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DISSERTATION

Titel der Dissertation

Characterization of the phosphatase Lip1, a novel determinant of *L. monocytogenes* virulence

Verfasser

Mag. Renate Kastner

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Abstract

Phosphorylation of proteins as well as inositides is a common means of regulating diverse cellular pathways in eukaryotic cells. However, it has not been until the mid-1990s that bacterial interference with host protein and lipid metabolism by secretion of phosphatases was discovered.

Remarkably, the bacterium *Listeria monocytogenes* expresses a protein, annotated lmo1800 and from now on designated Listeria phosphatase 1 (Lip1), that contains a conserved signature sequence motif of conventional protein tyrosine-phosphatases. Strikingly, Lip1 contains a signal peptide for membrane insertion or secretion, indicating that Lip1 might not be involved in the metabolism of the bacterium itself but could be associated with *Listeria* pathogenicity.

In vitro analysis revealed, that this putative phosphatase displays both, protein tyrosine and phosphatidylinositol phosphatase activities. To address the biological function of the listerial Lip1, a knockout strain was generated. Its virulence was compared to wildtype *L. monocytogenes* in different cell types and during *in vivo* infections of mice. The knockout of this gene leads to a significant reduction of bacteria in target organs after intragastric delivery of bacteria as well as after intraperitoneal infection of mice. Consistently, after infection with ΔLip1 bacteria a strong increase in survival of mice was detected, compared to infection with the wt strain. This observation goes in line with a reduction of serum IFNγ levels in mice infected with *L. monocytogenes* ΔLip1. *In vitro*, the major phases of *L. monocytogenes* intracellular life cycle were investigated for Lip1 contribution. No defects of the knockout strain were found concerning bacterial uptake, phagosomal escape, intracellular replication or virulence.

Taken together, the phosphatase Lip1 could be characterized as a new factor of *L. monocytogenes* virulence.

Zusammenfassung

Phosphorylierung von Proteinen und Inositiden dient in eukaryotischen Zellen der Regulation zahlreicher Signalübertragungswege. Es wurde jedoch erst Mitte der 1990er Jahre entdeckt, dass Bakterien ebenfalls mittels Phosphatasen in den Protein- oder Lipidmetabolismus der Wirtszellen eingreifen können.

Bemerkenswerterweise exprimiert das Bakterium *Listeria monocytogenes* ein Protein, annotiert *lmo1800*, das ab nun unter der Bezeichnung Listeria phosphatase 1 (Lip1) geführt wird, das eine konservierte Domäne enthält, die charakteristisch für konventionelle Tyrosin-Phosphatasen ist. Auffallend ist des Weiteren, dass Lip1 ein Signalpeptid für Membranverankerung oder Sekretion aufweist. Dies deutet darauf hin, dass Lip1 möglicherweise nicht für den Metabolismus des Bakteriums selbst gebraucht wird, sondern eine Rolle in der Pathogenität von *L. monocytogenes* spielen könnte.

Eine *in vitro* Analyse zeigte, dass Lip1 tatsächlich Phosphatase-Aktivität besitzt und sowohl phosphorylierte Tyrosine als auch Phosphatidylinositole als Substrat verwenden kann. Um die biologische Funktion von Lip1 untersuchen zu können, wurde ein knockout Stamm (ΔLip1) generiert. Die Virulenz dieses Stammes im Vergleich zum Wildtyp wurde in verschiedenen Zelltypen und in Mausinfektionen *in vivo* ermittelt. Der knockout von Lip1 bewirkt eine signifikante Reduktion der Bakterienzahlen in den betroffenen Organen Leber und Milz, sowohl nach intragastrischer als auch nach intraperitonealer Infektion. Dementsprechend ist nach einer Infektion mit ΔLip1 im Vergleich zu wildtyp Bakterien ein signifikanter Anstieg in der Überlebensrate von Mäusen zu beobachten. Mäuse, die mit dem ΔLip1 Stamm infiziert wurden, zeigen auch eine stark verminderte systemische IFNγ Produktion. *In vitro* wurde untersucht, ob Lip1 für den Durchlauf der wichtigsten Phasen im intrazellulären Leben von *L. monocytogenes* benötigt wird. Dabei konnten hinsichtlich der Aufnahme, des Entkommens aus dem Phagosom, der intrazellulären Vermehrung und der Virulenz des Bakteriums keine Defekte des knockout Stammes gefunden werden.

Zusammenfassend konnte die Phosphatase Lip1 als bisher nicht beschriebene Komponente der Virulenz von *L. monocytogenes* identifiziert werden.

Contents

1 Introduction

1.1 *Listeria*

The earliest report on *Listeria monocytogenes* goes back to the year 1926, when E. G. D. Murray discovered this Gram positive bacterium causing a lethal disease in their laboratory rabbit colony. Later on, *Listeria* have been described as human pathogens giving rise to listeriosis, a severe disease with a very high mortality rate. Immunocompetent persons are not in danger of developing life-threatening symptoms and usually the infection is asymptomatic or presents as a mild gastroenteritis. However, in pregnant women *Listeria* can cross the placental barrier and invade the foetus, leading to abortion. The second risk group are immunocompromised and elderly individuals, where infections can cause meningoencephalitis, bacteremia or septicemia. In 1983, *Listeria* infections were first related to the uptake of contaminated edibles (Schlech et al., 1983) and since then it became clear that uncooked food, soft cheeses made from raw milk and ready-to-eat meats are the major sources of infection. Following ingestion of contaminated food, bacteria can cross the intestinal barrier and spread via the blood and lymph to their main target organs, the spleen and the liver (figure 1.1). The immune response elicited by *Listeria* (see section 1.1.3) leads to eradication of the infection in immunocompetent or to the above mentioned symptoms in immunocompromised persons (Vázquez-Boland et al., 2001; Cossart, 2007).

The genus *Listeria* belongs to the phylum of firmicutes with low GC content, which also contains the genera *Bacillus, Clostridium, Enterococcus, Streptococcus* and *Staphylococcus*. The taxon currently includes six species: *L. monocytogenes, L. innocua, L. welshimeri, L. ivanovii, L. seeligeri*, and *L. grayi* with only *L. monocytogenes* and *L. ivanovii* being pathogenic (Collins et al., 1991; Hain et al., 2007). *Listeria* species are facultative anaerobic non spore forming rods that do not contain a capsule. Remarkably, they tolerate harsh environmental conditions like high salt concentration (10 % NaCl), a broad range of pH concentration (4,5–9) or temperature stress (0–45 °C), which turns them into a major safety issue in the food industry.

Naturally, *Listeria* occur in various habitats like soil, groundwater or decaying vegetation (Hain et al., 2007). From the existence as a saprophyte, *Listeria* can switch to a parasitic lifestyle as an intracellular pathogen (figure 1.2). *Listeria* can force their uptake into non phagocytic cells. Once ingested, bacteria evade the hostile environment of the phagosome by lysing the membrane of the surrounding vesicle. In the cytoplasm they rapidly multiply and move within the cell by exploiting the actin skeleton of the host cell. The directed actin polymerization also allows *Listeria* to spread into neighbouring cells by forming a filopod into the adjacent cell that is again ingested and lysed (Vázquez-Boland et al., 2001; Lambrechts et al., 2008). These processes require the expression of a set of virulence genes. The key virulence factors required for bacterial intracellular life are under the control of the transcription factor PrfA (positive regulatory factor A) (Freitag et al., 2009). The characteristics of the most important virulence factors and their regulation will be addressed in more detail in the following section.

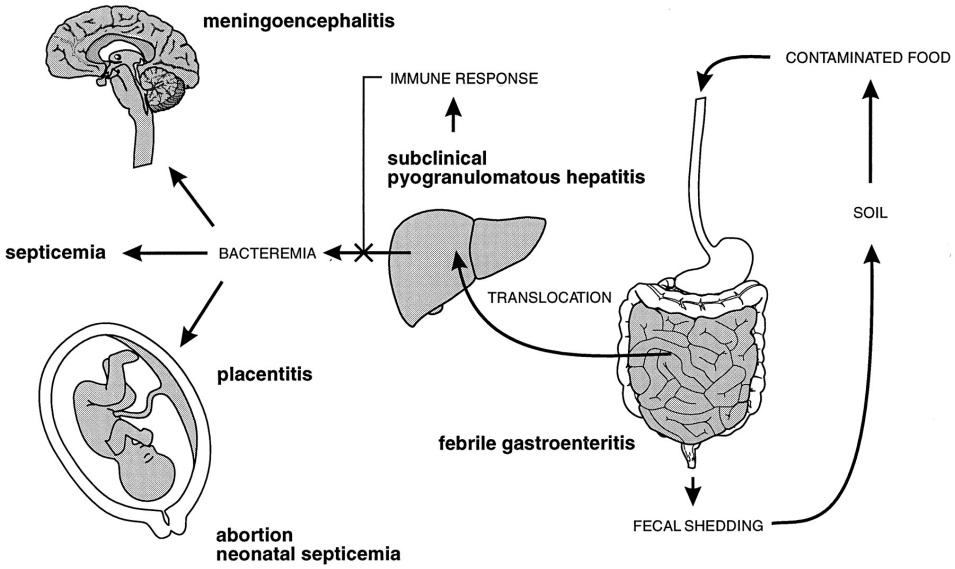


Figure 1.1: After uptake with contaminated edibles, *L. monocytogenes* survives the harsh conditions of the gastrointestinal tract. The bacteria cross the intestinal barrier and spread via the blood and the lymph to the spleen and the liver. Unless eradicated by the immune system, *Listeria* can further spread and cause meningoencephalitis, septicemia or abortions in pregnant women. Taken from Vázquez-Boland et al. (2001).

1.1.1 *Listeria* virulence factors

PrfA The transcription factor PrfA directly regulates ten genes (Milohanic et al., 2003; Marr et al., 2006) (figure 1.2). These genes are involved in uptake into host cells (*inlA* and *inlB*), escape from the phagosome, intracellular growth (*hpt*) and cell to cell spread (*actA, plcB, mpl* and *hly*). Six virulence genes including PrfA itself are located together in the virulence gene cluster LIPI-1 (listeria pathogenicity island-1), however *inlAB*, *inlC* and *hpt* loci are placed to different points in the chromosome (Chakraborty et al., 1992; Vázquez-Boland et al., 2001; Scortti et al., 2007).

The expression of PrfA is controlled at different levels. PrfA transcription is controlled by three promotor regions, leading to a monocistronic transcript (P1*PrfA* and P2*PrfA*) or a bicistronic transcript (P*plcA*). Translation of the P1*PrfA* driven transcript is regulated by a RNA thermosensor in the 5'UTR. This secondary structure blocks translation at low temperatures but melts down at 37 °C, making the ribosome binding site accessible (Johansson et al., 2002). The P2*PrfA* region contains a vegetative σ^a dependent promotor and a stress induced σ^b dependent promotor. Finally, PrfA can induce its own production by the PrfA dependent promotor *PplcA*, providing a positive feedback loop (Mengaud et al., 1991; Chakraborty et al., 1992; Scortti et al., 2007).

Recently, another mechanism of PrfA regulation has been described, namely the riboswitches SreA and SreB. Apart from regulating their own downstream gene, they also act as small noncoding RNAs, regulating the expression of PrfA in *trans*. SreA and SreB were shown to directly bind to the *prfA* 5'UTR leading to a decrease in PrfA expression. As high PrfA levels lead to SreA transcription, it seems that this mechanism presents a negative regulatory loop for modulating PrfA activity (Loh et al., 2009; Xayarath and Freitag, 2009).

Additionally, PrfA activity is regulated at a post-translational level. PrfA exists in a weakly and in a highly active state dependent on its conformation. It has been suggested, that this conformational change is induced by binding of a small molecular cofactor upon the entry into host cells (Gray et al., 2006; Scortti et al., 2007; Freitag et al., 2009).

Internalins Bacterial invasion into host cells is directly mediated by two members of the internalin family, namely Internalin A (InlA) and Internalin B (InlB).

InlA interacts with the host cell adhesion protein E-cadherin leading to cytoskeletal rearrangements necessary for the bacterial uptake. E-cadherin is a transmembrane protein that mediates cell to cell adhesion via homophilic interactions and is ex-

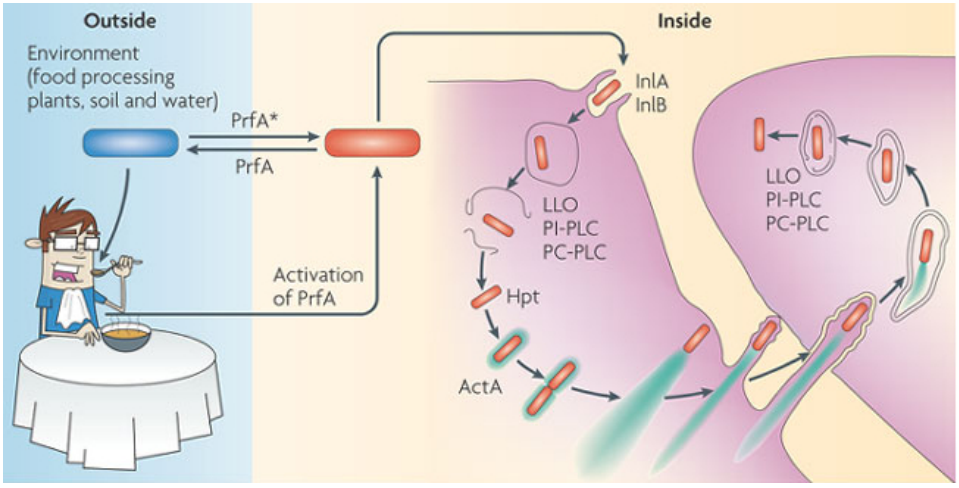


Figure 1.2: Commonly, *Listeria* are taken up with contaminated food. Upon entry of the host, the transcription factor PrfA is activated and induces expression of virulence factors required for forcing uptake into nonphagocytic cells (*inlA* and *inlB*), phagosomal escape (*hly, plcA* and *plcB*) and cell to cell spread (*actA, plcB, mpl* and *hly*). Taken from Freitag et al. (2009).

pressed at adherens junctions and the basolateral face of polarized epithelial cells (Cossart and Lecuit, 1998; Bierne et al., 2007). Internalin A binds with high affinity to human and guinea pig, however not to mouse or rat E-cadherin due to a requirement for a prolin on position 16 of the E-cadherin. To overcome this problem, a transgenic mouse expressing human E-cadherin was generated (Lecuit et al., 2001). An alternative approach was to increase bacterial affinity to the murine E-cadherin, which enhances bacterial colonization of target organs and virulence (Wollert et al., 2007). In the LO28 strain of *L. monocytogenes* the InlA contains a nonsense mutation that leads to a truncated version of the protein released into the medium (Jonquières et al., 1998).

The invasion protein InlB mediates entry into host cells by interacting with its main host receptor hepatocyte growth factor receptor (HGF-R or c-Met), but also glycosaminoglycans or the complement receptor gC1qR. The InlB dependent uptake is mediated by clathrins and apart from cytoskeletal rearrangements, interaction with c-Met induces signalling pathways like PLCγ, NFκB and the Ras-MAP kinase pathway (Bierne and Cossart, 2002; Hamon et al., 2006; Bierne et al., 2007). *Listeria* induced cellular signalling events will be covered in more detail in section 1.1.2.

Other internalins that do not act as invasins, but have been associated with virulence are InlC, InlH and InlJ.

Deletion of InlC leads to a significant increase in mouse LD_{50} compared to wt bacteria (Engelbrecht et al., 1996). Recently, a role of InlC has been suggested in relaxing cortical tension between polarized epithelial cells, which is required for cell to cell spread. InlC promotes formation of bacterial protrusions into neighbouring cells by inhibiting Tuba, a protein shown to regulate junctional configuration (Otani et al., 2006; Rajabian et al., 2009).

InlH expression occurs monocystronic and is, as an σ^b regulated gene, induced by stress. During systemic infection with InlH deficient bacteria, increased levels of interleukin 6 (IL-6) were found in infected tissues. The cell type important for this effect needs yet to be determined, as no role for InlH in controlling IL-6 levels in infected macrophages could be observed (Personnic et al., 2010).

InlJ is efficiently produced during *in vivo* infections with *Listeria*, however not in medium even under stress conditions, and expression could not be detected in any of the tested tissue culture cells. Expression of InlJ in *L. innocua* indicated, that InlJ promotes adherence of bacteria, thus acts as an adhesin (Sabet et al., 2008).

Listeriolysins The pore forming toxin Listeriolysin O (LLO) is encoded by the *hly* gene and is a member of the family of cholesterol dependent cytolysins (CDCs). It can bind to cholesterol rich regions of eukaryotic membranes, however not to bacterial membranes which lack sterols and consequently are protected from its cytolytic activity. Experiments with *hly*- mutant strains and neutralizing antibodies showed, that LLO is required for bacterial escape from the phagosome. Moreover, it was shown that LLO alone is sufficient to mediate escape from primary vacuoles, as LLO expression by *Bacillus subtilis* allows the bacteria to disrupt the phagosomal membrane (Bielecki et al., 1990). Hence LLO deficient bacteria are not able to replicate and are avirulent. Additionally, LLO is involved in lysis of the secondary phagosomes after *Listeria* spread to adjacent cells, facilitated by the bacterial phospholipases C (see also following subsection) (Portnoy et al., 1988; Gedde et al., 2000; Edelson and Unanue, 2001; Schnupf and Portnoy, 2007).

In their intracellular niche, *Listeria* are shielded from extracellular host defence. Indeed, increased cytotoxicity could be linked to reduced *in vivo* virulence in mice. To prevent lysis of the host plasma membrane, LLO activity is controlled by several mechanism. As already pointed out, *hly* expression is controlled by PrfA. LLO has an acidic pH optimum (Schnupf et al., 2006) and block of acidification of the lysosome inhibits LLO mediated membrane perforation (Beauregard et al., 1997). The N-terminus of LLO contains a PEST-like sequence, which in eukaryotes can target

proteins for degradation. Therefore it was initially speculated that this sequence targets LLO for degradation in the host cytoplasm. A hypothesis that was later proven wrong (Decatur and Portnoy, 2000; Lety et al., 2002; Schnupf et al., 2006; Schnupf and Portnoy, 2007). Rather, LLO can be targeted for degradation by the N-end rule pathway, which involves recognition of proteins with destabilizing N-terminal amino acids (Schnupf et al., 2007).

A subset of *Listeria* strains of the lineage I, the evolutionary lineage associated with the majority of epidemic outbreaks, encodes for a second haemolysin called Listeriolysin S (LLS). LLS is induced by oxidative stress and contributes to *Listeria* virulence and survival in polymorphnuclear neutrophils (Cotter et al., 2008).

Phospholipases There are two phospholipases C (PLC) secreted by *Listeria*, namely the phosphatidylinositol-specific PLC (PI-PLC) and the broad-range phosphatidylcholine-preferring PLC (PC-PLC). Both are secreted at low pH and enhance the LLO mediated lysis of vacuoles (Hybiske and Stephens, 2008). Mutants lacking PI-PLC show minor defects in lysing primary vacuoles that have a single membrane but have the same efficiency in cell to cell spread. Perforation of secondary double-membrane vacuoles requires PC-PLC activity. Double mutants for both PLCs display highly decreased virulence in mice and are less efficient in escape from primary vacuoles and cell to cell spread (Smith et al., 1995).

ActA *Listeria* exploit the host actin cytoskeleton to move within the cell and into neighbouring cells with the help of the virulence factor ActA (figure 1.3). Site directed polymerization of actin on one pole of the bacterium, the so called actin comet tail, pushes the bacteria forward. The N-terminus of ActA contains homologies to the eukaryotic WASP protein and directs binding to the actin nucleating Arp2/3 complex. Unlike WASP family proteins, ActA lacks regulation domains and is constitutively active (Lambrechts et al., 2008). Additionally, ActA binds to the host VASP protein that recruits the actin-binding protein profilin (Hamon et al., 2006).

Interestingly, it has been shown that activity of PI3-K is required for *Listeria* actin based motility and that *Listeria* concentrate PI(4,5)P_2 and PI(3,4,5)P_3 on their surface *in vivo* (Sidhu et al., 2005). However, the function of phosphoinositides in the process of actin based movement could not be elucidated so far.

FbpA Fibronectin-binding protein A (FbpA) is a multifunctional protein required for efficient colonization of the liver after intravenous infection. FbpA binds to

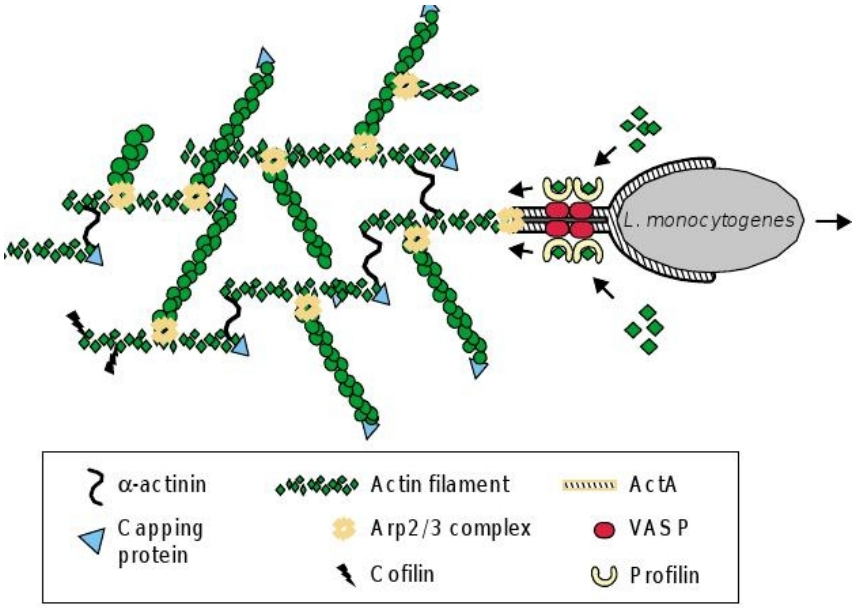


Figure 1.3: Actin based intracellular movement of *Listeria*. Taken from Cossart and Bierne (2001).

immobilized fibronectin and it contributes to cell adherence to human liver epithelial cell lines HEp-2. Furthermore, there are indications that FbpA modulates post-transcriptionally the levels of the virulence factors LLO and ActA (Dramsi et al., 2004).

p60 The 60 kilodalton protein p60 is encoded by the invasion associated protein (*iap*) gene. *Iap* knockout bacteria show slightly reduced invasiveness in 3T6 fibroblasts and Caco-2 epithelial cells and are highly attenuated in mice after intravenous injection. Furthermore, *iap*- bacteria cannot form actin tails and are reduced in their ability to spread within and into neighbouring cells (Pilgrim et al., 2003).

Mpl Transposon insertion into the *mpl* gene leads to reduced virulence and the loss of phospholipase activity (Raveneau et al., 1992). Further studies could show that the zinc metalloprotease Mpl is required for maturation of the PC-PLC. This phospholipase is produced as a proenzyme and needs activation via proteolytic cleavage. (Poyart et al., 1993).

Hpt Hexose phosphate transporter (Hpt) mediates the uptake of host glucose phosphate that is exploited as carbon source. Presence of Hpt is required for optimal in-

tracellular replication and normal proliferation in mouse organs (Chico-Calero et al., 2002).

1.1.2 *Listeria* induced signalling pathways in epithelial cells

Phagocytosis is a process only employed by specialized cells like macrophages or dendritic cells. Endocytosis, however, is common to all cell types. *Listeria* can enter cells via either mechanism, that is phagocytosis or forced uptake into nonphagocytic cells via the zipper mechanism mediated by internalins. During the entry into cells that are normally not phagocytic, *Listeria* trigger different signalling cascades (Bonazzi and Cossart, 2006).

InlA mediated entry Interaction of InlA with its host receptor E-cadherin leads to the recruitment of several molecules that anchor E-cadherin to the actin cytoskeleton, such as α catenin and β catenin (figure 1.4). However, α catenin cannot bind β catenin and actin concurrently, indicating that it regulates actin dynamics rather than stably linking E-cadherin/β catenin complexes to the actin cytoskeleton. Furthermore, ARHGAP10, myosin VIIa and vezatin are recruited to the *Listeria* entry site. ARHGAP10 is a GTPase activating protein (GAP) for RhoA and Cdc42, two proteins involved in actin polymerization. Also, ARHGAP10 was shown to be required for α catenin recruitment (Pizarro-Cerdá and Cossart, 2006; Ireton, 2007). The unconventional myosin VIIa and the transmembrane protein vezatin are also crucial for *Listeria* internalization. Vezatin acts as a link between the E-cadherin/β catenin/actin complex and the motor protein myosin VIIa. Myosin VIIa is suggested to produce the contractive force that together with the actin polymerization process mediates bacterial internalization (Sousa et al., 2004).

InlB mediated entry The interaction of InlB with its receptor Met resembles the physiological activation of Met by its natural ligand the hepatocyte growth factor (HGF). Engagement of Met by InlB leads to activation of the protein kinase activity of Met, activation of the host phosphatidylinositol 3-kinase (PI3-K) p110-p85 and the activation of the Ras mitogen-activated kinase (MAPK) (figure 1.5).

Upon InlB binding, Met dimerizes and is autophosphorylated, leading to the binding of the adaptors Cbl, Grb2, Shc and Gab1. The adaptor protein Gab1 is also recruited to the plasma membrane by a pleckstrin homology (PH) domain that interacts with phosphoinositides. Gab1 then recruits several SH2 containing proteins like the regulatory subunit p85 of PI3-K, the tyrosine phosphatase Shp2 and the

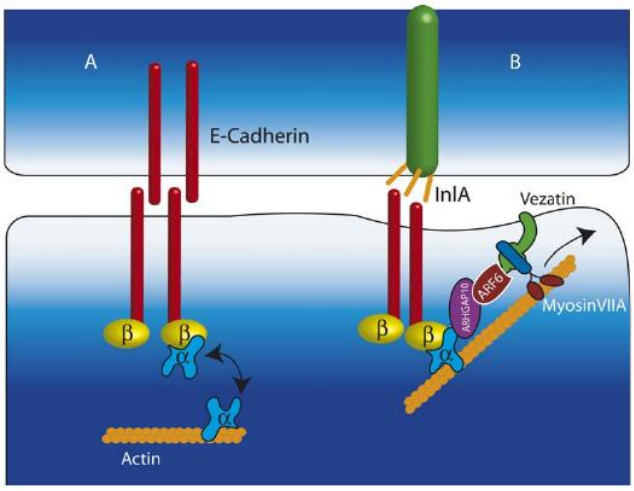


Figure 1.4: InlA mediated *Listeria* uptake. Taken from Bonazzi and Cossart (2006).

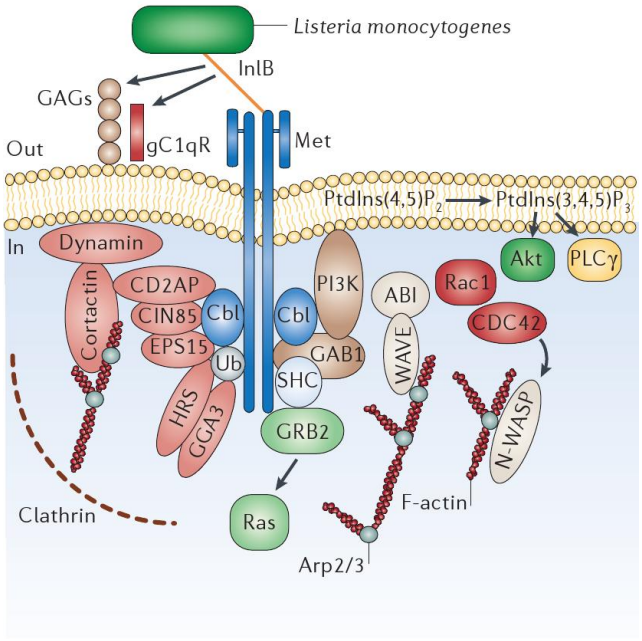


Figure 1.5: Met signalling pathways induced by InlB. Taken from Hamon et al. (2006).

adaptor CrkII (Sun et al., 2005; Hamon et al., 2006). CrkII stimulates PI3-K activity that promotes actin polymerization by a yet unclear mechanism. One possibility of PI3-K contribution to actin polymerization is that PI3-K mediated formation of phosphatidylinositol 3,4,5-tris phosphate (PI(3,4,5)P_3) is required, a phospholipid with the ability to uncap barbed actin filaments. Otherwise it could activate the Arp2/3 complex, the GTPase Rac or its effector WAVE2 (Ireton, 2007).

The cytoskeletal rearrangements required for *Listeria* uptake depend on the proteins Arp2/3, the Rho GTPases Rac1 and Cdc42, cofilin and the LIM kinase. The Arp2/3 complex facilitates actin nucleation and branching. Cofilin is an actin depolymerizing factor that severs actin filaments and is inactivated by serine phosphorylation mediated via LIM kinase. The cofilin phosphocycle helps to tightly regulate the actin polymerizing and depolymerizing steps required for internalization (Bierne et al., 2001). Recruitment and activation of the Arp2/3 complex requires proteins of the Wiskott-Aldrich syndrome protein (WASP)/ WASP family Verprolin-homologous protein (WAVE) family and seems to be cell-type dependent. WASP proteins are activated downstream of the Rho GTPase Cdc42, and WAVE proteins function downstream of Rac. Additionally, proteins of the Ena/VASP family are essential for cytosekeletal rearrangements by promoting elongation of actin filaments through protecting barbed filament ends from decapping (Hamon et al., 2006; Bierne et al., 2005).

It has been demonstrated that the internalization process triggered by InlB is clathrin-dependent, one of the best characterized endocytic routes. Also, dynamin is involved, a protein mediating the fission of vesicles from the plasma membrane during clathrine mediated uptake (Bonazzi and Cossart, 2006).

In the macrophage like cell line J774 recruitment of the adaptor Grb-2 to the Met receptor leads to Ras activation. Additionally, this study suggests that Ras is necessary for activation of PI3-K and the downstream effector Akt (also called protein kinase B, PKB). Akt is phosphorylated on Ser473 leading to the activation of the IκB kinase complex and eventually to the release of NFκB (Mansell et al., 2001). Activation of PI3-K leads to generation of the lipid second messenger PI(3,4,5)P_3 that is involved in a variety of processes that will be discussed in section 1.2.2. In the human epithelial cell line HEp-2 InlB mediated signaling leads to activation of phospholipase Cγ1 (Plcγ) downstream of PI3-K (Bierne et al., 2000).

1.1.3 Immune responses to *Listeria*

Innate immune responses to *Listeria* The intestinal tract is the primary entry site of *Listeria* into the host. It is not entirely clear, how bacteria translocate from the gut to deeper organs. Some studies in mice suggest, that *L. monocytogenes* enters passively via M cells and the underlying Peyer's patches (Jensen et al., 1998). However, other studies demonstrate that *L. monocytogenes* does not require M cells to cross the intestinal barrier (Pron et al., 1998; Daniels et al., 2000). In humans, *L. monocytogenes* were found to invade the intestine by specific interaction of InlA with host E-cadherin. In polarized epithelial monolayers E-cadherin is inaccessible as it is located to the basolateral face of the cells. At sites where senescent cells are extruded and removed from the epithelium, E-cadherin is accessible to the bacteria (Pentecost et al., 2006).

As a first line of defence, the innate immune response plays a crucial role in combating *Listeria* infections. Shortly after crossing the epithelial barrier of the intestinal tract *Listeria* colonize the liver leading to release of neutrophil chemoattractants by infected hepatocytes. Neutrophils can engulf and destroy bacteria by producing antimicrobial peptides, most importantly nitric oxide (NO) and oxygen radicals. Also, they are crucial for destruction of *Listeria* infected hepatocytes (Conlan and North, 1991; Zenewicz and Shen, 2007). The importance of neutrophils for early clearance of infection was shown by depletion of granulocytes, which leads to increased susceptibility of mice to *Listeria* infections (Rogers and Unanue, 1993; Conlan and North, 1994; Pamer, 2004).

Other inflammatory cells important for bacterial clearance are macrophages. Additional to resident macrophages, named Kupffer cells in the liver, blood monocytes are recruited to sites of infection by chemokines, such as CSF-1 and MCP-1 produced by neutrophils. IL-12 and TNF were found to be mainly produced by infected macrophages and these cytokines induce the synthesis of IFNγ by natural killer (NK) cells (Unanue, 1997). IFNγ in turn activates macrophages and increases their bactericidal activity (Zenewicz and Shen, 2007). Accordingly, mice lacking IFNγ, TNF or their receptors are highly susceptible to *Listeria* infections (Buchmeier and Schreiber, 1985; Huang et al., 1993; Pfeffer et al., 1993; Rothe et al., 1993).

Dendritic cells (DCs) are a group of professional antigen presenting cells that are important for linking innate and adaptive immune responses (Steinman and Hemmi, 2006). *In vivo* studies using a temporal deletion of CD11c$^+$ DCs with a diphtheria toxin-receptor based system showed that CD11c$^+$ DCs are required for priming cytotoxic T cell (CTL) precursors in response to *Listeria* infection (Jung

et al., 2002). It was speculated that $CD11c^+$ DCs are required for presenting antigen by MHC molecules to naive $CD8^+$ T cells. However, a recent publication suggests, that $CD11c^+$ DCs are crucial for bacterial uptake into the spleen and not strictly required for $CD8^+$ T cell proliferation (Neuenhahn et al., 2006). Another subset of DCs, the TNF/iNOS producing TipDCs, are essential for mediating innate immune responses *in vivo* by producing large amounts of TNF and NO in the spleen (Serbina et al., 2003b).

Recognition of *Listeria* by the immune system leading to an inflammatory response Detection of invading pathogens by the innate immune system ensues from recognition of conserved pathogen associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs). Several families of PRRs have been identified such as the Toll like receptors (TLRs), the Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and the Nucleotide-binding oligomerization domain (NOD)-like receptor (NLRs).

An essential adaptor molecule utilized by all TLRs except TLR3 is MyD88. The MyD88 dependent pathway eventually leads to activation of the transcription factors NFκB and AP-1 that initiate the transcription of inflammatory cytokines (Kumar et al., 2009). Mice deficient for MyD88 show increased susceptibility to primary infections with *Listeria* and the role of MyD88 could be linked to production of the proinflammatory cytokines TNF and IFNγ (Seki et al., 2002; Serbina et al., 2003a). *Listeria* express several potential TLR ligands like peptidoglycan and lipoproteins that can be recognized by TLR2, and flagellin, a TLR5 ligand. For example, early activation of NFκB and AP-1 in bone marrow derived macrophages (BMDMs) depends on recognition of a *Listeria* ligand by TLR2 (Stockinger et al., 2004; O'Connell et al., 2005). The importance of TLR2 dependent recognition of *Listeria* for protective immunity *in vivo* is controversial, as some studies show reduced survival in TLR2 deficient compared to wt mice (Torres et al., 2004), however others detect TLR2 independent immunity (Edelson and Unanue, 2002; Pamer, 2004). To investigate the role of TLR5 signaling *in vivo*, a *L. monocytogenes* strain with a deletion of the flagellin structural gene flaA was created. This flaA deficient strain did not show any defects in virulence. Together, the MyD88 dependency but the lack of clear dependence on TLR2 or TLR5 might suggest a functional redundancy between TLRs in recognizing *Listeria* infections *in vivo* (Way et al., 2004).

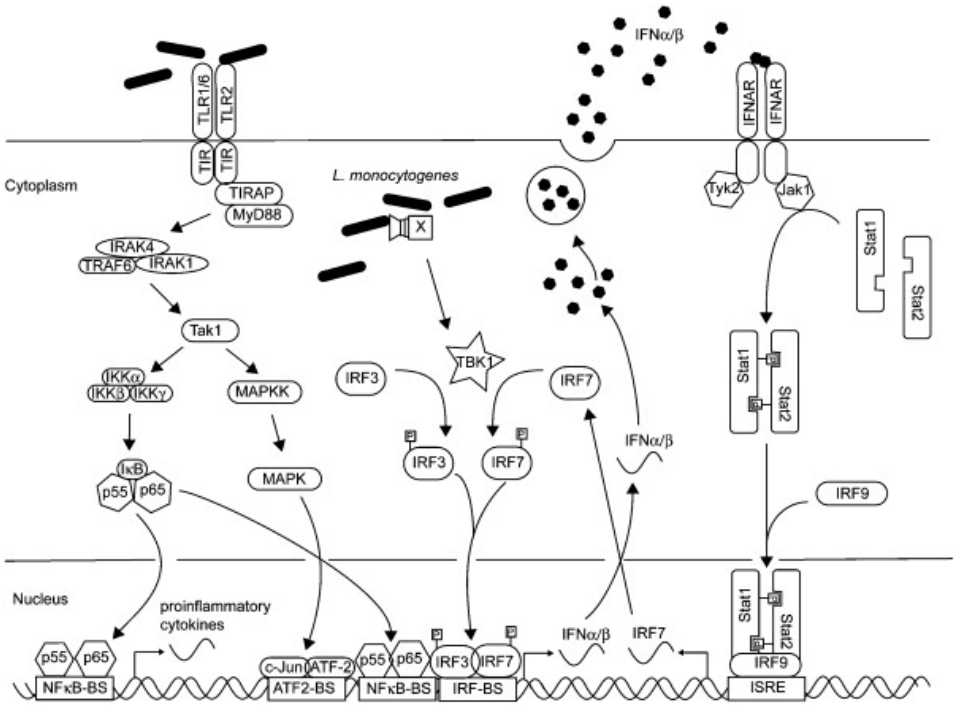


Figure 1.6: Extracellular recognition of *Listeria* by TLR2 leads to the recruitment of the bridging adaptor TIRAP and the adaptor protein MyD88. A signaling cascade involving IRAK1, IRAK4 and TRAF6 is activated. The protein kinase TAK1 is recruited which on the one hand activates the IKK-complex, eventually leading to NFκB activation, on the other hand activates the MAPK pathway (Kawai and Akira, 2007). Cytoplasmic recognition of *Listeria* by a yet unknown receptor leads to activation of the kinase TBK1. TBK1 then phosphorylates the transcription factor IRF3 that stimulates synthesis of IFNβ. IFNβ signals via its receptor, the IFNAR, resulting in activation of the kinases Jak1 and Tyk2. Subsequently, Stat1 and Stat2 are phosphorylated by the Jak kinases leading to heterodimer formation. IRF9 binds the Stat heterodimers, forming the ISGF3 transcription factor that binds to interferon stimulated response elements (ISREs) in interferon stimulated genes. Taken from Stockinger and Decker (2008).

The role of IFN-I in *Listeria* infections Cytokines that critically influence the course of *Listeria* infection are type I Interferons (IFN-I). IFN-I protect cells from viral infections, however during bacterial infections their role is less well defined. For instance in *Streptococcus pneumoniae* or *Salmonella typhimurium* infections IFN-I exert protective functions, yet in case of *Listeria* infections their production is detrimental for the host (Decker et al., 2005). Mice deficient for the IFN-I receptor (IFNAR) are highly resistant to *Listeria* infections (Auerbuch et al., 2004; Carrero et al., 2004). Likewise, BMDMs produce IFN-I upon *Listeria* infection which in turn sensitizes them to *Listeria* induced necrotic cell death (Barsig and Kaufmann, 1997; Stockinger et al., 2002; Zwaferink et al., 2008). Interestingly, it was shown that IFN-I production leads to downregulation of the IFNγ receptor in DCs and macrophages as well as to reduced responsiveness to IFNγ during systemic infection. Thereby IFN-I antagonises the host response to IFNγ (Rayamajhi et al., 2010).

Up to now the receptor recognizing *Listeria* infections for the production of IFN-I has not been identified. TLR4, TLR9 and the adaptors MyD88, TRIF and TRAM could be excluded from contributing to IFN-I production (Stockinger et al., 2004; O'Connell et al., 2005). Also, the intracellular receptors Nod1 and Nod2 do not play a direct role in *Listeria* induced IFN-I production (Stockinger et al., 2004; Werts et al., 2007; Stockinger and Decker, 2008). IFN-I production requires the escape of *Listeria* from the phagosome and cytoplasmic signal transduction dependent on IRF3 and its upstream kinase TBK1 (Stockinger et al., 2002, 2004; O'Connell et al., 2005). First hints towards DNA being the ligand that activates the unknown intracellular receptor were provided by transfection of BMDMs with RNAse or DNAse treated *Listeria* extracts (Stetson and Medzhitov, 2006).

Adaptive immune responses to *Listeria* Full clearance of infection requires adaptive immune responses, that are mainly mediated by CTLs due to the intracellular niche of *Listeria* (Zenewicz and Shen, 2007). For activating T cells, antigen has to be presented to T cells by MHC molecules in combination with costimulation. As already mentioned above, dendritic cells are thought to capture and present *Listeria* antigens to T cells (Lara-Tejero and Pamer, 2004). Lymphocyte deficient SCID mice can mount an early immune response to *Listeria*, however they develop chronic infections (Bhardwaj et al., 1998). The role of CD^8 T cells is on the one hand to kill infected cells via perforin and granzyme, leading to the release of bacteria. On the other hand, they secrete IFNγ to activate macrophages. Protective immunity to *Listeria* depends mainly on CD^8 T cells, as shown by adaptive transfer studies

(Czuprynski and Brown, 1990; Kaufmann et al., 1986; Zenewicz and Shen, 2007).

1.2 Phosphatases

1.2.1 Protein phosphorylation by bacteria

Phosphorylation and dephosphorylation as a mechanism to regulate protein activity in eukaryotes was discovered in the mid 1950s (Krebs and Fischer, 1956), but has been neglected for a long time in bacteria. However, it could be shown in the last decades that protein phosphorylation plays an important role in regulating cellular mechanisms in microorganisms and contributes to bacterial virulence.

In bacteria, there are three protein phosphorylation systems known: the two-component system in which a phosphoryl group is transferred from a histidyl residue of a sensor kinase to a aspartyl group of a response regulator. Secondly, the phosphoenol pyruvate:carbohydrate phosphotransferase (PTS) system, where a phosphoryl group is passed along protein intermediates and eventually transferred on a sugar. And finally protein O-phosphorylation occurs that resembles the phosphorylation system in eukaryotes, where a phosphomonoester is formed by phosphorylation of the side hydroxyl groups of serine, threonine or tyrosine (Cozzone et al., 2004).

Bacterial phosphatases

The main protein phosphatase families of prokaryotes are the Mg^{2+} or Mn^{2+}-dependent protein phosphatases (PPM) family, the phosphoprotein phosphatase family (PPP), the polymerase and histidinol phosphatase (PHP) family and the protein-tyrosine phosphatases (PTP). Members of the PPP and PPM family are serine/threonine phosphatases, at which the PPP phosphatases are more abundant in eukaryotes (Shi et al., 1998; Shi, 2004).

The largest family of protein phosphatases are the PTPs. The designation PTP is somewhat misleading, as some members of this group are not only specific to phosphotyrosyl residues, but remove phosphoryl groups from all three amino acids, serine, threonine and tyrosine. Those enzymes are called dual-specificity protein phosphatases (DSPs). The PTP family contains the low molecular weight (LMW) PTPs and the conventional PTPs/DSPs.

Tyrosine phosphatases characteristically contain the signature motive CX_5R, that forms the conformationally conserved phosphate-binding P-loop. The P-loop cystein functions as a nucleophil that attacks the protein-bound phosphoryl group. The de-

phosphorylated protein is displaced and a cysteinyl-phosphate reaction intermediate is formed. The conserved arginine builds salt bridges with the phosphoryl group to support substrate binding and stabilizes the reaction intermediate. A conserved aspartic acid (Asp) serves as a general acid and base. During the formation of the reaction intermediate, it protonates the oxygen of the leaving group in the tyrosine residue. In the second step, the Asp functions as a base and accepts a proton from the entering water molecule to enhance the hydrolysis of the phosphoenzyme intermediate. The LMW and the PTPs/DSPs differ in the relative location of the Asp to the active site CX_5R motive. In LMW PTPs the Asp is 80 to 110 residues distant on the C-terminal side of the active site cystein, whereas in the conventional PTPs/DSPs it is located 25 to 50 residues N-terminal of the cystein (Shi et al., 1998; Kennelly and Potts, 1999; Shi, 2004; Tabernero et al., 2008).

Bacterial phosphatases are known to be important virulence factors. On the one hand they are involved in the production or regulation of exopolysaccharides and capsular polysaccharides which are involved in bacterial pathogenicity (Cozzone et al., 2004). On the other hand, phosphatases can be directly translocated into the host cytoplasm and influence pathogen survival, internalization and replication by affecting host phosphorylation (DeVinney et al., 2000). Examples of secreted bacterial phosphatases will be discussed in the next section.

Secreted bacterial phosphatases in virulence

To date, few bacterial phosphatases have been identified that modulate cellular functions. For example the *Yersinia* protein tyrosine phosphatase YopH and the *Salmonella typhimurium* tyrosine phosphatase SptP are delivered to the host cytoplasm by the type III secretion system (T3SS) (Galán, 2009). Also, *Mycobacterium tuberculosis* encodes for secreted phosphatases, the tyrosine phosphatase MPtpA and the phosphatase MPtpB that exhibits triple phosphatase activity (Koul et al., 2000; Beresford et al., 2007).

***Yersinia* YopH** Early host colonization by the Gram negative bacterium *Yersinia* requires the invasion of host cells. Infection with *Yersinia* is initiated by M cell invasion by an integrin mediated mechanism and the colonization of ileal Peyer's patches (Vazquez-Torres and Fang, 2000). Later on, *Yersinia* survive in the host as extracellular pathogens (Pizarro-Cerdá and Cossart, 2009). Then, *Yersinia* can escape degradation by the host defence through blocking phagocytosis by macrophages. Apart from YopH, this process requires the cytotoxin YopE that exerts GAP func-

tion on Rho GTPases. Substrates dephosphorylated by YopH in different cell types are $p130^{Cas}$, focal adhesion kinase (FAK), paxillin and Fyn-binding protein (Fyb). Those substrates are involved in connecting the actin cytoskeleton and extracellular matrix proteins in focal adhesions. How YopH blocks the uptake of *Yersinia* has not been clarified. However, the cytoskeletal rearrangements induced by *Yersinia* invasion resembles focal adhesions and disruption of these structures could be an explanation (DeVinney et al., 2000; Aepfelbacher et al., 2007).

***Salmonella* SptP** The Gram negative *Salmonella* enter host cells via the zipper mechanism, characterized by profound rearrangements of the actin cytoskeleton. Membrane ruffling and lamellipodia formation leads to engulfment of bacteria into membrane bound vacuoles. The tyrosine phosphatase SptP possesses GTPase activating protein (GAP) activity and targets and inactivates the Rho GTPase Rac and Cdc42. Thereby it counters the cytoskeletal rearrangements induced by the invasion process and helps the cell to regain its normal cellular architecture (Galán, 2001).

***Mycobacterium tuberculosis* MPtpA** Like *Yersinia*, *M. tuberculosis* evades intracellular destruction in macrophages by blocking phagosome maturation. The lipid phosphatase SapM contributes to the inhibition of phagosome maturation by hydrolysing PI(3)P. MPtpA dephosphorylates VPS33B, a protein involved in vesicle trafficking and thereby inhibits phagosome-lysosome fusion (Bach et al., 2008). Interestingly, deletion of MPtpA leads to attenuation of *M. tuberculosis* growth in human macrophages, but seems to be dispensable for mouse infection models (Grundner et al., 2008).

1.2.2 Subversion of phosphoinositide metabolism by bacteria

Phosphoinositides

Phosphatidylinositides consist of a glycerol moiety that is esterified in position 1 and 2 with two fatty acids and on position 3 to a myo-inositol head-group connected to a diacylglycerol by a phosphodiester bound. The myo-inositol head-group contains five free hydroxyl groups, whereat only the 3, 4 and/or 5-positions can be reversibly phosphorylated. Phosphatidylinositides can be cleaved by phospholipase C, creating the second messengers inositol 1,4,5-trisphosphate ($Ins1,4,5P_3$) and diacylglycerol (DAG). Importantly, they can directly interact with proteins and alter their localization pattern, conformation or activity (Pendaries et al., 2005; Paolo and

Camilli, 2006).

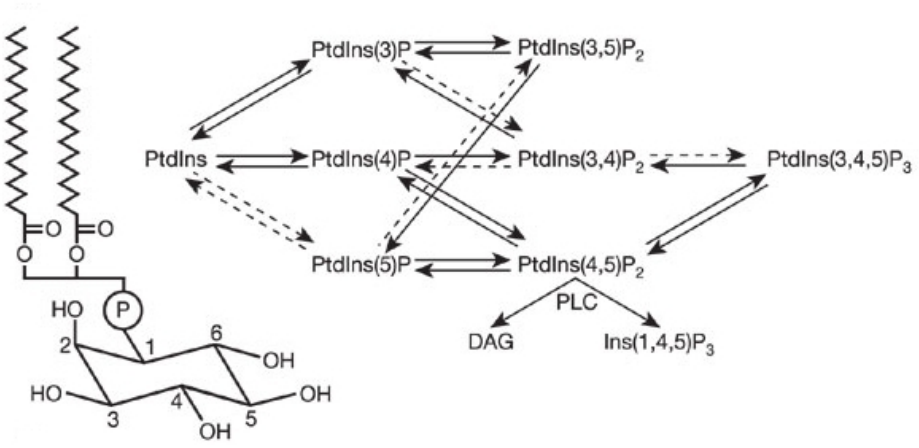


Figure 1.7: Structure of phosphatidylinositol and reactions leading to the generation of seven phosphatidylinositide species. Reactions shown with dashed lines have been shown *in vitro*, but their importance *in vivo* remain unclear. DAG, diacylglycerol; PLC, phospholipase C. Taken from Paolo and Camilli (2006).

Phosphatidylinositides in regulation of host cells

Turnover of phosphatidylinositol (PtdIns) and its phosphorylated products called phosphatidylinositides (PIPs) play an important role in regulating diverse cellular processes (figure 1.8). Their head groups can bind cytosolic proteins containing either clusters of basic residues within unstructured regions or modules such as the pleckstrin homology (PH), phox homology (PX) or Fab1p/YOTB/Vac1/eea1 (FYVE) domains. PIPs exert their functions by either regulating integral membrane proteins or recruitment of cytoskeletal and signalling components to the membrane (Paolo and Camilli, 2006; Pendaries et al., 2005).

PIPs can recruit members of the Ras superfamily of small GTPases, including proteins regulating signalling like the Ras family itself, proteins modulating the actin cytoskeleton such as the Rho family, or proteins implicated in organelle and vesicular transport for example the Arf or Rab family.

$PI(4,5)P_2$ is directly implicated in transduction of extracellular signals. Phospholipase C, which is activated during phagocytosis, can hydrolyse $PI(4,5)P_2$ into the second messenger diacylglycerol (figure 1.7). When converted into $PI(3,4,5)P_3$ by PI3-K, it mediates a multitude of effects like cell growth, migration, phagosome

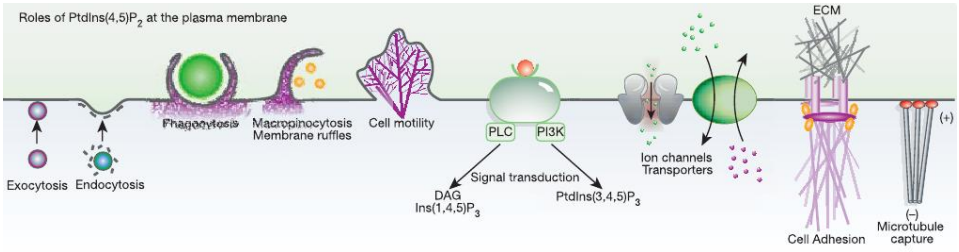


Figure 1.8: Roles of PI(4,5)P_2 at the plasma membrane. Taken from Paolo and Camilli (2006).

maturation and survival (Cantley, 2002; Paolo and Camilli, 2006). Importantly, recruitment of PDK1 and Akt to PI(3,4,5)P_3 via their PH domain leads to activation of the serine threonine kinase Akt by PDK1. Akt can phosphorylate Bad, thereby inducing survival of the cell. Another target of Akt is the glycogen synthase kinase 3 (GSK3) that is inhibited by phosphorylation. Thus, GSK3 inhibited proteins like cyclin-D or c-Myc become active (Paolo and Camilli, 2006).

PIPs also play a role in membrane dynamics, underlined by the fact that different PIPs preferentially localize to distinct subcellular compartments (figure 1.9). For example the plasma membrane is enriched in PI(4,5)P_2 and PI(4)P. During signaling events or phagocytosis, also PI(3,4,5)P_3 temporarily accumulates at the plasma membrane (Weber et al., 2009). Completion of phagocytosis requires activity of PI3-K and goes in line with disappearance of PI(4,5)P_2. Upon completion of phagocytosis, PI(3)P accumulates on the sealed phagosome and is required for phagosome maturation (Hilbi, 2006).

Modifications of host phosphoinositide metabolism by bacteria

There are several ways how bacteria modulate host phosphoinositide metabolism (figure 1.10). For example the *Legionella* effector proteins anchor to *Legionella* containing vesicle (LCV) membranes via PI(4)P. *Yersinia* uptake is mediated via interaction with β1 integrin leading to activation of Rac1 and local induction of PI(4,5)P_2 via PIP5K. Also *Listeria* uptake involves activation of PI3-K as pointed out in section 1.1.2. There are a few examples of virulence mechanisms that involve the introduction of bacterial phosphatidylinositide phosphatases into the host cytoplasm. In the following section, some examples will be discussed in more detail.

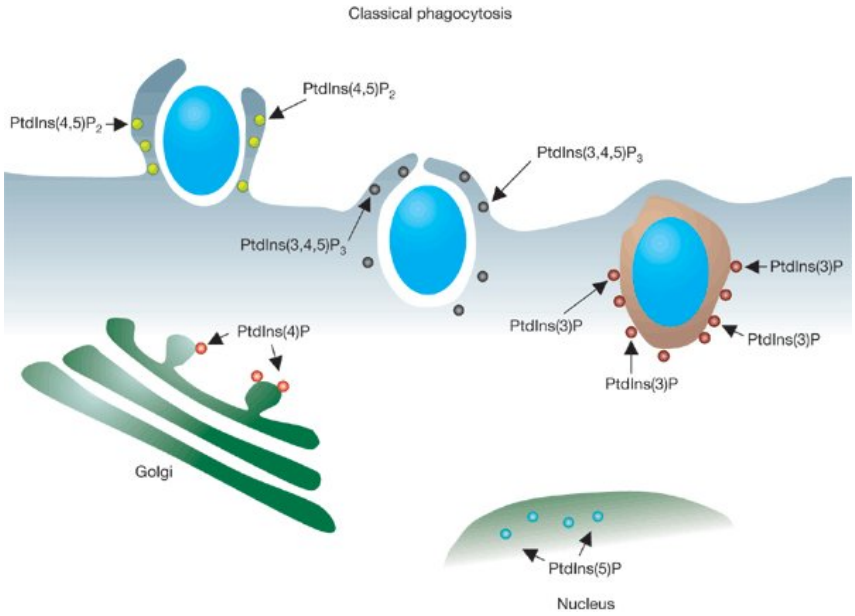


Figure 1.9: Phosphoinositides localize to different subcellular compartments and are involved in classical phagocytosis. Taken from Pizarro-Cerdá and Cossart (2006).

Shigella *Shigella* force their uptake into non phagocytic cells by the trigger mechanism and rapidly escape from the phagosome. Via the T3SS, they inject the inositol polyphosphatase IpgD into the host cytoplasm. *In vitro*, it targets several phosphatidylinositols. *In vivo* it specifically dephosphorylates PI(4,5)P_2 at the plasma membrane, generating PI(5)P (Weber et al., 2009; Hilbi, 2006). The *in vivo* function of IpgD is ill defined. It seems to be involved in the formation of fully structured entry sites and expression of IpgD in epithelial cells leads to morphological changes like membrane blebbing and actin filament remodelling due to a decrease in the membrane tether force (Niebuhr et al., 2000, 2002; Pendaries et al., 2006). However, IpgD deficient mutants show the same invasive phenotype compared to wt bacteria (Allaoui et al., 1993). Additionally, it was shown that IpgD is required for the activation of the host cell kinase Akt (Pendaries et al., 2006). Although it might have some prosurvival effects, IpgD dependent Akt activation is not sufficient to protect epithelial cells from staurosporine induced apoptosis (Clark and Maurelli, 2007).

Salmonella enterica The delivery of the phosphatase SigD (also named SopB) into the host cytoplasm by the T3SS promotes *Salmonella* uptake. It diminishes the cortical PI(4,5)P_2 pool in epithelial cells and thereby decreases membrane rigidity. This

promotes the fission of membrane invaginations and the sealing of phagosomes (Terebiznik et al., 2002; Weber et al., 2009). Apart from regulating membrane dynamics, SigD protects epithelial cells from apoptosis by activation of Akt (Steele-Mortimer et al., 2000; Knodler et al., 2005). Furthermore, SigD promotes fluid secretion and inflammatory responses in the infected ileum (Galyov et al., 1997; Weber et al., 2009).

Mycobacterium Live *Mycobacteria tuberculosis* enter macrophages in a PI3-K dependent manner and interfere with phagosome maturation by excluding PI(3)P from the phagosomal membrane. *Mycobacteria* secrete the lipid phosphatase SapM that specifically hydrolysis PI(3)P and thereby inhibits phagosome fusion with lysosomes. Also, the trafficking toxins lipoarabinomannan (LAM) and phosphatidylinositol mannoside (PIM) are involved in arresting the bactericidal endocytic pathway (Weber et al., 2009). Additional to SapM, *Mycobacteria* secrete the phosphatase MPtpB that shows broad substrate specificity *in vitro*. It dephosphorylates phospho-tyrosine, -serine and -threonine substrates, as well as phosphoinositides, thus exhibiting triple specificity phosphatase (TSP) activity (Beresford et al., 2007). A recent study shows that MPtpB inhibitors severely impair mycobacterial growth within macrophages (Beresford et al., 2009). However, the actual biologic role and *in vivo* substrates of MPtpB have yet to be determined.

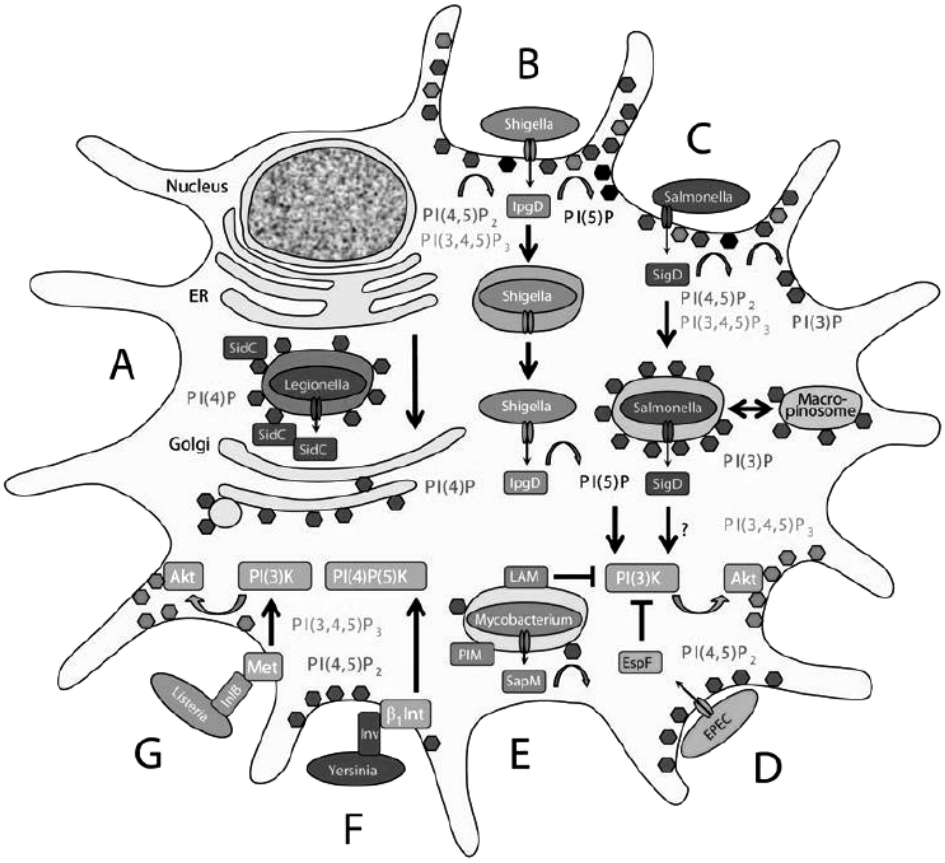


Figure 1.10: Modulations of host phosphoinositide metabolism by pathogenic bacteria. Taken from Hilbi (2006).

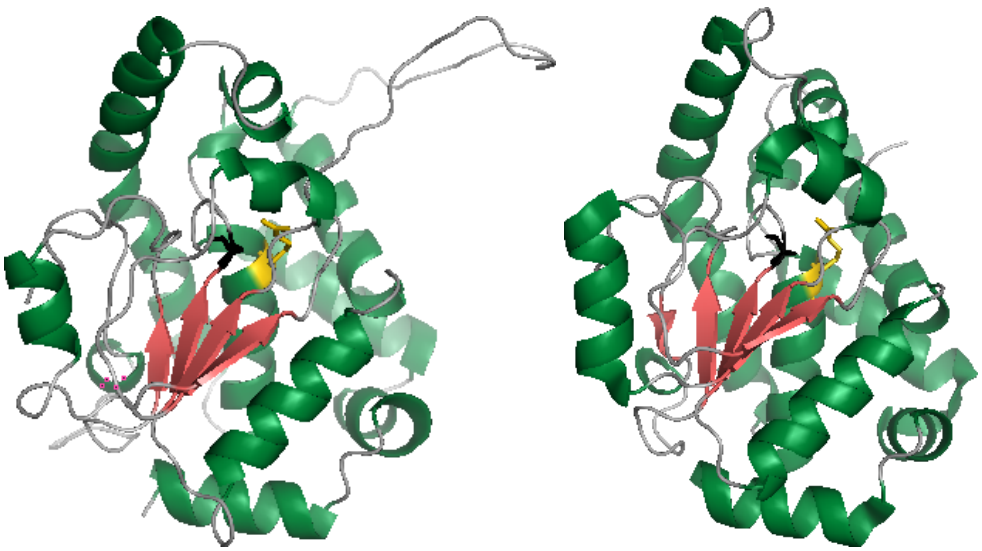
2 Aims

An *in silico* analysis of the *Listeria monocytogenes* genome revealed the presence of a hypothetical tyrosine phosphatase. The first aim of this study was to test, whether the protein possesses phosphatase activity and to identify specificity for *in vitro* substrates. Next, a knockout in *L. monocytogenes* should be established to assess a potential role of the phosphatase in virulence of *L. monocytogenes in vitro*. Herein, different steps of the intracellular infectious cycle of *L. monocytogenes* should be investigated. Finally, an important aim was to determine whether the protein contributes to *L. monocytogenes* virulence during infection of mice *in vivo*.

3 Results

3.1 *In silico* analysis of the *Listeria* genome

The *Listeria* genome contains two potential LMW PTPs *lmo2540* and *lmo0938* that display strong homologies to LMW PTPs of *Bacillus subtilis* or *Acinetobacter johnsonii*. Also, it includes two proteins with homology to classical tyrosine phosphatases, namely *lmo1800* and *lmo1935*. Of those four potential phosphatases only *lmo1800*, designated Listeria phosphatase 1 (Lip1), has a putative signal sequence for either secretion or membrane insertion. The predicted protein structure of Lip1 shows strong homology to the *Mycobacterium tuberculosis* phosphatase MPtpB. In the *Listeria* genome, no classical tyrosine kinase could be found by homology screens.



(a) (b)

Figure 3.1: (a) Predicted protein structure of Lip1 and (b) published 3D model of *Mycobacterium tuberculosis* MPtpB. The P-loop cysteine is marked in black and the aspartic acid in yellow.

3.2 Overproduction and purification of Lip1 *in vitro*

To test Lip1 enzyme activity, the *lip* gene was cloned into the expression vector pQE31 and expressed in *E. coli* XL1 Blue as a fusion protein with a N-terminal His_6-tag for subsequent affinity purification with Ni-NTA beads (see 5.1.2). Figure 3.2 shows the SDS-polyacrylamide gel of purified His_6-Lip1.

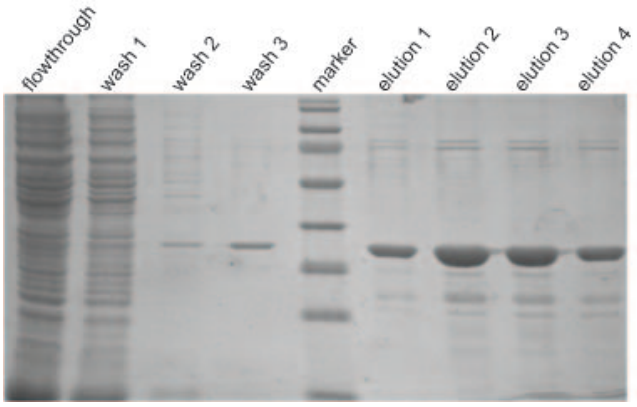


Figure 3.2: Overexpression of His_6-Lip1 in *E. coli*. SDS-PAGE gel of different fractions of purification.

3.3 Measurement of Lip1 phosphatase activity

Using *p*-nitro-phenyl-phosphate (*p*NPP), a chromogenic substrate for detection of phosphatase activity, we observed maximal Lip1 activity at a pH of 6 and a temperature of 30 °C (see figure 3.3). Furthermore, the phosphatase activity of the recombinant protein was tested in presence of phospho-serine (P-Ser), phospho-threonine (P-Thr) and phospho-tyrosine (P-Tyr), showing that Lip1 specifically dephosphorylates P-Tyr but does not have any activity on P-Ser or P-Tyr (see figure 3.3). Recombinant Lip1 was also used for testing activity towards phosphatidylinositol-phosphates. Lip1 readily dephosphorylated PI(3)P, PI(5)P and PI(3,5)P_2.

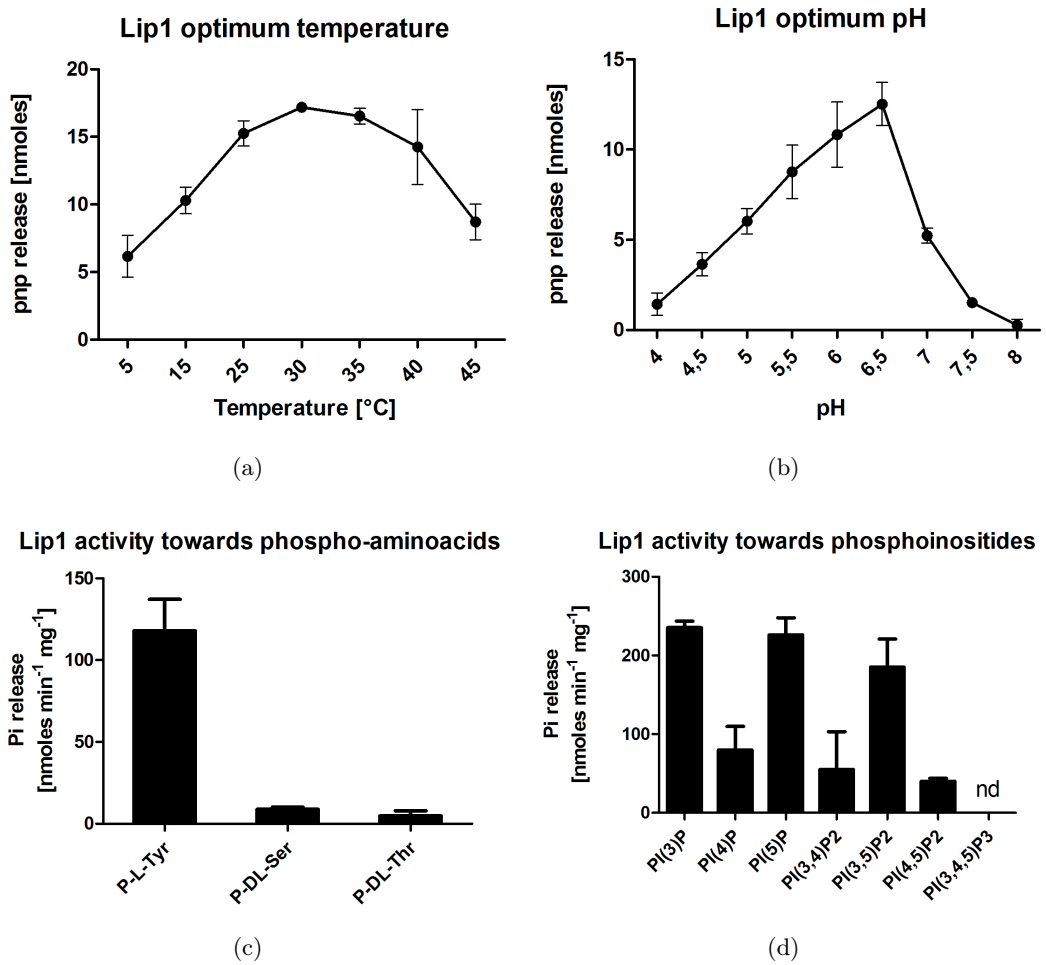
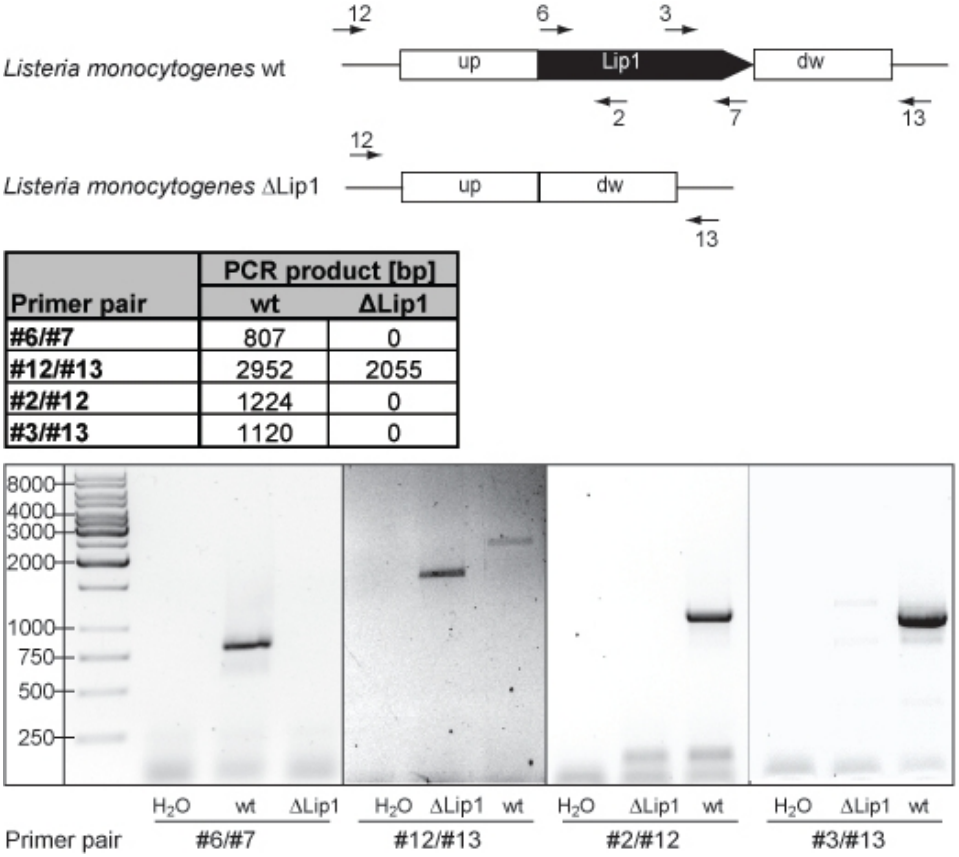


Figure 3.3: Phosphatase activity of Lip1. (a) Optimum temperature and (b) pH were tested using the chromogenic substrate *p*NPP. Specific activity of Lip1 towards (c) phosphorylated amino acids and (d) phosphatidylinositol-phosphates was assessed by measuring inorganic phosphate (Pi) release with a malachite green assay. nd, not detectable.

3.4 Construction of a ΔLip1 deletion strain of *L. monocytogenes* LO28

The *lip1* gene was deleted from the *L. monocytogenes* genome using the pEC84 based homologous integration and excision technique (see chapter 5.1.2). This deletion mutant, designated *L. monocytogenes* ΔLip1 was genotyped by PCR (see figure 3.4).

To test whether the ΔLip1 mutant displays defects in replication, growth at 37 °C was tested in different rich media (Brain Heart Infusion medium (BHi), Luria-Bertani broth (LB)), under conditions of osmotic stress (BHi supplemented with 5 % NaCl) and low pH (BHi with pH=5,5). The ΔLip1 mutant did not differ from the parental strain in the *in vitro* growth rate in any of the tested media (figure 3.5), suggesting that Lip1 is not required for viability or cell division in rich medium and under stress conditions.



Primer pair	PCR product [bp] wt	ΔLip1
#6/#7	807	0
#12/#13	2952	2055
#2/#12	1224	0
#3/#13	1120	0

Figure 3.4: Confirmation of Lip1 knockout by PCR. Primer binding sites are indicated in the graphical display of the genomic *lip1* locus of *L. monocytogenes* wt and ΔLip1 and the size of the PCR product in the respective genotype is listed in the table. Size marker: Generuler 1kB DNA Ladder.

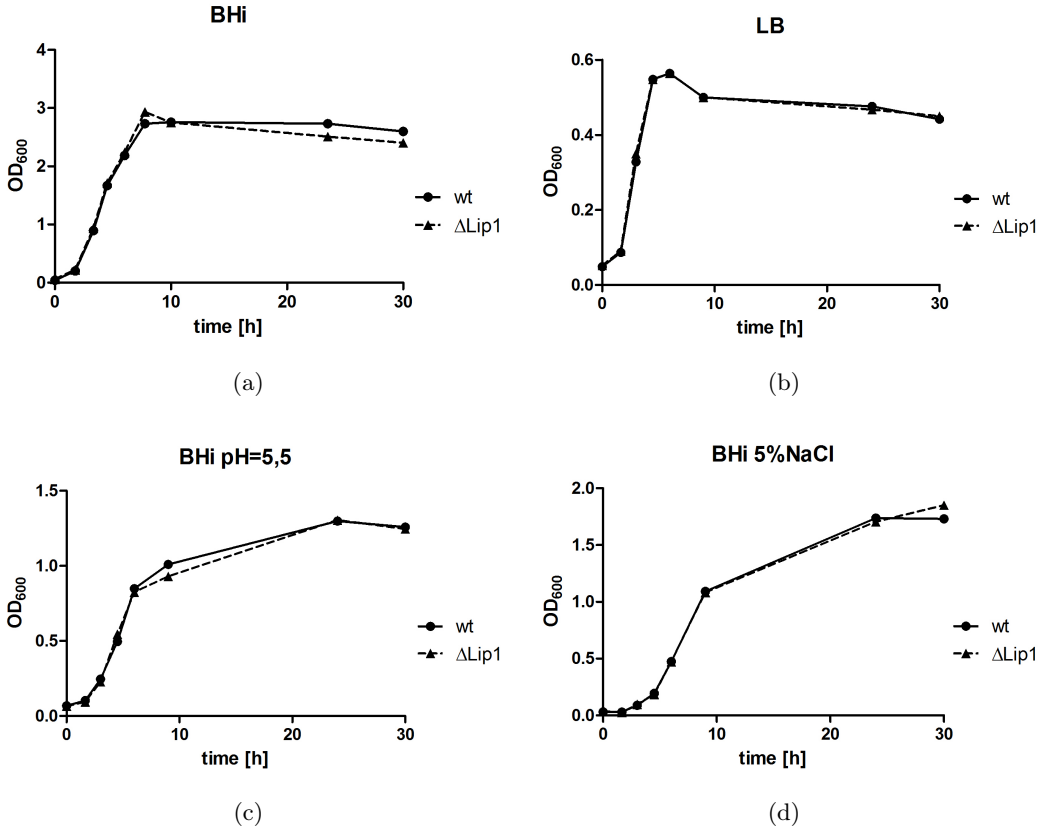


Figure 3.5: Comparison of *L. monocytogenes* LO28 wt and ΔLip1 growth in different media. BHi, Brain Heart Infusion medium; LB, Luria-Bertani broth.

3.5 Lip1 secretion by *L. monocytogenes* LO28

The ORF of *lip1* predicted a signal sequence for either secretion or membrane insertion. To investigate the relevance of this finding *in vivo*, we constructed a pAM401 based expression vector to fuse Lip1 and GFP. Said plasmid was also used to express GFP alone as a control. Then, strains of *L. monocytogenes* transformed with the plasmids pAM401-Lip1-GFP and pAM40-GFP were grown over night in BHi. The growth medium was precipitated and the cytoplasmic, as well as the membrane fraction were prepared from the cell pellet. Subsequent analyses by western blot for GFP showed, that the fusion protein was detectable in the cytoplasm, the cell membrane and in cellular supernatants, suggesting Lip1 contains a functional export signal (see figure 3.6). To check the purity of fractions, antibodies to listeriolysin O (LLO), a virulence factor secreted by *Listeria*, and to the bacterial ribosomal protein S9 were used. As expected, LLO but not S9 could be detected in the culture supernatant.

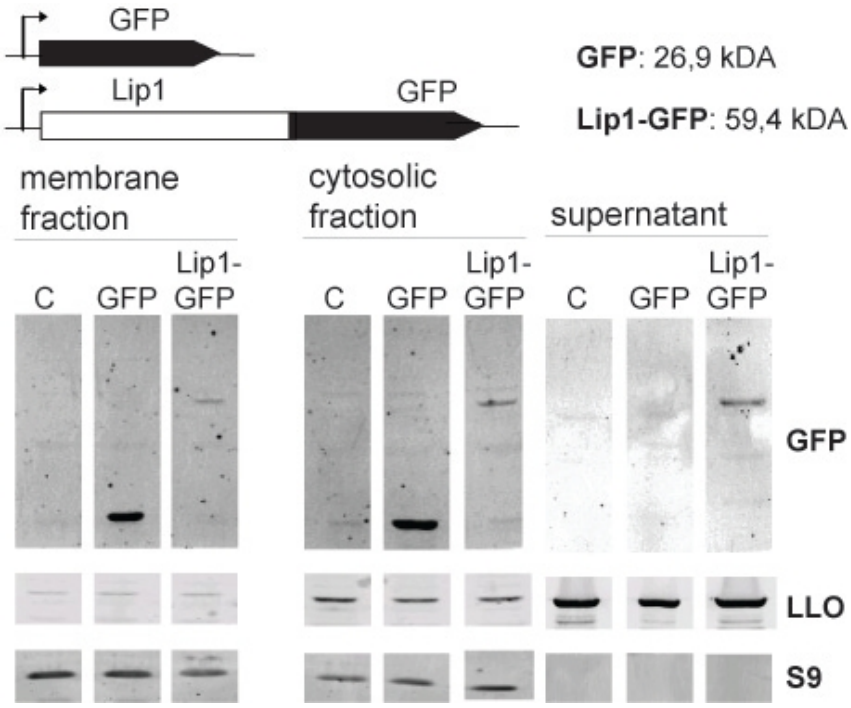
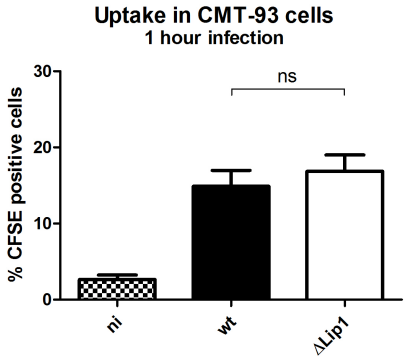


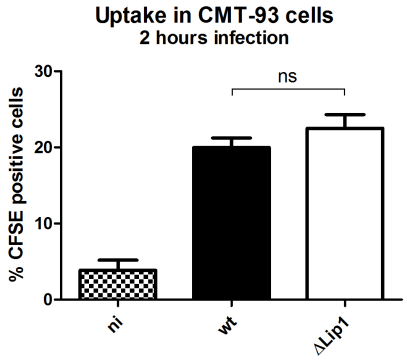
Figure 3.6: The supernatant, membrane and cytosolic fraction were collected from o/n cultures of different *L. monocytogenes* clones containing either the empty control plasmid pAM401 (C), the plasmid expressing the GFP control (GFP) or the plasmid expressing the fusion protein Lip1-GFP. Western blot analysis was performed, using antibodies to GFP, the secreted protein Listeriolysin O (LLO) and the ribosomal protein S9. The fusion protein Lip1-GFP could be detected in all three fractions.

3.6 Uptake of *L. monocytogenes* LO28 wt and ΔLip1 in BMDMs and CMT-93 epithelial cells

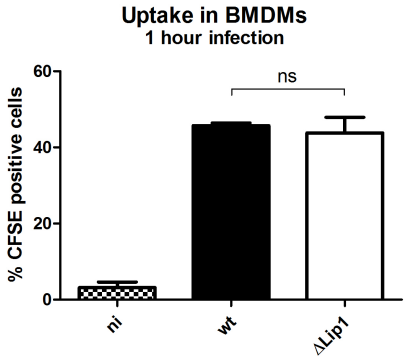
To test uptake of bacteria by host cells, *Listeria* were labelled with the fluorescent dye carboxyfluorescein succinimidyl ester (CFSE) and uptake was determined by flow cytometry. Due to cell type specific differences in the uptake mechanism, professional phagocytic BMDMs and the murine rectal epithelial cell line CMT-93 were tested. There was no difference detectable in uptake into either cell type (see figure 3.7).



(a)



(b)



(c)

Figure 3.7: Uptake of *L. monocytogenes* LO28 wt and ΔLip1 by (c) BMDMs and (a,b) CMT-93 cells was measured 1 or 2 hours after infection. Bacteria were labelled with CFSE and the percentage of CFSE positive cells was determined by flow cytometry. Signals of extracellular bacteria were quenched by addition of Trypan blue solution. ni, non-infected.

3.7 Cytoplasmic escape of *L. monocytogenes* LO28 wt and ΔLip1 in Raw264.7 cells

After internalization, *Listeria* can escape into the cytoplasm by forming pores in the phagosomal membranes. Cytoplasmic bacteria can be visualized by fluorescence microscopy when CFSE stained, green bacteria colocalize with the Phalloidin-Alexa-594 stained, red actin cytoskeleton and therefore appear yellow. The percentage of cytoplasmic bacteria surrounded by an actin cloud compared to the total bacterial number per cell was counted. The cytoplasmic escape was not significantly different between wt and ΔLip1 *Listeria* (see figure 3.8).

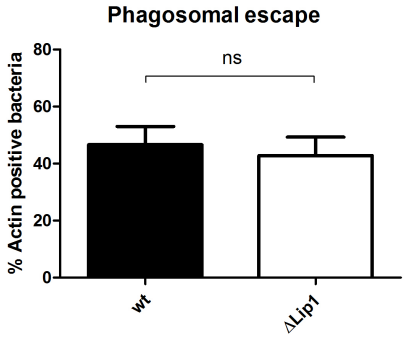


Figure 3.8: Phagosomal escape assay in Raw264.7 cells. Percent of actin positive bacteria were determined in 50 cells per bacterial strain and the mean of two independent experiments was calculated.

3.8 Intracellular replication of *L. monocytogenes* LO28 wt and ΔLip1 in different cell types

The intracellular growth of *Listeria* was surveyed by Colony Forming Units (CFU) assay. Infected cells were lysed at the indicated time points and serial dilutions of intracellular bacteria were plated. Again, to exclude cell type specific effects, professional phagocytic BMDMs and epithelial CMT-93 cells were used for this assay. In a preliminary CFU experiment, we observed that uptake of bacteria into epithelial cells after one hour is very low (see also uptake assay (figure 3.7). Therefore CMT-93 cells were incubated with bacteria for two hours before extracellular bacteria were killed with gentamicin. Also, mouse L2 fibroblasts were analysed, as they were used for a plaque assay to analyse cell to cell spread. To exclude that observed results in

cell to cell spread are influenced by differences in intracellular growth, CFUs were monitored in parallel. In none of the tested cell types, the intracellular growth of the wt and ΔLip1 *Listeria* differed significantly.

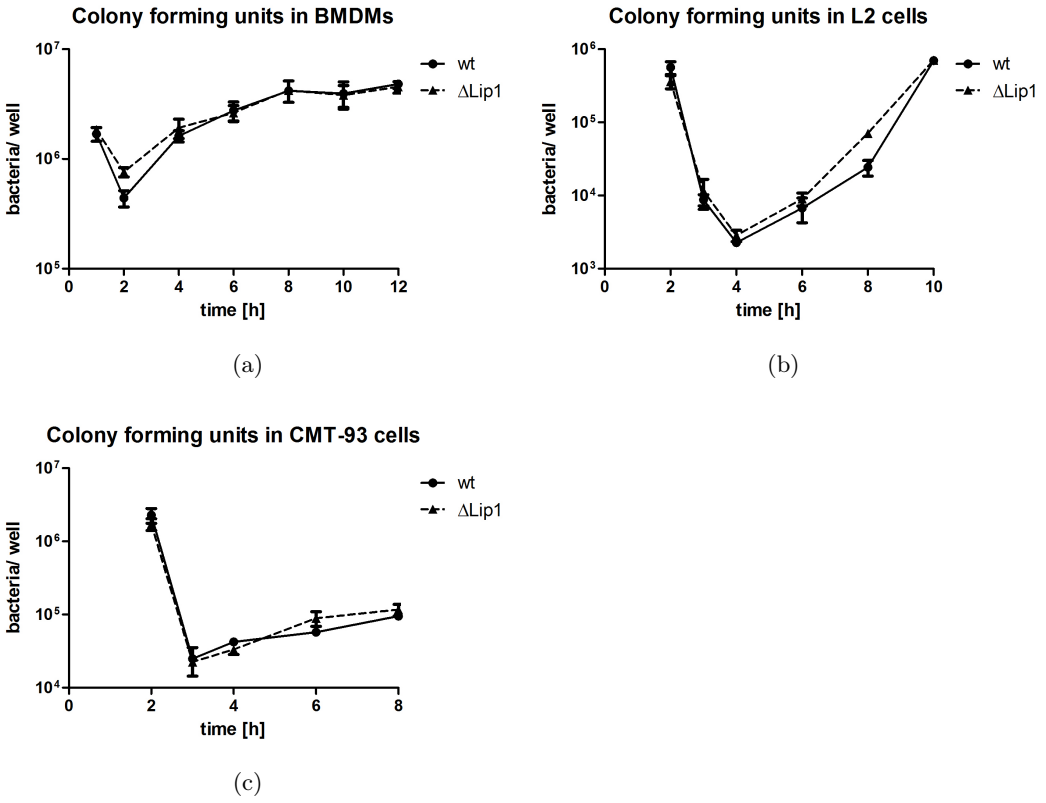
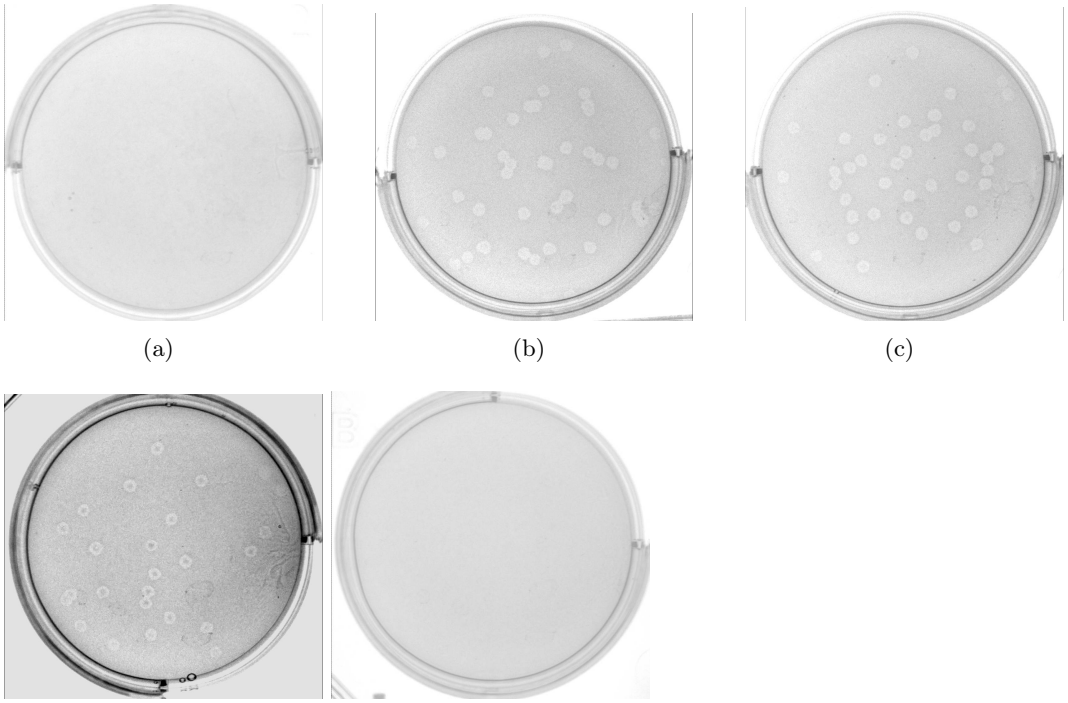


Figure 3.9: Colony Forming Units assay in (a) BMDMs, (b) L2 cells and (c) CMT-93 cells after infection at MOI 10, MOI 20 or MOI 50, respectively. Extracellular bacteria were killed with gentamicin 1 hour after infection of BMDMs or 2 hours after infection of L2 or CMT-93 cells. Cells were lysed at indicated time points and serial dilutions of cellular lysates were plated.

3.9 Ability of *L. monocytogenes* LO28 wt and ΔLip1 to spread from cell to cell

To test the ability of *Listeria* to spread from one cell to the neighbouring cells, plaque assays were performed. Semi-confluent layers of L2 fibroblast cells were infected at low MOI and overlayed with a mixture of tissue culture medium, agarose

and gentamicin 2 hours after infection. After an incubation time of 3 days, another overlay containing neutral red solution was applied. Neutral red is a stain for live host cells and clear plaques formed by lysis of infected cells are revealed. *L. monocytogenes* EGD wt and ΔPlcAB were included as a control. ΔPlcAB bacteria are not able to escape from secondary vacuoles and do not form plaques. Comparing the average surface area of plaques formed by wt or ΔLip1, no significant difference could be observed (see figures 3.10 and 3.11).



(a) (b) (c)

(d) (e)

Figure 3.10: Plaque formation in L2 cells by *L. monocytogenes* (b) LO28 wt (c) LO28 ΔLip1 (d) EGD wt (e) EGD ΔPlcAB (a) non-infected control. For quantification of relative plaque surface area see figure 3.11.

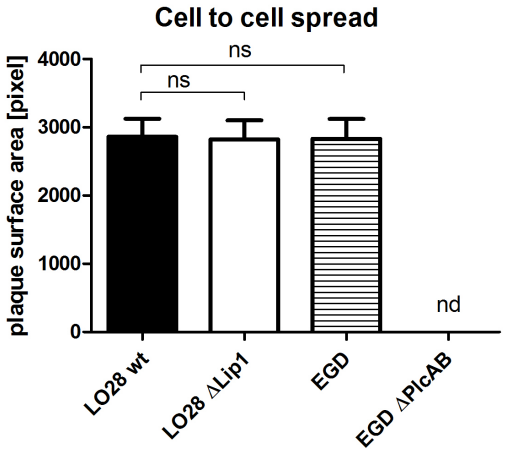


Figure 3.11: Detection of cell to cell spread in L2 cells by a plaque assay. Relative plaque surface area was calculated for 50 plaques per bacterial strain and the mean of two independent experiments was calculated. nd, not detectable.

3.10 Virulence of *L. monocytogenes* LO28 wt and ΔLip1 in BMDMs and CMT epithelial cells

The induction of cell death by *Listeria* in infected BMDMs was detected by propidium iodide (Pi) staining. Loss of membrane integrity leads to uptake of the fluorescent nucleic acid dye Pi and thereby live and dead cells can be distinguished. Infected cells and untreated control cells were detached from culture plates by citrate buffer and Pi uptake was measured by flow cytometry. As infected cells might be sensitive to citrate buffer treatment, cell death was additionally shown as percentage of lactic acid dehydrogenase (LDH) release from dead cells in infected BMDMs and CMT-93 cell cultures. Cell death induced by infection at different MOIs was compared to LDH release after total cell lysis. Wt and ΔLip1 *Listeria* did not differ in the induction of cell death in either of the tested cell types.

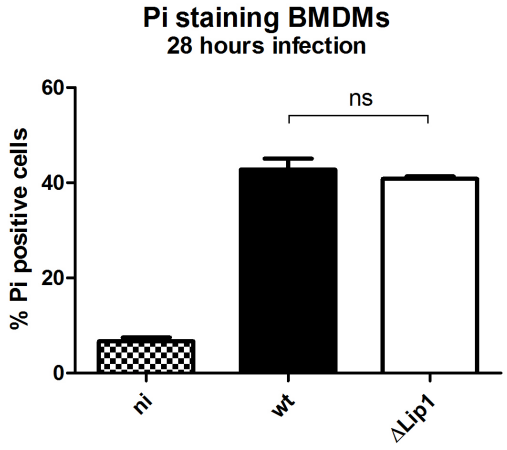
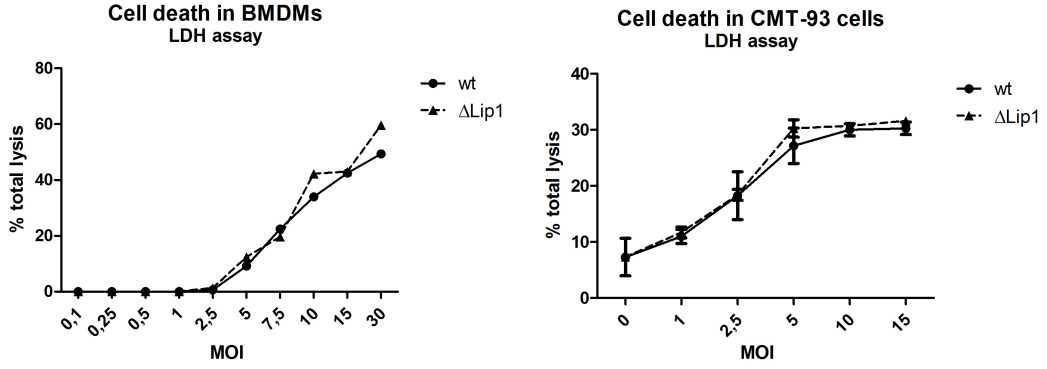


Figure 3.12: BMDMs were infected with *Listeria* wt and ΔLip1 at MOI 10. 28 hours after infection, cell death was assessed by Pi staining. ni, non-infected.

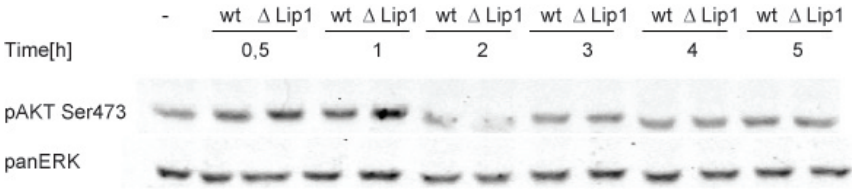


(a) (b)

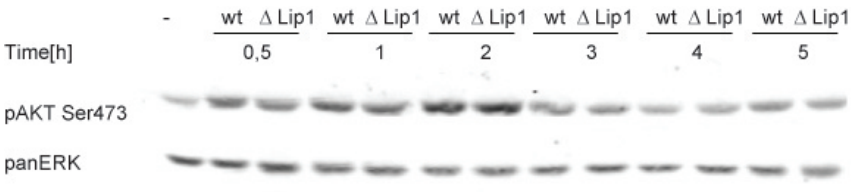
Figure 3.13: Cell death was assessed by measurement of LDH release in the supernatant (a) 24 hours after infection of BMDMs and (b) 26 hours after infection of CMT-93 cells at the indicated MOIs. Cell death is indicated as percentage of LDH release after total lysis.

3.11 Activation of the Akt pathway by *L. monocytogenes* LO28

The Akt pathway is targeted by at least two bacterial phosphoinositide phosphatases (Knodler et al., 2005; Pendaries et al., 2006). Phosphorylation of Akt serine 473 upon *Listeria* infection was analysed in CMT-93 epithelial cells and BMDMs. In BMDMs an increase in phosphorylation of Akt serine 473 could be observed after 1 hour of infection with both, wt and ΔLip1 bacteria. Also in CMT-93 cells no difference in the pattern of serine 473 phosphorylated Akt could be detected.



(a)



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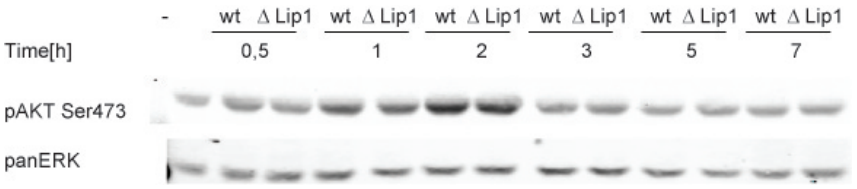
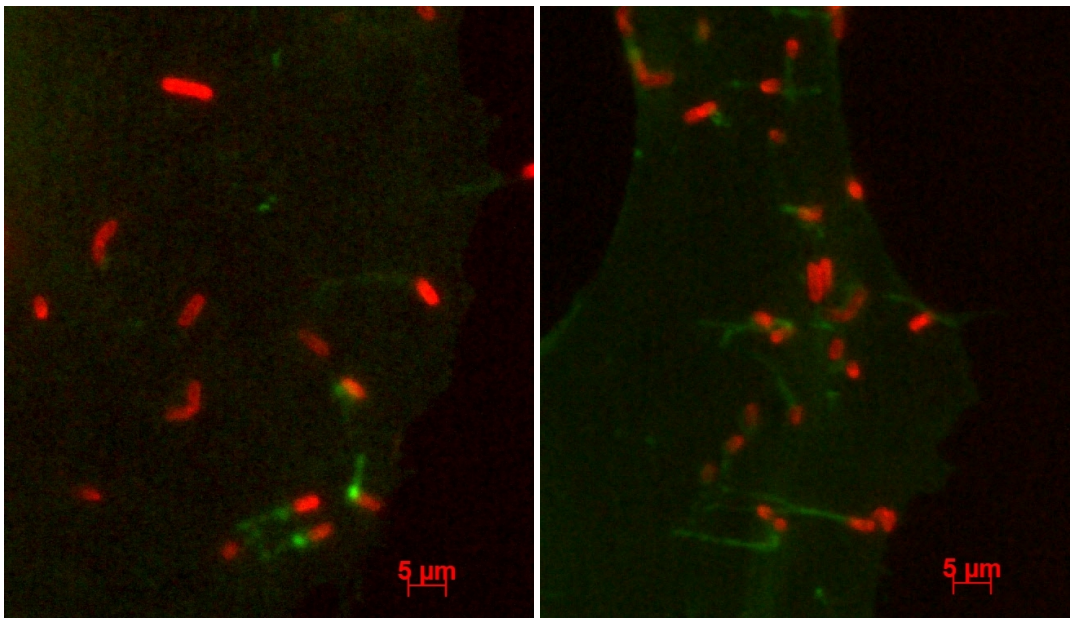


(c)

Figure 3.14: Phosphorylation of Akt on serine 473 after infection of (a) BMDMs at MOI 10 or (b,c) CMT-93 cells at MOI 100 with wt or ΔLip1 was determined at the indicated time points. Panel (c) shows an extended kinetic compared to (b). Western blots of whole-cell extracts were stained with phospho-Akt serine 473 (pAKT Ser473) antibody and probed with antibodies recognizing ERK 1 and 2 (panERK) for normalization.

3.12 Akt-PH-GFP recruitment by *L. monocytogenes*

Listeria that move within the cytoplasm accumulate $PI(3,4,5)P_3$ around their surface. Akt contains a PH domain that binds $PI(3,4)P_2$ and $PI(3,4,5)P_3$, and is concentrated to moving *Listeria* and the actin rich tails (Sidhu et al., 2005). We used a fusion protein of GFP linked to the PH domain of Akt to investigate Akt recruitment to *L. monocytogenes* wt and ΔLip1. Ptk2 cells were transfected for 48 hours with Akt-PH-GFP and infected with *L. monocytogenes* for 6 hours. Bacteria were stained with a primary antibody to *Listeria* and a red secondary Alexa-594 conjugated antibody. We could observe recruitment of the Akt-PH-GFP fusion protein to one pole of *L. monocytogenes* wt and ΔLip1. Akt-PH-GFP was also found in polarized tails, that presumably represent the actin rich tails formed by *L. monocytogenes*.



(a) (b)

Figure 3.15: Localization of Akt-PH-GFP (green) in infected Ptk2 cells. Cells were transfected with Akt-PH-GFP and infected with *L. monocytogenes* (a) wt and (b) ΔLip1 for 6 hours. Bacteria were visualized using an antibody to *Listeria* and a secondary Alexa-594 conjugated antibody (red).

3.13 Virulence of *L. monocytogenes* LO28 wt and ΔLip1 in mice after intraperitoneal infection

To determine whether the disruption of the *lip1* gene has an effect on bacterial pathogenicity *in vivo*, C57Bl/6 wt mice were infected intraperitoneally with wt and ΔLip1 *Listeria* and survival was monitored for ten days. After infection with 1×10^6 CFU, all mice infected with wt bacteria succumbed to the infection by day 6. Mice infected with ΔLip1 displayed significantly enhanced survival as 80 % of mice survived the infection (figure 3.16).

Additionally, bacterial burdens in the target organs spleen and liver were analysed on day one and three after *i. p.* infection. 24 hours after infection, no significant difference in CFUs could be observed. However, mice infected with ΔLip1 *Listeria* exhibited a significant reduction of bacterial loads in spleen and liver on day three post infection, compared to the wt strain (figure 3.17).

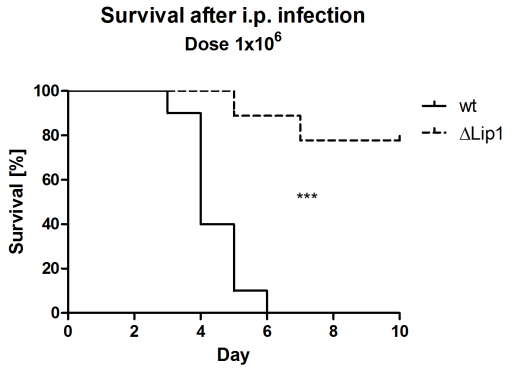
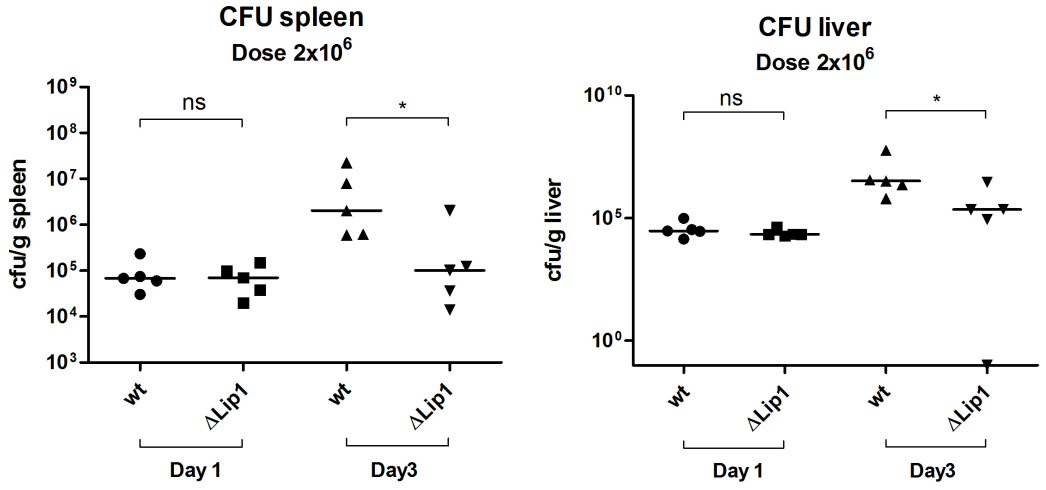


Figure 3.16: Survival test. Groups of 10 C57Bl/6 wt mice were infected intraperitoneally with the indicated dose of *Listeria* wt or ΔLip1. Survival was monitored for ten days. Data are depicted as Kaplan–Meier plots. Significance was calculated using the Gehan-Breslow-Wilcoxon test.

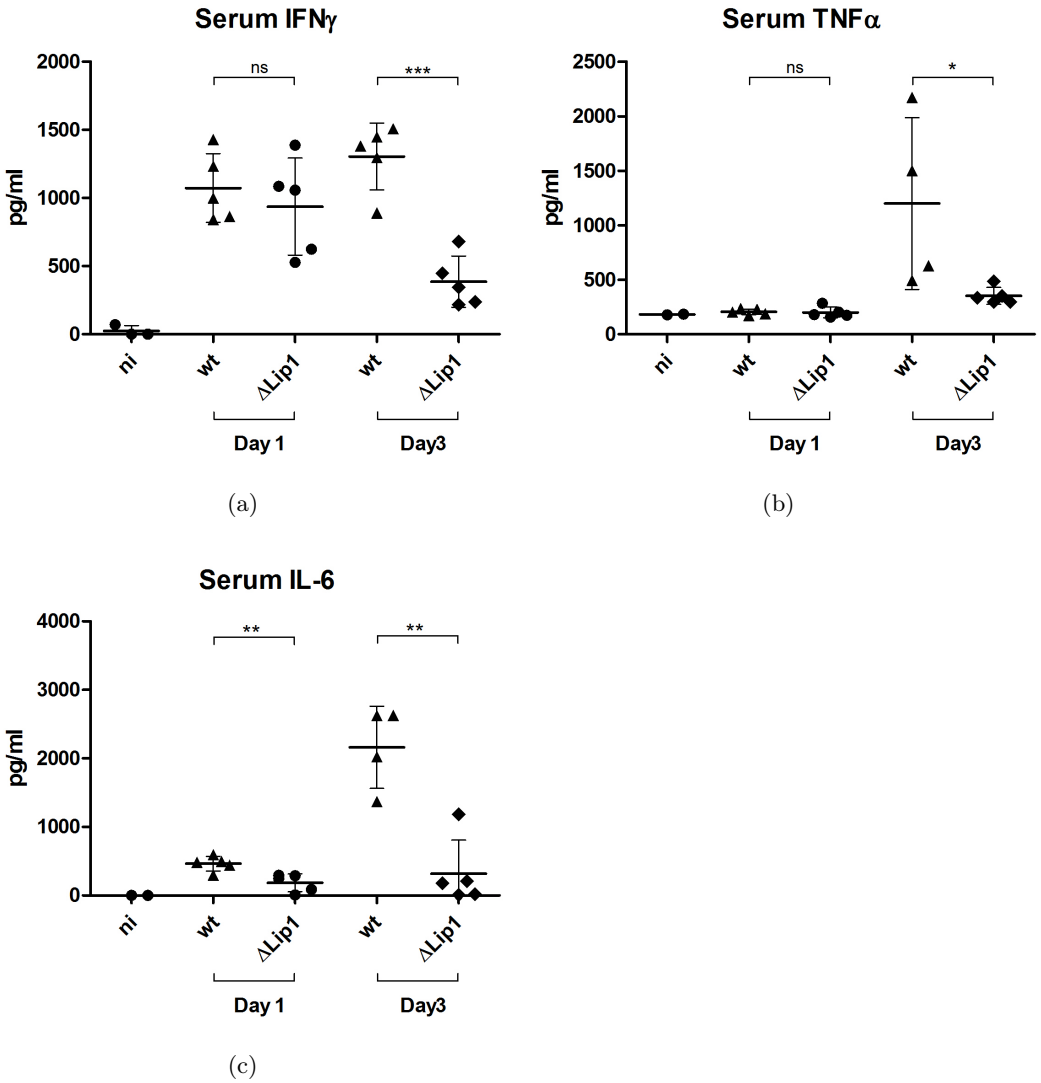


(a) (b)

Figure 3.17: Groups of 4 to 5 C57Bl/6 wildtype mice were infected intraperitoneally with the indicated dose of *Listeria* wt or ΔLip1. At day one or three, the target organs liver and spleen were homogenized and serial dilutions were plated on Oxford-Agar plates. Medians were plotted and statistical significance was calculated using the Mann-Whitney U test.

3.14 Detection of serum cytokine levels after intraperitoneal infection

To test if the reduced bacterial loads had an impact on cytokine production, serum levels of IFNγ, TNFα and IL-6 were measured with ELISA. IFNγ was robustly produced after infection with wt *Listeria* on day one and three after infection and a significant reduction in serum levels could be detected after infection with ΔLip1 bacteria on the third day after infection. Production of TNFα could be detected at low levels three days after infection with wt bacteria, however with a quite high variability between analysed mice. In mice infected with ΔLip1, TNFα levels were only marginally elevated compared to uninfected mice and significantly lower compared to infection with wt bacteria. Also serum IL-6 levels were highly reduced in infection with ΔLip1 compared to wt bacteria (figure 3.18).



(a) (b)

(c)

Figure 3.18: Cytokine levels in serum after intraperitoneal infection measured with ELISA. Individual mice are shown and means with standard deviation are depicted. Statistical significance was calculated using the Students unpaired t test. ni, non-infected.

3.15 Haematology analysis after intraperitoneal infection

Mice were infected *i. p.* with 5×10^5 *L. monocytogenes* wt or ΔLip1. On day four after infection, blood was collected and analysed with a haematology cell counter. Compared to the blood of uninfected control mice, numbers of white blood cells and lymphocytes dropped significantly in infected mice. We did not observe a difference in numbers of white blood cells, lymphocytes, monocytes or granulocytes in the blood of mice comparing infection with *L. monocytogenes* wt and ΔLip1.

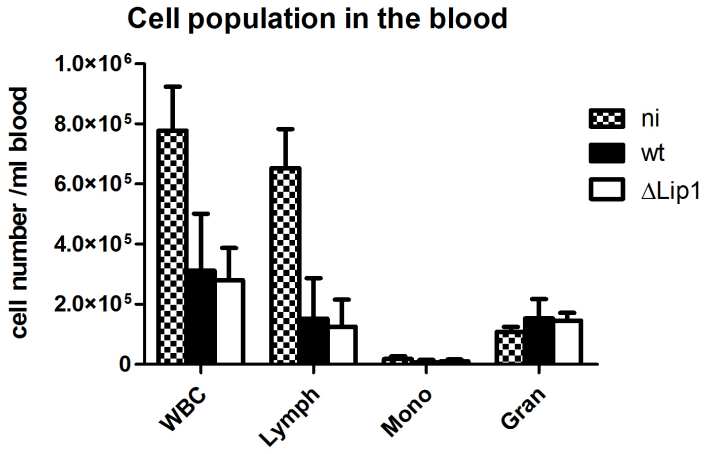
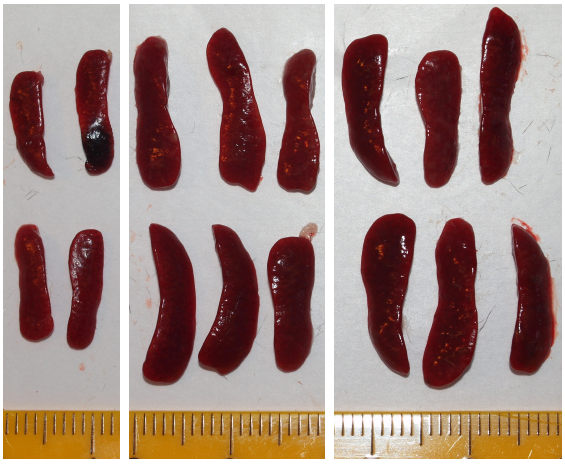


Figure 3.19: Groups of six mice were infected *i. p.* with *L. monocytogenes* wt or ΔLip1. Blood was collected four days after infection, and from four non-infected control mice. Cell numbers were determined using a haematology cell counter. WBC, white blood cells; Lymph, lymphocytes; Mono, monocytes; Gran, granulocytes; ni, non-infected.

3.16 Analysis of splenic cells after intraperitoneal infection

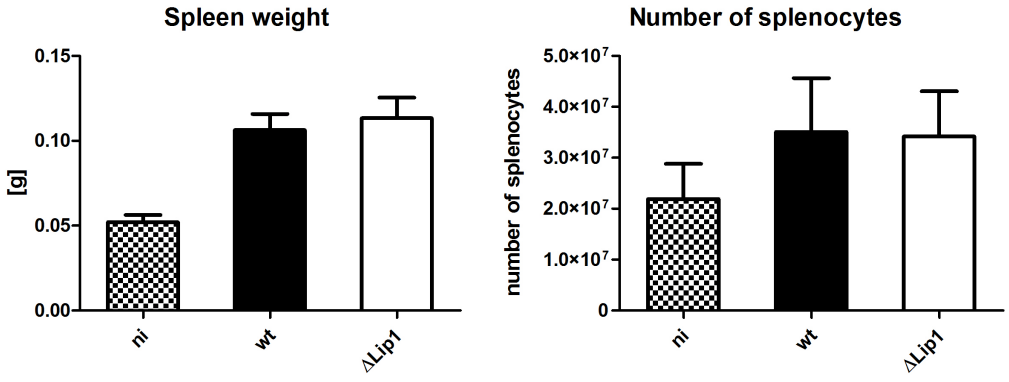
In preliminary experiments we observed, that mice infected with *L. monocytogenes* display splenomegaly. Therefore we compared the spleen weight of mice infected *i. p.* with *L. monocytogenes* wt and ΔLip1 and analysed percentages of different splenic cell populations. Spleens of mice infected with *L. monocytogenes* wt and ΔLip1 were removed, weighed and single cell suspensions were prepared. Red blood cell lysis was performed and the number of splenic cells was determined with a cell viability analyser (Vi-cell, Beckman Coulter). Antibody stainings used to distinguish

B cells, $CD4^+$ T cells, $CD8^+$ T cells, natural killer cells, dendritic cells, inflammatory monocytes and neutrophils are listed in table 3.1. We observed splenomegaly on day four in both, mice infected with *L. monocytogenes* wt and ΔLip1 (figure 3.20). The increased spleen size goes in line with an increase in splenic cell numbers. Concerning splenic cell populations, we observed an increase of inflammatory monocytes and neutrophils and a decrease in T cells in infected compared to non infected mice. However, we could not detect a significant difference in splenic cell populations between infection with *L. monocytogenes* wt and ΔLip1.



(a) (b) (c)

Figure 3.20: Spleens of (a) non-infected mice, and mice infected *i. p.* with *L. monocytogenes* (b) wt or (c) ΔLip1 for four days.



(a) (b)

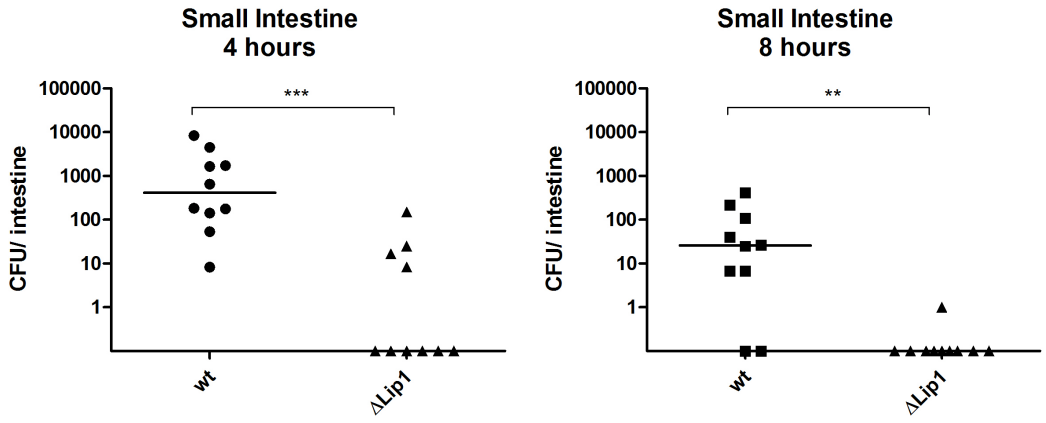
Figure 3.21: Groups of six mice were infected *i. p.* with *L. monocytogenes* wt or ΔLip1. A group of four control mice was left untreated. Spleens were removed at day 4 post infection and weighed. Single cell suspensions were prepared and number of splenocytes was determined using a cell viability analyser. ni, non-infected.

Staining	**ni**	**wt**	**ΔLip1**
$B220^+$ $CD19^+$ (B cells)	53,9 ± 1,9	59,1 ± 7,3	57,4 ± 2,0
$CD3^+$ $CD4^+$ $CD8^-$ ($CD4^+$ T cells)	20,8 ± 2,1	13,2 ± 2,1	12,4 ± 2,2
$CD3^+$ $CD8^+$ $CD4^-$ ($CD8^+$ T cells)	12,1 ± 1,5	7,6 ± 1,5	7,0 ± 1,8
$CD11c^+$ $CD11b^+$ $MHCII^+$ (DC)	0,6 ± 0,2	1,0 ± 0,2	0,9 ± 0,2
$NK1.1^+$ $CD3^-$ (NK cells)	3,1 ± 1,0	3,5 ± 1,2	4,0 ± 1,0
$CD11b^+$ $Ly6C^{hi}$ $Ly6G^{int}$ (Infl. mono.)	1,2 ± 0,2	4,5 ± 1,7	6,1 ± 0,8
$CD11b^+$ $Ly6C^{int}$ $Ly6G^{hi}$ (Neutrophils)	0,9 ± 0,1	5,6 ± 1,4	7,4 ± 2,4

Table 3.1: Percentage of splenic cell types with standard deviation. Groups of six mice mice were infected *i. p.* with *L. monocytogenes* wt or ΔLip1. A group of four control mice was left untreated. Spleens were removed at day 4 post infection and single cell suspensions were prepared. After red blood cell lysis, splenic cells were stained with indicated antibodies and subjected to FACS analysis. DC, dendritic cells; NK cells, natural killer cells; Infl. mono., inflammatory monocytes; ni, non-infected.

3.17 Virulence of *L. monocytogenes* LO28 wt and ΔLip1 in mice after intragastric delivery

Additional to *i. p.* infection studies, mice were infected via intragastric gavage to mimic natural infection with contaminated nourishments. Already 4 hours after infection, bacteria could be detected in the small intestine whereas significantly less bacteria were found after infection with ΔLip1 compared to wt bacteria (figure 3.22). Bacterial counts dropped at eight hours (figure 3.22) and were below detection limit after 24 hours (figure 3.23). Three days after infection numbers of wt bacteria again increased approximately to initial levels at 4 hours. Again, numbers of ΔLip1 bacteria were significantly decreased. A decrease in ΔLip1 bacterial counts three days after infection could also be observed in the spleen and liver. Whereas the difference was pronounced in the spleen it did not reach statistical significance in the liver (figure 3.23).



(a) (b)

Figure 3.22: CFU assays after intragastric delivery of 1×10^9 bacteria. 4 and 8 hours after infection, mice were killed and small intestines were removed and flushed with sterile PBS. The bacterial titre was determined by homogenisation of the small intestine and plating of serial dilutions on Oxford-Agar plates. Medians were plotted and statistical significance was calculated using the Mann-Whitney U test.

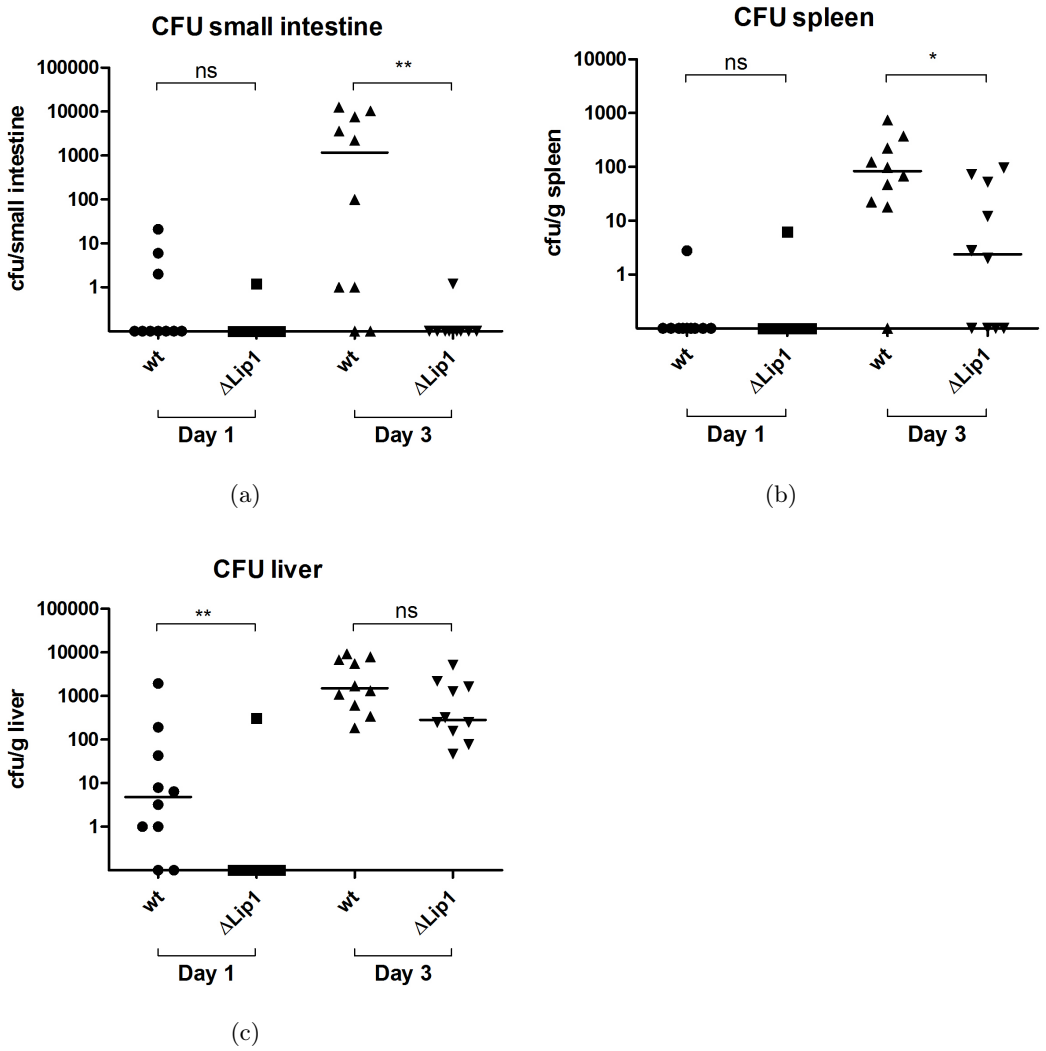


Figure 3.23: CFU assays after intragastric delivery of 1×10^9 bacteria. 24 and 72 hours after infection, mice were killed and the bacterial titre was determined by homogenisation of the respective target organ and plating of serial dilutions on Oxford-Agar plates. Small intestines were flushed with sterile PBS before homogenisation. Medians were plotted and statistical significance was calculated using the Mann-Whitney U test.

4 Discussion

L. monocytogenes is a well established model organism for investigating intracellular infections. Extensive studies on the infectious cycle of *Listeria* have provided us with profound knowledge of the virulence factors these bacteria require for successfully exploiting host cells as a replication niche. A mechanism of bacterial virulence that has only been discovered in the last decades, is the interference with the host metabolism by secretion of bacterial phosphatases.

So far, it has not been investigated, whether phosphatases also contribute to virulence of *L. monocytogenes*. Therefore, we performed a screen of the *L. monocytogenes* genome for putative phosphatases. The sequence analysis revealed two potential LMW phosphatases *lmo2540* and *lmo0938* and two potential classical tyrosine phosphatases, *lmo1800* (Lip1) and *lmo1935*. Of those genes only *lip1*, contains a putative signal sequence for either secretion or membrane insertion, suggesting that Lip1 might not be required for the metabolism of the bacterium itself but could target substrates in the host cell. The presence of the three tyrosine phosphatases *lmo2540*, *lmo0938* and *lmo1935*, that are unlikely to be secreted, suggest *L. monocytogenes* also encodes for a tyrosine kinase. However, we could not detect any bacterial tyrosine kinases in the *L. monocytogenes* genome.

Interestingly, the predicted protein structure of lmo1800 shows strong homology to the phosphatase MPtpB, a virulence factor of *Mycobacterium tuberculosis* (figure 3.1). MPtpB contains a flexible two helix-lid that protects the active site from oxidative inactivation. The resistance to oxidation mediated by the lid conformational dynamics might represent an adaptation to oxidative challenges *in vivo* (Flynn et al., 2010). Based on the predicted 3D model of Lip1, there is evidence that also Lip1 contains this flexible loop structure.

We characterized the enzymatic activity profile and substrate specificity of the putative phosphatase Lip1 *in vitro* using a His_6-Lip1 fusion protein. Testing Lip1 activity towards phosphorylated amino acids, we found that Lip1 specifically dephosphorylates phospho-tyrosine but does not have any activity towards phospho-serine or phospho-tyrosine (figure 3.3). The pH and temperature optimum were assayed

using *p*-nitrophenylphosphate (*p*NPP), a commonly used substrate for tyrosine phosphatases. Typically, tyrosine specific phosphatases display a bell shaped pH rate profile with a low pH optimum (Kolmodin and Aqvist, 2001). In line with this, Lip1 has optimal activity at a pH of 6,5 (figure 3.3). The optimal temperature for Lip1 phosphatase activity is around 30–35 °C (figure 3.3).

The *M. tuberculosis* phosphatase MPtpB displays dual specificity phosphatase activity towards phospho-tyrosine and phospho-serine/-threonine substrates and additionally phosphoinositide phosphatase activity for a broad range of lipids (Beresford et al., 2007). Due to the homology of Lip1 to the mycobacterial phosphatase, Lip1 activity on phosphoinositide phosphates was tested. This analysis showed, that Lip1 exhibits activity against PI(3)P, PI(5)P and PI(3,5)P_2 (figure 3.3). In contrast, it had little or no activity towards PI(4)P, PI(3,4)P_2, PI(4,5)P_2 and PI(3,4,5)P_3, apparently preferring a single phosphate or a combination of phosphates at the D3 and D5 position of the inositol head.

Next, we approached the question whether the putative signal peptide for secretion or membrane insertion, revealed by bioinformatics, is functional *in vitro*. Therefore, a plasmid for expression of a Lip1-GFP fusion protein by *Listeria* was created. We analysed supernatants, membrane and cytosolic fractions of bacterial over night cultures for the presence of the Lip1-GFP construct. Lip1-GFP was present in the supernatants, indicating that Lip1 is secreted by *L. monocytogenes* (figure 3.6). This finding contrasts a recent publication showing that Lip1 is lipidated by the prolipoprotein diacylglyceryl transferase (Lgt), leading to the anchorage of Lip1 to the bacterial surface (Baumgärtner et al., 2007). Possibly, the attachment of GFP used in our assay interferes with Lip1 localization.

To initiate a functional analysis *in vivo*, a Lip1 knockout strain of *L. monocytogenes* LO28 was created. We ruled out differences in the growth rate of the ΔLip1 and the parental strain in rich liquid media and under stress conditions (figure 3.5). This finding was essential as liquid cultures were used for performing *in vitro* and *in vivo* infection studies.

The intracellular life cycle of *L. monocytogenes* comprises the uptake of bacteria into the host cell, escape from the cytoplasm followed by replication within the cytoplasm and finally cell to cell spread, leading to a new round of infection. All steps were analysed *in vitro* for a contribution of Lip1. However, no defects of the ΔLip1 strain could be detected.

Phosphoinositides play an important role in membrane dynamics and are required for efficient phagocytosis (Haucke and Paolo, 2007). Indeed, some bacteria secrete

phosphatases that influence bacterial uptake or phagosomal maturation and thereby contribute to bacterial virulence. Examples are the phosphatase SopB of the intracellular bacterium *Salmonella enterica* that promotes bacterial uptake (Zhou et al., 2001) or the *Mycobacterium tuberculosis* phosphatase SapM that inhibits phagosomal maturation (Vergne et al., 2005). Also the tyrosine phosphatase YopH blocks the uptake of *Yersinia* by a yet unclear mechanism. In contrast to the above mentioned phosphatases that are either secreted or directly injected into the host cytoplasm, Lip1 is anchored to the surface of *L. monocytogenes*. While *Listeria* reside within the phagosome, Lip1 presumably cannot get into proximity of phosphoinositide on the outside of vesicles that regulate phagosome fission and maturation. Therefore a contribution of Lip1 to these events seems unlikely but cannot be ruled out. As the uptake mechanism of *L. monocytogenes* differs in cell types, we analysed professional phagocytic BMDMs, as well as the murine rectal epithelial cell line CMT-93, where *Listeria* force their uptake by an internalin mediated mechanism. Lip1 does not influence the bacterial uptake mechanism into either cell type (figure 3.7). As the *L. monocytogenes* LO28 strain contains a point mutation in the *inlA* gene leading to the release of the internalin (Jonquières et al., 1998), a contribution of Lip1 to InlA mediated uptake of other *Listeria* strains cannot be excluded formally.

Measuring phagosomal escape, ΔLip1 and wildtype bacteria were equally efficient in translocation from phagosomes into the cytosol (figure 3.8). Also, intracellular growth of *L. monocytogenes* was tested for a contribution of Lip1. To exclude cell type specific effects, BMDMs and CMT-93 cells were analysed. However, intracellular replication of *L. monocytogenes* did not significantly differ in any of the tested cell types (figure 3.9). We also examined the efficacy of ΔLip1 bacteria at spreading from cell to cell with a plaque forming assay in murine fibroblast L2 cells. The intracellular growth rate of wildtype and ΔLip1 bacteria is equal in L2 cells (figure 3.9) and therefore plaque size should only reflect transmission of bacteria from cell to cell. Wildtype and ΔLip1 form comparable numbers of plaques, indicating that the uptake into L2 cells is equal. Also, the average surface area of plaques formed by wildtype and ΔLip1 bacteria does not differ significantly, showing that Lip1 is not required for efficient *L. monocytogenes* cell to cell spread (figure 3.11).

Intracellular movement of *L. monocytogenes* critically depends on PI3-K and the recruitment of PI(4,5)P_2 and PI(3,4,5)P_3 to the bacterial surface (Sidhu et al., 2005). PI(4,5)P_2 is suggested to serve as substrate for formation of PI(3,4,5)P_3 by PI3-K. However, the role of PI(3,4,5)P_3 in *listerial* actin based motility is not understood. The host protein Akt contains a PH domain that binds PI(3,4)P_2 and PI(3,4,5)P_3,

and is concentrated to moving Listeria. We found that ΔLip1 bacteria do not have a defect in recruiting Akt-PH-GFP to their surface (figure 3.15), an indication that also PI(3,4,5)P_3 is present. This result is in line with efficient plaque formation by *L. monocytogenes* ΔLip1, as PI(3,4,5)P_3 recruitment is required for production of filopodia and therefore for cell to cell spread (Sidhu et al., 2005).

Cellular processes that are subverted by bacterial phosphatases include interference with uptake, block of phagocytosis or cytoskeleton rearrangements. Interestingly, some bacterial phosphoinositide-phosphatases modulate the Akt pathway, a key regulator of cell survival (Song et al., 2005). For instance activation of the Akt pathway by the phosphatase IpgD from *Shigella flexneri* leads to prolonged survival of the host cell and efficient replication of the bacteria (Pendaries et al., 2006). Akt is one of the main target molecules downstream of PI3-K. PI3-K is necessary for internalin mediated *L. monocytogenes* invasion (Ireton et al., 1996), and furthermore InlB has been shown to activate Akt (Mansell et al., 2001). We could detect activation of Akt one hour after infection of BMDMs with *L. monocytogenes* and up to two hours after infection of CMT-93 cells. The pattern of Akt activation showed no remarkable difference between the wildtype and the ΔLip1 strain. In line with equal intracellular growth and equal activation of the host Akt pathway, we did not find a difference in killing of infected BMDMs and CMT-93 cells by *L. monocytogenes* wt and ΔLip1.

Whether the physiologically relevant substrate of Lip1 *in vivo* represents a phosphoinositol or a tyrosine phosphate is unclear. To obtain evidence for interference of Lip1 with host cell tyrosine phosphorylation, we analysed whole cell extracts after infection with *L. monocytogenes* wt and ΔLip1. However, we did not find a difference in patterns of tyrosine phosphorylation of host proteins (data not shown). Likewise, tyrosine phosphorylation of Stat1 during infection was unaffected by the presence or absence of Lip1. These results hint towards phosphoinositol being the relevant substrate, but clearly, further effort has to be invested into this issue.

Strikingly, the virulence of *L. monocytogenes* ΔLip1 compared to the wt strain was profoundly reduced *in vivo*. Lethality caused by *L. monocytogenes* wildtype was significantly higher compared to ΔLip1 bacteria (figure 3.16) in mice infected intraperitoneally. The increased survival of the host correlated with reduced bacterial burdens of ΔLip1 bacteria in the target organs spleen and liver (figure 3.17). We also compared serum levels of cytokines, that play an important role in early stages of *Listeria* infection, namely IFNγ, TNFα and IL-6 (Unanue, 1997). We detected robust IFNγ production on day one after infection with both strains. However, IFNγ

levels dropped significantly on day three in the serum of mice infected with ΔLip1 compared to infection with the wt strain. Serum TNFα and IL-6 were increased on day three after infection with wt bacteria. In contrast, these cytokines were only marginally elevated in the serum of ΔLip1 infected compared to non infected control mice (figur 3.18). Most likely, the reduction in serum cytokines is a consequence of the decreased bacterial burden after infection with *L. monocytogenes* ΔLip1 compared to infection with the wt strain. This assumption is also supported by the fact that we did not detect changes in the quality of the immune response elicited by *L. monocytogenes* wt or ΔLip1, more specifically, the composition of cell populations in the blood and presence of immune cells in the spleen. The amount of lymphocytes in the blood dropped markedly in mice infected with either *L. monocytogenes* ΔLip1 or wt. In general, the amount of white blood cells, granulocytes and monocytes in the blood did not differ after infection with the two strains (figure 3.19). Also in the spleen, we did not observe a difference in numbers of the analysed immune cell populations (table 3.1).

To assess whether reduced *in vivo* virulence also occurs after a more natural route of infection, we administered *L. monocytogenes* via intragastric gavage. Interestingly, numbers of ΔLip1 compared to wt bacteria were significantly reduced in the intestine already 4 hours after infection (figure 3.22). Also three days after infection, we observed significantly less ΔLip1 than wt bacteria in small intestines and spleens of infected mice (figure 3.23).

In conclusion, we could characterize Lip1 as an important virulence factor of *L. monocytogenes*. However, the strong impairment in virulence observed *in vivo* could not be assigned to a defect in any *in vitro* cell culture model so far. As the reduction in ΔLip1 compared to wt bacteria occurs already a few hours after infection, it can be ruled out that the observed phenotype is due to an interference of Lip1 with adaptive immune responses. Rather, future experiments will focus on the ability of ΔLip1 bacteria to survive in host cells encountered early after infection. Although Lip1 is not needed for growth in BMDMs, it might be required for withstanding the enhanced bactericidal activities of granulocytes or activated macrophages.

Another future aim will be the identification of physiological substrates of Lip1. An elegant method for finding substrates of PTPs is substrate-trapping. This technique makes use of the stable intermediate formed between tyrosine phosphatases and their substrates. By mutating the catalytic cystein to serine, substrate release from the phosphatase is blocked and the enzyme-substrate interaction is stabilised. Subsequently, substrates can be identified by mass spectrometry. Finding physiolog-

ical substrates of Lip1 would be an essential step towards a complete understanding of Lip1 function *in vivo*.

Bacterial virulence factors provide important targets for antimicrobial drug development. Recently, specific inhibitors of the mycobacterial phosphatase MptpB have been identified. These compounds impair survival of *Mycobacterium tuberculosis* in macrophages and suggests a strong potential to be developed as drug candidates (Beresford et al., 2009). Also, the virulence factor Lip1 might be an interesting future drug target for the treatment of listeriosis.

5 Materials and Methods

5.1 Methods

5.1.1 Biochemical analysis

Phosphatase activity assay For testing Lip1 pH optimum, 1 µg purified protein was incubated with 10 mM *p*-nitrophenol phosphate (*p*NPP, Sigma, Catalogue # S0942) at 37 °C for 10 min in buffers adjusted to different pH. For buffers ranging from pH=4–6, 100 mM Citrate buffer with 1 mM EDTA was used. For buffers at pH=7–8, 100 mM Tris-HCl with 1 mM EDTA was used. After incubation, 4 N NaOH was added and pnp release was measured at OD_{405}. Temperature optimum was measured in 100 mM MES buffer, pH=6, using 0,5 µg protein and 10 mM *p*NPP at indicated temperatures.

To measure free amount of phosphate during dephosphorylation assays, a malachite green phosphate assay kit (Cayman Chemicals, Catalogue # 10009325) was used with a range of substrates: diC8-PtdIns3P (Catalogue # P-3008), diC8-PtdIns4P (Catalogue # P-4008), diC8-PtdIns5P (Catalogue # P-5008), diC8-PtdIns(3,4)P2 (Catalogue # P-3408), diC8-PtdIns(3,5)P2 (Catalogue # P-3508), diC8-PtdIns(4,5)P2 (Catalogue # P-4508), diC8-PtdIns(3,4,5)P3 (Catalogue # P-3908) (all obtained from Echelon), O-phospho-DL-serine (Catalogue # 79710), O-phospho-DL-threonine (Catalogue # 1003), O-phospho-L-tyrosine (Catalogue # 9405, all phosphorylated amino acids were obtained from Sigma). To measure activity towards phosphatidylinositol-phosphates 0,5 µg protein was incubated with 50 µM substrate for 10 minutes at 30 °C.

Western blot analysis Western blot analysis was performed as described (Kovarik et al., 1998), but fluorophore-linked secondary antibodies (Molecular Probes-Invitrogen (Lofer, Austria) and Rockland (Gilbertsville, PA)) and an Odyssey infrared imaging system (LI-COR Bioscience, Lincoln, NE) were used.

Antibodies for western blot analysis All primary antibodies were stored in PBS supplemented with 2 % BSA and 0,0 5% Sodium azide.
Rabbit polyclonal LLO antibody was (Diatheva, used 1 : 1500 (Catalogue # ANT0036)).
Mouse monoclonal GFP (B-2) antibody (Santa Cruz, used 1 : 200 (Catalogue # sc-9996)).
Mouse monoclonal panERK antibody (Beckton Dickinson, used 1 : 2000 (Catalogue # 610123)).
Rabbit polyclonal phosphoAKT (Ser473) antibody (Cell signalling, used 1 : 1000 (Catalogue # 9271)).
Antibody recognizing the bacterial ribosomal protein S9 was kindly provided by Isabella Moll (MFPL, University of Vienna, Austria).
Secondary anbibodies were diluted 1 : 20000 in PBS.
IRDye800 Conjugated anti-Rabbit IgG (Rockland, Catalogue # 611-132-1122).
IRDye800 Conjugated anti-Mouse IgG (Rockland, Catalogue # 610-143-121).
IRDye700 Conjugated anti-Rabbit IgG (Rockland Catalogue # 611-130-122).
Alexa Flour680 anti-Mouse IgG (Molecular probes Catalogue # A21058).

Colloidal Coomassie staining SDS-PAGE gels were fixed in Fixing solution for 30 minutes to 1 hour. Gels were washed two times with H_2O and stained with Colloidal Coomassie solution for about 3 hours. Gels were not destained afterwards.

5.1.2 Bacterial culture and cloning

Bacterial strains and growth in media *L. monocytogenes* LO28 wt (Kocks et al., 1992) and its deletion mutant strain (ΔLip1) were grown in Brain Heart infusion (BHi) (Difco) broth, on BHi or Oxford agar (Merck) plates. *Escherichia coli* DH5α or XL1BlueMRF′ were used as hosts for plasmid constructions. The strains were grown in Luria-Bertani medium (LB, either in liquid or on agar plates).

When required, LB medium for growth of *E. Coli* was supplemented with antibiotics to the following final concentration : ampicillin 100 µg/ml, chloramphenicol 15 µg/ml, tetracyclin 15 µg/ml, erythromycin 500 µg/ml, kanamycin 25 µg/ml.

When required, BHi medium for growth of *L. monocytogenes* was supplemented with chloramphenicol 3 µg/ml, erythromycin 3 µg/ml, kanamycin 300 µg/ml.

Liquid cultures were grown at 37 °C under shaking.

Transformation of *E. coli* For preparation of competent *E. coli*, overnight cultures were reinoculated 1 : 100 in 100 ml LB. At OD=0,35–0,4, cells were centrifuged at 5000 g for 15 minutes at 4 °C. The pellet was resuspended in 10 ml 100 mM $CaCl_2$ and kept on ice for 20 minutes. After centrifugation at 5000 g for 15 minutes at 4 °C, bacteria were resuspended in 2 ml of 100 mM $CaCl_2$ containing 15 % glycerol. Aliquots of 100 µl were stored at −80 °C.

For transformation of competent *E.coli*, bacteria were incubated with plasmid DNA on ice for 15–30 minutes and briefly heat shocked for 1 min at 42 °C. After a 2 minutes incubation step on ice, 1 ml LB was added and bacteria were incubated for 1 hour at 37 °C before plating on LB agar plates containing appropriate antibiotics.

Transformation of *L. monocytogenes* *Listeria* were transformed by electroporation of penicillin-treated cells. For preparation of competent *Listeria*, 2 ml of an overnight culture were reinoculated in 100 ml BHi supplemented with 0,5 M sucrose. At OD=0,2, penicillin G (or ampicillin) was added to the culture to a final concentration of 10 µg/ml and the culture was incubated for 2 hours. Then, the culture was centrifuged at 6000 g for 10 minutes at 4 °C. The pellet was resuspended in 45 ml Hepes/sucrose (1 mM Hepes pH=7,0 with 0,5 M sucrose). The pellet was again resuspended in 45 ml Hepes/sucrose and centrifuged as above. Then, the pellet was resuspended in 400 µl Hepes/sucrose and aliquots of 100 µl were stored at −80 °C.

DNA was added to competent *Listeria* and electroporated with a Gene Pulser (Biorad; parameters used: 1,5 kV, 400 ohms, 25 µFD; with a pulse duration of about 5 ms). After addition of 1 ml BHi supplemented with 0,5 M sucrose, the culture was incubated for 1 hour at 37 °C without shaking and plated on BHi plates containing appropriate antibiotics.

Overexpression and purification of Lip1 For high-level expression of a N-terminally His_6-tagged version, Lip1 was cloned into pQE31 (QIAGEN). Therefore, PCR on genomic DNA of *L. monocytogenes* (LO28) was performed using primers lmo1800_5' and lmo1800_3' (includes STOP codon of *lip1*). After restriction with the appropriate enzymes, the PCR fragment was inserted into pQE31 resulting in the plasmid pQE31-Lip1. pQE31-Lip1 was transformed into *E. coli* BL1Blue creating the strain BL1Blue-pQE31-Lip1. Primer sequences are listed in table 5.1.

BL1Blue-pQE31-Lip1 bacteria were grown over night in LB supplemented with ampicillin and tetracyclin and reinoculated 1 : 100 in 1 ml LB with Amp and Tet the following day. At OD=0,5 after approximately 3 hours of growth, Lip1 overexpression

was induced by addition of 1mM IPTG. Bacteria were centrifuged for 15 min at 6000 g and washed once with purification buffer. Purification with Ni-NTA beads was performed according to the manufacturer's protocol. The bacterial pellet was washed with 20 ml purification buffer supplemented with 10 mM imidazol, and the pellet was resuspended in purification buffer supplemented with 10 mM imidazol, 10 mg/ml lysozyme and 5 µl DNAseI (Fermentas). After incubation for 20 min at 4 °C on a rotating wheel, cells were lysed by sonication. The supernatant was incubated with Ni-NTA beads for 1 h at 4 °C and loaded on a purification resin (PerfectPrep Endofree Columns CB5-10, Qiagen). The resin was washed once with buffer alone and 2 times with purification buffer with increasing amounts of imidazole (10 mM, 20 mM). Protein was eluted 4 times with 1 ml purification buffer with 150 mM imidazole. Protein concentration was determined by the Bradford method (Kruger, 1994). To check purity of fractions, samples were analysed by SDS-PAGE. The acrylamide gel was stained with colloidal coomassie staining. The four eluted fractions were pooled and stored in purification buffer at −20 °C.

Chromosomal deletion of *lip1* The temperature sensitive plasmid pEC84 was used to delete *lip1* from *L. monocytogenes* LO28 genome. 1002 base upstream and 1001 downstream regions of the *lip1* gene were amplified by PCR using primers KOUP1 and KOUP2 and primers KODW1 and KODW2, respectively, from *L. monocytogenes* genomic DNA. Primer sequences are listed in table 5.1. After restriction with the appropriate enzymes, the two PCR fragments were ligated and cloned into the vector pEC84 resulting in the plasmid pEC84-ΔLip1. pEC84 contains a thermosensitve origin of replication that is active at 28 °C, but no at 37 °C. The resulting plasmid was introduced into *L. monocytogenes* LO28 by electroporation and chromosomal integration of the plasmid was gained by growing bacteria at 37 °C in the presence of kanamycin. The excision event was achieved by repeated passage of bacteria at the replication permissive temperature 28 °C, in absence of kanamycin. The chromosomal deletion of *lip1* in *L. monocytogenes* LO28, resulting in the strain *L. monocytogenes* LO28 ΔLip1, was verified by PCR. Primer sequences are listed in table 5.1. The plasmid pEC84 was kindly provided by Emmanuelle Charpentier, Umea University, Molecular Infection Medicine Sweden (MIMS).

Expression of Lip1-GFP fusion protein For expression of a Lip1-GFP fusion, an in frame fusion of Lip1 with GFP was expressed under the control of the *Listeria* promotor P*dlt* in the pAM401 backbone, a widely used shuttle vector (Fujimoto

and Ike, 2001). To amplify the P*dlt* promotor, as described (Abachin et al., 2002), the primers PDLT5 (adapted from oligo EA20, (Abachin et al., 2002)) and PDLT3 were used. The *lip1* gene, including the RBS, was amplified by PCR from genomic *L. monocytogenes* DNA, using oligos RBS59-5 and RBSFULL-3. The GFPmut2 gene was amplified using oligos GFP2-5 and GFP2-3. Fragments were ligated and inserted into pAM401, resulting in the plasmid designated pAM401-Lip1-GFP. As a control, GFP was cloned into pAM401, controlled by the same promotor region. The resulting plasmid was designated pAM401-GFP. Primer sequences are listed in table 5.1.

Fractionation of bacterial cells Stationary-phase cultures of *L. monocytogenes* pAM401, pAM401-GFP or pAM401-Lip1-GFP were grown over night. Concentration of bacteria was measured at OD_{600}. Same numbers of bacteria of each strain (40×10^9) were centrifuged at 6000 g for 15 minutes. The supernatant was passed through a 0,22 µM filter , protein was precipitated using 0,5 % TCA and 0,02 % DOC and washed with acetone. The pellet was resuspended in SDS sample buffer. The bacterial pellet was washed with PBS, resuspended in PBS adjusted to a final concentration of 10 mM Vanadat, 1 M DTT, 1× Proteinaseinhibitormix (Roche Diagnostics) and lysed by sonication. The solution was centrifuged at 15000 g for 30 min at 4 °C. Both, the supernatant, containing cytosolic protein, and the pellet, containing membrane proteins, were used for western blot analysis.

5.1.3 *In vitro* experiments

Cells RAW264.7 cells, CMT-93 and L2 cells were cultured in DMEM (Gibco, Invitrogen) supplemented with 10 % FCS (Gibco, Invitrogen). Ptk2 were cultured in MEM (Gibco, Invitrogen) supplemented with 20 % FCS (Gibco, Invitrogen) and 1 % L-Glutamine/GlutaMAX-I (GIBCO, Catalogue # 35050-038). Bone marrow-derived macrophages were obtained by culture of bone marrow of 7–10 week old mice and cultured in DMEM (Gibco, Invitrogen) supplemented with 10 % FCS (Gibco, Invitrogen) and L929 cell-derived CSF-1 as described previously (Baccarini et al., 1985). Cells were incubated at 37 °C in a humidified 5 %CO_2 atmosphere. All mice were in a C57BL/6 genetic background.

Infection of cells Cells were seeded in antibiotic free medium and grown over night. The OD_{600} of overnight cultures was measured and the number of bacteria was determined by use of the following equation: $OD_{600}1 = 2 \times 10^9\, bacteria/ml$. Cells

were infected with *L. monocytogenes* wt or ΔLip1 bacteria at the indicated MOI. Using BMDMs, extracellular bacteria were killed one hour after infection by addition of gentamicin containing medium (final concentration 50 µg/ml). Another hour later, medium was changed to a concentration of gentamicin of 10 µg/ml. When infecting CMT-93, L2 or Ptk-2 cells, medium was changed 2 hours after infection to medium containing 50 µg/ml gentamicin and one hour later to medium containing 50 µg/ml gentamicin.

Preparation of whole cell protein extracts Cells were washed once with cold PBS and scraped in 80 µl Frackelton buffer. After centrifugation for 5 min at 13000 g at 4 °C, the supernatant was combined with 30 µl SDS sample buffer, incubated at 95 °C for 10 min and subjected to western blot analysis.

Measurement of cell death BMDMs or CMT-93 cel, afterls were infected with *L. monocytogenes* LO28 wt and ΔLip1 at different multiplicities of infection (MOIs). Extracellular bacteria were killed with gentamicin-containing medium 1 hour after infection of BMDMs or 2 hours after infection of CMT-93 cells (final concentration 50 µg/ml).

For determining cell death by LDH release, medium was changed to medium containing 10 µg/ml gentamicin and 3 % FCS another 60 minutes later. Cells were incubated for a minimum of 24 hours. Release of Lactate dehydrogenase (LDH) was determined using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI, Catalogue # G1780) according to manufacturer's instructions.

Alternatively, cell death was measured by Pi staining. Culture supernatants containing cells in suspension were collected and adherent cells were detached with citrate buffer. Cells were washed once with sterile PBS and stained with propidium iodide at a final concentration of 5 µg/ml. Percentage of Pi positive cells was determined by flow cytometry with a FACSCalibur (Becton Dickinson). Data were acquired and analysed with the help of CellQuest or FlowJo software.

Determination of colony forming units *in vitro* BMDMs were infected at an MOI of 10. As described above, extracellular bacteria were killed with gentamicin containing medium 1 and 2 h after infection. At different time points, extracellular bacteria were removed by washing twice with PBS and cells were lysed with distilled water. Serial dilutions of lysates were plated on BHi agar plates and number of intracellular bacteria were determined by counting colony forming units (cfus).

Phagosomal escape assay Phagsosomal escape assay was performed as described (Reutterer et al., 2008). 3×10^5 RAW264.7 cells were seeded on coverslips in six-well dishes and infected with CFSE-labelled *L. monocytogenes* LO28 wt or ΔLip1 at an MOI of 10. Bacteria were stained with 10 µM CFSE in PBS for 20 minutes at 37°C under shaking. 3 hours after infection, cells were washed twice with PBS and fixed with 4 % para-formaldehyde in PBS for 10 min at RT. Afer two PBS washing steps, cells were permeabilized with 0,2 % Triton X-100 in PBS for 10 min at RT. Cells were again washed twice with PBS and incubated in $6,6 \times 10^{-6}\,M$ Phalloidin-Alexa 594 (Molecular probes, Invitrogen) in PBS for 40 min at RT. After two PBS washing steps, cells were counterstained with DAPI (final concentration 250 ng/ml). Coverslips were mounted on slides using Fluorescent Mounting Medium (Dako; Glostrup, Denmark, Catalogue # S3023). Samples were analysed with a Zeiss Axio Imager Fluorescence microscope.

Plaque assay Plaque assay in L2 mouse fibroblast cell line was performed as described (Marquis, 2006). 3×10^6 L2 cells were seeded in six-well plates and incubated for 40–48 hours. Cells were infected at different MOIs and 2 hours after infection, cells were washed three time with sterile PBS and overlayed with L2 cell overlay (see 5.2). 3 days post infection plaques were visualized by overlay with L2 staining overlay (see 5.2). Plates were scanned with AlphaImager (Biorad) using white transillumination light. The plaque diameter (pixel) was measured using the measure tool of GIMP 2.6 software. Due to the irregular shape of some plaques, the diameter was taken from left to right as well as from the top to bottom, and the mean diameter was used for calculating the relative plaque area.

Localization of Akt-PH-GFP in infected Ptk2 cells This assay was performed as described (Sidhu et al., 2005). 3×10^6 Ptk2 cells were seeded on coverslips in six-well dished and incubated for 40–48 hours. Cells were transfected with 1,2 µg Plasmid encoding Akt-PH-GFP using 10 µg/ml Lipofectamin reagent (Invitrogen, Catalogue # 50470) and 11 µg/ml Plus Reagent (Invitrogen, Catalogue # 10964-021) for 6 hours. 48 hours after transfection, cells were infected. 2 hours after infection, extracellular bacteria were killed by addition of gentamicin containing medium 2 and 3 h after infection. 6 hours after infection, cells were fixed with 4 % para-formaldehyde and permeabilized with 2 % BSA in PBS. Cells were stained with a 1 : 100 dilution of Rb pAb to Listeria monocytogenes (Abcam, Catalogue # ab35132-1) in Antibody Diluent (Dako, Glostrup, Denmark, Catalogue # S3022) for 1 hour at room tem-

perature or at 4 °C over night. A secondary Alexa Fluor594 chiken anti-rabbit IgG antibody (Molecular probes Catalogue # A-21442) was diluted 1 : 1000 in PBS and applied for 1 hour. Coverslips were mounted on slides using Fluorescent Mounting Medium (Dako; Glostrup, Denmark, Catalogue # S3023).

5.1.4 *In vivo* experiments

Ethics Statement All animal experiments were discussed and approved by the University of Veterinary Medicine of Vienna institutional ethics committee and carried out in accordance with protocols approved by the Austrian law (GZ 680 205/67-BrGt/2003).

Mouse infection studies To omit that *in vitro* growth may reduce bacterial virulence, the *L. monocytogenes* LO28 wt or ΔLip1 strains were passaged *in vivo*. Both strains were inoculated into 129/SV mice and recovered from livers of infected mice. Bacteria were prepared for infection as described (Stockinger et al., 2009). For infection, *L. monocytogenes* LO28 or Δlmo1800 were washed with PBS (endotoxin free, Sigma) and injected into the peritoneum of 8–10 week old mice. Alternatively, mice were infected with 2×10^9 cfu via intragastric gavage. The infectious dose was controlled by plating serial dilutions on Oxford agar plates. Survival of mice was monitored for 10 days and displayed as Kaplan-Meier plots. For determination of bacterial loads of liver spleen and intestine, mice were killed at the indicated time points. Respective organs were isolated, homogenized (Polytron system PT 2100 and dispersing aggregate PT-DA 12/2 EC-D, Kinematica, Switzerland) in PBS, serial dilutions were plated on Oxford agar plates and incubated at 37 °C for 48 h. Intestines were thoroughly washed with PBS before homogenization.

Haematology analysis Mice were infected *i. p.* with *L. monocytogenes* and blood was collected from the retroorbital sinus four days after infection. Blood was immediately transferred to EDTA coated tubes (MiniCollect EDTA, greiner bio-one, Catalogue # 450476) to omit coagulation and analysed with a Vet hematology analyzer (A. menarini diagnostics, Netherlands).

ELISA Blood was collected from the retroorbital sinus of mice. Blood was incubated at room temperature for approximately 10 to 15 minutes to allow coagulation, followed by centrifugation for 10 minutes at 10000 g. Serum was stored at −80 °C and cytokine levels were measured by ELISA using Mouse TNF-alpha DuoSet (R&D

Systems, Catalogue # DY410) or Mouse IL-6 DuoSet (R&D Systems, Catalogue # DY106) following manufacturer's recommendations. IFNγ levels were measured using the capture antibody (1 : 1000 dilution of anti-mouse IFNγ clone XMG1.2, eBioscience, Catalogue # 14-7311-85) and the detection antibody (1 : 500 dilution of anti-mouse IFNγ biotin conjugated clone R4-6A2, eBioscience, Catalogue # 13-7312-85) according to manufacturer's recommendations. For the standard curve, dilutions of mouse IFNγ (eBioscience, Catalogue # 39-8311-60) were used.

Characterization of splenic cell populations Spleens were isolated at the indicated time point and single cell suspensions were prepared using cell strainers with a 70 µm nylon mesh (cell strainer, BD bioscience, Catalogue # 352350). Erythrocytes were lysed with red blood cell lysis buffer for 90 seconds and remaining cells were washed with PBS. Cells were incubated with antiFC γIII/II antibody for 10 minutes at room temperature and washed with PBS. Cells were stained with 1 : 200 dilutions of indicated fluorescent antibodies for 30 minutes on ice. Cells were washed with PBS and analysed with a FACSCalibur (Becton Dickinson). Data were acquired and analysed with the help of CellQuest or FlowJo software.
Anti-mouse CD16/CD32 (Mouse Fc block) (BD Pharmingen, Catalogue # 553142)
FITC anti-mouse CD19 (BD Pharmingen, Catalogue # 557398)
FITC anti-mouse CD4 (BD Pharmingen, Catalogue # 553047)
FITC anti-mouse Cd11b (BD Pharmingen, Catalogue # 553310)
FITC anti-mouse Ly-6G (BD Pharmingen, Catalogue # 551460)
FITC anti-mouse CD8a (BD Pharmingen, Catalogue # 553030)
FITC anti-mouse CD69 (BD Pharmingen, Catalogue # 557392)
PE anti-mouse CD45R/B220 (BD Pharmingen, Catalogue # 553090)
PE anti-mouse CD8a (BD Pharmingen, Catalogue # 553032)
PE anti-mouse I-A^b (BD Pharmingen, Catalogue # 553552)
PE anti-mouse NK-1.1 (eBioscience, Catalogue # 12-5941-82)
PE-Cy7 anti-mouse CD11b (BD Pharmingen, Catalogue # 552850)
Pe-Cy7 anti-mouse CD11b (BD Pharmingen, Catalogue # 550993)
PerCP-Cy5.5 anti-mouse Ly-6C (eBioscience, Catalogue # 45-5932-82)
PerCP-Cy5.5 anti-mouse CD25 (BD Pharmingen, Catalogue # 551071)
APC anti-mouse CD11b (BD Pharmingen, Catalogue # 550261)
APC anti-mouse F4/80 (eBioscience, Catalogue # 17-4801-82)
APC anti-mouse CD3e (BD Pharmingen, Catalogue # 553066)

Primer Name	Primer Sequence
Primers for Lip1 overexpression	
lmo1800_5'	CGGGATCCAGAAGCAAATGTTAAAACCGAGC (*Bam*HI site)
lmo1800_3'	CCCAAGCTTTTAATAAAGATATGCTTTTTTTAG (*Hind*III site)
Primers for chromosomal *lip1* deletion	
KOUP1	AAAACTGCAGCCAAGTAAGCCCGGTCGAAATC (*Pst*I)
KOUP2	CGGGATCCTCTTTCTCCTCCTTTTTGATTG (*Bam*HI)
KODW1	CGGGATCCAAAAGTAAGCCCTTGAAAA AGG (*Bam*HI)
KODW2	GGGGTACCGTAGATACACTACCAACTCCGAC (*Kpn*I)
Primers for checking Lip1 knockout	
#2	GTCCATCCGTTGTTTTGTATCCG
#3	GCAAAATATGGTTCGATGGACAAC
#6	CGGGATCCAGAAGCAAATGTTAAAACCGAG
#7	CCCAAGCTTTTAATAAAGATATGCTTTTTTTAG
#8 (=KOUP1)	AAAACTGCAGCCAAGTAAGCCCGGTCGAAATC
#11	GGGGTACCGTAGATACACTACCAACTCCGA
#12	GATATGCCGGTATTTTCCCTAGG
#13	CTAGCAAATCAATTGTCAAAG TG
Primers for expression of lmo1800-GFP fusion	
PDLT5	AAAACTGCAGCCCGTTCCAAGACCCACAAC (*Pst*I)
PDLT3	CGGGATCCGTTGTAAATGAATTTCGCACC (*Bam*HI)
RBS59-5	CGGGATCCAGCAATCAAAAAGGAGGAGAAAG (*Bam*HI)
RBSFULL-3	AATTGGGAGCTCATAAAGATATGCTTTTTTTAGTTG (*Sac*I)

Table 5.1: Primer list. Restriction sites are underlined.

5.2 Media and solutions

General

10 × PBS

81 mM	Na_2HPO_4 x $2H_2O$
25 mM	KCl
1,4 M	NaCl
15 mM	KH_2PO_4
	pH=7,3

Cell culture

1000 × Penicillin & Streptomycin

0,6 g	Penicillin
1 g	Streptomycin
10 ml	H_2O

Red blood cell lysis buffer

0,0372 g/l	EDTA
8,29 g/l	NH_4Cl
1 g/l	KHCO3

2 × DMEM

24 g/l	DMEM (Gibco D-MEM:F-12 (1 : 1))
10 g/l	BSA
2,4 g/l	$NaHCO_3$

L2 cell overlay

add to 2× DMEM before use:

20 %	FCS
20 µg/ml	Gentamicin
2 %	Glutamine

mix medium 1 : 1 with 1,4 % Agarose

L2 staining overlay

add to 2× DMEM before use:

20 %	FCS
20 µg/ml	Gentamicin
2 %	Glutamine
35 µl/10 ml	1N HCl
600 µl/10 ml	Neutral red solution (Sigma)

mix medium 1 : 1 with 1,4 % Agarose

10 × Citrate buffer

100,7 g/l	Potassium chloride (1,35 M)
44 g/l	Sodium citrate (0,15 M)

Bacterial cultures

Brain Heart Infusion medium

37 g/l	BHi powder

Brain Heart Infusion Agar

37 g/l	BHi powder
1 %	Agar

LB

10 g/l	Trypton
5 g/l	Yeast extract
10 g/l	NaCl

LB Agar

10 g/l	Trypton
5 g/l	Yeast extract
10 g/l	NaCl
1 %	Agar

Oxford Agar

58,5 g/l	Oxford-Listeria-Selective Agar (MERCK)
10 ml/l	Oxford-Listeria-Selective-Supplement (MERCK)

RNA/DNA

1,5 × RNA loading buffer

80 %	Formamide
0,01 M	EDTA
0,002 %	Bromphenol blue

6 × DNA loading buffer

0,25 %(w/v)	Bromphenolblue
0,25 %(w/v)	Xylene cyanol FF
30 %(v/v)	Glycerol in H_2O
	store at 4 ℃

50 × TAE buffer

2 M	Tris
1 M	Acetic acid
0,05 M	EDTA

25 × RNA running buffer

0,25 M	Na_2HPO_4
0,25 M	NaH_2PO_4

Protein

Frackelton buffer

10 mM	Tris
50 mM	NaCl
30 mM	$NaPP_i$
1 %	Triton X-100
	pH=7–7,5, store at 4 °C

add to Frackelton buffer before use

40 µl/ml	25× Protease inhibitor mix in H_2O (Roche Diagnostics)
10 µl/ml	100 mM PMSF (final: 1 mM; stock solution in 2-propanol)
10 µl/ml	10 mM Sodium vanadate (final: 100 µM)
1 µl/ml	1 M DTT (final: 1 mM)

10 mM sodium vanadate

18 mM	Na_3O_4V
20 μl	$HCl : H_2O$ in the ratio 1 : 1

boil the solution in the water bath at 85 °C
until the yellow colour of the solution disappears
store at −20 °C

10 % (w/v) SDS

10 g	SDS
100 ml	H_2O

7,5 % separating gel

2 ml	40 % Acrylamide/Bisacrylamide Solution
2 ml	Tris/HCl pH=8,8
4 ml	H_2O
80 µl	10 % SDS
24 µl	TEMED
24 µl	10 % APS

4 % collection gel

0,5 ml	40 % Acrylamide/Bisacrylamide Solution
1 ml	Tris/Hcl pH=6,8
2,5 ml	H_2O
40 µl	10 % SDS
12 µl	TEMED
12 µl	10 % APS

10 × running buffer for SDS page

0,25 M	Tris
1,92 M	Glycine
1 %(w/v)	SDS

Ponceau staining solution

0,2 % (w/v)	Ponceau S
3 % (w/v)	Trichloratic acid (TCA)

SDS sample buffer

9,5 ml	H_2O
6 ml	0,5 M Tris-HCl, pH=6,8
2,5 ml	Glycerol
4 ml	10 % SDS
2 ml	2-β-mercaptoethanol
0,5 % (w/v)	Bromphenolblue
	store at −20 ℃

Anode buffer I

36,34 g/l	Tris
20 %	Methanol
	pH=10,4

Anode buffer II

0,302 g/l	Tris
20 %	Methanol
	pH=10,4

Cathode buffer

5,25 g/l	6-Aminocaproic Acid
20 %	Methanol
0,01 %	SDS

10 × TBST

12 g/l	Tris
88 g/l	NaCl

5 ml	Tween

Stripping buffer

25 g/l	Glycine
8,79 g/l	NaCl
0,5 ml	Tween 20
	pH=2,8

Colloidal Coomassie staining

Fixing solution

40 %	Ethanol (416 ml per litre)
10 %	Acetic acid (98 ml conc. Ch_3COOH per litre)

Coomassie Brilliant Blue stock

5 g	Coomassie Brilliant Blue G-250
100 ml	H_2O
	store at RT

Colloidal Coomassie Dye stock

50 g	Ammonium sulphate
6 ml	85 % phosphoric acid
490 ml	H_2O
10 ml	Coomassie Brilliant Blue stock
	store at RT

Colloidal Coomassie solution

200 ml	Colloidal Coomassie Dye stock
50 ml	methanol
	prepare freshly

FACS

FACS buffer

0,2 %	BSA
0,02 %	Sodium azide
	in PBS

Trypan blue solution

1 mg/ml	Trypan blue
	in Citrate buffer, pH=4

ELISA

Wash buffer

0,05 %	Tween in PBS, pH=7,2–7,4

Reagent diluent

1 %	BSA in PBS, pH=7,2-7,4 in PBS, pH=7,2–7,4

TMB Substrate Reagent (BD OptEIA, Catalogue # 555214)

1 part	Reagent A
1 part	Reagent B

Stop Solution

2 N	H_2SO_4

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Curriculum vitae

Personal data

Name	Renate Kastner
Date of birth	23. November 1981
Place of birth	St. Pölten
Nationality	Austria

Education

1988–1992	Elementary school, Melk
1992–2000	Comprehensive school, Stiftsgymnasium Melk
Mai 2000	A-Levels ("Matura")

University studies

2000–2003	Basic Studies of Biology at the University of Vienna
2003–2007	Studies of Genetics and Microbiology at the University of Vienna
Oct 2004–Feb 2005	Studies at the University of Antwerp (Erasmus Programme)
Dec 2005–Mar 2007	Diploma thesis at the University of Vienna in the group of Prof. Dr. Thomas Decker Title: "Response of dendritic cells to intracellular bacterial infection"
Apr 2007–current	PhD thesis at the University of Vienna in the group of Prof. Dr. Thomas Decker Title: "Characterization of the phosphatase Lip1, a novel determinant of *L. monocytogenes* virulence"

Scientific Meetings

Jun 17–19, 2009	YSA PhD Symposium of the Medical University of Vienna, Vienna, Austria. Poster presentation
Apr 23–26, 2009	Semmering Vaccine Symposium 2009, Baden, Austria. Poster presentation
Nov 18th, 2008	VBC Student Symposium II, Vienna, Austria. Oral presentation
Sep 3–6, 2008	ÖGAI, Joint annual meeting of Immunology of the Austrian and German Societies, Vienna, Austria. Poster presentation
Sep 13–15, 2007	EMDS Innsbruck, 21st Annual Meeting, Innsbruck, Austria. Poster presentation
Aug 27–31, 2006	6th Joint Meeting of the International Cytokine Society, the International Society for Interferon and Cytokine Research and the European Cytokine Society, Vienna, Austria.

Publications

Characterization of the interferon-producing cell in mice infected with *Listeria monocytogenes*. Stockinger S, Kastner R*, Kernbauer E*, Pilz A, Westermayer S, Reutterer B, Soulat D, Stengl G, Vogl C, Frenz T, Waibler Z, Taniguchi T, Rülicke T, Kalinke U, Müller M, Decker T.. PLoS Pathog. 2009 Mar;5(3):e1000355. (Authors marked with * contributed equally to this study.)

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Lebenslauf

Persönliche Daten

Name	Renate Kastner
Geburtsdatum	23. November 1981
Geburtsort	St. Pölten
Nationalität	Österreich

Ausbildung

1988–1992	Volksschule Melk
1992–2000	Stiftsgymnasium Melk
Mai 2000	Matura am Stiftsgymnasium Melk

Universitätsstudium

2000–2003	Biologiestudium, Universität Wien
2003–2007	Biologiestudium, Studienzweig Genetik und Mikrobiologie, Universität Wien
Okt. 2004–Feb. 2005	Studium an der Universität Antwerpen, Belgien (Erasmus Programm)
Dez. 2005–März 2007	Diplomarbeit an der Universität Wien in der Gruppe von Prof. Dr. Thomas Decker Titel: "Response of dendritic cells to intracellular bacterial infection"
Seit Apr. 2007	Doktorarbeit an der Universität Wien in der Gruppe von Prof. Dr. Thomas Decker Titel: "Characterization of the phosphatase Lip1, a novel determinant of *L. monocytogenes* virulence"

Konferenzen

17.–19. Juni, 2009	YSA PhD Symposium of the Medical University of Vienna, Wien, Österreich. Posterpräsentation
23.–26. Apr., 2009	Semmering Vaccine Symposium 2009, Baden, Österreich. Posterpräsentation
18. Nov., 2008	VBC Student Symposium II, Wien, Österreich. Vortrag
3.–6. Sept., 2008	ÖGAI, Joint annual meeting of Immunology of the Austrian and German Societies, Wien, Österreich. Posterpräsentation
13.–15. Sept., 2007	EMDS Innsbruck, 21st Annual Meeting, Innsbruck, Österreich. Posterpräsentation
27.–31. Aug., 2006	6th Joint Meeting of the International Cytokine Society, the International Society for Interferon and Cytokine Research and the European Cytokine Society, Wien, Österreich.

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