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Šantić, Marina; Asare, Rexford; Dorić, Miljenko; Abu Kwaik, Yousef

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Host-Dependent Trigger of Caspases and Apoptosis by *Legionella pneumophila*▽†

Marina Santic,[1,2] Rexford Asare,[2] Miljenko Doric,[1] and Yousef Abu Kwaik[2]*

Department of Microbiology and Parasitology, University of Rijeka, Rijeka, Croatia,[1] and Department of Microbiology and Immunology, Room 406, University of Louisville College of Medicine, 319 Abraham Flexner Way 55A, Louisville, Kentucky 40202[2]

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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 nonpermissive 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 monocyte-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 to 8 h, but apoptosis is delayed until late stages of infection. While *L. pneumophila* triggers a Dot/Icm-dependent activation of caspase-1 in nonpermissive BALB/c mouse-derived macrophages, caspase-3 is not activated at any stage of 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 nonpermissive 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.

The ability of *Legionella pneumophila* to cause pneumonia is dependent on its capacity to invade and replicate within alveolar macrophages, monocytes, and potentially alveolar epithelial cells (1, 38, 43). Upon entry into the host cell, *L. pneumophila* modulates the 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 *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 rough endoplasmic reticulum (RER) derived within minutes of its biogenesis from the plasma membrane (16, 18, 39, 41). Upon activation of human macrophages by gamma interferon, the LCP fuses to the lysosomes and fails to be remodeled by the RER (33). In contrast to what was found for *L. pneumophila*, recent studies have shown that *Legionella longbeachae* is trafficked to, and replicates within, a nonacidified, late-endosome-like phagosome that is remodeled by the RER (5). The Dot/Icm type IV secretion system of *L. pneumophila* is essential for evasion of endocytic fusion and for remodeling of the LCP into an 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 *Acanthamoeba polyphaga*, the bacteria escape into the host cell cytosol, where they reside for 2 to 6 h prior to lysis of the host cell plasma membrane (24).

In addition to evasion of vesicle traffic by *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 (3, 25), which is a novel modulation of caspase-3 activity that halts it from the 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 antiapoptotic signals that are mediated by NF-κB and non-NF-κB signaling mechanisms (2, 20).

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Room 406, University of Louisville College of Medicine, 319 Abraham Flexner Way 55A, Louisville, KY 40202. Phone: (502) 852-4117. Fax: (502) 852-7531. E-mail: abukwaik@louisville.edu.

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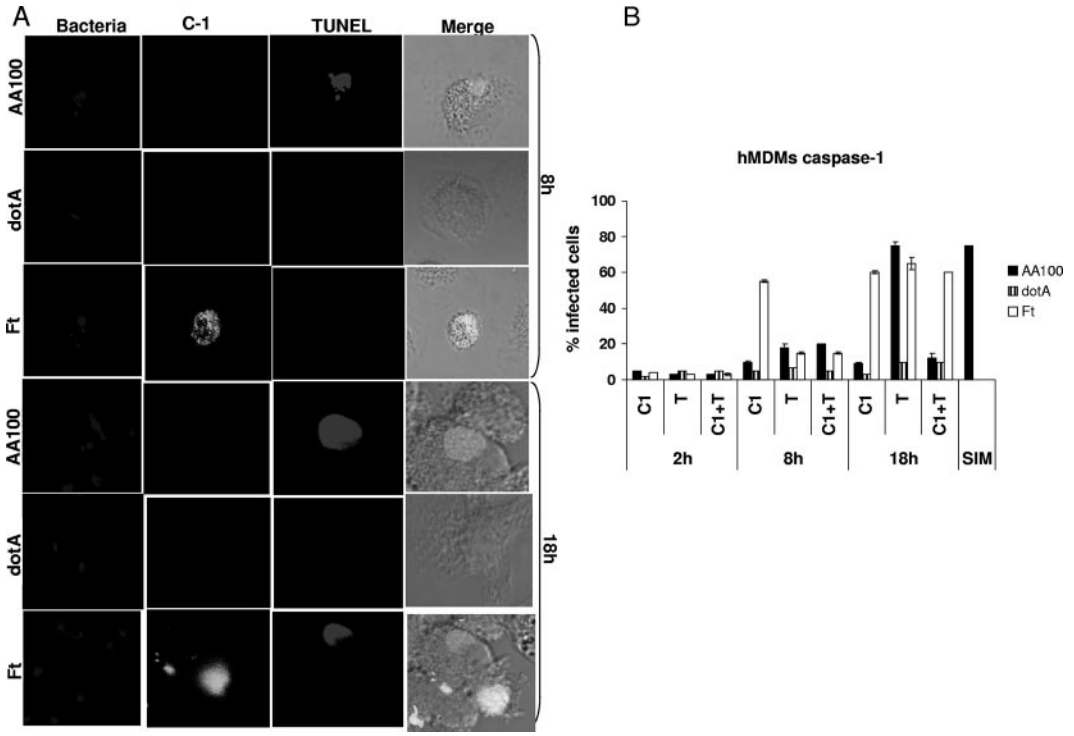

FIG. 1. Temporal activation of caspase-1 in hMDMs is triggered by *L. pneumophila* during late but not early stages of infection. Human macrophages were infected with *L. pneumophila* AA100 or the *dotA* mutant. *F. tularensis* (Ft) was used as a positive control. Representative confocal microscopy images of hMDMs are shown. The cells were stained for active caspase-1 (C-1) and for apoptosis by TUNEL (T) assays. Quantification of the percentages of cells with active caspase-1 (C1) in addition to the double positives (C1+T) is shown in panel 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.

Among inbred mouse 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 antiapoptotic activity, due to inhibition of caspase-3 and caspase-7 (10, 21). However, caspase-3 is not required for the infection of mouse macrophages by *L. pneumophila* (26, 31), which is distinct from that of human macrophages (25). In mouse macrophages that are nonpermissive for intracellular proliferation of *L. pneumophila*, the bacterial flagellin (FlaA) triggers caspase-1-mediated proinflammatory rapid cell death/pyropoptosis (26, 31). The mechanism and the role of Naip5 in activation of caspase-1 by *L. pneumophila* are 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 nonpermissive mouse 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 human monocyte-derived macrophages (hMDMs) and A/J mouse macrophages, *L. pneumophila* does not trigger caspase-1 activation throughout the intracellular infection, despite the escape of highly flagellated *L. pneumophila* bacteria 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 that in hMDMs, but caspase-3 is not triggered in the resistant BALB/c mouse-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. The mice were housed in specific-pathogen-free conditions within the animal care facility. The virulent clinical isolate of *L. pneumophila* strain AA100 and its isogenic *dotA* mutant (GL10) have been described previously (49). The wild-type *Francisella 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 72 h. The plates for *gfp*-transformed AA100 or its isogenic *dotA* mutant were supplemented with 5 μg/ml chloramphenicol. After cultivation, the bacteria were washed by centrifugation and resuspended in sterile saline.

To prepare mouse bone marrow-derived macrophages (mBMDM), bone marrow samples were 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 volunteers were 25 to 45 years old, with no history of pneumonia or any underlying chronic disease.

Inoculation of animals. Mice were inoculated intratracheally, as we described previously (4, 23, 29). Briefly, the mice were anesthetized by intraperitoneal injection of ketamine (2.5 mg/mouse). A total of 50 μl of the *L. pneumophila*

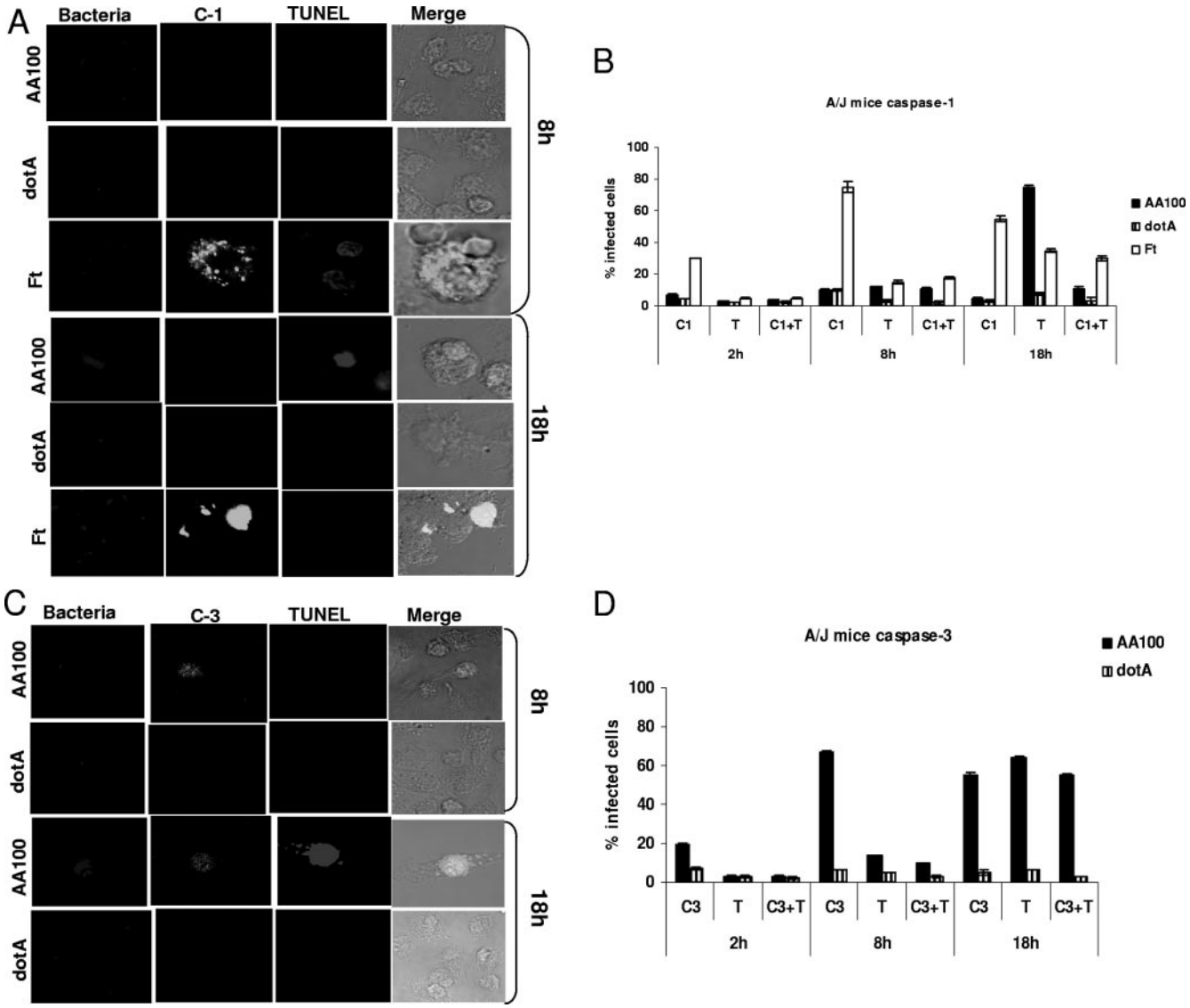

FIG. 2. Dot/Icm-mediated early activation of caspase-3 but not caspase-1 in A/J mouse-derived mBMDM upon infection by *L. pneumophila*. A/J mouse mBMDM were infected with *L. pneumophila* AA100 or the *dotA* mutant or with *F. tularensis* (Ft) as a positive control. Representative confocal microscopy images at 8 and 18 h after infection are shown in panels A and C. The cells were stained for active caspase-1 (C-1) or C-3 and apoptosis by TUNEL (T) assays. Quantification of the percentages of cells with active caspase-1 (C1) and C3 in addition to the double positives (C1+T or C3+T) is shown in panels 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.

suspension (10^6 CFU) in sterile water was inoculated directly into the trachea by 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* bacteria 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 numbers of CFU of *L. pneumophila* AA100 or the *dotA* mutant strain in the lungs were determined by a plate dilution method using buffered charcoal yeast extract agar. After 3 days of incubation at 37°C, the colonies were enumerated and the results were expressed as numbers of CFU per lung.

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 48 h after inoculation, the mice were humanely sacrificed using CO_2 asphyxiation. Before lung removal, the pulmonary vasculature was perfused with 10 ml of saline containing 5 mM 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 for analyses of the 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) with an in situ cell death detection kit as recommended by the manufacturer (Roche, Indianapolis, IN). The histopathology of the lung tissue stained with eosin and hematoxylin was analyzed by light microscopy. An analysis of the histology of the lung tissue in the presence of intracellular green fluorescent protein (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.).

TEM. For examination of apoptosis in the lungs of A/J mice by transmission electron microscopy (TEM), mouse lungs were removed and placed in 2.5% glutaraldehyde as described previously (24). Briefly, the lungs were postfixed by immersion in 2% osmium tetroxide in 0.1 M sodium Sorenson's buffer for 1 h,

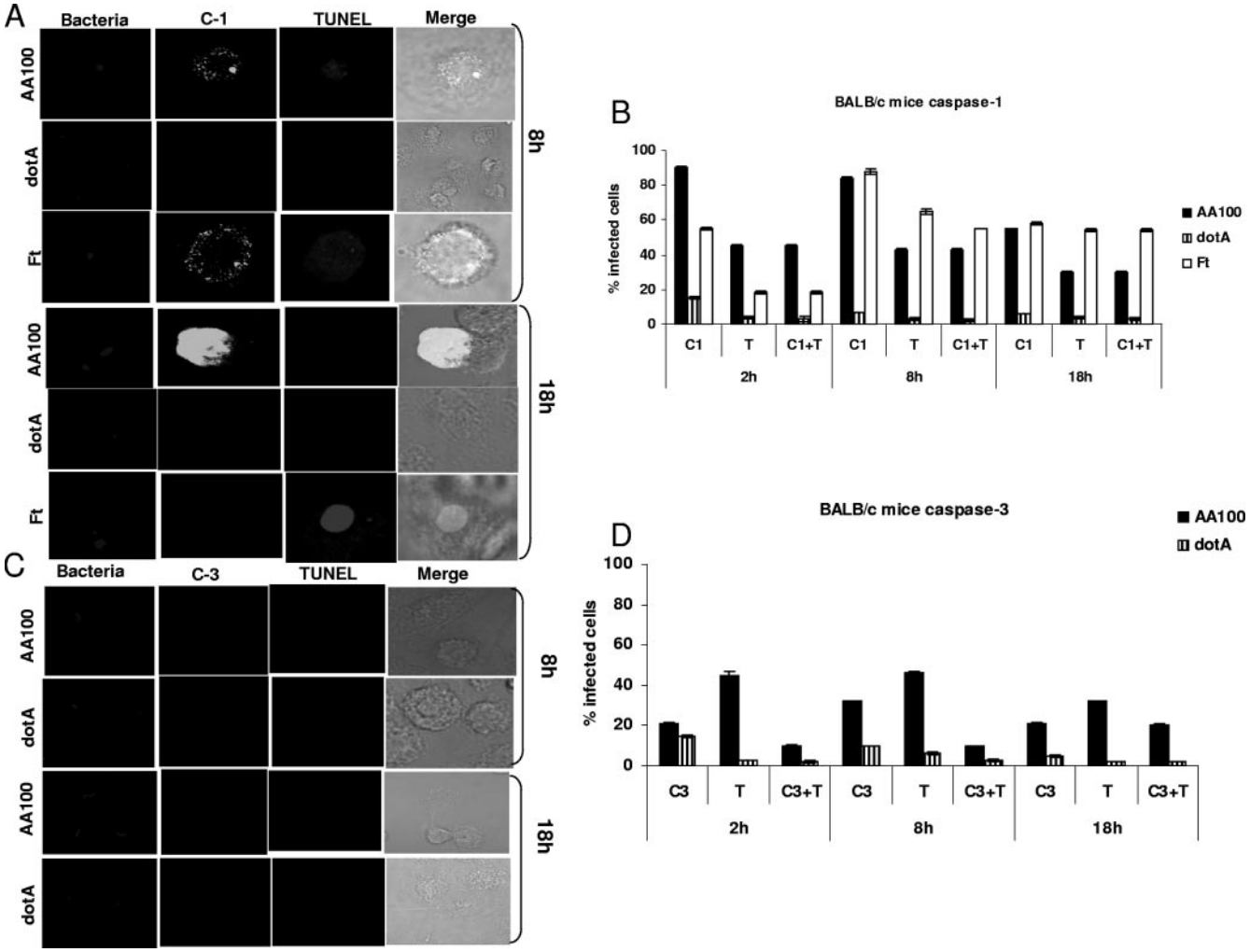

FIG. 3. Dot/Icm-mediated early activation of caspase-1 but not caspase-3 in BALB/c mouse-derived mBMDM upon infection by *L. pneumophila*. BALB/c mouse mBMDM were infected with *L. pneumophila* AA100 or the *dotA* mutant or with *F. tularensis* (Ft) as a positive control. Representative confocal microscopy images are shown in panels A and C. The cells were stained for active caspase-1 (C-1) or C-3 and apoptosis by TUNEL (T) assays. Quantification of the percentages of cells with active caspase-1 (C1) and C3 in addition to the double positives (C1+T or C3+T) is shown in panels 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.

followed by dehydration in acetone and infiltration and embedment in Epon 12 epoxy resin (24). Ultrathin sections (0.1 μm) were then cut, stained with uranyl acetate and lead citrate, and examined with a Philips TEM (Morgagni 268D; Philips, The Netherlands) at 80 kV.

Caspase activation and TUNEL assays. To assess activation of caspase-1 and caspase-3 by confocal microscopy, 2.5×10^5 mBMDM or hMDMs on glass coverslips were infected with *L. pneumophila* AA100, the *dotA* mutant, or *F. novicida* at a multiplicity of infection of 10 for 1 h, followed by incubation for 2, 8, and 18 h. For caspase-1 activation, macrophages were stained for 1 h 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, OR).

Apoptotic nuclei were labeled with TUNEL according to the manufacturer's instructions (Boehringer Mannheim Corporation, Indianapolis, IN). 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 were performed at least three times, and the data shown are representative of one experiment. Statistical analyses were performed using the two-tailed 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 mouse-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 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 ac-

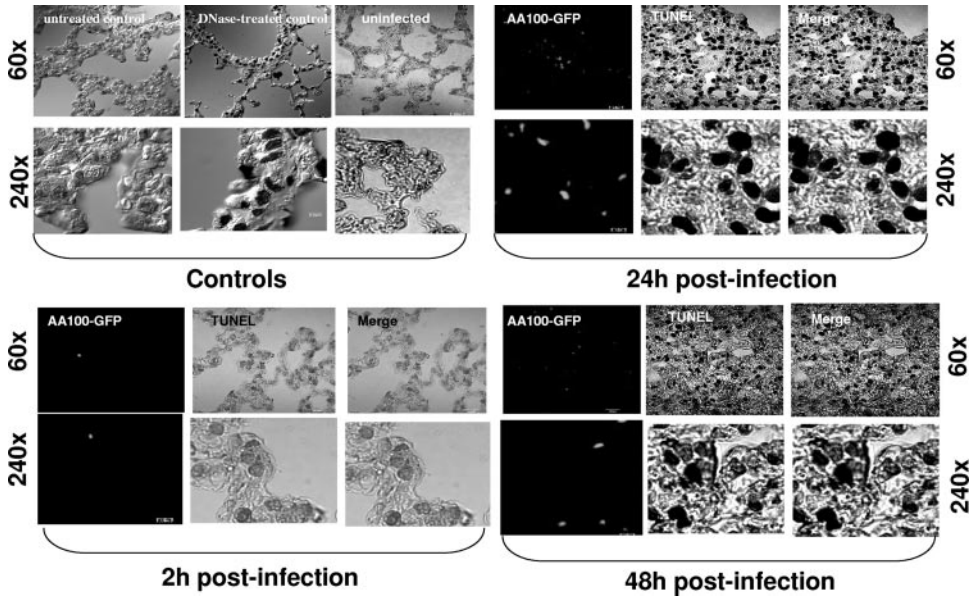

FIG. 4. *L. pneumophila* induces pulmonary apoptosis in A/J mice during late stages of infection. Representative laser scanning confocal microscopy images of lung tissues of A/J mice infected with 10^6 CFU/mouse of *L. pneumophila* AA100 are shown. At 2, 24, and 48 h after infection, lungs were processed to be sectioned and labeled (see Materials and Methods). Lung tissues of uninfected A/J mice and DNase-treated and -untreated lung tissues were used as controls. Apoptotic cells were labeled using TUNEL (black), and the bacteria are visualized by use of GFP. The experiments were done in triplicate, using five 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.

tivation, cells were pretreated with Simvastatin or infected by *Francisella tularensis*. By 2 h after infection of hMDMs with all the strains, caspase-1 activation was minimal, which was not significantly different from that for uninfected cells (Student's *t* test, $P > 0.2$) (Fig. 1). By 8 h after infection of hMDMs with *L. pneumophila* AA100, only ~10% of the infected cells were positive for caspase-1 and also apoptotic (Student's *t* test, $P > 0.1$) (Fig. 1). While ~80% of *L. pneumophila*-infected hMDMs were apoptotic by 18 h postinfection, only ~10% of them exhibited caspase-1 activation (Fig. 1). Similar results were obtained for hMDMs (data not shown). Therefore, the large number of infected hMDMs that became apoptotic at 18 h were 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 laboratories (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 were not significantly different from those for uninfected cells at all time points after infection (Student's *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 by *L. pneumophila* in permissive mouse 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 mouse-derived macrophages. We utilized single-cell analyses to examine the kinetics of potential activation of caspase-3 and apoptosis in primary mBMDM obtained form A/J permissive and BALB/c nonpermissive 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 mBMDM 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 2 h after infection, which was significantly different from what was found for cells infected by the *dotA* mutant or uninfected cells (Student's *t* test, $P < 0.01$), but only a few cells were positive for TUNEL (Fig. 2). At 8 h postinfection, ~70% of *L. pneumophila*-infected A/J mBMDM exhibited caspase-3 activation, which was significantly different from what was found for cells infected by the *dotA* mutant or uninfected cells (Student's *t* test, $P < 0.003$), but only a few infected cells underwent apoptosis (Fig. 2). By 18 h after infection with *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's *t* test, $P < 0.001$) (Fig. 2). Apoptosis was inhibited when the infected cells were pretreated with the caspase-3 inhibitor but not when the infected cells were pretreated with the caspase-1 inhibitor (data not shown). The *dotA* mutant control neither activated caspase-3 nor triggered apoptosis at any time point after

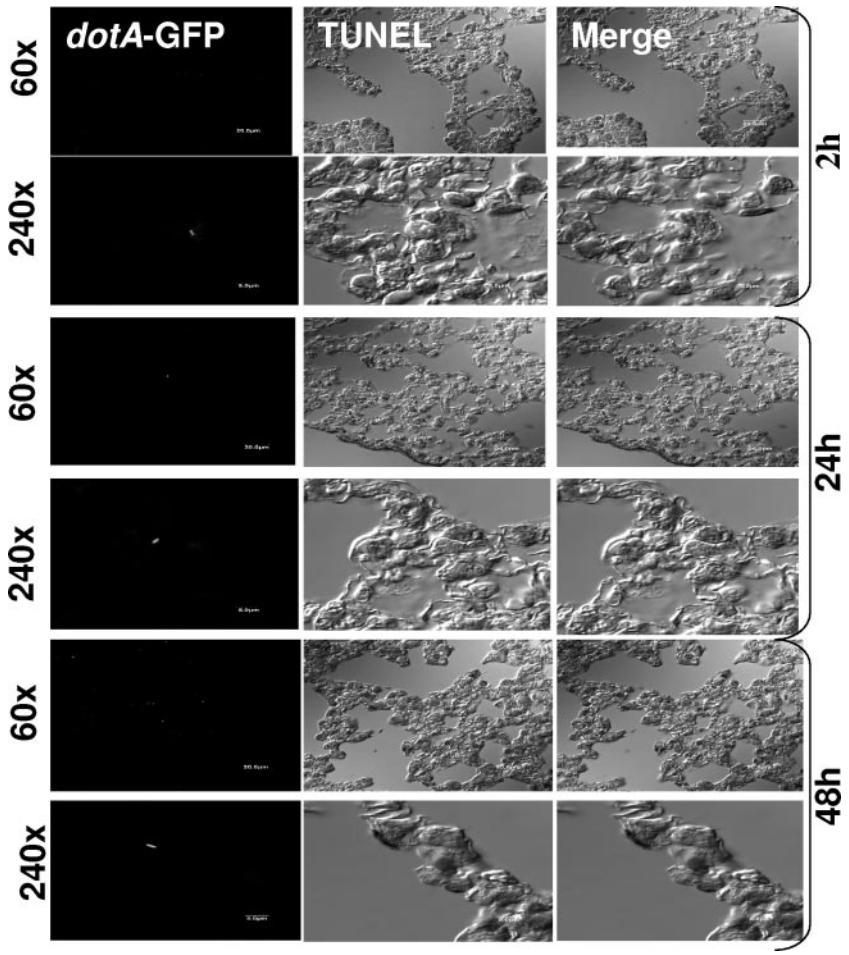

FIG. 5. The Dot/Icm secretion system is essential for the induction of pulmonary apoptosis by *L. pneumophila*. Representative laser scanning confocal microscopy images of lung tissues of A/J mice infected with 10^6 CFU/mouse of the *dotA* mutant are shown. At 2, 24, and 48 h after infection, lungs were processed to be sectioned and labeled (see Materials and Methods). Apoptotic cells were labeled using TUNEL (black), and the bacteria are visualized by use of GFP. The experiments were done in triplicate, using five 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.

infection of A/J mBMDM, which was not significantly different from what was found for uninfected cells (Student's *t* test, $P < 0.3$) (Fig. 2).

At 2 to 8 h after infection of A/J mBMDM with *L. pneumophila* AA100, caspase-1 activation and apoptosis were not significantly different from what was found for cells infected by the *dotA* mutant or uninfected cells (Student's *t* test, $P > 0.2$) (Fig. 2). By 18 h postinfection, ~80% of the *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 a few cells underwent apoptosis at all time points after infection (Fig. 2), which was not significantly different from what was found for uninfected cells (Student's *t* test, $P > 0.2$). Infection of A/J mBMDM by the *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 mouse-derived macrophages.** Although caspase-1 has been shown to be triggered by *L. pneumophila* within nonpermissive mouse-derived macrophages, it is not known whether activation of caspase-3 is also triggered by *L. pneumophila* at any stage of 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 to 8 h after infection of BALB/c mBMDM, when most infected cells were positive for caspase-1 activity and were also apoptotic (Student's *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, a result not significantly different from what was found for uninfected cells (Student's *t* test, $P > 0.1$) (Fig. 3). The cells infected by *F. tularensis* exhibited time-dependent activation of caspase-1 (Fig. 3).

In contrast, caspase-3 activation was minimal at 2 h after infection of BALB/c mBMDM by *L. pneumophila* AA100, which was not significantly different from what was found for *dotA*-infected and uninfected cells (Student's *t* test, $P > 0.3$). At 8 h after infection with the wild-type strain of *L. pneumophila* ~30% of infected cells exhibited activation of caspase-3

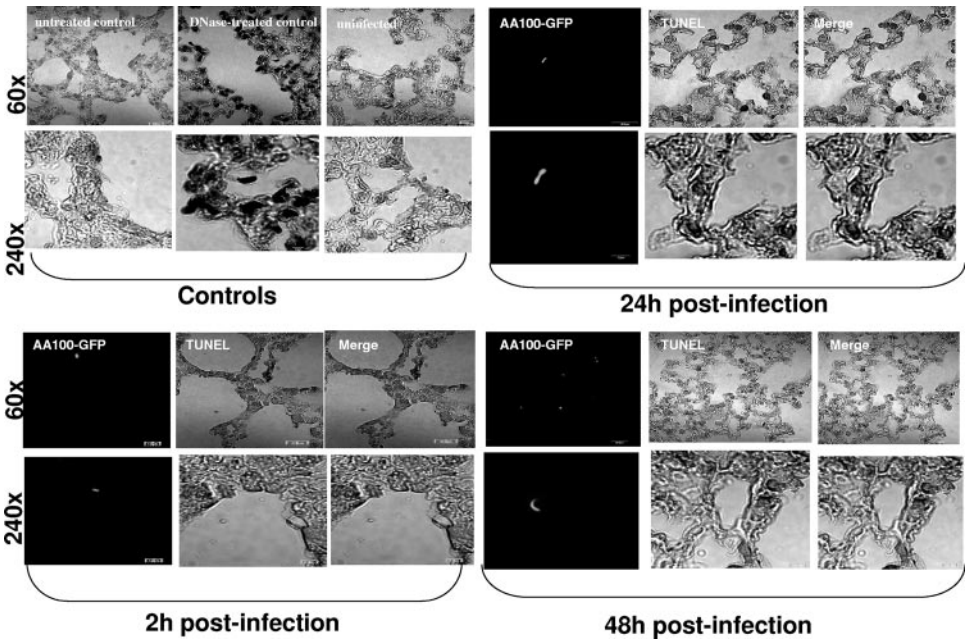

FIG. 6. Failure of *L. pneumophila* to induce pulmonary apoptosis in nonpermissive BALB/c mice. Representative laser scanning confocal microscopy images of lung tissues of BALB/c mice infected with 10^6 CFU/mouse of *L. pneumophila* AA100 are shown. At 2, 24, and 48 h after infection, lungs were processed to be sectioned and labeled (see Materials and Methods). Lung tissues of uninfected BALB/c mice and DNase-treated and -untreated lung tissues were used as controls. Apoptotic cells were labeled using TUNEL (black), and the bacteria are visualized by use of GFP. The experiments were done in triplicate, using five 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.

(Student's t test, $P < 0.05$) and ~10% of these were also positive for TUNEL. Similar results were obtained at 18 h after infection by *L. pneumophila* AA100 (Fig. 3). The *dotA* mutant triggered very low levels of activation of caspase-3 at all time points after infection, which was similar to what was found for uninfected macrophages (Student's t test, $P > 0.2$). Taken together, our results show that *L. pneumophila* does not trigger caspase-3 in nonpermissive BALB/c mouse-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 48 h 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 (see Fig. S1 in the supplemental material). Inflammatory infiltration was first evident in lung tissue from *L. pneumophila* AA100-infected A/J mice at 24 h after infection and became more severe by 48 h postinfection (see Fig. S2 in the supplemental material). 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 (see Fig. S3 in the supplemental material).

We examined by in situ cell analyses the kinetics of apoptosis in the lung tissues 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 the intensity of the staining.

At 2 h after infection of A/J mice with *L. pneumophila* AA100, no apoptotic cells were detected in the lung tissues of infected mice (Fig. 4). However, at 24 to 48 h 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, or 48 h) 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 2 h after infection, most of the cells revealed normal morphologies (Fig. 7). At 24 and 48 h, typical morphological features of programmed cell death, including condensed nuclear chromatin and apoptotic bodies, could be detected (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 what was found for uninfected lung tissue (Fig. 8). We conclude that *L. pneumophila* triggers Dot/Icm-dependent pulmonary apoptosis in permissive A/J mice by 24 to 48 h postinfection. Taken together, these results show that 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.

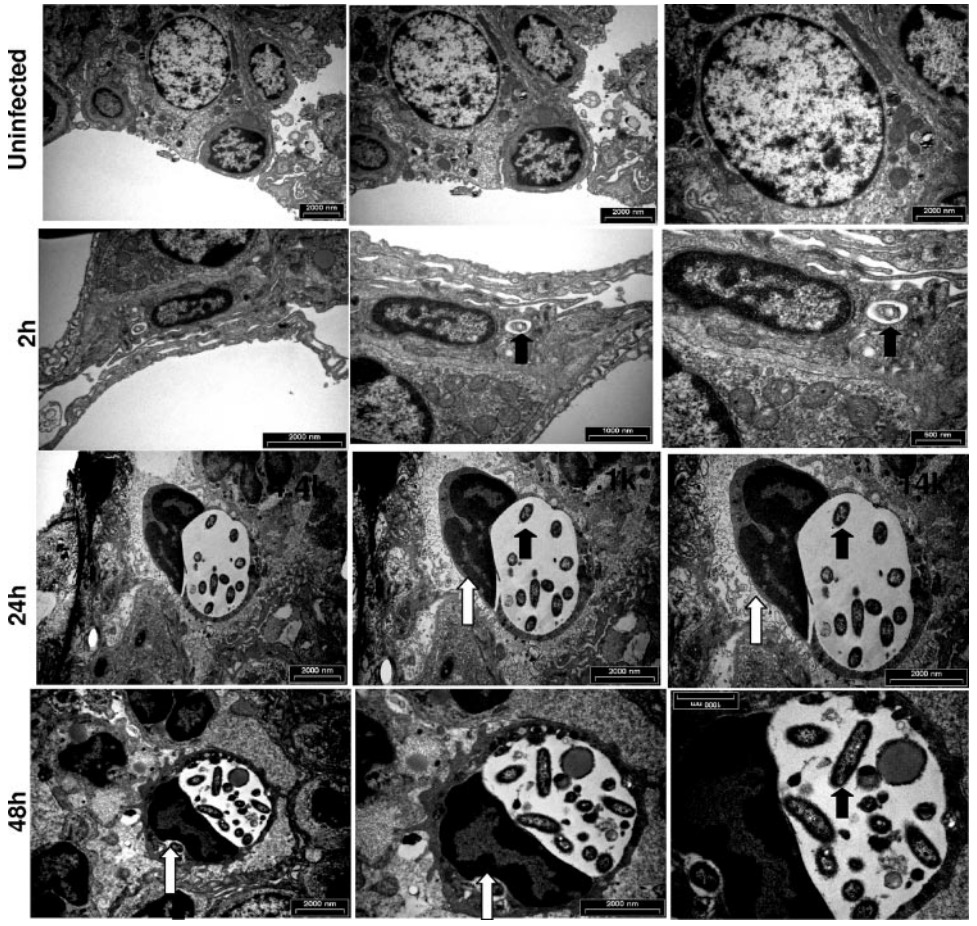

FIG. 7. Transmission electron micrographs of the lungs of A/J mice infected with *L. pneumophila* AA100. Representative TEM images of lung tissues of A/J mice infected with 10^6 CFU/mouse of *L. pneumophila* AA100 are shown. At 2 h after infection, the morphological changes were very similar to those for the uninfected lung tissues of the A/J mice. At 24 and 48 h postinfection, 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 three mice for each time point, and the images are representative of 10 ultrathin sections from each animal.

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 (3, 25). The novel delay in apoptosis despite robust caspase-3 activation is associated with induction of several NF-κB-dependent and NF-κB-independent antiapoptotic mechanisms during early and exponential replication, and the infected cells are remarkably resistant to external potent apoptotic stimuli (2, 20). Our current studies show that, similarly to hMDMs, caspase-3 is also triggered early within *L. pneumophila*-infected A/J mouse macrophages but the infected cells do not undergo apoptosis until late stages of infection, and these processes are Dot/Icm dependent (3, 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 (model in Fig. 9). 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 until late stages of infection, similar to that for human macrophages. The delayed apoptosis is likely due to the potent antiapoptotic stimuli triggered by *L. pneumophila* in primary mouse and human macrophages (2, 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 that 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) (model in Fig. 9). Although FlaA of *L. pneumophila* is the trigger of caspase-1 in mouse macrophages (26, 31), escape of flagellated bacteria into the cytoplasm of

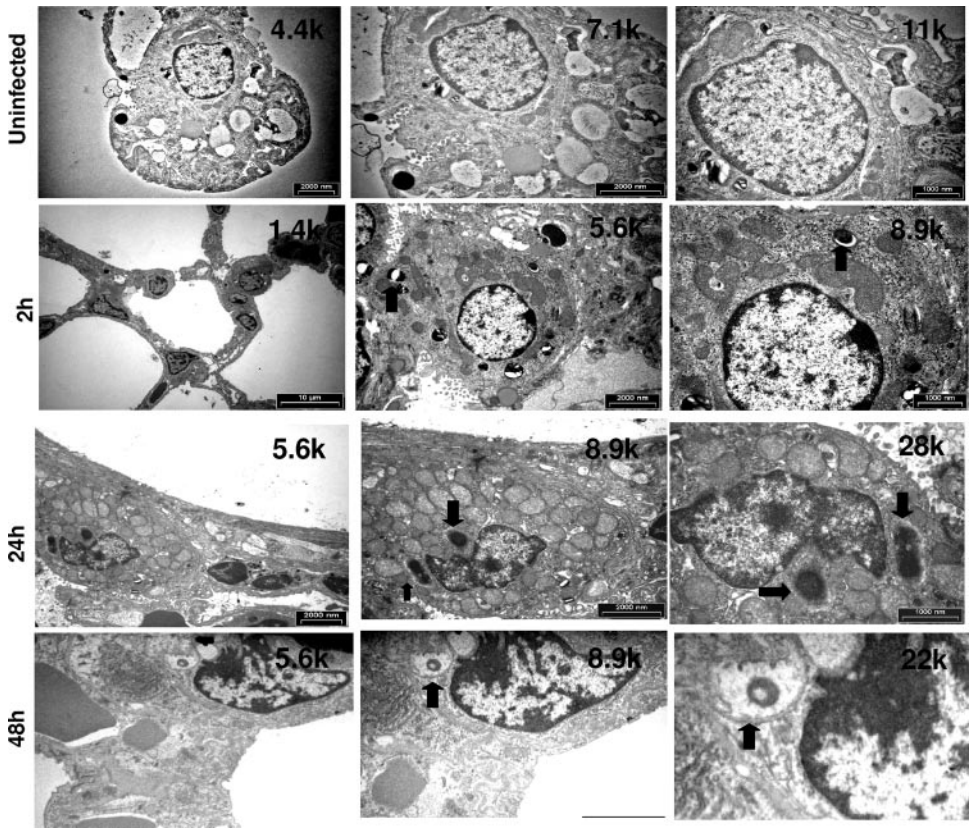

FIG. 8. Transmission electron micrographs of the lungs of A/J mice infected with the *dotA* mutant of *L. pneumophila*. Representative TEM images of lung tissues of A/J mice infected with 10^6 CFU/mouse of the *dotA* mutant of *L. pneumophila* AA100 are shown. The black arrows indicate bacteria. The experiments were done in triplicate, using three mice for each time point, and the images are representative of 10 ultrathin sections from each animal.

hMDMs during late stages of infection (12 to 18 h) (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. Pretreatment 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 infection is independent of caspase-1. Taken together, these results indicate that it is more likely that apoptosis detected during late stages of infection of human and A/J mouse macrophages in vitro and in the pulmonary cells of mice is mediated by caspase-3 (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 48 h postinfection 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 section 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 macrophage tissue culture systems (3, 12, 13, 15, 25, 27, 49) and with our current data for A/J mouse macrophages in vitro. Our data have also shown that there is no detectable pulmonary apoptosis in 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 infection. It is possible that other innate immunity mechanisms are involved in vivo to block caspase-1-mediated apoptosis and inflammation in the 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 to 48 h postinfection, 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 mouse-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, sim-

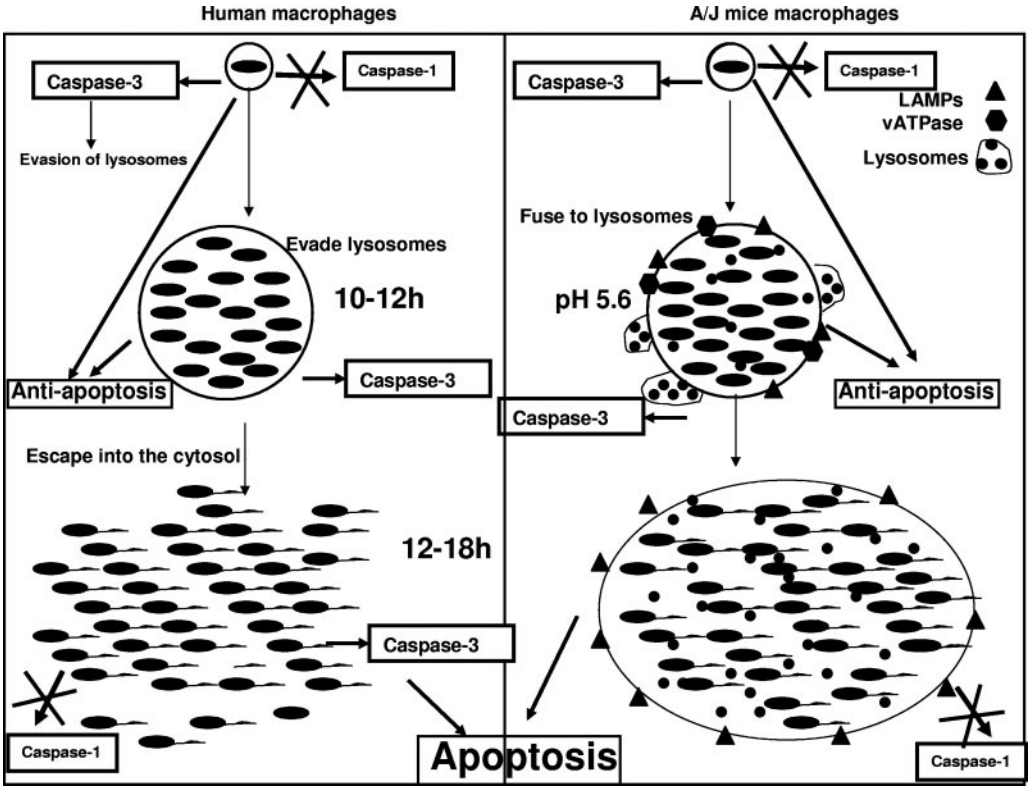

FIG. 9. Model contrasting the infection of human versus A/J mouse macrophages by *L. pneumophila* (see text for details).

ilar to what occurs with hMDMs, *L. pneumophila* triggers differential and temporal early activation 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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