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Temporal and spatial trigger of post-exponential virulence-associated regulatory cascades by *Legionella pneumophila* after bacterial escape into the host cell cytosol

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Summary

During late stages of infection and prior to lysis of the infected macrophages or amoeba, the *Legionella pneumophila*-containing phagosome becomes disrupted, followed by bacterial escape into the host cell cytosol, where the last few rounds of bacterial proliferation occur prior to lysis of the plasma membrane. This coincides with growth transition into the post-exponential (PE) phase, which is controlled by regulatory cascades including RpoS and the LetA/S two component regulator. Whether the temporal expression of flagella by the regulatory cascades at the PE phase is exhibited within the phagosome or after bacterial escape into the host cell cytosol is not known. We have utilized fluorescence microscopy-based phagosome integrity assay to differentiate between vacuolar and cytosolic bacteria/ or bacteria within disrupted phagosomes. Our data show that during late stages of infection, expression of FlaA is triggered after bacterial escape into the macrophage cytosol and the peak of FlaA expression is delayed for few hours after cytosolic residence of the bacteria. Importantly, bacterial escape into the host cell cytosol is independent of flagella, RpoS, and the two component regulator LetA/S, which are all triggered by *L. pneumophila* upon growth transition into the PE phase. Disruption of the phagosome and bacterial escape into the cytosol of macrophages is independent of the bacterial pore-forming activity, and occurs prior to the induction of apoptosis during late stages of infection. We conclude that the temporal and spatial engagement of virulence-associated regulatory cascades by *L. pneumophila* at the PE phase is temporally and spatially triggered after phagosomal escape and bacterial residence in the host cell cytosol.

Keywords

Legionnaires'; lysosomes; intracellular; endosome; trafficking

Introduction

Legionella pneumophila, the causative agent of Legionnaire's disease, replicates within alveolar macrophages in humans and in amoebae in the environment (Swanson & Hammer, 2000, Fields, 1996, Molmeret *et al.*, 2004b, Molmeret *et al.*, 2005). The cellular and molecular aspects of the intracellular fate of *L. pneumophila* in both of these evolutionarily

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distant host cells are similar (Molmeret et al., 2004b, Molmeret *et al.*, 2004a, Molmeret et al., 2005, Segal & Shuman, 1999) where the *Legionella*-containing phagosome (LCP) is blocked from maturation through the endosomal-lysosomal pathway and becomes remodeled by the rough endoplasmic reticulum (RER) (Abu Kwaik, 1996, Horwitz, 1983b, Horwitz, 1983a, Bozue & Johnson, 1996, Tilney *et al.*, 2001, Al-Khodori *et al.*, 2008b). These modulations of phagosome biogenesis are controlled by the Dot/Icm type IV secretion apparatus (Isberg *et al.*, 2009, Vogel *et al.*, 1998, Segal *et al.*, 1998). Interestingly, trafficking of other species of *Legionella* is different from *L. pneumophila*; such as the *L. longbeachae*-containing phagosome is remodeled into a late endosome-like phagosome surrounded by the RER, and bacterial proliferation occurs in a late endosome-like phagosome (Asare & Abu Kwaik, 2007, Asare *et al.*, 2007). Despite its different trafficking, *L. longbeachae* also escapes into the cytosol prior to lysis of the host cell, similar to *L. pneumophila* (Asare & Abu Kwaik, 2007, Asare *et al.*, 2007). Egress of *L. pneumophila* into the cytosol is independent of the hydrolytic enzymes secreted through the type II secretion system (Molmeret et al., 2004a, Hales & Shuman, 1999, Banerji *et al.*, 2008, Banerji *et al.*, 2005, Rossier *et al.*, 2008, DebRoy *et al.*, 2006).

A biphasic life cycle associated with bacterial differentiation governs the life cycle of *L. pneumophila*. After a lag phase of ~4 h within macrophages and protozoa, exponential replication of *L. pneumophila* is initiated (Horwitz, 1983a, Rowbotham, 1986). Upon entry into the post-exponential (PE) growth phase, *L. pneumophila* undergo phenotypic modulations and differentiation (Faulkner & Garduno, 2002, Garduno *et al.*, 2002, Hiltz *et al.*, 2004) and exhibits several virulence-related traits: including, motility, and a pore-forming activity essential for lysis of the host cell and bacterial egress into the extracellular environment (Bachman & Swanson, 2001, Hammer *et al.*, 2002, Molofsky & Swanson, 2003, Bachman & Swanson, 2004, Dalebroux *et al.*, 2009, Alli *et al.*, 2000, Byrne & Swanson, 1998, Gao & Abu Kwaik, 2000a, Molmeret *et al.*, 2002a). Phenotypic transition of *L. pneumophila* into the transmission phenotype at the PE phase is highly controlled by regulatory cascades, including RelA, SpoT, PmrA/B, RpoS and the two component regulatory system LetA/S (Dalebroux *et al.*, 2009, Al-Khodori *et al.*, 2008a, Zusman *et al.*, 2007, Hammer & Swanson, 1999, Bachman & Swanson, 2001, Hammer *et al.*, 2002, Byrne & Swanson, 1998, Abu-Zant *et al.*, 2006, Bruggemann *et al.*, 2006, Hovel-Miner *et al.*, 2009).

Upon termination of the intracellular infection, the pore-forming activity of *L. pneumophila* facilitates bacterial escape into the cytosol by ~12h where the last few rounds of bacterial replication occur prior to lysis of the host cell by ~18h (Alli *et al.*, 2000, Molmeret & Abu Kwaik, 2002, Molmeret *et al.*, 2002a, Molmeret *et al.*, 2002b, Bitar *et al.*, 2005). A mutant of *L. pneumophila* defective in the pore-forming activity is defective in egress from the host cell, and therefore has been designated rib, for release of intracellular bacteria (Alli *et al.*, 2000). The rib mutant has a spontaneous deletion that results in truncation of IcmT, which is thought to be an inner membrane structural component of the Dot/Icm secretion apparatus (Molmeret *et al.*, 2002a, Molmeret *et al.*, 2002b, Bitar *et al.*, 2005). Despite its trafficking and prolific intracellular replication similar to the wild type strain, the rib mutant fails to lyse the host cell at the post-exponential phase (Alli *et al.*, 2000, Molmeret *et al.*, 2002a, Molmeret *et al.*, 2002b, Bitar *et al.*, 2005). It is not known whether the pore-forming activity defective in the rib mutant is involved in disruption of the phagosomal membrane or in lysis of the plasma membrane by the cytosolic bacteria. Whether the regulatory cascade triggered by *L. pneumophila* at the post-exponential phase is involved in bacterial escape into the cytosol or lysis of the plasma membrane is not known either. Interestingly, although *L. pneumophila* triggers activation of caspase-3 during early stages of infection of human macrophages (Muller *et al.*, 1996, Gao & Abu Kwaik, 1999a, Gao & Abu Kwaik, 1999b, Gao & Abu Kwaik, 2000b, Zink *et al.*, 2002, Neumeister *et al.*, 2002, Walz *et al.*, 2000,

Molmeret *et al.*, 2004c, Abu-Zant *et al.*, 2005), apoptosis is not triggered till late stages of the infection, and the delay in apoptosis is mediated by the induction of various anti-apoptotic pathways, some of which are mediated by NF- κ B and SdhA (Losick & Isberg, 2006, Abu-Zant *et al.*, 2007, Laguna *et al.*, 2006). The induction of late stage apoptosis coincides with phenotypic transition at the post-exponential phase (Faulkner & Garduno, 2002, Garduno *et al.*, 2002, Hiltz *et al.*, 2004, Hammer & Swanson, 1999, Bachman & Swanson, 2001, Hammer *et al.*, 2002, Byrne & Swanson, 1998), but the kinetics of these temporal and spatial events and their coordination at the PE phase are not known. Whether apoptosis of human macrophages during late stages of infection is involved in bacterial egress is not known.

In this paper, we show that expression of flagellin, which is an indicator of genes triggered by the regulatory cascades at the PE phase, occurs after disruption of the phagosomal membrane and bacterial escape into the cytosol. Importantly, flagellin, and major regulators at the PE phase such as RpoS, LetA and LetS are dispensable for disruption of the *Legionella*-containing phagosome (LCP) and bacterial escape into the cytosol. Disruption of the LCP and bacterial escape into the cytosol is independent of the pore-forming activity, and is also independent of the induction apoptosis during late stages of infection.

Results and Discussion

Expression of flagellin by *L. pneumophila* after bacterial escape into the cytosol

Although expression of *flaA* and flagellation has been shown to be triggered at the PE phase (Hammer & Swanson, 1999, Byrne & Swanson, 1998), it is not known whether this temporal expression is triggered by signals that are transduced to the bacteria within the phagosome or after escape into the host cell cytosol (Molmeret *et al.*, 2004a). Therefore, we first examined the kinetics of temporal and regulation of *flaA* at different stages of the intracellular infection. We utilized Real-Time PCR on the intracellular bacteria harvested from hMDMs to determine the level of *flaA* mRNA at various stages of the infection. The data showed that expression of *flaA* was baseline up to 12h post-infection and was triggered by ~4-fold by 16h and up to 12-fold by 20h post-infection (Fig. 1). These data suggested that it is most likely that this trigger of *flaA* expression is exhibited by cytosolic bacteria (Molmeret *et al.*, 2004a).

Previous studies electron microscopy studies have shown that the phagosome is intact up to 8h post-infection, while integrity of the phagosomes is disrupted by 12h, and the majority of the phagosomes are disrupted by 16-20h post-infection (Molmeret *et al.*, 2004a). We have recently established a fluorescence-based phagosome integrity assay, which allows better and more reliable quantitation of cytosolic vs. vacuolar bacteria (Molmeret *et al.*, 2007, Santic *et al.*, 2007b, Santic *et al.*, 2008). Therefore, we utilized this assay to determine more accurately whether expression of *flaA* correlated with localization of the bacteria within intact phagosomes or in the cytosol. In the fluorescence microscopy-based phagosome integrity assay (Molmeret *et al.*, 2007, Santic *et al.*, 2007b, Santic *et al.*, 2008), the plasma membrane is selectively and transiently permeabilized using the glass beads 'loading technique' (McNeil & Warder, 1987, Reddy *et al.*, 2001), and the macrophage cytosol is loaded with specific anti-bacterial antibodies (Molmeret *et al.*, 2007, Santic *et al.*, 2007b, Santic *et al.*, 2008). The plasma membrane of the macrophage is "wounded" by glass beads for a few seconds, during which antibodies can pass through the lesions of plasma membrane into the cytosol. This is followed by removal of the glass beads and the antibodies, and further incubation for a few minutes to allow the plasma membrane to repair the "wounds", which occurs within 1-3 minutes in most cells (McNeil & Warder, 1987, Reddy *et al.*, 2001). If the bacteria are surrounded by an intact limiting membrane, the cytosolic polyclonal antibodies will not have access to them, but if the bacteria are free

within the cytoplasm or within severely disrupted phagosomes, they would readily bind the antibodies. The hMDMs are then fixed and processed for confocal microscopy for integrity of the phagosome. All the bacteria are labelled after permeabilization by Triton X-100 with a monoclonal antibody. For a negative control, infected cells were treated with monoclonal antibody without permeabilization of the plasma membrane by the glass beads, which showed no binding of the antibody to intracellular bacteria for any of the strains tested (data not shown). For the positive control, the cells were permeabilized by Triton-X100, which allowed the antibodies to bind to all intracellular bacteria for all the strains. Our data were very clear that at 8h post-infection by the wild type strain AA100, there was minimal disruption of the phagosomes, but a gradual disruption of majority of the phagosomes (>70%) was clearly evident by 12h, which peaked (>90%) by 18-20h post-infection (Fig. 2). These data suggested that expression of *flaA* was correlated with the presence of the bacteria in the cytosol after disruption of the phagosomal membrane.

Next we determined, simultaneously, and within the same infected cells the kinetics of expression of *flaA*, and whether that expression was triggered by bacteria within intact phagosomes or by cytosolic bacteria. To examine differential and temporal expression of *flaA* at the single cell level, we utilized a plasmid construct that expressed GFP under the control of the promoter for *flaA*, as an indicator of genes triggered at the PE phase (Hammer & Swanson, 1999, Byrne & Swanson, 1998). The data were very clear that triggering of *pflaA-gfp* expression was exhibited by cytosolic bacteria, and not by the bacteria within intact phagosomes (Fig. 3). The data confirmed our data above that *pflaA-gfp* was triggered after bacterial escape into the cytosol as the number of GFP-positive cytosolic bacteria increased at 12-16h (~35%) and peaked at 20h (~55%) (Fig. 3). The large number of cytosolic bacteria that did not express *pflaA-gfp* at 20h indicated that bacterial presence in the cytosol for sometime was essential to trigger *pflaA-gfp*. Note that lysis of the host cell was exhibited within few hours of bacterial escape into the cytosol, indicating that host cell lysis was manifested rather rapidly once the PE regulatory cascade was triggered by the cytosolic bacteria. Taken together, we conclude that after bacterial escape into the cytosol, the bacteria senses cytosolic signals that trigger expression of the PE regulatory cascade that results in rapid lysis of the host cell plasma membrane and bacteria egress. The cytosolic signals transduced to the bacteria are not known but they likely include a signal of low level of nutrients that are rapidly consumed by the large number of cytosolic bacteria. Our data do not exclude the possibility that the trigger is initiated in the phagosome just prior to escape and is amplified once the bacteria sense the cytosolic signals.

We examined whether cytosolic extracts of U937 macrophages would trigger *in vitro*-grown E phase bacteria to express *flaA*. In this experiment, we used equivalent number of intracellular bacteria and host cells to what is routinely done in these experiments during infection. Broth-grown bacteria at the lag, E, and PE phases were incubated for different time intervals (5 min-4h) with cytosolic extracts. Our results showed that there was no detectable effect of the cytosolic extract on expression of *flaA* at any stage tested when examined by microscopy for motility or by detection of expression of FlaA-GFP fusion by fluorescence microscopy. However, interpretation of the results of these studies might be complicated, since this approach is an artificial condition that does not mimic the signaling events within the intact host cell. During infection, vesicles and organelles may interact with the bacteria or the vacuole and that interaction would not exist when the cell is lysed. In addition, at 16-20h post-infection when FlaA is triggered, the host cells are wasted and the cytosol is most likely different than the cytosol of lysed non-infected cells. It is likely the cytosolic signals that trigger virulence traits at the PE phase are present in the cytosol of wasted cells but not in healthy cytosolic extracts.

Escape of *L. pneumophila* into the cytosol of macrophages is independent of flagellation

One of the phenotypic traits that are triggered by regulators of the PE phase is flagellation and motility, which are major indicators of phenotypic transition at the PE phase (Hammer & Swanson, 1999, Byrne & Swanson, 1998). It is not known whether flagellation is involved in disruption of the phagosome and escape of the bacteria into the cytosol. We constructed a *flaA* mutant of strain AA100 to study the role of temporal trigger of flagellation in the kinetics of disruption of the phagosome and bacterial escape into the cytosol. To differentiate between vacuolar vs. cytosolic bacteria, we utilized the fluorescence microscopy-based phagosome integrity, described above (Molmeret et al., 2007, Santic et al., 2007b, Santic et al., 2008). Our data showed that at 8, 12, 16 and 20 h post-infection of hMDMs, there was no significant difference between the wild type strain and the *flaA* mutant in the kinetics of disruption of the phagosome and bacterial escape into the cytosol (Fig. 2 and data not shown). These data are consistent with our data above that flagellation was triggered after bacterial escape into the cytosol. We conclude that disruption of the phagosome and bacterial escape into the macrophage cytosol is independent of flagellation.

Taken together, these data show that expression of *flaA*, as an indicator of the PE regulatory cascade, is triggered after bacterial escape into the cytosol. Since a large number of cytosolic bacteria do not express *pflaA* by 12-20h post-infection, it is most likely that bacterial residence within the cytosol for few hours is essential for the cytosolic signals to be transduced to the bacteria to trigger the PE regulatory cascade. Thus, the PE phase signals that are transduced to the intracellular bacteria are encountered by the bacteria after their phagosomal escape and cytosolic residence.

Role of the post-exponential phase regulatory cascades in disruption of the LCP

Phenotypic modulation and differentiation by *L. pneumophila* from the replication phase into the transmission phase at the PE phase (Faulkner & Garduno, 2002, Garduno et al., 2002, Hiltz et al., 2004) is highly regulated by several regulators, including the RpoS stationary phase transcription factor, and the two component regulatory system LetA/S (Hammer & Swanson, 1999, Bachman & Swanson, 2001, Hammer et al., 2002, Byrne & Swanson, 1998, Bruggemann et al., 2006). Since escape of *L. pneumophila* from the LCP into the cytosol coincides with growth transition into the PE phase (Molmeret & Abu Kwaik, 2002, Molmeret et al., 2004a), we examined the role of few crucial components of this regulatory cascade in disruption of the LCP and bacterial escape into the cytosol. We utilized isogenic mutants of the parental strain AA100 in the *rpoS*, *letA*, and *letS* genes to examine their roles in the kinetics of disruption of the LCP and bacterial escape into the macrophage cytosol. Since these mutants are not defective in intracellular replication within U937 macrophages, we examined the kinetic of their escape from the phagosome into the cytosol within these cells. We utilized the fluorescence microscopy-based phagosome integrity to examine escape of the regulatory mutant's *rpoS*, *letA* and *letS* to the cytosol at different stages of infection. Our data showed that ~10% of the *rpoS*, *letA* and *letS* mutants disrupted their LCPs at 8 h post-infection, similar to the wild-type strain (Fig. 2) (Student *t*-test, $p>0.2$). At 12 and 18 h post-infection, 75-90% of all the mutants were cytosolic, which was not significantly different (Student *t*-test, $p>0.2$) from the wild-type strain (Fig. 2).

Disruption of the LCP and bacterial escape into the cytosol of macrophages and amoebae (Molmeret et al., 2004a) coincide with triggering the post-exponential regulatory cascade that governs phenotypic transition into the transmission phenotype of *L. pneumophila* (Hammer & Swanson, 1999, Bachman & Swanson, 2001, Hammer et al., 2002, Byrne & Swanson, 1998). Our data above show that RpoS, LetA, and LetS, which are major regulators of this regulatory cascade (Hammer & Swanson, 1999, Bachman & Swanson,

2001, Hammer et al., 2002, Byrne & Swanson, 1998), are not involved in disruption of the LCP and bacterial escape into the cytosol. We conclude that the major components of the regulatory cascades that trigger flagellation and other virulence-related traits are transduced to the intracellular bacteria after their escape into the cytosol and not in the intact phagosome.

Bacterial escape into the cytosol is independent of the pore-forming activity

Lysis of the host cell and bacterial egress from human macrophages after termination of intracellular replication is preceded by the escape of *L. pneumophila* into the cytosol, where the last few rounds of replication occur (Molmeret et al., 2004a). The pore-forming activity, which is defective in the rib mutant, who expresses a truncated IcmT, is essential for lysis of the host cell and subsequent bacterial egress (Alli et al., 2000, Molmeret et al., 2002a, Molmeret et al., 2002b). It is not known whether the pore-forming activity was required for disruption of the LCP and bacterial escape into the cytosol, or for subsequent lysis of the macrophages plasma membrane. Therefore, we examined whether the rib mutant was capable of disruption of the LCP and escape into the cytosol of hMDMs despite its defect in escape into the extracellular environment (Alli et al., 2000, Molmeret et al., 2002a, Molmeret et al., 2002b). We utilized the fluorescence microscopy-based phagosome integrity assay in which the vacuolar and cytosolic bacteria are differentially labeled. Our data showed that at 8 h post-infection of hMDMs, the wild-type bacteria in ~10% of infected macrophages bound the anti-bacterial antibodies loaded into the macrophage cytosol (Fig. 4), which is consistent with previous findings (Molmeret & Abu Kwaik, 2002, Molmeret et al., 2004a). At 12 h and 18 h post-infection, the wild-type bacteria bound the anti-bacterial antibodies in more than 80 and 90 % of infected macrophages, respectively. (Fig. 4) While at 8 h post-infection, ~10 % of the rib mutant bound the antibodies loaded into the macrophage cytosol, ~70 and 95 % of the mutant bacteria bound the antibodies at 12 and 18h post-infection, respectively (Fig. 4), which was not significantly different from the wild type strain (Student *t*-test, $p>0.2$). These results indicate that bacterial escape into the cytosol is independent of the pore-forming activity of *L. pneumophila* (Alli et al., 2000, Molmeret et al., 2002a, Molmeret et al., 2002b).

Macrophage apoptosis is triggered after bacterial escape into the cytosol

Despite early activation of caspase-3 by *L. pneumophila* during early stages of infection of human macrophages, apoptosis is not triggered till late stages of infection (Molmeret et al., 2004c, Abu-Zant et al., 2005, Abu-Zant et al., 2007), and this delay in apoptosis is mediated by SdhA and triggering several anti-apoptotic pathways in the infected cells (Laguna et al., 2006, Losick & Isberg, 2006, Abu-Zant et al., 2007, Abu-Zant et al., 2005). Inhibition of caspase-3 but not caspase-1 blocks the *L. pneumophila*-induced late stage apoptosis (Gao & Abu Kwaik, 1999b, Asare et al., 2007, Santic et al., 2007a, Molmeret et al., 2004c). Apoptosis of human macrophages infected by *L. pneumophila* coincides with disruption of the LCP and bacterial escape into the cytosol where the final few rounds of bacterial replication occur (Molmeret et al., 2004a). Therefore, we examined whether bacterial escape into the cytosol is a consequence of triggering the apoptotic program within infected hMDMs after waning of the anti-apoptotic stimuli (Losick & Isberg, 2006, Abu-Zant et al., 2007, Abu-Zant et al., 2005). To test this hypothesis, we examined whether inhibition of apoptosis would block disruption of the LCP and bacterial escape into the cytosol. At 1h after infection of hMDMs with *L. pneumophila*, macrophages were treated with the caspase-3 inhibitor (Molmeret et al., 2004c), and examined for integrity of the LCPs at the ultra-structural level using transmission electron microscopy, as we described previously (Molmeret et al., 2004a). Our data showed that there was no significant difference (Student *t*-test, $p>0.1$) between integrity of the LCPs in cells treated with the caspase-3 inhibitor compared to untreated cells at different intervals post-infection (Fig. 5). Most of the

untreated cells had apoptotic nuclei by 18h post-infection, and almost none of the treated cells were apoptotic (data not shown) (Gao & Abu Kwaik, 1999b). Inhibition of caspase-3 after establishment of the LCP, at 1h post-infection, had no detectable effect on the number of intracellular bacteria (Fig. 5), consistent with previous observations (Molmeret et al., 2004c, Abu-Zant et al., 2007).

To confirm these observations, we utilized the fluorescence-based phagosome integrity assay for differential labeling of vacuolar vs. cytosolic bacteria to determine the kinetic of the temporal and spatial disruption of the LCP and the trigger of apoptosis in the infected cells. The cells were labeled for apoptosis using TUNEL assays. Our data showed that by 8h post-infection, ~20% of the infected cells were apoptotic, and fewer than 5% of the LCPs were disrupted. By 12h post-infection, approximately 80% of the LCPs were disrupted, but fewer than 20% of these cells were apoptotic (Fig. 6). By 18h post-infection, 90% of the phagosomes were disrupted, consistent with previous observations (Molmeret et al., 2004c, Abu-Zant et al., 2005). However, only ~30% of the infected cells that harbored large numbers of cytosolic bacteria were apoptotic (Fig. 6). Similar results were also observed for the rib mutant (data not shown), which triggers apoptosis similar to the wild type strain but fails to lyse the host cell. Our data show disruption of the LCP precedes apoptosis by at least 6h.

Our data have shown that bacterial escape into the cytosol during late stages of infection is independent of the induction of apoptosis. It is possible that the LCP becomes disrupted due to the physical pressure on the phagosomal membrane by the large number of bacteria, and the failure to expand the phagosomal membrane to accommodate the increasing number of bacteria. It is possible that bacterial escape into the cytosol is the signal that triggers the infected macrophages to undergo apoptosis by 18h post-infection, at which time the macrophage harbors a large number of bacteria free in the cytosol. It is also possible that weakening of the anti-apoptotic stimuli that are exhibited during early and exponential replication may tip the balance in favor of apoptosis in the presence of activated caspase-3 throughout the infection (Laguna et al., 2006, Abu-Zant et al., 2007, Abu-Zant et al., 2005, Molmeret et al., 2004c). We speculate that multiple of these events are involved in lysis of the host cell. Thus, disruption of the LCP and bacterial escape into the cytosol precedes macrophage apoptosis.

Taken together, our data show that escape of *L. pneumophila* into the macrophage cytosol during late stages of infection precedes flagellation and triggering of the virulence-associated regulatory cascades at the PE phase. Our data indicate that triggering of the regulatory cascades is not exhibited by the vacuolar bacteria but by the cytosolic organisms and the peak of this trigger is evident by 20h post-infection, which is few hours after cytosolic residence of the bacteria, indicating that the signals for phenotypic modulation are transduced to the intracellular bacteria within the macrophage cytosol. Our data indicate that lysis of the host cell plasma membrane is likely to be rapid after triggering of the PE phase traits by the cytosolic bacteria. Our data show that bacterial escape into the cytosol precedes triggering macrophage apoptosis. Our studies also show that disruption of the LCP and escape of *L. pneumophila* into the macrophage cytosol during late stages of infection are independent of the bacterial pore-forming activity.

Experimental Procedures

Bacterial strains, mutant construction and plasmids

The clinical isolate *L. pneumophila* serogroup I strain AA100/130b (ATCC BAA-74) and its isogenic mutants, *dotA*, and the rib mutant GN229 have been described previously (Pedersen et al., 2001, Zink et al., 2002). The *rpoS* isogenic mutant has been described previously

(Abu-Zant et al., 2006). To construct the *flaA*, *letA* and *letS* isogenic mutants, the corresponding genes were cloned by PCR and a kanamycin resistance cassette was introduced into the genes using an Epicenter in vitro transposome kit (Molmeret et al., 2002b). The mutated loci were introduced into the chromosome of the parental strain AA100 by allelic exchange using natural transformation and confirmed by PCR, as we described previously (Stone & Abu Kwaik, 1999). The *pflaA-gfp* construct was a gift from Michele Swanson (University of Michigan) (Byrne & Swanson, 1998). Bacteria were grown on buffered charcoal-yeast extract (BCYE) plates, supplemented with 5 µg/ml of chloramphenicol for *Legionella* strains when GFP-encoding plasmid or complementing plasmids were used. The mutants were grown in the presence of 50 µg/ml of kanamycin.

Macrophages and Microscopy

Quiescent human monocyte derived macrophages (hMDMs) were prepared as we described previously (Santic *et al.*, 2005b, Santic *et al.*, 2005a). Monolayers of hMDMs were washed three times with the tissue culture medium prior to infection. Unless specified, all infections were carried out using MOI of 10 for 1 h followed by washing off the extracellular bacteria, and the infected cells were incubated for the indicated periods of infection specified for each experiment. Infected monolayers were fixed, dehydrated, processed, stained and examined with a Philips CM-12 Transmission Electron Microscope (TEM) as we described previously (Molmeret et al., 2004a). Infection of adherent cells on circular glass cover slips (VWR) in 24-well culture plates, their fixation and preparation for confocal microscopy were performed as previously described (Molmeret et al., 2004c). Inhibitor of caspase-3 (Z-DEVD-fmk) was purchased from Biovision and was used at 100 µM concentrations. The secondary conjugated antibodies were purchased from Molecular Probes. The cells were analyzed using an Olympus Fv500 laser scanning confocal microscope. TUNEL assays were performed and analyzed by confocal microscopy, exactly as described previously (Gao & Abu Kwaik, 1999a, Gao & Abu Kwaik, 1999b). On average, 15-20 1 µm serial sections of each image were captured and analyzed using Adobe Photoshop 6.0 (Adobe Photoshop, Inc). Approximately 100 individual cells were analyzed from different sections or coverslips, and the experiments were repeated 3 times.

Quantitative Real Time PCR

For analysis of gene expression *in vitro*, samples of bacterial cultures were grown in BYE medium to an optical density (OD₅₅₀) of 0.8-1 (E phase) and 2.0-2.2 (PE phase) and from intracellular bacteria at 8, 12, 16, and 20h post-infection. The RealTime PCR procedures were exactly as we described recently (Habyarimana *et al.*, 2008, Al-Khodori et al., 2008b). Total RNA was extracted using RNeasy Mini Kit (Qiagen, Valencia, CA) as recommended by the manufacturer. RNA integrity was assessed by visualizing ethidium bromide-stained 0.8% agarose gel. Total RNA was treated with DNase I (Ambion, Austin, TX) at 37°C for 30 min. Equal amount of bacterial RNA from all conditions was used for cDNA synthesis with Superscript III Plus RNase H⁻ reverse transcriptase (RT) (Invitrogen, California) and random primers. The generated cDNA was diluted fivefold with RNase free water. Real-time qPCR was done using the DNA Engine Opticon System (MJ Research), and carried out in triplicates using the DyNAmo SYBR Green qPCR Kit in 20 µl reaction volume, as recommended by the manufacturer (New England Biolabs, Ipswich, MA). The primers (F: GTTACCCACAGAAGAAGCAC, R: CCACTACCCTCTCCCATACT) were used to amplify the 16sRNA. The *flaA* gene was amplified by the two primers (F: CGATGGTTCTTTCTCTGG, R: GCTACTTCTGTTCCTGTTG). PCR conditions were 5 min at 94°C, 15 s at 96°C and 15 s at 72°C for 30 cycles. The concentration was determined by the comparative C_T method (threshold cycle number at the cross-point between amplification plot and threshold) and normalized values to the 16sRNA. Relative

quantitation by quantitative reverse transcriptase PCR was validated by equivalent and linear amplification of 16sRNA and the studied gene at the assay concentrations.

Differential labeling of vacuolar vs. cytosolic bacteria

The cytoplasm of live macrophages was loaded with anti-bacterial antibodies using the glass beads loading technique (McNeil & Warder, 1987, Reddy et al., 2001) to temporarily permeabilize the cell membranes, as we described previously (Molmeret et al., 2007, Santic et al., 2007b, Santic et al., 2008). After 1 h infection, cells were washed 3 times with PBS and 400 µl of anti-bacterial monoclonal antibodies were added on top of the coverslips, along with an aliquot of 0.5 g of acid washed sterile glass beads (Sigma, 425-600 Microns). The beads were rolled over the cells 12 times, which had no detectable effect on viability of cells as confirmed by trypan blue exclusion (data not shown). The glass beads and antibodies were immediately washed off with PBS and the cells were incubated at 37°C for 1 h to allow sufficient time for the loaded antibodies to bind the cytoplasmic bacteria. The cells were then fixed and processed for confocal microscopy. Controls were either cells subjected to the same treatment without the glass beads or cells that were fixed and permeabilized with 0.05% Triton X-100 for 15 min on ice. Approximately 100 individual cells were analyzed from different coverslips, and the experiments were repeated 3 times.

Statistical analysis

All experiments have been performed at least three times and the data shown are representatives of one experiment. To analyze for statistical significant differences between different sets of data, student two-tail Student *t*-test was used and the *P* value was obtained. Some of the standard deviations are too small to be displayed by the software.

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Fig. 1. Expression of *flaA* is triggered at the PE phase

Real- Real Time (qRT-PCR) was used to determine regulation of expression of *flaA* by intracellular bacteria at different time points after infection. Identical amounts of RNA were used after isolation from an equivalent number of bacteria (see experimental procedures). The data are representative of three independent experiments. Error bars represent standard deviations of triplicate samples.

Fig. 2. Disruption of the phagosome and bacterial escape into the cytosol by 12h post-infection and its independence of the regulators at the PE phase

(A) Representative confocal microscopy images at 8, 12, 18 h post-infection of macrophages infected by GFP-expressing wild-strain strain, *rpoS*, *letA* and *letS* mutants. After infection, the macrophage cytosol was loaded with specific anti-bacterial Mabs that differentially label cytosolic bacteria (red). Quantitative analysis of disruption of the phagosome is shown in panel B. The data shown are representative of three independent experiments.

Fig. 3. Expression of *pflaA-gfp* is triggered after bacterial escape into the macrophage cytosol but not within intact phagosomes

The macrophages were infected by *L. pneumophila* strain AA100 harboring *pflaA-gfp*. At different time points after infection, cytosolic bacteria were labeled with specific rabbit polyclonal anti-bacterial antiserum (red) after preferential permeabilization of the plasma membrane the glass beads “loading technique”. After permeabilization with Triton X-100, all bacteria were detected using monoclonal antibodies (blue). Quantitative analysis of the % of cells harboring cytosolic bacteria that expressed GFP is shown in panel B.

Fig. 4. Disruption of the phagosome and escape of *L. pneumophila* into the macrophage cytosol is independent of the pore-forming activity

(A) Representative confocal microscopy images at 8, 12, and 18 h post-infection of macrophages by the GFP-expressing wild-type (WT) strain, the rib mutant, or heat-killed (HK) WT strain control. After infection, specific anti-bacterial Mabs (red) were loaded into the cytoplasm of live infected macrophages using the glass beads “loading technique”. Quantitative analysis of co-localization of bacterial GFP expression with binding to anti-bacterial antibodies is shown in panel B. The data shown are representative of three independent experiments and the error bars represent standard deviation.

Fig. 5. Inhibition of apoptosis does not block disruption of the LCP and bacterial escape into the cytosol

Macrophages were untreated or treated with the caspase-3 inhibitor (C3I) at 1h post-infection. At 18h post-infection, the cells were processed for transmission electron microscopy. The % of disrupted LCPs is shown in (A), and the average number of bacteria/phagosome is shown in (B). The data shown are representative of three independent experiments and the error bars represent standard deviation.

Fig. 6. Disruption of the LCP and bacterial escape into the cytosol precedes the induction of macrophage apoptosis

Monolayers of GFP-expressing wild type strain-infected macrophages were labeled at different time intervals using the glass bead loading technique (see Fig. 1 legend) and TUNEL assays. Representative confocal microscopy images are shown in A while quantitation is shown in B. DP indicates disrupted phagosomes. The data shown are representative of three independent experiments and the error bars represent standard deviation.