Cellular Microbiology (2004) **6**(1), 33–48 doi:10.1046/j.1462-5822.2003.00335.x

Activation of caspase-3 by the Dot/Icm virulence system is essential for arrested biogenesis of the *Legionella*-containing phagosome

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Summary

The Dot/Icm type IV secretion system of *Legionella pneumophila* is essential for evasion of endocytic fusion and for activation of caspase-3 during early stages of infection of macrophages, but the mechanisms of manipulating these host cell processes are not known. Here, we show that caspase-3 activation by *L. pneumophila* is independent of all the known apoptotic pathways that converge on the activation of caspase-3. The cytoplasmic proteins IcmS, IcmR and IcmQ, which are involved in secretion of Dot/Icm effectors, are required for caspase-3 activation. Pretreatment of U937 macrophages and human peripheral blood monocytes (hPBM) with the capase-3 inhibitor (DEVD-fmk) or the paninhibitor of caspases (Z-VAD-fmk) before infection blocks intracellular replication of *L. pneumophila* in a dose-dependent manner. Inhibition of caspase-3 results in co-localization of the *L. pneumophila*-containing phagosome (LCP) with the late endosomal/lysosomal marker Lamp-2, and the LCP contains lysosomal enzymes, similar to the *dotA* mutant, which is defective in caspase-3 activation. However, activation of caspase-3 before infection does not rescue the replication defect of the *dotA* mutant. Interestingly, inhibition of caspase-3 after a 15 or 30 min infection period by the parental strain has no detectable effect on the formation of a replicative niche. The Dot/Icm-mediated activation of caspase-3 by *L. pneumophila* specifically cleaves, in a dose- and time-dependent manner, the Rab5 effector Rabaptin-5, which maintains Rab5-GTP on the endosomal membrane. In addition, PI3 kinase, which is a crucial effector of Rab5 downstream of Rababptin-5, is not required for intracellular replication. Using single-cell analysis, we show that apoptosis is not evident in the infected cell until bacterial replication results in >20 bacteria per cell. We conclude that activation of caspase-3 by the Dot/Icm virulence system of *L. pneumophila* is essential for halting biogenesis of the LCP through the endosomal/lysosomal pathway, and that this is associated with the cleavage of Rabpatin-5.

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Introduction

There are at least 14 caspases (cysteine proteases) that play distinct roles in apoptosis (Stegh and Peter, 2001; Shi, 2002a). Diverse extracellular and intracellular signals can activate initiator caspases (caspase-8 and -9), leading to activation of two distinct signalling pathways designated extrinsic and intrinsic pathways respectively (Strasser *et al.*, 2000; Chen and Wang, 2002; Shi, 2002a,b). The extrinsic pathway is mediated by ligation of a death receptor upon its binding to a death-inducing ligand that results in the activation of caspase-8, which activates caspase-3 (Ashkenazi and Dixit, 1998). The intrinsic pathway is mediated by agents such as stress stimuli and toxins that cause the release of cytochrome *c* from mitochondria into the cytoplasm (Cai *et al.*, 1998; Shi, 2002b) and result in activation of caspase-9, which activates caspase-3 (Cai *et al.*, 1998; Shi, 2002b). Therefore, both apoptotic pathways converge on the activation of caspase-3, which is the executioner of apoptosis (Enari *et al.*, 1998; Sakahira *et al.*, 1998; Porter and Janicke, 1999).

After its biogenesis from the plasma membrane, the nascent phagosome harbouring an invading bacterium

undergoes a series of maturation steps starting with an early endosome controlled by Rab-5 (Duclos and Desjardins, 2000; Woodman, 2000; Pedersen *et al*., 2001), followed by a late endosome regulated by Rab-7 and subsequent fusion to lysosomes where the organism is degraded (Sturgill-Koszycki *et al*., 1996; Meresse *et al*., 1999a,b; Duclos and Desjardins, 2000; Fratti *et al*., 2001; Mukherjee *et al*., 2001). The ability of many intracellular pathogens to cause disease is totally dependent on halting endocytic maturation of their phagosomes at a distinct stage along the endosomal–lysosomal degradation pathway (Meresse *et al*., 1999b; Duclos and Desjardins, 2000). Although the stage of phagosomal maturation has been unravelled for various intracellular pathogens, the molecular mechanisms are not well understood (Meresse *et al*., 1999b; Duclos and Desjardins, 2000).

The intracellular bacterium *Legionella pneumophila* is the causative agent of Legionnaires' disease, a potentially fatal pneumonia (Abu Kwaik, 1998). The ability of *L. pneumophila* to cause pneumonia is dependent on its capacity to invade and replicate within alveolar macrophages, monocytes and, potentially, alveolar epithelial cells (Abu Kwaik, 1998; Vogel and Isberg, 1999; Swanson and Hammer, 2000). Upon entry into the host cell, *L pneumophila* modulates the biogenesis of the phagosome into a replicative niche that excludes Rab5 and is halted from maturation through the 'default' endosomal–lysosomal degradation pathway (Horwitz, 1983a; Roy *et al*., 1998; Vogel *et al*., 1998; Wiater *et al*., 1998). The *Legionella*-containing phagosome (LCP) intercepts early secretory vesicles from the endoplasmic reticulum (ER) exit sites, which allows the organism to remodel the LCP membrane that becomes rough ER-derived within 15 min of its biogenesis from the plasma membrane (Horwitz, 1983b; Swanson and Isberg, 1995; Tilney *et al*., 2001; Kagan and Roy, 2002). In addition to evasion of vesicle traffic by *L. pneumophila* during the early stages of infection, the bacterium also induces caspase-3 activation in macrophages, monocytes and alveolar epithelial cells (Muller *et al*., 1996; Gao and Abu Kwaik, 1999a,b; 2000; Zink *et al*., 2002). The Dot/Icm type IV secretion system (Segal *et al*., 1998; Vogel *et al*., 1998) of *L. pneumophila* is essential for evasion of endocytic fusion (Roy *et al*., 1998; Vogel *et al*., 1998; Wiater *et al*., 1998), recruitment of the RER (Horwitz, 1983b; Swanson and Isberg, 1995; Tilney *et al*., 2001; Kagan and Roy, 2002) and activation of caspase-3 (Zink *et al*., 2002). These manipulations of host cell processes are thought to be mediated by the injection of effectors by the Dot/Icm transporter directly from the bacterium into the host cell (Nagai *et al*., 2002). Two effectors, RalF and LidA, exported by the Dot/Icm system have been reported (Nagai *et al*., 2002; Conover *et al*., 2003). In addition, some of the *dot/icm*-encoded proteins have been shown to be cytoplasmic and are involved in secretion of Dot/Icm effectors. IcmS and IcmW have been shown to form a complex in the bacterial cytoplasm, and this complex is involved in the export of specific effectors that are essential for phagosomal biogenesis (Zuckman *et al*., 1999; Coers *et al*., 2000). IcmR is believed to be a chaperone that forms a complex with IcmQ in the bacterial cytoplasm, and both proteins are important for Dot/Icm secretion (Coers *et al*., 2000). However, the Dot/Icm effectors with which IcmS, R, Q and W interact remain unknown.

The mechanism and biological significance of caspase-3 activation during the early stages of infection on intracellular trafficking of the LCP is not known. Although a recent report has concluded that caspase-3 is activated by *L. pneumophila* through the intrinsic pathway, these conclusions are based on using a multiplicity of infection (MOI) of 100 (Neumeister *et al*., 2002) that leads to cytotoxicity, which enhances apoptosis (Husmann and Johnson, 1994; Byrne and Swanson, 1998; Kirby *et al*., 1998; Alli *et al*., 2000; Zink *et al*., 2002).

In this report, we show that the Dot/Icm-mediated activation of caspase-3 by *L. pneumophila* in the host cell during the early stages of infection is independent of the extrinsic and intrinsic pathways of apoptosis. We show that inhibition of caspase-3 results in inhibition of intracellular replication as a result of maturation of the LCP through the endosomal–lysosomal degradation pathway, similar to the *dot/icm* mutants. The activated caspase-3 cleaves the Rab5 effector Rabaptin-5, and inhibition of caspase-3 blocks the cleavage of Rabaptin-5. Our data show that activation of caspase-3 by the Dot/Icm virulence system is essential for evasion of endocytic fusion and is associated with the cleavage of Rabaptin-5.

Results

Novel caspase-3 activation by L. pneumophila

Legionella pneumophila activates caspase-3 in macrophages, which is independent of the host cell protein synthesis (Gao and Abu Kwaik, 1999a,b), and a functional Dot/Icm secretion system is essential to transmit the signal that activates caspase-3 (Zink *et al*., 2002). The two main cellular pathways that result in activation of caspase-3 are mediated by caspase-8 or cytochrome *c*-mediated activation of caspase-9 (Ashkenazi and Dixit, 1998). We examined the role of these initiators in the activation of the effector caspase-3 by *L. pneumophila*. We used a *dotA* mutant defective in the expression of a cytoplasmic membrane structural component of the Dot/Icm secretion apparatus as a negative control (Roy and Isberg, 1997), and staurosporin, which triggers both

pathways, was used as a positive control. Infections were carried out using an MOI of 10 for 1 h followed by washing off the extracellular bacteria and further incubation for 3 h. Activation of caspases and the release of cytochrome *c* into the cytoplasm was examined by immunoblots of cellular extracts probed with the corresponding antibodies. Although caspase-3 was activated (Fig. 1A), neither caspase-8 (Fig. 1B) nor caspase-9 (Fig. 1C) was activated upon infection by *L. pneumophila*. Consistent with lack of activation of caspase-9, cytochrome *c* was restricted to the mitochondria and was not detectable in the host cell cytoplasm (Fig. 1D). In contrast, both caspase-8 and -9 were activated, and mitochondrial cytochrome *c* was released into the cytoplasm in staurosporin (ST)-treated control cells (Fig. 1). Importantly, when the cytochrome *c* immunoblot (Fig. 1D) of the cytoplasmic fraction was reprobed with anticaspase-3, caspase-3 was activated by the wild-type strain and in the ST-treated cells, but not by the *dotA* mutant (data not shown). These data indicated that caspase-3 was activated by *L. pneumophila* independent of the extrinsic and intrinsic apoptotic pathways that converge on the activation of caspase-3.

Fluorogenic substrates specific for caspase-3, -8 and -9 were used to determine the activity of these caspases after 3 h incubation, following 1 h infection at an MOI of 10 and compared with uninfected and staurosporin-treated cells. Caspase-3 showed a 63-fold increase in fluorescence units in the AA100 infected cells when compared with uninfected ones, whereas the *dotA*-infected cells were similar to the uninfected control cells (data not shown). Importantly, neither caspase-8 nor caspase-9 was activated in the AA100-infected cells despite the activation of caspase-3 in these cells. Activity of caspase-3, -8, and -9 was increased by 72-, 84- and 58-fold in staurosporin-treated cells compared with untreated ones. These data confirmed the immunoblot data that caspase-3 was activated by *L. pneumophila* independent of caspase-8 or caspase-9.

Cell lines defective in specific parts of the apoptotic machinery have been created previously. We used two such cell lines to confirm that activation of caspase-3 by

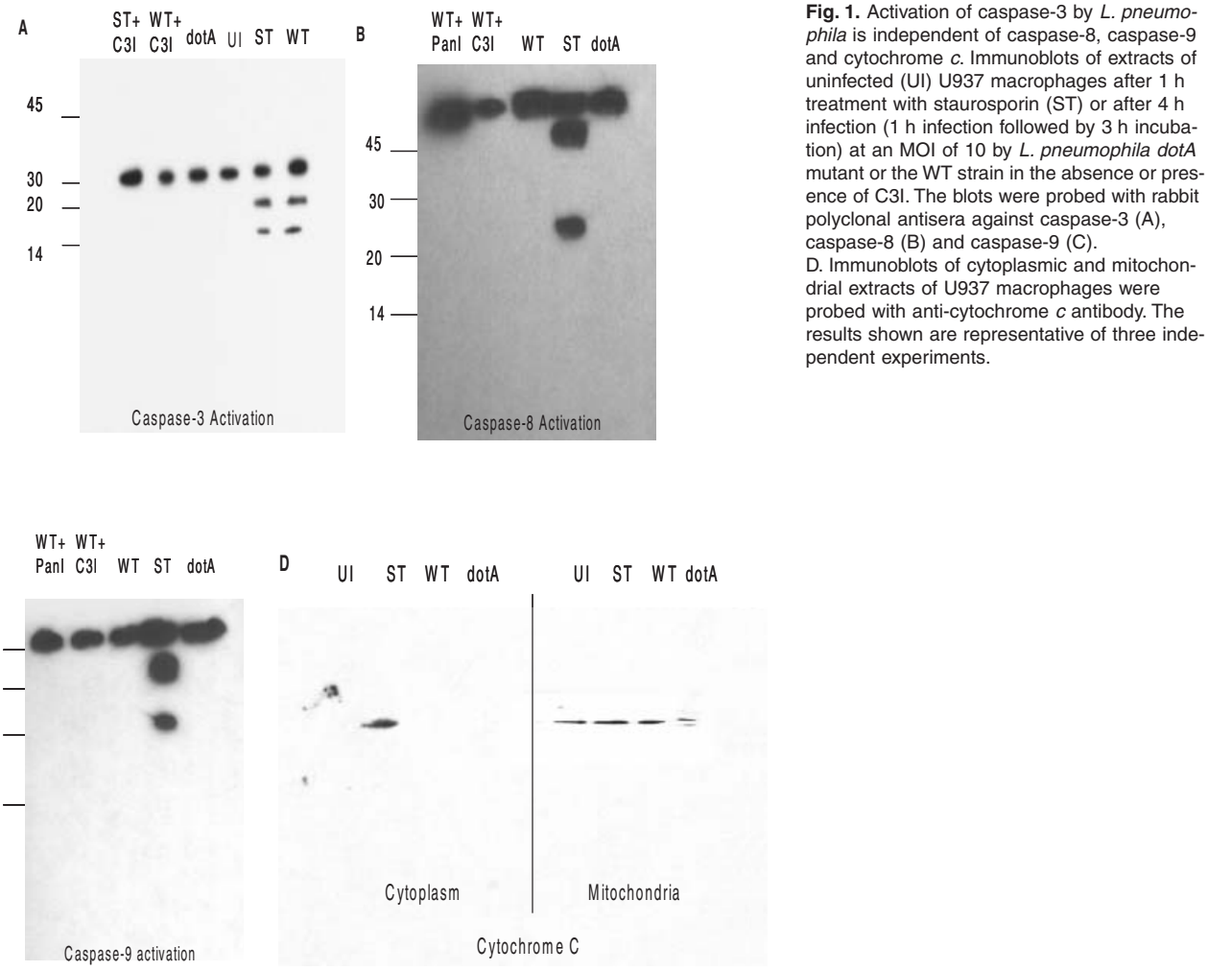

Fig. 1. Activation of caspase-3 by *L. pneumophila* is independent of caspase-8, caspase-9 and cytochrome *c*. Immunoblots of extracts of uninfected (UI) U937 macrophages after 1 h treatment with staurosporin (ST) or after 4 h infection (1 h infection followed by 3 h incubation) at an MOI of 10 by *L. pneumophila dotA* mutant or the WT strain in the absence or presence of C3I. The blots were probed with rabbit polyclonal antisera against caspase-3 (A), caspase-8 (B) and caspase-9 (C).
D. Immunoblots of cytoplasmic and mitochondrial extracts of U937 macrophages were probed with anti-cytochrome *c* antibody. The results shown are representative of three independent experiments.

L. pneumophila was independent of the intrinsic and extrinsic pathways of caspase activation. One cell line is a Jurkat cell line expressing a dominant-negative FADD protein that has been shown to be completely defective for the capsase-8 cascade of the extrinsic pathway. The second cell line is the LNCP cell line overexpressing Bcl_{xL}, a protein that regulates the release of cytochrome *c* and, when overexpressed, blocks cytochrome *c* release, preventing the activation of caspase-9 through the intrinsic pathway. The data showed that, similar to U937 macrophages, caspase-3 was activated by *L. pneumophila* in cells expressing a dominant-negative mutant of FADD in which caspase-8 activation is blocked (Fig. 2). In addition, *L. pneumophila* induced caspase-3 activation within LNCP cells overexpressing Bcl_{xL}, which blocks cytochrome *c* release from the mitochondria and subsequent activation of caspase-9 (Fig. 2). These observations further confirmed that *L. pneumophila* activated caspase-3 independently of release of cytochrome *c* and activation of caspase-8.

It is possible that *L. pneumophila* triggers a calcium ion influx that results in apoptosis through the activation of calpain (Squier and Cohen, 1997), similar to what has been described for *Neisseria* (Muller *et al*., 1999). To test this possibility, inhibitors specific for calmodulin (calmodulin inhibitor II) and calpain (calpeptin and calpain inhibitor AA) were examined for the ability to block caspase-3 activation by *L. pneumophila*. Ionomycin treatment, which results in calcium influx into the cells, was used as a positive activator of the calcium-induced signalling calpain pathway. Neither the calmodulin inhibitor nor the calpain inhibitor had any effect on the ability of *L. pneumophila* to induce activation of caspase-3 (data not shown). Thus, the activation of caspase-3 by *L. pneumophila* was independent of the calpain pathway that converges on the activation of caspase-3.

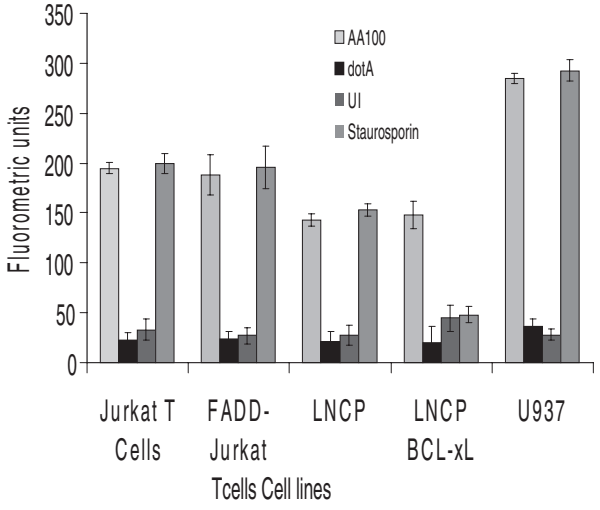

Fig. 2. FADD dominant-negative mutant or overexpression of Bcl_{xL} have no effect on caspase-3 activation by *L. pneumophila*. The activity of caspase-3 was determined upon infection of LNCP cell line, Bcl_{xL} overexpressing LNCP cells, Jurkat cells and FADD dominant-negative Jurkat cells. Infection of U937 macrophages and staurosporin (St)-treated cells were used as controls. The caspase-3 activity was determined using fluorogenic substrate and is presented as fluorometric units. All samples were run in triplicate in each experiment; the results shown are representative of three independent experiments, and error bars represent standard deviation.

Activity of various caspases upon infection by L. pneumophila

Our data above clearly showed that caspase-8 and caspase-9 were not activated despite the activation of caspase-3 by *L. pneumophila*. Next, we examined whether other capsases, in addition to caspase-3, were activated. Fluorogenic substrates specific for caspase-1, -2, -3, -5, -6, -8 and -9 were used to determine the activity of different caspases after 3 h incubation following 1 h infection at an MOI of 10. Caspase-3 showed an ≈60-fold increase in fluorescence units compared with uninfected cells, whereas caspase-2 showed an 18-fold increase in fluorescence units compared with uninfected cells (data not shown). Activity of caspase-3 and caspase-2 was blocked by the caspase-3-specific inhibitor (C3I), suggesting that caspase-3 was playing a role in the modest activation of caspase-2. This was not surprising, as caspase-2 has been shown to be a downstream effector of caspase-3 and can form a feedback loop with caspase-3 (Cai *et al*., 1998). All other caspases had background activities upon infection by *L. pneumophila* similar to uninfected cells, and any slight increase in their activity was blocked by the C3I (data not shown). We have also shown previously that caspase-1 is not activated by *L. pneumophila* (Gao and Abu Kwaik, 1999a). In staurosporin-treated cells, all the caspases, with the exception of caspase-1, were activated 80- to 140-fold, and a *dotA* mutant did not activate any of the caspases (see Fig. 1; data not shown). Taken together, these data further confirmed that activation of caspase-3 by *L. pneumophila* was independent of upstream initiators of its activation, including caspase-8 and -9 and release of cytochrome *c*, which are the initiators for the extrinsic and intrinsic pathways of apoptosis.

IcmS, IcmR and IcmQ are required for activation of caspase-3

We have shown previously that structural components of the Dot/Icm secretion system are essential for activation of caspase-3 during the early stages of infection (Zink *et al*., 2002). These data suggested that an effector exported by the Dot/Icm system was likely to be involved in the activation of caspase-3. A few Dot/Icm proteins

have been shown to be located in the cytoplasm and provide a chaperone-like function in the export of Dot/Icm effectors. IcmS and IcmW have been shown to form a complex in the bacterial cytoplasm, and this complex is essential for phagosomal biogenesis (Zuckman *et al*., 1999; Coers *et al*., 2000). IcmR is believed to be a chaperone that forms a complex with IcmQ in the bacterial cytoplasm, and both proteins are important for Dot/Icm secretion (Coers *et al*., 2000). Therefore, we examined the role of IcmS, IcmR and IcmQ in the activation of caspase-3. Null mutants in each of the three genes were constructed, and intracellular growth kinetics in hPBM showed that the three mutants were defective in intracellular replication (Fig. 3A), consistent with previous reports (Segal and Shuman, 1997; Coers *et al*., 2000), and the defect was complemented by the corresponding wild-type genes (data not shown). Measurement of caspase-3 activation by the three mutants showed that the *icmR* and *icmQ* mutants were both totally defective in caspase-3 activation, similar to *dotA* (Fig. 3B). The *icmS* mutant was partially defective in caspase-3 activation, which correlated with its partial defect in intracellular replication (Fig. 3). These data showed that IcmS, IcmR and IcmQ are involved in caspase-3 activation in the host cell.

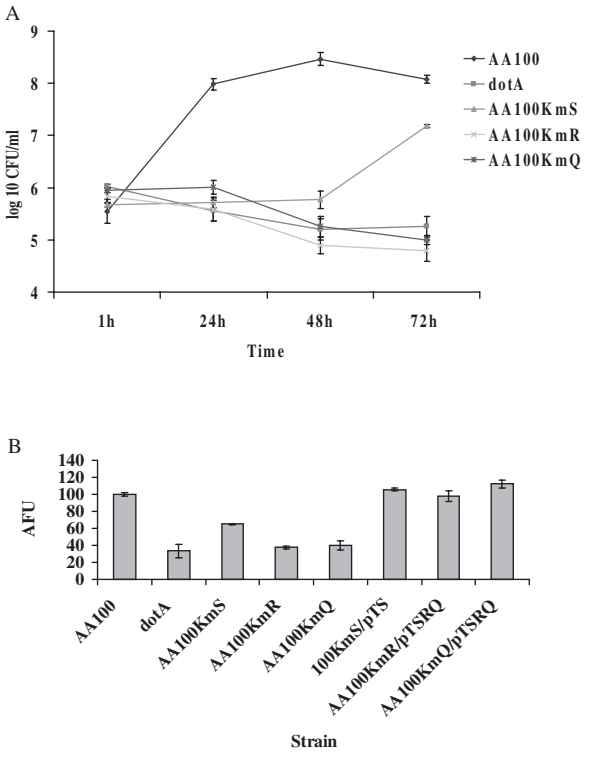

Fig. 3. The cytoplasmic proteins IcmS, IcmR and IcmQ are required for activation of caspase-3.
A. Growth kinetics of the wild-type strain AA100 and the *icmS* (AA100KmS), *icmR* (AA100KmR) and *icmQ* (AA100KmQ) isogenic mutants in hPBM. The defect in intracellular growth of the three mutants was complemented by the corresponding wild-type gene (data not shown).
B. The caspase-3 activity of the different strains was determined using fluorogenic substrate specific for caspase-3 and is expressed as arbitrary fluorescence units (AFU). The plasmids pTS and pTSRQ contain *icmTS* and *icmTSRQ* respectively. All samples were run in triplicate in each experiment; the results shown are representative of three independent experiments, and error bars represent standard deviation.

Activation of caspase-3 is essential for intracellular replication

As the Dot/Icm secretion apparatus and many of its cytoplasmic components are essential for evasion of endocytic fusion and for activation of caspase-3 (Kirby *et al*., 1998; Roy *et al*., 1998; Segal *et al*., 1998; Wiater *et al*., 1998), we first examined the effect of the paninhibitor of caspases (PanI) and the caspase-3-specific inhibitor (C3I) on intracellular replication in U937 macrophages and hPBM. Both inhibitors blocked intracellular replication almost completely at a concentration of 10 μM in both U937 macrophages and hPBM (Fig. 4A and B). Inhibition of intracellular replication by the two inhibitors was dose dependent, in that 2 μM had no detectable effect whereas >10 μM abolished intracellular replication completely (Fig. 4A and B). In contrast, inhibitors of caspase-1, -6, -8 and -9 had no detectable effect, and the caspase-2 inhibitor had only a very mild effect on intracellular replication within U937 macrophages (data not shown). There was no effect of DMSO, which was used to dissolve the caspase inhibitors, on intracellular replication or viability of macrophages (data not shown). Preincubation of the bacteria for 2 h with 100 μM PanI or C3I had no effect on bacterial viability or intracellular replication in U937 macrophages (data not shown), consistent with previous observations (Gao and Abu Kwaik, 1999a,b). We conclude that inhibition of caspase-3 blocks intracellular replication of *L. pneumophila*.

Activation of caspase-3 is essential for evasion of endocytic fusion

Our data showed that activation of caspase-3 by *L. pneumophila* is essential for intracellular replication, and inhibition of caspase-3 blocked intracellular replication. We tested the hypothesis that inhibition of intracellular replication upon inhibition of caspase-3 resulted from maturation of the LCP through the 'default' endosomal–lysosomal degradation pathway. We used two independent strategies to analyse maturation of the LCP through the endosomal–lysosomal degradation pathway. First, we examined co-localization of the bacterial phagosome with the late endosomal/lysosomal marker Lamp-2 by confocal laser scanning microscopy using samples that were coded, and co-localization was examined as described

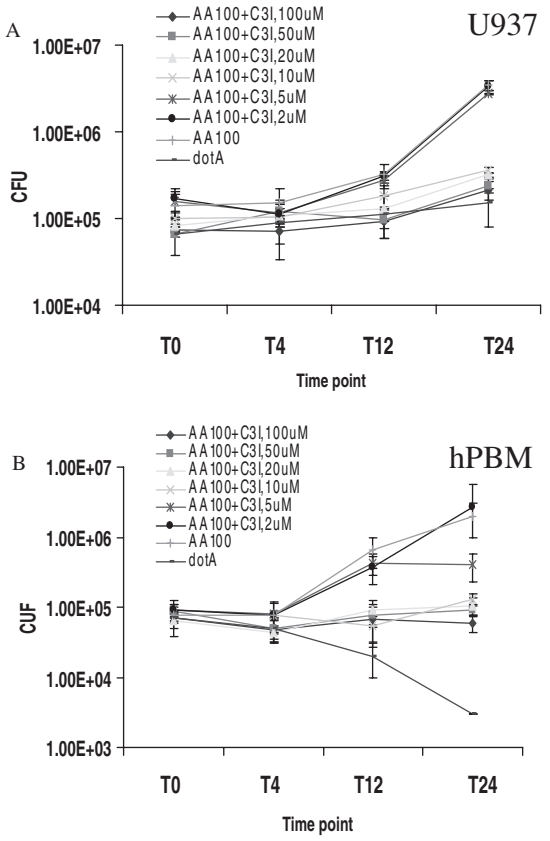

Fig. 4. The caspase-3 activity is essential for intracellular replication of *L. pneumophila*. Intracellular growth kinetics of the *dotA* mutant or the wild-type strain AA100 of *L. pneumophila* within U937 macrophages (A) or hPBM (B) infected at an MOI of 10 for 1 h in the absence or presence of different concentrations of C3I. The number of cfus at different time points after the 1 h infection is shown. The data shown are representative of five independent experiments, and error bars represent standard deviation.

previously (Harb and Abu Kwaik, 2000; Pedersen *et al*., 2001). In cells infected by the wild-type (WT) strain, <10% of the bacterial phagosomes co-localized with the late endosomal/lysosomal marker Lamp-2 (Fig. 5). When infections by the WT strain were performed in the presence of C3I or PanI, >80% of the bacterial phagosomes co-localized with Lamp-2, similar to the *dotA* mutant (Fig. 5; data not shown). Importantly, preincubation of the bacteria for 2 h with 100 μM C3I had no effect on the ability of the WT strain to exclude Lamp-2 (≈ 15% co-localization).

To confirm alteration in trafficking of the LCP upon inhibition of caspase-3, our second strategy was to use transmission electron microscopy to examine fusion of the LCP to lysosomes by detection of the lysosomal enzyme acid phosphatase within the phagosome, as described previously (Molmeret *et al*., 2002a). The data showed that ≈20% of the WT strain LCPs contained the lysosomal enzyme acid phosphatase at 1 h and 2 h after infection (Fig. 5). In the presence of C3I, ≈75% of the WT strain LCPs contained acid phosphatase at 1 h and 2 h after infection, similar to the *dotA* mutant (Fig. 5). Therefore, C3I treatment rerouted the LCP trafficking and caused fusion of the LCP to lysosomes. Therefore, inhibition of caspase-3 rendered the WT strain traffic as the *dotA* mutant whose, the phagosome matured through the 'default' endosomal–lysosomal degradation pathway.

It is well documented that Rab5 is excluded from the LCP, even when Rab5 is overexpressed in the infected cells (Roy *et al*., 1998; Clemens *et al*., 2000). Our data

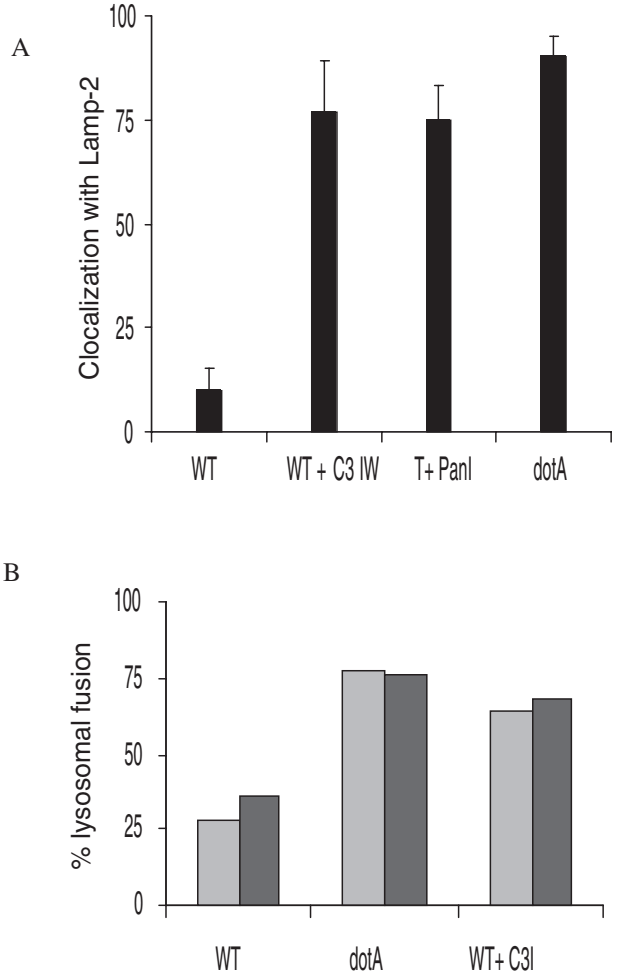

Fig. 5. The caspase-3 activity is essential for exclusion of Lamp-2 from the LCP and for evasion of fusion of the LCP to lysosomes.
A. Quantification of co-localization of the LCP with Lamp-2 at 1 h after infection by the *dotA* mutant or the wild-type (WT) strain in the absence or presence of C3I or the caspase PanI at 10 μM concentrations, determined by examination of 150 phagosomes using confocal laser scanning microscopy.
B. Quantification of lysosomal fusion to the LCP detected by the presence of the lysosomal enzyme acid phosphatase in the bacterial phagosome at 1 h (light bars) and 2 h (dark bars) after infection by electron microscopy. Quantification was determined by examination of 150 phagosomes. The data shown are representative of three independent experiments.

showed that, when U937 macrophages were pretreated with C3I, the WT LCPs co-localized with late endosomal and lysosomal markers, similar to the *dotA* mutant LCPs in untreated cells. To mature to late endosomes that fuse to lysosomes upon C3I treatment, the LCP must have become an early endosome that acquired Rab5. Earlier studies that could not detect Rab5 on the LCP, even though ≈20% of the WT LCPs mature to late endosomes and fuse to lysosomes, are likely to result from the speed and transient acquisition and loss of Rab5 (≈ 1 min) during endosomal biogenesis (Rybin *et al*., 1996). This clearly showed that the caspase-3 activity was essential for the exclusion of endosomal markers from the LCP. Taken together, inhibition of caspase-3 resulted in endocytic maturation of the LCP that subsequently fused to lysosomes.

We also examined the effect of caspase-3 inhibition on recruitment of the RER around the LCPs by electron microscopy, as described previously (Abu Kwaik, 1996; Gao *et al*., 1998; Molmeret *et al*., 2002a). The data showed that ≈80% of the WT LCPs and ≈20% of the *dotA* mutant LCPs were surrounded by the RER. Upon caspase-3 inhibition, only ≈24% of the WT LCPs were surrounded by the RER, while no effect was detected for the *dotA* mutant LCPs. These data showed that inhibition of caspase-3 blocked recruitment of the RER around the LCP, which is very consistent with numerous previous observations that alteration in endocytic trafficking of the LCP blocks recruitment of the RER.

Legionella pneumophila *induces caspase-3-mediated cleavage of Rabaptin-5*

The LCP is completely blocked from endocytic fusion (Horwitz, 1983a; Clemens and Horwitz, 1995; Roy *et al*., 1998; Clemens *et al*., 2000). In several model systems of caspase-3-mediated apoptosis, endocytic fusion, as well as endocytosis of transferrin and fluid phase tracers, is inhibited, and this inhibition is mediated by the caspase-3-mediated cleavage of Rabaptin-5 (Cosulich *et al*., 1997; Swanton *et al*., 1999). As inhibition of caspase-3 resulted in maturation of the LCP through the endosomal–lysosomal pathway, we examined whether Rabaptin-5 was cleaved by caspase-3 upon infection by *L. pneumophila*. Our data showed that Rabaptin-5 was specifically cleaved by caspase-3 upon infection by *L. pneumophila* (Fig. 6). Importantly, caspase-3-mediated cleavage of Rabpatin-5 was blocked by the specific caspase-3 inhibitor (Fig. 6). The *dotA* mutant was completely defective in the caspase-3-mediated cleavage of Rabaptin-5, consistent with its failure to activate caspase-3 (Zink *et al*., 2002). Importantly, cleavage of Rabpatin-5 upon infection by *L. pneumophila* was dose

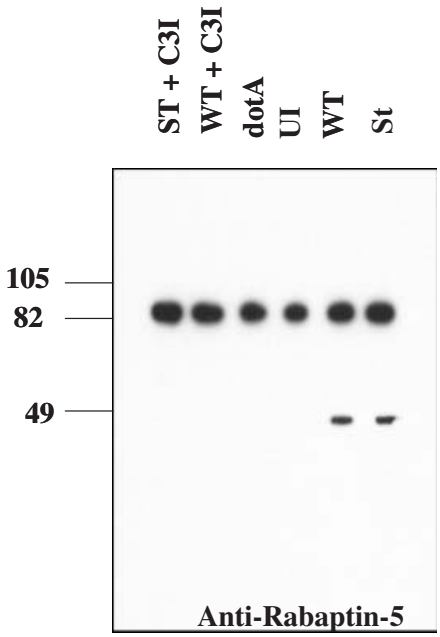

Fig. 6. Caspase-3-mediated cleavage of Rabaptin-5 is dependent on the signal transmitted by the Dot/Icm virulence system. Immunoblot of extracts of uninfected (UI) U937 macrophages, ST-treated or infected by *dotA* or the WT strain in the presence or absence of C3I, as described in the legend to Fig. 1. The blot was probed with anti-Rabaptin-5 N-terminus antibody. The results shown are representative of three independent experiments.

(Fig. 7A) and time dependent (Fig. 7B), which correlated with the dose- (Fig. 7C) and time-dependent activation of caspase-3 (Fig. 7D). Although cleavage of Rabaptin-5 by caspase-3 is the only principal factor responsible for inhibition of endocytic fusion in several *in vitro* and *in vivo* apoptosis model systems (Stenmark *et al*., 1995; Cosulich *et al*., 1997; Swanton *et al*., 1999), it is possible that other unknown effectors of Rab-5 may also be involved in the process. Thus, the Dot/Icm virulence system of *L. pneumophila* transmitted a signal that activated caspase-3, which cleaved the Rab5 effector Rabaptin-5.

PI3 kinase is not required for intracellular replication

Cleavage of Rabaptin-5 results in blocking subsequent recruitment of other downstream effectors of Rab5, leading to dissociation of Rab5 from the endosome and subsequent inhibition of endocytic fusion (Stenmark *et al*., 1995; Cosulich *et al*., 1997; Swanton *et al*., 1999). PI3 kinase is the main effector of Rab5 (Simonsen *et al*., 1998) that is downstream of Rabaptin-5 and is involved in subsequent recruitment of ≈20 Rab5 effectors to create a Rab5-rich domain on early endosomes, which is essential for endosomal fusion (Zerial and McBride, 2001). Previous studies have shown that the PI3 kinase inhibitor wartmannin has

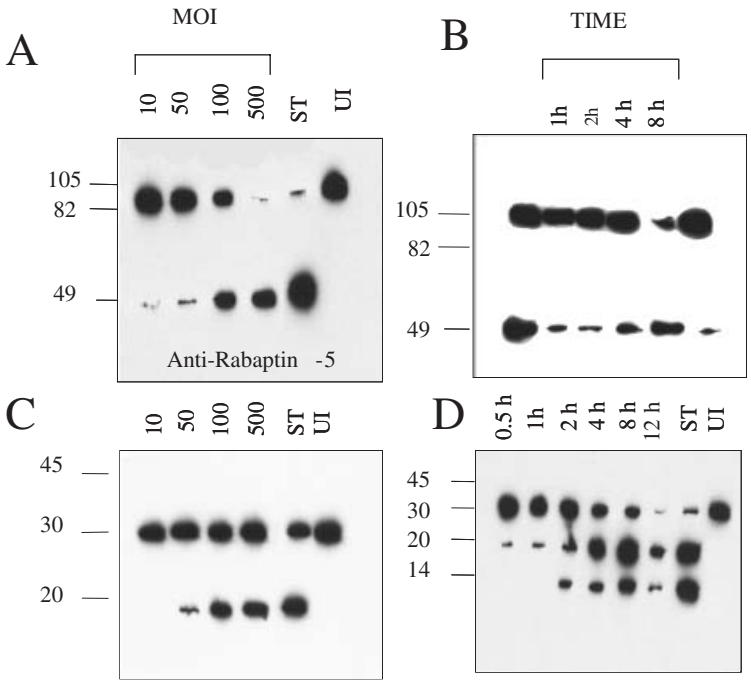

Fig. 7. Caspase-3-mediated cleavage of Rabaptin-5 is dose and time dependent, and is correlated with activation of caspase-3. Immunoblots of extracts of uninfected (UI) U937 macrophages, ST-treated or infected by the WT strain, as described in the legend to Fig. 1. A and C. Dose-dependent (MOI = 10–500) cleavage of Rabaptin-5 (A) and caspase-3 (C). B and D. Time-dependent (MOI of 10) cleavage of Rabaptin-5 (B) and caspase-3 (D). Note correlation of cleavage of rabaptin-5 and caspase-3. The results shown are representative of three independent experiments.

no effect on phagocytosis and entry of *L. pneumophila* into U937 macrophages (Khelef *et al*., 2001). In this study, we examined whether blocking of endocytic fusion and exclusion of Rab5 by the LCP was mediated by a step upstream of PI3 kinase. To test this hypothesis, we examined the effect of methyl adenine (0.1 and 1 mM), wartmannin (1, 10 and 100 nM) and LY294002 (1, 10 and 100 μM), as specific inhibitors of PI3 kinase, on the ability of *L. pneumophila* to form a replicative niche permissive for intracellular replication. Growth kinetics of *L. pneumophila* within hPBM and U937 macrophages was examined in the presence of several concentrations of each inhibitor. The data showed that, even at the highest concentrations, none of the inhibitors had any effect on the ability of the bacteria to replicate intracellularly (data not shown). These data showed that PI3 kinase was not required for the formation of the replication niche by *L. pneumophila*. The data supported the hypothesis that exclusion of Rab5 from the LCP was mediated at a step upstream of PI3 kinase.

Induction of apoptosis does not rescue the replication defect of the dotA *mutant*

We hypothesized that, if the induction of caspase-3 was the main function of the Dot/Icm secretion system responsible for evasion of endocytic fusion and intracellular replication, then preinduction of the caspase-3 activity could rescue the *dotA* mutant from its intracellular replication defect. Therefore, we monitored the caspase-3 activity and examined intracellular replication of the *dotA* mutant in hPBM that had been pretreated for 2 h with several concentrations of staurosporin (0.1–100 μM) to induce the caspase-3 activity. Our data showed that, despite activation of caspase-3, the *dotA* mutant was not rescued for intracellular replication by preactivation of caspase-3 (data not shown). These data indicated that the caspase-3 activity alone was not sufficient to compensate for the loss of function of the secretion apparatus, which is thought to be exporting many effectors required for intracellular replication.

The caspase-3 activity is essential during the first few minutes of infection

We examined whether the caspase-3 activity was essential throughout the intracellular infection or was only required during early stages of establishment of the LCP and its fusion with the RER, which has been shown to be completed within the first 15 min in U937 macrophages (Tilney *et al*., 2001). U937 macrophages and hPBM were preincubated with a 10 μM concentration of C3I, or the C3I was added at 5, 15 and 30 min after the addition of the bacteria, and the effect on intracellular replication was examined within the first 24 h. The 10 μM concentration was used because it was the threshold concentration that showed submaximal inhibition of intracellular replication to allow us to recognize small differences between the treatments. The data showed that addition of the inhibitor at 5 min after initiation of the infection caused inhibition of intracellular replication similar to preincubation of the cells with the inhibitor (Fig. 8; data not shown). Interestingly, addition of the inhibitor at 15 or 30 min after initiation of

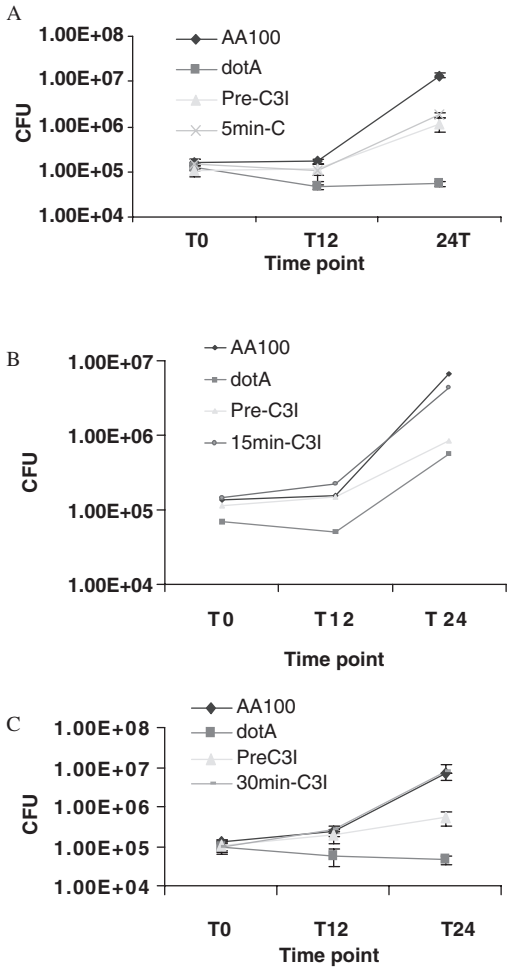

Fig. 8. The role of caspase-3 in establishment of a replicative niche is only required during the first few minutes of infection. Growth kinetics in hPBM was examined for the WT strain AA100 in the presence of C3I under conditions in which the monocytes were pre-treated with 10 μM C3I (pre-C3I) or the C3I was added at 5 (A), 15 (B) or 30 min (C) after infection. The *dotA* mutant was included as a negative control defective in intracellular replication. The data shown are representative of four independent experiments, and error bars represent standard deviation.

the infection had no effect on intracellular replication (Fig. 8; data not shown). Therefore, the caspase-3 activity was essential only during the first few minutes of internalization to establish the LCP but, by 15 min, the caspase-3 activity was dispensable for intracellular replication. The first 15 min interval coincides with transformation of the LCP membrane into a RER (Tilney *et al*., 2001).

Apoptosis of infected cells during late stages of infection

It has been a puzzling issue to us why *L. pneumophila* induces caspase-3 activation at such an early stage of the infection (Gao and Abu Kwaik, 1999a,b). One would predict that, once the bacteria trigger the cell to undergo apoptosis, the host cell would not be able to support intracellular bacterial replication. Previous studies from our laboratory and others on the induction of apoptosis used an MOI of 50–100 (Muller *et al*., 1996; Hägele *et al*., 1998; Gao and Abu Kwaik, 1999a,b; Neumeister *et al*., 2002). We thought that this high MOI may be responsible for the early trigger of apoptosis and that, at lower MOIs with limited activation of caspase-3, apoptosis may be delayed long enough to allow intracellular replication before killing of the host cell. Therefore, we used single-cell analysis of the infection at an MOI of 5 to examine the kinetics of induction of apoptosis in the infected cells and to determine the effect of apoptosis on bacterial replication. The apoptotic cells were labelled by TUNEL, and the direct comparison of the number of replicating bacteria in apoptotic cells versus non-apoptotic cells was analysed by quantification of intracellular bacteria per cell (Fig. 9). At 2 h after infection, 6% of the cells were apoptotic, similar to the uninfected cells at this time point. At 2 h after infection, the cells contained one to five bacteria per cell (>90% of infected cells had one bacterium), and none of the macrophages with intracellular bacteria was apoptotic (Fig. 9). At 4 h after infection, none of the cells was apoptotic, and the number of intracellular bacteria had increased with 27% of infected cells containing 5–10 bacteria and 73% of the cells containing one to five bacteria (Fig. 9). After 8 h of incubation, 40% of infected cells were apoptotic, and the number of intracellular bacteria increased with 14% of infected cells containing >20 bacteria, 44% containing 10–20, 12% containing 5–10 and 30% containing one to five bacteria (Fig. 9). In macrophages containing >10 bacteria, 40% of infected cells were apoptotic, and all macrophages with >20 bacteria were apoptotic at 8 h (Fig. 9). At 16 h after infection, ≈90% of the infected cells contained >20 bacteria, and all of these cells were apoptotic (Fig. 9). A small number of cells at 16 h had only a few intracellular bacteria, which probably represented secondary infections, and the higher level of apoptosis in these cells probably results from extracellular bacteria released from infected cells (Gao and Abu Kwaik, 1999b). Therefore, *L. pneumophila* replicated in cells that subsequently underwent apoptosis during late stages of the infection.

Discussion

Previous studies have shown that the Dot/Icm virulence system is essential for activation of caspase-3 upon infection by *L. pneumophila* (Gao and Abu Kwaik, 1999a,b; Zink *et al*., 2002). Our current data show that the three cytoplasmic proteins IcmS, IcmR and IcmQ, which are involved in secretion of Dot/Icm effectors, are also required for caspase-3 activation. The mechanism by which caspase-3 is activated upon infection is not known at this time, but it is possible that the Dot/Icm type IV

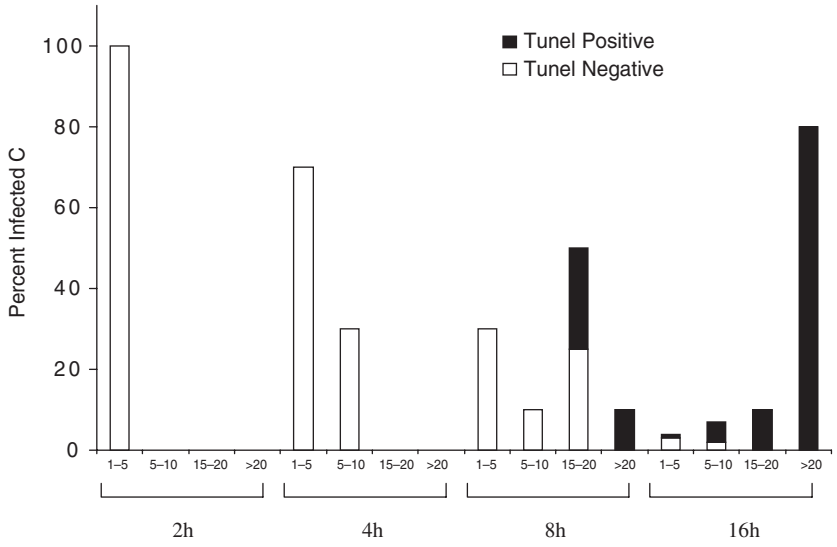

Fig. 9. DNA fragmentation in the *L. pneumophila*-infected cells is not triggered until late stages of intracellular replication. Single-cell analysis of AA100-infected hPBM was performed by confocal laser scanning microscopy. DNA fragmentation was examined bUNEL staining, and the average number of bacteria per cell, labelled with an antibody, was examined in 300 infected cells. All samples were run in triplicate in each experiment, and the results shown are representative of three independent experiments.

secretion system exports an effector molecule that specifically activates caspase-3.

Here, we show that activation of caspase-3 by *L. pneumophila* is independent of the intrinsic and extrinsic pathways of the caspase cascade. We have provided several lines of evidence that caspase-8 and caspase-9 are not activated and cytochrome *c* is not released from mitochondria, despite the activation of caspase-3. Our findings that cytochrome *c* is not released from the mitochondria is in direct discrepancy with a recent report in which it has been concluded that cytochrome *c* is released during infection by *L. pneumophila* (Neumeister *et al*., 2002). The reason for this discrepancy may be due to one or more of the following. First, our studies are performed with a low MOI of 0.1–10 in which the infection is carried out for 1 h followed by extensive removal of the extracellular bacteria to avoid cytotoxicity (Zink *et al*., 2002). In contrast, Neumeister *et al*. (2002) have used an MOI of 100, and the infection was performed in the presence of this high MOI for the total period of the experiments of 2–24 h, which probably causes cytotoxicity (Husmann and Johnson, 1994; Byrne and Swanson, 1998; Kirby *et al*., 1998; Hammer and Swanson, 1999; Alli *et al*., 2000; Bachman and Swanson, 2001; Joshi *et al*., 2001). Our data also show that an MOI of 100 or more causes the release of cytochrome *c* from mitochondria before activation of caspase-3 (data not shown). Importantly, caspase-3 activation precedes the release of cytochrome *c* reported by Neumeister *et al*. (2002). In addition, a few contradictory observations have been reported in their study, including activation of caspase-8 despite their findings that caspase-8 or FADD is not involved in the activation of caspase-3 by *L. pneumophila*. Although many *dot/icm* mutants have been shown to be defective in the induction of capase-3 activation (Zink *et al*., 2002), a *dot/icm* mutant or killed *L. pneumophila* have not been used as negative controls in the studies reported by Neumeister *et al*. (2002). Therefore, in addition to the cytotoxic MOI used, the specificity of their observations is difficult to interpret without these controlled studies.

Rabaptin-5 is a crucial effector of Rab5, and the Rab5–Rabaptin-5 interaction is essential for the recruitment of ≈20 downstream effectors of Rab5 to create a Rab5-rich domain essential for fusion to endosomes (Horiuchi *et al*., 1997; Christoforidis *et al*., 1999; McBride *et al*., 1999; Nielsen *et al*., 2000; Woodman, 2000; Zerial and McBride, 2001). In several model systems of apoptosis, activation of caspase-3 results in the cleavage of Rabaptin-5 into three polypeptides of 47, 53 and presumably 9 kDa that dissociate from the membrane into the cytosol (Cosulich *et al*., 1997; Swanton *et al*., 1999). This results in inhibition of endocytic fusion, which can be reversed by purified Rabaptin-5 (Stenmark *et al*., 1995; Cosulich *et al*., 1997; Horiuchi *et al*., 1997; McBride *et al*., 1999; Swanton *et al*., 1999; Zerial and McBride, 2001). Our data show that activation of caspase-3 by the Dot/Icm virulence system is essential for evasion of endocytic fusion to the LCP, and for the cleavage of Rabaptin-5.

Whether the caspase-3-mediated cleavage of Rabaptin-5 is responsible for evasion of vesicle trafficking by the LCP is not known. A potential primary role for the cleavage of Rabaptin-5 in evasion of vesicle trafficking by the LCP is contradicted by the documented observations that Rab5 is not detectable on the LCP and, therefore, Rabaptin-5 may not even be recruited to the LCP (Roy *et al*., 1998; Coers *et al*., 1999; Clemens *et al*., 2000). However, the lack of detection of Rab5 on the LCP may be due to one of two possibilities. First, the LCP blocks the recruit-

ment of Rab5. If this is the case, then the caspase-3-mediated cleavage of Rabaptin-5 may serve as a secondary back-up strategy to block endocytic fusion to the LCP. The second possibility that may explain the lack of detection of Rab5 on the LCP is that most of the LCPs transiently acquire Rab5, but Rab5 is rapidly dissociated from the LCPs as a result of the cleavage of Rabaptin-5. This speculation is supported by several lines of indirect and circumstantial evidence. First, the lack of detection of Rab5 on the LCP, even when Rab5 is overexpressed in infected cells (Roy *et al*., 1998; Coers *et al*., 1999; Clemens *et al*., 2000). Secondly, exclusion of Rab5 from the LCP, which is predicted to be reversed when caspase-3 is inhibited and cleavage of Rabaptin-5 is blocked. Thirdly, our observations that PI3 kinase, which acts downstream of Rabaptin-5 in the assembly of the Rab5 domain on early endosomes, is not required for the establishment of a replicative niche by *L. pneumophila*. Fourthly, the ability of the wild-type strain to evade endocytic fusion is enhanced in macrophages expressing the GDP-locked dominant-negative mutant of Rab5 (Rab5S34N) that is not recruited to endosomes (Roy, 2002), indicating that exclusion of Rab5 enhances the ability of the LCP to form a replicative niche. It is important to note that, although the four lines of evidence mentioned above may support the possibility of acquisition and rapid loss of Rab5 by the LCP, each of these lines of evidence can be explained by other processes. It remains to be determined whether cleavage of Rabaptin-5 plays a direct role in evasion of vesicle trafficking by the LCP or whether other events upstream of Rabaptin-5, or the exclusion of Rab5 itself, are primarily responsible for evasion of vesicle trafficking by the LCP. The possible acquisition and dissociation of Rab5 and Rabaptin-5 by the LCP will be addressed in future studies aimed at determination of the kinetics of acquisition and loss of Rab5 and Rabaptin-5 by the LCP in live cells.

Superinfection of WT-infected macrophages with a *dot* mutant results in fusion of the *dotA* LCP to lysosomes, whereas the WT LCP in the same cell is blocked from lysosomal fusion (Coers *et al*., 1999). These observations indicate that the effect of the Dot/Icm system in evading vesicle fusion is a *cis*-acting effect and is limited to the LCP, whereas other fusion events in the infected cell are not affected (Coers *et al*., 1999). Therefore, it is tempting to speculate whether activation of caspase-3 by the Dot/Icm signal and cleavage of Rabaptin-5 occurs within the vicinity of the LCP.

Our observations of the crucial role of caspase-3 for the ability of *L. pneumophila* to evade endocytic fusion and to replicate intracellularly may be consistent with the genetic studies on the mammalian *lgn1* locus that confers susceptibility to infection by *L. pneumophila* (Beckers *et al*., 1995; 1997; Dietrich *et al*., 1995; Endrizzi *et al*., 1999; Growney and Dietrich, 2000). The *lgn1* locus encodes for the neuronal apoptosis inhibitory protein 5 (Naip-5) (Charmaillard *et al*., 2003; Diez *et al*., 2003; Wright *et al*., 2003), which is a member of the family of apoptosis inhibitor proteins (IAPs), such as XAIP that has been shown to be a direct inhibitor of caspase-3 (Deveraux *et al*., 1997; Goyal, 2001; Suzuki *et al*., 2001; Maier *et al*., 2002; Salvesen and Duckett, 2002). XIAP binds to the active site of caspase-3 and blocks the groove of its substrate, similar to the interaction of caspase-3 with its tetrapeptide inhibitor (DEVD) (Chai *et al*., 2001; Huang *et al*., 2001; Riedl *et al*., 2001). However, whether Naip-5 functions as an inhibitor of caspase-3, similar to its homologue XIAP, is still to be determined.

Using single-cell analysis, we have shown that, despite the activation of caspase-3 by *L. pneumophila* during early stages of infection, apoptosis is not triggered in the infected cell until the terminal stages of the infection when the cell harbours >20 organisms. Therefore, activation of caspase-3 may serve two purposes in the intracellular life cycle of *L. pneumophila*. First, during early stages of the intracellular infection, caspase-3 activation is essential for evasion of endocytic fusion and for establishment of the intracellular infection. Secondly, during late stages of the intracellular infection, the increased level of capase-3 activation as a result of bacterial replication results in apoptosis of the infected cell, which may facilitate bacterial egress. It has been also shown that the pore-forming activity of *L. pneumophila*, which is induced upon termination of intracellular replication, contributes to cytolysis of the host cell (Alli *et al*., 2000; Molmeret and Abu Kwaik, 2002; Molmeret *et al*., 2002a,b). It is possible that the inductions of both apoptosis and the pore-forming activity during late stages of the infection are two primary factors leading to cytolysis of the host cell and bacterial egress.

In summary, we have shown that activation of caspase-3 by *L. pneumophila* during early stages of infection is independent of upstream initiators of caspase-3 activation. We have described a crucial role for caspase-3 in arrested biogenesis of the LCP and its subversion of vesicle traffic. Activation of caspase-3 results in the cleavage of Rabaptin-5. Although the cleavage of Rabaptin-5 may be sufficient to halt maturation of the LCP, other upstream events such as the exclusion of Rab5 itself may be primarily responsible for evasion of vesicle trafficking by the LCP. The caspase-3 activity is dispensable after the first few minutes of infection, and this correlates with transformation of the LCP into a RER-derived replication niche. We have also shown that caspase-3 activation results in apoptosis during late stages of the infection when the infected cell harbours >20 bacteria, which may contribute to bacterial egress from the host.

Experimental procedures

Bacterial and eukaryotic cells

The virulent clinical isolate of *L. pneumophila* strain AA100 and its isogenic *dotA* mutant (GL10) have been described previously (Zink *et al*., 2002). The isogenic *icmS*, *icmR* and *icmQ* mutants of AA100 have been described previously (Molmeret *et al*., 2002b). U937 macrophage-like cells were maintained at 37°C and 5% CO_2 in RPMI-1640 tissue culture medium (Gibco BRL) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco BRL). For infections, U937 cells (1×10^5 cells per well in 96- or 24-well plates) were differentiated for 48 h using phorbol 12-myristate 13-acetate (PMA) as described previously (Zink *et al*., 2002). Differentiated cells are non-replicative adherent macrophage-like cells. Monolayers were washed three times with the tissue culture medium before infection. Jurkat and FADD dominant-negative Jurkat cells were obtained from ATCC. The cell line overexpressing Bcl_{xL} was a kind gift from V. Ragnekar (Chakraborty *et al*., 2001). Peripheral blood monocytes were isolated from volunteers who had no history of Legionnaires' disease. The monocytes were obtained using the Ficoll Hypaque gradient as described previously (Gao *et al*., 1998).

Infections

Unless specified, all infections were carried out for 1 h followed by washing off the extracellular bacteria, and the infected cells were incubated for the indicated periods of infections specific for each experiment. Unless specified, infections for immunoblot analysis were performed at an MOI of 10 for 1 h followed by 3 h incubation. Unless specified, an MOI of 10 was used for intracellular growth kinetics, confocal laser scanning microscopy and electron microscopy. Superinfection of AA100-infected cells by the *dotA* mutant was performed as described previously (Coers *et al*., 1999).

To measure activity of various caspases, differentiated U937 cells in 96-well plates with 1×10^5 cells per well were infected as stated above with an MOI of 10 for 1 h followed by removal of extracellular bacteria and further incubation for 3 h. The cells were then lysed with 50 μl of chilled lysis buffer (Biovision) on ice for 10 min. To the lysates, 50 μl of 2× reaction buffer containing 10 mM dithiothreitol (DTT) and 5 μl of 1 mM fluorogenic substrates (see below) was added. The samples were then incubated at 37°C for 2 h. Activation was determined using a fluorescent plate reader (Perkin-Elmer) with an excitation at 400 nm and an emission at 505 nm. All samples were tested in triplicate and compared with uninfected cells to determine the amount of caspase activity above background.

Reagents

Fractionation of cytoplasmic and mitochondrial cytochrome *c* was performed as described previously (Fan *et al*., 1998). Staurosporin was obtained from Sigma and used at 1 μg ml^{-1} for 1 h. The primary rabbit polyclonal antisera used in Western blots against caspase-3 are from R and D systems, while antisera against caspase-8 and -9 were from Santa Cruz Biotechnology. Anti-cytochrome *c* antibodies were from Pharmingen. Rabbit antisera against the C-terminus of Rabaptin-5 were kind gifts from Marino Zerial (Dresden, Germany) and Philip Woodman (Manchester, UK). Anti-Rab5 monoclonal antibody was purchased from Signal Transduction Laboratories. All secondary antibodies conjugated to horseradish peroxidase (HRP) used for Western blots were obtained from Sigma.

The activation of the individual caspases within U937 cells was accomplished using fluorogenic substrates obtained from Biovision. The substrates were oligopeptides fused to 7-amino-4-trifluoromethyl coumarin (AFC). Caspase substrates are as follow: caspase-1 (YVAD-AFC), caspase-2 (VDVAD-AFC), caspase-3 (DEVD-AFC), caspase-5 (WEHD-AFC), caspase-6 (VEID-AFC), caspase-8 (IETD-AFC), caspase-9 (LEHD-AFC) and caspase-10 (AEVD-AFC). Inhibitors of caspase-3 (Z-DEVD-fmk) and the paninhibitor of caspases (Z-VAD-fmk) and other caspase inhibitors were all from Biovision. Unless indicated, the caspase inhibitors were used at 100 μM concentrations. Ionomycin, calmodulin inhibitor II, calpeptin and calpain inhibitor AA were obtained from Sigma and used at a concentration of 2 μm. The PI3 kinase inhibitors methyl adenine, wartmannin and Ly294002 were purchased from Sigma and used at the concentrations specified for each experiment.

Detection of apoptosis by TUNEL assays

TUNEL assays were performed exactly as described previously (Gao and Abu Kwaik, 1999b). Briefly, cells attached to coverslips in 12-well plates were infected for 1 h with *L. pneumophila* at an MOI of 10 to achieve apoptosis, followed by washing for removal of extracellular bacteria and further incubation for various time intervals. For labelling of apoptotic nuclei, the cells were subjected to fluorescein isothiocyanate (FITC)-conjugated terminal deoxytransferase-mediated dUPT nick end-labelling (TUNEL) using an apoptosis detection kit, according to the manufacturer's instructions (Boehringer Mannheim). Cells were examined by confocal laser scanning microscopy (see below). A minimum of 200 cells per sample were examined, and apoptosis was quantified as the percentage of apoptotic cells (TUNEL-positive nuclei). Multiple independent samples were examined.

Confocal laser scanning and electron microscopy

Approximately 5×10^5 U937 cells were differentiated on 18 mm (0.13–0.17 mm thick) circular glass coverslips (VWR) in 12-well culture plates. After infections, cells were washed three times with warm culture medium and fixed at 37°C in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 1 h. Paraformaldehyde was removed by washing wells three times with PBS, pH 7.5. All subsequent steps were performed at room temperature, and each step was followed by three washes in PBS. Blocking was performed using 3% bovine serum albumin (BSA) for 1 h. The anti-LAMP-2 (H4B4) monoclonal antibody (developed by J. T. August and J. E. K. Hildreth) was obtained from the Developmental Studies Hybridoma Bank (University of Iowa, IA, USA). Anti-rabbit conjugated to Alexa red and the DNA dye TOTO-3 were from Molecular Probes. Infections for confocal microscopy were processed and analysed using a Leica TCS Sp laser scanning confocal microscope as described previously (Pedersen *et al*., 2001). On average, 8–15 0.2 μm serial sections of each image were captured and stored for further analyses, using Adobe PHOTOSHOP 6.0.

Fusion of lysosomes to *L. pneumophila* phagosomes was

determined by detection of the lysosomal enzyme acid phosphatase by electron microscopy as described previously (Molmeret *et al*., 2002a). Briefly, the acid phosphatase-specific substrate, β-glycerolphosphate, was used, and lead nitrate was the capture metal, before fixation and processing (Molmeret *et al*., 2002a).

Immunoblot analyses

Six-well plates containing 1–10^6 U937 cells were used in these assays. Uninfected or infected cells at an MOI of 10 in the presence or absence of the caspase-3-specific inhibitor, pancaspase inhibitor or treated for 1 h with staurosporin were used. The cells were incubated for 3 h after the 1 h infection period, then resuspended at several intervals in cold PBS containing a protease inhibitor cocktail at a concentration of 1 μg μl^{-1} (Sigma). Cells (1–10^6) were pelleted by centrifugation at 5000 *g* for 10 min at 4°C and lysed in 50 μl of warm lysis buffer (1% SDS, 10%glycerol, 10% β-mercaptoethanol, 0.01% bromophenol blue, 8% 0.5 M Tris, pH 6.8). Equivalent amounts of proteins (equivalent number of cells) were resolved on sodium dodecyl sulphate (SDS)-10% polyacrylamide gel and electrotransferred onto Immobilon-P (Millipore) membranes that were probed with the respective rabbit polyclonal antisera at a dilution of 1:2000 in blocking solution (3% BSA in 3 M Tris, pH 7.5, NaCl, 0.5 M EDTA, Tween 20). Secondary anti-rabbit antibody conjugated to HRP (Sigma) was used at 1:12 000 dilution. The HRP was detected using Supersignal chemiluminescent detection reagents (Pierce). The blots were subsequently stripped in 30% hydrogen peroxide for 60 min at 25°C and reprobed when necessary.

Cytochrome *c* release analysis was accomplished using the mitochondria/cytosol fractionation kit from BioVision. U937 cells were infected in six-well tissue culture plates at an MOI of 10 for 1 h followed by a 3 h incubation in six-well plates containing 1×10^6 cells per well. Two wells were used for each sample for a total of 2×10^6 cells. After the infection, the cells were washed three times with cold PBS to remove extracellular bacteria. The cells were detached from the well using a cell scraper and resuspended in 1 ml of cytosol extraction buffer that contains DTT and protease inhibitors. The suspension was then homogenized on ice and examined by microscopy to confirm cell lysis. The lysates were centrifuged at 10 000 *g* for 30 min at 4°C. The supernatant was removed as the cytosolic fraction, and the remaining pellet was resuspended in 100 μl of mitochondrial extraction buffer. The two fractions from each sample were loaded onto an SDS-polyacrylamide gel and processed for immunoblot analysis as stated above.

Data analysis

Percentage co-localization for all confocal and electron microscopy results were derived from random examination of 150–200 infected cells from each of the triplicate samples in each experiment. All samples were coded, and the codes were not revealed until all data analysis was completed. All experiments were performed three to five times in triplicate, and the results shown are from one representative experiment.

Acknowledgements

We thank Vivik Raganekar for valuable cell lines. We thank Marino Zerial and Philip Woodman for the anti-Rabaptin-5 antisera. Y.A.K. is supported by Public Health Service grant RO1AI43965.

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