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Caspase-11 promotes the fusion of phagosomes harboring pathogenic bacteria with lysosomes by modulating actin polymerization

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Summary

Inflammasomes are multiprotein complexes that include members of the NLR (nucleotide-binding domain leucine-rich repeat containing) family and caspase-1. Once bacterial molecules are sensed within the macrophage, the inflammasome is assembled mediating the activation of caspase-1. Caspase-11 mediates caspase-1 activation in response to lipopolysaccharide and bacterial toxins. Yet, its role during bacterial infection is unknown. Here, we demonstrated that caspase-11 was dispensable for caspase-1 activation in response to *Legionella, Salmonella, Francisella* and *Listeria*. We also determined that active mouse caspase-11 was required for restriction of *L. pneumophila* infection. Similarly, human caspase-4 and 5, homologs of mouse caspase-11, cooperated to restrict *L. pneumophila* infection in human macrophages. Caspase-11 promoted the fusion of the *L. pneumophila*- vacuole with lysosomes by modulating actin polymerization through cofilin. However, caspase-11 was dispensable for the fusion of lysosomes with phagosomes containing non-pathogenic bacteria, uncovering a fundamental difference in the trafficking of phagosomes according to their cargo.

Introduction

The inflammasome complex includes members of the NLR (nucleotide-binding domain leucine-rich repeat containing) family, the adaptor molecule apoptosis-associated speck-like protein containing a caspase recruitment domain (Asc), and caspase-1 (Martinon et al., 2002). The inflammasome is assembled when microbial molecules or danger signals are sensed by members of the NLR within the macrophage cytosol. Once assembled, the inflammasome mediates the cleavage and activation of caspase-1 with the subsequent

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processing and secretion of interleukin-1β (IL-1β) and IL-18 (Martinon et al., 2002). Murine caspase-11 contributes to caspase-1 activation in response to lipopolysaccharide (LPS) and bacterial toxins (Kayagaki et al., 2011). Mice lacking caspase-11 (*Casp4*$^{-/-}$) fail to produce mature IL-1β or active caspase-1 and are resistant to endotoxic shock induced by bacterial toxins (Wang et al., 1998). Caspase-11 interacts with Aip1 to promote cofilin-mediated actin depolymerization (Li et al., 2007). However, role of caspase-11 during intracellular infection remains to be elucidated. Based on expression profiles, caspase-4 and caspase-5 are the human homologs of mouse caspase-11 (Mariathasan and Monack, 2007; Martinon et al., 2002). Human caspase-5 is also a component of the NLRP1 inflammasome, suggesting that caspase-5 activates caspase-1 (Martinon et al., 2002). Yet, the roles of human caspase-4 and -5 during bacterial infection are unknown.

Caspases are a family of cysteine proteases that play a distinct role in apoptosis and inflammation (Salvesen and Ashkenazi, 2011; Siegel, 2006; Stennicke and Salvesen, 1998). Caspases are synthesized as inactive single-chain zymogens and typically activated by cleavage. However, this cleavage appears to have a modest effect on the catalytic activity of initiator caspases, such as caspase-8, caspase-9 and caspase-11 (Srinivasula et al., 1999; Stennicke and Salvesen, 2000). *Legionella pneumophila* (*L. pneumophila)* is the causative agent of Legionnaires' pneumonia, a severe disease in the elderly and immuno-compromised patients (Horwitz and Silverstein, 1980, 1981). Replication of *L. pneumophila* within human macrophages is critical for the disease and requires a functional bacterial type IV secretion (Dot) system (Vogel and Isberg, 1999). In wild-type (WT) murine macrophages, *L. pneumophila* flagellin leaks through the Dot system and is recognized by the NLR Nlrc4 leading to caspase-1 then caspase-7 activation that restricts *L. pneumophila* infection by promoting the fusion of the *L. pneumophila* containing vacuole with the lysosome (Akhter et al., 2009; Amer et al., 2006; Case et al., 2009). Naip5 (nucleotide oligomerization domain-like receptor family apoptosis inhibitory protein) is another NLR that restricts *L. pneumophila* infection. Therefore, host Nlrc4, caspase-1, caspase-7, Naip5 and bacterial flagellin are required for restriction of *L. pneumophila* infection in WT murine macrophages. Consequently, macrophages lacking Nlrc4 (*Nlrc4*$^{-/-}$), caspase-1 (*Casp1*$^{-/-}$), caspase-7 (*Casp7*$^{-/-}$), functional Naip5 (A/J) are permissive to infection. Likewise, the isogenic *L. pneumophila* mutants lacking flagellin (Fla) replicate readily in WT murine macrophages (Amer et al., 2006; Ren et al., 2006). On the other hand, *L. pneumophila* mutants lacking a functional Dot system (*dotA*$^{-/-}$) fail to secrete essential virulence factors, thus they traffic to the lysosome in WT and in permissive macrophages as well (Amer et al., 2006; Ren et al., 2006).

In this report, we have shown that caspase-11 was a component of the Nlrc4 inflammasome, yet non-essential for the activation of caspase-1 in response to *L. pneumophila*, *Salmonella typhimurium* (*Salmonella*), *Francisella novicida* (*Francisella*), or *Listeria monocytogenes* (*Listeria*) infection. In addition, caspase-11 controlled the fusion of *L. pneumophila*-containing phagosome with the lysosome independently of caspase-1. Caspase-11 promoted this fusion event by mediating actin remodeling whereby the assembly of F-actin facilitated phagosome-lysosome fusion and mediated the clearance of *L. pneumophila*. In addition, caspase-11 was dispensable for the delivery of the non-pathogenic *dotA*$^{-/-}$ *L. pneumophila* mutant to the lysosomes. Likewise, caspase-11 was not required for the clearance of non-pathogenic *Escherichia coli* (*E. coli*), thus uncoupling these two fundamental fusion modes at the molecular level. On the other hand, human macrophages, which are permissive to *L. pneumophila* infection, do not activate caspase-1 in response to this pathogen. However, we demonstrated in this study that ectopic expression of both caspase-4 and -5 in human macrophages restricted *L. pneumophila* infection and was accompanied by caspase-1 activation.

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Caspase-11 protein was undetected in uninfected murine macrophages but its expression was induced during *L. pneumophila* infection independently of the host Nlrc4, Asc, Naip5 and of bacterial flagellin. However, caspase-11 interaction with the Nlrc4 inflammasome members and its activation required bacterial flagellin. Therefore, these findings provide a molecular framework to understand the complexity of the inflammasome and the role of caspase-11 in the innate immune response to bacterial infection.

RESULTS

Caspase-1 is activated in the absence of caspase-11 within macrophages infected with *L. pneumophila*, *Salmonella, Francisella* and *Listeria*

To evaluate the contribution of murine caspase-11 to caspase-1 activation upon *L. pneumophila* infection, we compared caspase-1 cleavage in WT, caspase-11-deficient (*Casp4*$^{-/-}$) and caspase-1-deficient (*Casp1*$^{-/-}$) bone marrow-derived macrophages (BMDMs) infected with *L. pneumophila* for 2 hrs. In WT macrophages, the bacterium induced proteolytic activation of pro-caspase-1 as determined by the detection of the mature 20-kDa subunit in cell extracts by Western blots (Figure 1a). Proteolytic processing of pro-caspase-1 in response to *L. pneumophila* was also detected in caspase-11-deficient macrophages (Figure 1a). Infection of WT macrophages with the *L. pneumophila* mutant lacking flagellin (Fla) did not lead to proteolytic activation of pro-caspase-1 (Figure 1a). These data demonstrated that caspase-11 was dispensable for caspase-1 activation in response to *L. pneumophila*. Quantitative polymerase chain reaction with reverse transcription (RT-PCR) showed that *Casp4* (which encodes for mouse caspase-11) was induced in WT macrophages in response to *L. pneumophila* infection (Supplementary Figure 1a). Notably, *Casp4* expression was induced independently of bacterial flagellin, the host Nlrc4 and the adaptor molecule Asc (encoded by *Pycard*). A functional Naip5 (A/J mice express a nonfunctional Naip5) (Wright et al., 2003) was also dispensable for caspase-11 induction in response to *L. pneumophila* (Figures 1a, b and Supplementary Figure 1a, b and c). Caspase-11 was also induced in response to *Salmonella*, *Francisella* and *Listeria* (Figure 1c).

Then, to discern if caspase-11 is dispensable for caspase-1 activation with other inflammasome-engaging intracellular organisms, we examined the activation of caspase-1 in caspase-11-deficient macrophages infected with *Listeria*, *Francisella*, and *Salmonella*. Infection of WT macrophages and macrophages lacking caspase-11 with any of these organisms led to the cleavage of caspase-1 (Figure 1c). Together, these data indicate that caspase-11 is not required for the activation of caspase-1 in response to *L. pneumophila*, *Salmonella*, *Francisella*, and *Listeria*.

Given that *L. pneumophila* is sensed by Nlrc4 inflammasome suggests that caspase-11 is a member of the Nlrc4 inflammasome assembled during *L. pneumophila* infection. To test for this possibility, we next examined if caspase-11 interacted with components of the Nlrc4 inflammasome (Poyet et al., 2001; Sutterwala and Flavell, 2009). WT and caspase-11-deficient macrophages were infected with *L. pneumophila*, then endogenous caspase-11 was immuno-precipitated with specific caspase-11 antibodies attached to magnetic beads. Caspase-11 precipitated with endogenous pro-caspase-1, Nlrc4 and Asc exclusively in WT macrophages and only in the presence of *L. pneumophila* (Figure 1d). Therefore, caspase-11 interacts with members of the Nlrc4 inflammasome in the presence of *L. pneumophila* infection and this interaction is specific because members of the inflammasome did not precipitate in caspase-11-deficient macrophages (Figure 1d). It is possible that the lack of precipitation of the inflammasome members in uninfected macrophages is merely due to the lack of caspase-11 expression. To test this possibility, we next examined if caspase-11 interacted with members of the Nlrc4 inflammasome during infection with the *L.*

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pneumophila Fla mutant which induces caspase-11 expression but does not activate caspase-1 (Amer et al., 2006; Case et al., 2009). Despite its induction by the Fla mutant, caspase-11 did not interact with members of the Nlrc4 inflammasome (Figure 1 e). Therefore, bacterial flagellin was necessary of the interaction of caspase-11 with Nlrc4 inflammasome.

Caspase-11-deficient mice and their derived macrophages are permissive to *L. pneumophila*

Macrophages from the great majority of mouse strains restrict *L. pneumophila* replication (Brieland et al., 1994; Derre and Isberg, 2004; Yamamoto et al., 1988). However, *Nlrc4*$^{-/-}$, *Casp1*$^{-/-}$ and *Casp7*$^{-/-}$ mice and their derived macrophages are permissive to this bacterium (Akhter et al., 2009; Case et al., 2009). To determine if caspase-11 modulates the growth of *L. pneumophila*, we tested the ability of caspase-11-deficient *(Casp4*$^{-/-}$*)* macrophages to support bacterial replication in comparison to restrictive WT macrophages. Macrophages from caspase-1 or caspase-11-deficient mice supported significant *L. pneumophila* replication over 72 hrs of infection (Figure 2a). Notably, caspase-11-deficient macrophages allowed *L. pneumophila* growth, but less than *Casp1*$^{-/-}$ macrophages (Figure 2a). In contrast, as expected the bacterial growth in WT macrophages was controlled (Figure 2a). The difference in intracellular bacterial replication between WT and caspase-11-deficient cells was not due to differential uptake of *L. pneumophila* because at 1 hr post infection, the number of *L. pneumophila* associated with different macrophages was comparable (Figure 2a). To visualize the pathogenic organism inside the cells, WT, and caspase-11-deficient macrophages were infected with *L. pneumophila* that constitutively express green fluorescent protein (GFP). The number of bacteria associated with macrophages was monitored by confocal laser scanning fluorescence microscopy (Figure 2b). At 24 hr post infection, only a few individual bacteria were identified inside WT macrophages, whereas expanded compartments packed with *L. pneumophila* were observed within caspase-11-deficient macrophages (Figure 2b). Both WT and caspase-11-deficient macrophages restricted the replication of *L. pneumophila dotA*$^{-/-}$ mutant (Supplementary Figure 4). Therefore, intracellular *L. pneumophila* replication which requires a functional Dot system is modulated by caspase-11.

To confirm the role of caspase-11 in *L. pneumophila* restriction, caspase-11-deficient macrophages were complemented with a plasmid carrying the *Casp4* which encodes caspase-11 (PL- Casp11) and the correlation between caspase-11 expression and bacterial replication was examined (Figure 2c and d). Ectopic expression of caspase-11 was sufficient to restore the ability of caspase-11-deficient murine macrophages to restrict *L. pneumophila* growth (Figure 2c and d). The expression of the control vector by the same technique did not alter the permissiveness of the caspase-11-deficient macrophages to *L. pneumophila* (Figure 2c).

To determine if caspase-11 is activated during *L. pneumophila* infection, macrophages were infected with native *L. pneumophila* or its corresponding mutant lacking flagellin. Then macrophage lysates were mixed with biotinylated-YVAD-CMK and the presence of caspase-11 within the precipitated complex was determined by Western blots. Caspase-11 precipitated with the YVAD-biotin-coated beads only during *L. pneumophila* infection (Figure 3a). The interaction of caspase-11 with the substrate required bacterial flagellin (Figure 3a). Therefore, caspase-11 is activated during *L. pneumophila* infection and such activation requires flagellin.

Next, to test whether the enzymatic activity of caspase-11 is required for the restriction of *L. pneumophila* infection, caspase-11-deficient macrophages were transfected with a plasmid (pCAGGS-*Casp4*m2) carrying a catalytically inactive mutant of caspase-11 (PL-inactive

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Casp11). The mutant caspase-11 failed to control *L. pneumophila* infection despite its comparable expression to native caspase-11 (Supplementary Figure 3a and b). Together, these results indicate that caspase-11 activity is required for the restriction of *L. pneumophila* growth within macrophages.

To ascertain the role of caspase-11 in restriction of *L. pneumophila*, caspase-11 was depleted from WT macrophages by siRNA specific to *Casp4* (which encodes for murine caspase-11) (Figure 3b). The intracellular growth of *L. pneumophila* was evaluated (Figure 3c). WT macrophages treated with siRNA specific to *Casp4* but not siRNA control allowed more *L. pneumophila* growth (Figure 3c).

Since Legionnaires' disease is caused by the replication of *L. pneumophila* in the lungs (Horwitz, 1983b; Horwitz and Silverstein, 1980), we investigated if caspase-11 regulates bacterial growth within murine lungs *in vivo*. WT and caspase-11-deficient ($Casp4^{-/-}$) mice were infected intratracheally with *L. pneumophila* and the bacterial load in the lungs was determined. Bacterial counts after 4 hrs of infection reflect the initial bacterial load in the lungs (Figure 3d), whereas bacterial counts at 48 hrs denote bacterial growth (Figure 3e). After 48 hrs of infection, significantly more *L. pneumophila* were recovered from the lungs of caspase-11-deficient mice compared with counts obtained from WT mice (Figure 3e). Together, these results indicate that caspase-11 restricts *L. pneumophila* replication *in vitro* and *in vivo*.

Because human caspase-4 and -5 are homologs of murine caspase-11, we determined if they contribute to *L. pneumophila* restriction in permissive human macrophages. The THP-1 macrophage cell line which is permissive to *L. pneumophila*, was transfected with caspase-4 (*CASP4*) and -5 (*CASP5*) plasmids individually and in combination. Then, macrophages were infected with *L. pneumophila*. The ectopic expression of each caspase alone partially restricted bacterial growth (Supplementary Figure 2a and b). Ectopic expression of both caspases together restricted *L. pneumophila* growth (Supplementary Figure 2 a and b). Notably, expression of both caspase-4 and -5 provoked caspase-1 activation upon *L. pneumophila* infection (Supplementary Figure 2 c). Thus, like murine caspase-11, human caspase-4 and -5 can together restrict *L. pneumophila* infection in human macrophages.

Ectopic expression of caspase-11 in $Casp1^{-/-}$ macrophages partially restricts the growth of *L. pneumophila*

Given that both caspase-1 and caspase-11-deficient macrophages allow *L. pneumophila* replication, we tested if caspase-11 is expressed in $Casp1^{-/-}$ macrophages. As reported previously (Kayagaki et al., 2011; Li et al., 2007; Wang et al., 1998), caspase-11 protein was undetectable in $Casp1^{-/-}$ macrophages (Figure 1a). In addition, Casp4 mRNA was undetectable by quantitative RT PCR in $Casp1^{-/-}$ macrophages (Supplementary Figure 1a). Therefore, both caspase-11- and caspase-1-deficient macrophages lack caspase-11 expression (Figure 1a). Thus, we examined if the lack of caspase-11 in $Casp1^{-/-}$ macrophages contributed to their permissiveness to *L. pneumophila* growth. Distinctly, the re-establishment of caspase-11 protein with an exogenous plasmid restored caspase-11 expression in $Casp1^{-/-}$ macrophages and was accompanied by partial restriction of *L. pneumophila* infection (Figure 2d and 4a). These data suggest that the absence of caspase-11 contributes to the permissiveness of $Casp1^{-/-}$ macrophages to *L. pneumophila*.

Caspase-11 mediates the fusion of the *L. pneumophila*-containing phagosome with the lysosome in restrictive WT macrophages

To visualize *L. pneumophila* within WT and caspase-11-deficient macrophages, we examined *L. pneumophila*-infected macrophages by transmission electron microscopy. Only

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a few bacteria were detected within WT macrophages with signs of degradation such as irregular edges (Figure 4b arrow). In caspase-11-deficient macrophages, many *L. pneumophila* were identified and did not show signs of degradation (Figure 4b arrow head). We quantified the percentage of *L. pneumophila* showing signs of degradation by confocal microscopy using specific antibodies against *L. pneumophila*. Around 60–80% of *L. pneumophila* were degraded in WT macrophages at 30 min and 6 hrs after infection, respectively (Figure 4c). The number of degraded *L. pneumophila* within caspase-11-deficient macrophages did not surpass 45% at any time point (Figure 4c). To corroborate these results, WT and caspase-11-deficient macrophages were infected with the *L. pneumophila* strain (SSK) that expressed GFP in the presence of IPTG. The GFP-expressing *L. pneumophila* were considered to be live (Sturgill-Koszycki and Swanson, 2000). In WT macrophages, up to 80% of *L. pneumophila* failed to express GFP, whereas, in caspase-11-deficient macrophages 45% *L. pneumophila* lacked GFP expression (Figure 4d and Supplementary Figure 4a). These results indicate that more *L. pneumophila* survive and respond to IPTG within macrophages lacking caspase-11 compared to WT macrophages.

Because caspase-11 is required for the proper function of the cytoskeleton machinery (Li et al., 2007; Wang et al., 1998), we next examined if the fusion of the *L. pneumophila*-containing phagosome with the lysosome is defective in the absence of caspase-11, thus allowing the pathogen to avoid degradation within macrophages. The incidence of lysosome fusion was scored by following the number of *L. pneumophila*-containing phagosomes acquiring lysotracker red, a dye that traffics to acidic vacuoles (Figure 5a and b). In WT macrophages, the majority (55–65%) of phagocytosed *L. pneumophila* were contained inside phagosomes that efficiently fused with lysosomes (Figure 5a and b). In caspase-11-deficient macrophages, only 40% of the *L. pneumophila* containing vacuoles acquired the lysotracker within 6 hrs of infection (Figure 5a and b). Similar results were obtained using LAMP-1, a marker for late endosomes (Figure 5c). Therefore, these data indicate that caspase-11 is required for the proper fusion of *L. pneumophila*-containing phagosomes with the lysosomes but does not address if caspase-11 activity is required for this function. Thus, we examined the acquisition of the lysotracker by the *L. pneumophila*-phagosomes in caspase-11-deficient macrophages after their transfection with plasmids expressing either the native caspase-11 or mutant (inactive) caspase-11. Only macrophages expressing a functional caspase-11 delivered *L. pneumophila* to the lysosomes (Figure 5d).

To examine if these results reflect an inherent defect in phagosome-lysosome fusion in caspase-11-deficient macrophages, we examined the trafficking of the *L. pneumophila* *dotA*$^{-/-}$ which is known to co-localize with the lysosomes in restrictive WT and in permissive *Casp1*$^{-/-}$ macrophages (Supplementary Figure 4b). Caspase-11-deficient macrophages delivered most of the *dotA*$^{-/-}$ mutant to lysotracker-labeled vacuoles within 1 hr of infection (Supplementary Figure 4b). In agreement with these results, phagosomes harboring *E. coli* were effectively (>95%) and promptly (1 hr post infection) fused with the lysosomes in macrophages lacking caspase-11 similar to WT macrophages (Figure 5e and f). Hence, caspase-11 modulates the fusion of phagosomes harboring intracellular pathogens such as *L. pneumophila* but not those enclosing non-pathogenic bacteria.

L. pneumophila replicates in endoplasmic reticulum (ER)-labeled vacuoles (Vogel and Isberg, 1999), thus, we examined the recruitment of the ER marker calreticulin to the *L. pneumophila* vacuole (Supplementary Figure 4c and d). In caspase-11-deficient macrophages, 30% of the *L. pneumophila* localized with calreticulin within 6 hrs of infection, whereas in WT macrophages, less than 5% bacteria did so (Supplementary Figure 4c and d). Therefore, caspase-11-deficient macrophages allow *L. pneumophila* replication within ER-labeled vacuoles.

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F-actin network formation around *L. pneumophila*-containing vacuole is essential for fusion with the lysosome and requires caspase-11

Phagosomes bind and move along microtubules and actin filaments to encounter and interact with other compartments within the cell. Thus, F-actin remodeling promotes the fusion of specific vesicular compartments including lysosomes (Desjardins et al., 1994; Jahraus et al., 2001; Kjeken et al., 2004; Marion et al., 2011; Stockinger et al., 2006; Tjelle et al., 2000). To determine whether caspase-11 mediates actin remodeling in macrophages infected with *L. pneumophila,* the amount of red fluorescent phalloidin, which reflects the quantity of polymerized F-actin, was determined by confocal microscopy (Figure 6a). Polymerized F-actin was higher in WT macrophages than in caspase-11-deficient macrophages throughout 2 and 6 hrs of infection (Figure 6a and c). These findings prompted us to determine whether *L. pneumophila*-containing phagosomes are surrounded by polymerized actin. Notably, we found that *L. pneumophila*-containing phagosomes are frequently surrounded by polymerized F-actin structures in WT macrophages (Figure 6b and c). This actin staining around the *L. pneumophila*-containing vacuoles correlated with *L. pneumophila* degradation (Figure 6c, white arrow heads, and Supplementary Figure 4a). In stark contrast, scarce amounts of F-actin were found around the *L. pneumophila*-containing phagosomes in caspase-11-deficient macrophages (Figure 6b and c). Notably, the failure to form the F-actin network was accompanied by a prominent defect in *L. pneumophila* clearance and the accumulation of replicative vacuoles. To investigate if actin remodeling is needed for the proper fusion of the *L. pneumophila* vacuoles with lysosomes, WT macrophages were treated with cytochalasin-D after 30 min of *L. pneumophila* infection to allow the uptake of the organism. Cytochalasin-D hindered the acquisition of lysotracker by *L. pneumophila* vacuoles, indicating that actin remodeling is required for the proper fusion of the *L. pneumophila*-containing vacuole with the lysosome (Supplementary Figure 5a and c).

The nucleation of actin on the phagosomal membrane requires flotillin-1 (Dermine et al., 2001; Desjardins et al., 1994). Supplementary Figure 6 demonstrated that in caspase-11-deficient macrophages, flotilin-1 expression is too scarce to promote actin nucleation which is required for the fusion of the *L. pneumophila*-containing vacuole with the lysosome.

The change of phosphorylation state of cofilin during *L. pneumophila* infection requires caspase-11

Dynamic phosphorylation and dephosphorylation of cofilin mediates cyclic actin polymerization and depolymerization that promotes phagosome-lysosome fusion (Bamburg and Bernstein, 2010; Ghosh et al., 2004). To determine the mechanism by which caspase-11 modulates actin remodeling, we first examined the phosphorylation of cofilin in WT and caspase-11-deficient macrophages. Uninfected WT macrophages allowed the phosphorylation of basal amounts of cofilin (Figure 7a). Then, *L. pneumophila* infection of WT macrophages led to gradual dephosphorylation of cofilin (Figure 7a). Nevertheless, cofilin was unphosphorylated in uninfected caspase-11-deficient macrophages and remained unphosphorylated throughout *L. pneumophila* infection (Figure 7a), thus maintaining actin in the depolymerized form (Figure 7a). Second, we determined if caspase-11 interacted with actin in the presence of *L. pneumophila* infection. Immunoprecipitation of caspase-11 was accompanied by the precipitation of actin only during *L. pneumophila* infection (Figure 7b). Thus, caspase-11 interacts with actin and is required for modulation of the phosphorylation state of cofilin during infection with pathogenic *L. pneumophila*.

To determine if the human homologs of mouse caspase-11, caspase-4 and -5 alter cofilin phosphorylation in human macrophages, THP-1 cells were transfected with the empty plasmid (vector) or plasmids incorporating caspase-4 (*CASP4*) and -5 (*CASP5*), then infected with *L. pneumophila*. THP-1 cells transfected with vector alone maintained cofilin

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in the phosphorylated form before and during *L. pneumophila* infection. Cofilin was dephosphorylated only when caspases-4 and -5 were ectopically expressed in THP-1 cells before *L. pneumophila* infection (Supplementary Figure 2 d). Thus, alteration of the phosphorylation state of cofilin during *L. pneumophila* infection requires the expression of caspase-11 in the mouse and caspase-4 and -5 in human macrophages.

Discussion

Inflammasomes are protein complexes that include members of the NLR family of proteins and lead to caspase-1 activation when assembled (Lamkanfi and Dixit, 2009). Murine caspase-11 contributes to caspase-1 activation in response to bacterial toxins and LPS and seems to be induced through Toll-Like receptor 4 (TLR4) during LPS treatment (Choi et al., 2009; Kayagaki et al., 2011; Wang et al., 1998). However, little is known about its role in response to pathogenic bacteria. We have demonstrated that caspase-1 is activated in caspase-11-deficient macrophages by *L. pneumophila*, *Salmonella*, *Francisella* and *Listeria* suggesting the existence of another protease mediating caspase-1 activation at least during intracellular infection (Mueller et al., 2002). However, it is still possible that caspase-1 is autoactivated upon assembly and oligomerization (Mariathasan et al., 2004; Yu and Finlay, 2008).

Caspase-11 expression is undetectable and is inducible by stress or apoptotic signals (Kang et al., 2002). Here, we have shown that endogenous caspase-11 was induced upon *L. pneumophila* infection, then interacted with the members of the inflammasome such as Nlrc4, caspase-1, Asc, and also with actin. This interaction required bacterial flagellin. Therefore, restriction of *L. pneumophila* was not mediated by the mere induction of caspase-11 but by its interaction with the inflammasome complex. Yet, caspase-11 was not a prerequisite for inflammasome assembly since caspase-1 was activated in the absence of caspase-11. Flagellin, however, was required for caspase-1 activation by the inflammasome whether caspase-11 is included in the complex or not. Therefore, although caspase-11 seemed to be a member of the Nlrc4 inflammasome complex, it was not required for caspase-1 activation during infection with pathogenic bacteria.

As an initiator caspase, caspase-11 is predicted to undergo autocatalytic intrachain cleavage that may have only a modest effect on its catalytic activity (Srinivasula et al., 1999; Stennicke et al., 1999; Stennicke and Salvesen, 2000). Here we have shown that caspase-11 enzymatic activity was required for restriction of *L. pneumophila* infection. Furthermore, the ectopic expression of native caspase-11 in *Casp1*$^{-/-}$ macrophages partially restricted *L. pneumophila* infection. These data suggested that caspase-11 function did not require caspase-1. Similarly, depletion of caspase-11 from WT macrophages allowed moderate *L. pneumophila* growth. Together, these results indicated that whereas the activity of both caspase-1 and caspase-11 efficiently suppressed *L. pneumophila* replication, the absence of either caspases allowed for bacterial growth. These data also suggested that many phenotypes observed in *Casp1*$^{-/-}$ macrophages may actually be due to the lack of caspase-11.

Murine macrophages lacking caspase-11 are defective in migration and in phagocytosis (Li et al., 2007). This observation suggests that the uptake of *L. pneumophila* may be impaired in caspase-11-deficient macrophages. However, *L. pneumophila* uptake was not affected and the final bacterial burden in caspase-11-deficient macrophages and mice was higher than that in WT counterparts.

Intracellular growth of *L. pneumophila* requires halting of phagosome-lysosome fusion (Horwitz, 1983a; Vogel and Isberg, 1999). This trafficking defect is observed in permissive

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macrophages, whereas in restrictive WT macrophages, most *L. pneumophila*-containing vacuoles fuse with lysosomes and the bacteria are degraded (Coers et al., 2000; Horwitz, 1983a). However, the mechanism by which phagosome-lysosome fusion is modulated upon *L. pneumophila* infection is not fully understood. Therefore, one could propose that the reason for the permissiveness of caspase-11-deficient macrophages is due to a defect in phagosome maturation. It is unlikely that caspase-11 controls *L. pneumophila*-phagosome fusion with the lysosome through controlling the activation of caspase-7 (Akhter et al., 2009) since caspase-7 and caspase-3 were activated in caspase-11-deficient macrophages in response to *L. pneumophila*. Notably, phagosome-lysosome fusion required proper dynamic actin polymerization and depolymerization (Desjardins et al., 1994; Jahraus et al., 2001; Kjeken et al., 2004; Marion et al., 2011; Stockinger et al., 2006; Tjelle et al., 2000). Sustained accumulation of polymerized actin during *Salmonella* or *Leishmania donovani* infection prevents phagosome-lysosome fusion (Meresse et al., 2001). Disorganization of the F-actin network during *Mycobacterium avium* infection also prevents the fusion of its enclosing vacuole with the lysosome (Guerin and de Chastellier, 2000). Thus, it is possible that caspase-11 modulated phagosome-lysosome fusion by affecting actin polymerization since the lack of caspase-11 maintained cofilin in the unphosphorylated active form, sustaining actin depolymerization which hinders proper phagosome-lysosome fusion. Accordingly, we found that the low amount of polymerized actin in the vicinity of the *L. pneumophila*-containing phagosome in caspase-11-deficient macrophages was associated with defective fusion with the lysosome. This conclusion was further corroborated by the fact that caspase-11 interacted with actin upon infection with *L. pneumophila* that expressed flagellin. Interestingly, *L. pneumophila*-containing phagosomes in *Casp1*$^{-/-}$ macrophages (which also lack caspase-11) failed to acquire phalloidin staining and do not fuse with lysosomal compartments. However, phagosomes enclosing non-pathogenic bacteria such as *E. coli* acquired phalloidin staining regularly and fused with the lysosome in WT and caspase-11-deficient macrophages. Therefore, caspase-11 separates phagosomal fusion with lysosomes according to their cargo, uncoupling their trafficking pathways. It is also possible that endocytic pathways followed by pathogenic bacteria are distinct from general phagocytic pathways leading to lysosomal degradation.

Taken together, in WT murine macrophages, intracellular *L. pneumophila* replication was regulated by caspase-11 and caspase-1, and required a functional Dot system. This information in addition to the existence of an interaction between caspase-11 and members of the Nlrc4 inflammasome only in the presence of bacterial flagellin, led us to propose a working model where monomeric flagellin leaking through the Dot system engaging caspase-1 and caspase-11 within the inflammasome complex (Santic et al., 2007; Silveira and Zamboni, 2010). Both caspase-1 and caspase-11 converge on the fusion of the *L. pneumophila* vacuole with the lysosome yet function independently of each other. Nevertheless, in human macrophages, ectopic expression of caspase-4 and caspase-5 cooperated to activate caspase-1 and dephosphorylate cofilin during *L. pneumophila* infection thus, restricting bacterial growth. Therefore, the recapitulation of events taking place in restrictive murine macrophages such as caspase-1 activation (Abdelaziz et al., 2011a) and dynamic alteration of cofilin phosphorylation state renders permissive human macrophages restrictive to *L. pneumophila*.

Our study is not the first report describing the encounter between the inflammasome and cytoskeletal signaling (Waite et al., 2009a; Waite et al., 2009b) however, the contribution of caspase-11 to phagosome-lysosome fusion in the context of intracellular infection has not been previously reported. Our results also demonstrate the intriguing possibility of biological functions of caspase-11 during bacterial infections.

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Material and Methods

Preparation of bone-marrow-derived macrophages (BMDM)

All animal experiments were performed according to protocols approved by the Animal Care Use Committee of The Ohio State University College of Medicine. Wild-type (WT) C57BL/6 and A/J mice were purchased from Jackson. Caspase-11-deficient (*Casp4*$^{-/-}$) mice on C57BL/6 background were obtained from Dr. Yang at Harvard University. Caspase-1-deficient (*Casp1*$^{-/-}$) mice on C57BL/6 background were obtained from Dr. Hise at Case Western University. BMDMs were prepared as previously described (Abdelaziz et al., 2011a; Abdelaziz et al., 2011b; Abdulrahman et al., 2011; Akhter et al., 2009; Amer et al., 2006; Kotrange et al., 2011).

Bacterial growth *in vitro*

L. pneumophila strain Lp02, the Dot type IV secretion mutant (*dotA*$^{-/-}$) and SSK strain were previously described (Amer and Swanson, 2005; Brieland et al., 1994; Sturgill-Koszycki and Swanson, 2000). Infections and quantification of colony-forming-units (CFUs) was previously described. *Escherichia coli* strain DH5α, *Listeria monocytogenes*, *Francisella novicida* and *Salmonella Typhimurium* were grown as previously described (Abdelaziz et al., 2011a; Abdelaziz et al., 2011b; Abdulrahman et al., 2011; Akhter et al., 2009; Amer et al., 2006; Kotrange et al., 2011).

Immunoblotting

Proteins on Western blots were detected with specific antibodies against caspase-11 (Sigma Aldrich), caspase-1 (Cell Signaling), caspase-3 (Cell Signaling), caspase-7 (Cell Signaling), Asc (Alexis Biochemicals), Nlrc4 (Novus Biologicals), cofilin (Cell Signaling), phosphorylated-cofilin (Cell Signaling), flotillin-1 (Santa Cruz), Flag (Sigma) and actin (Abcam). Corresponding bands were visualized as previously described (Abdelaziz et al., 2011a; Abdelaziz et al., 2011b; Akhter et al., 2009).

Transmission Electron Microscopy

WT and caspase-11-deficient *(Casp4*$^{-/-}$*)* primary murine macrophages were processed as previously described. (Abdelaziz et al., 2011a; Abdelaziz et al., 2011b; Abdulrahman et al., 2011; Akhter et al., 2009; Amer et al., 2006; Kotrange et al., 2011).

Fluorescence microscopy

Lysotracker Red (Invitrogen) was used to stain acidic vesicles. Calreticulin antibody (Stressgen) and *Legionella* antibody (Abcam) were used as previously described. Polymerized F-actin structures were visualized by staining with rhodamine-phalloidin (1:100 dilution, Molecular Probes) for 30 min (Li et al., 2007). Images were taken using laser scanning confocal fluorescence microscope using a 60x objective as previously described (Abdelaziz et al., 2011a; Abdelaziz et al., 2011b; Abdulrahman et al., 2011; Akhter et al., 2009; Amer et al., 2006; Kotrange et al., 2011)

Transfection of primary macrophages with small interfering RNA (siRNA)

siRNA treatment was performed using siRNA against mouse *Casp4* (Dharmacon): GUGCAACAAUCAUUUGAAA, AAGCUAAUCUGGAAAUGGA, CGAAAGGCUCUUAUCAUAU, GAUGUGCUACAGUAUGAUA. siRNA was nucleofected into primary macrophages using Lonza Nucleofection kit and Amaxa equipment as described previously and according to the manufacturer's protocol (Abdelaziz et al., 2011a; Abdelaziz et al., 2011b; Abdulrahman et al., 2011; Akhter et al., 2009; Gavrilin et al., 2006; Gavrilin et al., 2009; Kotrange et al., 2011).

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Plasmids and transfection

Mouse caspase-11 plasmid (pCASGGS-*Casp4*), inactive caspase-11 plasmid (pCAGGS-*Casp4*m2) and pCAGGS vector (LMBP 3818) (PL- Casp11, PL-inactive Casp11, and PL respectively) were purchased from Gent University (Belgium). Plasmids encoding human caspase-4 (*CASP4*) and caspase-5 (*CASP5*) were purchased from Origene. THP-1 monocytes were treated with 200nM of phorbol-12-myristate-13-acetate (PMA) for 3 hrs to transform them to macrophage-like adherent cells. Plasmids were nucleofected (Lonza) into murine BMDMs and THP-1 human monocytes using Y-01 or V-01 program respectively and as described previously (Abdelaziz et al., 2011a; Abdelaziz et al., 2011b; Abdulrahman et al., 2011; Gavrilin et al., 2009; Hall et al., 2007; Kotrange et al., 2011). Bacteria were added after 16 hrs of recovery from nucleofection.

RT-PCR

Quantification of *Casp4* expression was performed with SYBR Green I PCR Master Mix in the StepOne Plus Real Time PCR System (both, Applied Biosystems) and expressed in relative copy numbers (RCN) as we described earlier (Abdelaziz et al., 2011a; Abdelaziz et al., 2011b; Abdulrahman et al., 2011; Hughes et al., 2010; Kotrange et al., 2011). The following primers were used for murine *Casp4*: CATCACTAGACTCATTTCCTGCTT and CTGGAATTTCAGGAATAGAATGTG.

Immunoprecipitation of active caspases

Mouse macrophages were infected with *L. pneumophila* for 4 hrs. To label active caspase-11, 5×10^7 cells were lysed in KPM buffer in the presence of biotinylated-YVAD-CMK (AnaSpec, Frement, CA) as previously described (Fahy et al., 1999; Shoma et al., 2008). Next, lysates were incubated and immunoprecipitated using Streptavidin-beads (Thermo Fisher Scientific). Immunoprecipitates were analyzed by western blots. Membranes were immunoblotted using caspase-11 antibodies.

Statistical Analysis

All experiments were performed at least three independent times. Comparisons of groups for statistical difference were analyzed using Student's two-tailed *t*-test. P value ≤ 0.05 is considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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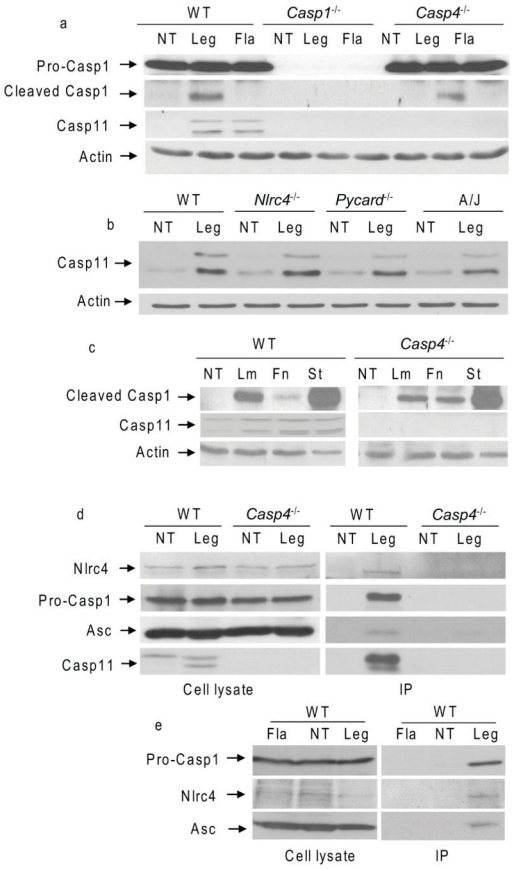

Figure 1. Caspase-11 is dispensable for caspase-1 activation and interacts with members of the Nlrc4 inflammasome

(a) Wild-type (WT), caspase-1-deficient (*Casp1*$^{-/-}$) and Caspase-11-deficient (*Casp4*$^{-/-}$) BMDMs were infected with *L. pneumophila* (Leg), its corresponding flagellin mutant (Fla) for 2 hrs, or left untreated (NT). Cell lysates were immunoblotted for pro-, cleaved (caspase-1) Casp1, (caspase-11) Casp11 and actin. (b) WT, *Nlrc4*$^{-/-}$, *Pycard*$^{-/-}$ (Asc-deficient) and A/J (express mutant Naip5) BMDMs were uninfected (NT) or infected with Leg and the expression of caspase-11 was examined by Western blot. (c) WT and *Casp4*$^{-/-}$ BMDMs were infected with *Listeria monocytogenes* (Lm), *Francisella novicida* (Fn), and *Salmonella Typhimurium* (St) for 2 hrs or left untreated (NT). (a, b, and c) Cell lysates were immunoblotted for pro-, and cleaved Casp1, and Casp11 and actin. (d) WT and *Casp4*$^{-/-}$ BMDMs were untreated (NT) or infected with Leg for 4 hrs. (e) WT macrophages were untreated (NT) or infected with Leg, Fla for 4 hrs. (d and e) Casp11 was then immunoprecipitated from cell lysates. The Western blots of cell lysates and of immune-complexes (IP) were probed with Nlrc4, Casp1, Asc and Casp11 antibodies. Blots are representative of three independent experiments.

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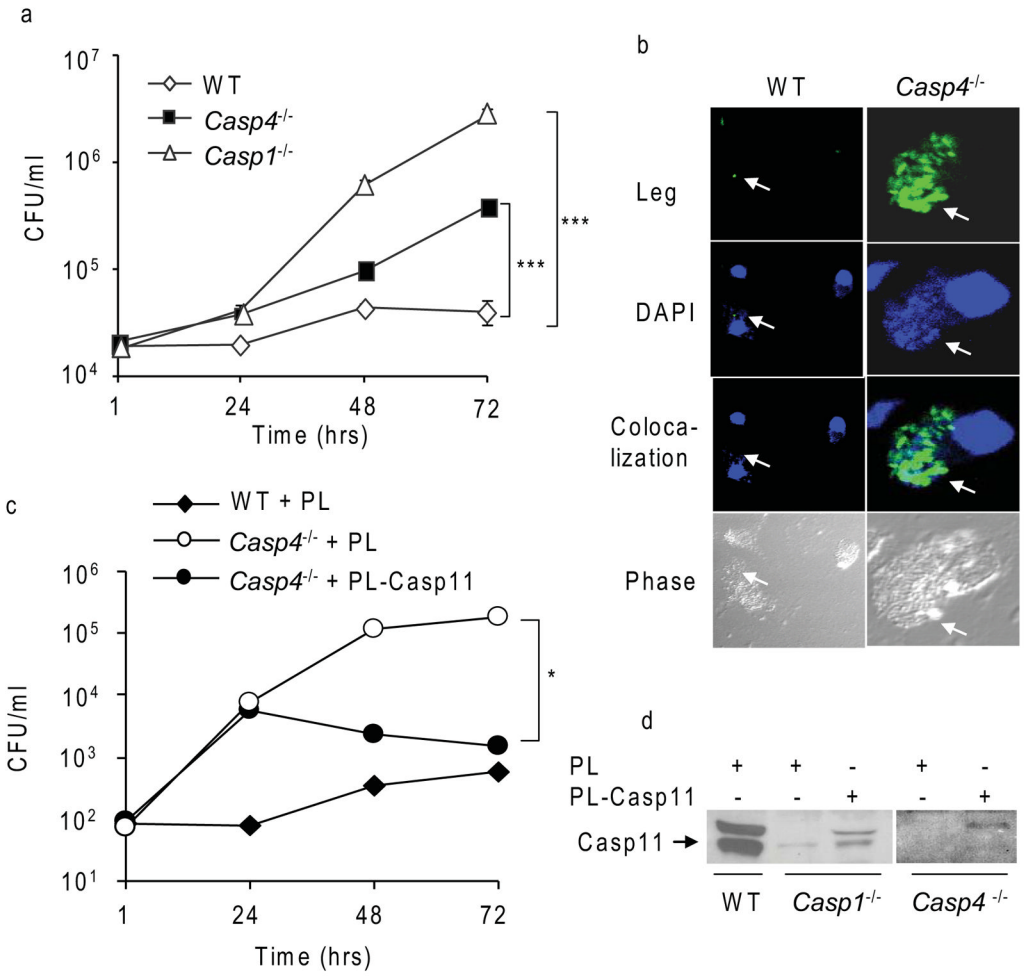

Figure 2. Caspase-11-deficient *(Casp4$^{-/-}$)* macrophages allow *L. pneumophila* intracellular replication

(a) Wild-type (WT), caspase-11-deficient (*Casp4*$^{-/-}$) and caspase-1-deficient (*Casp1*$^{-/-}$) murine BMDMs were infected with *L. pneumophila* (Leg) and colony forming units (CFUs) were enumerated at 1, 24, 48 and 72 hrs. Data are representative of three independent experiments and presented as means ± S.D. Asterisks indicate significant differences from WT macrophages (***P<0.001). (b) Confocal microscopy of Leg-infected WT or *Casp4*$^{-/-}$ BMDMs after 24 hrs. Nuclei are stained blue with DAPI and Leg express green florescent protein (GFP). White arrows indicate the sites of Leg. (c and d) WT, *Casp1*$^{-/-}$, and *Casp4*$^{-/-}$ BMDMs were nucleofected with plasmid harboring *Casp4* (PL- Casp11) or empty vector (PL) for 24 hrs. (c) BMDMs were infected with *L. pneumophila* and CFUs were enumerated at 1, 24, 48 and 72 hrs. (d) Samples were lysed and immunoblotted for caspase-11 expression.

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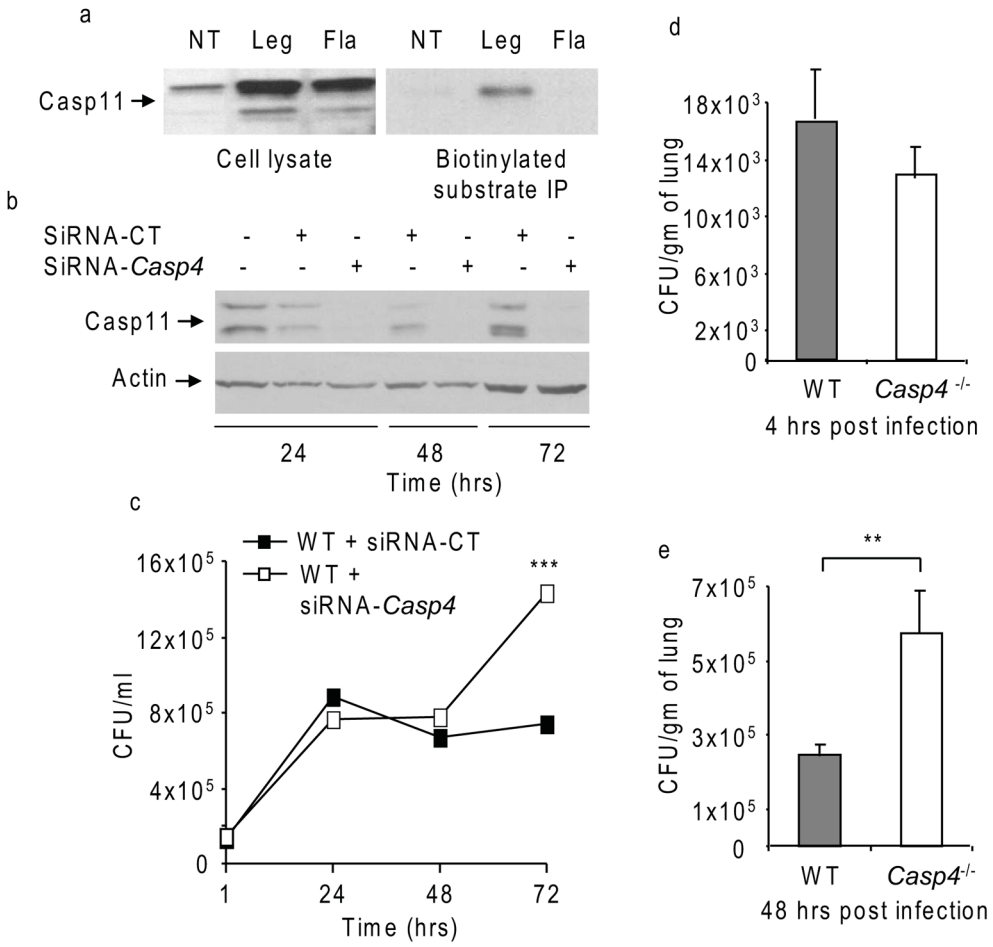

Figure 3. Caspase-11 is activated during *L. pneumophila* infection and restricts infection *in vivo*

(a) Wild-type (WT) BMDMs were infected with *L. pneumophila* (Leg) or its isogenic flagellin mutant (Fla), lysed (cell lysates), then mixed with biotinylated-YVAD-CMK, immunoprecipitated (IP), processed for Western blot with caspase-11 antibodies. (b) WT macrophages were nucleofected with siRNA specific to *Casp4* (siRNA-*Casp4*) or siRNA control (siRNA-CT) then lysed and processed for Western blots to detect the expression of caspase-11 (Casp11) protein. (c) Macrophages were treated as in (b) then, infected with *L. pneumophila* and colony forming units (CFUs) were enumerated at 1, 24, 48 and 72 hrs. Data are representative of three independent experiments ± S.D. (d and e) WT and caspase-11-deficient *(Casp4*$^{-/-}$*)* mice were infected intratracheally with *L. pneumophila* then CFUs recovered from homogenized lungs were enumerated and expressed as CFU per gram of lung tissue at 4 hrs (d) and 48 hrs (e). Data are represented as the means of data obtained from 4 mice ±S.D. Asterisks indicate significant differences (**P<0.01; ***P<0.001).

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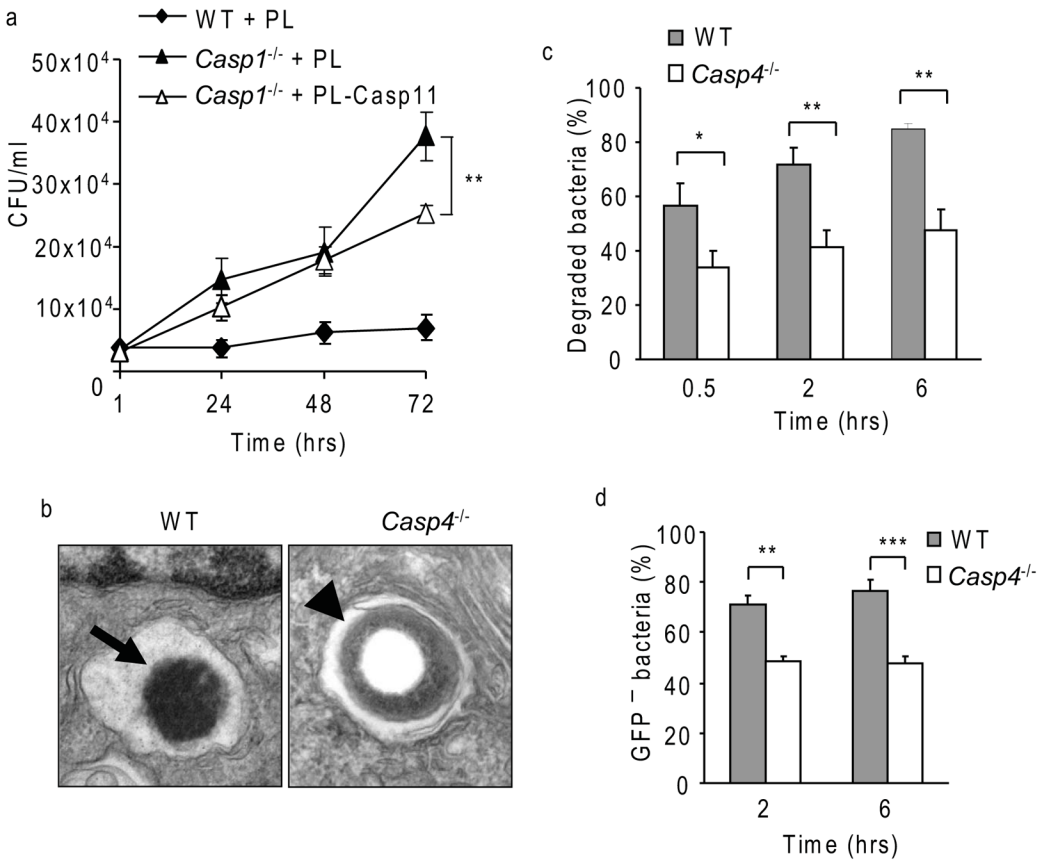

Figure 4. Caspase-11 promotes *L. pneumophila* degradation in macrophages

(a) Wild-type (WT) and caspase-1-deficient (*Casp1*$^{-/-}$) BMDMs were nucleofected with plasmid carrying *Casp4* gene (PL- Casp11) or vector alone (PL), and infected with *L. pneumophila* and colony forming units (CFUs) were enumerated at 1, 24, 48, and 72 hrs. (b) WT and caspase-11-deficient *(Casp4*$^{-/-}$*)* macrophages were infected with *L. pneumophila* for 4 hrs and processed for electron microscopy. Black arrow indicates internalized *L. pneumophila* showing irregular contour, and black arrowhead indicates intact *L. pneumophila* (magnification 80,000). (c) The percent of degraded bacteria in WT and *Casp4*$^{-/-}$ BMDMs were quantified by confocal microscopy using specific *L. pneumophila* antibody. (d) WT and *Casp4*$^{-/-}$ macrophages were infected with the SSK strain of *L. pneumophila* that responds to IPTG by expressing GFP. The percentage of *L. pneumophila* not responding to IPTG (GFP$^{-/-}$) was quantified by confocal microscopy. Data in a, c and d are representative of three independent experiments ±S.D. Asterisks indicate significant differences (**P*< 0.05; ***P*<0.01; ****P*<0.001).

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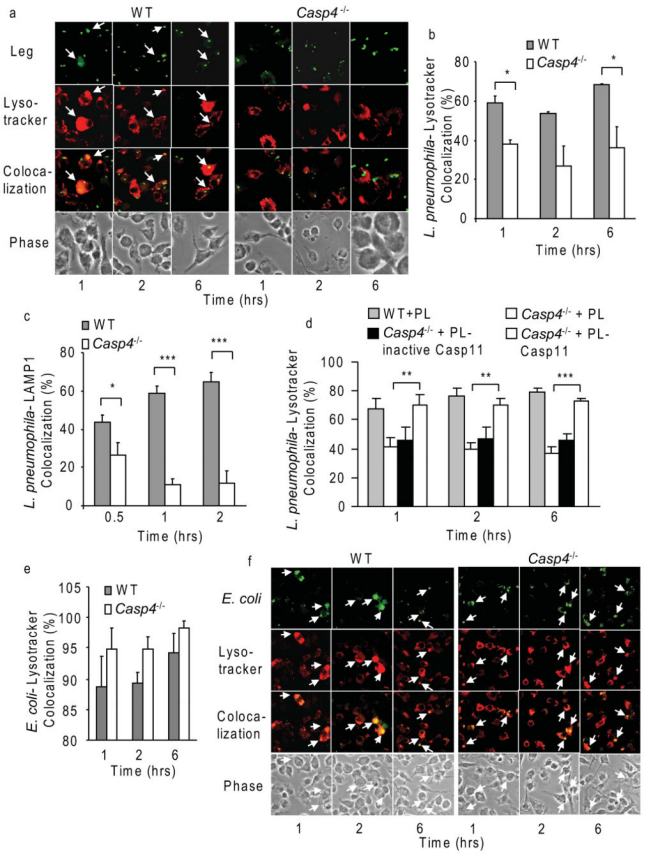

Figure 5. Caspase-11 activity is required to promote the fusion of the lysosome with phagosomes harboring *L. pneumophila* but not those harboring *E. coli*

(a) WT and caspase-11-deficient *(Casp4*$^{-/-}$*)* BMDMs were infected with *L. pneumophila* (Leg) constitutively expressing GFP. Fixed samples were processed for confocal microscopy. (b and e) The colocalization of the bacteria with lysotracker red was enumerated. The sites of colocalization are indicated with white arrows (a and f). (c) Cells treated as in (a) were fixed and colocalization of Leg with the endocytic marker LAMP-1 was quantified. (d) WT and *Casp4*$^{-/-}$ BMDMs were nucleofected with vector alone (PL), plasmid carrying native *Casp4* gene (PL- Casp11), or plasmid carrying mutant *Casp4* gene (PL-inactive Casp11), and infected with Leg. (e and f) WT and *Casp4*$^{-/-}$ BMDMs were infected with GFP constitutively expressing *Escherichia coli* (*E. coli*). Data are representative of three independent experiments and presented as the means ± S.D. Asterisks indicate significant differences (**P*<0.05; ***P*<0.01; ****P*<0.001).

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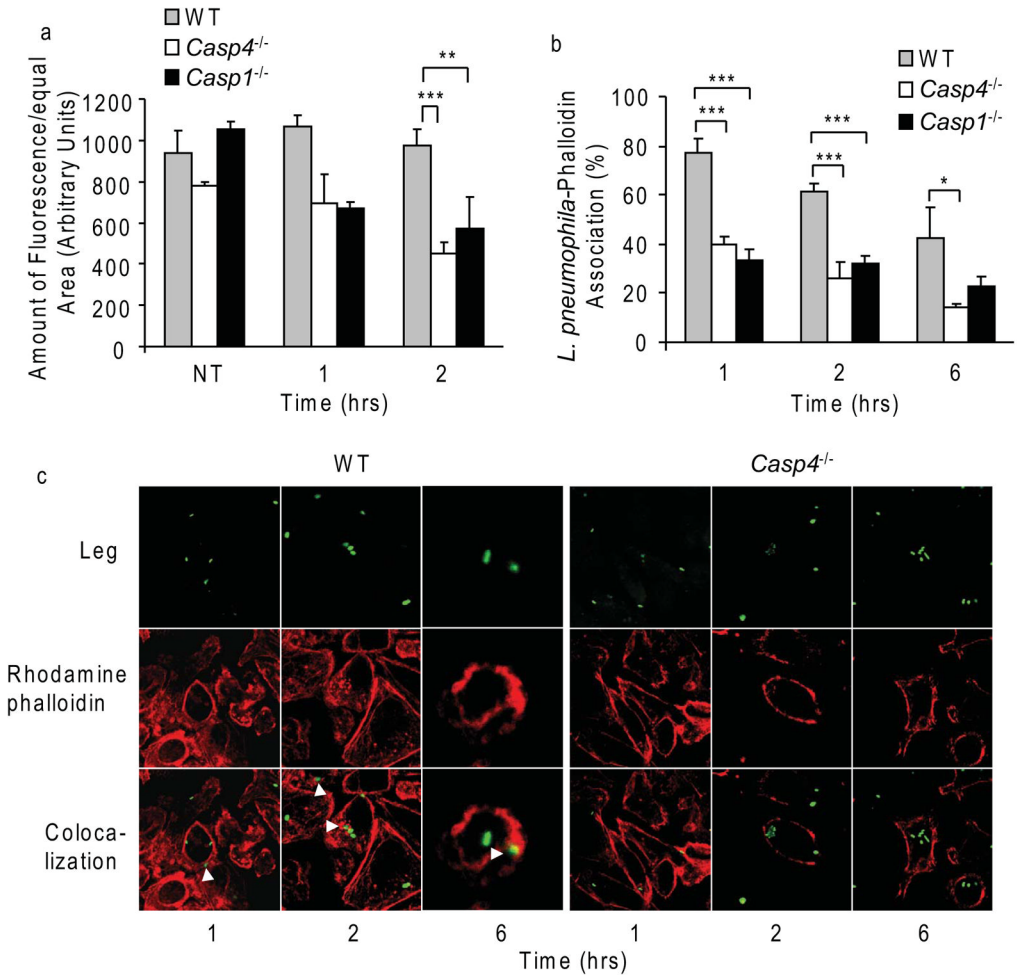

Figure 6. Caspase-11 is required for the dynamic formation of polymerized actin around phagosomes

(a–c) WT, caspase-11-deficient (*Casp4*$^{-/-}$) (a and b), and *Casp1*$^{-/-}$ BMDMs (c) were infected with *L. pneumophila* (Leg) constitutively expressing GFP. Polymerized actin was stained with rhodamine-phalloidin. (a) The amount of rhodamine-phalloidin within equal areas was quantified by confocal microscopy and expressed as arbitrary units. (b) The percentage of phalloidin-labelled Leg-containing phagosomes was quantified by confocal microscopy. (c) Confocal microscopy showing rhodamine-phalloidin staining (red) around GFP-expressing (green) Leg. Phagosomes containing degraded bacteria are heavily labeled for polymerized actin (white arrow heads). Data in a and b are representative of three independent experiments and presented as the means ±S.D. Asterisks indicate significant differences (*P<0.05; **P<0.01; ***P<0.001).

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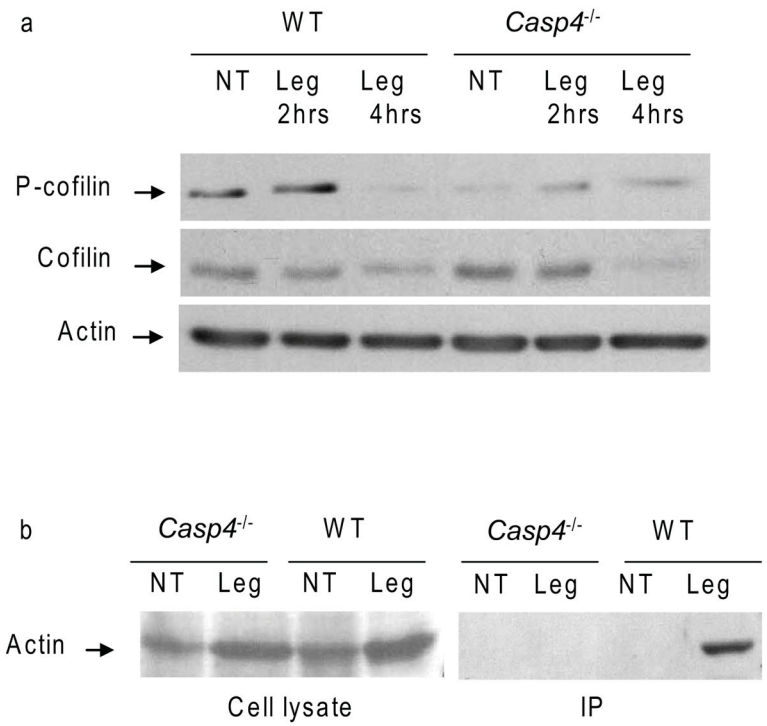

Figure 7. Caspase-11 is required for the phosphorylation of cofilin and interacts with actin upon *L. pneumophila* infection

(a) WT and caspase-11-deficient (*Casp4*$^{-/-}$) BMDMs were infected with *L. pneumophila* (Leg) for 2 and 4 hrs or left untreated (NT). BMDMs lysates were immunoblotted with antibodies against phosphorylated cofilin (P-cofilin), cofilin and actin. (b) WT and *Casp4*$^{-/-}$ BMDMs were infected with Leg or left NT, lysed (cell lysates), and immunoprecipitated (IP) with beads coated with caspase-11 antibody and immunoblotted with actin antibody.