Sensing Cytosolic RpsL by Macrophages Induces Lysosomal Cell Death and Termination of Bacterial Infection

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Abstract

The intracellular bacterial pathogen Legionella pneumophila provokes strong host responses and has proven to be a valuable model for the discovery of novel immunosurveillance pathways. Our previous work revealed that an environmental isolate of L. pneumophila induces a noncanonical form of cell death, leading to restriction of bacterial replication in primary mouse macrophages. Here we show that such restriction also occurs in infections with wild type clinical isolates. Importantly, we found that a lysine to arginine mutation at residue 88 (K88R) in the ribosome protein RpsL that not only confers bacterial resistance to streptomycin, but more importantly, severely attenuated the induction of host cell death and enabled L. pneumophila to replicate in primary mouse macrophages. Although conferring similar resistance to streptomycin, a K43N mutation in RpsL does not allow productive intracellular bacterial replication. Further analysis indicated that RpsL is capable of effectively inducing macrophage death via a pathway involved in lysosomal membrane permeabilization; the K88R mutant elicits similar responses but is less potent. Moreover, cathepsin B, a lysosomal protease that causes cell death after being released into the cytosol upon the loss of membrane integrity, is required for efficient RpsL-induced macrophage death. Furthermore, despite the critical role of cathepsin B in delaying RpsL-induced cell death, macrophages lacking cathepsin B do not support productive intracellular replication of L. pneumophila harboring wild type RpsL. This suggests the involvement of other yet unidentified components in the restriction of bacterial replication. Our results identified RpsL as a regulator in the interactions between bacteria such as L. pneumophila and primary mouse macrophages by triggering unique cellular pathways that restrict intracellular bacterial replication.
Author Summary

The death of the host cell during infection can be triggered by one or more microbial molecules; this “live or die” selection provides effective means for the dissection of immune recognition mechanisms as well as for the identification of the microbial molecules responsible for such responses. We found that infection of primary mouse macrophages by Legionella pneumophila strains harboring wild type RpsL, the S12 component of the bacterial ribosome, causes macrophage death by a mechanism independent of the three inflammatory caspases, caspase 1, 7 and 11. Importantly, although both confer resistance to streptomycin at indistinguishable effectiveness, the K88R, but not the K43N mutation in RpsL enables L. pneumophila to replicate in macrophages. Purified RpsL and RpsL\textsubscript{K43N} physically delivered into macrophages cause cell death by inducing damage to lysosomal membranes and the release of cathepsins. We also found that the lysosomal protease cathepsin B is required for efficient RpsL-induced cell death but its absence is not sufficient for macrophages to support intracellular bacterial replication. Thus, RpsL functions as an immune induction molecule to trigger one or more signaling cascades that leads to lysosomal cell death as well as the termination of bacterial replication.

Introduction

Pattern recognition receptors (PRRs) sense pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) generated by infection or endogenous cellular injury or tissue damage to initiate immune responses [1]. The Toll-like receptors (TLRs) were the first identified PRRs that recognize PAMPs and induce the expression of pro-death cytokines and pro-inflammatory molecules through the nuclear factor \(\kappa B\) (NF-\(\kappa B\)) signaling pathway [1]. These molecules could orchestrate efficient defense against invading pathogens through the induction of cell death, which is an effective means of defense against infections in many microbe-host interaction systems. For example, TNF-\(\alpha\) engages the cellular apoptosis or necroptosis pathway to defend against infection [1]. The second group of PRRs contains the NOD-like receptor (NLR), the retinoic-acid inducible gene-I (RIG-I)-like helicase, and the PYHIN (pyrin and HIN200 domain—containing proteins; also known as p200 or HIN200 proteins) protein families [2]. These structurally and functionally heterologous proteins recognize more diverse ligands (including PAMPs) and can be generally divided into two categories based on their downstream signaling events. The first category of receptors promote transcriptional activation of cytokines through pathways controlled by the transcriptional activator NF-\(\kappa B\) or IRF3 [2], whereas the second group of receptors initiate the assembly of large cytoplasmic signaling complexes known as the inflammasomes [2]. The inflammasome senses microbial infection and/or danger-associated molecules and activate caspase-1/11-dependent cytokine production and inflammatory cell death (pyroptosis), which is believed to be important in the removal of the replicative niche of intracellular pathogens [3].

Recent studies have identified the ligands for several of these receptors. For example, NAIP5 and NAIP6 are the receptors for bacterial flagellin, and NAIP1 and NAIP2 sense the needle and rod proteins of bacterial type III secretion systems, respectively [4–6]. Cytosolic DNA directly binds to the AIM2 inflammasome [7–9] and intracellular lipopolysaccharides (LPS) directly activate caspase 11, a key component of inflammatory response [10]. However, the ligands for other members of the NAIP family of NLR proteins remain elusive. Similarly, our understanding of the broader mechanisms underlying infection-induced death of immune cells is limited [11].
The pathogen *Legionella pneumophila* replicates within amoebae hosts in the environment; it also is able to grow in alveolar macrophages in the human lung, which causes Legionnaires’ disease. Intracellular replication of *L. pneumophila* requires the Dot/Icm type IV secretion system, which delivers hundreds of proteins into host cells to construct a niche supportive of bacterial growth [12–14]. Because its primary evolutionary pressure for virulence derives from life in amoebae hosts, *L. pneumophila* does not seem to have evolved sophisticated immune-evasive mechanisms. As a result, challenge of immune cells such as macrophages with this bacterium leads to strong immune responses often not seen with more finely-adapted pathogens [15]. Thus, *L. pneumophila* has emerged as a powerful tool in dissecting novel host immune recognition mechanisms [16]. For example, characterization of mutants capable of replicating in macrophages from incompatible mice led to the identification of flagellin as the bacterial factor responsible for triggering the strong immune response [17,18], which functioned by directly engaging NAIP5 to activate the NLRC4 inflammasome [4,5]. Similarly, mutants lacking effectors with similar biochemical activity have allowed the identification of protein synthesis inhibition as a signal for immune induction [19].

We recently found that an environmental *L. pneumophila* strain induces extensive cell death in mouse bone marrow-derived macrophages (BMDMs) that are permissive for commonly used laboratory strains [20]. Furthermore, the cell death differed from canonical apoptosis, necrosis or the caspase-1/11-dependent pyroptosis [20]. Here we found that clinical *L. pneumophila* strains induce similar responses. By identifying mutants capable of replicating in BMDMs, we found that the ribosomal protein RpsL is responsible for the restriction of replication. Although two mutations of RpsL both conferred bacterial resistance to the antibiotic streptomycin, one (K88R) allowed *L. pneumophila* isolates (both clinical and environmental) to grow in BMDMs, while the other (K43N) did not. Further cell biological studies revealed that RpsL induced cell death by triggering signaling cascades that led to lysosomal membrane permeabilization and subsequent cell death. Our results established RpsL as a ligand capable of triggering a unique immune recognition by inducing lysosomal cell death.

**Results**

**A K88R mutation in rpsL allows strain LPE509 to replicate in mouse primary macrophages**

To approach the mechanism underlying cell death induction by strain LPE509 [20], we hypothesized that this strain codes for unique factor(s) capable of triggering a signaling cascade that leads to cell death, and that mutants lacking such factor(s) may enable the bacterium to replicate in macrophages. To identify such factor(s), we attempted to generate an insertion mutant library of LPE509 with a transposon introduced by bi-parental mating. To acquire an antibiotic resistance marker for eliminating the *Escherichia coli* donor cells used to deliver the transposon, we first isolated spontaneous streptomycin resistant (StrepR) mutants of LPE509, and the transposition mutant pools generated from two such mutants were then tested for intracellular replication in BMDMs from A/J mice, which are permissive for intracellular replication of laboratory *L. pneumophila* strains. Unexpectedly, robust intracellular growth was observed in all mutant pools derived from one particular StrepR strain. Further analyses indicated that the parental strain used for mutant production had gained the ability to grow in BMDMs (Fig 1A). Sequencing analysis revealed a K88R mutation in the *rpsL* (lpg0324) gene of the StrepR mutant. To rule out the possibility that additional mutations contributed to this phenotype, we introduced the *rpsL*K88R allele into strain LPE509 by gene replacement to produce LPE509*<sup>rpsLK88R</sup>; in BMDMs from A/J mice, this strain grew at rates comparable to the laboratory strain Lp02*<sup>rpsLK88R</sup>, which increased almost 1000-fold in 72 hrs (Fig 1B). Consistent with
earlier observations [20], wild type bacteria were cleared by macrophages within the experimental duration (Fig 1B). These results indicate that RpsL may regulate intracellular replication of *L. pneumophila*. Because multiple mutations in the RpsL or the 16S rRNA can result in resistance to streptomycin [21], we assessed the intracellular growth of several independent streptomycin-resistant mutants for intracellular growth in BMDMs to determine whether all mutations that confer StrepR property of the bacteria.

Among 8 such mutants tested, 4 have overcome the restriction whereas the other 4 still were unable to replicate in macrophages (Figs 1B and 1C). Sequencing analysis of the *rpsL* gene in these mutants revealed that in each of the 4 mutants capable of replicating in BMDMs, an A to G mutation occurred at nucleotide 263, causing a Lys to Arg substitution in the 88th residue (K88R). In each of the 4 StrepR mutants still defective in replicating in BMDMs, an A to T mutation at nucleotide 129 that led to a Lys to Asn substitution (K43N) was detected (Fig 1C). In agreement with the fact that the K88R and the K43N mutations confer indistinguishable resistance to streptomycin in bacteria such as *E. coli* [22], *L. pneumophila* mutants harboring these two mutations exhibited similar levels of streptomycin-resistance: both with a minimal inhibitory concentration (MIC) greater than 100 μg/ml (S1 Table). These results suggest that the K88R mutation in RpsL (RpsLK88R) is specific for overcoming the growth restriction of strain LPE509 in BMDMs permissive for commonly used laboratory *L. pneumophila* strains.

To further determine whether StrepR *per se* is sufficient to permit intracellular growth in BMDMs, we introduced a plasmid expressing a streptomycin adenyltransferase [23] into strains LPE509. This strain has an MIC of 30 μg/ml (S1 Table), but cannot productively replicate in BMDMs (S2 Fig). Taken together, these results establish that a K88R mutation in the ribosome protein RpsL is sufficient to allow strain LPE509 to replicate in BMDMs and that...
resistance to Strep alone does not confer on *L. pneumophila* the ability to overcome the growth restriction imposed by these macrophages.

**RpsL is responsible for intracellular growth restriction of *L. pneumophila* in mouse primary macrophages**

Our discovery that the *rpsL*\_K88R mutation is required for *L. pneumophila* to grow in BMDMs is consistent with the fact that commonly used laboratory strains such as Lp02 [24] and JR32 [25] are resistant to streptomycin and that both harbor a K88R mutation in *rpsL* [26]. These results predicted that the parental streptomycin-sensitive strain Philadelphia 1 (Phil-1) [24] might be unable to replicate in BMDMs. Indeed, unlike its derivatives Lp02 (referred to as Lp02\_rpsL\_K88R for clarity), strain Phil-1 was unable to replicate in BMDMs (Fig 2A). Importantly, robust growth occurred when the *rpsL* gene of strain Phil-1 was replaced by homologous recombination with *rpsL*\_K88R but not with *rpsL*\_K43N (Fig 2A). Similar growth restriction was observed in strains Paris [27] and Thunder Bay [28], in which a K88R mutation in RpsL allowed both strains to grow robustly in BMDMs (S3 Fig). Furthermore, strain Lp02\_rpsL\_WT derived from Lp02\_rpsL\_K88R has lost the ability to grow in BMDMs (Fig 2B). This strain, however, retained the ability to replicate in both Hela cells and the human macrophage cell line U937 (S4 Fig). Again, introduction of the streptomycin adenyltransferase gene into strain LPE509 or Lp02\_rpsL\_WT conferred antibiotic resistance but not intracellular replication (S1 Table; S2 Fig), further supporting the notion that wild type RpsL is responsible for the restriction of bacterial replication in BMDMs. Taken together, these results indicate that wild type RpsL is the genetic determinant that governs the outcome of *L. pneumophila* infection in BMDMs; this protein restricts intracellular growth of *L. pneumophila* in BMDMs and that such restriction occurs in both clinical and environmental isolates. For clarity, we used strains Lp02\_rpsL\_K88R and Lp02\_rpsL\_WT in all subsequent experiments.

**Infection by *L. pneumophila* expressing wild type RpsL leads to macrophage death**

Because host cell death accompanied the restriction of intracellular growth in infections with strain LPE509 [20], we determined whether strain Lp02\_rpsL\_WT induces a similar phenotype. Compared to infections with strain Lp02\_rpsL\_K88R, significantly higher levels of lactate dehydrogenase (LDH) was detected in culture supernatant of BMDMs challenged with strain Lp02\_rpsL\_WT (Fig 3A). Single cell analysis by the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining revealed that at 12 hrs post-infection, close to 50% of the macrophages infected with Lp02\_rpsL\_WT stained positively for apoptosis, whereas only about 10% Lp02\_rpsL\_K88R infected cells appeared apoptotic (Fig 3B). At this time point, a fraction of the bacterial phagosomes have developed into vacuoles containing more than 10 bacteria. However, unlike Lp02\_rpsL\_K88R that can be distinctly stained by the anti-*Legionella* antibody, the staining signals of Lp02\_rpsL\_WT appeared diffuse, which could be the debris from dying bacterial cells detected by the polyclonal antibodies raised against fixed bacterial cells [29] (Fig 3C). Furthermore, although the presence of phagosomes with multiple bacteria is readily detectable in single cell analysis (Fig 3C and S5 Fig), the bacteria cells in these vacuoles appeared not to be viable as the total bacterial counts in samples infected with strains expressing wild type RpsL decreased at the 15 hrs or 24 hrs postinfection time points (Figs 1, 2 and S6).

Since inflammatory caspases such as caspase-1, 7 and 11 play important roles in macrophage death induced by bacteria infection [30,31], we thus examined whether these caspases play a role in the cell death induced by *L. pneumophila* expressing wild type RpsL. We infected macrophages from mice lacking caspase-1, 7 and 11 (caspase-1/7/11\(^{-/-}\)) and found that the
challenge of macrophages from these mice with strain Lp02rpsLWTΔflaA still led to cell death as measured by TUNEL staining. The reason to delete the flaA gene is that the genetic background of the knockout mice is C57BL/6, which is sensitive to flagellin produced in strain Lp02rpsLWT [17,18]. Again, infection by strain Lp02rpsLK88RΔflaA caused significantly less death in these cells than did infection with strain Lp02rpsLWTΔflaA (Fig 3D). Consistent with the observation of cell death, BMDMs lacking caspase-1, 7 and 11 did not support the growth of strain L. pneumophila expressing wild type RpsL cause macrophage death, which may contribute to the restriction of intracellular growth. Moreover, this cell death occurred in a mechanism independent of caspases-1, 7 and 11.

RpsL directly induces cell death in macrophages

Because the K43N mutation in RpsL imposes higher translation accuracy on the ribosome [32,33], it is unlikely that the cell death is caused by factors such as the potential increase in mistranslated bacterial proteins. The high level of conservation of RpsL among taxonomically diverse bacteria prompted us to hypothesize that this protein is a ligand capable of inducing macrophage death. Thus, we purified recombinant His6-RpsL and His6-RpsLK88R with a procedure employing the extensive isopropanol washing (methods) to exhaustively remove endotoxin (LPS) and delivered the proteins into macrophages by transfection [5]. When directly added to the BMDMs culture, neither protein caused detectable cell death (Fig 4A–B). However, when a transfection reagent was included, samples receiving His6-RpsL released significantly higher amount of LDH than samples receiving His6-RpsLK88R (Fig 4A). Similar results were obtained when cell death was evaluated by TUNEL staining in which close to 30% of cells receiving wild type RpsL were apoptotic, which is significantly higher than the <10% seen in samples transfected with His6-RpsLK88R (Fig 4B–C). Further, adding RNAase III to the protein did not alter its cell death induction activity, indicating that dsRNA [34] did not contribute to the observed phenotype (Fig 4D). On the other hand, treatment of protease K abolished the
cell death-inducing ability of the protein (Fig 4D). Consistent with the fact that strains expressing RpsL-K43N cannot replicate within BMDMs, His6-RpsL-K43N induced macrophage death at rates comparable to those of wild type protein (Fig 4E). In addition, that wild type RpsL but not the K88R mutant from E. coli also induced robust cell death in macrophages (Fig 4F), which is consistent with the high-level conservation (91% identity) of this protein between these two bacteria. Furthermore, the cell death induction is specific for RpsL, as another similarly purified ribosomal protein, His6-RpsM was unable to induce macrophage death (Fig 4F). Finally, consistent with the observation that macrophage death induced by L. pneumophila strains harboring wild type RpsL is independent of caspases 1/7/11 (Fig 3D), His6-RpsLWT induced cell death in macrophages lacking these caspases at rates comparable to those of C57BL/6 (Fig 4G–H). Because caspase-11 directly senses intracellular LPS [10], these results also indicate that the cell death was not caused by LPS potentially present in the RpsL protein preparations. Moreover, the fact that transfection is required for RpsL to exert its effects, this protein must be active only when it is present in the cytosol; this observation also suggested that RpsL

Fig 3. L. pneumophila strains harboring wild type rpsL caused cell death in infected macrophages. Bone marrow-derived macrophages from A/J mice were infected by the indicated bacterial strains for 14 hrs and health status of infected cells were evaluated by measuring the release of lactate dehydrogenase (LDH) (A) and by TUNEL staining (B) following by differently labeling extra- and intracellular bacterial by immunostaining. Infections were performed in triplicate and similar results were obtained in at least 3 independent experiments. For the TUNEL results, at least 300 infected cells were scored in each sample. C. Representative images of TUNEL signals and of bacterial phagosomes. Intracellular L. pneumophila bacteria were immunolabeled (red) with specific antibodies in BMDMs infected for 14 hrs; the status of host cell death was by TUNEL staining (green). Note the debris-like signals around the bacteria in samples infected with the strain harboring wild type RpsL. Images were acquired by a CCD camera with an Olympus IX-81 fluorescence microscope. Bar, 10 μm. D. Cell death caused by L. pneumophila occurs independent of the three inflammatory caspases, caspase-3, 7 and 11. BMDMs prepared from mice lacking caspases-3, 7 and 11 or its parental line C57BL/6 were infected with indicated L. pneumophila strains for 14 hrs and samples were processed to determine cell death as described in B. Note that the strain expressing wild type RpsL caused cell death in BMDMs from both mouse lines. The deletion of the flaA gene is to eliminate the effects caused by the NAIP5 allele in these mouse lines in response to flagellin.

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was delivered into the cytoplasm of the host cell by *L. pneumophila* during infection. However, we were unable to detect such translocation using the β-lactamase reporter [12,35] (S8 Fig).

The fact that *L. pneumophila* strains harboring wild type *rpsL* were able to replicate in alternative hosts such as Hela and U937 cells (S4 Fig) suggested that RpsL did not induce death in these cells. Indeed, Hela cells transfected with His6-RpsL did not detectably stain positively for apoptosis (S9A Fig). The lack of cell death is not due to deficiency in protein delivery because a similar procedure successfully introduced purified β-galactosidase into these cells (S9B–S9C Fig). Taken together, these results establish that wild type RpsL directly induces cell death and such induction is specific for primary mouse macrophages; the K88R mutant triggers similar responses but in a less potent manner.

**RpsL causes lysosome membrane permeabilization in macrophages**

Since macrophages from mice lacking the three inflammatory caspases are still sensitive to RpsL, it is very unlikely that the cell death is caused by canonical pyroptosis. We thus explored the involvement of other cell death pathways [36]. The lysosome, an organelle of low pH which contains a variety of hydrolases, has been implicated to play important roles in cell death in response to
certain stimuli [37]. Such stimuli could trigger lysosome membrane permeabilization (LMP), leading to the release into the cytosol of hydrolases such as cathepsins (e.g. cathepsin B, D, and L) from the lysosomal lumen, where they induce apoptotic or pyroptotic cell death through caspase activation, or necroptosis when caspase activation was inhibited [38]. If RpsL activates the lysosomal cell death pathway, then inhibiting lysosomal acidification should reduce its cytotoxicity. To test this hypothesis, we inhibited lysosomal acidification by bafilomycin A1 (BFA1), which targets the vacuolar ATPase responsible for regulating organelle pH [39] prior to RpsL transfection. Cell death was almost completely abrogated in samples pre-incubated with BFA1 (Fig 5A).

We next assessed the integrity of lysosomal membranes in macrophages receiving RpsL using acridine orange (AO), a lysotrophic dye that when concentrated in lysosome, emits orange fluorescence upon blue light excitation, but green fluorescence when the loss of lysosome membrane integrity redistributes it into the cytosol and nucleus [40]. In samples transfected with RpsL, about 40% of the cells had lost lysosomal membrane integrity, as indicated by the emission of green fluorescence (Fig 5B&D); this dropped to approximately 15% of the cells when RpsL_K88R was delivered into the cells (Fig 5B&D). In controls not transfected with the protein, essentially all macrophages emitted orange but not green fluorescence (Fig 5B&D). Similar results were obtained in infections in which strain Lp02_rpsLWT caused significantly higher levels of lysosome damage than strain Lp02_rpsLK88R (Fig 5C&E). Taken together, we conclude that RpsL induces LMP.

**Cathepsin B is required for efficient RpsL-induced macrophage cell death**

A direct result of LMP is the release of catabolic hydrolases, particularly cathepsin proteases, which have distinct roles in initiating cell death in different cell types [38]. We first used specific inhibitors to identify which cathepsin(s) are important for RpsL-induced cell death. CA074-Me, an inhibitor for cathepsin B (CtsB) but not pepstatin A, an inhibitor against cathepsin D antagonized RpsL-induced cell death (Fig 6A). Consistently, the cytosolic activity of cathepsins significantly increased upon RpsL delivery in a mechanism that required vacuolar acidification (Fig 6B). Notably, such increase also occurred in response to RpsL_K88R but at a significantly lower magnitude (Fig 6B). The strong inhibitory effects of CA074-Me on the cathepsin activity induced by RpsL suggest the selectivity of CtsB release, at least within our experimental duration.

To further examine the role of this protease in RpsL-induced cell death, we prepared macrophages from mice deficient in CtsB (ctsB⁻/⁻) or CtsH (ctsH⁻/⁻) and transfected them with recombinant RpsL. Six hours after transfection, cell death occurred in macrophages from both wild type and the ctsH⁻/⁻ mice, although the rates in cells from the latter mouse line were significantly lower (Fig 6C). At the 6-hr time point, consistent with the abolishment of RpsL-induced cell death by inhibition of CtsB, almost no TUNEL positive cells were detected in macrophages from ctsB⁻/⁻ mice (Fig 6C). These macrophages were not generally defective in apoptosis as staurosporine treatment induced extensive cell death (Fig 6C). Similar results were obtained in infections in which strain Lp02_rpsLWT induced significantly less cell death in BMDMs from ctsB⁻/⁻ mice 14 hrs after uptake (Fig 6D). To determine whether BMDMs that received RpsL maintained their plasma membrane integrity, we measured the level of LDH in similarly treated samples. Intriguingly, although wild type RpsL is consistently more potent than the K88R mutant in inducing LDH release, similar levels of LDH were detected in BMDMs from the three different mouse lines after RpsL transfection (Fig 6E). In light of this observation, we determined the kinetics of RpsL-induced cell death in ctsB⁻/⁻ BMDMs by TUNEL staining. Again, few TUNEL positive cells were detected in these cells within 6 hrs after transfection (Fig 6F). However, extensive cell death was observed in ctsB⁻/⁻ BMDMs 12 hrs after transfection; at this
time point, the rates of TUNEL positive cells among BMDMs from these three mouse lines became indistinguishable (Fig 6F). Consistent with these results, RpsL is able to induce the release of cathepsins such as aspartic cathepsin into the cytosol in ctsB−/− BMDMs (S10 Fig). These cathepsins may be responsible for the cell death observed when extended induction time was allowed after transfection. Taken together, these results establish that the lack of CtsB does not block but does significantly delays the cell death induced by RpsL.

Cathepsins in the cytoplasm are able to activate cell death by cleaving the BH3-Only protein Bid to produce truncated Bid (tBid), which subsequently inserts into the mitochondrial membranes, leading to cytochrome c (Cyto c) release and caspase activation [41] (Fig 7A).
with this notion, tBid was significantly more abundant in macrophages infected with strain Lp02rpsLWT than in those infected with strain Lp02rpsLK88R or samples infected with the dotA mutant (Fig 7B). Activation of caspase-3 and the subsequent cleavage of poly(ADP-ribose) polymerase (cPARP) also occurred more robustly in macrophages infected with Lp02rpsLWT (Fig 7B). Caspase-3 is activated in permissive macrophages infected with strains harboring RpsLK88R [12,42]; In agreement with the notion that RpsL induces cell death, such activation was significantly more robust in BMDMs infected with strains expressing wild type RpsL (Fig 7B). Furthermore, significantly more activated caspase-3 and cPARP can be detected in macrophages that received recombinant RpsL than those that received RpsL<sub>K88R</sub> (Fig 7C). Consistent with the involvement of the activation of caspase-3 here, both DEVD-FMK, a cell-permeable, irreversible caspase-3-specific inhibitor [43] and the pan caspase inhibitor z-VAD-FMK [44] significantly dampened the RpsL-induced cell death within the first 6 hrs of protein transfection (Fig 7D–E). Thus, the canonical apoptosis pathway contributes to the cell death induced by RpsL.
We next examined the ability of BMDMs from ctsB<sup>−/−</sup> mice to support replication of the strain Lp02<sub>rpsLWTΔflaA</sub>. Consistent with earlier observations [17,18], deletion of flaA allowed strain Lp02<sub>rpsLK88R</sub> to replicate in BMDMs from the C57BL/6 mouse background. Despite the lack of cell death induced by strain Lp02<sub>rpsLWTΔflaA</sub> at 12 hrs post infection (Fig 6D), ctsB<sup>−/−</sup> BMDMs were unable to support intracellular growth of this strain (S11 Fig). This suggests that the cell death occurred after bacterial replication has been aborted or that the cell death is one of the branches of the signaling cascade induced by RpsL, which is consistent with the plasma membrane damage observed in ctsB<sup>−/−</sup> BMDMs that received recombinant RpsL (Fig 6E).

**Discussion**

Genetically tractable pathogens are effective tools for probing host immune recognition mechanisms. By exploiting the cell death phenotype that restricts the replication of *L. pneumophila* in primary macrophages, we revealed that such cell death occurs in infections with essentially all wild type (streptomycin-sensitive) *L. pneumophila* strains, clinical or environmental. More importantly, we found that the ribosomal protein RpsL is directly responsible for such
restriction. Our results supports a model in which RpsL activates a pathway that leads to lysosomal membrane permeabilization (LMP) and the subsequent apoptotic cell death, which is accompanied by the termination of intracellular bacteria replication.

It seems clear that RpsL is responsible for the macrophage death caused by L. pneumophila infection. A K88R mutation in RpsL allows the bacteria to replicate proficiently in these cells and the replacement of such mutations with the wild type allele in a widely used replication-competent laboratory strain abolished its ability to grow intracellularly in BMDMs. Although RpsL_{K88R} confers resistance to streptomycin, several lines of evidence indicate that this antibiotic apparently plays no role in intracellular replication. First, although the rpsL_{K43N} mutation confers resistance to streptomycin indistinguishably to that of the K88R mutation, mutants harboring RpsL_{K43N} were unable to grow in BMDMs. Second, expression of a streptomycin inactivation enzyme did not allow the bacteria to grow intracellularly. Third, purified RpsL but not RpsM, another ribosomal protein, induces cell death in macrophages. Fourth, purified RpsL but not RpsM, another ribosomal protein, induces cell death in macrophages. Fourth, purified RpsL but not RpsM, another ribosomal protein, induces cell death in macrophages. Fourth, purified RpsL but not RpsM, another ribosomal protein, induces cell death in macrophages. Fourth, purified RpsL but not RpsM, another ribosomal protein, induces cell death in macrophages. Fourth, purified RpsL but not RpsM, another ribosomal protein, induces cell death in macrophages. Fourth, purified RpsL but not RpsM, another ribosomal protein, induces cell death in macrophages. Fourth, purified RpsL but not RpsM, another ribosomal protein, induces cell death in macrophages. Fourth, purified RpsL but not RpsM, another ribosomal protein, induces cell death in macrophages. Fourth, purified RpsL but not RpsM, another ribosomal protein, induces cell death in macrophages. Fourth, purified RpsL but not RpsM, another ribosomal protein, induces cell death in macrophages. Fourth, purified RpsL but not RpsM, another ribosomal protein, induc...
significantly better than their wild type counterparts in macrophages [50], likely due to less immune activation by RpsL$_{K88R}$ from mutant bacteria. The K88R mutation in RpsL may represent an evolved mechanism that enables the bacteria to gain resistance to both antibiotics and the innate immunity. Alternatively, antibiotics and host innate immune sensors could have converged to recognize the same constrained epitope of the pathogen.

The lysosome is emerging as a signal integration center for various stresses, including those that lead to cell death [38]. Our results indicate that sensing of RpsL leads to the activation of the lysosomal cell death pathway and the induction of LMP, which in turn releases the lysosomal hydrolases, particularly cathepsin B. In agreement with this notion, the cell death cannot be blocked by inhibitors of necrosis or classical apoptosis [20] but can be abrogated by agents targeting vacuolar acidification or cathepsins. LMP can be caused by diverse molecules such as reactive oxygen species (ROS), lipids and certain activated receptors such as TNF-α [38]. It is becoming evident that lysosomes do not indiscriminately release their contents, but rather, certain signals cause selective release of proteins such as cathepsins. For example, selective release of CtsB in response to different signals in different contexts leads to either tumor growth or apoptosis [51–54]. In neutrophils, cytosolic cathepsin D plays a major role in activating caspase-8 to resolve inflammation [55]. One challenge for future studies is to determine the mechanisms that govern the selectivity of cathepsin release in response to specific cues such as RpsL.

The inability of BMDMs from ctsB$^{-/-}$ mice to support intracellular replication of L. pneumophila strains expressing wild type RpsL is consistent with the apparent multiple effects of CtsB in cell death, including its role in the amplification of events of apoptosis [38] and, in some cell types, the timing of apoptosis [56]. It also agrees well with the fact that the plasma membranes of BMDMs from ctsB$^{-/-}$ can still be damaged after RpsL is introduced. CtsB itself is capable of directly causing cell death directly or by serving as a signal amplifier, which has been documented in TNF-α-associated LMP [57]. Alternatively, in addition to cell death, sensing RpsL by BMDMs may activate other yet unidentified pathways that lead to the arrest of bacterial replication. This potential multifaceted mechanism of action differs from that of a terminal cell death enzyme such as caspase-1, whose absence leads to limited growth of L. pneumophila strains expressing flagellin [58]. It is also possible that the arrest of bacterial replication occurs prior to macrophage death. One focus of the future studies will be the mechanism underlying both the cell death and the intracellular replication phenotypes.

That the cell death occurs only in primary mouse macrophages suggests that RpsL does not directly damage the lysosomal membranes and that its putative receptor clearly is more abundant in or specifically functions in these cells. Whether similar responses occur in immortalized mouse macrophage lines remains to be determined. Furthermore, it is possible that the pathway activated by RpsL exists only in mouse cells, perhaps because human cells are defective in one or more components of the pathway such as the putative receptor. Such potential differences may explain the fact that L. pneumophila strains expressing wild type RpsL are able to cause successful infections in humans. It is likely that RpsL engages the putative receptor to activate or to initiate the production of molecules capable of causing LMP. That RpsL-induced macrophage death occurs independent of several inflammatory caspases is consistent with the observation that we did not detect significant induction of inflammatory cytokines in macrophages upon RpsL delivery. The lysosomal cell death process is involved in the activation of the NLRC4 and NLRP3 inflammasomes that are induced by flagellin and cholesterol crystals, respectively [36,59]; whether RpsL-induced cell death is accompanied by any inflammation awaits further investigation. Clearly, identification of the putative receptor for RpsL will greatly facilitate the elucidation of the signaling pathway that leads to LMP. Such results will also aid in the study of the mechanism underlying the differences between mouse and humans in their response to wild type L. pneumophila.
Materials and Methods

Ethics statement

Primary murine macrophages were prepared from indicated mice lines, and the experiments were performed in strict accordance with the regulations of the Public Health Services (PHS) Policy on Humane Care and Use of Laboratory Animals. All animal procedures were performed according to a protocol approved by the Purdue Animal Care and Use Committee (protocol number: 04–081).

Bacterial strains, plasmids and media

*Legionella pneumophila* strain Philadelphia 1 [24], a generous gift from Dr. Isberg (Tufts Medical School, Boston, MA), its derivatives Lp02 (dot/icm<sup>+</sup>) (Lp02<sub>rpsLK88R</sub>) [24], Lp03(dotA<sup>+</sup>) (rpsLK88R) [24], JR32 [25] were used. Strains Paris [27] and Thunder Bay [28] were from Dr. Alex W. Ensinger (University of Toronto). For strains Lp02 and Lp03 and their derivatives that are thymidine auxotrophic [24], we introduced plasmid pJB908 expressing thyA [60] to make them thymidine autotrophic for all experiments. The environmental strain LPE509 was described earlier [20]. L. pneumophila was cultured on charcoal-yeast extract (CYE) plates or in ACES-buffered yeast extract (AYE) broth [61]. *Escherichia coli* strains were grown on L-agar plates or L broth, antibiotics were used at the following concentrations: For *E. coli*, Amp, 100 μg/ml; Km, 30 μg/ml; streptomycin 50 μg/ml. For *L. pneumophila*, Km was used at 20 μg/ml, Amp was used at 100 μg/ml and streptomycin was used at 100 μg/ml.

Spontaneous streptomycin resistant mutants were isolated by plating cultures of strain LPE509 onto AYE plates containing 100 μg/ml streptomycin. Colonies from independent cultures were purified and used for described experiments. The rpsL locus of each mutant was amplified by PCR and the PCR products were sequenced to determine the mutation. The sequence of the 16S rRNA was also determined via similar methods.

Allele exchange of the rpsL gene was performed using the standard method [62]. Briefly, the open reading frame of rpsL (lpg0324) together with a 300-bp flanking sequence was amplified from genomic DNA of wild type or rpsL<sub>K88R</sub> mutant strain of LPE509. The DNA digested with appropriate restriction enzymes was ligated to the *pir* protein-dependent plasmid pSR47s [63], to produce pZL<sub>rpsL</sub> and pZL<sub>rpsL<sub>K88R</sub></sub>, respectively. The primers used were: 5′-CTGGAGCTCAAAAGAAAACGTGATGGTA-3′ and 5′-CTGGTCGACGTACTTCCACTTGGGCGA-3′. Plasmids were introduced into appropriate *L. pneumophila* strains by electroporation, and transformants were streaked onto CYE plate supplemented with 5% sucrose and the desired strains were screened by the gain or loss of resistance to streptomycin followed by DNA sequencing of the locus.

Mice, bone marrow-derived macrophage, cell culture and bacterial infection

A/J and C56BL/6 mice were purchased from the Jackson laboratory (Bar Harbor, Maine). Caspase 1/11<sup>-/-</sup> [64] and the corresponding C56BL/6 control mice were similarly maintained. Mice lacking caspase 1, 7 and 11 were generated by intercrossing Casp1/11<sup>-/-</sup> mice [65] and Casp7<sup>-/-</sup>-mice [66] (purchased from Jackson Labs, Bar Harbor, ME). The cathepsin B [52] and cathepsin H [67] knock mice (cstB<sup>−/−</sup>, cstH<sup>−/−</sup>) and the control mice were maintained in the animal facility of New York University School of Medicine. Bone marrow-derived macrophages were prepared from 6–10-week old female mice with L-supernatant as described previously [68]. Macrophages were seeded in 24-well plate the day before infection. Cell density of 4x10<sup>5</sup>/well was used for intracellular bacterial growth and LDH release assay, whereas 2x10<sup>5</sup>/well was used for immunofluorescence staining and other single cell-based assays. *L. pneumophila* used for infection was grown in AYE broth to post-exponential phase based on both optical value (OD<sub>600</sub> = 3.4–4.0) and bacterial motility.
Intracellular bacterial growth

For intracellular growth curve in mouse macrophages, we infected the cells plated on 24-well at a multiplicity of infection (MOI) of 0.05. Two hours after adding the bacteria, we synchronized the infection by washing the cells with PBS for three times. Infected macrophages were incubated at 37°C with 5% CO₂. At the desired time point, the cells were lysed with 0.02% saponin, diluted lysate was plated on CYE plates, and CFU were determined from triplicate infections of each strain.

Antibodies, immunoblotting and immunostaining of infected macrophages

Mouse macrophages seeded on coverslips were infected with *L. pneumophila* at an MOI of 1, extracellular bacteria were removed by washing the cells with PBS for three times. Infections were terminated at appropriate time points by fixing with 4% paraformaldehyde. After fixation, samples were first stained for extracellular or total bacteria using anti-*L. pneumophila* antibodies [29] at a dilution of 1:10,000 and appropriate secondary antibodies conjugated to distinct fluorescent dyes. Processed coverslips were mounted on glass slides with an anti-fade reagent (Vector laboratories, CA). Samples were examined by visual inspection with an Olympus IX-81 fluorescence microscope.

For western blotting, anti-cleaved PARP was purchased from Abcam (ab2317). Anti-mouse-BID was purchased from R&D System (MAB860). Anti-cleaved caspase-3 was purchased from Cell Signaling Technology (#9664). Anti-tubulin was purchased from Developmental Studies Hybridoma at University of Iowa.

TUNEL staining

2x10⁵ mouse macrophages were infected with relevant *L. pneumophila* at an MOI of 1, and incubated for 12 hours after infection. Samples were fixed and stained with intracellular and extracellular bacteria as described above, and then stained with TUNEL using the *In Situ* Cell Death Detection Kit (Roche Diagnostics, Indianapolis). Fifty microliter TUNEL reaction mixture was added to each coverslip and incubated at 37°C for 1 hr. After 3x washes with PBS, coverslips were mounted with anti-fade reagent.

Intracellular protein delivery

Recombinant proteins were purified as previously described [69]. Briefly, recombinant proteins bound to Ni-NTA columns were subjected to extensive organic solvent wash (50x bed volume with wash buffer containing 60% isopropanol) to remove the majority of endotoxin contaminants [29]. β-galactosidase was purchased from Clontech. If necessary, BMDMs were treated with lysosome acidification inhibitor bafilomycin A1 (20 nM), cathepsin B inhibitor CA-074ME (25 μM), cathepsin D inhibitor pepstatin A (25 μM) or DMSO (vehicle) for 1 hour before protein transfection. The purified proteins were then introduced into macrophages using Lipofectamine 2000 as follows: For transfection of 2x10⁵ macrophages, 12.5-μg recombinant protein suspended in 50-μl RPMI-1640 and 2-μl Lipofectamine 2000 suspended in 50-μl RPMI-1640 were equilibrated at RT for 5 min, then mixed at RT for 30 min. The mixture was then directly applied to the macrophage cultures.

Acridine orange staining

BMDMs plated on glass coverslips at a density of 2x10⁵/well were either transfected with appropriate proteins or infected with different *L. pneumophila* strains at an MOI of 1. For infection, the samples were washed 3 times with warm PBS to synchronize the infection 2 hrs after
adding the bacteria. The transfected/infected samples were incubated for 8 hrs. After centrifugation at 200g for 5 min, the culture medium was removed, and 0.5 ml of the dye solution containing acridine orange (5 μg/ml) was added into each well. The plate was incubated at 37°C for 15 min before imaging using a fluorescence microscope (Olympus IX-81).

**LDH release assay**

LDH release during infection was determined using CytoTox 96 Assay (Promega, Madison, WI). 4×10^5 BMDMs were either transfected with appropriate proteins or infected with appropriate *L. pneumophila* strains at an MOI of 1. Four or six hours post transfection/infection, cell culture was centrifuged at 200g for 5 min, and 50-μl supernatant of each well was transferred to a new 96-well enzymatic assay plate. Fifty microliter reconstituted Substrate Mix was then added to each well of enzymatic assay plate, and incubated in dark at room temperature for 30 min. The enzymatic reaction was stopped by adding 50-μl stop solution to each well. The absorbance at 490 nm was measured using a Biotek microplate reader. Total LDH release was determined by complete lysis of the cells using a lysis solution provided by the manufacturer, and spontaneous LDH release was determined by using the supernatant of cells without infection/transfection. The percentage of LDH release was calculated with the follow formula: LDH release (%) = (Experimental LDH release-Spontaneous LDH release)/(Total LDH release-Spontaneous LDH release)x100.

**Cytosolic cathepsin activity**

4×10^5 BMDMs from A/J mice were plated in 24-well plates. RpsL proteins were delivered into the cells as described above. 6 hours post protein transfection, cytosolic cathepsin B activity was determined as previously described [70]. Briefly, macrophages were washed with phenol red and HEPES free DMEM (Invitrogen, OR). 100μg/ml saponin was added to each well and samples are incubated for 10 min on ice. Cell lyzates was cleared at 500xg for 10 min at 4°C in flat-bottom 96-well plates. 10 μl of the cell lysate was then added to 90 μl of the cathepsin B substrate buffer (100 μM zRR-AMC, 50 mM sodium acetate [pH 6.0], 4 mM EDTA, 10 mM dithiothreitol, 1 mM Pefablock). The generation of free AMC was determined by recording fluorescence (excitation, 355 nm; emission, 460 nm) at 30° in a TECAN multiplate reader. Cathepsin B activity was determined by measuring the increase in AMC fluorescence. Aspartic cathepsin activity was determined by measuring the aspartic cathepsin activity in total (0.02% Triton) or cytosolic fraction using Mca-KPLGL-Dpa-AR-NH2 Fluorogenic Peptide (R&D, cat# ES010), in a buffer containing 0.1 M NaOAc and 0.2 M NaCl, pH 3.5.

**Supporting Information**

S1 Table. The minimal inhibition concentration (MIC) of streptomycin for *L. pneumophila* strains.

(DOCX)

S1 Fig. Intracellular replication of *L. pneumophila* streptomycin resistance mutants in mouse bone marrow-derived macrophages. Eight spontaneous streptomycin resistance mutants isolated by plating bacterial cultures onto bacteriological medium grown to post exponential phase were used to infect BMDMs from A/J mice at an MOI of 0.05. After synchronization of the infections 2 hrs post infection, total bacterial counts were determined at the indicated time points. Infections were performed in triplicate and results shown are one of two experiments done independently.

(TIFF)
S2 Fig. Resistance to streptomycin did not allow *L. pneumophila* strains harboring wild type *rpsL* to replicate in A/J mouse macrophages. A plasmid expressing a streptomycin adenyltransferase that confers resistant to the antibiotic was introduced into *L. pneumophila* strain LPE509 and the Lp02 derivative harboring wild type RpsL(Lp02rpsLWT). The resulting bacterial strains were used to infect BMDMs from A/J mice together with relevant controls strains at an MOI of 0.05. After synchronization at 2 hrs psi, total bacterial counts (colony-forming-unit) were determined at indicated time points by plating appropriately diluted saponin solubilized infected cells onto bacteriological medium. Infections were performed in triplicate and data shown were from one representative of three independent experiments with similar results.

(TIFF)

S3 Fig. Primary macrophages from A/J mice do not support replication of wild type clinic strains. Clinic *L. pneumophila* strain Paris, Thunder Bay and relevant control strains were used to infect primary macrophages from A/J mice at an MOI of 0.05. Total bacterial counts were determined following standard procedures. Infections with each strain were performed in triplicate and similar results were obtained in two independent experiments.

(TIFF)

S4 Fig. Laboratory strain Lp02 expressing wild type RpsL replicates robustly in two human cell lines. Strain Lp02rpsLWT and Lp02rpsLK88R were used to infect Hela cells (A) or the human macrophage cell line U937 (B) at an MOI of 0.05. After 2 hrs incubation, infections were synchronized by washing 3 times with warm PBS. Total bacterial counts were determined at indicated time points by spreading appropriately diluted lysates of infected samples onto bacteriological medium. Similar results were obtained in two independent experiments.

(TIFF)

S5 Fig. *L. pneumophila* strains expressing wild type RpsL is able to form phagosomes with multiple bacteria. BMDMs from A/J mice were infected with the indicated strains of *L. pneumophila* at an MOI of 1 for 14 hrs and the infected samples were fixed for immunostaining. Extracellular and intracellular bacteria were sequentially labeled Legionella-specific antibodies and secondary antibodies conjugated with distinct fluorophores. The size distribution of the phagosomes was scored by counting the number of bacteria in the vacuoles. Phagosome categories: 1–3 bacteria, small vacuoles; 4–9 bacteria, medium vacuole; more than 10 bacteria, large vacuoles. At least 150 phagosomes were scored from each infection done in triplicate. Similar results were obtained in two independent experiments.

(TIFF)

S6 Fig. Viable bacteria of strains expressing wild type RpsL or the K43N mutant decrease in the first infection cycle in macrophages. Indicated *L. pneumophila* strains grown to post-exponential phase were used to infect BMDMs from A/J mice at an MOI of 0.05. After synchronization at 2 hrs after adding the bacteria to the cell culture, total bacterial counts were determined every 3 hours by spreading saponin lysates of infected samples onto bacteriological medium. Note that only the strain expressing RpsL_K88R displayed significant increase in total viable bacterial counts. On the other hand, the number of colony-forming unit for both strain Lp02rpsLWT and Lp02rpsLK43N decreased in the experimental duration.

(TIFF)

S7 Fig. Caspase-1, 7 and 11 are dispensable for growth restriction of *L. pneumophila* bearing wild type *rpsL*. Bone marrow-derived macrophages from C57L/B6 (A) or caspase-1/7/11−/− (B) mice were challenged with indicated bacterial strains grown to post-exponential phase at
an MOI of 0.05. After synchronization 2 hrs postinfection, total bacterial counts (colony-forming-unit) were determined at indicated time points. Infections were performed in triplicate and data shown were from one representative of three experiments with similar results.

(TIFF)

S8 Fig. RpsL is not detectably translocated into host cells by *L. pneumophila*. A. RpsL and RpsL*K88R* were fused to β-lactamase on a plasmid, respectively and the resulting constructs were transformed into *L. pneumophila* strains. The bacterial strains were used to determine Dot/Icm-dependent protein translocation with the CCF4-AM substrate with an established protocol. Strains expressing RalF or FabI were used as positive and negative controls, respectively. Note the robust translocation by the RalF construct indicated by the appearance of blue cells after bacterial infection. B. Expression of the relevant fusions in *L. pneumophila*. The bacterial cells used for infection in (A) were probed for the expression of the fusion with a β-lactamase specific antibody. The isocitrate dehydrogenase (ICDH) was probed as a loading control.

(TIFF)

S9 Fig. RpsL does not induce cell death in Hela cells. Hela cells seeded on coverslips were transfected with His6-RpsL, His6-RpsL*K88R* (A-B) or their mixture with β-galactosidase (C). Samples treated with staurosporine were established as positive controls. The samples were processed for TUNEL staining or for β-galactosidase staining with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (B-C) to evaluate the effectiveness of transfection. Assays were performed in triplicate and similar results were obtained in two independent experiments. Note the X-gal staining of transfected β-galactosidase (blue) in C. Bar, 20 μm.

(TIFF)

S10 Fig. RpsL induces the release of aspartic cathepsins into the cytosol in BMDMs from ctsB−/− mice. BMDMs from the indicated mouse lines were transfected with RpsL or RpsL*K88R* for 6 hrs; samples receiving only the transfection reagent were also included as controls. The activity was determined by measuring the aspartic cathepsin activity in total (0.02% Triton) or cytosolic fraction using the Mca-KPLGL-Dpa-AR-NH2 Fluorogenic Peptide (R&D Systems, cat# ES010). Similar results were obtained in two independent experiments.

(TIFF)

S11 Fig. BMDMS from ctsB−/− mice do not support intracellular replication of *L. pneumophila* strains expressing wild type RpsL. Bone marrow-derived macrophages from C57L/B6 (A), ctsB−/− (B) or ctsH−/− (C) mice were challenged with indicated bacterial strains grown to post-exponential phase at an MOI of 0.05. After synchronization 2 hrs postinfection, total bacterial counts (colony-forming-unit) were determined at indicated time points. Infections were performed in triplicate and data shown were from one representative of three experiments with similar results.

(TIFF)

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Author Contributions
Conceived and designed the experiments: WZ LT ZQL. Performed the experiments: WZ LT. Analyzed the data: WZ LT ZQL. Contributed reagents/materials/analysis tools: MLQ JAJ JMQ. Wrote the paper: WZ ZQL.

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