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Listeria monocytogenes, a model in infection biology

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Running head: a model in infection biology

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Abstract:

Listeria monocytogenes causes listeriosis, a systemic infection which manifests as bacteremia, often complicated by meningoencephalitis in immunocompromised individuals and the elderly, and fetal-placental infection in pregnant women. It has emerged over the past decades as a major foodborne pathogen, responsible for numerous outbreaks in Western countries, and more recently in Africa. *L. monocytogenes*' pathogenic properties have been studied in detail, thanks to concomitant advances in biological sciences, in particular molecular biology, cell biology and immunology. *L. monocytogenes* has also been instrumental to basic advances in life sciences. *L. monocytogenes* therefore stands both a tool to understand biology and a model in infection biology. This review briefly summarizes the clinical and some of the pathophysiological features of listeriosis. In the context of this special issue, it highlights some of the major discoveries made by Pascale Cossart in the fields of molecular and cellular microbiology since the mid-eighties regarding the identification and characterization of multiple bacterial and host factors critical to *L. monocytogenes* pathogenicity. It also briefly summarizes some of the key findings from our laboratory on this topic over the past years.

Listeria monocytogenes and listeriosis

Listeria monocytogenes (*Lm*) is a ubiquitous bacterium which can be isolated from soil and water. It can also colonize plants, be ingested by herbivorous animals, reach their intestinal lumen, compete with their intestinal microbiota and asymptomatically be shed back in the environment via the feces. It may also invade their intestinal tissue, replicate in mesenteric lymph nodes, spleen and liver, be released in the intestinal lumen by the biliary tract and direct release from infected intestinal tissue (Bakardjiev, Theriot, & Portnoy, 2006; Zhang et al., 2017). A large number of mammals and birds have been shown to carry *Lm* in their stool. Dairy cattle can develop invasive infection of the central nervous system and fetal-placental unit, leading to circling disease and abortion, respectively (Gill, 1933; McDonald, 1967; Oevermann, Zurbriggen, & Vandevelde, 2010). *Lm* was discovered in the early 1920s, as a cause of systemic infection in wild gerbils and captive guinea pigs and rabbits (Murray, Webb, & Swann, 1926; Pirie, 1927). Although it was rapidly identified as a potential human pathogen, it is only after the second world war that it started to be considered as a major human pathogen responsible for deadly fetal-placental and central nervous system infections. Its foodborne origin was formally proven in the mid 1980s, in the context of the epidemiological investigations of a large outbreak in Canada, which was linked to the ingestion of contaminated coleslaw (Schlech et al., 1983). Multiple concomitant factors have contributed to the emergence of *Lm* as a major foodborne pathogen in Western countries in the second half of the twentieth century: (*i*) the industrialization of food production and the resulting large distribution of contaminated food, (*ii*) the generalization of food refrigeration which allows *Lm* selective growth, and (*iii*) the development of at risk segments of the population, such as patients under immunosuppressive therapies they were being made available, and the prolonged survival of patients with chronic immunosuppressive conditions.

In middle to low income countries, in which food production has started to be industrialized without strict microbiological surveillance of food production plants, and where the prevalence of immunosuppressed individuals is high in the context of the HIV pandemic, *Lm* is now emerging as a major foodborne pathogen, as recently highlighted by the world largest reported outbreak that happened in South Africa, via the contamination of a large processed meat production plant (Thomas et al., 2020). This underlines the necessity of proper microbiological and epidemiological surveillance programs, which can be highly effective in preventing large outbreaks, as illustrated in Western countries which have implemented them. This also underlines the importance of diagnosing listeriosis, which is based on bacterial culture, which is not routinely performed in many countries with little clinical microbiology resources. This likely leads to an underestimation of the actual medical and economic burden associated with *Lm* worldwide. Currently, in the United States and Europe, where *Lm* food and epidemiological surveillance programs have been implemented, the incidence of listeriosis ranges between 2 to 5 cases per year per million population (ECDC, 2018; Pohl et al., 2019; Silk et al., 2012; Voetsch et al., 2007). Human listeriosis manifests as septicemia, central nervous system and maternal-fetal infections, as well as rare forms of localized infections (Charlier et al., 2014; Charlier et al., 2012; Chersich et al., 2018)

(Danion et al., 2017; Morgand et al., 2018; Pilmis et al., 2019; Shoai-Tehrani et al., 2019). Upon ingestion of a large inoculum, *Lm* also induces a benign and spontaneously resolutive gastroenteritis in immunocompetent individuals (Aureli et al., 2000; Dalton et al., 1997). In France, where a very systematic surveillance is implemented, septicemia represents roughly 50% of cases, neurolisteriosis 30%, maternal-fetal infections 10%, and all types of rare and localized infections combined 10 % (Charlier et al., 2017). There has been a sharp decline in the number of maternal-neonatal cases, in conjunction with the enhanced surveillance of food contamination and information campaigns on the preventive measures to avoid listeriosis over the past three decades (Girard et al., 2014). The overall median incubation period of invasive listeriosis differs significantly by clinical form of the disease: a longer incubation period is observed for pregnancy-associated cases (median: 27.5 days) than neurolisteriosis (median: 9 days), bacteremia cases (median: 2 days) and gastroenteritis (median: 24 hours) (Goulet, King, Vaillant, & de Valk, 2013). As illustrated by the MONALISA prospective study, the prognosis of listeriosis is bleak, with a 3-month mortality rate of 46% in bacteremia cases and 30% in neurolisteriosis cases, and only 40% of patients with neurolisteriosis who survive fully recovering (Charlier et al., 2017). Whereas maternal-fetal listeriosis never leads to maternal mortality and is virtually never associated with maternal neurolisteriosis, more than 80% of infected mothers experience major fetal or neonatal complications (fetal loss, very high prematurity, early or late onset disease) (Charlier et al., 2017). This underlines that in contrast to maternal-fetal listeriosis, bacteremia and neurolisteriosis are almost constantly associated with immunosuppressive comorbidities, including cirrhosis, diabetes mellitus, end-stage renal disease, solid organ cancer, haematological malignancy, haemopoietic stem-cell or solid organ transplantation, asplenia, neutropenia, lymphopenia, HIV infection, inflammatory bowel diseases, inflammatory rheumatic disorders and other autoimmune diseases, congenital immune deficiency, prescription of corticosteroids or other immunosuppressive therapies in the past 5 years, and age older than 70 years. While active antiretroviral therapies and antimicrobial prophylaxis by cotrimoxazole both mitigate the risk for listeriosis associated with HIV infection (Jurado et al., 1993; Fernandez-Sabe et al., 2009), the recent outbreak in South Africa has dramatically emphasized that uncontrolled HIV infection constitutes a major risk factor for listeriosis (Thomas et al., 2020).

Listeria monocytogenes entry and survival into eukaryotic cells

Lm is able to survive in professional phagocytes, by escaping its internalization vacuole via the action of the pore-forming toxin listeriolysin O, which also provides this Gram-positive bacillus with its beta-hemolytic activity on blood agar plates, a key phenotypic identification criterion (Cossart et al., 1989; Gaillard, Berche, Mounier, Richard, & Sansonetti, 1987). Once in the cytosol, *Lm* can polymerize actin via its surface protein ActA, and this propels bacteria intracellularly and from one cell to its neighbors, allowing the propagation of the infection into foci without contact with the extracellular milieu (Kocks et al., 1992; Tilney & Portnoy, 1989). LLO and ActA are part of *Lm* core genome, and their deletion

leads to several orders of magnitude loss of virulence in animal models of infection, highlighting the critical importance of *Lm* intracellular survival in the pathogenesis of listeriosis (Gaillard, Berche, & Sansonetti, 1986; Portnoy, Jacks, & Hinrichs, 1988; Levraud et al., 2009). These two genes are part of *Listeria* pathogenicity island-1 (LIPI-1) and co-regulated by the transcriptional regulator PrfA, the master regulator of *Lm* virulence genes (Cossart & Lecuit, 1998). The identification and characterization of these genes and their products in the late 1980s and early 90s resulted from the collective efforts from scientists in the US and Europe, with Pascale Cossart and her team at Institut Pasteur playing a leading role.

Another key property of *Lm* is its capacity to induce its internalization into non-professional phagocytes, such as epithelial cells. By screening a library of mutants, the *inlAB* operon, which encodes two surface proteins named internalin, abbreviated InlA, and InlB, was identified and characterized (Gaillard, Berche, Frehel, Gouin, & Cossart, 1991). The receptor for InlA is E-cadherin (Ecad), a transmembrane adherent junction protein expressed in epithelial cells and some subsets of myeloid cells. It was identified by Jerome Mengaud when I joined Pascale Cossart's laboratory for my master degree (Mengaud, Ohayon, Gounon, Mege, & Cossart, 1996). InlA is sufficient to induce *Lm* internalization, with its N-terminal leucine-rich repeat region playing a critical role in the internalization process (Lecuit, Ohayon, Braun, Mengaud, & Cossart, 1997). In order to understand mechanistically how InlA-Ecad interaction leads to *Lm* internalization, cells expressing various Ecad constructs were needed. As mouse E-cadherin had been the most intensively studied, most constructs were derived from this species. A major problem was that the expression of none of these constructs, including full-length mouse Ecad, conferred permissiveness to InlA-mediated *Lm* internalization when expressed in cultured fibroblasts. The most likely explanation was that the new comer, myself, was not able to get the system work. However, it turned out that in contrast to chicken and human E-cadherin, mouse Ecad is actually not a receptor for InlA. Indeed, InlA interaction with Ecad directly involves Ecad 16th amino acid, which is a proline in permissive species (human, guinea pig, rabbit, gerbils) and a glutamic acid in non-permissive murine species (mouse and rats) (Lecuit et al., 1999). Having uncovered InlA-Ecad interaction species specificity, the cell biology of InlA-mediated internalization could be worked out. The cytoplasmic domain of Ecad, which links via beta- and alpha-catenins Ecad to the actin cytoskeleton, was shown to be critical for internalization (Lecuit et al., 2000), and follow-up studies in Pascale Cossart's laboratory identified key factors involved in InlA-mediated internalization, including ARHGAP10, vezatin, Myosin 7, caveolin and clathrin (Bonazzi, Veiga, Pizarro-Cerda, & Cossart, 2008; Sousa et al., 2005; Sousa et al., 2007; Sousa et al., 2004; Veiga & Cossart, 2005).

Listeria monocytogenes crossing of the intestinal barrier

Identifying that murine Ecads were not receptors for *Lm* helped answer two puzzling questions: (*i*) why are mice and rats so poorly permissive to listeriosis upon oral inoculation?; (*ii*) why does InlA play no role *in vivo* in a mouse model of infection. Because guinea pig enterocytes had been shown to be infected

by *Lm in vivo* upon oral inoculation (Racz, Tenner, & Mero, 1972), and InlA mediates entry into guinea pig epithelial cells, which express an Ecad with a proline at position 16th (Lecuit et al., 1999), the role of InlA was tested in this species. It was also tested in a humanized mouse model expressing human Ecad specifically in enterocytes (iFABP-hEcad mice). Both in guinea pigs and iFABP-hEcad mice, InlA mediates *Lm* breaching of the intestinal barrier (Lecuit et al., 2001). This result paved the way to further study *Lm* interactions with the intestine, studies that we are pursuing as of today; it also allowed me to defend my PhD.

Ecad constituting *adherens* junctions, which are situated beneath tight junctions, a major issue was to figure out how could *Lm* located in the intestinal lumen access Ecad. By whole-mount three-dimensional tissue imaging, Ecad was shown to be luminally accessible on extruding cells at the tip of intestinal villi, on villus foldings, and most strikingly around goblet cells. Goblet cells actually constitute a major site of translocation of *Lm* from the intestinal lumen to the lamina propria, in a strict InlA-dependent manner (Nikitas et al., 2011). Unexpectedly, bacteria were shown to translocate across goblet cells within a few minutes, with no contribution of either LLO and ActA, arguing that bacteria remain in their internalization vacuole for translocation. Indeed, a non-pathogenic non-invasive *Listeria* species, *Listeria innocua* (*Li*), translocates as efficiently as *Lm* when expressing InlA heterologously. Moreover, *Lm* translocation is microtubule-dependent, and involves the exocytic machinery, as one would expect for the apical-basal transfer of an intravacuolar cargo (Nikitas et al., 2011). These unexpected results exemplify that the intracellular fate of *Lm* when studied in a tissue context may differ from what observed in cultured cell lines. We are now in the process of dissecting the cell biology of *Lm* translocation across the gut barrier in intestinal organoids, which contain globlet cells permissive to InlA-mediated translocation and are genetically amenable.

In order to study the tissue response to *Lm* intestinal infection, the iFABP mouse model permissive to *Lm* upon oral inoculation was rederived as axenic, in the context of my postdoctoral studies in Jeffrey Gordon's laboratory in Washington University in Saint Louis, USA. Surprisingly, the intestinal tissue response to *Lm* is independent of InlA-mediated invasion of intestinal villi (Lecuit, Sonnenburg, Cossart, & Gordon, 2007). It fully depends on LLO, which triggers intestinal host tissue response in Peyer's patches, where *Lm* translocates across the intestinal barrier in an InlA-independent manner via M cells. While the absence of response to *Lm* at the villus level is consistent with the observation that *Lm* translocates across goblet cells within a vacuole, the relative absence of lamina propria villus response is intriguing. The mechanism and significance of this unanticipated observation is currently being investigated.

Listeria monocytogenes systemic dissemination

After crossing the intestinal barrier, *Lm* reaches the liver via the portal vein. Kupffer cells, the resident macrophages of fetal origin that line liver sinusoids, are key contributors of host defense against enteroinvasive bacteria. Once *Lm* reaches the liver, it is rapidly phagocytized by Kupffer cells and

induces their early necrostatin-1s-dependent death (Bleriot et al., 2015). This triggers monocyte recruitment which orchestrates an anti-bacterial type-1 inflammatory response. Kupffer cell death also triggers a type-2 response that involves the hepatocyte-derived alarmin interleukin-33 and basophil-derived interleukin-4. This alternative activation of monocyte-derived macrophages recruited to the liver allows them to replace ablated Kupffer cells and restore liver homeostasis. *Lm*-associated Kupffer cell death therefore appears as a key signal orchestrating not only type-1 microbicidal inflammation, but also type-2-mediated tissue repair upon infection (Bleriot et al., 2015).

***Listeria monocytogenes* crossing of the placental barrier**

Pregnant women are at high risk for fetal-placental listeriosis, yet they only extremely rarely (<1%) develop neurolisteriosis. This suggests that *Lm* placental infection does not only result from *Lm* bacteremia which may be favored by pregnancy-associated immune suppression, but is also a consequence of a specific tropism of *Lm* for the placenta (Charlier et al., 2017). Whereas *Lm* strains that express a truncated and non-functional InlA are isolated in up to 42% of isolates of food origin, only a small minority (4.4%) of clinical strains express a truncated InlA. This epidemiological observation strongly supports a role for InlA in human listeriosis and is consistent with the experimental results that have shown a key role for InlA in *Lm* breaching of the intestinal barrier (Lecuit et al., 2001). While up to 4.2% of clinical strains isolated from cases of bacteremia express truncated InlA, less than 1% strain of maternal-neonatal origin do ((Jacquet et al., 2004) and our unpublished observations), strongly arguing for a role of InlA in placental invasion. It is indeed the case, as *Lm* invasion of 3rd trimester human placental explants is InlA-dependent, consistent with the fact that placental cells situated at the maternal-fetal interface and bathed in maternal blood, the syncytiotrophoblast and cytotrophoblasts, are of epithelial origin and therefore express InlA receptor Ecad (Lecuit et al., 2004). Human placental explant invasion by *Lm* is also InlB-dependent. InlB, which gene is situated downstream of *inlA* on the *inlAB* operon, is a *Lm* surface protein which mediates entry into a wide range of cultured cells (Dramsi et al., 1995), upon interaction with its ubiquitously expressed receptor cMet, the hepatocyte growth factor receptor (Shen, Naujokas, Park, & Ireton, 2000). As for InlA interaction with Ecad, InlB interaction with cMet is species-specific: it occurs in human, rats and mice, but not in guinea pigs and rabbits (Khelef, Lecuit, Bierne, & Cossart, 2006). The complementary observations that (*i*) InlA plays not role in placental infection of guinea pig, a species permissive to InlA-Ecad but not InlB-cMet (Bakardjiev, Stacy, Fisher, & Portnoy, 2004), and that (*ii*) InlB plays not role in placental infection of mice (Le Monnier et al., 2007), a species permissive to InlB-cMet but not to InlA-Ecad, suggested that InlA and InlB may act in a conjugated manner for *Lm* invasion of the placenta. It is indeed the case. *Lm* being pathogenic for wild gerbils (Pirie, 1927), it was hypothesized and experimentally proven that gerbils are permissive to both InlA and InlB (Disson et al., 2008). Moreover, in pregnant gerbils, as well as in humanized knock-in mice in which mouse Ecad has been punctually mutated so that it expresses

an E16P Ecad permissive to InlA, both InlA and InlB act in a conjugated manner and mediate placental infection (Disson et al., 2008).

While both InlA and InlB are required for *Lm* crossing of the placental barrier (Disson et al., 2008), InlB is not involved in *Lm* crossing of the intestinal barrier (Khelef et al., 2006). Phosphoinositide 3-kinase (PI3-K) is involved in both InlA- and InlB-dependent pathways. Indeed, InlA-dependent internalization requires PI3-K activity but does not activate it, whereas InlB-cMet interaction activates PI3-K (Ireton et al., 1996; Ireton, Payrastre, & Cossart, 1999). Because goblet cells, which are targeted in an InlA-dependent manner, exhibit a constitutive PI3-K activity, InlB-dependent activation of PI 3-kinase is dispensable for InlA-dependent *Lm* intestinal barrier crossing. In contrast, the placental barrier does not exhibit constitutive PI3-K activity, rendering InlB necessary for InlA-dependent *Lm* placental invasion. These results provide a molecular explanation for the respective contributions of InlA and InlB to *Lm* host barrier invasion, and reveal the critical role of InlB in rendering cells permissive to InlA-mediated invasion (Gessain et al., 2015). They also show that PI3-K activity is critical to host barrier permissiveness to microbes, and that pathogens exploit both similarities and differences of host barriers to disseminate.

Uneven virulence of *Listeria monocytogenes* strains

Microbial pathogenesis studies are typically performed with so-called “reference strains”, to ease experimental reproducibility between laboratories. However, the use of these reference strains overlooks within-species heterogeneity in microbial virulence. By integrating human epidemiological and clinical data with bacterial population genomics, the biodiversity of *Lm* can be harnessed. Taking advantage of the clonal structure of this bacterial species, clones epidemiologically associated weither with a food origin (namely CC9 and CC121) or with human central nervous system (CNS) and maternal-neonatal (MN) listeriosis (namely CC1, CC4 and CC6) were identified (Maury, Tsai et al., 2016). These CNS- and MN-associated clones (CC1, CC4, CC6) happen to be most prevalent in patients without immunosuppressive comorbidities, and, consistent with this observation, are hypervirulent in a humanized mouse model of listeriosis. In contrast, the clones most prevalent in food and least prevalent in patients (CC9 and CC121) are hypovirulent (Maury, Tsai et al., 2016). Comparative genomics between hypervirulent and hypovirulent clones led to the identification of multiple new putative virulence factors. One gene clusters, which encodes a putative PTS, has been shown to be involved in the enhanced neural and placental tropism of CC4, by a mechanism that remains to be identified (Maury, Tsai et al., 2016). Interestingly, the clones which are the most prevalent in human patients are also the ones most frequently associated with listeriosis in dairy cattle, and also those most associated with contaminated dairy products. This suggests that *Lm* virulence in human may be linked to its ability to be associated with dairy cattle (Maury et al., 2019). In the same way that Pascale Cossart and colleagues have shown that comparative genomics between *Lm* and *Li* can lead to the identification of multiple *Lm*

genes involved in its pathogenic potential (Glaser et al., 2001), comparative genomics of multiple *Lm* isolates from various sources is expected to be exceptionally powerful to identify clinically relevant microbial virulence attributes.

Listeria monocytogenes in the genomics and post-genomics era

Lm microbiological surveillance is now based on whole genome sequencing (WGS) and the use of universally applicable genome-wide strain genotyping approach such as core genome multi locus sequence typing (cgMLST) (Moura et al., 2016). WGS-based cgMLST *Lm* typing not only greatly improves the identification of clusters of listeriosis cases which are linked microbiologically, but also the identification of the food sources of these clusters (Moura et al., 2017). It also offers the academic community with a large number of sequenced *Lm* genomes (more than 40,000 in Institut Pasteur database https://bigsdb.pasteur.fr/listeria/) from which the population structure of *Lm* can be studied, its evolution, transmission routes and worldwide spread investigated (Moura et al., 2016). The analysis of the sequence diversity of *Lm* genome and its association with temporal, geographical, epidemiological, clinical and experimental metadata by mean of genome wide association studies (GWAS) is expected to shed light on the evolution of *Lm* and the forces that shape its genome. In addition, the prospective MONALISA cohort, which is ongoing but has already recruited more than 1,200 patients with listeriosis, the corresponding sequenced clinical strains, as well as detailed patient biological data including their DNA (Charlier et al., 2017), will not only help determine the detailed clinical features of listeriosis and refine its treatment, but also help identify the bacterial and host factors associated with listeriosis and its outcome.

Afterword

As illustrated by the examples cited in this review, the understanding of *Lm* pathogenicity and of the disease it causes has dramatically progressed over the past decades, yet many important scientific advances regarding *Lm*, many of which made by Pascale Cossart (Radoshevich & Cossart, 2018), are not cited in this review. Her contribution in establishing *Lm* as a model for infection biology is immense, and showcased by their mutual fame worldwide. Pascale has always conveyed and shared her enthusiasm about science and *Lm* in particular, as illustrated on Figure 1. In the context of this issue dedicated to her, I wish to take the opportunity to thank her: it has really been a pleasure, an honor and an extraordinary opportunity to have her as a mentor. While being a scientist is undoubtedly a demanding job, it is arguably one of the most exciting one, and Pascale has certainly not missed this aspect, and transmitted this feeling around her, including for sure to me. Given her legendary energy, I am sure she would agree that there is much to be done before *Lm* has revealed all its secrets. Among other things, we indeed still do not precisely know what are its natural reservoirs, the evolutionary forces that have shaped its genome and led to the acquisition and evolution of the genes that mediates its virulence in mammals. We also ignore the phylogeography of *Lm*, and the evolution history of *Listeria*

genus. The observation that *Lm* may naturally loose its hemolytic activity (Maury et al., 2017), whilst its closest non-pathogenic species *Li* may rarely be hemolytic (Moura et al., 2019) illustrate that studying *Listeria* genus and species in light of the forces that shape their evolution may lead to more unexpected and likely exciting discoveries.

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Figure 1: Pascale's laboratory members gathered for a "pot" in 2005, in the mythical "greniers" of the Duclaux Building at Institut Pasteur, where her laboratory was located. The happy faces may reflect enthusiasm for science, and also be related to what contained the empty glasses in the foreground.

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