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A bacterial protein promotes the recognition of the *Legionella pneumophila* vacuole by autophagy

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Abstract

Legionella pneumophila (L. pneumophila) is an intracellular bacterium of human alveolar macrophages that causes Legionnaires' disease. In contrast to humans, most inbred mouse strains are restrictive to *L. pneumophila* replication. We demonstrate that autophagy targets *L. pneumophila* vacuoles to lysosomes and that this process requires ubiquitination of *L. pneumophila* vacuoles and the subsequent binding of the autophagic adaptor p62/SQSTM1 to ubiquitinated vacuoles. The *L. pneumophila legA9* encodes for an ankyrin-containing protein with unknown role. We show that the *legA9* mutant is the first *L. pneumophila* mutant to replicate in wild-type (WT) mice and their bone marrow derived macrophages (BMDMs). Less *legA9* mutant-containing vacuoles acquired ubiquitin labeling and p62/SQSTM1 staining, evading autophagy uptake and avoiding lysosomal fusion. Thus, we describe a bacterial protein that targets the *L. pneumophila* -containing vacuole for autophagy uptake.

Keywords

Autophagy; Ubiquitination; Lysosomes; Trafficking; Macrophage

Introduction

L. pneumophila, the causative agent of Legionnaires' disease, is a intracellular pathogen that replicates within human alveolar macrophages [1]. Upon internalization by human macrophages, *L. pneumophila* disrupts normal endocytic pathway trafficking to establish a replicative vacuole, this specialized niche allows the bacterium to multiply exponentially [1, 2]. In contrast to human, most inbred mice strains are restrictive to *L. pneumophila*

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Ethic statements: This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institute of health and The Ohio State University. The Institutional Animal Care and Use Committee (IACUC) have approved our protocol number 2007A0070. Human cells were obtained from anonymous samples from Red Cross.

replication [3-7]. Autophagy is among various mechanisms that restrict *L. pneumophila* growth [5-9]. Autophagy is a lysosomal degradation pathway that maintains cellular homeostasis by degradation of the cell's internal components in response to starvation and stress [10, 11]. Further, autophagy contributes to the control of variety of bacterial infections [12-16]. Central to this pathway is formation of the autophagosome, a double membrane sac-containing the sequestered cytoplasmic material. Atg8-microtubule associated protein1 light chain 3 (LC3), is the hallmark of autophagosome formation [12, 17-19]. Autophagolysosomes are generated by fusion of autophagosomes and lysosomes, where the sequestered cargo is degraded [8, 11, 12]. Autophagic clearance of protein aggregates requires ubiquitin-binding proteins such as p62/SQSTM1 [20, 21]. Using separate domains, p62 binds ubiquitin and LC3, therefore delivering ubiquitinated targets to the autophagosome for degradation [11, 21].

The genome of *L. pneumophila* contains several genes predicted to encode homologous eukaryotic-like protein domains and some of them have been implicated in *L. pneumophila* pathogenesis [22-24]. Ankyrin proteins are distributed within the nucleus and cytoplasm of eukaryotic cells, and play an essential role in cell cycle, motility, oncogenesis and transcriptional regulation [25-27]. *LegA9* was identified among other *L. pneumophila* eukaryotic-like genes in a bioinformatics screen of the *L. pneumophila* Philadelphia-1 genome and encodes for an ankyrin-containing protein [28]. However, the role of LegA9 in *L. pneumophila* pathogenesis is not known.

Here we show that in the restrictive BMDMs, the *legA9* mutant replicates substantially. This is the first *L. pneumophila* mutant to be found to replicate in WT BMDMS. In contrast to WT, *legA9* mutant vacuoles showed significant reduction in ubiquitin labeling and colocalization with p62, thereby avoiding uptake by autophagosomes, and enabling intracellular growth. Our data support a mechanism whereby LegA9 facilitates recruitment of the autophagic machinery to *L. pneumophila* vacuoles resulting in clearance of infection. This study provides a mechanistic link between LegA9 and clearance of *L.* p*neumophila* in WT macrophages by autophagy.

Results

WT mice and their BMDMs are restrictive to parent *L. pneumophila* intracellular replication but permissive to *legA9* mutant strain

To characterize the role of LegA9 in *L. pneumophila* pathogenesis, we examined the intracellular replication of the JR32 and *legA9* mutant in WT BMDMs. In contrast to the restriction of JR32 in WT macrophages, the *legA9* mutant showed a 10 fold increase in the colony forming units (CFUs) over time (48-72 hr) (Fig. 1A). Complementation of *legA9* mutation on *LegA9* + *LegA* pBC-KS+ plasmid restricted the replication of this strain in a comparable manner to JR32 in the presence of IPTG (Fig. 1B).

Since Legionnaires' disease is caused by replication of *L. pneumophila* in alveolar macrophages [1, 29], we investigated the role of LegA9 for bacterial replication within the lungs of live mice. C57BL/6 mice were infected with 1×10^6 JR32 or the *legA9* mutant intratracheally [5]. CFUs after 4 hr of infection denoted the initial bacterial load in the lungs (Fig. 1C); whereas the lungs of infected mice harbored 3 fold more *legA9* mutant bacteria compared to the JR32 strain 48 hr post infection (Fig. 1D).

Flagellin mediates restriction of *L. pneumophila* in murine macrophages and *flaA* mutant bacteria replicate significantly more than the parent strain [5]. Western blot analysis of *legA9* mutant bacterial lysate shows that it contains flagellin comparable to WT (Supplementary 1A). Furthermore, the *legA9* mutant bacteria express a functional Dot/Icm

system as shown by the normal lysis of sheep RBCs (Supplementary 1B). Together, these data indicate that LegA9 plays a role in restricting *L. pneumophila* infection *in vitro* and *in vivo*.

The *legA9* mutant replicates substantially in human monocyte-derived macrophages (hMDMs)

Human are permissive to *L. pneumophila* replication [30]. To characterize the role of LegA9 in the intracellular replication of *L. pneumophila* in human, we tested the intracellular growth of JR32 and *legA9* mutant in acute monocytic leukemia cell line (THP-1). THP-1 derived macrophages are permissive to parental strain JR32 and were more permissive to *legA9* mutant at 48 and 72 hr post infection (Supplementary 2A). In addition, we evaluated the intracellular growth of *legA9* mutant in hMDMs. The *legA9* mutant replicated a 100 fold more than JR32 in hMDMs (Supplementary 2B). Therefore, both JR32 and *legA9* mutant replicated in hMDMs yet, *legA9* mutant replicated significantly more than the JR32 strain.

Vacuoles harboring *legA9* mutant bacteria evade fusion with lysosomes and escape recognition by autophagy in WT BMDMs

It is possible that the *legA9* mutant replicated in WT macrophages because it evaded lysosomal fusion [8, 9, 31]. Thus, we examined the colocalization of JR32 and *legA9* mutant-containing vacuoles with lysotracker red, a dye that stains acidic compartments in live cells and is retained after aldehyde fixation (Fig. 2A). Approximately, 60% of JR32 bacteria were delivered to lysotracker-labeled compartments within 1 hr of infection. In contrast, only ∼30% of *legA9* mutant bacteria colocalized with lysotracker-labeled compartments even at 6 hr post infection (Fig. 2B). Therefore, vacuoles containing *legA9* mutant bacteria evade lysosomal fusion.

In restrictive murine macrophages, autophagic machinery recognizes the *L. pneumophila* phagosome as cargo [8, 32, 33]. Autophagosomes harboring *L. pneumophila* fuse with lysosomes and within the autophagolysosome, *L. pneumophila* is degraded and the infection is contained [8]. It is possible that *legA9*-containing vacuoles escape recognition by the autophagy machinery and therefore are not delivered to lysosomes for degradation. Consequently, acquisition of endogenous autophagy marker LC3 by JR32 or *legA9*-containing vacuoles was examined. In contrast to JR32, *legA9*-containing vacuoles showed less colocalization with LC3 (Fig. 2C and D). Approximately 55% of JR32-containing vacuoles acquired LC3 staining; whereas ∼30% of *legA9*-containing vacuoles acquired the autophagy marker. Together, *legA9* mutant vacuoles evade the recognition by autophagy and lysosomal fusion resulting in growth in murine macrophages.

Rapamycin treatment increases LC3 acquisition by the *legA9*-containing vacuole and restricts its intracellular replication

The above data suggested that the *legA9*-containing vacuoles are not recognized as readily as the parent strain as autophagic cargo for lysosomal delivery. Accordingly, we determined the effect of autophagy induction on trafficking of *legA9*-containing vacuoles [8, 34]. Rapamycin inhibits the mammalian target of rapamycin (mTOR) pathway and has been used to stimulate autophagy [35]. BMDMs were pre-treated with rapamycin for 1 hr prior to infection with JR32 and *legA9* mutant. CFUs enumerated at 1 hr (Fig. 3A) denote equal initial inocula. Induction of autophagy by rapamycin resulted in a significant decrease in the CFUs of the *legA9* mutant as compared to non-treated macrophages at 24 hr (Fig. 3B). The decrease in bacterial number is not a result of rapamycin or DMSO-mediated toxicity (Supplementary 3). Furthermore, rapamycin treatment increased co-localization of the *legA9*-containing vacuoles with lysotracker (Fig. 3C) and LC3 (Fig. 3D) at 2 hr post infection. To confirm the role of autophagy in *L. pneumophila* pathogenesis, we examined

the effect of bafilomycin, an inhibitor of lysosomal acidification, on *L. pneumophila* replication. Our data demonstrate that bafilomycin treatment resulted in an increase in JR32 CFUs 72 hr post infection (Supplementary 4). Moreover, 3-methyladenine, an inhibitor of PI3 Kinases increased JR32 CFUs 24 hr post infection (Supplementary 5). Therefore, drugs that induce or inhibit autophagy modulate the ability of *L. pneumophila* to replicate within macrophages.

To further confirm the role of autophagy in controlling *L. pneumophila* infection in macrophages, we compared the growth rate of JR32 in WT and $Atg5^{-/-}$ derived BMDMs. Atg5 is an essential molecule required for autophagy [10], and functions as an essential mediator of LC3-I to LC3-II conversion [19]. $Atg5^{-/-}$ cells are unable to execute autophagy as a result of a defect in autophagosome formation [19]. Both the JR32 and *legA9* mutant CFUs were higher at 72 hr (Fig. 4A). These data suggest that a defect in autophagy in the $Atg5^{-/-}$ mice compromised the clearance of *L. pneumophila*. We confirmed the lack of Atg5 in the $Atg5^{-/-}$ macrophages by Western blot (Fig. 4B). Furthermore, treatment of infected $Atg5^{-/-}$ BMDMs with JR32 or the *legA9* mutant with rapamycin did not alter the bacterial number at 1 hr (Supplementary 6A and C) or 24 hr (Supplementary 6B and D) post infection. These data suggest that rapamycin is not able to induce autophagy in the absence of the essential autophagy protein Atg5.

It is possible that LegA9 exert its effect locally on *L. pneumophil*a vacuole or globally affecting autophagy throughout the macrophages. To differentiate between these possibilities, we infected WT macrophages with the JR32 and *legA9* mutant bacteria and examined the endogenous LC3-I conversion to LC3-II by Western blotting before and after rapamycin treatment. Rapamycin induced autophagy as determined by increase in the level of LC3-II in 2, and 4 hr post infection in both JR32 and l*egA9* infected macrophages (Supplementary 7A). Despite the induction of autophagy by rapamycin in both JR32 and *legA9* infected cells, there were no difference in the level of LC3-II between the JR32 and the *legA9* infected macrophages (Supplementary 7B). These data suggest that rapamycin induced autophagy to the same level in both the JR32 and the *IegA9* infected macrophages.

Significantly fewer vacuoles-containing the *legA9* mutant acquire ubiquitin compared to vacuoles harboring JR32

Ubiquitinated cargos are delivered to the autophagic machinery through a variety of adaptor proteins [11, 21, 36]. The specificity and targeting mechanisms of *L. pneumophila*-containing vacuoles by autophagy are yet to be elucidated. *L. pneumophila*-containing vacuoles are decorated with polyubiquitinated proteins in murine BMDMs [37, 38]. We hypothesize that ubiquitination of *L. pneumophila* vacuoles tags them for autophagosome uptake. Accordingly, we examined the acquisition of ubiquitin by vacuoles-containing JR32 or *legA9* mutant. Approximately 70-75% of vacuoles-containing JR32 associated with ubiquitin in murine BMDMs at all time points. In contrast, only 45% of vacuoles containing *legA9* mutant bacteria were associated with ubiquitin (Fig. 5A and B). These data indicate that fewer *legA9*-containing vacuoles acquire ubiquitin. Since it is possible that infection with the *legA9* mutant affects the ubiquitination level within the cell, we examined total ubiquitination via Western blotting (Supplementary 8) and found that the defect in colocalization of the *legA9* mutant vacuoles with ubiquitin is not a consequence of ubiquitin depletion.

To examine the role of the proteosomal pathway is controlling *L. pneumophila* growth, we inhibited proteasome function with (MG-132). Proteasomal inhibition did not affect CFUs recovered after infection with JR32 and *legA9* mutant bacteria at 1hr (Supplementary 9A and C) or 24 hr (Supplementary 9B and D). These data suggest that the proteasomal pathway is not required for clearance of *L. pneumophila*.

p62/SQSTM1 targets *L. pneumophila*-containing vacuoles to autophagosomes for degradation in WT BMDMs

Vacuoles-containing JR32 readily colocalize with ubiquitin; whereas the *legA9*-containig vacuoles do so less frequently. The selectivity of this process can be mediated via several adaptor proteins that link ubiquitination to autophagy [20]. Among these adaptors is the p62/SQSTM1, which is able to bind ubiquitin and LC3, therefore delivering ubiquitinated targets to the autophagosome for degradation [11, 21, 36]. Thus, we examined the colocalization of vacuoles-containing JR32 and *legA9* mutant with p62. The *legA9*-containing vacuoles colocalize less frequently with p62 when compared to the JR32 strain (Fig. 6A and B). It is plausible that infection with the *legA9* mutant results in downregulation of p62. Therefore, we examined the level of p62 by ELISA in total cell lysates from BMDMs infected with JR32 or the *legA9* mutant for 6 hr. The level of p62 increased following infection with JR32 or the *legA9* mutant compared to non-treated macrophages (Fig. 6C). These results indicate that *legA9*-containing vacuoles are defective in colocalization with p62 despite its abundance in macrophages.

To determine if p62 targets *L. pneumophila* vacuoles for degradation, p62 level was downregulated in murine macrophages with specific siRNA. Twenty four hr post nucleofection macrophages were infected with the JR32 or the *legA9* mutant. Equivalent numbers of bacteria were recovered after one hour of infection (Fig. 6D). After 24 hr of infection, WT BMDMs did not allow *L. pneumophila* replication, yet, downregulation of p62 resulted in a significant increase in CFUs (Fig. 6E). Downregulation of p62 did not result in an increase in CFUs of the *legA9* mutant (Fig. 6F and G). The downregulation of p62 was confirmed by Western blot analysis of SiRNA-treated macrophage with the p62 antibody (Fig. 6H). Therefore, p62/SQSTM1 targets *L. pneumophila*-containing vacuoles to the autophagosomes to restrict its growth in WT murine macrophages. NDP52 is another adaptor protein that acts cooperatively with other adaptor proteins to drive efficient antibacterial autophagy of *Salomonella*, *Shigella* and *Lesteria*[39-41]. To examine the role of NDP52 in delivering *L. pneumophila* vacuoles to autophagosomes, we nucleofected WT BMDMs with siRNA against NDP52 prior to infection with JR32 or *legA9* mutant bacteria (Supplementary 10). Our data show that down-regulation of NDP52 does not alter CFUs recovered 24 hr post infection.

The permissiveness of BMDMs to *legA9* mutant bacteria is independent of caspase-1 or caspase-7 activation

Caspase-1 mediated caspase-7 activation restricts *L. pneumophila* by promoting fusion of *L. pneumophila* vacuoles with lysosomes [5, 8, 9]. Replication of the *legA9* mutant could be a result of impaired activation of caspase-1. Casp-$1^{-/-}$ BMDMs allow for growth of the JR32 strain [6]. Yet, replication of the *legA9* mutant surpassed that of JR32 at 24, 48, and 72 hr post infection (Fig. 7A). Caspase-1 activation is associated with cleavage and maturation of IL-1β. Our data show that there is no significant difference in the level of IL-1β produced from WT macrophages infected with JR32 or the *legA9* mutant for 24 hr (Fig. 7B). To determine if the replication of *legA9* is due to a lack of caspase-1 activation, detection of the mature subunits of casp-1 was performed by Western blot. Infection of WT BMDMs with JR32 or the *legA9* mutant was accompanied by caspase-1 activation (Fig. 7C). Taken together, these data suggest that the ability of the *legA9* mutant to replicate within WT macrophages is independent of caspase-1 activation.

At physiological levels of infection, *L. pneumophila* activates caspase-7 in a caspase-1-dependent manner [6]. Caspase-7 activation during *L. pneumophila* infection restricts growth by promoting early macrophage death and efficient delivery of the bacterium to the lysosome [6, 42]. It was possible that replication of the *legA9* mutant is due to diminished

caspase-7 activation. Therefore, activation of caspase-7 was assessed by Western blot. The absence of *legA9* did not compromise caspase-7 activation in WT macrophages (Fig. 7D). Therefore, our data demonstrate that replication of the *legA9* mutant occurs irrespective of caspase-1 or caspase-7 activation.

The permissiveness of BMDMs to *legA9* mutant is independent of host cell death

Early macrophage death terminates the replication cycle of *L. pneumophila* and prevents the establishment of infection [43]. It is plausible that the *legA9* mutant manipulates the macrophage life span to maintain replication. Accordingly, the role of LegA9 in induction of cell death was examined. We measured apoptosis in the overall population of macrophages by determining the cytoplasmic apoptosis histone-associated-DNA-fragments at MOI of 0.5, 1 and 5. Our results demonstrate that infection by JR32 and the *legA9* mutant led to comparable cell death at low and high MOIs (Fig. 8A).

To understand the role of LegA9 in *L. pneumophila*-mediated host cell necrosis, we measured the level of LDH release in culture supernatants of WT BMDMs infected with JR32 or the *legA9* mutant. Our data demonstrated that at an MOI of 0.5 or 5, comparable LDH is released after 24 hr of infection with JR32 and the *legA9* mutant (Fig. 8B). Together, our data demonstrate that the ability of *legA9* to replicate in WT macrophages is not due to extension of macrophage survival.

Discussion

LegA9 (lpp2058) was identified among *L. pneumophila* eukaryotic-like genes in a bioinformatics screen of the *L. pneumophila* Philadelphia-1 genome and encodes for an ankyrin-containing protein with unidentified role [28]. We showed that *L. pneumophila* lacking *legA9* replicate in WT mice and their derived macrophages. Several eukaryotic pathways contribute to the restriction of *L. pneumophila* growth in WT BMDMs [5-7], among which WT macrophages efficiently activate autophagy and deliver *L. pneumophila* vacuoles for lysosomal degradation. However, it is still unclear what tags the *L. pneumophila* vacuole for autophagy uptake.

Our data suggests that LegA9 protein labeled the *L. pneumophila* vacuole for autophagy uptake but does not alter cellular autophagy activity since LC3II is produced upon both JR32 and *legA9* mutant infections. Additionally, rapamycin treatment activated autophagy in both JR32 and *legA9* infected macrophages, denying a role for LegA9 in autophagy activation.

We showed that ubiquitination of *L. pneumophila* vacuoles targets them to autophagy in a process that is dependent upon recognition of the ubiquitinated vacuoles by the adaptor p62, thus delivering them to autophagosomes for degradation. In contrast to JR32, *legA9* mutant vacuoles significantly evade recognition by p62 and autophagosomes thus escaping delivery to lysosomes. AJ derived murine macrophages are permissive to *L. pneumophila* and used to study pathogenesis. Interestingly, vacuoles harboring WT *L. pneumophila* derived from AJ macrophages exhibits K48 and K63 polyubiquitinated proteins [37, 38]. Generally, lysine 48 conjugated ubiquitin moieties to proteins target them to proteasomal degradation while lysine 63 modulates protein activity and trafficking in the cell [44, 45]. Host proteasomal degradation of Lys^{48}-linked polyubiquitinated proteins, assembled on the *L. pneumophila* vacuole generate amino acids required for intracellular bacterial survival and growth [46]. However, we showed that ubiquitination of *L. pneumophila* vacuoles in restrictive macrophages targets them to autophagy machinery for degradation of their contents. Thus ubiquitination can be useful for the bacteria to provide nutrition within the vacuole but could also be detrimental by tagging the vacuole for autophagosome recognition. Thus it is

necessary for *L. pneumophila* to delay the maturation of its containing vacuole [8] by inhibiting autophagy at a specific stage [47]. *L. pneumophila* express RavZ, (a cysteine protease that specifically deconjugates Atg8 proteins from phospholipid membranes, thus inhibiting autophagy [47]. We think that LegA9 performs functions beyond vacuole tagging since *L. pneumophila* lacking *legA9* replicates more than the JR32 strain in the Atg5-/- macrophages with defective autophagy system.

It seems counterintuitive that *L. pneumophila* would retain *legA9* in the genome, even though it is not required for replication in *Acanthamoeba castellanii*. It has been suggested that maintaining redundant genes may prevent single genetic lesions from enabling the bacteria to multiply intracellularly [28]. Our study shows that when *L. pneumophila* lacks LegA9 it replicates more efficiently than the parental strain in human macrophages, thus making the bacterium more pathogenic. Furthermore, cycling of *L. pneumophila* through multiple protozoan hosts in the environment prevents *L. pneumophila*-host specialization by maintaining pathways that are required for growth in macrophages and other hosts. However, growth restriction of *L. pneumophila* in the murine model or under laboratory conditions is sufficient to change the fitness and host range of this bacterium [48]. Restriction of *L. pneumophila* replication in murine macrophages by acquisition of the LegA9 factor could provide an example of selective pressure to increase adaptability of this bacterium to infect different hosts.

Materials and methods

Bacterial strains

L. pneumophila JR32 [49] and the *legA9* mutant [28] were kindly provided by Dr. Howard Shuman, University of Chicago. *L. pneumophila* strains were grown as previously described [6, 50].

Cell culture and infections

WT mice were purchased from Jackson laboratory. C57BL/6 BMDMs were prepared from the femurs of five to eight-week-old mice as previously described [6, 7]. Isolation and preparation of the human monocyte-derived macrophages (hMDMs) from peripheral blood was carried out as previously described [50-52]. Acute monocytic leukemia cell line (THP-1) was maintained as previously described [53]. Infections were performed as previously described [5-7, 53]. The $Atg5^{-/-}$ mice [54] were obtained from Herbert W. Virgin from Washington University with permission of Noboru Mizushima.

Western blot analysis

30μg/ml of protein was loaded on 12% SDS polyacrylamide gel and immunoblotted with antibodies that recognize caspase-1, caspase-7 (cell signaling), p62 (Sigma), mono and polyubiquitinated conjugates, mAb FK2 (Enzo) and LC3 (Sigma). The α-flaA polyclonal Ab was obtained from Dr. Howard Shuman, University of Chicago. The primary antibodies were followed by appropriate secondary rabbit or mouse antibodies as described in [6, 7, 53].

In vitro drug treatment of macrophages

Rapamycin (Sigma-Aldrich, R0395) was dissolved in DMSO (Sigma-Aldrich, D2650) at 1 mg/ml. Rapamycin was used at a concentration of 0.2 μg/ml [8]. Murine macrophages were either treated or untreated with rapamycin for 1 hr before they were infected. Rapamycin was maintained throughout the infection. Bafilomycin A1 (sigma) was added 1 hr after infection at final concentration of 12.5 nM. The 3-methyladenine (sigma) was dissolved in

heated dH_2O at 65°C, added 1hr before infection at a concentration of 10 mM and kept throughout the infection [8]. The proteasome inhibitor MG-132 (Biovision, Mountain view, CA) was dissolved in DMSO and maintained throughout the infection [46].

Macrophage cytotoxicity assay

Quantification of apoptosis was performed using the Cell Death Detection ELISA (Roche Applied Science) to the specification of the manufacturer. The fold change of macrophage necrosis was determined by measuring the release of host cell cytoplasmic lactate dehydrogenase (LDH) using the cytotoxicity detection kit (Roche Applied Science) to the specification of the manufacturer.

Confocal laser scanning microscopy (CLSM)

Colocalization experiments were performed as previously described [5-7]. Antibodies against *L. pneumophila* (Abcam), Atg8/LC3 (Abgent, Ap1805a), polyubiquitin FK2 (Enzo) or p62 (BD bioscience) were used. Images were captured with the Olympus Fluoview Fv10i.

Contact-dependent hemolysis

One × 10^8 sheep RBCs (sRBCs) were incubated with *L. pneumophila* at an MOI of 25 for 3 hrs, as previously described [55, 56].

Enzyme-Linked Immunosorbent Assay (ELISA) for p62

Two × 10^6 of BMDMs were either un-infected or infected with JR32 or the *legA9* mutant for 6 hr. The levels of p62 or IL-1β were determined by a sandwich ELISA according to the manufactures specification.

SiRNA treatment

siRNA treatment was performed using siRNA against P62 (Dharmacon-18412):ACAGAUGCCAGAAUCGGAA, CUGCUCAGGAGGAGACGAU,GAACAGAUGGAGUCGGGAA, CCAUGGGUUUCUCGGAUGA. NDP52 (Dharmacon-76815) CAACACAGAGGGUCAAUAA, CAGAAGAGGACAUCCGGAU, CCAAGGAUGUGGAGCGCUA, GAGUUGAGGUGUCCGUGUA, siRNA was nucleofected into primary macrophages using Lonza Nucleofection kit and Amaxa equipment as we described previously [57].

Statistical analysis

All experiments were performed at least 3 independent times and yielded similar results. Comparisons of groups for statistical difference were done using student's two tailed t test. P value ≤ 0.05 was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

L. pneumophila	*Legionella pneumophila*
BMDMs	Bone marrow derived macrophages
hMDMs	human monocyte derived macrophages
CFUs	colony forming units

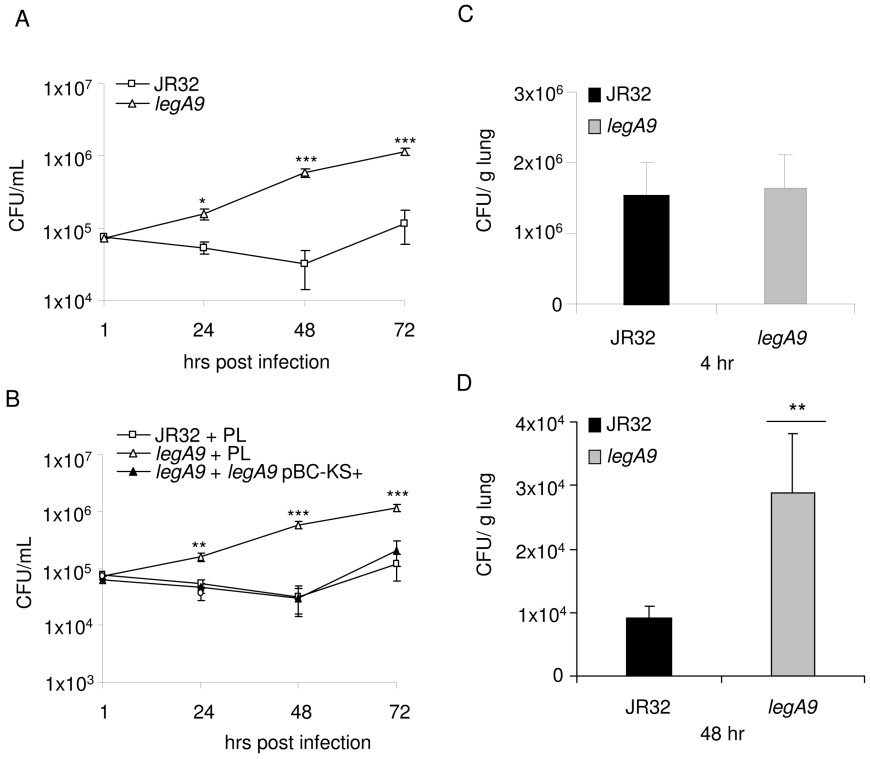

Figure 1.

WT mice and their BMDMs are restrictive to parent *L. pneumophila* intracellular replication but permissive to *legA9* mutant strain, (A) BMDMs were infected with *L. pneumophila* JR32 or the *legA9* mutant with MOI of 0.5. CFUs were scored at 1, 24, 48 and 72 hr. (B) BMDMs were infected with JR32 or the *legA9* mutant harboring empty plasmid (PL), or *legA9*+ *legA9* pBC-KS+ plasmid. Data are presented as means ± S.D of at least three independent experiments. Asterisks indicate significant differences (**P*<0.05, ***P*<0.01, ****P*<0.001). Four female C57BL/6 mice/group received 1×10^6 of JR32 or *legA9* mutant bacteria intratracheally. Lungs were homogenized and plated for CFUs counting at (C) 4 or (D) 48 hr post infection. Data are representative of one of two independent experiments and presented as means ± S.D. Asterisks indicate significant differences (***P*<0.01).

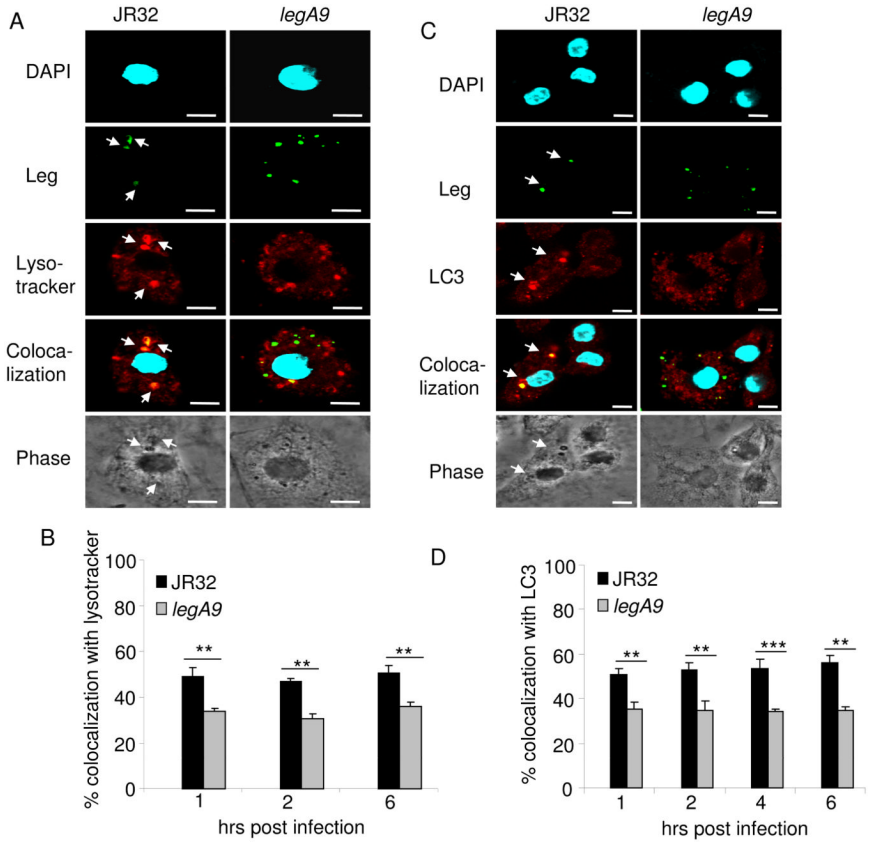

Figure 2.

Vacuoles harboring *legA9* mutant bacteria significantly evade fusion with lysosomes and escape recognition by autophagy in WT BMDMs. (A) Representative images from 6 hr infected WT BMDMs with JR32 or *legA9* mutant bacteria. Nuclei were stained blue with DAPI and *L. pneumophila* stained green with *L. pneumophila* antibody. Lyso-tracker red was used to stain acidified lysosomes. White arrows show *L. pneumophila* colocalization with lysotracker. (B) Percent colocalization of *L. pneumophila* with lyso-tracker. (C) Representative images from 6 hr infected WT BMDMs with JR32 or *legA9* mutant bacteria. LC3 is stained red with LC3 antibody. White arrows show *L. pneumophila* colocalization with LC3. (D) Percent colocalization of *L. pneumophila* with LC3. Hundred bacteria were scored from each coverslips. Images were captured with the 60X objective and magnified 3×, scale bar = 10μm. Data are presented as means ± S.D of three independent experiments. Asterisks indicate significant differences (***P*<0.01, ****P*<0.001).

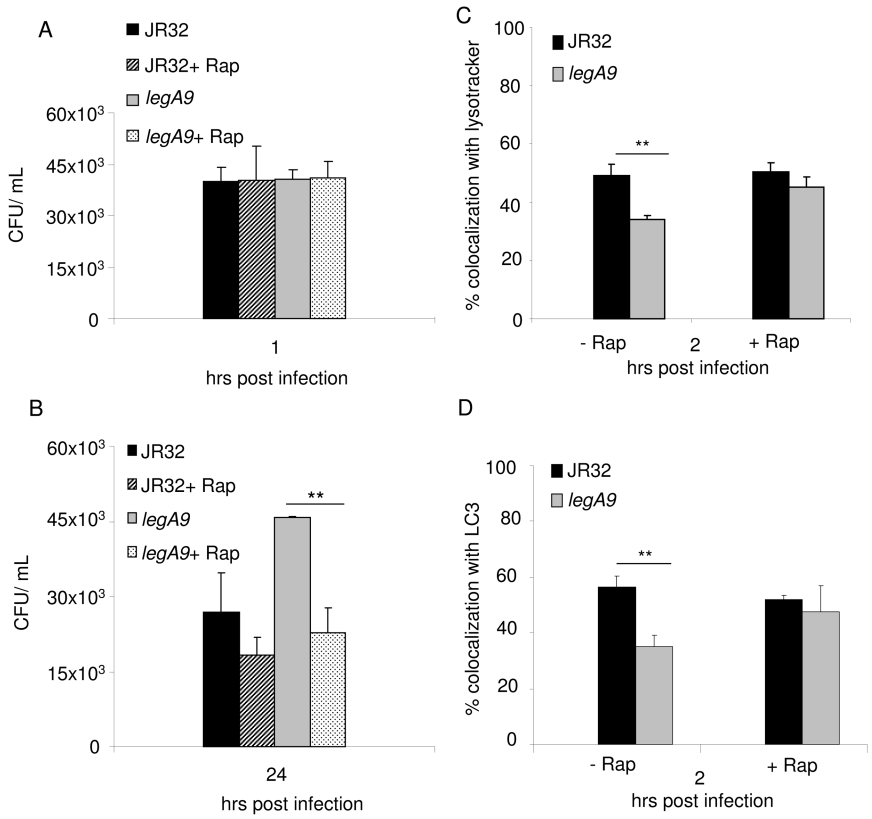

Figure 3.

Induction of autophagy in macrophages restricts *legA9* mutant replication, and rescues colocalization with LC3 and fusion with lysosomes. BMDMs were untreated or pre-treated with rapamycin (Rap) for 1 hr and kept throughout the infection. BMDMs were infected with *L. pneumophila* JR32 or the *legA9* mutant for (A) 1 or (B) 24 hr with an MOI of 0.5. CFUs were scored at these time points. (C) Percent co-localization of *L. pneumophila* with (C) lyso-tracker (D) and (D) LC3. Hundred bacteria were scored from each coverslips. Images were captured with the 60X objective and magnified 3×, scale bar = 10μm. Data are presented as means ± S.D of three independent experiments. Asterisks indicate significant differences (**P<0.01).

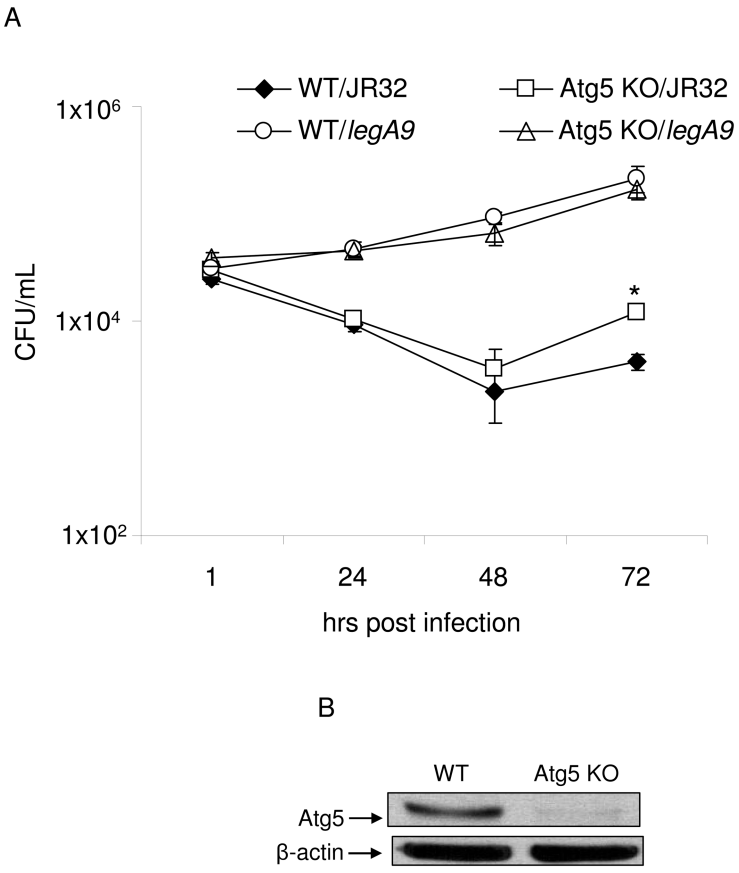

Figure 4.

BMDMs derived from Atg5$^{-/-}$ mice are defective in clearing the WT *L. pneumophila* and the *legA9* mutant. BMDMs derived from WT and Atg5$^{-/-}$mice were infected with *L. pneumophila* JR32 or *legA9* mutant at an MOI of 0.5 (A). Data are representative of two independent experiments performed in triplicate and presented as means ± S.D. Asterisks indicate significant differences (*P<0.05). (B) Western blot showing the absence of Atg5 in the knockout.

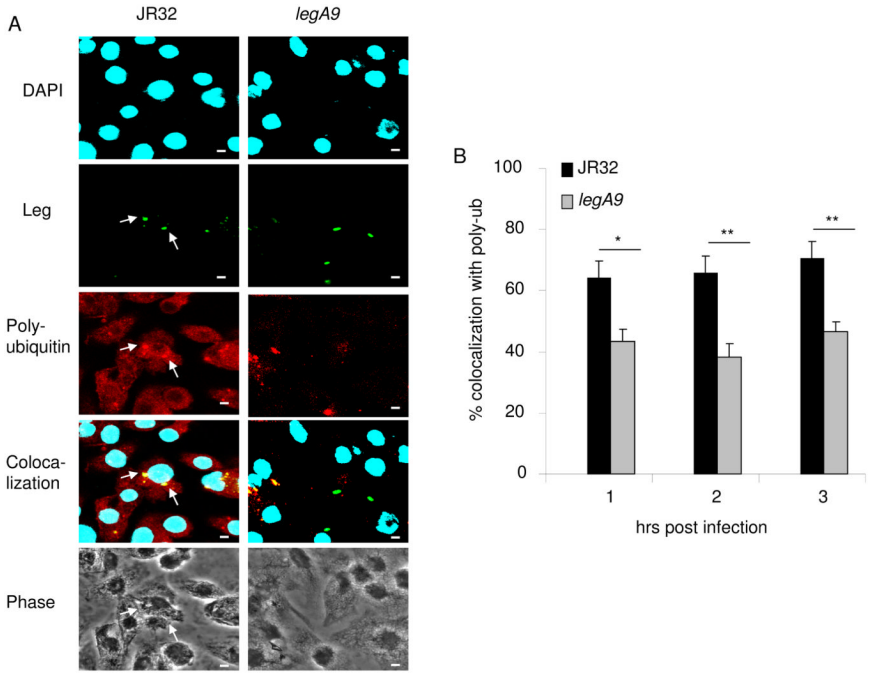

Figure 5.

Macrophage vacuoles-containing the *legA9* mutant, have reduced ubiquitin compared to vacuoles harboring parent *L. pneumophila*. (A) Representative images from 6 hr infected BMDMs with JR32 or *legA9* mutant bacteria. Poly-ubiquitin is stained red. White arrows show *L. pneumophila* colocalization with poly-ubiquitin. (B) Percent colocalization of *L. pneumophila* with poly-ubiquitin. Hundred bacteria were scored from each coverslips. Images were captured with the 60X objective and magnified 3×, scale bar = 10μm. Data are presented as means ± S.D of two independent experiments. Asterisks indicate significant differences (*P<0.05, **P<0.01, ***P<0.01).

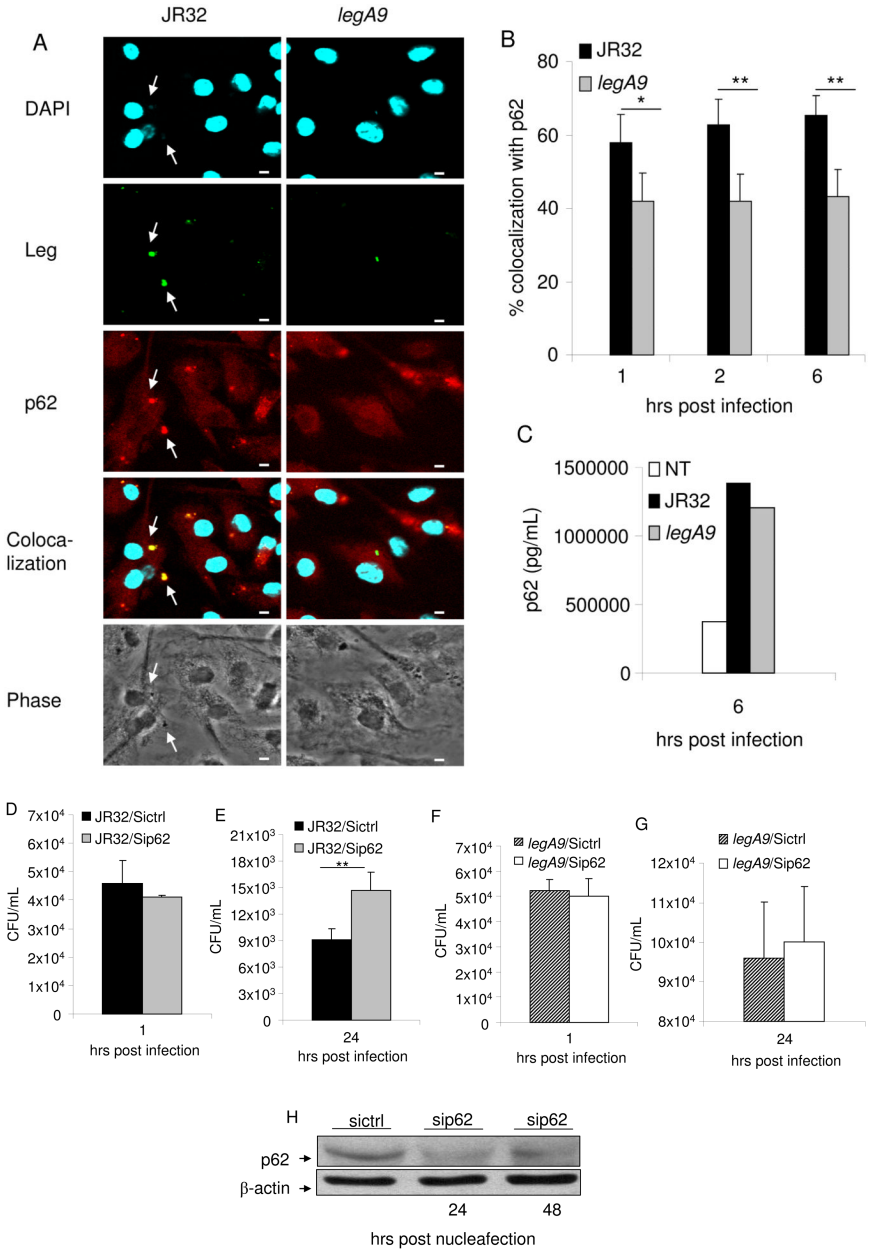

Figure 6.

p62/SQSTM1 targets *L. pneumophila*-containing vacuoles to the autophagosomes for degradation in WT murine macrophages. (A) Representative images from 6 hr infected BMDMs with JR32 or *legA9* mutant bacteria. P62 is stained red with p62 antibody. White arrows show *L. pneumophila* colocalization with p62. (B) Percent colocalization of *L. pneumophila* with p62. Hundred bacteria were scored from each coverslips. Images were captured with the 60X objective and magnified 3×, scale bar = 10μm. Data are presented as means ± S.D of three independent experiments. Asterisks indicate significant differences (**P*<0.05, ***P*<0.01). (C) BMDMs were infected with JR32 or the *legA9* mutant for 6 hr and the level of p62 protein was quantified in cell lysates by ELISA (performed in triplicate and the S.D.s are too small to detect). BMDMs were nucleofected with scrambled control (sictrl) or siRNA against p62 (sip62). Twenty four hrs after nucleafection, murine macrophages were infected with JR32 or *legA9* mutant with an MOI of 0.5 for 1 hr (D and

F) or 24hr (E and G). (H) Western blot analysis showing the downregulation of p62 at 24 and 48 hr post nucleofection. Data are representative of three independent experiments and presented as means ± S.D. Asterisks indicate significant differences (*P<0.05, **P<0.01).

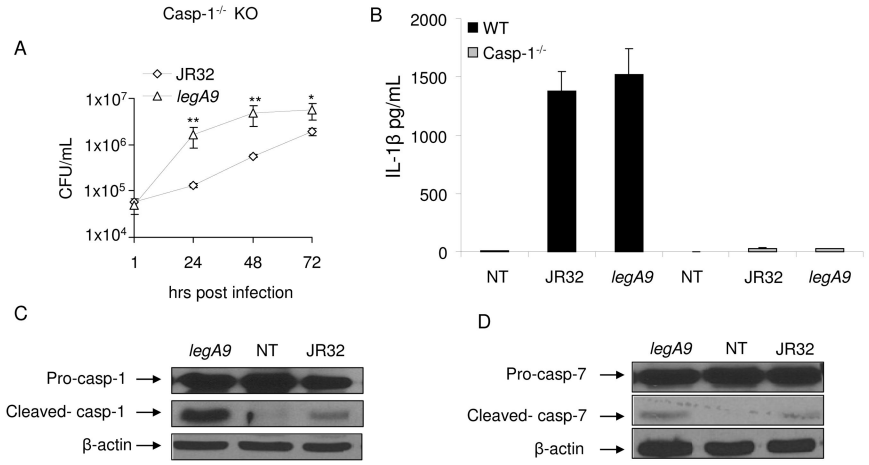

Figure 7.

The permissiveness of BMDMs to legA9 mutant bacteria is independent of caspase-1 or caspase-7 activation. (A) Caspase-1 KO (casp-$1^{-/-}$) macrophages were infected with L. *pneumophila* JR32 or *legA9* with an MOI of 0.5 for 1, 24, 48 and 72 hr. (B) CFUs were measured at the indicated time points. Levels of IL-1β were detected in supernatants of WT or casp-$1^{-/-}$ BMDMs infected with JR32 or the *legA9* mutant after 24 hr. WT BMDMs were either not treated (NT) or infected with *L. pneumophila* JR32 or *legA9* mutant bacteria for 2 hr. Activation of casp-1 or casp-7 was detected in cell extracts using (C) casp-1 or (D) casp-7 antibodies, respectively.

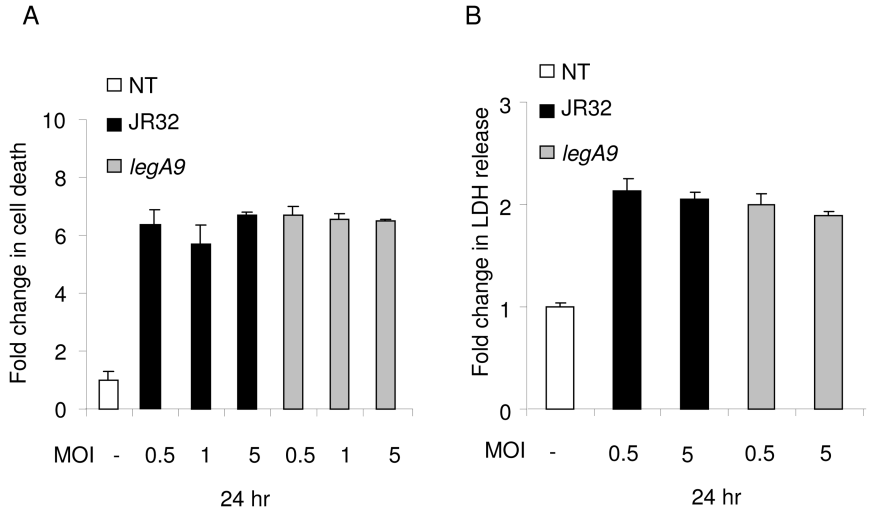

Figure 8.

Replication of the *legA9* mutant is not due to extended macrophage survival. (A) BMDMs were not infected (NT) or infected with *L. pneumophila* JR32 or the *legA9* mutant at an MOI of 0.5, 1 or 5 for 24 hr. Apoptosis was quantified by Photometric enzyme immunoassay analysis of cytoplasmic (apoptosis) histoneassociated-DNA fragments (ELISA). (B) BMDMs were either not treated (NT) or treated with *L. pneumophila* JR32 or the *legA9* mutant at an MOI of 0.5 or 5 for 24 hr. The fold change in LDH release was measured from the overall population of macrophages.