Phosphocholine may allow for Listeriolysin-mediated escape of phagocytized *Listeria* from vacuolar compartments into the host cytosol while protecting against overt destruction of the infected cell.

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On: “Phosphocholine antagonizes listeriolysin O-induced host cell responses of Listeria monocytogenes (JID-67560)” by Luigi La Pietra *et al*.

In this issue of *the Journal of Infectious Diseases*, La Pietra and colleagues discuss the role of phosphocholine, the product liberated from phosphatidyl choline by the phosphatidylcholine-specific phospholipase C (PC-PLC), as an inhibitor of Listeriolysin O (LLO) activity. This investigation opens important avenues and potential developments in our understanding of how bacterial toxins disrupt the membranes of eukaryotic cells and thereby trigger lethal processes in the host. LLO, also known as hemolysin,[1] is one of the many cholesterol-dependent cytolysins (CDCs) produced by several important Gram-positive bacterial pathogens.

Listeria is among those bacteria that are quickly phagocytized and avoid being killed by extracellular immune defense mechanisms. But the intracellular journey of *Listeria* does not stop there. From the pioneering work of Portnoy, Berche, and many other researchers, we know that LLO produced by *Listeria* will form pores in the phagosomal membrane, allowing the bacteria to escape from the vacuolar compartment and access the cytosol. Once there, *Listeria* will quickly replicate, and then initiate a new round of infection in adjacent cells, thereby spreading the infection.[2,3] In this process, the pathogen remains in its protective niche, explaining why the infection remains unabated.

Although showing a number of activities on host cells as well as extracellularly,[4,5] intracellularly released LLO is considered as major virulence factor for *Listeria* since LLO-deficient mutants cannot egress from the phagosome, where they are eventually killed or otherwise kept under check by cell host defenses.[6] Translationally inhibiting LLO production with luteolin causes reduction in phagosome escape of *Listeria* and the ensuing cytoplasmic growth and cytotoxicity of infected macrophages.[7]

One may, of course, ask why virulent *Listeria* that have reached the cytosol will not also destroy the cellular membrane, thereby losing their protective intracellular niche. Thus, uncontrolled LLO activity and/or synthesis indiscriminately occurring in cytosol as well as in phagosomes would be highly detrimental to the survival of the bacteria. A wide range of host

cell regulatory mechanisms that negatively affect LLO-dependent pore formation in the cytosol (such as enhanced Ca^{++} flux), as well as impaired translation of LLO during growth the cytosol, have been proposed.

La Pietra et al., however, have presented an indirect but interesting mechanism. They based their study on the observation that *Listeria* expresses a non-specific phospholipase (PC-PLC) that acts broadly on phosphatidylcholine and other phospholids,[8] and that is associated with the escape of *Listeria* from phagosomes,[9] contributing to cell-to-cell spread to neighboring cells. PC-PLC is produced as an inactive pro-enzyme, but a *Listeria* strain constitutively producing a mature active form of PC-PLC is markedly attenuated for virulence.[10] An inactive pro-enzyme form of PC-PLC also protects cells against mitochondrial damage caused by the egress of *Listeria* from phagosomes and its spread in the cytosol,[11] a point relevant for the experimental design. La Pietra et al. show that it is phoshocholine (ChoP), the product of the enzymatic cleavage of phosphatidylcholine by PC-PLC, that blunts LLO-dependent host cell responses through a direct interaction between ChoP and LLO. In support of this hypothesis, the authors offer a series of interesting demonstrations.

First, they focused on mitochondria and showed that reactive oxygen species (ROS) generation in cells infected with the PC-PLC-negative mutant is significantly higher than in cells infected with a wild type *Listeria* (in vitro, a purified PC-PLC inhibited LLO-induced mitochondrial ROS production). PC-PLC also antagonized LLO-induced mitochondrial fragmentation in cells (which, again, could also be reproduced in vitro). Additional studies examining damage caused on mitochondrial tubular structures by LLO revealed that PC-PLC has a protective effect. Next, they showed that PC-PLC impaired LLO induced Ca^{++} flux in treated HeLa cells and that PC-PLC antagonizes LLO-induced apoptosis in a cytochrome *c*- and caspase-dependent manner. They also showed a strong increase in caspase 3/7 activation in cells infected with a *Listeria* PC-PLC-

defective mutant (Lm ΔPC-PLC). In vitro, LLO caused an increase in caspase 3/7 activity, which was significantly reduced by the addition of purified PC-PLC.

Next, they looked at a direct effect of ChoP (the product of PC-PLC) on LLO-increased mitochondrial ROS production, but this experiment led to unclear results, since PC-PLC it-self had similar effects. Yet, parallel studies of the hemolytic activity of LLO showed a significant negative effect not only in the presence of PC-PLC but also with ChoP alone if mixed to LLO prior of starting the experiment. This effect was pH- and redox-status-independent. This finding led the authors to look for direct effects of ChoP on LLO in the presence of lipid bilayers. They used large unilamellar vesicles (LUV), which represent to some extent the natural form of lipid membranes on which LLO is thought to act. ChoP-pretreated LLO bound to LUVs to a lower extent than untreated LLO. The analysis showed that ChoP binds to LLO in a concentration-dependent manner and, while not directly inhibiting the interaction of LLO with the membrane, increases its dissociation, thereby reducing its pore-forming ability.

While PC-PLC of *Listeria* was long thought to be an essential virulence factor, allowing the escape of the bacteria from phagosomes and cell-to-cell spread, through its capacity to degrade host membrane phospholipids,[9] it was also recognized that this enzyme is secreted in an inactive form, making some sort of activation necessary. Showing that mutants in which the enzyme is constitutively active are not virulent eventually disproved this hypothesis. What La Pietra et al. show here is that it is actually the product of this enzyme that is the regulator, and acts by decreasing LLO activity. Importantly, ChoP concentrations are highest in the endoplasmic reticulum membrane and lowest in plasma membranes, of which the phagosomal membrane

derives.[12] *Listeria* exploits these compartmental differences, and LLO will easily disrupt the membrane surrounding phagocytized I*Listeria*, while it may not act easily on other membranes, i.e., once released into the cytosol. But other mechanisms must also be considered. Thus, the expression of the PC-PLC gene is favored by neutral pH, making it active only when the bacterium is present in the host cell cytoplasm. By releasing ChoP after activation, it will block LLO activity. It remains to be seen, however, whether the conversion of PC-PLC from its inactive precursor form will effectively produce the necessary amount of ChoP. In addition, there is no indication in the article why LLO produced by *Listeria*, once in the cytosol, would not be able to act on the cellular membrane from its inner leaflet (a key point, for which Portnoy's group has recently proposed a mechanism by which LLO present in the cytosol will co-opt the host endocytosis machinery to protect the integrity of the host plasma membrane).[13] Thus, the story is probably still incomplete and other factors acting upstream and regulating multiple steps may also play a critical role.[14] We also know that different bacterial virulence factors act in a complementary fashion during infection of host cells, a point that has not been addressed here. Moreover, the data, as presented, do not provide the necessary molecular explanation as to how ChoP causes an increased dissociation of LLO from bilayers, which clearly calls for additional biochemical and biophysical studies based on and perhaps beyond what is already known about the cellular and molecular mode of action of LLO.[15,16] However, the article calls our attention to modulation of LLO binding to membranes by a simple enzyme product acting as a downstream regulator. In this context, it may be worthwhile to mention that targeting LLO is being used as means of reducing *Listeria* virulence, with a recent successful

attempt in cultured cells as well as laboratory animals using botulin, a naturally occurring lupane-structured pentacyclic triterpenoid.[17] Thus, antivirulence strategies aiming at impairing LLO activity may be useful auxiliary approaches when resistance of *Listeria* to traditional antibiotics appears, as proposed for other pathogens when targeting the corresponding virulence factors.[18] It remains to be seen whether this mechanism of escape from phagosomes to spread the infection is unique to *Listeria monocytogenes* or could also operate and be under similar control for other bacteria during their intracellular voyages. While xenophagy protects the host cytosol from phagocytized bacteria other than *Listeria monocytogenes*, such as *Streptococcus pyogenes*, *Mycobacterium tuberculosis*, *Shigella flexneri*, *Salmonella typhimurium*, and *Legionella pneumophila*, by entrapping and delivering these microbes to a degradative compartment,[19] their most successful forms may actually circumvent this process by escaping in the cytosol to establish replicative niches inside different cell types.

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