Published in final edited form as:

Microb Pathog. 2009 January ; 46(1): 1–5. doi:10.1016/j.micpath.2008.09.008.

The ability of *Listeria monocytogenes* PI-PLC to facilitate escape from the macrophage phagosome is dependent on host PKC β

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Abstract

Listeria monocytogenes are facultative intracellular pathogenic bacteria that can infect macrophages as well as non-professional phagocytes. After entry in the host cell, the bacteria escape from the phagosome into the cytoplasm. In murine macrophages and in cell lines derived from these cells, escape of *L. monocytogenes* from the phagosome is absolutely dependent on listeriolysin O (LLO) and facilitated by a secreted phosphatidylinositol-specific phospholipase C (PI-PLC). Work in this laboratory has previously demonstrated a LLO and PI-PLC-dependent translocation of host PKC β isoforms. Pharmacological inhibition of PKC β resulted in a significant reduction in permeabilization of the phagosome, and in the number of bacteria reaching the cytosol. These findings led to the prediction that the bacterial PI-PLC promotes escape through the production of diacylglycerol leading to the activation of host PKC β . To test this hypothesis, bone marrow-derived macrophages (BMM ϕ) obtained from PKC β knockout (PKC β KO) or C57Bl/6 mice were infected with *L. monocytogenes*. We observed that wild type *L. monocytogenes* escapes from the phagosome of PKC β KO BMM ϕ as well as they do from C57Bl/6 BMM ϕ . However, in PKC β KO BMM ϕ , *L. monocytogenes* uses a PI-PLC-independent, but phosphatidylcholine-preferring PLC (PC-PLC)-dependent pathway to facilitate escape. These findings strongly support the hypothesis that PI-PLC promotes escape through mobilization of host PKC β .

Keywords

Listeria monocytogenes; PKC β ; PI-PLC; listeriolysin O; PC-PLC; knockout

1. Introduction

Listeria monocytogenes, a gram-positive facultative intracellular bacterium, is a food-borne pathogen with worldwide distribution [1–3]. Its intracellular life cycle consists of internalization by professional phagocytes and other cells, escape from the phagosome, cytoplasmic multiplication, intracellular mobility by means of bacteria-induced host actin polymerization and cell-to-cell spread [4]. After entry into the phagosome, the production and activity of proteins needed for optimal escape from the phagosome are upregulated under the

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control of the protein PrfA [5–7]. In murine macrophages escape of *L. monocytogenes* from the phagosome requires the bacterial pore-forming cytolysin listeriolysin O (LLO) [8] and is aided by bacterial phosphatidylinositol-specific phospholipase C (PI-PLC) encoded by *plcA* [9]. The exact mechanisms leading to escape from the phagosome are not understood. Structural studies have shown that LLO can polymerize and form pores in the vacuolar membrane [10]. These pores are too small to allow *L. monocytogenes* to cross the phagosome membrane and reach the cytoplasm [11], but allow the diffusion of electrolytes and proteins between phagosome and cytoplasm [10;12].

Bacterial PI-PLCs are 30–35 kDa soluble enzymes that catalyze the cleavage of the membrane lipid phosphatidylinositol (PI) into inositol phosphate and diacylglycerol (DAG). PI-PLCs are produced by various gram-positive bacteria, including the pathogens *L. monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus* and *Bacillus anthracis*, for which these PI-PLCs are known to be, or are considered to be potential virulence factors [13]. *Listeria monocytogenes* PI-PLC has very low activity on glycosyl-PI-anchored proteins unlike the classical PI-PLCs secreted by *B. cereus*, *B. anthracis* and *S. aureus* [14;15]. Thus, its main target is PI in the host cell.

Previous studies from our laboratory using murine-macrophage derived cell lines have shown that, even before its entry, *L. monocytogenes* acts on the future host cell and induces the translocation of PKC δ to the periphery of the cell as well as the translocation of PKC β isoforms to early endosomes which coalesce. These studies using deficient *L. monocytogenes* strains have demonstrated a crucial role for LLO and PI-PLC in both these translocations [16]. Studies with the calcium channel blocker SK&F96365 revealed the importance of elevated Ca²⁺ for PKC β II translocation and escape from the phagosome [16;17]. Host PLC is stimulated in a LLO-dependent process, thus providing a second source of DAG for PKC β I translocation [18]. In a bacteria-independent system, activation of PKC by PMA leads to translocation of PKC α and PKC β II to a recycling endosome compartment in a juxtanuclear position. This translocation involves phospholipase D [19;20]. These diverse findings lead to a model in which rapid activation of PKC β in macrophages by the combined actions of LLO and PI-PLC leads to the sequestration of proteins along PKC β with in endosomal compartments; this is hypothesized to interfere with the normal endosomal recycling needed for maturation of the phagolysosome, a hallmark of *L. monocytogenes* infections.

In this study, to avoid the potential problem that PKC β inhibitors could inhibit proteins other than PKC β , we infected bone marrow-derived macrophages obtained from PKC β knockout mice [21] and compared their susceptibility to infection with that of wild type C57Bl/6, PKC β -producing, mice. These studies have shown that in the absence of PKC β escape from the phagosome is independent of PI-PLC.

2. Results and discussion

2.1. Infection of macrophages derived from C57Bl/6 and PKC β KO mice with wild type *L. monocytogenes*

Bone marrow-derived macrophages (BMM ϕ) were differentiated *in vitro* from cells obtained from PKC β KO-restored or control C57Bl/6-restored femurs as described in the Materials and methods section. These macrophages were first infected with wild type *L. monocytogenes* 10403S (see Table I for bacterial strains). Entry of *L. monocytogenes* was equivalent in BMM ϕ from C57Bl/6 or PKC β KO mice (data not shown). We observed escape of *L. monocytogenes* from the phagosome of BMM ϕ from both PKC β KO and C57Bl/6 restored mice (Figure 1). Although escape of wild type *L. monocytogenes* from the phagosomes appears slightly lower in PKC β KO BMM ϕ , the difference from C57Bl/6 BMM ϕ was not significant (Figure 2).

2.2. Infection of BMMφ with mutant strains of *L. monocytogenes*

A strain lacking LLO was unable to escape from the phagosomes of either wild type or PKCβKO BMMφ (data not shown). In C57Bl/6 BMMφ, a *plcA* mutant of *L. monocytogenes* (PI-PLC⁻) exhibited a significant reduction in escape compared to the wild type (Figure 2), as previously observed [9;22]. To assess the role of bacterial phospholipases in escape from PKCβKO BMMφ, cells from C57Bl/6 and PKCβKO mice were infected with wild type, $\Delta plcA$ (PI-PLC⁻), the broad range PLC-deficient (PC-PLC⁻, $\Delta plcB$) or the double-deficient *L. monocytogenes* lacking both PI-PLC and PC-PLC (PI-PLC⁻/PC-PLC⁻, $\Delta plcA \Delta plcB$). As noted above, when infected with the PI-PLC⁻ strain, C57Bl/6 BMMφ exhibited a significant reduction in escape, however strikingly there was no significant difference in escape between wild-type and PI-PLC⁻ bacteria in PKCβKO BMMφ (Figure 2). When BMMφ from C57Bl/6 were infected with PI-PLC⁻/PC-PLC⁻ *L. monocytogenes*, escape was reduced compared to the wild type and similar to that of PI-PLC⁻ *L. monocytogenes*. When PKCβKO BMMφ were infected with the PI-PLC⁻/PC-PLC⁻ *L. monocytogenes* strain, escape from the phagosome was significantly reduced compared to wild type and PI-PLC⁻ strains (Figure 2). This result revealed an unexpected effect of loss of PC-PLC on escape from the phagosome of BMMφ from PKCβKO mice. Infection of BMMφ from both C57Bl/6 and PKCβKO with a strain deleted in PC-PLC confirmed this result. Although the results with BMMφ from C57Bl/6 mice were somewhat variable, they confirmed the previous observation that loss of PC-PLC has no measurable effect on escape from the macrophage phagosome [7]. With BMMφ from the PKCβKO strain, escape of the PC-PLC deletion strain compared to the wild type *L. monocytogenes* was reduced to the level observed with the double phospholipase mutant (Figure 2). These results indicate that PC-PLC, which plays a role in escape of *L. monocytogenes* from the phagosome of certain human epithelial cell lines [23], can promote bacterial escape in BMMφ in the absence of PKCβ. In the absence of PI-PLC and PC-PLC, LLO presumably mediates escape by virtue of its ability to form pores and thereby equilibrate the contents of the phagosome and the cytosol, thus inhibiting maturation of the phagosome [24].

A recent study has shown that mice deficient in PKCdelta are highly susceptible to infection with *L. monocytogenes*. In macrophages from PKCdelta ^{-/-} mice *L. monocytogenes* escaped more efficiently from the phagosome into the cytoplasm than in wild type macrophages [25]. Thus it appears that whereas PKCbeta promotes listerial infection, PKCdelta controls it.

2.3 Effects of cell signaling inhibitors

We have previously shown that the PKCβ inhibitor Gö 6983 (Calbiochem) significantly inhibited escape of wild type *L. monocytogenes* from the phagosomes of the murine macrophage cell line J774 [26]. Similarly, pretreatment with Gö 6983, 10 μM, inhibited escape of wild type *L. monocytogenes* from C57Bl/6 BMMφ phagosomes, but it did not affect *L. monocytogenes* escape in PKCβKO BMMφ (Figure 3A). This is consistent with the postulated involvement of PKCβ in escape of *L. monocytogenes* in C57Bl/6 BMMφ and indicates that the PKCβ inhibitor is specific as it does not exhibit an inhibitory activity in PKCβKO BMMφ. It also confirms that in the absence of PKCβ, *L. monocytogenes* can use a PKCβ-independent pathway. As could be expected, treatment with the PKCβ inhibitor Gö 6983 had no effect on escape of PI-PLC⁻ *L. monocytogenes* from either the C57Bl/6 or the PKCβKO BMMφ (data not shown).

In J774 cells, pretreatment with the calcium channel blocker SK&F96365 (Calbiochem) revealed the critical importance of elevated Ca²⁺ for PKCβII translocation, and bacterial escape [18]. As shown in Figure 3B, SK&F 96365, 25 μM, strongly inhibited escape of wild type *L. monocytogenes* in both C57Bl/6 and PKCβKO BMMφ, indicating that an elevation of intracellular Ca²⁺ is required for escape in both the PKCβ-dependent and the PKCβ-independent pathways.

3. Conclusions

In this study, we show that wild type *L. monocytogenes* enters and escapes from the phagosome with the same efficiency in PKC β KO and C57Bl/6 BMM ϕ . This indicates that host PKC β is not essential for the escape of wild type *L. monocytogenes*. Similar to previous observations, PI-PLC $^{-}$ *L. monocytogenes* exhibits reduced escape in C57Bl/6 BMM ϕ . However, the escape of PI-PLC $^{-}$ *L. monocytogenes* is not reduced in PKC β KO BMM ϕ . These findings strongly support the hypothesis that PI-PLC promotes escape from the phagosome of macrophages through redistribution of host PKC β .

Although PI-PLC-deficient *L. monocytogenes* escaped from the phagosome similarly to wild type *L. monocytogenes* in the absence of host PKC β , escape of the single PC-PLC $^{-}$ mutant and the double PI-PLC $^{-}$ /PC-PLC $^{-}$ mutant was reduced. These results indicate that the “compensatory pathway” used by *L. monocytogenes* in the absence of host PKC β is dependent on bacterial PC-PLC, which is known to be involved in escape from the secondary vacuole after cell-to-cell spread [7;27;28]. PC-PLC is involved in escape of *L. monocytogenes* from the primary phagosome in the epithelial cell line Henle 407 [23] as well as in the human cell lines HEp-2 and HeLa [28]. The ability of *L. monocytogenes* PC-PLC to promote escape from the double membrane vacuole formed after cell-to-cell spread has been attributed to its general attack on membrane phospholipids [29] and to its ability to mediate membrane fusion by virtue of its phospholipase/sphingomyelinase activities [30]. How it mediates escape from the primary vacuole of certain non-phagocytic cells is not clear at this time.

Recent studies have shown that activation of classical PKC isoforms α and β by phorbol myristate acetate (PMA) resulted in their translocation to large juxtanuclear vesicles [19;20]. Further studies have shown that this translocation results in sequestration of proteins (CD59 and caveolin) and lipid cargo [31]. These findings suggest that *L. monocytogenes* PI-PLC through the formation of diacylglycerol activates a pathway that affects endosomal recycling pathways, which could interfere with phagosome maturation. The different pathways leading to *L. monocytogenes* escape in macrophages and human cell lines are not understood. A better understanding of signaling pathways in these cells should be the goal of future research in this emerging area.

4. Materials and methods

4.1 Cells

Two-month-old C57Bl/6 mice were lethally irradiated (8.5–9.5 Gy) and restored with 1×10^7 donor bone marrow cells (obtained from C57Bl/6 or PKC β KO mice [21] by injection via the tail vein. Bone marrow-derived macrophages (BMM ϕ) were differentiated *in vitro* from cells obtained from PKC β KO-restored or control C57Bl/6-restored femurs following the protocol described by Lutz et al [32]. BMM ϕ differentiation was stimulated with M-CSF (PeproTech Inc) throughout the culture [33].

4.2 Bacteria

The list of *L. monocytogenes* strains used in this study appears in Table I

4.3. Bacterial entry

After infection, bacterial entry was measured as described previously [17]. Briefly, *L. monocytogenes* were labeled with fluorescein isothiocyanate (FITC: 1 mg/ml in PBS, 45 min. at 37°C) and used to infect bone marrow-derived macrophages. At various times during a 45-min. infection, cells were washed with PBS and extracellular bacteria stained with ethidium bromide (25 mg/ml, 1 min.). Cells were then washed with PBS and fixed with 4%

paraformaldehyde. The number of extracellular bacteria was subtracted from the number of bacteria (FITC-stained) to determine the percentage of entry.

4.4 Escape

Escape of *L. monocytogenes* from the phagosome: To measure escape of the bacteria from the phagosome, we used a longer incubation time (90 min.) after infection with the FITC-labeled bacteria before fixation, the cells were stained with Alexa568-Phalloidin to detect actin polymerization around cytosolic bacteria [17;33].

Acknowledgements

The authors are greatly indebted to Lauren Zenewicz for help with mouse irradiation and bone marrow restoration. This study was supported by NIH Grant AI-45153 to H. G.

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Figure 1. Infection of bone marrow-derived macrophages by wild type and mutant strains of *L. monocytogenes*

BMMφ obtained from C57Bl/6 (A) and PKCβKO (B) restored mice were infected with FITC-labeled wild type *L. monocytogenes* and fixed after 90 min. Cellular actin was then stained using Alexa568-phalloidin (red). Pictures taken with both filters were overlaid. Escaped *L. monocytogenes* which polymerize actin appear yellow surrounded by a red halo (some are indicated by arrowheads) while non-escaped *L. monocytogenes* appear green.

Figure 2. Effect of PKC β constitutive deficiency on escape of wild type and PLC deficient *L. monocytogenes*

BMM ϕ from C57Bl/6 or PKC β KO restored mice were infected with either wild type, PI-PLC⁻, PC-PLC⁻ or PI-PLC⁻/PC-PLC⁻ FITC-labeled *L. monocytogenes*. After 90 min., cells were fixed and actin was stained with Alexa568-phalloidin. The percentage of escape was determined by counting the bacteria inducing actin polymerization and dividing this number by the total number of intracellular bacteria. Data represent the mean $\pm$ SEM of 2 to 4 experiments. NS: not significant, *: $p < 0.01$ using Student's T test when compared to the wild type strain 10403S. The error bar (± 0.03) for escape for the PC-PLC⁻ strain in PKC β KO is too small to be seen.

Figure 3. Effect of inhibitors on escape of *L. monocytogenes* from macrophages from wild type and PKC β KO mice

Cells from wild type or PKC β KO restored mice were infected with wild type FITC-labeled *L. monocytogenes*. Where indicated, cells were treated with Gö 6983 10 μ M(A), a PKC β inhibitor, or SK&F96365 25 μ M(B), a Ca²⁺ channel blocker, 10 min. before infection to the end of the experiments. Ninety minutes after infection, cells were fixed and actin was stained with Alexa568-phalloidin. The percentage of escape was determined as described in the legend to Fig. 2. Data represent the mean $\pm$ SEM of 3 experiments. NS, not significant; NT, not treated. *: $p < 0.05$ using Student's T test to compare treated and non treated samples.

Table I

L. monocytogenes
strains used in this study

Designation	Genotype	Phenotype	Reference:
10403S		Wild type	[35]
DP-L1552	$\Delta plcA$	PI-PLC ⁻	[9]
DP-L1935	$\Delta plcB$	PC-PLC ⁻	[7]
DP-L1936	$\Delta plcA \Delta plcB$	PI-PLC ⁻ /PC-PLC ⁻	[7]
DP-L2161	Δhly	LLO ⁻	[34]