Host-dependent trigger of caspases and apoptosis by *Legionella pneumophila*

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Abstract

The Dot/Icm system of Legionella pneumophila triggers activation of caspase-3 during early stages of infection of human macrophages, but apoptosis is delayed until late stages of infection. During early stages of infection of mouse macrophages the organism triggers rapid caspase-1-mediated cytotoxicity, which is mediated by bacterial flagellin. However, it is not known whether caspase-1 is triggered by L. pneumophila in human macrophages, or whether caspase-3 is activated in permissive or non-permissive mouse macrophages. Using single cell analyses we show that the wild type strain of L. pneumophila does not trigger caspase-1 activation throughout the intracellular infection of human monocytes-derived macrophages (hMDMs), even when the flagellated bacteria escape into the cytoplasm during late stages. Using single cell analyses we show that the Dot/Icm system of L. pneumophila triggers caspase-3 but not caspase-1 within permissive A/J mouse bone marrow-derived primary macrophages by 2-8h, but apoptosis is delayed till late stages of infection. While L. pneumophila triggers a Dot/Icm-dependent activation of caspase-1 in non-permissive BALB/c mouse-derived macrophages, caspase-3 is not activated at any stage of the infection. We show that robust intrapulmonary replication of the wild type strain of L. pneumophila in susceptible A/J mice is associated with late stage Dot/Icm-dependent pulmonary apoptosis and alveolar inflammation. In the lungs of non-permissive BALB/c mice, L. pneumophila does not replicate and does not trigger pulmonary apoptosis or alveolar inflammation. Thus, similar to hMDMs, L. pneumophila does not trigger caspase-1 but triggers caspase-3 activation during early and exponential replication in permissive A/J mouse-derived macrophages, and apoptosis is delayed until late stages of infection. The Dot/Icm type IV secretion system is essential for pulmonary apoptosis in the genetically susceptible A/J mice.
Introduction

The ability of \textit{L. pneumophila} to cause pneumonia is dependent on its capacity to invade and replicate within alveolar macrophages, monocytes, and potentially alveolar epithelial cells (3, 38, 43). Upon entry into the host cell, \textit{L pneumophila} modulates biogenesis of the phagosome into a replicative niche that is halted from maturation through the “default” endosomal-lysosomal degradation pathway (17, 32, 42, 44). The \textit{L. pneumophila}-containing phagosome (LCP) intercepts early secretory vesicles from the endoplasmic reticulum exit sites, which allows the organism to remodel the LCP membrane to become RER-derived within minutes of its biogenesis from the plasma membrane (16, 18, 39, 41). Upon activation of human macrophages by IFN-\(\gamma\), the LCP fuses to the lysosomes and fails to be remodeled by the RER (33). In contrast to \textit{L. pneumophila}, recent studies have shown that \textit{L. longbeachae} is trafficked to, and replicates within, a non-acidified late endosome like phagosome that is remodeled by the RER (5). The Dot/Icm type IV secretion system of \textit{L. pneumophila} is essential for evasion of endocytic fusion and for remodeling of the LCP into a rough endoplasmic reticulum (RER)-derived compartment (16, 18, 32, 35, 39, 41, 42, 44). These manipulations of host cell processes during early stages are thought to be mediated by the injection of effectors by the Dot/Icm transporter directly from the bacterium into the host cell (8, 28). During late stages of infection of human macrophages and \textit{Acanthamoeba polyphaga}, the bacteria escape into the host cell cytosol where they reside for 2-6 hours prior to lysis of the host cell plasma membrane (24).

In addition to evasion of vesicle traffic by \textit{L. pneumophila} during early stages of infection, the bacterium also induces Dot/Icm-dependent activation of caspase-3 in
human macrophages (12-14, 25, 27, 49). There are at least 14 caspases (cysteine proteases) that trigger the activation of two distinct apoptosis signaling pathways designated the extrinsic and intrinsic pathways that converge on the activation of caspase-3, which is the executioner of rapid apoptosis (30, 36). Interestingly, the Dot/Icm-mediated activation of caspase-3 by *L. pneumophila* in human macrophages during early stages of infection seems to be novel, since it is independent of the extrinsic and intrinsic pathways of apoptosis (25). Interestingly, despite the robust activation of caspase-3 during early and exponential replication of *L. pneumophila* within human macrophages, apoptosis is not triggered until termination of intracellular replication (2, 25), which is a novel modulation of caspase-3 activity that halts it from rapid dismantling of the cell. Recent data have shown that the delay in apoptosis of *L. pneumophila*-infected human macrophages is associated with induction of strong Dot/Icm-dependent anti-apoptotic signals that are mediated by NF-κB and non-NF-κB signaling mechanisms (1, 20).

Among inbred mice strains, the A/J strain is the only one susceptible to *L. pneumophila* infection, while all the other strains are relatively resistant (46-48). This genetic susceptibility is attributed to a polymorphism in the gene encoding the neuronal apoptosis inhibitory protein (naip5) (11, 45). The naip family of genes are evolutionary conserved from viruses to humans and some encode proteins that possess anti-apoptotic activity, due to inhibition of caspase-3 and caspase-7 (10, 21). However, caspase-3 is not required for the infection of mice macrophages by *L. pneumophila* (26, 31), which is distinct from human macrophages (25). In mice macrophages that are non-permissive for intracellular proliferation of *L. pneumophila*, the bacterial flagellin (FlaA) triggers caspase-1-mediated pro-inflammatory rapid cell death/pyropoptosis (26, 31). The
mechanism and the role of Naip5 in activation of caspase-1 by L. pneumophila is not known.

It is not known whether caspase-1 is triggered by L. pneumophila in human macrophages, or whether caspase-3 is activated in permissive or non-permissive mice macrophages. It is also not known whether similar kinetics of apoptosis in tissue culture systems is also exhibited in the lungs of animal models. Here we show that within hMDMs and A/J mice macrophages, L. pneumophila does not trigger caspase-1 activation throughout the intracellular infection, despite the escape of highly flagellated L. pneumophila into the cytosol of hMDMs during late stages of infection. L. pneumophila triggers differential and temporal early activation of caspase-3 in A/J mouse-derived macrophages, similar to hMDMs, but caspase-3 is not triggered in the resistant BALB/c mice-derived macrophages. Our data show that Dot/Icm-mediated pulmonary apoptosis is triggered during late stages of intrapulmonary replication in susceptible A/J mice. In contrast, L. pneumophila fails to induce pulmonary apoptosis in BALB/c mice, despite rapid caspase-1-mediated cell death in primary macrophages in vitro.
Materials and Methods

Animals, bacteria and macrophages

Female pathogen-free A/J and BALB/c mice, 8 to 9 weeks of age, were used in all experiments. Mice were housed in specific pathogen-free conditions within the animal care facility. The virulent clinical isolate of \textit{L. pneumophila} strain AA100 and its isogenic \textit{dotA} mutant (GL10) have been described previously (49). The wild type \textit{F. tularensis} subsp. novicida strain U112 has also been described previously (19). The bacteria were maintained frozen at -80°C and, prior to use, were grown on buffered charcoal yeast extract agar for 72h. The plates for \textit{gfp}-transformed AA100 or its isogenic \textit{dotA} mutant were supplemented with 5µg/ml chloramphenicol. After cultivation, bacteria were washed by centrifugation and resuspended in sterile saline.

To prepare mBMDM (mouse bone marrow derived macrophages), bone marrow was isolated from healthy A/J or BALB/c mice and were prepared as described previously (26). To prepare hMDMs, peripheral blood monocytes were isolated from healthy volunteers with no history of Tularemia or Legionnaires’ disease and hMDMs were prepared as we described previously (33). The age of volunteers was 25-45 years old with no history of pneumonia or any underlying chronic disease.

Inoculation of animals

Mice were inoculated intratracheally (i.t.), as we described previously (4, 23, 29). Briefly, the mice were anesthetized by intraperitoneal (i.p.) injection of ketamine (2.5 mg/mouse). A total of 50µl of the \textit{L. pneumophila} suspension ($10^6$ cfu) in sterile water was inoculated directly into the trachea using a 26-gauge needle followed by 10 to 20 µl
of air. Control animals were inoculated with saline only and were sacrificed at different time points.

**Quantitation of *L. pneumophila* in pulmonary tissues of mice**

At different time points after inoculation of bacteria, the mice were humanely sacrificed. The lungs were aseptically excised, finely minced, and homogenized in a tissue homogenizer with 5 ml of sterile distilled water. The number of cfu of *L. pneumophila* AA100 or *dotA* mutant strain in the lungs was determined by the plate dilution method using BCYE agar. After 3 days of incubation at 37°C the colonies were enumerated and the results were expressed as the number of cfu per lungs.

**Pulmonary histopathology**

The histological changes and apoptosis in the lungs of A/J and BALB/c mice in response to *L. pneumophila* were assessed by light and confocal microscopy. At 2, 24 and 48h after inoculation, the mice were humanely sacrificed using CO₂ asphyxiation. Before lung removal, the pulmonary vasculature was perfused with 10 ml of saline containing 5mM EDTA, via the right ventricle. The excised lungs were inflated and fixed in 10% neutral formalin for 24 h, dehydrated, and embedded in paraffin. Sections were cut and stained with eosin and hematoxylin (EH) for analyses of infiltration process in the lungs of infected mice. In addition, sections (5 µm) were cut and labeling of apoptotic cells was carried out using terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) using an in situ cell death detection kit, as
recommended by the manufacturer (Roche, Indianapolis, IN). Histopathology of the lung tissue stained with EH was analyzed by light microscopy. An analysis of histology of the lung tissue in the presence of intracellular GFP-expressing bacteria, and apoptotic cells (TUNEL positive) was carried out using laser scanning confocal microscopy. On average, 10 0.2-µm thick serial sections of each image were captured and stored for further analyses, using Adobe Photoshop CS version 8.0 (Adobe Photoshop, Inc.).

**Transmission Electron Microscopy**

For examination of apoptosis in the lungs of A/J mice by transmission electron microscopy, mice lungs were removed and placed in 2.5% gluteraldehyde, as described previously (24). Briefly, lungs were post fixed by immersion in 2% osmium tetroxide in 0.1 M sodium Sorenson’s buffer for 1 h, followed by dehydration in acetone and infiltration and embedding in Epon 12 epoxy resin (24). Ultrathin sections (0.1 µm) were then cut, stained with uranyl acetate and lead citrate, and examined in Philips transmission electron microscope (Philips, Morgagni 268D, Netherlands) at 80 kV.

**Caspase activation and TUNEL assays**

To assess activation of caspase-1 and caspase-3 by confocal microscopy, $2.5 \times 10^5$ mBMDMs or hMDMs on glass coverslips were infected with *L. pneumophila* AA100, the *dotA* mutant or *F. tularensis* subsp. *novicida* at MOI of 10 for 1h followed by incubation of 2, 8 and 18 h. For caspase-1 activation, macrophages were stained for 1h with FAM–YVAD–FMK (Immunochemistry Technologies, Bloomington, IN) as recommended by the manufacturer. As a positive control for caspase-1 activation in hMDMs, macrophages were treated with 10 mM Simvastatin (Calbiochem, San Diego, CA) (9). For caspase-3
activation, after infection and fixation, the cells were incubated with anti-active caspase-3 rabbit polyclonal antiserum for 1 h, followed by a goat anti-rabbit immunoglobulin G secondary antibody conjugated to Alexa red (Molecular Probes, Inc., Eugene, Oregon).

Apoptotic nuclei were labeled with TUNEL according to manufacturer's instructions (Boehringer Mannheim Corporation, Indianapolis, Ind.). Cells were examined with a Zeiss Axiophot Photomicroscope Leica TCS NT confocal laser scanning microscope. A minimum of 100 cells per sample were examined, and apoptosis was quantified as the percentage of apoptotic cells (TUNEL-positive nuclei).

**Statistical analyses**

All experiments have been performed at least three times and the data shown are representative of one experiment. Statistical Analyses were performed using student two-tail Student t-test.
Results

*L. pneumophila* does not trigger activation of caspase-1 throughout the infection of primary human macrophages

Although caspase-1 has been shown to be triggered by *L. pneumophila* within mice-derived macrophages, it is not known whether caspase-1 is triggered by *L. pneumophila* during any stage of the infection of human macrophages. We utilized primary human monocytes-derived macrophages (hMDMs) to examine potential activation of caspase-1 by *L. pneumophila* AA100 or the dotA mutant throughout the intracellular infection. As a positive control for caspase-1 activation, cells were pretreated with Simvastatin or infected by *F. tularensis*. By 2h after infection of hMDMs with all the strains caspase-1 activation was minimal, which was not significantly different from uninfected cells (Student *t*-test, *p* > 0.2) (Fig. 1). By 8h after infection of hMDMs with *L. pneumophila* AA100 only ~10% of the infected cells were positive for caspase-1 and also apoptotic (Student *t*-test, *p* > 0.1) (Fig. 1). While ~80% of *L. pneumophila*-infected hMDMs were apoptotic by 18h post-infection, only ~10% of them exhibited caspase-1 activation (Fig. 1). Similar results were obtained for hMDMs derived from four different donors (data not shown). Therefore, the large number of infected hMDMs that became apoptotic at 18h was not associated with activation of caspase-1. When the cells were labeled for active caspase-3, *L. pneumophila* triggered time-dependent early activation of caspase-3 and late stage apoptosis was inhibited by the caspase-3 inhibitor (data not shown), consistent with many previously published data from independent labs (12-14, 25, 27, 49). Caspase-1 activation was exhibited in control hMDMs treated with Simvastatin or infected by *F. tularensis* as positive controls (Fig. 1).
The caspase-1 activity and apoptosis in hMDMs infected with the dotA mutant was not significantly different from uninfected cells at all time points after infection (Student $t$-test, $p > 0.3$) (Fig. 1). We conclude that *L. pneumophila* does not trigger caspase-1 activation throughout the infection of human macrophages while caspase-3 is highly activated throughout the intracellular infection. Importantly, late stage apoptosis in hMDMs is not associated with activation of caspase-1.

**Differential activation of caspases and apoptosis *L. pneumophila* in permissive mice macrophages**

Although caspase-3 has been shown to be triggered by *L. pneumophila* within human macrophages, it is not known whether *L. pneumophila* triggers caspase-3 in mice-derived macrophages. We utilized single cell analyses to examine the kinetics of potential activation of caspase-3 and apoptosis in primary bone marrow-derived macrophages (mBMDM) obtained form A/J permissive and BALB/c non-permissive mice. We also examined the kinetics of caspase-1 activation throughout the infection to decipher whether apoptosis triggered during late stages of infection was mediated by caspase-1 or caspase-3. We used *Francisella tularensis* as a positive control for caspase-1 activation (22).

A/J mBMDMs infected by *L. pneumophila* AA100 or its dotA mutant were examined for the kinetics of caspase-3 activation and apoptosis using single cell analyses by confocal microscopy. Approximately 20% of the cells infected by *L. pneumophila* AA100 exhibited activation of caspase-3 at 2h after infection, which was significantly different from the infection by the dotA mutant or uninfected cells (Student $t$-test, $p <$
0.01), but only few cells were positive for TUNEL (Fig. 2). At 8h post infection, ~70% of \textit{L. pneumophila}-infected A/J mBMDMs exhibited caspase-3 activation, which was significantly different from the infection by the dotA mutant or uninfected cells (Student $t$-test, $p < 0.003$), but only few infected cells underwent apoptosis (Fig. 2). By 18h after infection with \textit{L. pneumophila} AA100, a large number of the cells were lysed, and ~60% of the remaining infected cells exhibited caspase-3 activation and were also apoptotic (Student $t$-test, $p < 0.001$) (Fig. 2). Apoptosis was inhibited when the infected cells were pre-treated with the caspase-3 inhibitor but not when the infected cells were pre-treated with the caspase-1 inhibitor (data not shown). The dotA mutant control neither activated caspase-3 nor did it trigger apoptosis at any time point after infection of A/J mBMDM, which was not significantly different from uninfected cells (Student $t$-test, $p < 0.3$) (Fig. 2).

At 2-8h after infection of A/J mBMDM with \textit{L. pneumophila} AA100, caspase-1 activation and apoptosis was not significantly different from the infection by the dotA mutant or uninfected cells (Student $t$-test, $p > 0.2$) (Fig. 2). By 18h post-infection, ~80% of the \textit{L. pneumophila} AA100-infected cells were TUNEL positive but only 10% of them exhibited caspase-1 activation. The dotA mutant did not activate caspase-1 in A/J mBMDM and only few cells underwent apoptosis at all time points after infection (Fig. 2), which was not significantly different from uninfected cells (Student $t$-test, $p > 0.2$). Infection of A/J mBMDM by the \textit{F. tularensis} control triggered time-dependent activation of caspase-1.
*L. pneumophila* triggers temporal and differential rapid activation of caspase-1 and apoptosis in resistant BALB/c mice-derived macrophages

Although caspase-1 has been shown to be triggered by *L. pneumophila* within non permissive mice-derived macrophages, it is not known whether activation of caspase-3 is also triggered by *L. pneumophila* at any stage of the infection. Therefore, we used single cell analysis to examine the temporal kinetics of activation of caspase-1 and caspase-3 in BALB/c mBMDM. The data showed that *L. pneumophila* AA100 triggered robust activation of caspase-1 at 2-8h after infection of BALB/c mBMDM, where most infected cells were positive for caspase-1 activity and were also apoptotic (Student *t*-test, *p* < 0.001) (Fig. 3). In contrast, infection of BALB/c mBMDM with the *dotA* mutant triggered minimal caspase-1 activation and apoptosis, which was not significantly different from uninfected cells (Student *t*-test, *p* > 0.1) (Fig. 3). The cells infected by *F. tularensis* exhibited time-dependent activation of caspase-1 (Fig. 3).

We examined the kinetics of activation of caspase-3 in upon infection. In contrast, caspase-3 activation was minimal at 2h after infection of BALB/c mBMDM by *L. pneumophila* AA100, which was not significantly different from the *dotA*-infected and uninfected cells (Student *t*-test, *p* > 0.3). At 8h after infection with the wild type strain of *L. pneumophila* ~30% of infected cells exhibited activation of caspase-3 (Student *t*-test, *p* < 0.05) and ~10% of these were also positive for TUNEL. Similar results were obtained at 18h after infection by *L. pneumophila* AA100 (Fig. 3). The *dotA* mutant triggered very low level of activation of caspase-3 at all time points after infection, which was similar to uninfected macrophages (Student *t*-test, *p* > 0.2). Taken together, our results show that *L.*
pneumophila does not trigger caspase-3 in non-permissive BALB/c mice-derived primary macrophages.

**L. pneumophila induces Dot/Icm-dependent pulmonary inflammation and apoptosis in A/J mice**

*L. pneumophila* AA100 replicated in the lungs of A/J mice, where the number of bacteria peaked at 48h after infection but did not replicate within the lungs of BALB/c mice, and the *dotA* mutant did not replicate in any mice, consistent with previous published observations (Supplementary Fig. S1). Inflammatory infiltration was first evident in lung tissue from *L. pneumophila* AA100 infected A/J mice at 24h after infection, and became more severe by 48h post-infection (Supplementary Fig. S2). However, there was no detectable inflammatory infiltration in the lungs of A/J mice infected with the *dotA* mutant or BALB/c mice infected with *L. pneumophila* AA100 at any time points after infection (Supplementary Fig. S3).

We examined by in situ cell analyses the kinetics of apoptosis in the lung tissue of A/J and BALB/c mice infected with *L. pneumophila* AA100 and the *dotA* mutant by laser scanning confocal microscopy. As a positive control, we used DNase-treated sections of lung tissue. As negative controls, we used DNase-untreated sections and lung tissue sections of uninfected mice inoculated with saline (Fig. 4). Any nuclei stained black by TUNEL were considered apoptotic, regardless of intensity of the staining.

At 2h after infection of A/J mice with *L. pneumophila* AA100 no apoptotic cells were detected in the lung tissue of infected mice (Fig. 4). However, at 24-48h after infection large numbers of pulmonary cells were apoptotic (Fig. 4). At all time intervals
examined, there was no detectable pulmonary apoptosis in the dotA mutant-infected mice (Fig. 5). In addition, *L. pneumophila* AA00 did not trigger pulmonary apoptosis in BALB/c mice at any time point (2, 24, and 48h) after infection (Fig. 6).

We used TEM to examine the apoptotic process in the lungs of A/J mice infected with the wild type strain of *L. pneumophila* or the dotA mutant. At 2h after infection, most of the cells revealed normal morphology (Fig. 7). At 24 and 48h typical morphological features of programmed cell death could be detected, including condensed nuclear chromatin and apoptotic bodies (Fig. 7). In contrast, in the lungs of A/J mice infected with the dotA mutant, condensation of chromatin was rarely seen (Fig. 8), which was similar to uninfected lung tissue (Fig. 8). We conclude that *L. pneumophila* triggers Dot/Icm-dependent pulmonary apoptosis in permissive A/J mice by 24-48h post-infection. Taken together, the Dot/Icm transport system plays an essential role in intracellular replication and induction of pulmonary apoptosis and inflammation during experimental Legionnaires’ disease in genetically-susceptible but not resistant mice.
Discussion

*L. pneumophila*-infected human macrophages exhibit robust activation of caspase-3 during early and exponential intracellular replication but apoptosis is not triggered until late stages of infection, which is a novel modulation of caspase-3 activity (2, 25). The novel delay in apoptosis despite robust caspase-3 activation is associated with induction of several NF-κB-dependent and NF-κB-independent anti-apoptotic mechanisms during early and exponential replication and the infected cells are remarkably resistant to external potent apoptotic stimuli (1, 20). Our current studies show that similar to hMDMs, caspase-3 is also triggered early within *L. pneumophila*-infected A/J mouse macrophages but the infected cells do not undergo apoptosis till late stages of infection, and these processes are Dot/Icm-dependent (2, 25). However, while early activation of caspase-3 is essential for evasion of endocytic fusion and replication within human macrophages (26, 31), caspase-3 is dispensable for the infection of A/J mouse macrophages (see model in Fig. 9). Once the bacterial effector involved in caspase-3 activation in human macrophages is identified and characterized, we speculate that interference with the function of such an effector should target the organism to degradation within the phagolysosomes. We conclude that *L. pneumophila* triggers caspase-3 activation during early stages of infection of permissive A/J mouse-derived macrophages but apoptosis is delayed till late stages of infection, similar to human macrophages. The delayed apoptosis is likely due to the potent anti-apoptotic stimuli triggered by *L. pneumophila* in primary mouse and human macrophages (1, 20) (Fig. 9). It is likely that the induction of late stage apoptosis enables the intracellular bacteria to escape and disseminate in the lungs of the host, and thus, amplify the infection. However, it is also
possible the apoptotic infected macrophages are recognized by phagocytic cells that engulf them and degrade them along with the intracellular bacteria.

During late phases of the infection of mouse macrophages by *L. pneumophila*, the bacterial phagosome becomes acidified, and fuses to lysosomes during mid-late exponential replication, and the bacteria continue to proliferate in the acidic phagolysosomes (34, 37). In contrast, in human macrophages, the LCP does not fuse to the lysosomes throughout the intracellular infection (6, 7, 34), but the bacteria disrupt the phagosome and escape into the cytosol where they finish the last few rounds of proliferation (24) (see model in Fig. 9). Although FlaA of *L. pneumophila* is the trigger of caspase-1 in mice macrophages (26, 31), escape of flagellated bacteria into the cytoplasm of hMDMs during late stages of infection (12-18h) (26, 31) does not trigger the activation of caspase-1. We conclude that *L. pneumophila* does not trigger caspase-1 activation at any stage of infection of human macrophages.

Although caspase-3 is activated, caspase-1 is not triggered throughout the infection of hMDMs and A/J mouse macrophages. Pre-treatment of A/J mouse macrophages or hMDMs with the caspase-3 inhibitor blocks apoptosis, while the caspase-1 inhibitor has no effect on apoptosis, consistent with the lack of caspase-1 activation and the robust activation of caspase-3. Thus, apoptosis during late stages of the infection is independent of caspase-1. Taken together, it is more likely that apoptosis detected during late stages of infection of human and A/J mouse macrophages in vitro and in pulmonary cells of mice is mediated by caspase-3 (see model in Fig. 9). However, our data may not exclude additional apoptotic processes.
The *dot/icm* mutants are completely defective in induction of caspase-3 activation and apoptosis *in vitro* (49). A previous study has shown that the wild type strain of *L. pneumophila* triggers pulmonary apoptosis in A/J mice at 48h post-infection when the mice are exposed to hypoxia (40), but whether apoptosis is triggered at an earlier stage or whether it is *dot/icm*-dependent is not known. Our current data show that the *dot/icm* type IV secretion system is essential for the late stage induction of pulmonary apoptosis in the permissive A/J mice. This is consistent with the role of the *dot/icm* system in triggering apoptosis in human macrophages tissue culture systems (2, 12, 13, 15, 25, 27, 49), and with our current data in A/J mouse macrophages in vitro. Our data have also shown that there is no detectable pulmonary apoptosis in the resistant BALB/c mice infected with *L. pneumophila*. It is not known why the *dot/icm* system of the wild type strain does not trigger caspase-3 in BALB/c mice but it is likely that the Naip5 cytosolic protein is involved in counteracting the bacterial effectors involved in caspase-3 activation.

Interestingly, despite the robust activation of caspase-1 in BALB/c mouse-derived macrophages *in vitro*, there was minimal or no detection of pulmonary inflammation or apoptotic pulmonary cells *in vivo* at any stage of the infection. It is possible that other innate immunity mechanisms are involved *in vivo* to block caspase-1-mediated apoptosis and inflammation in pulmonary cells of BALB/c mice. We conclude that the *dot/icm* secretion system is essential for the induction of pulmonary apoptosis of susceptible A/J mice by 24-48h post-infection, but the *dot/icm* system is unable to trigger pulmonary apoptosis in the resistant BALB/c mice despite the robust activation of caspase-1 in BALB/c mice-derived macrophages *in vitro*. 
In summary, *L. pneumophila* does not trigger caspase-1 activation throughout the intracellular infection of hMDMs and permissive A/J mouse macrophages. Our data show that similar to hMDMs, *L. pneumophila* triggers differential and temporal early trigger of caspase-3 in permissive A/J mouse-derived macrophages but not in BALB/c mouse-derived macrophages. We show that *L. pneumophila* induces Dot/Icm-dependent pulmonary inflammation and apoptosis *in vivo* during experimental Legionnaires’ disease in the susceptible A/J mice but not in the resistant BALB/c mice.
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References


Figure legends

Fig. 1. Temporal activation of caspase-1 in hMDMs is triggered by *L. pneumophila* during late but not early stages of infection. Human macrophages were infected with *L. pneumophila* AA100 or the *dotA* mutant. The *F. tularensis* (Ft) was used as a positive control. Representative confocal microscopy images of hMDMs are shown. The cells were stained for active caspase-1 (C-1) and for apoptosis by TUNEL (T) assays. Quantification of the % of cells with active caspase-1 (C-1) in addition to the double positives (C1+T) is shown in B and is based on examination of 100 infected cells from three different coverslips. The data are representative of three experiments, and error bars represent standard deviations.

Fig. 2. Dot/Icm-mediated early activation of caspase-3 but not caspase-1 in A/J mice-derived mBMDM upon infection by *L. pneumophila*. A/J mice mBMDM were infected with *L. pneumophila* AA100 or with the *dotA* mutant or with *F. tularensis* (Ft) as a positive control. Representative confocal microscopy images at 8 and 18h after infection are shown in A and C. The cells were stained for active caspase-1 (C-1) or caspase-3 (C-3) and apoptosis by TUNEL (T) assays. Quantification of the % of cells with active caspase-1 (C-1) and caspase-3 (C-3) in addition to the double positives (C1+T or C3+T) are shown in B and D and is based on examination of 100 infected cells from three different coverslips. The data are representative of three experiments, and error bars represent standard deviations.
Fig. 3. Dot/Icm-mediated early activation of caspase-1 but not caspase-3 in BALB/c mice-derived mBMDM upon infection by *L. pneumophila*. BALB/c mice mBMDM were infected with *L. pneumophila* AA100 or with the dotA mutant or with *F. tularensis* (Ft) as a positive control. Representative confocal microscopy images are shown in (A) and (C). The cells were stained for active caspase-1 (C-1) or caspase-3 (C-3) and apoptosis by TUNEL (T) assays. Quantification of the % of cells with active caspase-1 (C-1) and caspase-3 (C-3) in addition to the double positives (C1+T, or C3+T) are shown in B and D and is based on examination of 100 infected cells from three different coverslips. The data are representative of three experiments, and error bars represent standard deviations.

Fig. 4. *L. pneumophila* induces pulmonary apoptosis in A/J mice during late stages of infection. Representative laser scanning confocal microscopy images of lung tissue of A/J mice infected with $10^6$ cfu/mouse of *L. pneumophila* AA100. At 2, 24, and 48 hours after infection lungs were processed to be sectioned and labeled (Material and Methods). Lung tissue of uninfected A/J mice, DNase-treated, and untreated lung tissues were used as controls. Apoptotic cells were labeled using TUNEL (black), and the bacteria are indicated by the green color (GFP). The experiments were done in triplicate using 5 mice for each time point, and the images are representative of 20 microscopic fields from each animal. The results are representative of three independent experiments.

Fig. 5. The Dot/Icm secretion system is essential for the induction of pulmonary apoptosis by *L. pneumophila*. Representative laser scanning confocal microscopy
images of lung tissue of A/J mice infected with $10^6$ cfu/mouse of the dotA mutant. At 2, 24, and 48 hours after infection lungs were processed to be sectioned and labeled (Material and Methods). Apoptotic cells were labeled using TUNEL (black), and the bacteria are indicated by the green color (GFP). The experiments were done in triplicate using 5 mice for each time point, and the images are representative of 20 microscopic fields from each animal. The results are representative of three independent experiments.

Fig. 6. Failure of \textit{L. pneumophila} to induce pulmonary apoptosis in non-permissive BALB/c mice. Representative laser scanning confocal microscopy images of lung tissue of BALB/c mice infected with $10^6$ cfu/mouse of \textit{L. pneumophila} AA100. At 2, 24, and 48 hours after infection, lungs were processed to be sectioned and labeled (Material and Methods). Lung tissue of uninfected BALB/c mice, DNase-treated, and untreated lung tissues were used as controls. Apoptotic cells were labeled using TUNEL (black), and the bacteria are indicated by the green color (GFP). The experiments were done in triplicate using 5 mice for each time point, and the images are representative of 20 microscopic fields from each animal. The results are representative of three independent experiments.

Fig. 7. Transmission electron micrographs of the lungs of A/J mice infected with \textit{L. pneumophilla} AA100. Representative TEM images of lung tissue of A/J mice infected with $10^6$ cfu/mouse of \textit{L. pneumophila} AA100. At 2h after infection morphological changes were very similar to uninfected lung tissues of the A/J mice. At 24 and 48h post-infection, condensation of chromatin was evident in pulmonary cells. The black and white arrows indicate bacteria and the apoptotic nuclei, respectively. The experiments
were done in triplicate using 3 mice for each time point, and the images are representative of 10 ultrathin sections from each animal.

**Fig. 8.** Transmission electron micrographs of the lungs of A/J mice infected with the *dotA* mutant of *L. pneumophilla*. Representative TEM images of lung tissue of A/J mice infected with 10⁶ cfu/mouse of the *dotA* mutant of *L. pneumophila* AA100. The black arrows indicate bacteria. The experiments were done in triplicate using 3 mice for each time point, and the images are representative of 10 ultrathin sections from each animal.

**Fig. 9.** A model contrasting the infection of human vs. A/J mice macrophages by *L. pneumophila* (see text for details).
Fig. 1

hMDMs caspase-1

% infected cells

AA100

dotA

Ft

C-1 TUNEL

Merge

Bacteria

2h 8h 18h SIM

100

80

60

40

20

0

C1

T

C1+T

C1

T

C1+T

10

18h

SIM

AA100

dotA

Ft
Fig. 2

A/J mice caspase-1

A/J mice caspase-3

% infected cells

% infected cells

Fig. 2
Fig. 3

A. Bacteria C-1 TUNEL Merge

B. BALB/c mice caspase-1

C. BALB/c mice caspase-3

D. BALB/c mice caspase-3

% infected cells

2h 8h 18h

BALB/c mice caspase-1

BALB/c mice caspase-3

2h 8h 18h

% infected cells
Fig. 4

Controls

24h post-infection

2h post-infection

48h post-infection
Fig. 5
Human macrophages

- Caspase-3 → Caspase-1
- Evasion of lysosomes
- Evade lysosomes
- 10-12h
- Anti-apoptosis
- Escape into the cytosol
- 12-18h
- Apoptosis

A/J mice macrophages

- Caspase-3 → Caspase-1
- LAMPs
- ATPase
- Lysosomes
- Fuse to lysosomes
- pH 5.6
- Anti-apoptosis
- Caspase-3
- Caspase-1

Fig. 9