Legionella pneumophila S1P-lyase targets host sphingolipid metabolism and restrains autophagy

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Autophagy is an essential component of innate immunity, enabling the detection and elimination of intracellular pathogens. Legionella pneumophila, an intracellular pathogen that can cause a severe pneumonia in humans, is able to modulate autophagy through the action of effector proteins that are translocated into the host cell by the pathogen’s Dot/Icm type IV secretion system. Many of these effectors share structural and sequence similarity with eukaryotic proteins. Indeed, phylogenetic analyses have indicated their acquisition by horizontal gene transfer from a eukaryotic organism (3, 4). One of these proteins exhibits a high degree of homology to eukaryotic SPLs (sphingosine-1-phosphate lyase (SPL)). We determined the majority of its effectors are encoded by eukaryotic-like genes acquired through horizontal gene transfer. We determined the crystal structure of one of these effectors, sphingosine-1-phosphate lyase (LpSpl), and show that it has high similarity with its eukaryotic homologue. We demonstrate that LpSpl possesses SLP lyase activity that was abrogated by mutation of the catalytic site residues. L. pneumophila triggers the reduction of several sphingolipids critical for macrophage function in an LpSpl-dependent and -independent manner. LpSpl activity alone was sufficient to prevent an increase in sphingosine levels in infected host cells and to inhibit autophagy during macrophage infection. LpSpl was required for efficient infection of A/J mice, highlighting an important virulence role for this effector. Thus, we have uncovered a previously unidentified mechanism used by intracellular pathogens to inhibit autophagy, namely the disruption of host sphingolipid biosynthesis.

Legionella pneumophila | sphingosine-1-phosphate lyase | autophagy | sphingolipids | virulence

The Gram-negative intracellular bacterium Legionella pneumophila is an opportunistic human pathogen responsible for Legionnaires’ disease. The bacteria are naturally found in freshwater systems where they replicate within protozoan hosts (1). It is thought that the adaptation to replication within amoebas has equipped L. pneumophila with the factors required to replicate successfully within human macrophages following opportunistic infection (2). Through genome sequencing, we have discovered that L. pneumophila encodes a high number and variety of proteins similar in sequence to eukaryotic proteins that are never or rarely found in other prokaryotic genomes (3). Subsequent phylogenetic analyses have suggested that many of these proteins were acquired by horizontal gene transfer (3, 4). One of these proteins exhibits a high degree of similarity to eukaryotic sphingosine-1-phosphate lyase (SPL). The L. pneumophila SPL homolog (LpSpl encoded by gene lpp2128, lpp2176, or lpp22) is conserved in all L. pneumophila strains sequenced to date, but absent from Legionella longbeachae (SI Appendix, Table S1). Phylogenetic analysis of SPL sequences showed that the L. pneumophila spl gene was most likely acquired early during evolution by horizontal gene transfer from a protozoan organism (4, 5). With the increase in genome sequences available, SPL homologs have now been identified in other bacteria such as Roseofexus, Myxococcus, Stigmatella, and Symbiobacterium (6).

Eukaryotic SPL tightly regulates intracellular levels of sphingosine-1-phosphate (S1P). Sphingolipids are ubiquitous building blocks of eukaryotic cell membranes, and the sphingolipid metabolites ceramide, ceramide-1-phosphate, sphingosine, and S1P are key signaling molecules that regulate many cellular processes important in immunity, inflammation, infection, and cancer (7). SPL uses pyridoxal 5'-phosphate (PLP) as a cofactor to reversibly degrade S1P into phosphoethanolamine and hexadecanol.
Structural analysis of SPL from *Symbiobacterium thermophilum* (SISPL) and *Saccharomyces cerevisiae* (Dpl1p) identified the residues involved in activity and proposed a mechanism for S1P cleavage (8). Structural elucidation of human SPL (hSPL) showed that the yeast and the human enzymes adopt largely the same structures (9).

Recent work suggests a possible link between the role of lipids in the regulation of apoptosis and autophagy (10). Autophagy is an evolutionary conserved pathway controlling the quality and quantity of eukaryotic organelles and the cytoplasmic biomass. Double-membrane vesicles called “autophagosomes” engulf nonfunctional or damaged cellular components and deliver them to lysosomes, where the content is degraded (11). Furthermore, it has been shown that autophagy acts as a cell-autonomous defense mechanism against intracellular bacteria, contributing to antibacterial immunity by regulating the inflammatory immune response and routing engulfed intracellular bacteria toward lysosomal degradation (12, 13). Many pathogenic are able to evade autophagy, although the molecular mechanisms at play remain largely uncharacterized (14–20). Among these pathogens *L. pneumophila* is known to escape cellular attack by blocking autophagy defenses (21).

Although it has been reported that *L. pneumophila* interferes with the autophagy machinery and with host factors that play a role in the cellular defense (22), only two *L. pneumophila* proteins that target the autophagy machinery, RavZ and LegA9, have been identified (23, 24). The bacterial effector RavZ is a cysteine protease that cleaves and causes delipidation of the autophagosome protein LC3, thereby dampening the autophagy process (23, 25). Interestingly, RavZ is not present in all strains of *L. pneumophila*, but in all strains tested, *L. pneumophila*-mediated disruption of autophagosomal maturation delays and/or prevents the Legionella-containing vacuole (LCV) from fusing with lysosomes (26). Thus, *L. pneumophila* likely also employs other mechanisms to restrain autophagy, and LpSPL, present in all strains sequenced to date, is a good candidate. A first characterization of LpSPL in strain JR32 showed that this protein is secreted by the Dot/Lcm type IV secretion system and that it complements the sphingosine-sensitive phenotype of a *S. cerevisiae* SPL-null mutant, suggesting that it indeed has SPL activity. However, no functional analyses were reported (5). Thus, we aimed to understand the function of this SPL homolog in *L. pneumophila*.

Here we report the crystal structure of LpSPL, identify the active site, and show that LpSPL indeed confers SPL lyase activity to *L. pneumophila*. Its activity during infection leads to the specific reduction of cellular levels of sphingosine, whereas other sphingolipids are down-regulated in a LpSPL-independent manner. Furthermore, we confirm that LpSPL plays a role in delaying the autophagy response of the cell during infection and is important for infection in a pulmonary mouse model of Legionnaires’ disease.

**Results and Discussion**

**Crystal Structure of LpSPL Reveals a Dimeric Multidomain Architecture.** hSPL is a single-pass type I integral membrane protein. The region N-terminal to the transmembrane helix is located in the lumen of the endoplasmic reticulum (ER), whereas the C-terminal region faces the cytosol and contains a large enzymatically active domain (27). We were successful in obtaining well-diffracting crystals of the cytoplasmic fragment of LpSPL spanning residues 56 to the C terminus (*Lp* SPl56–605). The *Lp* SPl56–605 structure was solved by single-wavelength anomalous dispersion phasing, using a Se-Met–derivatized protein crystal (8) (SI Appendix, Table S2). Traceable electron density in the asymmetric unit was observed for two polyepptide chains; however, missing or poor electron density was found in regions throughout each of the chains (Fig. 1A). The overall structure of the LpSPL protomer could be subdivided into three main regions: an N-terminal region (*Lp*SPl107–108–57), which was missing in our model due to poor electron density; a large cofactor-binding domain (*Lp*SPl158–430) named the “core” domain, which was largely complete in our structure; and a small C-terminal domain (*Lp*SPl342–527–528). We did not observe electron density for the N- and C-terminal extremities of *Lp*SPl56–605, including the above-mentioned residues 56–106 and residues 533–605. The basic architecture of LpSPL was typical of type 1 PLP-dependent enzymes (28), and the presence of an N-terminal region clusters LpSPL with the other characterized SPL enzymes (Fig. 1 B and C; *SI Appendix, S2A and S2B*). A single chain of LpSPL had a boomerang-like shape, with the N-terminal region and cofactor-binding domains at either end and the C-terminal domain at the apex. The two chains in the asymmetric unit superimposed with an rmsd of 0.5 Å over 328 matching Ca atoms, which excluded the poorly assigned N-terminal domain. LpSPL monomers associated into a homodimer that buried ~2500 Å2 of solvent-inaccessible surface area (Fig. 1B). The dimer was formed through interactions between the cofactor-binding domains and could be described as a diamond structure with the C-terminal domains at either apex.

As mentioned above, the N-terminal domain in the LpSPL structure was largely disordered except for one helix packed against the solvent-exposed face of the partner chain’s cofactor-binding domain away from the active site region (Fig. 1B). The LpSPL cofactor-binding domain featured a mixed α/β fold (29) and was centered around a seven-stranded β-sheet with two and three helices on either side of the β-sheet. The C-terminal helix of the cofactor-binding domain connected to the C-terminal domain. The C-terminal domain had an α/β structure, composed of a three-stranded β-sheet and three α-helices packed against one face of the β-sheet. Notably, in the human SPL structure, the C-terminal domain featured a two-helix extension connected to the last α-helix of the catalytic domain. This latter element is missing in the crystallized LpSPL fragment due to protolytic cleavage during crystallization. In accordance with previously characterized SPL structures, the LpSPL structure features a cavity between the N-terminal domain, the cofactor-binding domain, and the C-terminal domain, which corresponded to the PLP-binding active center (Fig. 1B). However, we did not find any additional density corresponding to a PLP molecule in this region of the LpSPL structure.
the cofactor-binding domain (sequence and conformation of the loop at the N-terminal end of the S1P substrate (SI Appendix, Fig. S2D). To investigate whether L. pneumophila exhibited lyase activity during infection, we generated an SPL mutant strain (Δspl) and infected mouse embryonic fibroblasts lacking the endogenous Sgpl gene (MEFspl−/−). Whereas cells infected with the Δspl strain did not exhibit lyase activity, the WT bacteria, the complemented strain (Δspl +pKS-LpSPL), as well as the LpSPL-overexpressing strain (WT +pKS-LpSPL) showed lyase activity (Fig. 2D).

We then undertook mutational analyses of the conserved active site residues to provide evidence into their individual roles in the catalytic activity of LpSPL. We introduced individual alanine substitutions of the active site residues and quantified their in vivo enzymatic activity (SI Appendix, Fig. S2C). The M228A mutation did not affect SPL activity in agreement with the structure, as this residue is buried against the seven-stranded β-sheet of the cofactor-binding domain and is thus not expected to participate in the enzymatic reaction. In contrast, the mutations C326A and K366A dramatically decreased the enzymatic activity of the enzyme (Fig. 2E). C328 is solvent-exposed in the active site of LpSPL, and its side chain is closely associated with the side chain of D326 and the backbone oxygen of G220 (Fig. 2F). The residues adjacent to the active site region in hSPL with the corresponding residues in hSPL (G210, C317, and K353) bound to the PLP molecule in the hSPL structure, with pairwise rmsd values between 2.0 and 2.1 Å over 347–350 matching Co atoms (e.g., LpSPL116–326 and hSPL222–314) (SI Appendix, Fig. S2 A and B). The high level of structural conservation between the structure of LpSPL and eukaryotic SPLs allowed detailed analysis of the LpSPL active center localized at the interface between the two monomers in the dimer (Fig. 24). The active center of the LpSPL structure was better resolved for one of the monomers (chain A), whereas several active site residues in the corresponding region in the other monomer (chain B) appear disordered. Of the resolved active site residues, the LpSPL active site was well conserved in amino acid composition and placement compared with the hSPL structure (Fig. 2B). Specifically, the positions of 11 catalytically important residues were absolutely conserved between the LpSPL and hSPL structures with the 12th residue showing a conservative (Leu to Ile) substitution (Fig. 2B). The LpSPL K360 residue aligned well with the PLP-conjugating lysine (K353) bound to the PLP molecule in the hSPL structure, suggesting that this LpSPL residue plays a similar functional role. Comparisons of the active site of LpSPL with those from other type 1 PLP-dependent enzymes that showed lower degrees of statistical significance in the Dali search revealed a higher level of sequence and structural divergence. This was seen especially in the sequence and conformation of the loop at the N-terminal end of the cofactor-binding domain (LpSPL148–164).

LpSPL Exhibits Sphingosine-1-Phosphate Lyase Activity During Infection.

The structural analyses (Fig. 1 B and C; SI Appendix, Fig. S2A and B) and sequence comparison of LpSPL with eukaryotic SPL proteins (SI Appendix, Fig. S2A) revealed that LpSPL has a conserved active site compared with other SPL enzymes (Fig. 2 B and C), strongly suggesting similar enzymatic activity for LpSPL. We therefore analyzed whether LpSPL exhibited SPL lyase activity by using the camoinaric sphinganine 1-phosphate analog, a fluorogenic substrate that is cleaved by SPL in a highly specific manner, producing umbelliflorone whose fluorescence was quantified (30). Expression of LpSPL in HEK-293T cells induced significant lyase activity as did mouse SPL (mSPL) used as positive control (Fig. 2C). We confirmed these results by using a commonly used radiolabeled S1P substrate (SI Appendix, Fig. S2D). To investigate whether L. pneumophila exhibited lyase activity during infection, we generated an SPL mutant strain (Δspl) and infected mouse embryonic fibroblasts lacking the endogenous Sgpl gene (MEFspl−/−). Whereas cells infected with the Δspl strain did not exhibit lyase activity, the WT bacteria, the complemented strain (Δspl +pKS-LpSPL), as well as the LpSPL-overexpressing strain (WT +pKS-LpSPL) showed lyase activity (Fig. 2D).

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LpSPL Shows a Conserved Domain Architecture and Active Site Composition Typical for SPL Enzymes. A search for structural homologs using the Dali server confirmed the structures of hSPL (9), yeast Dpl1p, and eukaryotic SPLs (8) as the closest matches for the LpSPL protomer. Each of these structures closely matched the LpSPL structure, with pairwise rmsd values between 2.0 and 2.1 Å over 347–350 matching Co atoms (e.g., LpSPL116–326 and hSPL222–314) (SI Appendix, Fig. S2 A and B). The high level of structural conservation between the structure of LpSPL and eukaryotic SPLs allowed detailed analysis of the LpSPL active center localized at the interface between the two monomers in the dimer (Fig. 24). The active center of the LpSPL structure was better resolved for one of the monomers (chain A), whereas several active site residues in the corresponding region in the other monomer (chain B) appear disordered. Of the resolved active site residues, the LpSPL active site was well conserved in amino acid composition and placement compared with the hSPL structure (Fig. 2B). Specifically, the positions of 11 catalytically important residues were absolutely conserved between the LpSPL and hSPL structures with the 12th residue showing a conservative (Leu to Ile) substitution (Fig. 2B). The LpSPL K360 residue aligned well with the PLP-conjugating lysine (K353) bound to the PLP molecule in the hSPL structure, suggesting that this LpSPL residue plays a similar functional role. Comparisons of the active site of LpSPL with those from other type 1 PLP-dependent enzymes that showed lower degrees of statistical significance in the Dali search revealed a higher level of sequence and structural divergence. This was seen especially in the sequence and conformation of the loop at the N-terminal end of the cofactor-binding domain (LpSPL148–164).

LpSPL Targets the Sphingolipid Metabolism of the Host Cell. Based on the structural and functional similarity between LpSPL and eukaryotic S1P lyases, we hypothesized that delivery of LpSPL to the host cytoplasm might impact host-cell sphingolipid metabolism. In eukaryotic cells, SPL cleaves S1P to phosphoethanolamine and a fatty aldehyde, which are subsequently reincorporated into glycerolipid metabolic pathways (8) (SI Appendix, Fig. S1). To determine whether LpSPL could mimic eukaryotic SPL, we first determined its subcellular localization. Efforts to directly determine the subcellular localization of LpSPL in the host cell were unsuccessful, consistent with previous studies showing that the levels of secretion of Dot/Icm effectors are very low (31–33). However, when transfected with an Xpress-tagged form of LpSPL or a FLAG-tagged form of mSPL, both clearly localized to the ER, as has been reported for hSPL. This finding contrasts with previous reports of the mitochondrial localization of LpSPL (5); nevertheless, coexpression with the ER marker Sec61α and mitotracker staining confirmed localization to the ER (SI Appendix, Fig. S3). On the other hand, it has recently been proposed that mammalian SPL might localize to mitochondrial-associated membranes (MAM), specific membrane domains involved in the
transfer of de novo-synthesized phospholipids from the ER to the mitochondria (34). Thus, Spl may also localize to MAMs. Furthermore, given the important role of the ER in the LCV formation, as well as the role of such membranes in autophagosome formation (35), Spl might play a role in driving autophagosome relocation during LCV formation.

As the enzymatic activity and the localization determined here suggested that Spl acts as eukaryotic SPL, we sought to measure the sphingolipid levels upon infection with L. pneumophila. We hypothesized that the irreversible catalysis of SIP by LpSPL would affect the major sphingolipids to compensate the loss of SIP. THP-1 cells were infected with wild-type L. pneumophila (WT) or the LpSPL mutant strain (Δspl), and the major sphingolipids were quantitatively analyzed by mass spectrometry. After 6 h of infection, several lipid species such as sphingomyelin (SM), ceramide, and glycosphingolipids, were reduced compared with uninfected macrophages. Furthermore, phosphatidylcholine was also reduced, likely due to the choline headgroup exchange compensating for loss of SM (Fig. 3A). However, the reduced levels of these lipids were not due to LpSPL activity, as macrophages infected with Δspl showed similar levels of lipids as WT-infected cells (Fig. 3A). It is thus possible that Legionella uses other mechanisms to reduce cellular sphingolipids, which may depend on TLR signaling and/or the presence of additional effectors as at least one gene shows high similarity to mammalian sphingomyelinase (SI Appendix, Fig. S1). Although we failed to detect SIP in THP-1 cells (likely due to very low cellular levels), sphingolipid levels in macrophages infected with WT and Δspl-pKS-LpSPL were similar to uninfected cells (Fig. 3B). In contrast, Δspl-infected macrophages showed significantly increased sphingolipid levels compared with uninfected or WT-infected macrophages (Fig. 3B). Thus, we show here, to our knowledge for the first time, that L. pneumophila-infected macrophages show decreased levels of major sphingolipids and that L. pneumophila specifically secretes LpSPL to prevent increased levels of sphingosine.

**LpSPL Decreases Starvation-Induced Autophagy.** Sphingolipids including ceramide, sphingosine, and SIP have been shown to stimulate macroautophagy (hereafter referred to as “autophagy”) (36–38). As autophagy is a cell-autonomous defense mechanism against intracellular bacteria (39) and a relationship between autophagy and sphingosine metabolism is actively discussed in the field (40), we wondered whether LpSPL would modulate autophagy in human cells. Moreover, it has been reported that autophagosomes form at the ER–mitochondria contact sites (35), which is also the localization that we proposed for LpSPL. Furthermore, our data show that infected macrophages show decreased sphingolipid levels, which are impeded by the expression of LpSPL (Fig. 3A). We thus hypothesized that L. pneumophila employs its LpSPL to dampen autophagy and to promote intracellular survival (21, 41).

To investigate whether the increased levels of sphingosine in Δspl-infected macrophages resulted in activation of autophagy, we first measured the accumulation of the conjugated form of LC3 (LC3II) in LpSPL-transfected cells (Fig. 3C). As we have previously demonstrated that SIP contributes to the stimulation of autophagy during starvation (42), we determined the effect of LpSPL expression on starvation-induced autophagy by quantifying the LC3II/actin ratio in starved cells (EBSS) supplemented or not with Bafilomycin-A1 (BafA1). BafA1 prevents maturation of autophagic vacuoles by inhibiting lysosome–autophagosome fusion and blocking LC3II degradation (43). Indeed, we detected a lower amount of LC3II in cells expressing LpSPL or mSPL, compared with cells transfected with the empty vector, suggesting that LpSPL impairs the formation of autophagosomes (Fig. 3C). Ectopic expression of LpSPL in L. pneumophila Δspl-pKS-LpSPL (mean ± SD) was confirmed by counting the GFP-LC3-positive cells. The number of LC3-positive cells was reduced in both cells transfected with LpSPL or with mSPL compared with starved control cells (Fig. 3D). In accordance with these results, the degradation of long-lived proteins in starved cells, which largely depends on autophagy (44), was severely impaired in cells expressing LpSPL (SI Appendix, Fig. S4B). The inhibitory effect of LpSPL on LC3-II accumulation is in agreement with the function of mSPL, as its ectopic expression also resulted in a decreased starvation-induced autophagy as detected both by counting LC3-positive cells and by LC3II accumulation (Fig. 3D; SI Appendix, S4B). Taken together, our data show that LpSPL activity inhibits autophagosome formation and not autophagosome maturation, as RaoZ does (25). To further analyze this finding, we studied autophagosome maturation in transfected cells by using an RFP-GFP-LC3 tandem system (SI Appendix, Figs. S5 A and B), which allows us to follow autophagosome-to-autolysosome conversion under starvation conditions (SI Appendix, Fig. S5C). Ectopic expression of either LpSPL or mSPL in the absence of BafA1 did not impact the ratio between autophagosomes and autolysosomes, confirming that SPL acts at the stage of autophagosome biogenesis (SI Appendix, Fig. S5D). This result is in line with a recent report that suggests that SIP is an initiator of autophagosome formation and SPL an inhibitor of autophagy in primary cells (45). Thus, ectopic expression of LpSPL can prevent autophagosome formation under nutrient limiting conditions.

![Fig. 3. LpSPL targets host sphingosine to impair starvation-induced autophagy.](image-url)

(A) Relative levels of total lipids from THP-1 cells infected with WT L. pneumophila or the Δspl mutant and analyzed by LC-MS (mean ± SD). (B) Levels of sphingosine were determined in uninfected THP-1 cells or infected with the WT or Δspl mutant carrying the empty vector (wt, Δspl) or the mutant expressing LpSPL (Δspl-LpSPL) (mean ± SD). (C) (Top) Immunoblot visualizing the LC3-I/LC3-II conversion in HEK-293T cells transfected for 48 h with empty vector (MOCK) or LpSPL kept in complete medium (CM) or minimum medium (EBSS) with or without BafA1. β-Actin was used as loading control. (Bottom) LC3II/Actin signal quantification (mean ± SD; n = 3). (D) Quantitative high-content analysis of GFP-LC3 puncta of cells transiently transfected with the empty vectors (MOCK), LpSPL, or mSPL expressing vectors in EBSS and BafA1. (Top) Representative pictures of GFP-LC3 puncta. (Scale bar, 10 μm.) (Bottom) Percentage of cells positive for GFP-LC3 puncta (mean ± SD; n = 3). (E) p62/Actin signal quantification of Western blot analysis of p62 degradation in HEK-293T cells transfected for 48 h with empty vectors (MOCK), mSPL, or LpSPL wild type (wt) or carrying single amino acid mutations, as indicated (mean ± SD; n = 3).
To understand at which stage *L. pneumophila* affects the autophagy pathway, we analyzed the activation of the kinase TOR (mTOR in mammals), which forms a large complex called mTORC1 that assembles under the tight control of cellular nutrient availability and negatively controls autophagy (46). Phosphorylation of 4EBP1 and S6, direct effectors of the mTORC1 complex, was unaffected upon transfection of eukaryotic cells with either *L. pneumophila* or mSPL, suggesting that SPL activity impacts the autophagy pathway downstream from the mTOR pathway (SI Appendix, Fig. S6A). To confirm that the action of *Lp*SPL required its enzymatic activity, we ectopically expressed mutant forms of *Lp*SPL (K366A, C328A, and M228A; Fig. 2E) in cells and measured both LC3 II accumulation (SI Appendix, Fig. S6B) and degradation of the autophagy cargo p62/SQSTM1. Autophagic clearance of protein aggregates containing LC3 puncta are shown (>300 individual cells analyzed/condition) in the presence of BafA1 (mean ± SD; n = 3). (B) LC3 (green), nuclei (cyan), *L. pneumophila* (red), and F-actin (gray). Representative images of uninfected and 20 h infected cells in the same field. (Scale bar, 20 μm.) (C) Quantitative high-content analysis of the number of cells with LC3 puncta in the presence or not of BafA1 at 20 h postinfection (open circles: pooled single-well replicates; red line: mean ± SD). (D) Quantitative high-content analysis of LC3 puncta as in C, 8 h of infection with *L. pneumophila* strain Philadelphia wt, ΔravZ, Δspl, and Δspl/ravZ in the presence of BafA1. (E) Competitive infection of A/J mice measured after simultaneous intratracheal inoculation of 10^7 cfu of wt and Δspl mutant. Mice were killed 72 h following inoculation to examine the bacterial content of their lungs, and the competitive index was calculated (mean = 0.2588; P < 0.0001).

**Lp*SPL Restrains the Host Autophagy Response During *L. pneumophila* Infection.** To determine the impact of *Lp*SPL activity during infection, we quantified the inhibition of autophagy in infected human macrophages. We performed high-content image-based assays to automatically quantify the number of cells with LC3 puncta in a high number of cells (SI Appendix, Fig. S7). THP-1 cells were infected with either WT or the Δspl mutant strain at different time points, and the accumulation of LC3 puncta was quantified as a function of time (Fig. 4A). We observed a reduction of LC3 accumulation in WT-infected cells from 8 to 20 h of infection (Fig. 4A). In contrast, at 20 h postinfection, single-cell analysis of cells infected with the Δspl strain showed, despite a large variance and a wide distribution in the data, a significant increase of cells with LC3 puncta compared with WT-infected cells (Fig. 4B and C), pointing to a role of *Lp*SPL in restraining autophagy during infection of human macrophages. These results are in agreement with previous observations that *L. pneumophila* tends to limit the autophagy response of the host cell during intracellular replication (21, 49) and identified a *L. pneumophila* effector implicated in modulating autophagy. To understand the interplay of the different effectors that manipulate autophagy, we analyzed also the *L. pneumophila* strain Philadelphia that encodes RavZ and *Lp*SPL. We constructed a ΔravZ Δspl, and a Δspl/ravZ double mutant in the strain Philadelphia and analyzed the accumulation of LC3 puncta for these strains. As expected the contribution of RavZ to inhibiting the autophagy machinery is much stronger than that of *Lp*SPL probably because RavZ is directly targeting LC3 (Fig. 4D). However, ravZ is present neither in the *L. pneumophila* strain Paris nor in about 40% of the *L. pneumophila* clinical isolates, suggesting that *L. pneumophila* has evolved several effectors, including *Lp*SPL, that inhibit the autophagy machinery at different stages.

To investigate whether *Lp*SPL played a role in the virulence of *L. pneumophila*, we analyzed the intracellular replication of the WT or the Δspl strain in amoeba (Acanthamoeba castellanii) (SI Appendix, Fig. S8A). We observed a slightly impaired infection capacity but similar replication of the Δspl as also reported previously (5). This result is comparable to that observed for RavZ, as its deletion had no measurable impact on intracellular replication (23). Interestingly, intracellular replication assays in human-derived macrophages (THP-1) suggested that the Δspl strain replicates slightly better than the WT strain (SI Appendix, Fig. S8B), similarly to what was observed for a deletion mutant of Leg9, which also plays a role in the autophagy response (24). Thus, *L. pneumophila* has evolved several strategies to restrain autophagy. However, the important role of autophagy in controlling *L. pneumophila* infection is evident when comparing the growth rate of *L. pneumophila* in WT and Atg9−/− Dictyostelium discoideum. *L. pneumophila* replicated better when the autophagy machinery was impaired by disrupting ATG9 in *D. discoideum* (SI Appendix, Fig. S9A) (24, 50). Similarly, mSPL also contributes to restriction of *L. pneumophila* growth, as deletion of the gene encoding mSPL enhances intracellular replication of *L. pneumophila* (SI Appendix, Fig. S9B). Thus, *L. pneumophila* possesses different effectors modulating the autophagic machinery, each having a distinct role in modulating the fine balance of autophagy. Furthermore, each one of these effectors alone does not show a strong replication phenotype when deleted, further suggesting that interference with the autophagy response is particularly fine-tuned and that our infection assays are too crude to accurately measure these small differences or that these differences can be observed only in in vivo infection models. This hypothesis is supported by the fact that *Lp*SPL is required for optimal replication of *L. pneumophila* in a pulmonary mouse model of infection (Fig. 4E). Intratracheal colonization of A/J mice revealed that Δspl bacteria were outcompeted by WT *L. pneumophila* 72 h after infection with a competitive index < 0.5 (mean 0.26 ± 0.35, P < 0.0001, one sample t test with theoretical mean of 1.00). Thus, *L. pneumophila* lacking *Lp*SPL was not able to establish an efficient infection in competition with WT *L. pneumophila*.

In conclusion, we report here the structure and function of, to our knowledge, the first translocated bacterial effector that directly targets the host-cell sphingolipid metabolism to change the fine balance of the different signaling molecules. The crystal structure of *Lp*SPL identified the active site, and its comparison...
with hSPL together with the characterization of its enzymatic activity revealed that \textit{L. pneumophila} uses molecular mimicry of eukaryotic SPLs to alter sphingolipid levels of the infected host through its S1P lyase activity. \textit{L. pneumophila} effector RavZ, which is acquired from \textit{Legionella} and the \textit{Imagopole-CiTech} (part of the FranceBioImaging infrastructure supported by ANR-10-INSB-04-01, Conseil de la Region Ile-de-France, Fondation pour la Recherche M\'edicale), contributes to counteracting the antibacterial response of the host cell.  

### Materials and Methods

The methods and materials are described in length in \textit{SI Appendix}.

#### Materials

- Protein purification and crystallography, lipidomics, SPL activity assays, cloning, bacterial strain and mutant constructions, cell culture and infection assays, pulmonary infection of \textit{A. alvei} mice (all animals were handled in strict accordance with animal ethics guidelines and approved by the Melbourne University Animal Ethics Committee).

#### Methods

- \textit{S. cerevisiae} S1P lyase activities were assayed with 
- \textit{N. crassa} S1P lyase activities were assayed with

#### Acknowledgments

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- The crystal structure was solved in part by National Institutes of Health Grants GM074942 and GM094585 (to A.S. through Midwest Center for Structural Genomics) and by the US Department of Energy, Office of Biological and Environmental Research, under Contract DE-AC02-06CH11357. P.C. and T.L. laboratories were supported by INSERM.
Supplementary Information

*Legionella pneumophila* S1P-lyase targets host sphingolipid metabolism and restraints autophagy

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**Table S1.** Distribution of the SPL encoding gene in different *Legionella* strains

**Table S2:** X-ray diffraction data collection and structure refinement statistics

**Figure S1:** Schematic overview of the sphingolipid pathway.

**Figure S2:** Comparison of the crystal structure and the sequence of *Lp*SPL with known eukaryotic SPLs and lyase activity determined using a radiolabelled substrate

**Figure S3:** Subcellular localization of *Lp*Spl and mSPL (A to D).

**Figure S4:** *Lp*Spl expression decreases proteolysis and mSPL impacts starvation induced autophagy.

**Fig. S5:** *Lp*SPL and mSPL expression modulates the starvation-induced autophagic flux.

**Fig. S6:** Impact of *Lp*SPL and mSPL expression on the mTor pathway and analysis of the activities of *Lp*SPL catalytic mutants on LC3 conversion.

**Figure S7:** Scheme of the high Content Analysis of LC3-puncta.

**Figure S8:** Intracellular replication of wt and Δspl *L. pneumophila* strains.

**Figure S9:** Autophagy and SPL pathways contribute to the restriction of intracellular growth of *L. pneumophila*
Supplementary Material and Methods

Protein expression and purification. Various MBP-His6-tagged constructs of \textit{LpSpl} used in this study were amplified from genomic DNA of \textit{L. pneumophila} strain Paris and cloned into the p19MBP-Lic T7 expression vector downstream of a DNA fragment encoding an N-terminal MBP-TMVV-His6 tag followed by a TEV protease recognition and cleavage site. MBP-His6-tagged \textit{LpSpl} proteins were expressed and purified as previously described (1). Briefly, the expression plasmid for each polypeptide was transformed into \textit{Escherichia coli} BL21(DE3)/pRK1037 (Stratagene), which harbors an extra plasmid (pRK1037) encoding the Tobacco Vein Mottling Virus (TVMV) protease. \textit{E. coli} was then cultured in 1L of Studier medium supplemented with appropriate antibiotics and incubated at 37°C until an OD$_{600}$ ~1.2. The culture was shifted to 18°C and protein expression induced by the addition of 0.3 mM isopropyl b-D-thiogalactoside (IPTG) followed by overnight incubation. Cells were harvested by centrifugation, disrupted by sonication, and the insoluble material was removed by centrifugation. His6-tagged proteins were purified using nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography, and dialyzed in a buffer containing 10 mM HEPES, pH 7.5, 300 mM NaCl and 0.5 mM tris-2-carboxyethyl phosphine (TCEP). The His6-tag was cleaved by the addition of His6-TEV protease and passed through a second Ni-NTA column. The purified protein was concentrated to less than 10 ml final volume and further purified via size exclusion chromatography on a HiLoad 26/60 Superdex 200 column (Amersham Pharmacia) developed with 10 mM HEPES, pH 7.5, 300 mM NaCl and 0.5 mM tris-2-carboxyethyl phosphine (TCEP). Selenomethionine-enriched proteins were prepared after growth of bacteria in SeMet high-yield media (Shanghai Medicilon) and purified as described above.

Crystallography and structure determination of \textit{LpSpl}. Crystallization of \textit{LpSpl}_{56-605} was performed using SeMet labeled protein treated with thermolysin. Initial crystals were obtained by sitting drop vapor diffusion at room temperature (20–24°C) using an optimized sparse matrix crystallization screen (2). Optimized crystals were grown at 22°C using the sitting drop method, with protein at 10 mg/mL over reservoir solution containing 25% PEG3350, 0.1M NaCl, 0.1M KCl, 0.1M NH$_4$SO$_4$, 0.1 M Hepes pH 7.5. Crystals were cryo-protected with reservoir solution supplemented with paratone prior to flash freezing in liquid nitrogen. Diffraction data at 100 K at the wavelength corresponding to the anomalous scattering peak wavelength of seleno-methionine (0.97924 Å) were collected at beamline 19-BM at the Structural Biology Center, Advanced Photon Source, Argonne National...
Laboratory. Diffraction data were reduced with HKL3000 (3). The structure was solved by single-wavelength anomalous dispersion (SAD) using the AutoSol module of Phenix (4). Twelve Se-Met sites in the asymmetric unit were found as judged by occupancy values greater than 0.50. We reasoned that two proteolytically cleaved fragments of LpSpl_{56-605}, each containing six Se-Met sites, would explain the observed Matthew’s coefficient calculation of approximately 650 amino acids in the asymmetric unit of the crystal. An initial model of the two protein molecules was built using Phenix AutoBuild, followed by rounds of manual model building and refinement with Coot (5) and Phenix.refine with TLS parameterization. The final atomic model includes residues 108-529 of chain A of LpSpl and 107-526 of chain B. Many residues were not built due to missing or poor density, including: chain A residues 126-157, 181-191, 387-397, 402-404, 486-487, 494-495; chain B residues 123-124, 134-159, 180-190, 386-398, 402-403, 448-450, 475-478, 486-492. Structure similarity searches of the PDB were performed using the Dali-lite (6) and PDBePISA servers (7). The homodimer interface was analyzed using the PDBePISA server. Structure superpositions were performed with Coot or the Dali-lite server. Protein structure images were produced with PyMOL. The crystal structure of Se-Met LpSpl_{56-605} was submitted to the PDB with the accession number 4W8I.

Lipidomics analyses. Macrophage cultures were washed in ice-cold phosphate buffered solution and lipids extracted on ice using monophasic extraction with chloroform:methanol:water (1:2:0.6, v/v) containing 250 nM dipalmitoylphosphatidylcholine (dPPC) as internal standard. After removal of insoluble material by centrifugation (15,000g, 15 min), extracts were dried under nitrogen and resuspended in 100 µL of butanol/methanol (1:1, v/v) containing 5µM ammonium formate. Cellular lipids were separated by injecting 5-µL aliquots onto a 50 mm x 2.1 mm x 2.7 µm Ascentis Express RP Amide column (Supelco, Sigma) at 35°C using an Agilent LC 1200. Lipids were eluted at 0.18 mL·min⁻¹ over a 5 min gradient of water/methanol/tetrahydrofuran (50:20:30, v/v) to water/methanol/tetrahydrofuran (5:20:75, v/v), with the final buffer held for 3 min. Lipids were also analyzed by electrospray ionisation-mass spectrometry (ESI-MS) using an Agilent Triple Quad 6460 (Mulgrave, Australia). Lipid species from each lipid class were identified using precursor ion scanning from 100 -1000 m/z, in positive ion mode, sphingomyelins (SM, m/z 184.1) and ceramides (CER, m/z 264.6). Identified lipid species were quantified using multiple reaction monitoring (MRM) with a 10 ms dwell time for the simultaneous measurements of ~20 to 50 compounds and the chromatographic peak width of 30 sec to 45
sec, a minimum data points collected across the peak was 12 to 16. Optimized parameters for capillary, fragmentor, and collision voltages were 4000 V, 140 - 380, and 15–60 V, respectively. In all cases, the collision gas was nitrogen at 7 Lmin⁻¹. ESI-MS data was processed using Agilent Mass Hunter (Mulgrave, Australia) and normalized to dPC. Cellular sphingosine was separated by injecting 5-µL aliquots onto a 50 mm x 2.1 mm x5 µm SeQuantR PHILIC column (Supelco, Sigma) at 20 °C using an Agilent LC 1290. Sphingosine was eluted at 0.6 mLmin⁻¹ over a 14 min isocratic gradient of 25% of water and 75% of acetonitrile with 0.1% Formic acid (8). Sphingosine (Sph) was analyzed by electrospray ionisation-mass spectrometry (ESI-MS) positive mode using an Agilent QTOF 6550 (Mulgrave, Australia). Sphingosine was quantified according to extracted peak area using accurate mass of Sph. Optimised parameters for capillary and fragmentor were 4000 V and 380, respectively. In all cases, the collision gas was nitrogen at 11 Lmin⁻¹. ESI-MS data was processed using Agilent Mass Hunter (Mulgrave, Australia).

**SPL activity assays.** SPL activity was measured as previously described (9). Briefly, 48h after transfection of HEK-293T cells with empty vectors (MOCK), LpSpl or mouse SPL (mSPL) or 8 h after infection at an MOI=30 of MEF spl⁻/⁻ cells with wt, Δspl, Δspl+LpSpl, wt+LpSpl strains Spl activity was measured. In a 96-well plate, the fluorogenic substrate (125 µM) was incubated with 100 µL of infected cells lysates (9 mg/ml) in 0.5M potassium phosphate buffer pH 7.5 containing 25 µM Na₃VO₄ and 0.25 mM pyridoxal phosphate at 37ºC for 6 h. Cleavage of the fluorogenic substrate (S1P analogue) was measured by quantifying the released fluorescent product umbelliferone. The fluorescence was measured with a Tecan Infinite M200 (excitation 355nm; emission 460 nm). The lyase assay using a radiolabelled substrate was performed as described above. Briefly, 300 ug protein/sample were incubated with [4,5-³H]dihydrosphingosine-1-phosphate and the SPL activity was calculated by taking into account the production of palmitic acid, 1-hexadecanol and hexadecanal. After enzyme incubation and lipid extraction, the aldehyde was separated by thin-layer chromatography and quantified by using liquid-scintillation counting.

**Cloning.** The *L. pneumophila* spl gene (*lpp2128*) was PCR amplified using genomic DNA of *L. pneumophila* strain Paris (fw: 5'- cgggatccatgttcggttttatttcagattt rev: 5'- cctgctcgagtcaaagcgaggtttgcactt) and cloned *BamHI*-XhoI into pCDNA4-HisMax-C (Invitrogen). Catalytically inactive mutants of LpSpl (M228A, C328A and K366A) were
obtained by site direct mutagenesis using the QuickChange Site Directed Mutagenesis kit (Stratagene). Mouse SPL was cloned in pCDNA5 vector in frame with a FLAG tag.

**Bacterial strains and culture conditions.** *L. pneumophila* was cultured in *N*- (2-acetamido)-2-aminoethanesulfonic acid (ACES)-buffered yeast extract broth or on ACES-buffered charcoal-yeast (BCYE) extract agar (10). *E. coli* strains were cultured aerobically in Luria–Bertani (LB) broth or on LB agar. DsRed bacteria were obtained by introducing pSW001 (11). When required, antibiotics were used: ampicillin (100 µg/ml), kanamycin (50 µg/ml for *E. coli*, 12.5 µg/ml for *L. pneumophila*), chloramphenicol (20 µg/ml for *E. coli* and 10 µg/ml for *L. pneumophila*). All strains were grown at 37°C. The Δspl deletion mutant was constructed as previously described (12). Briefly, the spl gene was inactivated by introducing a kanamycin resistance cassette into the chromosomal gene. The chromosomal region containing the spl gene was PCR-amplified (fw: GAGATTATCCGGGTCAAGCA; rev: TTGCAAGGCAGCATTTAAAAA) and the PCR product cloned into the pGEMT-easy vector (Promega). An inverse PCR was performed (fw: CGGGATCCTCTTTGGAAGAGGGATG; rev: CGGGATCCATCCCCCTCTTCCAACAAGA) and the amplified pGEMT-easy backbone with 500 bp flanking regions were BamHI-digested and ligated with kan^R^ cassette. ravZ was inactivated by introducing a apramycin resistance cassette. The mutant allele was constructed using a 3-steps PCR. Briefly, three overlapping fragments: i) lpg1683 upstream region (fw: CGGGATCTCCTCTTTGGAAGAGGGATG; rev: CGGGATCCATCCCCCTCTTCCAACAAGA), ii) antibiotic cassette (fw: TGCAGCTCCATCAGCAAA; rev: CCCTCACAAGTCATCTCG); iii) lpg1683 downstream region (fw: CGAGATGACGTTGGAGGGACAGGGGAAGAGCTTGCC; rev: CCAATAACATGACTTATTAGGATACAATAG) were amplified independently and purified on agarose gels. The three resulting PCR products were mixed at the same molar concentration (15nM) and a second PCR with flanking primer pairs (fw: CGTACTAAGCTATCTATCGAATTACC; rev: CGTACTAAGCTATCTATCGAATTACC) was performed. For recombination, the constructs were introduced into *L. pneumophila* strains Paris and Philadelphia by natural competence. Bacteria were grown in liquid media at 37°C during 24 hours until the transition phase and the bacterial pellet was incubated with at least 500ng of linear, purified DNA during 24h at 30°C without shaking. The strains that had undergone allelic exchange were selected by plating on BCYE agar plates containing antibiotics. Each mutant was verified by
PCR and sequencing. Δspl mutant was complemented with the full-length spl gene cloned into pBC-KS downstream its own promoter (fw: CTGCAGACTTCTGCAAGGTTTGTC; rev: TCTAGATCAAAGCGAGGTTTGCAC).

**Cell culture and infection assays.** Cell lines used in this study were HeLa cells (ATCC CCL-2), HeLa cells stably transfected with GFP-LC3 or RFP-GFP-LC3, HEK293T cells (ATCC CLR-11268), U2OS cells stably transfected with the ER marker Sec61β-GFP (kind gift of Dr. Fabrizia Stavru), the human monocytic leukemia cell line THP-1 (ATCC TIB-202) and mouse embryonic fibroblasts (MEFs) wt and knockout for the spl gene (MEFs-spl⁻/⁻) (13). Cells was maintained in 5% CO₂ at 37°C in supplemented 10% fetal bovine serum (BIOWEST), DMEM GlutaMAX medium and RPMI 1640 medium GlutaMAX for THP-1 (Invitrogen). When indicated, cells are treated with minimum medium (EBSS, Invitrogen) or Bafilomycin A1 (BafA1, 200 nM; Enzo Life Science). *A. castellanii* (ATCC50739) was cultured in PYG 712 medium [2% proteose peptone, 0.1% yeast extract, 0.1M glucose, 4 mM MgSO₄, 0.4 M CaCl₂, 0.1% sodium citrate dihydrate, 0.05 mM Fe(NH₄)₂(SO₄)₂ ¥ 6H₂O, 2.5 mM NaH₂PO₃, 2.5 mM K₂HPO₃] at 20°C for 72 h prior to harvesting for *L. pneumophila* infection. Infection assays were performed as previously described (14). Briefly, cells were seeded in 12-well tissue culture trays (Falcon, BD) at a density of 2x10⁵ cells/well. THP-1 cells were pre-treated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) for 72h to induce differentiation into macrophage-like adherent cells. Stationary phase *L. pneumophila* were re-suspended in serum free medium and added to cells monolayer at different multiplicities of infection (MOI), as indicated. After 2 hours of incubation cells were washed with PBS before incubation with serum-free medium. At different time points as indicated, cells were lysed with PBS- TritonX-100 0.1% and the amount of bacteria was monitored counting the number of colony-forming units (cfu) determined by plating on BCYE agar (14). *D. discoideum* wild-type or ATG9 knock-out (kindly provided by L. Eichinger, University of Cologne, Germany) were grown and infected at 25 °C as previously described (15). Briefly, the amoebae were grown in HL-5 medium and infected (MOI=1) in MB medium with *L. pneumophila* grown to stationary phase in AYE medium. At the time points indicated, the amoebae were lysed with 0.8% saponin and CFU were determined.

**Transfections.** Cells were transiently transfected using Fugene HD Transfection Reagent (Roche). The expression vectors used were: pCDNA4/5 (MOCK), pCDNA4-LpSPL (Xpress tag), pCDNA4-LpSPL with single amino acid mutations (Xpress tag), pCDNA5-mSPL.
(FLAG tag). In all experiments cells were transfected 48h before analysis and maintained in complete medium (CM). When indicated cells were treated 4h and 2h before analyses with minimum medium (EBSS) and BafA1, respectively.

**Western Blotting.** Equal amounts of proteins were loaded on polyacrylamide gels and transferred to PVDF membranes (Perkin-Elmer). Primary antibodies: anti-LC3 (NanoTools; clone 5F10), anti-β-actin (SIGMA; clone AC-74), anti-p62 (Abnova; H0008878-M01), anti-4EBP1 (Cell Signaling Technology ; CS9452), anti-6P and anti-6 protein (Cell Signaling Technology; CS221 and CS2311). Anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies (DAKO) were used, followed by chemiluminescence using ECL Plus Western Blotting Detection System (GE Healthcare). Chemiluminescence signals were recorded on a G:BOX (Syngene).

**Immunofluorescence microscopy.** For high content analysis of LC3-puncta, 48h after transfection cells were subjected to starvation (EBSS) and/or BafA1 treatment and an image-based high content assay was performed on living cells grown in µClear 96-well plates (Greiner Bio-One). Images of 30 fields of each well were acquired at 40X magnification using an Opera High Throughput spinning-disk microscope (Perkin Elmer) and 4 excitation lasers (405, 488, 561 and 633nm). Automatic analysis of a high number of cells (between 300 to 700 cells per condition) was performed using Columbus software (Perkin Elmer) and an in-house developed script (shared upon request and schematically explained in Fig. S7). THP-1 cells were infected and fixed in 4 % paraformaldehyde (Electron Microscopy Science). After permeabilization (0.075% Saponin) and blocking (1% BSA), cells were stained with anti-human-LC3 primary antibodies (MBL; clone 4E12) and stained with Alexa488-conjugated secondary antibodies, DAPI and Phalloidin-633 (Molecular Probes). Images of 30 fields of each well were acquired at 40X magnification using the Opera High Throughput spinning-disk microscope (Perkin Elmer) and 4 excitation lasers (405, 488, 561 and 633nm). Automatic analysis of a high number of cells (between 125 to 1000 cells per condition) was performed using Columbus software (Perkin Elmer) and an in-house developed script (shared upon request and schematically explained in Fig. S7). Cells were scored as positive for LC3 puncta if they contained more than 5 puncta. For subcellular localization of LpSPL and mSPL transfected U2OS-Sec61β-GFP cells were plated in µClear 96-well plates (Greiner Bio-One), stained for mitochondria (Mitotracker Orange, M7510, Molecular Probes) and fixed in 4 % paraformaldehyde (Electron Microscopy Science) at 48h post-infection. After
permeabilization (0.075% Saponin) and blocking (1% BSA), cells were stained using anti-
Xpress (Invitrogen; R910-25) or Anti-Flag (Thermo; cloneFG4R) primary antibodies,
washed, and stained with Alexa-Fluor-633 secondary antibody and DAPI (All from
Molecular probes). Images were acquired at 63X magnification using the Opera High
Throughput spinning-disk microscope (Perkin Elmer) and 4 excitation lasers (405, 488, 561
and 633nm). RGB profiles were calculated using ImageJ software.

**Measurement of the Degradation of Long-lived Proteins.** We followed the previously
reported method (16). Briefly, cells were incubated for 18 h at 37°C with 0.2 µCi/mL-[14C]
valine. Unincorporated radioisotopes were removed by three rinses with phosphate-buffered
saline (pH 7.4). Cells were then incubated in nutrient- and serum-free medium (without amino
acids and in the absence of fetal calf serum) plus 0.1% bovine serum albumin and 10 mM
unlabeled valine. After the first hour of incubation, at which time short-lived proteins were
being degraded, the medium was replaced with the appropriate fresh medium, and the
incubation was continued for an additional 4h period. Cells and radiolabeled proteins from the
4h chase medium were precipitated in 10% (v/v) trichloroacetic acid at 4°C. The precipitated
proteins were separated from soluble radioactivity by centrifugation at 600 × g for 10 min and
then dissolved in 250 µl Soluene 350. The rate of protein degradation was calculated as acid-
soluble radioactivity recovered from both cells and the medium

**Pulmonary infection of A/J mice with L. pneumophila.** The competitive virulence assays
for L. pneumophila strain Paris wt and the Δspl mutant within A/J mice was studied as
described previously (14). Briefly, 6- to 8-week-old female A/J mice (Jackson Laboratory,
Maine, USA) were anaesthetized and inoculated intra-tracheally with 10^5 bacteria of each L.
pneumophila strain. 72 hours following inoculation, mice were sacrificed and their lung
tissues were isolated. The 72 h time point was chosen as after this length of time the innate
immune response begins to control bacterial load and hence mutant attenuation can be
revealed. Tissue was homogenized and complete host cell lysis achieved by incubation in
0.1% saponin for 15 min at 37°C. Serial dilutions of the homogenate were plated onto both
plain and antibiotic selective BCYE agar to determine the number of viable bacteria and the
ratio of wt to mutant bacteria colonizing the lung.
### Supplementary Table 1: Distribution of the SPL encoding gene in different *Legionella* strains

<table>
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<th><em>Legionella pneumophila</em> Strains</th>
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Supplementary Table 2: X-ray diffraction data collection and structure refinement statistics

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<td>Redundancy</td>
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| Refinement                      |               |
| Resolution range (Å)            | 19.71 – 2.85   |
| Number of reflections           |               |
| Working set                     | 16318         |
| Test set                        | 1471          |
| Rwork/Rfree                     | 0.260/0.315 (0.327/0.360) |
| Number of non-hydrogen atoms    |               |
| Protein                         | 5499          |
| Water                           | 16            |
| Root mean square distance       |               |
| Bond length (Å)                 | 0.006         |
| Bond angle (°)                  | 1.277         |
| Average thermal factor (Å²)     |               |
| Protein                         | 68.6          |
| Water                           | 45.8          |
| Ramachandran analysis (%)       |               |
| Most favoured regions           | 84.1          |
| Additionally allowed regions    | 15.5          |
| Generously allowed regions      | 0.3           |
| Disallowed regions              | 0             |

*Values in parantheses refer to highest resolution shells.
*Rfree was calculated by randomly omitting 5% of the observed reflections from the refinement.
*According to the Ramachandran plot in Procheck PDB Validation Server.
Supplementary Figure Legends

**Figure S1:** Schematic overview of the sphingolipid pathway. (A) Schematic overview; lipid products are boxed; enzymes are in blue and the *L. pneumophila* genes encoding for putative enzymatic activities that are part of this pathway are in red.

**Figure S2:** Comparison of the crystal structure and the sequence of *Lp*SPL with known eukaryotic Spls. (A) Superposition of dimeric structures of *Lp*SPL and *Symbio bacterium thermophilum* *St*SPL colored by chain. The superposition of the full structure of *Lp*SPL onto *St*SPL showed the two proteins have a RMSD of 2.3 Å over 347 residues (*Lp*SPL residues 107-526 and *St*SPL residues 67-472) and 34% sequence identity. (B) Superposition of dimeric structures of *Lp*SPL and *Saccharomyces cerevisiae* Dpl1p colored by chain. (C) Top panel: Schematic representation of cytosolic domain of *Lp*Spl. Site-specific mutations are indicated in color: M228 and C328 (corresponding to C218 and C317 in human SPL and known to be important for proper folding) are in green. Purple box indicates predicted co-factor binding domain, comprising the K360 and K366 residues (corresponding to K353 and K359 in human SPL). Bottom panel: Sequence alignment: *Drosophila melanogaster* [NP_652032.1]; *Mus musculus* [NP_033189.2]; *Rattus norvegicus* [NP_775139.1]; *Homo sapiens* [NP_003892.2]; *Caenorhabditis elegans* [NP_499913.1]; *Dictyostelium discoideum* [XP_639378.1]; *Saccharomyces cerevisiae* [NP_010580.1]; *Leishmania major* [XP_001684840.1]. (D) *Lp*SPL and mSPL activities determined by using a radiolabelled S1P substrate. Lyase activity was measured on total protein extract (300 µg protein/sample) of HEK293T untransfected (MOCK) or transfected with expression vectors carrying *Lp*SPL or mSPL. The SPL activity was calculated by taking into account the production of palmitic acid, 1-hexadecanol and hexadecanal. This assay was done once to confirm SPL activity. For all other assays the fluorogenic substrate was used (Figure 2C, 2D, 2E), as it is is much easier to use, less expensive and avoids manipulation of radioactive material.

**Figure S3:** Subcellular localization of *Lp*Spl and mSPL (A to D). U2OS cells stably transfected with the ER marker Sec61β (GFP, green) were transiently transfected 48h with Xpress-tagged *Lp*SPL or FLAG-tagged mSPL. (A) *Lp*SPL transfected cells were stained stained for anti-Xpress (yellow), mitochondria (Mitotracker, red) and nucleus (DAPI, cyan). Insets show details of the specific fluorescence (boxed) at higher magnification. Scale bar: 10µm. (B) Merged images corresponding to (A) of ER (Sec61β, green) and *Lp*SPL (anti-
Xpress, red). Colocalization appears in yellow. Right graph corresponds to the RGB profile of green and red signals from left panels across the yellow line. **(C)** mSPL transfected cells were stained for anti-FLAG (yellow), mitochondria (Mitotracker, red) and nucleus (DAPI, cyan). Insets show details of the specific fluorescence (boxed) at higher magnification. Scale bar: 10 µm. **(D)** Merged images corresponding to (C) of ER (Sec61β, green) and mSPL (anti-FLAG, red). Co-localization appears in yellow. Right graph corresponds to the RGB profile of green and red signals from left panels across the yellow line.

**Figure S4: LpSPL expression decreases proteolysis and mSPL impacts starvation induced autophagy.** **(A)** LC3-I/LC3-II conversion in HEK-293T cells transfected with the empty vector (MOCK) or the vector expressing the mouse SPL (mSPL) and treated in complete medium (CM) or minimum medium (EBSS), with or without Bafilomycin-A1 (BafA1). β-actin was used as loading control. LC3II/Actin ratio was indicated below. Mean ± SD; n=3. **(B)** The rate of [14C]valine-labelled long-lived proteins was determined in starved cells transfected with the empty vector (MOCK) or the LpSPL expressing vector.

**Figure S5: LpSPL and mSPL expression modulates the starvation-induced autophagic flux.** **(A)** Schematic presentation of a RFP-GFP-LC3 reporter assay that allows studying the autophagic flux induced by starvation (EBSS treatment). Autophagosomes (green, GFP) and (red RFP). Once autophagosomes fuse with lysosomes to form autolysosomes, acid sensitive GFP green fluorescence disappears and only red fluorescence (RFP) remains, a step that can be inhibited by BafA1 treatment causing an accumulation of autophagosomes and a reduction of autolysosomes. **(B)** Representative images showing the work flow of the script developed to automatically quantify autophagosomes and autolysosomes in living HeLa cells stably expressing RFP-GFP-LC3 (HeLa-LC3-tandem cells). HeLa-LC3-tandem cells were imaged for RFP and GFP fluorescence in starved conditions (EBSS treatment), with and without BafA1. The contour of the cell was detected from cellular background due to the GFP expression. RFP+GFP+ puncta (yellow; autophagosomes) and RFP+GFP- puncta (red; autolysosomes) were detected and quantified in each cell. BafA1 treatment induces an accumulation of autophagosomes and a reduction of autolysosomes. Scale bars: 20 µm. **(C)** The chart (top) summarizes the results when monitoring the autophagic flux using HeLa-LC3-tandem cells. The graph (bottom) shows the results from automatic quantification of autophagosomes and autolysosomes in non-starved (complete medium) and starved (EBSS) cells, with or without BafA1. **(D)** HeLa-LC3-tandem cells were transiently transfected with
empty vector (MOCK), LpSPL or mSPL and the autophagosome to autolysosome conversion was assessed.

**Figure S6: Impact of LpSPL and mSPL expression on the mTor pathway and analysis of the activities of LpSPL catalytic mutants on LC3 conversion.** (A) Western blots showing the endogenous levels of phosphorylate S6 protein (S6-P), total S6 protein and total 4EBP1 protein in HEK-293T cells transfected 48h with empty vector (MOCK), LpSpl or mSPL and treated in complete medium (CM), minimum medium (EBSS) or minimum medium followed by complete medium (EBSS+CM). The smear signal in the 4EBP1 western blots indicates the phosphorylated forms of the protein. (B) Top panel: Immunoblotting visualizing the LC3-I/LC3-II conversion in HEK-293T cells transfected 48h with empty vector (MOCK) or single amino acid mutants of LpSpl (as indicated) and treated in complete medium (CM) or minimum medium (EBSS), with or without Bafilomycin-A1 (BafA1). β-actin was used as loading control. Bottom panel: LC3II/Actin signal quantification; mean ± SD; n=3.

**Figure S7: Scheme of the high Content Analysis of LC3-puncta.** The script developed to automatically measure LC3-puncta during the course of Legionella infection of THP-1 cells uses a sequential logical work-flow where the input image (1) is composed by signals from 4 different channels: DAPI (405, showed in blue), LC3-Alexa488 (488, green), DsRed Legionella (561, red) and Phalloidin-633 (633, white). A representative complete field is shown, showing details in the inserts. Nuclei were detected in the input image using DAPI signal (2), and then the cytoplasm associated to each nucleus was detected using Phalloidin-633 signal that stains F-Actin (3). Legionella was then detected in the cytoplasm of cells using the DsRed signal (4), allowing the discrimination of infected (5, ICs) and non-infected cells (5, NICs). Finally, LC3-puncta were detected in each single cell as spots in the cytoplasm. LC3-puncta were counted and cells were scored as positive for LC3 puncta if they contained more than 5 puncta.

**Figure S8: Intracellular replication of wt and Δspl L. pneumophila strains.** A. castellanii (A) and THP-1 cells (B) were infected with wt and a Δspl strain and intracellular replication was determined by recording the number of colony-forming units (CFU) (MOI of 0.1 for A. castellanii and of 10 for THP-1; mean ± SD; n=3).
Figure S9: Autophagy and SPL pathways contribute to the restriction of intracellular growth of *L. pneumophila*. Intracellular replication of *L. pneumophila* strain Paris in *D. discoideum* wt and ATG9<sup>−/−</sup> (A) or in MEFs wt and SPL<sup>−/−</sup> (B) was determined by recording the number of colony-forming units (CFU) over 7 days (MOI 0.1) in *D. discoideum* or 72 hours (MOI of 10) in MEFs (mean ± SD; n=3).
References


Figure S1

Sphingomyelin (SM)

Sphingomyelinase

Glycosyleramidase synthase

Glycosieramide (Glc-ceramide)

Sphingosine

Sphingosine kinase

Sphingosine-1-phosphate (S1P)

S1P lyase (SPL)

Hexadecenal + Phosphoethanolamine

Phosphatidylcholine (PC)
Figure S2

A

D. melanogaster
M. musculus
R. norvegicus
L. corby
L. Paris
L. Lens
L. Philadelphia
C. elegans
D. discoideum
S. cerevisiae
L. major

B

Figure S2

C

LpSpl vs StpSPL
LpSpl vs Dp11p

18

DE

Spt Activity (pmol/min)

0 1 2 3 4 5

MOCK LpSPL MOCK mSPL

Figure S2
Figure S3

A

B

C

D
Figure S4

A

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<tr>
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MOCK     CM   EBSS  CM   EBSS
CM       CM   EBSS

% proteolysis / hour

MOCK     LpSPL

B

% proteolysis / hour

MOCK     LpSPL
Figure S5

A

EBSS

Isolation membrane

Autophagosome
RFP+GFP+ LC3 punctum

EBSS + BafA1

Autolysosome
RFP+GFP- LC3 punctum

pH<5

Lysosome

B

EBSS

GFP

RFP

Merge

Cell detection

Autophagosomes
RFP+GFP+ LC3 punctum

EBSS + BafA1

EBSS

EBSS + BafA1

Cell detection

Autolysosomes
RFP+GFP- LC3 punctum

C

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D

![Graph showing LC3 puncta per cell for different treatments.](image-url)
Figure S6

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B

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Graph showing LC3II/Actin ratios for each condition.
Figure S7

1. Input Image
2. Detection of nuclei
3. Detection of cytoplasm
4. Detection of intracellular bacteria
5. Selection of infected cells (ICs)
6. Detection of LC3-puncta in ICs
5. Selection of non-infected cells (NICs)
6. Detection of LC3-puncta in NICs
Figure S9

A

B