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.

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 (Huynh & 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*
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; Indrigó 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 (30 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, 0.003% 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 Kantonalen 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 monocye–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-nylonic 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)-carboxyhydamin 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 spectrophotometer (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 baflomycin 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 *IspA::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 \( M. \) \( \text{tuberculosis} \) 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 microorganisms 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 \( M. \) \( \text{tuberculosis} \) 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) 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.

\textbf{\( lspA::aph \) is attenuated in macrophages}

We next studied whether the \textit{in vivo} attenuation of the \( lspA::aph \) mutant (Fig. 1; Sander et al., 2004) correlated with attenuation of the mutant in \textit{in vitro} models of infection. We infected resting BMDM of BALB/c mice with \( M. \) \( \text{tuberculosis} \) 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-\( \gamma \) reduced intracellular multiplication of wild-type bacteria by 26-fold. Likewise, the intracellular growth of the \( lspA::aph \) knockout mutant was impaired upon IFN-\( \gamma \) activation (Fig. 2b). The relative difference in intracellular multiplication between the wild type and \( lspA::aph \) mutant remained similar in resting and IFN-\( \gamma \)-activated BMDM (~10-fold). Complementation restored wild-type growth (see Supplementary Fig. S2). The phenotype of \textit{in vitro} infections paralleled the \textit{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.

\textbf{LspA and TNF-\( \alpha \) secretion by BMDM}

Upon phagocytosis, \( M. \) \( \text{tuberculosis} \) induces TNF-\( \alpha \) in macrophages, and host immunity against tuberculosis is crucially linked to this cytokine. An inverse correlation between TNF-\( \alpha \) secretion and intracellular growth rate of clinical isolates has been reported (Theus et al., 2007). To study whether interference with lipoprotein maturation in \( M. \) \( \text{tuberculosis} \) directly or indirectly affects macrophage activation we infected BMDM with \( M. \) \( \text{tuberculosis} \) wild-type or \( lspA::aph \) mutant. We measured the amount of TNF-\( \alpha \) released 5–72 h post-infection. Over the time period studied TNF-\( \alpha \) 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-\( \alpha \) secretion of BMDM upon infection with the \( lspA::aph \) mutant indicates that

![Graph](image-url)
Phagosome maturation and growth restriction

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

**IspA::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 *IspA::aph* is associated with phagosome maturation. The phagosome acquires LAMP-1 during maturation, resulting in peak LAMP1 levels in late phagosomes and phag-lysosomes. We infected macrophages with fluorescently labelled latex beads, *M. tuberculosis* wild-type or *IspA::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 *IspA::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 *IspA::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 IspA::aph phagosomes**

We wanted to further characterize the *M. tuberculosis* *IspA::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 *IspA::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 *IspA::aph* phagosomes was 6.3, which is not significantly different from that of *M. tuberculosis* wild-type phago-
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
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 ΔlspA 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; Sassetti & 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 and untreated controls, 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 showed 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

**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 ± 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≤0.01; ***P<0.001.
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 (Sassetti & 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*
attemptuation (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 phagosom-
al/lysosomal 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
maturaton (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
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 dimylocero-
sates 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; Sassetti
et al., 2001; Sassetti & Rubin, 2003). Overall, a significant
overlap between genes required for survival of *M. tuberculosis*
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 contradic-
tory 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 phagosomal acidifica-
tion. 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-lysosomal fusion.

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