Cutting Edge: A Novel Nonoxidative Phagosomal Mechanism Exerted by Cathepsin-D Controls *Listeria monocytogenes* Intracellular Growth


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Deciphering how \textit{Listeria monocytogenes} exploits the host cell machinery to invade mammalian cells is a key issue in understanding the pathogenesis of this food-borne pathogen, which can cause diseases ranging from gastroenteritis to meningitis and abortion. In this study, we show that the lysosomal aspartyl-protease cathepsin-D (Ctsd) is of considerable importance for nonoxidative listericidal defense mechanisms. We observed enhanced susceptibility to \textit{L. monocytogenes} infection of fibroblasts and bone-marrow macrophages and increased intraphagosomal viability of bacteria in fibroblasts isolated from Ctsd-deficient mice compared with wild type. These findings are further supported by prolonged survival of \textit{L. monocytogenes} in Ctsd-deficient mice after infection. Transient transfection of Ctsd in wild-type cells was sufficient to revert these wild-type phagosomes back to microbicidal compartments. Based on infection experiments with mutant bacteria, in vitro degradation, and immunoprecipitation experiments, we suggest that a major target of cathepsin D is the main virulence factor listeriolysin O. The Journal of Immunology, 2006, 176: 1321–1325.

\textit{Listeria monocytogenes} (LM) is able to infect different cell lineages. The number of viable intraphagosomal LM determines the success of an intracellular LM infection (1). Two major listericidal mechanisms are functional within phagosomes: 1) oxidative and 2) nonoxidative processes (2–6). Several indirect observations suggested the participation of the aspartyl-protease cathepsin-D (Ctsd) as a nonoxidative lysosomal bactericidal mechanism. First, acid sphingomyelinase knockout mice were highly susceptible to LM infection (7). Because ceramide is the product of ASMase and activates Ctsd (8, 9), the lack of both ceramide and Ctsd activation in ASMase−/− mice might correlate with LM survival. Second, LM permissive phagosomes lacked lysosomal proteases (10), whereas listericidal phagosomes contained lysosomal proteases (6).

To analyze the role of Ctsd in LM clearance, we used gain and loss of function experiments in fibroblasts and macrophages (MAC) followed by studies on the intracellular growth of LM, phagosome isolation, and evaluation of the viability of intraphagosomal LM. Fibroblasts and MAC from Ctsd-deficient mice were highly susceptible to LM infection and exhibited an enhanced intraphagosomal viability of this bacterium. Ctsd−/− mice contained 10-fold more bacteria compared with Ctsd+/+ mice.

\textbf{Materials and Methods}

\textbf{Chemicals, media, and Abs}

Biotinylation reagents (Pierce), transfection antibiotics (Clontech), and Abs: rabbit anti-Ctsd (11), polyclonal rabbit anti-LLO specific (Dathave) and HRP-conjugated Abs (Jackson ImmunoResearch Laboratories). Tissue culture media were DMEM (D) supplemented with 10 mM L-glutamine, 100 mM nonessential amino acids, 50 μg/ml gentamicin, 50 μg/ml vancomycin, and 5% (D5), 10% (D10), or 20% (D20) of FCS.

\textbf{Bacteria and infection in mice}

LM strains used were as follows: 10403S wild-type LM, \(\Delta LLO\) (LLO-deficient mutant, DP-L2161); \(\Delta plcA/plcB\) (plcA and plcB-deficient mutant, DP-L1936) and \(\Delta LLO\Delta plcA/plcB\) (LLO, plcA and plcB-deficient mutant, DP-L2319); \textit{Escherichia coli}-producing recombinant N-terminal-tagged His-LLO bacteria (12); and \textit{E. coli}-producing recombinant N-terminally His-tagged EEA1 (13). LM were FITC-labeled (0.5 mg/ml FITC-PBS, 1 h, 4° C), and CFU were enumerated. Mice and cells used correspond to the Ctsd+/+ or Ctsd−/− allelic phenotype and were called for simplicity, Ctsd+/+ or Ctsd−/− for Ctsd-deficient or wild-type mice, respectively.

\textbf{Acknowledgments}

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Cells, constructs, and transfections

Mouse embryonic fibroblasts (11, 14) were cultured in D10. Bone marrow
MAC (BM-MAC) were cultured in D20 with 25 ng/ml macrophage-CSF for 7
days. Vectors were as follows: pcDNA3.1/Ctsd-hygro, Ctsd/pEFP, and
pEFP were transfected and selected as described previously (15).

Bacterial infection assays

The infection experiments were performed as previously reported (2). Intrapha-
gosomal viability was evaluated in isolated phagosomes as CFU and expressed as
described elsewhere (6, 15).

Immunoblots, in vivo and in vitro degradation assays

Immunoblots of isolated phagosomes (30 μg/lane) (10) were incubated with
rabbit anti-Ctsd (1/1,000) or monoclonal anti-Rab5a (1/5,000) Abs. Biotiny-
lated was performed with EZ-link-LC-biotin (Pierce) (1 mg/ml, 1 h, 37°C,
and 4-h incubation in D10). Immunoprecipitations were performed with a rab-
bit anti-LLO Ab and developed with streptavidin-HRP (1/20,000). For Rab5a-
GTP assays, phagosomes were incubated with 3 μg of His-EEA1 to recover
exclusively Rab5a-GTP (13), and immunoblots were developed with anti-
Rab5a Ab. For in vitro degradation assays of His-LLO, 5 μg of purified Ctsd
was incubated with 5 μg of His-LLO in the presence or absence of 1 mM pep-
statin A.

Isolation of phagosomal membrane fractions

Fibroblasts and CHO cells were infected with LM (ratio of 20:1 bacteria:cells,
20 min) in the presence or absence of pepstatin A (5 μM) and processed as
described elsewhere (6, 15). Fibroblast phagosomes contained ≤2% of
plasma membrane, 0.2% of Golgi, and 0.25% of endosomal contamination.
For calculations of percentages of phagosomal and cytosolic bacteria, LM in-
fec tion was conducted at 0 h. Postnuclear supernatants (PNS) were obtained,
and CFU values were expressed as the total of internalized LM. Phagosomes were
isolated, and CFU values were expressed as percentages of the total LM. The per-
centage of cytosolic bacteria was calculated as (PNS — phagosomal numbers/ PNS) × 100.

Fluorescence microscopy

Conventional immunofluorescence microscopy was performed with cells fixed
in cold methanol. Confocal microscopy was used for visualization of BM-MAC
infected with FITC-LM.

Statistical analysis

For statistical analysis, the Student’s t test was applied.

Results

Ctsd controls the intracellular growth of LM in fibroblasts

We first analyzed the intracellular survival of LM in Ctsd+/− fibroblasts and observed exponential growth especially between 8 and 18 h postinfection (Fig. 1A). Ctsd played a crucial role in LM infection because the intracellular growth indexes of LM in Ctsd−/− fibroblasts were 4- to 6-fold higher than in wild-type Ctsd+/+ cells at 8 h postinfection (Fig. 1B). Moreover, transfection of Ctsd−/− cells with a Ctsd expression vector (Ctsd) reduced LM intracellular growth to values observed in control fibroblasts. Transfection of wild-type Ctsd+/+ fibroblasts with Ctsd decreased LM intracellular growth indexes by 4-fold (CFU ratio 9.8 ± 0.3 compared with 2.2 ± 0.5 in transfected cells). The specificity of the role of Ctsd was confirmed using fibroblasts deficient in cathepsin-L (Ctsd−/−) (Fig. 1B).

The microbicidal effect of Ctsd is localized to phagosomes and linked to

Rab5a

Phagosomes are critical compartments in the control of LM growth (1, 15). The number of viable LM (CFU) was 4-fold higher within Ctsd−/− phagosomes compared with control phagosomes (Fig.

FIGURE 1. Ctsd controls the intracellular growth of LM within phagosomes, and its listericidal effect is targeted to LLO and linked to Rab5a. A, Ctsd+/+ fibroblasts were infected with wild-type LM (0, 4, 8, or 18 h). Results are expressed as CFU ± SD of triplicates. B, Fibroblasts (Ctsd+/+, Ctsd−/+ or Ctsd−/− and Ctsd added, Ctsd+/+ or Ctsd−/−) were infected 8 h with wild-type LM (■), ΔplcB (■), or ΔLLOΔplcBΔplcB mutants (■). Ctsd added (+) bars show the transient transfection of fibroblasts with a Ctsd cDNA. Results are expressed as the ratio of CFU at 8 h to CFU at 0 h ± SD of triplicates. C, Phagosomes, 30 μg of protein, from fibroblasts of Ctsd+/+ and Ctsd−/− mice were obtained, and CFU from control cells were expressed as 100% of viable LM ± SD of three different experiments. D, Immunoblot of 30 μg of the purified phagosomes were loaded per lane and visualized with Abs against Ctsd and Rab5a. Ponceau staining was used as a loading control. Immunoblots of Ctsd show the immature (53 kDa, labeled as im) and mature (34 kDa, labeled as m) forms of the protein (11). Immunoblots also show the 25-kDa Rab5a protein. E, CHO cells were transfected with Ctsd-pcDNA3.1-hygro (Ctsd bars), Ctsd-pEFP (Ctsd-GFP bars), and pEFP (control-GFP bars) by electroporation, and purified phagosomes were obtained in the presence (■) or absence of 1 mM pepstatin A (■) (15). Intraphagosomal viabilities were expressed as in C and corresponded to the mean ± SD of three independent experiments. Thirty micrograms of the purified phagosomes were loaded per lane, and total Rab5a levels were determined. Rab5a-GTP levels were obtained after incubation with 3 μg of His-EEA1, and immunoblots were developed with anti-Rab5a Ab.
Phagosomes from Ctsd<sup>+/−</sup> cells transfected with Ctsd contained numbers of viable LM similar to those found in wild-type phagosomes. Phagosomes from Ctsd<sup>+/−</sup> fibroblasts transfected with Ctsd contained 8-fold fewer viable intraphagosomal bacteria compared with phagosomes from mock-transfected cells (Fig. 1C). Ctsd protein levels in wild-type Ctsd<sup>+/−</sup> phagosomes, as well as in Ctsd<sup>−/−</sup>-deficient fibroblasts re-expressing Ctsd, showed low amounts of the active 34-kDa mature Ctsd form (Fig. 1D). Phagosomes with low levels of intraphagosomal viable LM in Ctsd<sup>−/−</sup> fibroblasts transfected with Ctsd displayed 15-fold higher levels of active 34-kDa Ctsd. Interestingly, Ctsd<sup>−/−</sup> phagosomes exhibited very low levels of Rab5a compared with wild-type phagosomes. Moreover, transfection of Ctsd<sup>+/−</sup> fibroblasts with Ctsd resulted in a significant increase in Rab5a levels (Fig. 1D). These results suggest that intraphagosomal viability of LM inversely correlated with active Ctsd and high levels of Rab5a in phagosomes. Phagosomes from CHO cells (15) transfected with Ctsd displayed a 5-fold lower intraphagosomal CFU compared with control cells. The listerial potential of phagosomes from Ctsd-transfected CHO was dependent on the presence of active Ctsd, because it was peptatin-A sensitive (Fig. 1E). These phagosomes showed high total Rab5a and Rab5a-GTP levels (Fig. 1E). These results indicate that Rab5a activation was required for the listerial ability of Ctsd.

The listerial effect of Ctsd is targeted to listeriolysin O

We next analyzed whether the main virulence factors of LM with reported phagosomal activity, LLO or phospholipases C (PLCs) (1, 16), are targets for Ctsd. ΔpelA/plcB is a mutant deficient in plcA/plcB (PLCs), and ΔLLOΔpelA/plcB is deficient in LLO and the two PLCs (17). A ΔpelA/plcB LM mutant showed a 4-fold higher LM intracellular growth index in Ctsd<sup>−/−</sup> cells as compared with Ctsd<sup>+/−</sup> cells (Fig. 1B). No enhancement of LM replication indexes in Ctsd<sup>−/−</sup> cells was observed with ΔLLOΔpelA/plcB-deficient mutants (Fig. 1B). This suggests that the high LM susceptibility of Ctsd<sup>−/−</sup> cells was LLO-specific and renders the involvement of PLCs unlikely.

The calculated percentages of phagosomal vs cytosolic bacteria at 0 h reflected the compartment in which each LM bacterial mutant was able to survive (1,18) (Table I). In Ctsd<sup>+/−</sup> cells, 30% of total wild-type LM were localized within the phagosomes, and 70% of total viable LM were in the cytosol. In Ctsd<sup>−/−</sup> cells, the number of viable wild-type LM found within the phagosomes decreased to 10% of total viable LM, whereas the percentages of cytosolic bacteria increased to 90%. With ΔpelA/plcB-deficient mutants, phagosomal and cytosolic percentages of viable LM were similar to wild-type bacteria. However, ΔLLOΔpelA/plcB-deficient mutants exhibited significantly reduced numbers of viable LM, all of which were phagosomally localized in both Ctsd<sup>+/−</sup> and Ctsd<sup>−/−</sup> cells. ΔLLO-deficient mutants did not grow intracellularly in Ctsd<sup>+/−</sup> cells. In Ctsd<sup>−/−</sup> cells, they showed an unexpected vigorous intracellular growth, which was similar to that of wild-type LM. In fact, 99% of viable ΔLLO-deficient mutants remained phagosomal, and only 1% localized in the cytosol.

To further analyze whether Ctsd hydrolyzed LLO, we performed an in vivo LLO-degradation analysis using Ctsd<sup>+/−</sup> or Ctsd<sup>−/−</sup> fibroblasts infected with wild-type LM or ΔLLO-deficient mutants. Intact LLO protein could be isolated from Ctsd<sup>+/−</sup> fibroblasts infected with wild-type LM, but it was not detected in Ctsd<sup>+/−</sup> fibroblasts, suggesting in vivo degradation of LLO by Ctsd (Fig. 2A).

Direct incubation of recombinant His-LLO with purified human Ctsd revealed a full-length LLO and a cleaved LLO form in the absence of pepstatin A (Fig. 2B). In the presence of pepstatin A, only the full-length LLO was observed. Both forms of LLO, were stained with an anti-His Ab. Because the LLO recombinant protein was tagged at its N terminus, these results suggest that Ctsd cleavage takes place at the C terminus of LLO (data not shown). In contrast, Ctsd did not cleave a purified Listeria phosphatidylinositol-PLC (data not shown).

Ctsd is involved in the early MAC resistance to LM

MAC are responsible for the early resistance to LM (19). Within BM-MAC of Ctsd<sup>−/−</sup> mice, wild-type LM displayed an exponential growth preferentially between 10 and 18 h postinfection (Fig. 3A). After infection with wild-type LM, BM-MAC generated from Ctsd<sup>−/−</sup> mice exhibited 4- to 5-fold higher LM replication indexes as compared with BM-MAC from Ctsd<sup>+/−</sup> mice (Fig. 3B). ΔpelA/plcB LM mutants also showed increased replication indexes, but growth of ΔLLOΔpelA/plcB mutants was fully abrogated (Fig. 3B), supporting the notion that LLO is a putative substrate for Ctsd. We observed high numbers of intracellular wild-type FITC-labeled LM in the perinuclear regions of BM-MAC from Ctsd<sup>−/−</sup> mice (Fig. 3, E, G, I, and K). Confocal microscopy (Fig. 3, D and E) suggested that LM were freely localized within the cytosol as FITC-LM colocalized with tetramethylrhodamine isothiocyanate (TRITC)-phalloidin. The number of FITC-LM in BM-MAC from Ctsd<sup>−/−</sup> mice was significantly lower (Fig. 3, D, F, H, and J). We performed in vivo experiments with Ctsd<sup>−/−</sup> mice (11). Ctsd<sup>−/−</sup> and Ctsd<sup>+/−</sup> p17 mice were inoculated i.p. with a 1 LD<sub>50</sub> LM dose of ∼1×10<sup>8</sup> CFU. Three different experiments were performed with a total of 14 Ctsd<sup>−/−</sup> mice and 15 Ctsd<sup>+/−</sup> littermates (p < 0.01). The number of CFU per spleen observed in Ctsd<sup>−/−</sup> mice

### Table I. Percentages of cytosolic and phagosomal bacteria in fibroblasts infected with different LM strains

<table>
<thead>
<tr>
<th>Cells</th>
<th>Bacterial strain</th>
<th>Ctsd&lt;sup&gt;+/−&lt;/sup&gt; (CFU × 10&lt;sup&gt;3&lt;/sup&gt;)</th>
<th>Ctsd&lt;sup&gt;−/−&lt;/sup&gt; (CFU × 10&lt;sup&gt;3&lt;/sup&gt;)</th>
<th>Phago (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Ctsd&lt;sup&gt;+/−&lt;/sup&gt; (CFU × 10&lt;sup&gt;3&lt;/sup&gt;)</th>
<th>Ctsd&lt;sup&gt;−/−&lt;/sup&gt; (CFU × 10&lt;sup&gt;3&lt;/sup&gt;)</th>
<th>Phago (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Ctsd&lt;sup&gt;+/−&lt;/sup&gt; (CFU × 10&lt;sup&gt;3&lt;/sup&gt;)</th>
<th>Ctsd&lt;sup&gt;−/−&lt;/sup&gt; (CFU × 10&lt;sup&gt;3&lt;/sup&gt;)</th>
<th>Phago (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Ctsd&lt;sup&gt;+/−&lt;/sup&gt; (CFU × 10&lt;sup&gt;3&lt;/sup&gt;)</th>
<th>Ctsd&lt;sup&gt;−/−&lt;/sup&gt; (CFU × 10&lt;sup&gt;3&lt;/sup&gt;)</th>
<th>Phago (%)&lt;sup&gt;a&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>Wild type</td>
<td></td>
<td>2.1 ± 0.20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.5 ± 0.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30</td>
<td>70</td>
<td>10</td>
<td>90</td>
<td>30</td>
<td>70</td>
<td>10</td>
<td>90</td>
<td>30</td>
<td>70</td>
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<tr>
<td>ΔpelA/plcB</td>
<td></td>
<td>13 ± 0.35&lt;sup&gt;b&lt;/sup&gt;</td>
<td>55 ± 0.20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30</td>
<td>70</td>
<td>10</td>
<td>90</td>
<td>30</td>
<td>70</td>
<td>10</td>
<td>90</td>
<td>30</td>
<td>70</td>
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<tr>
<td>ΔLLOΔpelA/plcB</td>
<td>1.8 ± 0.37&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.3 ± 0.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>53 ± 0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100</td>
<td>0</td>
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<tr>
<td>ΔLLOΔpelA/plcB</td>
<td>1.0 ± 0.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.9 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>35 ± 0.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100</td>
<td>0</td>
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<tr>
<td>ΔpelA/plcB</td>
<td>1.0 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.9 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>35 ± 0.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100</td>
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<sup>a</sup> Fibroblasts (Ctsd<sup>+/−</sup>; Ctsd<sup>−/−</sup>) were infected for 0 h with wild-type LM, ΔpelA/plcB, ΔLLOΔpelA/plcB, or ΔLLO-deficient LM mutants, and isolated phagosomes (50 µg) were examined for CFU and results expressed as CFU × 10<sup>3</sup> ± SD. Phago, Phagosomes.

<sup>b</sup> Fibroblasts (Ctsd<sup>+/−</sup> or Ctsd<sup>−/−</sup>) were infected for 8 h as in rows. Results were expressed as ratio of CFU at 8 h divided by the CFU at 0 h.

<sup>c</sup> Phagosomes (Phago) or PNS (50 µg) were obtained 90 min after infection, sonilized, and viable LM quantified as CFU to calculate percentages of phagosomal and cytosolic bacteria as described in Material and Methods.
shown as a yellow fluorescence (white arrows). Rounded by polymerized actin. Free-cytosolic bacteria are FITC-LM for 1 h and incubated with TRITC-phalloidin. CFU titers for individual spleens (n) as well as the mean ESD (n/5000) of the same internal layer (a section located 3 μm from the top of the cell). These results underline the in vivo role of Ctsd among the innate mechanisms that restrict LM exponential growth at early phases of listeriosis.

**Discussion**

Early listeriosis is characterized by the exponential intracellular growth of LM observed in permissive cells of the liver. Growth control of LM occurs in the spleen (1, 19). The exponential LM growth observed in permissive cells of tissues from infected animals is not accompanied by massive cell destruction. Therefore, these cells might regulate LM growth by as yet still incompletely understood listericidal mechanisms. Due to the early postnatal lethality of Ctsd knockout mice (11, 14), we studied embryonic fibroblasts (permissive cells) and BM-MAC (restrictive cells) (18, 20). The lack of Ctsd in fibroblasts rendered them 4- to 6-fold more susceptible to LM infection, because isolated phagosomes exhibited higher intraphagosomal LM viability possibly linked to a lack of Rab5a. Using LM-permissive endothelial CHO cells transfected either with Ctsd (this work) or with Rab5a-inactive mutants (15), we observed that Ctsd-mediated listerical activity required the previous activation of Rab5a. In permissive cells, early interactions of phagosomes with endosomes transport Rab5a and Ctsd precursor to LM phagosomes (10) before LLO and PLCs become active (1). The low pH environment (10, 21) and Rab5a may activate certain amounts of Ctsd to cleave membrane-inserted LLO. In support of this model, LLO is degraded in Ctsd−/− fibroblasts as well as in an in vitro degradation assay. This mechanism could diminish LM intraphagosomal viability to some extent, although the remaining viable LM might rapidly inactivate Rab5a and Ctsd (15), allowing LM escape into the cytosol. In Ctsd−/− fibroblasts, the lack of Ctsd and Rab5a confers a more permissive environment and LLO action takes place without restriction, supported by the observation of higher numbers of viable intraphagosomal LM and of LM growing in the cytosol. These phagosomes resembled those of Rab5a-deficient cells that allowed growth of ΔLLO-deficient mutants (10) in a similar manner to Ctsd−/− cells.

Because MAC are critical effector cells of the innate response to LM (19), our findings in vivo indicate that Ctsd has an important and novel role in early listeriosis. Ctsd−/− mice were 10-fold more susceptible to LM infection, compared with Ctsd+/+ littermates.

**FIGURE 2.** Ctsd specifically degrades listeriolysin O both in vivo and in vitro. A. Fibroblasts were infected with wild-type LM (LLO+) or a ΔLLO-deficient mutant (LLO−). Intracellular proteins were biotinylated as described in Material and Methods. Controls were uninfected Ctsd+− cells. Unspecific bands are shown (bacteria: −lanes). B. Purified Ctsd was incubated with HisLLO in the presence or absence of 1 mM pepstatin A (pepA). Gels were stained with Coomassie.
This work on the role of Ctsd in the clearance of LM constitutes the first in vivo study demonstrating Ctsd as a bactericidal agent effective against intracellular bacteria.

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**Disclosures**

The authors have no financial conflict of interest.

**References**


