and monensin (4 µM) (Fluka) to equilibrate the intracellular and extracellular pH. Addition of bafilomycin A1 (40 µM) (Sigma-Aldrich) at the beginning of the incubation period dissipated the intraphagosomal pH measured at 150 min, indicating that the signal originated from intracellular bacilli (data not shown).

RESULTS

Attenuation of *lspA::aph* does not involve TLR2

M. tuberculosis lipoproteins trigger a TLR2 host response, which contributes to control of *M. tuberculosis* infection

(Reiling *et al.*, 2002) and phagosome maturation (Blander & Medzhitov, 2006). We have recently shown that the *lspA::aph* mutant, which is impaired in lipoprotein synthesis, is severely attenuated in both resistant and susceptible mouse strains (Sander *et al.*, 2004). To investigate the role of TLR2 in attenuation of the mutant, we infected TLR2^{-/-} mice and mice of the isogenic parental strain C57BL/6 by aerosol with *M. tuberculosis* wild-type, *lspA::aph* mutant and *lspA::aph-lspA* complemented mutant (Fig. 1). The lungs of mice infected with the parental strain exhibited the characteristic mode of bacterial growth: after initial multiplication during the first weeks, the number of c.f.u. reached a constant plateau over time. Following replication in the lungs the micro-organisms disseminated into the spleen. As reported previously (Sander *et al.*, 2004), the *lspA::aph* mutant showed a 2.5–3.5 log reduction in c.f.u. in lungs (Fig. 1) and was markedly impaired in dissemination to the spleen (see Supplementary Fig. S1) compared with the wild-type. Complementation of the mutant restored the wild-type phenotype. For wild-type *M. tuberculosis* the bacterial burden of infected organs was similar in wild-type and TLR2^{-/-} mice. Growth of the *lspA::aph* mutant was likewise independent of the TLR2 genotype of the host, i.e. upon infection of TLR2^{-/-} mice, the *lspA* knockout mutant showed a similar reduction in c.f.u. in the lungs

Fig. 1. Kinetics of *in vivo* growth of *M. tuberculosis* wild-type and mutants in lungs over a 108 day period. C57BL/6 mice (filled symbols) and C57BL/6 TLR2^{-/-} mice (open symbols) were infected with *M. tuberculosis* wild-type (squares), *lspA::aph* mutant (triangles) and *lspA::aph-lspA* complemented mutant (circles). The data are expressed as the mean $\pm$ SD (error bars) of c.f.u. obtained with five mice at each time point. Data were analysed by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test; *M. tuberculosis* wild type c.f.u. compared with *lspA::aph* c.f.u. in C57BL/6 and in TLR2^{-/-} mice were significantly different ($P < 0.001$); c.f.u. of *M. tuberculosis* wild type in C57BL/6 compared with c.f.u. in TLR2^{-/-} mice were not significantly different ($P > 0.05$); c.f.u. of *lspA::aph* in C57BL/6 compared with c.f.u. in TLR2^{-/-} mice were not significantly different ($P > 0.05$).

(Fig. 1) and impaired dissemination to the spleen (Supplementary Fig. S1) as upon infection of parental C57BL/6 mice. Complementation of *lspA::aph* restored wild-type virulence in TLR2^{-/-} mice.

lspA::aph is attenuated in macrophages

We next studied whether the *in vivo* attenuation of the *lspA::aph* mutant (Fig. 1; Sander *et al.*, 2004) correlated with attenuation of the mutant in *in vitro* models of infection. We infected resting BMDM of BALB/c mice with *M. tuberculosis* wild-type or *lspA::aph* mutant. BMDM were lysed at different time points post-infection and plated on agar to count c.f.u. (Fig. 2a). c.f.u. of the wild type increased about 800-fold over the time period studied, which is in the range reported by others for strain H37Rv in mouse BMDM (Berthet *et al.*, 1998; Hunt *et al.*, 2008; Rooyackers & Stokes, 2005). The increase in the number of c.f.u. was due to intracellular multiplication, as the bacteria in the cell medium account for less than 10 % of the total bacterial counts (data not shown) and DMEM does not support mycobacterial replication (Bryk *et al.*, 2008). The increase in c.f.u. for the *lspA::aph* mutant was approximately one-tenth of that of the wild type at day 9 after infection (Fig. 2a, inset). Activation of BMDM with IFN- γ reduced intracellular multiplication of wild-type bacteria by 26-fold. Likewise, the intracellular growth of the *lspA::aph* knockout mutant was impaired upon IFN- γ activation (Fig. 2b). The relative difference in intracellular multiplication between the wild type and *lspA::aph* mutant remained similar in resting and IFN- γ -activated BMDM (~ 10 -fold). Complementation restored wild-type growth (see Supplementary Fig. S2). The phenotype of *in vitro* infections paralleled the *in vivo* phenotype of the mutant, which already exhibited a growth defect in the initial phase of infection (Fig. 1). Multiplication in the mycobacterial growth media 7H9-ADC (see Supplementary Fig. S3) and 7H9-OADC (Sander *et al.*, 2004) was not impaired.

LspA and TNF- α secretion by BMDM

Upon phagocytosis, *M. tuberculosis* induces TNF- α in macrophages, and host immunity against tuberculosis is crucially linked to this cytokine. An inverse correlation between TNF- α secretion and intracellular growth rate of clinical isolates has been reported (Theus *et al.*, 2007). To study whether interference with lipoprotein maturation in *M. tuberculosis* directly or indirectly affects macrophage activation we infected BMDM with *M. tuberculosis* wild-type or *lspA::aph* mutant. We measured the amount of TNF- α released 5–72 h post-infection. Over the time period studied TNF- α secretion of both strains increased in parallel as compared with untreated controls, and there was no significant difference between wild-type and *lspA::aph* infected cells at any time (see Supplementary Fig. S4). Wild-type-like TNF- α secretion of BMDM upon infection with the *lspA::aph* mutant indicates that

Fig. 2. Multiplication of *M. tuberculosis* wild-type and *lspA::aph* mutant in (a) resting and (b) IFN- γ -activated BMDM at m.o.i. 0.5. Main panels show fold increase in c.f.u. of *M. tuberculosis* wild-type (■) and *lspA::aph* mutant (▲). Insets show relative growth of *lspA::aph* mutant compared with wild-type as a percentage. Values represent the mean $\pm$ SD (error bars) of four (resting BMDM) and three (activated BMDM) independent experiments. Statistical analysis was done by unpaired *t* test; wild-type compared with *lspA::aph* was significantly different ($P < 0.05$) at days 5, 7 and 9 in unstimulated BMDM, and at days 7 and 9 in IFN- γ -stimulated BMDM.

attenuation is not due to gross changes in macrophage bacterial sensing and signal transduction, corroborating the lack of TLR2-dependence evident *in vivo*.

lspA::aph phagosomes and LAMP1

Phagosome maturation arrest is a striking feature of pathogenic mycobacteria and for some *M. tuberculosis* mutants a correlation between attenuation and loss of phagosome maturation arrest exists (Pethe *et al.*, 2004). This prompted us to investigate whether the attenuated

phenotype of *lspA::aph* is associated with phagosome maturation. The phagosome acquires LAMP-1 during maturation, resulting in peak LAMP1 levels in late phagosomes and phago-lysosomes. We infected macrophages with fluorescently labelled latex beads, *M. tuberculosis* wild-type or *lspA::aph*, and quantified co-localization of phagosomes with LAMP1 at different time points post-infection using confocal microscopy (Fig. 3a). Latex beads served as a positive control to demonstrate phagosome maturation. They rapidly and progressively co-localized with LAMP1 (Fig. 3c). Wild-type bacteria co-localized with LAMP1 to about 20 % at 5, 20 and 60 min post-infection. Maximum co-localization of ~40 % was reached at the late time point (20 h), which is consistent with prior reports (Clemens & Horwitz, 1995; Ramachandra *et al.*, 2001; Xu *et al.*, 1994). The *lspA::aph* mutant showed wild-type-like characteristics with respect to degree and kinetics of LAMP1 co-localization (Fig. 3a, c).

IFN- γ activation of macrophages promotes phagosome maturation in cells infected with *M. tuberculosis*. To study whether inactivation of LspA affects IFN- γ -mediated phagosome maturation, we quantified co-localization of mycobacterial phagosomes with LAMP1 in IFN- γ -activated BMDM (Fig. 3b). As early as 20 min after infection, ~50 % of *M. tuberculosis* wild-type phagosomes acquired LAMP1, and this percentage remained unchanged during the course of the experiment. The *lspA::aph* mutant phagosomes showed similar kinetics of LAMP1 acquisition to wild-type bacterial phagosomes (Fig. 3b, c). Thus, LspA inactivation has no effect on LAMP1 acquisition of *M. tuberculosis*-containing phagosomes in resting and activated macrophages.

Acidification of *lspA::aph* phagosomes

We wanted to further characterize the *M. tuberculosis* *lspA::aph* phagosome and to corroborate the finding that phagosome maturation arrest is not affected by inactivation of LspA. Concurrent with phagosome maturation is acidification of the phagosomes (Crowle *et al.*, 1991; Hackam *et al.*, 1998; Oh & Straubinger, 1996; Sturgill-Koszycki *et al.*, 1994). Thus, we determined the intraphagosomal pH of *lspA::aph* phagosomes compared with *M. tuberculosis* wild-type phagosomes in BMDM by ratio-metric measurement (Fig. 4a). The intraphagosomal pH was calculated from fluorescence ratio analysis after excitation at 485/530 nm and measurement at 528/590 nm with a spectrofluorimeter. The intraphagosomal pH of all strains started to decline within minutes, and at 150 min after infection wild-type phagosomes were modestly acidified with a mean pH of 6.4, while phagosomes containing heat-inactivated wild-type bacteria were acidified to pH 5.8. These levels of intraphagosomal pH for live and dead mycobacteria are consistent with data reported elsewhere (de Chastellier & Thilo, 2006; Russell *et al.*, 2005; Sturgill-Koszycki *et al.*, 1994). The pH of the *lspA::aph* phagosomes was 6.3, which is not significantly different from that of *M. tuberculosis* wild-type phago-

Fig. 3. Kinetics of co-localization of *M. tuberculosis* wild-type (■) and *LspA::aph* mutant (▲) with late phagosomal marker LAMP1 in (a) resting and (b) IFN- γ -activated BMDM. Latex beads (●) served as a positive control. Values represent the means $\pm$ SD (error bars) of three independent experiments. Data were analysed by one-way ANOVA followed by Bonferroni's multiple comparison test; wild-type compared with *LspA::aph* was not significantly different ($P > 0.05$). (c) Representative confocal immunofluorescence images are shown of non-activated macrophages infected with FITC-labelled latex beads (top row), *M. tuberculosis* wild-type (second row), *LspA::aph* mutant (third row) or IFN- γ -activated macrophages infected with *M. tuberculosis* wild-type (bottom row) and immunostained for LAMP1 60 min after infection. Co-localization is indicated by arrows.

somes. Activation of BMDM with IFN- γ reduced the intraphagosomal pH to ~ 5.7 , independent of the strain used for infection (Fig. 4b). Intraphagosomal pH measurements in unstimulated mouse monocyte-macrophage cell line J774A.1 revealed results similar to those in unstimulated BMDM: a significantly lower pH of phagosomes containing heat-killed mycobacteria as compared with live *M. tuberculosis*, and no difference between wild-type and the *LspA::aph* mutant (Fig. 4c).

DISCUSSION

We have previously shown that the *M. tuberculosis* *LspA::aph* mutant has a strongly attenuated phenotype in a mouse infection model (Sander *et al.*, 2004). Here we investigated putative mechanisms involved in attenuation of this mutant at a cellular level. We found that a TLR2-dependent host response does not contribute to growth restriction of the *LspA::aph* mutant. We also found that mature lipoproteins are dispensable for phagosome

maturation arrest but required for intracellular survival. Our results demonstrate that intracellular survival of the *LspA::aph* mutant and phagosome maturation arrest are not necessarily linked but can be dissociated.

The *M. tuberculosis* genome encodes approximately 100 lipoproteins (Rezwan *et al.*, 2007a; Sutcliffe & Harrington, 2004). Some contribute to immunopathogenesis of tuberculosis via TLR2 (Bigi *et al.*, 2004; Brightbill *et al.*, 1999), while other lipoproteins have protective properties and are considered to be tuberculosis vaccine candidates (Romano *et al.*, 2006; Wang *et al.*, 2007). *LspA* mutants are unable to cleave off the signal peptide from pro-lipoproteins. The presence of a signal peptide inhibits the subsequent modification step and the transport of lipoproteins (Masuda *et al.*, 2002). In *LspA* mutants, protein release (shedding) and proteolytic cleavage of immature lipoproteins (shaving) by proteases with other specificities has been reported (Antelmann *et al.*, 2001). Both release and cleavage affect localization, function and antigenicity of proteins and may alter the protective or anti-protective

Fig. 4. Intraphagosomal pH of *M. tuberculosis* wild-type or *lspA::aph* phagosomes in (a) resting BMDM, (b) IFN- γ -activated BMDM and (c) resting J774 at 150 min after infection. Values represent the mean pH of triplicates $\pm$ SD (error bars) measured in six independent experiments. Dead (heat-inactivated) *M. tuberculosis* served as a control. Data were analysed by one-way ANOVA followed by Bonferroni's multiple comparison test; n.s., not significant ($P > 0.05$); ** $P \leq 0.01$; *** $P < 0.001$.

affect of lipoproteins. Under certain conditions, TLR2 contributes to control of *M. tuberculosis* infection (Reiling *et al.*, 2002); therefore, we investigated growth of wild type and *lspA::aph* mutant strains in TLR2^{-/-} and congenic C57BL/6 mice. We investigated virulence in a low-dose aerosol mouse model. Under these conditions the TLR2 genotype of the host does not affect control of *M. tuberculosis* wild-type (Fig. 1). Likewise, we observed

attenuation of the *lspA::aph* mutant in TLR2^{-/-} mice similar to that in congenic C57BL/6 mice. In addition, we measured a similar secretion of TNF- α by BMDM following *in vitro* infection with either mutant or wild-type (Supplementary Fig. S4). Thus, lipoprotein processing by LspA is not critical for TNF- α secretion or TLR2 responses to mycobacterial infection *in vivo*. Our *in vitro* findings are in agreement with those of others (Doz *et al.*, 2007; Gilleron *et al.*, 2003), who have reported that besides lipoproteins additional mycobacterial components such as PIM activate TLR2 in primary macrophages. In contrast, Banaiee *et al.* (2006) found a modestly reduced TNF- α secretion in RAW264.7 cells and defective TLR2 stimulation in HEK293-TLR2 reporter cells when exposed to a Δ *lspA* knockout mutant. This discrepancy may be explained by the different experimental settings. Our *in vitro* and *in vivo* data effectively rule out a significant involvement of TLR2 in virulence attenuation of the *lspA::aph* mutant.

A number of studies demonstrate a correlation between *in vivo* attenuation of mycobacterial mutants and impaired *in vitro* survival in macrophages (Camacho *et al.*, 1999; Sasseti & Rubin, 2003). The *in vivo* phenotype of the *lspA::aph* mutant is characterized by a pronounced reduction in c.f.u., which is already prominent soon after infection. We therefore studied whether this was concurrent with a reduced survival in macrophages. Macrophages are major effector cells involved in control of bacterial infection, but are also a major niche for multiplication of pathogenic mycobacteria. In *in vitro* macrophage infection experiments a variety of *M. tuberculosis* growth/survival rates, ranging from complete growth restriction (Vandal *et al.*, 2006) to extensive multiplication (100-fold in 6 days; 1000-fold in 9 days) (Berthet *et al.*, 1998; Rooyakkers & Stokes, 2005), have been described. In our studies, *M. tuberculosis* multiplied ~800-fold over 9 days. Stimulation of macrophages with IFN- γ consistently restricts growth of *M. tuberculosis* in relation to unstimulated cells. Again, huge differences exist in the literature with respect to absolute growth rates in IFN- γ -activated cells, ranging from 90 % killing (MacMicking *et al.*, 2003) to more than a 20-fold increase in 7 days (Rooyakkers & Stokes, 2005). In our experiments IFN- γ reduced growth of *M. tuberculosis* as compared with untreated controls 26-fold, but still allowed multiplication of *M. tuberculosis*. The reasons for the inter-laboratory growth differences of *M. tuberculosis* in both unstimulated and stimulated macrophages are not entirely clear. Compared with *M. tuberculosis* wild-type the growth of the mutant was significantly reduced in resting as well as in IFN- γ -activated BMDM (Fig. 2). Wild-type and *lspA::aph* show an identical growth rate in culture broth (Supplementary Fig. S3), excluding a simple growth deficit of the *lspA::aph* mutant. Thus, inactivation of LspA causes an intracellular fitness reduction of the bacteria upon both *in vivo* and *in vitro* infection.

The ability to arrest phagosome maturation is a hallmark of *M. tuberculosis* (Armstrong & Hart, 1971). Besides

avoidance of phago-lysosomal fusion, relatively little is known about how *M. tuberculosis* manages its intracellular lifestyle. Few of the various *M. tuberculosis* mutants which exhibit *in vivo* attenuation (Sasseti & Rubin, 2003; Smith, 2003) have been characterized with respect to intracellular localization. Likewise few of the mutants displaying a trafficking phenotype have been characterized for *in vivo* attenuation (MacGurn & Cox, 2007; Pethe *et al.*, 2004). While lipids of the mycobacterial cell wall are reportedly involved in phagosome maturation arrest, the role of lipoproteins is less clear. When studying co-localization of wild-type and *lspA::aph* mutant bacteria with the phagosomal/phagolysosomal marker LAMP1 we found little co-localization for both strains in resting macrophages (Fig. 3). In contrast, a mutant deficient in zinc metalloprotease *zmp1* shows a high degree of co-localization (Master *et al.*, 2008). To corroborate *lspA::aph* localization in an early, i.e. a LAMP-negative, phagosome we determined phagosomal pH, which is considered to be the ultimate marker for phagosome maturation (Huynh & Grinstein, 2007). We measured intraphagosomal pH of wild-type and *lspA::aph* mutant phagosomes (Fig. 4). The intraphagosomal pH was ~6.4 for both strains, demonstrating that there is no apparent difference between the *lspA::aph* phagosome and the *M. tuberculosis* wild-type phagosome. Thus, both strains localize to a compartment with early endosomal characteristics, including absence of LAMP1 and mildly acidic pH. In contrast, phagosomes containing *zmp1* mutants show a reduced pH (Master *et al.*, 2008). Following IFN- γ activation of the host cell, the mycobacteria-containing compartment acidifies to a pH of about 5.7. IFN- γ -induced acidification was also found in cells infected with the *lspA::aph* mutant and was similar to that in cells infected with the wild-type strain. We conclude that inactivation of LspA does not affect phagosome-maturation arrest, indicating that attenuation of the mutant takes place in a prelysosomal compartment. This suggests that LspA is required for multiplication of *M. tuberculosis* in early phagosomes. The early phagosome is thought to be a nutrient-restricted environment, which is important in limiting the availability of nutrients to phagocytosed bacteria (De Voss *et al.*, 2000; Wagner *et al.*, 2005). Despite some research in this field the key elements critical for adaptation of mycobacteria to this prelysosomal environment are unknown.

Approximately 100 *M. tuberculosis* proteins have been annotated as lipoproteins. Several of these lipoproteins are involved in uptake of nutrients or ions (PstS, ModA, FecB, LpqZ, LpqY; Sutcliffe & Harrington, 2004). Thus, the limited growth of the *lspA::aph* mutant in the nutrient-poor phagosome may be due to mislocation or malfunction of transport-associated lipoproteins. These uptake mechanisms may only be important in the nutrient-limited milieu of the early phagosome and not in nutrient-rich broth medium. Alternatively, the *lspA::aph* mutant may be more susceptible to direct host attacks. The physiology of *M. tuberculosis* is intimately linked to integrity of the cell wall, and some lipoproteins are involved in cell wall metabolism

(LppX, LpqK, LppW; Sutcliffe & Harrington, 2004). LppX is required for the translocation of phthiocerol dimycoserates to the outer membrane of *M. tuberculosis*, and disruption of *lppX* is accompanied by attenuation of the tubercle bacillus (Sulzenbacher *et al.*, 2006). At a gross level, i.e. by Ziehl-Neelsen staining, mycolic acid analysis and inspection by electron microscopy (Sander *et al.*, 2004), the cell wall of the *lspA* mutant is indistinguishable from that of the parental strain. However, minor alterations not detected by these investigations may increase the susceptibility of the mutant to antibacterial mechanisms of the host. Finally, lipoproteins of unknown functions (e.g. Mpt83) which are highly upregulated during all stages of *in vitro* and *in vivo* infection (Schnappinger *et al.*, 2003) may be required for intracellular survival and multiplication.

Deciphering the *M. tuberculosis* genome has facilitated the development of screens designed to identify genes implicated in the infection process (Lamichhane *et al.*, 2003; Sasseti *et al.*, 2001; Sasseti & Rubin, 2003). Overall, a significant overlap between genes required for survival of *M. tuberculosis* in macrophages and those required for full-blown virulence in *in vivo* infection of mice has been found. With respect to a correlation between intracellular survival and phagosome acidification, the results from genetic screens are contradictory and inconsistent. In a *Mycobacterium bovis* BCG transposon library screen designed to identify mutants defective in intracellular survival or in prevention of phagosomal acidification, little overlap between the two phenotypes was observed (Stewart *et al.*, 2005). Similarly, MacGurn & Cox (2007) observed little correlation between the degree of attenuation *in vivo* and phagosome maturation of the screened mutants. In contrast, the transposon mutagenesis screen reported by Pethe *et al.* (2004) found a correlation between mutants with increased localization to lysosomes and mutants with reduced intracellular survival (Pethe *et al.*, 2004). Surprisingly, no matches among the mutants identified to be deficient in phagosome maturation arrest exist between the three screens. In addition, neither screen picked up factors known to be involved in phagosome maturation arrest, e.g. lipoarabinomannan (LAM).

Phagosome maturation arrest is an active process requiring live bacteria. Our findings disprove the hypothesis that *lspA::aph* attenuation *in vivo* parallels phagosome acidification. Our results point to a mechanism restricting intracellular growth of *M. tuberculosis* that acts prior to the conversion of early phagosomes to late phagosomes. They indicate a role for the early mycobacterial phagosome in growth restriction of intracellular *M. tuberculosis* and of non-killing mechanisms in the control of tuberculosis infection, mechanisms that are unrelated to phagosome maturation arrest and avoidance of phago-lysosome fusion.

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REFERENCES

- Anes, E., Kuhnel, M. P., Bos, E., Moniz-Pereira, J., Habermann, A. & Griffiths, G. (2003). Selected lipids activate phagosome actin assembly and maturation resulting in killing of pathogenic mycobacteria. *Nat Cell Biol* 5, 793–802.
- Antelmann, H., Tjalsma, H., Voigt, B., Ohlmeier, S., Bron, S., van Dijk, J. M. & Hecker, M. (2001). A proteomic view on genome-based signal peptide predictions. *Genome Res* 11, 1484–1502.
- Armstrong, J. A. & Hart, P. D. (1971). Response of cultured macrophages to *Mycobacterium tuberculosis*, with observations on fusion of lysosomes with phagosomes. *J Exp Med* 134, 713–740.
- Banaiee, N., Kincaid, E. Z., Buchwald, U., Jacobs, W. R., Jr & Ernst, J. D. (2006). Potent inhibition of macrophage responses to IFN- γ by live virulent *Mycobacterium tuberculosis* is independent of mature mycobacterial lipoproteins but dependent on TLR2. *J Immunol* 176, 3019–3027.
- Berthet, F. X., Lagranderie, M., Gounon, P., Laurent-Winter, C., Ensergueix, D., Chavarot, P., Thouron, F., Maranghi, E., Pelicic, V. & other authors (1998). Attenuation of virulence by disruption of the *Mycobacterium tuberculosis* *erp* gene. *Science* 282, 759–762.
- Bigi, F., Gioffre, A., Klepp, L., Santangelo, M. P., Alito, A., Caimi, K., Meikle, V., Zumárraga, M., Taboga, O. & other authors (2004). The knockout of the *lprG-Rv1410* operon produces strong attenuation of *Mycobacterium tuberculosis*. *Microbes Infect* 6, 182–187.
- Blander, J. M. & Medzhitov, R. (2004). Regulation of phagosome maturation by signals from Toll-like receptors. *Science* 304, 1014–1018.
- Blander, J. M. & Medzhitov, R. (2006). On regulation of phagosome maturation and antigen presentation. *Nat Immunol* 7, 1029–1035.
- Brightbill, H. D., Libraty, D. H., Krutzik, S. R., Yang, R. B., Belisle, J. T., Bleharski, J. R., Maitland, M., Norgard, M. V., Plevy, S. E. & other authors (1999). Host defense mechanisms triggered by microbial lipoproteins through Toll-like receptors. *Science* 285, 732–736.
- Bryk, R., Gold, B., Venugopal, A., Sing, J., Samy, R., Pupek, K., Cao, H., Popescu, C., Gurney, M. & other authors (2008). Selective killing of nonreplicating mycobacteria. *Cell Host Microbe* 3, 137–145.
- Camacho, L. R., Ensergueix, D., Perez, E., Gicquel, B. & Guilhot, C. (1999). Identification of a virulence gene cluster of *Mycobacterium tuberculosis* by signature-tagged transposon mutagenesis. *Mol Microbiol* 34, 257–267.
- Chua, J., Vergne, I., Master, S. & Deretic, V. (2004). A tale of two lipids: *Mycobacterium tuberculosis* phagosome maturation arrest. *Curr Opin Microbiol* 7, 71–77.
- Clemens, D. L. & Horwitz, M. A. (1995). Characterization of the *Mycobacterium tuberculosis* phagosome and evidence that phagosomal maturation is inhibited. *J Exp Med* 181, 257–270.
- Clemens, D. L. & Horwitz, M. A. (1996). The *Mycobacterium tuberculosis* phagosome interacts with early endosomes and is accessible to exogenously administered transferrin. *J Exp Med* 184, 1349–1355.
- Clemens, D. L., Lee, B. Y. & Horwitz, M. A. (2000). *Mycobacterium tuberculosis* and *Legionella pneumophila* phagosomes exhibit arrested maturation despite acquisition of Rab7. *Infect Immun* 68, 5154–5166.
- Crowle, A. J., Dahl, R., Ross, E. & May, M. H. (1991). Evidence that vesicles containing living, virulent *Mycobacterium tuberculosis* or *Mycobacterium avium* in cultured human macrophages are not acidic. *Infect Immun* 59, 1823–1831.
- de Chastellier, C. & Thilo, L. (2006). Cholesterol depletion in *Mycobacterium avium*-infected macrophages overcomes the block in phagosome maturation and leads to the reversible sequestration of viable mycobacteria in phagolysosome-derived autophagic vacuoles. *Cell Microbiol* 8, 242–256.
- Dev, I. K. & Ray, P. H. (1984). Rapid assay and purification of a unique signal peptidase that processes the prolipoprotein from *Escherichia coli* B. *J Biol Chem* 259, 11114–11120.
- De Voss, J. J., Rutter, K., Schroeder, B. G., Su, H., Zhu, Y. & Barry, C. E., III (2000). The salicylate-derived mycobactin siderophores of *Mycobacterium tuberculosis* are essential for growth in macrophages. *Proc Natl Acad Sci U S A* 97, 1252–1257.
- Doz, E., Rose, S., Nigou, J., Gilleron, M., Puzo, G., Erard, F., Ryffel, B. & Quesniaux, V. F. J. (2007). Acylation determines the Toll-like receptor (TLR)-dependent positive versus TLR2-, mannose receptor-, and SIGNR1-independent negative regulation of pro-inflammatory cytokines by mycobacterial lipomannan. *J Biol Chem* 282, 26014–26025.
- Fratti, R. A., Backer, J. M., Gruenberg, J., Corvera, S. & Deretic, V. (2001). Role of phosphatidylinositol 3-kinase and Rab5 effectors in phagosomal biogenesis and mycobacterial phagosome maturation arrest. *J Cell Biol* 154, 631–644.
- Fratti, R. A., Chua, J., Vergne, I. & Deretic, V. (2003). *Mycobacterium tuberculosis* glycosylated phosphatidylinositol causes phagosome maturation arrest. *Proc Natl Acad Sci U S A* 100, 5437–5442.
- Gehring, A. J., Dobos, K. M., Belisle, J. T., Harding, C. V. & Boom, W. H. (2004). *Mycobacterium tuberculosis* LprG (Rv1411c): a novel TLR-2 ligand that inhibits human macrophage class II MHC antigen processing. *J Immunol* 173, 2660–2668.
- Gilleron, M., Quesniaux, V. F. J. & Puzo, G. (2003). Acylation state of the phosphatidylinositol hexamannosides from *Mycobacterium bovis* bacillus Calmette Guérin and *Mycobacterium tuberculosis* H37Rv and its implication in Toll-like receptor response. *J Biol Chem* 278, 29880–29889.
- Hackam, D. J., Rotstein, O. D., Zhang, W., Gruenheid, S., Gros, P. & Grinstein, S. (1998). Host resistance to intracellular infection: mutation of natural resistance-associated macrophage protein 1 (Nramp1) impairs phagosomal acidification. *J Exp Med* 188, 351–364.
- Hasan, Z., Schlax, C., Kuhn, L., Lefkovits, I., Young, D., Thole, J. & Pieters, J. (1997). Isolation and characterization of the mycobacterial phagosome: segregation from the endosomal/lysosomal pathway. *Mol Microbiol* 24, 545–553.
- Hostetter, J. M., Steadham, E. M., Haynes, J. S., Bailey, T. B. & Cheville, N. F. (2002). Cytokine effects on maturation of the phagosomes containing *Mycobacterium avium* subspecies paratuberculosis in J774 cells. *FEMS Immunol Med Microbiol* 34, 127–134.
- Hunt, D. M., Saldanha, J. W., Brennan, J. F., Benjamin, P., Strom, M., Cole, J. A., Spreadbury, C. L. & Buxton, R. S. (2008). Single nucleotide polymorphisms that cause structural changes in the cyclic AMP receptor protein transcriptional regulator of the tuberculosis vaccine strain *Mycobacterium bovis* BCG alter global gene expression without attenuating growth. *Infect Immun* 76, 2227–2234.
- Huynh, K. K. & Grinstein, S. (2007). Regulation of vacuolar pH and its modulation by some microbial species. *Microbiol Mol Biol Rev* 71, 452–462.

- Indrigo, J., Hunter, R. L., Jr & Actor, J. K. (2003). Cord factor trehalose 6,6'-dimycolate (TDM) mediates trafficking events during mycobacterial infection of murine macrophages. *Microbiology* **149**, 2049–2059.
- Jin, M. S., Kim, E., Heo, J. Y., Lee, M. E., Kim, H. M., Paik, S. G., Lee, H. & Lee, J. O. (2007). Crystal structure of the TLR1–TLR2 heterodimer induced by binding of tri-acylated lipopeptide. *Cell* **130**, 1071–1082.
- Kanzler, H., Barrat, F. J., Hessel, E. M. & Coffman, R. L. (2007). Therapeutic targeting of innate immunity with Toll-like receptor agonists and antagonists. *Nat Med* **13**, 552–559.
- Kelley, V. A. & Schorey, J. S. (2003). *Mycobacterium's* arrest of phagosome maturation in macrophages requires Rab5 activity and accessibility to iron. *Mol Biol Cell* **14**, 3366–3377.
- Lamichhane, G., Zignol, M., Blades, N. J., Geiman, D. E., Dougherty, A., Grosset, J., Broman, K. W. & Bishai, W. R. (2003). A postgenomic method for predicting essential genes at subsaturation levels of mutagenesis: application to *Mycobacterium tuberculosis*. *Proc Natl Acad Sci U S A* **100**, 7213–7218.
- MacGurn, J. A. & Cox, J. S. (2007). A genetic screen for *Mycobacterium tuberculosis* mutants defective for phagosome maturation arrest identifies components of the ESX-1 secretion system. *Infect Immun* **75**, 2668–2678.
- MacMicking, J. D., Taylor, G. A. & McKinney, J. D. (2003). Immune control of tuberculosis by IFN- γ -inducible LRG-47. *Science* **302**, 654–659.
- Master, S. S., Rampini, S. K., Davis, A. S., Keller, C., Ehlers, S., Springer, B., Timmins, G., Sander, P. & Deretic, V. (2008). *Mycobacterium tuberculosis* prevents inflammasome activation. *Cell Host Microbe* **3**, 224–232.
- Masuda, K., Matsuyama, S. & Tokuda, H. (2002). Elucidation of the function of lipoprotein-sorting signals that determine membrane localization. *Proc Natl Acad Sci U S A* **99**, 7390–7395.
- Mawuenyega, K. G., Forst, C. V., Dobos, K. M., Belisle, J. T., Chen, J., Bradbury, E. M., Bradbury, A. R. & Chen, X. (2005). *Mycobacterium tuberculosis* functional network analysis by global subcellular protein profiling. *Mol Biol Cell* **16**, 396–404.
- Noss, E. H., Pai, R. K., Sellati, T. J., Radolf, J. D., Belisle, J., Golenbock, D. T., Boom, W. H. & Harding, C. V. (2001). Toll-like receptor 2-dependent inhibition of macrophage class II MHC expression and antigen processing by 19-kDa lipoprotein of *Mycobacterium tuberculosis*. *J Immunol* **167**, 910–918.
- Oh, Y. K. & Straubinger, R. M. (1996). Intracellular fate of *Mycobacterium avium*: use of dual-label spectrofluorometry to investigate the influence of bacterial viability and opsonization on phagosomal pH and phagosome-lysosome interaction. *Infect Immun* **64**, 319–325.
- Pethe, K., Swenson, D. L., Alonso, S., Anderson, J., Wang, C. & Russell, D. G. (2004). Isolation of *Mycobacterium tuberculosis* mutants defective in the arrest of phagosome maturation. *Proc Natl Acad Sci U S A* **101**, 13642–13647.
- Ramachandra, L., Noss, E., Boom, W. H. & Harding, C. V. (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.
- Reiling, N., Holscher, C., Fehrenbach, A., Kroger, S., Kirschning, C. J., Goyert, S. & Ehlers, S. (2002). Cutting edge: Toll-like receptor (TLR)2- and TLR4-mediated pathogen recognition in resistance to airborne infection with *Mycobacterium tuberculosis*. *J Immunol* **169**, 3480–3484.
- Rezwan, M., Grau, T., Tschumi, A. & Sander, P. (2007a). Lipoprotein synthesis in mycobacteria. *Microbiology* **153**, 652–658.
- Rezwan, M., Laneelle, M. A., Sander, P. & Daffé, M. (2007b). Breaking down the wall: fractionation of mycobacteria. *J Microbiol Methods* **68**, 32–39.
- Rock, F. L., Hardiman, G., Timans, J. C., Kastelein, R. A. & Bazan, J. F. (1998). A family of human receptors structurally related to *Drosophila* Toll. *Proc Natl Acad Sci U S A* **95**, 588–593.
- Romano, M., Roupie, V., Wang, X. M., Denis, O., Jurion, F., Adnet, P. Y., Laali, R. & Huygen, K. (2006). Immunogenicity and protective efficacy of tuberculosis DNA vaccines combining mycolyl-transferase Ag85A and phosphate transport receptor PstS-3. *Immunology* **118**, 321–332.
- Rooyackers, A. W. J. & Stokes, R. W. (2005). Absence of complement receptor 3 results in reduced binding and ingestion of *Mycobacterium tuberculosis* but has no significant effect on the induction of reactive oxygen and nitrogen intermediates or on the survival of the bacteria in resident and interferon-gamma activated macrophages. *Microb Pathog* **39**, 57–67.
- Russell, D. G. (2001). *Mycobacterium tuberculosis*: here today, and here tomorrow. *Nat Rev Mol Cell Biol* **2**, 569–577.
- Russell, D. G., Purdy, G. E., Owens, R. M., Rohde, K. H. & Yates, R. M. (2005). *Mycobacterium tuberculosis* and the four-minute phagosome. *ASM News* **71**, 459–463.
- Saleh, M. T. & Belisle, J. T. (2000). Secretion of an acid phosphatase (SapM) by *Mycobacterium tuberculosis* that is similar to eukaryotic acid phosphatases. *J Bacteriol* **182**, 6850–6853.
- Sander, P., Rezwan, M., Walker, B., Rampini, S. K., Kroppenstedt, R. M., Ehlers, S., Keller, C., Keeble, J. R., Hagemeyer, M. & other authors (2004). Lipoprotein processing is required for virulence of *Mycobacterium tuberculosis*. *Mol Microbiol* **52**, 1543–1552.
- Sankaran, K. & Wu, H. C. (1994). Lipid modification of bacterial prolipoprotein: transfer of diacylglycerol moiety from phosphatidylglycerol. *J Biol Chem* **269**, 19701–19706.
- Sasseti, C. M. & Rubin, E. J. (2003). Genetic requirements for mycobacterial survival during infection. *Proc Natl Acad Sci U S A* **100**, 12989–12994.
- Sasseti, C. M., Boyd, D. H. & Rubin, E. J. (2001). Comprehensive identification of conditionally essential genes in mycobacteria. *Proc Natl Acad Sci U S A* **98**, 12712–12717.
- Schaible, U. E., Sturgill-Koszycki, S., Schlesinger, P. H. & Russell, D. G. (1998). Cytokine activation leads to acidification and increases maturation of *Mycobacterium avium*-containing phagosomes in murine macrophages. *J Immunol* **160**, 1290–1296.
- Schnappinger, D., Ehrh, S., Voskuil, M. I., Liu, Y., Mangan, J. A., Monahan, I. M., Dolganov, G., Efron, B., Butcher, P. D. & other authors (2003). Transcriptional adaptation of *Mycobacterium tuberculosis* within macrophages: insights into the phagosomal environment. *J Exp Med* **198**, 693–704.
- Smith, I. (2003). *Mycobacterium tuberculosis* pathogenesis and molecular determinants of virulence. *Clin Microbiol Rev* **16**, 463–496.
- Stewart, G. R., Patel, J., Robertson, B. D., Rae, A. & Young, D. B. (2005). Mycobacterial mutants with defective control of phagosomal acidification. *PLoS Pathog* **1**, 269–278.
- Sturgill-Koszycki, S., Schlesinger, P. H., Chakraborty, P., Haddix, P. L., Collins, H. L., Fok, A. K., Allen, R. D., Gluck, S. L., Heuser, J. & Russell, D. G. (1994). Lack of acidification in *Mycobacterium tuberculosis* phagosomes produced by exclusion of the vesicular proton-ATPase. *Science* **263**, 678–681.
- Sturgill-Koszycki, S., Schaible, U. E. & Russell, D. G. (1996). *Mycobacterium*-containing phagosomes are accessible to early endosomes and reflect a transitional state in normal phagosome biogenesis. *EMBO J* **15**, 6960–6968.

- Sulzenbacher, G., Canaan, S., Bordat, Y., Neyrolles, O., Stadthagen, G., Roig-Zamboni, V., Rauzier, J., Maurin, D., Laval, F. & other authors (2006).** LppX is a lipoprotein required for the translocation of phthiocerol dimycocerosates to the surface of *Mycobacterium tuberculosis*. *EMBO J* **25**, 1436–1444.
- Sutcliffe, I. C. & Harrington, D. J. (2004).** Lipoproteins of *Mycobacterium tuberculosis*: an abundant and functionally diverse class of cell envelope components. *FEMS Microbiol Rev* **28**, 645–659.
- Sutcliffe, I. C. & Russell, R. R. (1995).** Lipoproteins of Gram-positive bacteria. *J Bacteriol* **177**, 1123–1128.
- Theus, S., Eisenach, K., Fomukong, N., Silver, R. F. & Cave, M. D. (2007).** Beijing family *Mycobacterium tuberculosis* strains differ in their intracellular growth in THP-1 macrophages. *Int J Tuberc Lung Dis* **11**, 1087–1093.
- Underhill, D. M., Ozinsky, A., Hajjar, A. M., Stevens, A., Wilson, C. B., Bassetti, M. & Aderem, A. (1999).** The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. *Nature* **401**, 811–815.
- Vandal, O. H., Gelb, M. H., Ehrt, S. & Nathan, C. (2006).** Cytosolic phospholipase A2 enzymes are not required by mouse bone marrow-derived macrophages for the control of *Mycobacterium tuberculosis* in vitro. *Infect Immun* **74**, 1751–1756.
- Vergne, I., Chua, J. & Deretic, V. (2003).** Tuberculosis toxin blocking phagosome maturation inhibits a novel Ca^{2+} /calmodulin-PI3K hVPS34 cascade. *J Exp Med* **198**, 653–659.
- Vergne, I., Fratti, R. A., Hill, P. J., Chua, J., Belisle, J. & Deretic, V. (2004).** *Mycobacterium tuberculosis* phagosome maturation arrest: mycobacterial phosphatidylinositol analog phosphatidylinositol mannoside stimulates early endosomal fusion. *Mol Biol Cell* **15**, 751–760.
- Vergne, I., Chua, J., Lee, H.-H., Lucas, M., Belisle, J. & Deretic, V. (2005).** Mechanism of phagolysosome biogenesis block by viable *Mycobacterium tuberculosis*. *Proc Natl Acad Sci U S A* **102**, 4033–4038.
- Via, L. E., Deretic, D., Ulmer, R. J., Hibler, N. S., Huber, L. A. & Deretic, V. (1997).** Arrest of mycobacterial phagosome maturation is caused by a block in vesicle fusion between stages controlled by rab5 and rab7. *J Biol Chem* **272**, 13326–13331.
- Via, L. E., Fratti, R. A., McFalone, M., Pagan-Ramos, E., Deretic, D. & Deretic, V. (1998).** Effects of cytokines on mycobacterial phagosome maturation. *J Cell Sci* **111**, 897–905.
- Vieira, O. V., Botelho, R. J. & Grinstein, S. (2002).** Phagosome maturation: aging gracefully. *Biochem J* **366**, 689–704.
- Wagner, D., Maser, J., Moric, I., Boechat, N., Vogt, S., Gicquel, B., Lai, B., Reyat, J. M. & Bermudez, L. (2005).** Changes of the phagosomal elemental concentrations by *Mycobacterium tuberculosis* Mramp. *Microbiology* **151**, 323–332.
- Walburger, A., Koul, A., Ferrari, G., Nguyen, L., Prescianotto-Baschong, C., Huygen, K., Klebl, B., Thompson, C., Bacher, G. & Pieters, J. (2004).** Protein kinase G from pathogenic mycobacteria promotes survival within macrophages. *Science* **304**, 1800–1804.
- Wang, B., Henao-Tamayo, M., Harton, M., Ordway, D., Shanley, C., Basaraba, R. J. & Orme, I. M. (2007).** A Toll-like receptor-2-directed fusion protein vaccine against tuberculosis. *Clin Vaccine Immunol* **14**, 902–906.
- Xu, S., Cooper, A., Sturgill-Koszycki, S., van Heyningen, T., Chatterjee, D., Orme, I., Allen, P. & Russell, D. G. (1994).** Intracellular trafficking in *Mycobacterium tuberculosis* and *Mycobacterium avium*-infected macrophages. *J Immunol* **153**, 2568–2578.
- Yates, R. M. & Russell, D. G. (2005).** Phagosome maturation proceeds independently of stimulation of Toll-like receptors 2 and 4. *Immunity* **23**, 409–417.

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