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Claire Maudet[1,2], Sylvain Levallois[1,2,3], Olivier Disson[1,2], Marc Lecuit[1,2,3,4,5,*]

[1] Institut Pasteur, Biology of Infection Unit, Inserm U1117, 75015, Paris, France

[2] Inserm U1117, 75015, Paris, France

[3] Université de Paris, 75006, Paris, France

[4] National Reference Centre and WHO Collaborating Centre *Listeria*, Institut Pasteur, 75015, Paris, France

[5] Necker-Enfants Malades University Hospital, Division of Infectious Diseases and Tropical Medicine, APHP, Institut Imagine, 75006, Paris, France

* Correspondence to: marc.lecuit@pasteur.fr

Abstract

Listeria monocytogenes (*Lm*) is a foodborne bacterial pathogen that causes listeriosis, a severe infection that manifests as bacteremia and meningo-encephalitis mostly in immunocompromised individuals, and maternal-fetal infection. A critical pathogenic determinant of *Lm* relies on its ability to actively cross the intestinal barrier, disseminate systemically and cross the blood-brain and placental barriers. Here we illustrate how *Lm* both evades innate immunity, favoring its dissemination in host tissues, and triggers innate immune defenses that participate to its control.

Highlights

- *Lm* has evolved a propensity to evade innate immune defenses.
- The intestinal phase of listeriosis is mostly silent.
- *Lm* placental infection triggers innate immune responses that can alter the immune balance needed for placental fetal survival and development.
- Little is known regarding *Lm* innate immune responses in the CNS and their impact on infection and its control.

Introduction

Listeria monocytogenes (*Lm*) is a Gram-positive facultative intracellular bacterial pathogen. It causes listeriosis, a severe foodborne infection leading to bacteremia and meningo-encephalitis, particularly in immunocompromised individuals, and mother-to-child infections [1-4]. Upon ingestion of contaminated food, *Lm* crosses the intestinal barrier. This process is clinically silent or manifests as mild gastroenteritis. *Lm* can then disseminate systemically and can actively cross the blood-brain and placental barriers, thereby infecting the central nervous system (CNS) and feal-placental unit, respectively [2,3].

Lm crossing of the intestinal barrier requires the expression of internalin (InlA) on *Lm* surface, which mediates, upon the interaction with its receptor E-cadherin (Ecad) [5-7], its translocation across mucus-secreting goblet cells (GCs) of intestinal villi [8,9]. The conjugated action of InlA and InlB, another *Lm* surface protein whose receptor is the hepatocyte growth factor receptor Met [10], is required for *Lm* crossing of the placental barrier [11,12]. The interaction of InlA and InlB with their respective receptors are species-specific: InlA can interact with human, guinea pig and gerbil Ecad but not with mouse or rat Ecad [7,13], while InlB can interact with human, mouse and gerbil Met receptor but not with guinea pig or rabbit Met [10,14].

Because of the species-specificity of InlA-Ecad interaction, most studies aimed at deciphering innate immune responses to *Lm* in mice have been performed upon intravenous inoculation and thus focused on listeriosis systemic phase [15-17]. To circumvent InlA-Ecad species-specificity, our lab developed humanized mouse lines expressing human Ecad or a humanized E16P mouse Ecad, allowing the study of human listeriosis in mouse models permissive to InlA [9,11]. Wollert et al. also "murinized" InlA ($InlA^m$) so that it can interact with mouse Ecad, providing an alternative tool to study mouse *Lm* infection at the gut and placenta levels [18,19]. However, $InlA^m$ also interacts with mouse N-cadherin, and this alters *Lm* cell tropism and host responses [18,19].

Lm is a prototypic inducer of the cellular adaptive responses, whose antigen-specific effectors are cytotoxic CD8+ T cells [15,20]. Innate immune responses also play a role in modulating the efficiency of adaptive cellular immune responses [21]. Deciphering innate immune responses to *Lm*, and their impact on its dissemination and persistence in host tissues is important to fully decrypt the pathophysiology of listeriosis.

Innate immune responses at the gut level

In contrast to other enteropathogens such as *Shigella* or *Salmonella* [22,23], and consistent with the paucity of intestinal symptoms in patients with listeriosis, *Lm* does not induce overt inflammation in the gut [1,24]. Moreover, oral infection with *Lm* leads to little if any barrier damages [9,18,24].

On top of the antibacterial activity of stomach acidity and biliary salts, *Lm* is faced upon ingestion to the gut microbiota which exerts a colonization resistance effect through competition for space, food and by producing bacteriocins that act as antimicrobial peptides [25]. Paneth cells and intestinal epithelial cells (IECs) also produce antimicrobial peptides such as the bactericidal lectin RegIIIγ, in a MyD88 dependent manner [26-28], and some alpha-defensins like cryptdins, in a NOD2-dependent manner [29]. These peptides accumulate in the mucus layer, killing *Lm* before it can reach the intestinal villus surface. Mucus also constitutes a physical barrier that prevents *Lm* from reaching the intestine cell wall and its thickness is controlled by innate immune pathways. Indeed, upon infection, type 3 innate lymphoid cells (ILC3) signal via lymphotoxin-β to GCs, leading to mucus secretion that protects epithelial cells [30].

Lm has two intestinal portals of entry: one is specific and relies on active InlA-dependent cell targeting of GCs cells, which express luminally-accessible Ecad [8], and one is non-specific and relies on the phagocytic activity of M cells in Peyer's patches (PPs) (Figure 1). Crossing of GCs by *Lm* has been shown to occur *via* transcytosis, and to be rapid [8]. That *Lm* does not seem to exit the vacuole in these cells may be due to a lack of time for LLO to lyse the vacuole, a too low level of LLO expression and/or activity, as the gamma-interferon-inducible lysosomal thiol reductase GILT necessary to activate LLO is not expressed fast enough [31] and/or at a sufficient level in those cells [32]. This is expected to account for the almost complete absence of InlA-dependent intestinal tissue response, considering the limited capacity of *Lm* to activate intracytosolic PRRs during intravacuolar transport [8,24]. Moreover, *Lm* peptidoglycan N-deacetylation by *pgdA* and O-acetylation by *oatA* also mediates *Lm* resistance and evasion from innate immune defenses [33,34] together with InlC and InlH, which dampen NF-κB signaling and IL-6 production, respectively [35,36]. Accordingly, neutrophils are only very modestly recruited in the intestinal villus *lamina propria* upon oral infection with *Lm* [18]. Indeed, host responses to *Lm* in the gut are

fully dependent on the infection of PPs, in an LLO-dependent and InlA-independent manner [24]. These conclusions have been drawn from experiments involving reference laboratory strains, and further studies with clinically-relevant strains are therefore needed to determine their relevance to human listeriosis [37].

Once translocated across PPs' M cells, *Lm* infects professional phagocytes, in particular CX3CR1$^+$ macrophages, and reaches their cytoplasm through the action of the pore-forming toxin LLO [38]. In response to *Lm* infection of PPs, CX3CR1$^+$ cells express IL-12, leading to the production of IFNγ by small intestine NK cells and ILC1, in a STAT4-dependent manner [39], inducing a local inflammatory response and the recruitment of neutrophils and monocytes that will control the infection [40]. IFNγ is a critical cytokine involved in host response to *Lm,* most likely by enhancing bactericidal activities of myeloid cells [41,42]. Inhibiting its action, by a blocking antibody or gene knock-out, renders mice highly susceptible to orally acquired listeriosis in mice non permissive to InlA, in which *Lm* enters host tissues exclusively through PP [38,40]. Infected PPs' CX3CR1$^+$ cells also express IL-23, triggering to the production of IL-22 by ILC3s [38-40] and IL-11 by GP38$^+$ stromal cells, which were recently identified as participating in innate immune responses in the gut [38]. The conjugated activation of STAT1 by IFNγ and STAT3 by IL-22 and IL-11 in the intestinal epithelium leads to an accelerated IEC renewal and a consequent decrease in the number of mature GCs, thus limiting *Lm* InlA-dependent translocation across these cells and the resulting systemic dissemination [38] (Figure 1). This reveals an unsuspected functional link between the sensing of *Lm* infection at the PPs and the inhibition of *Lm* entry at the villus level. This innate immune response towards *Lm* has a cost for the host, since IL-22 and IL-11 synthesis in the colon tissue upon *Lm* infection [38,39] leads to a decrease in mucus thickness, and a corresponding increase in host susceptibility to colitis [38]. IFNγ has been shown to control mucus secretion upon infection by other pathogens such as *Salmonella* [43-45] and *Citrobacter rodentium* [46]. However, since *Lm* crosses the intestinal barrier through GCs, IFNγ also has a specific effect on *Lm* entry, by locking its portal of entry [38]. Once *Lm* has reached the *lamina propria*, it can disseminate to the liver and spleen, and in the pregnant host to the placenta.

Innate immune responses at the placenta level

Maternal-neonatal listeriosis has very severe consequences on pregnancy outcome, as 95% result in fetal/neonatal adverse effects [1]. It occurs mostly sporadically [1,47], yet large outbreaks linked to a single contaminating source can occur in countries where listeriosis surveillance is not as strict as in Western countries. The largest outbreak reported so far occurred in South Africa in 2017, where almost 500 cases of maternal-neonatal listeriosis were reported (50% of total cases) [48]. Infection is thought to occur at any time during pregnancy but is most often diagnosed in its second and third trimesters [49]. Depending on pregnancy stage, it can lead to abortion, stillbirth, preterm labor and/or fetal/neonatal infection [1,47]. Almost no maternal symptoms are present before fetal-placental infection manifests [1].

The placenta is made of fetal cells that organize a selective barrier that either blocks or actively transports the molecules needed for fetal growth and development. It blocks maternal and fetal cells from mixing and acts as an immune barrier to ensure immune tolerance towards the fetus. Maternal immune cells are found in the decidua, at the maternal-fetal interface. They consist mainly of NK cells (around 70%), macrophages (around 20%) and T cells [50].

During the second and third trimesters, a symbiotic balance is reached between the mother and the fetal-placental unit. Decidual immune cells shift from an inflammatory state during implantation and placentation to an anti-inflammatory (T_H2) state along pregnancy. This T_H2 environment is critical for healthy pregnancy as inflammation at this stage can result in miscarriage, stillbirth or preterm birth [50]. Upon infection, immune cells in the placenta will have to maintain fetal tolerance while also preventing infectious pricesses to reach the fetus.

As a facultative intracellular bacterium, *Lm* accesses the placenta free or in circulating myeloid cells. *Lm* is able to infect the syncytiotrophoblast, the most outer cells of the placenta (Figure 2) [11,51]. The syncytiotrophoblast is an epithelial syncytium that derives from the fusion of trophoblastic cells, is bathed in maternal blood and materializes the placental barrier. *Lm* crosses the placental barrier through the conjugated action of InlA and InlB *ex vivo* in human placental explants and *in vivo* in permissive rodent models such as gerbils and KIE16P mice [11,51]. This indicates that extracellular bacteria can interact with their host cell receptors Ecad and Met at the placental barrier level. InlA-mediated *Lm* internalization into cells requires PI3-kinase [52]. In trophoblast cells, PI3K basal activity is low, and InlB, acting as an

agonist of HGF on its receptor Met, activates PI3K, allowing InlA-mediated entry in the syncytiotrophoblast [12]. In contrast, at the intestinal level, GCs exhibit a constitutive PI3K activity, accounting for the absence of contribution of InlB in InlA-dependent translocation across GCs *in vivo* [12]. Upon infection, trophoblasts produce CSF-1 and MCP-1 leading to the recruitment of maternal neutrophils and macrophages [53,54]. What triggers expression of CSF-1 and MCP-1 in infected trophoblast is currently unknown. In the spleen, induction of MCP-1 by *Lm* requires its access to the cytosol (LLO-dependent) and is MyD88-independent [55]. Whether a similar process occurs in the placenta remains to be investigated. Macrophages, through TNF-α and IL-12, signal to decidual NK cells to produce IFNγ, increasing their bactericidal activity against *Lm* [56] (Figure 2). In decidual macrophages, *Lm* infection induces the expression of Perforin-2, a membrane-attack-complex-perforin-containing factor, that mediates the killing of intracytoplasmic *Lm* [57]. However, Perforin-2 can also trigger miscarriage. Therefore, Perforin-2 contributes to protect both the mother and the fetus in case of mild infection, but can trigger fetal expulsion in case of an uncontrolled infection, resulting in maternal protection at the expense of fetal survival. Infected placental cells also produce IL1-β and activate the inflammasome in recruited monocytes, helping clear *Lm* [58]. Infection of cultured trophoblast cells by *Lm* also leads to the induction of the type III interferons (IFNλ) [59]. *Ifnλ2 and Ifnλ3* transcripts are expressed in placental homogenates from infected KIE16P mice, activating the transcription of interferon stimulated genes (ISG) [59]. IFITM is an ISG which has been shown to block syncytiotrophoblast formation, impair placental development and function, and favor fetal growth retardation and miscarriage [60]. Yet, the contribution of IFITM production in *Lm*-infected placenta to placental dysfunction and miscarriage remains to be established. Decidual NK cells can also selectively kill *Lm* directly in the infected trophoblast without killing infected cells [61]. Indeed, they can transfer granulysin, an antimicrobial peptide, via nanotubes directly into infected cells (Figure 2), and granulysin can kill cytosolic *Lm* present without damaging the infected cell. Here, innate immune responses are expected to allow controlling infection without altering the placental barrier. Indeed, transgenic mice expressing granulysin show a lower bacterial burden in the placenta compared to wild-type mice upon *Lm* infection and are resistant to *Lm*-induced abortion [61].

Innate immune responses at the blood brain barrier and in the CNS

In elderly people or immunocompromised individuals, *Lm* can also cross the blood-brain barrier and induce neurolisteriosis, characterized by meningo-encephalitis [1]. As for maternofetal infection, neurolisteriosis is rare, yet its mortality rate is high, up to 30%, with almost 50% of surviving patients presenting with neurological sequelae [1], a frequency amongst the highest for CNS infections [62]. How *Lm* invades the CNS and the nature and impact of the resulting immune responses remain poorly understood. Experiments in mice treated with gentamicin suggest that bloodborne *Lm* reaching the blood-brain barrier are located intracellularly [63]. Leukocytes transfer experiments have shown that infected inflammatory monocytes can carry *Lm* to the CNS [64,65]. The targeting of *Lm*-infected monocytes to the CNS is IFNγ-dependent but CCR2-independent [66]. Once at the endothelial barrier level, it remains unclear if *Lm* reaches the parenchyma by cell-to-cell spread in an ActA-dependent manner, or by transmigration of infected monocytes through the endothelial barrier [67]. In *ex vivo* organotypic slices and *in vitro* models of infection, *Lm* can infect microglial cells, the resident macrophages of the brain [68,69]. Infected microglial cells secrete inflammatory cytokines such as TNF-α and MCP-1 upon infection. The consequences of cytokines production by microglia as well as the impact of infection on the fate of these cells remain unknown. In the liver, resident macrophages known as Kupffer cells, actively phagocyte *Lm*, leading to their rapid death, triggering the recruitment of inflammatory monocytes. *Lm* infection of the liver is first controlled by an M1 pro-inflammatory response, followed by a M2 polarization of infiltrating monocytes allowing their proliferation and differentiation into macrophages [70]. Whether a similar process takes place in the CNS during neurolisteriosis remains to be investigated. Pro-inflammatory signaling in the CNS might be deleterious and induce long-term neuronal damages [71] and the renewal of microglial cells may lead to altered immune functions in the CNS [72].

The fact that reference lab strains are poorly neuroinvasive have so far prevented detailed mechanistic *in vivo* studies [37]. CNS infection occurs with these strains only in immunocompromised animals or upon systemic injection of a very large inoculum. Conclusions obtained with these strains are therefore not necessarily transposable to clinical neuroinvasive and clinically relevant isolates, and the reasons for their enhanced neuroinvasiveness remains poorly understood. Characterization of CNS invasion by these neuroinvasive isolates and the resulting immune responses will

bring important insights into neurolisteriosis, a deadliest infection in human and a model infection [1].

Conclusions

Lm is a model microorganism that played a critical role in the discovery and the characterization of the adaptive immune system [15]. From its ingestion with contaminated food up to its final target organs the CNS and the fetal-placental unit, it is exposed to innate immune defenses. Deciphering the impact of innate immune responses on *Lm* in the *in vivo* context is key to fully understand the biology of listeriosis. As illustrated in this non-exhaustive review, *Lm* has a propensity to evade innate immune responses, which favors its silent dissemination in host tissues. Yet, innate immune responses play a key role in orchestrating host defenses against *Lm*, both in a direct way by the action of antibacterial effectors, and indirectly as a trigger of anti-*Lm* sterilizing and protective adaptive cell-based immunity.

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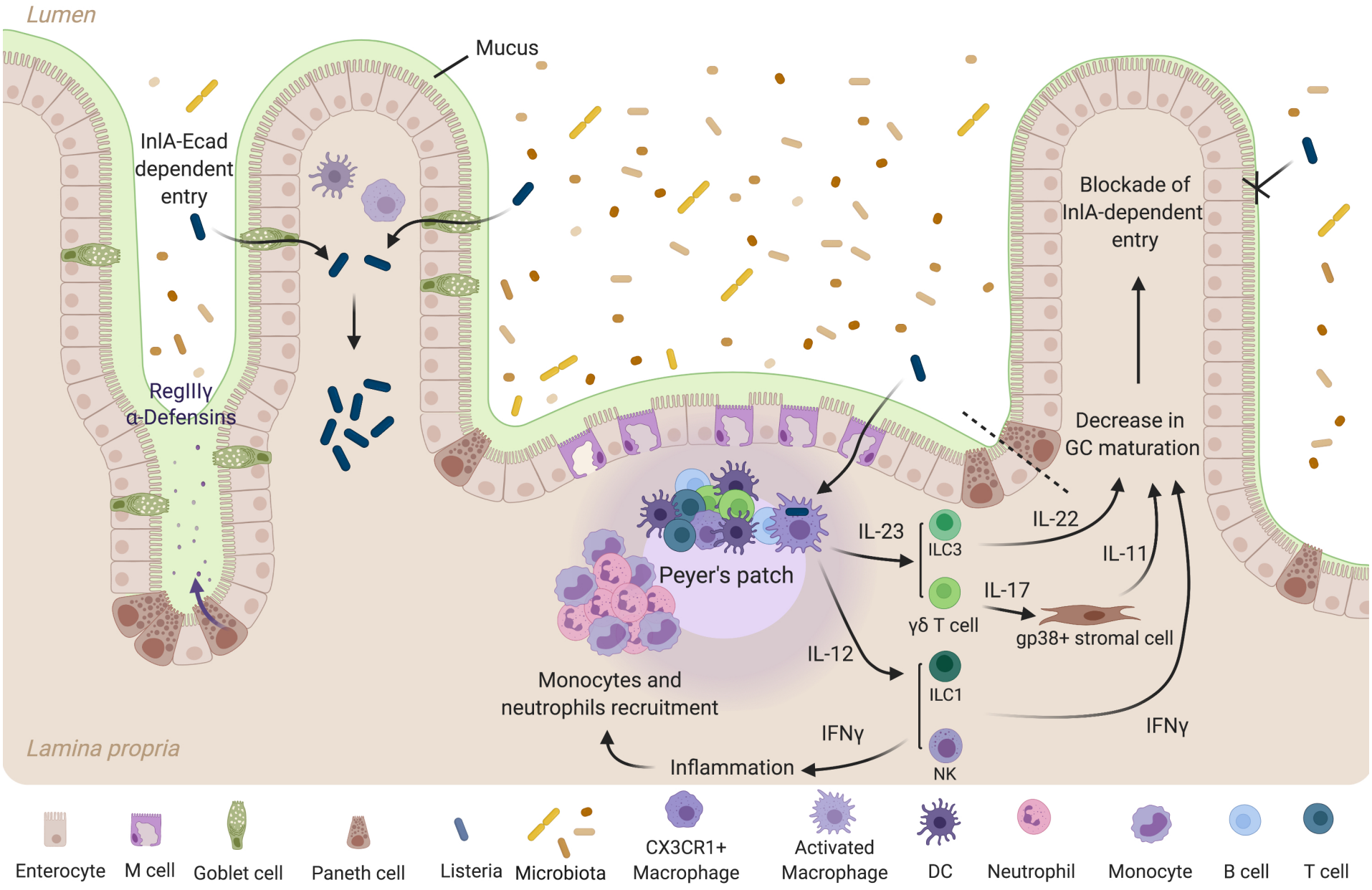
Figure legends:

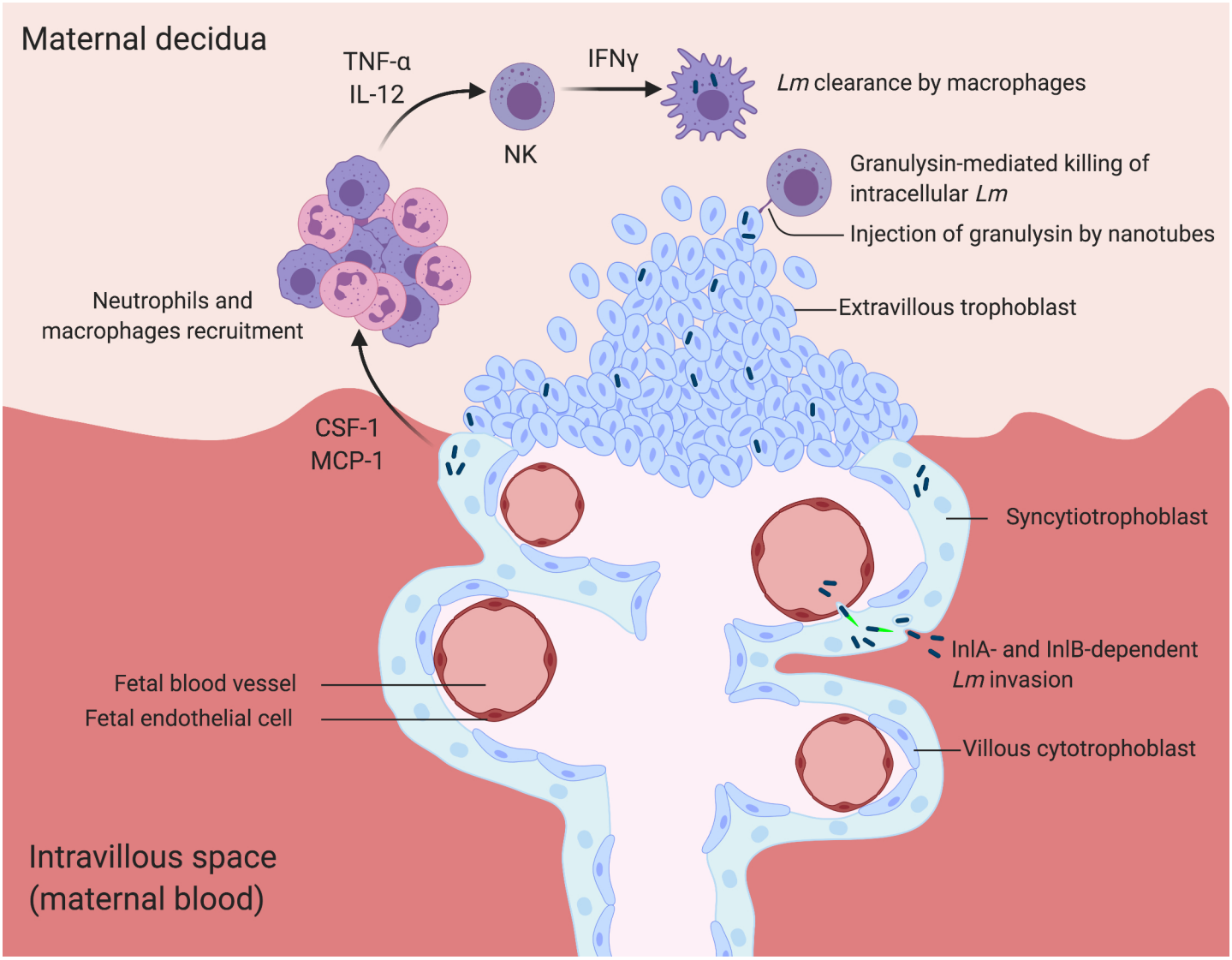
Figure 1: Innate immune responses in the gut

Lm can cross the intestinal barrier via two portals of entry. *Lm* crosses villi in an InlA-dependent manner, through mucus-secreting globlet cells, replicate in the *lamina propria* and disseminates into deeper organs. *Lm* can also be phagocytosed by M cells in PPs and infect CX3CR1^{+} macrophages. This leads to the blocking of InlA-dependent entry of *Lm*. In addition, IFNγ is involved in the recruitment of neutrophils and inflammatory monocytes in PPs.

Figure 2: Innate immune responses at the placental level

Maternal blood-borne *Lm* infects the syncytiotrophoblast in an InlA- and InlB-dependent manner, allowing its crossing of the placental barrier and access to fetal circulation. Infected syncytiotrophoblast produces CSF-1 and MCP-1 which lead to the recruitment of neutrophils and macrophages. These cells, through TNF-α and IL-12 signaling, induce the production of IFNγ by decidual NK cells, the major decidual innate immune effector cell. IFNγ increases the bactericidal activity of decidual macrophages, and *Lm* killing. *Lm* can also infect extravillous trophoblast present in the decidua. NK cells can directly kill *Lm* without killing these infected extravillous trophoblast, via the delivery, through nanotubes, of granulysin, which acts as an antimicrobial peptide.


Lumen
Mucus
InlA-Ecad dependent entry
RegIIIγ α-Defensins
Blockade of InlA-dependent entry
Decrease in GC maturation
Peyer's patch
IL-23
ILC3
IL-22
IL-11
IL-17
γδ T cell
gp38+ stromal cell
IL-12
ILC1
Monocytes and neutrophils recruitment
IFNγ
IFNγ
Inflammation
NK
Lamina propria
Enterocyte
M cell
Goblet cell
Paneth cell
Listeria
Microbiota
CX3CR1+ Macrophage
Activated Macrophage
DC
Neutrophil
Monocyte
B cell
T cell


Maternal decidua
TNF-α
IL-12
NK
IFNγ
Lm clearance by macrophages
Granulysin-mediated killing of intracellular Lm
Injection of granulysin by nanotubes
Extravillous trophoblast
Neutrophils and macrophages recruitment
CSF-1
MCP-1
Syncytiotrophoblast
InlA- and InlB-dependent Lm invasion
Fetal blood vessel
Fetal endothelial cell
Villous cytotrophoblast
Intravillous space (maternal blood)

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