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Für alle Frauen, die einen Kindsverlust
aufgrund von Listeriose zu beklagen haben.

Abstract

The foodborne pathogen *Listeria monocytogenes* (Lm) is a ubiquitously distributed bacterium causing severe morbidity and mortality in the fetus (fetal listeriosis). Lm can overcome the maternal-fetal barrier by passing the placental trophoblast layer leading to fetal infection and frequently to spontaneous pregnancy loss.

Several hypotheses have been suggested how Lm is able to migrate through the trophoblast layer, but detailed molecular mechanisms have not been elucidated so far. In this project, the aim is to discover the exact mechanisms of placental and fetal infection and identify new targets to treat pregnancy-associated listeriosis.

To do this, *in vitro* and *in vivo* experiments were combined with fluorescence-based imaging methods, to decipher the initial invasion steps of Lm at the maternal-fetal barrier. For this purpose, fluorescence reporter strains of Lm were used together with a humanized mouse model expressing human E-Cadherin (E-Cad), suggested to play a key role in cellular adhesion of Lm. Mechanisms of Lm invasion and migration as well as identification of responsible immunological interaction partners were uncovered by performing two-photon laser scanning microscopy (2PM), fluorescence activated cell sorting (FACS) analysis, and a variety of modified gentamicin protection assays *in vitro* and *in vivo*.

In vitro experiments identified neutrophils as a shuttle for Lm to the placenta. Uptake of Lm by neutrophils was increased by complement factor C3.

Neutrophils act as a survival niche and as a viability factor for Lm in the intravascular compartment and mediate the ‚transfer' of Lm into trophoblasts (HTR8 cells), discovering an interesting aspect of neutrophil function.

Blocking adhesion of neutrophils or their depletion *in vivo* impaired placental and fetal infection in the humanized E-Cad mouse model, demonstrating that neutrophils are crucial for placental and fetal infection with Lm. Using a Lm mutant strain defective in binding to human E-Cad also showed a decrease of placental and fetal infection in the humanized E-Cad mouse pointing to a substantial function of human E-Cad for Lm infection of placenta and fetus.

In conclusion, this work postulates a trojan horse mechanism by Lm which succeed in hijacking neutrophils enabling infection of the placenta. These findings help to understand of how Lm overcome the placental barrier and might lead to the development of new therapeutic approaches to minimize listeriosis in pregnant women and fetuses.

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Abbreviations

Aa	Amino acid
ActA	Actin assembly-inducing protein
ANOVA	Analysis of Variance
APC	Allophycocyanin
Arp2/3 complex	Actin Related Protein 2/3 complex
BHI	Brain heart infusion
BLP	Lactobacillus casei probiotic
BSA	Bovine serum albumine
BV	Brilliant violet
CEA	Carcinoembryonic antigen
CEACAM	Carcinoembryonic antigen-related cell adhesion molecule
CFU	Colony forming unit
CM	Chloramphenicol
CMV	Cytomegalovirus
C1q	Complement component C1q
CR	Complement receptor
CTB	Cytotrophoblast
CVF	Cobra venom factor
D	Decidual
DAMPS	Damage-associated molecular patterns
DAPI	4,6-Diamidino-2-phenylindole
DMSO	Dimethyl sulfoxid
dNKs	Decidual natural killer cells
ePE	Early onset preeclampsia
EDTA	Ethylendiaminetetraacetic acid
E-Cad	E-Cadherin
ECM	Extracellular matrix
EGD	Everitt George Dunne
EVT	Extravillous cytotrophoblast
EC1	Extracellular domain EC1
Fab	Antigen binding fragment
FACS	Fluorescence associated cell sorting

FITC	Fluorescein isothiocyanate
FCS	Fetal calf serum
FcyR	Fcy receptor
GPCR	G-protein coupled receptor
H	Hour
HBSS	Hanks balanced salt solution
HIV	Human immunodeficiency virus
Hpt	Hexose phosphatase translocase
HUVECs	Human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecule-1
iCTB	Invasive cytotrophoblast
Ig	Immune globulins
IL	Interleukin
Inl	Internalin
InlA	Internalin A
InlB	Internalin B
InlC	Internalin C
InlP	Internalin P
Interferon	IFN
i.p.	Intraperitoneal
i.v.	Intravenous
IVM	Intravital microscopy
IVS	Intervillous space
Ki	Knock-in
LFA-1	Leukocyte function-associated antigen-1
LLO	Listeriolysin O
LLRs	Leucine-rich repeats
Lm	Listeria monocytogenes
LPS	Lipopolysaccharide
Mac-1	Macrophage-1 antigen
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
min	minute
MIP	Monocyte inflammatory protein

MNT	Mononucleated trophoblast
MoDC	Monocyte derived dendritic cells
Mpl	Metalloprotease
NETs	Neutrophil extracellular traps
NK cells	Natural killer cells
OatA	O-acetyltransferase A
OD	Optical density
ON	Over night
PAMPS	Pathogen-associated molecular patterns
PgdA	Peptidoglycan N-deacetylase
Plc	Phospholipase C
PB	Pacific blue
PBMC	Peripheral blood mononuclear cells
PE	Phycoerythrin
PECAM-1	Platelet endothelial adhesion molecule-1
PFA	Paraformaldehyde
PFU	Placental fetal unit
PGN	Peptidoglycan
PI3-K	Phosphoinositide 3-kinase
PIP2	Phosphoinositide-4,5-bisphosphate
PIP3	Phosphoinositide-3,4,5-trisphosphate
PMA	Phorbol-12-myristate-13-acetate
PMN	Polymorphonuclear cell
PP13	Placental protein 13
PRR	Pattern recognition receptor
PSG	Pregnancy specific glycoprotein
PVDF	Polyvinylidene fluoride
RIPA	Radioimmunoprecipitation assay
RT	Room temperature
RTE	Ready to eat
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Sec	Seconds
SEM	Standard error of the mean
SCR	Short consensus repeat

SNPs	Single nucleotide polymorphisms
SpT	Spongiotrophoblast
SYN	Syncytiotrophoblast
TGC	Trophoblast giant cell
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α
TRITC	Tetramethylrhodamine
2PLSM	Two-photon laser scanning microscopy
WBC	White blood cell count
WT	Wild type

1. Introduction

1.1. Listeria

Listeria are Gram-positive, facultative anaerobic rods (1-2 µm in length) with a low guanine-cytosine content (Schardt *et al.*, 2017). They are acapsular and non-spore forming bacteria (Vázquez-Boland *et al.*, 2001). The genus Listeria consist of 17 members. Depending on their bacterial characteristics, they can be divided into two groups (Schardt *et al.*, 2017). The first one is called Listeria sensu lato, which includes 11 Listeria species that were described since 2009 (*L. marthii*, *L. rocourtiae*, *L. weihenstephanensis*, *L. grandensis*, *L. riparia*, *L. booriae*, *L. fleischmannii*, *L. floridensis*, *L. aquatica*, *L. newyorkensis*, and *L. cornellensis*) and are predicted to occur in the environment and are non-zoonotic and non-pathogenic organisms (Orsi and Wiedmann, 2016). The second group, Listeria sensu strictu, comprises the other six members *L. monocytogenes*, *L. seeligeri*, *L. marthii*, *L. ivanovii*, *L. welshimeri*, and *L. innocua* (Orsi and Wiedmann, 2016). Two of them, *L. monocytogenes* and *L. ivanovii*, are infectious pathogens of which the latter´s host are ruminants (Rocha *et al.*, 2017; Schardt *et al.*, 2017). The other one, *Listeria monocytogenes* (Lm), is a human pathogenic bacterium, whose molecular characteristics and relationship are used to classify Listeria species into the two groups (Chiara *et al.*, 2015; Orsi and Wiedmann, 2016).

1.1.1. *Listeria monocytogenes* strains EGD and EGDe

In 1924 Professor Everitt George Dunne (E.G.D) Murray and colleagues isolated the first Listeria strain from infected laboratory rabbits and guinea pigs. They named the unknown Gram-positive Bacillus *Bacterium monocytogenes* due to the clinical observation of mononuclear leukocytosis (Murray, Webb and Swann, 1926). It was H. Pirie who changed the name to *Listeria monocytogenes* (Pirie, 1940). For this thesis text, I will use the term Listeria or Lm for *Listeria monocytogenes*.

Because E.G.D Murray discovered the first Listeria strain, it was termed ‘EGD’. EGD strains from the United States-based Trudeau Institute were passaged through mice by scientists to keep up the virulence (Bécavin *et al.*, 2014). In 2001, that EGD strain was chosen for sequencing by the European consortium in the course of the genome sequencing project (Glaser *et al.*, 2001). Afterwards, Trinad Chakraborty tested again its virulence in mice and named that strain EGDe, in which the ‘e’ refers to ‘European’ (Bécavin *et al.*, 2014).

Worldwide, EGD and EGDe are the most commonly used Listeria strains to study host pathogen interactions. Both strains are of the same serovar (1/2a) but are described as ‘genetically highly

distinct' regarding their amount of single nucleotide polymorphisms (SNPs) (Bécavin *et al.*, 2014). In contrast to EGDe, EGD harbors a point mutation in the transcriptional PrfA regulon, so consequently major virulence genes are constitutively overexpressed (Bécavin *et al.*, 2014). It is further described that this overexpression enhances EGD invasiveness in human trophoblast cells (e.g. JEG3), whereas EGDe shows a higher bacterial burden in murine organs or blood counts after infection (Bécavin *et al.*, 2014).

Based on these findings, in this project, the EGD strain was used for human *in vitro* experiments and EGDe for murine *in vivo* studies.

1.1.2. Environmental resistance of *Listeria monocytogenes*

Listeria are highly resistant to environmental factors (e.g osmotic stress, bacteriocins, high hydrostatic pressure) and can tolerate very low and high temperatures (-0.4 °C – +45 °C) as well as a broad pH range (Bucur *et al.*, 2018). The fact that they are motile at temperatures from 10 °C to 25 °C underlines their ability of survival in many enviromental niches (Vázquez-Boland *et al.*, 2001). One can find them ubiquitously distributed in e.g. soil, water, feces, food and the intestinal tract of mammals (Vivant, Garmyn and Piveteau, 2013).

Using soil as a reservoir (agricultural areas) point to their saprophytic existence (Welshimer and Donker-Voet, 1971). As saprophytes they developed sophisticated mechanisms and were able to transfer from soil to animals and humans via contaminated food (Drolia and Bhunia, 2019). Despite food processing Listeria contaminated food is a complex problem not solved yet because it is hard and expensive to control conditions of the whole food chain (Vivant, Garmyn and Piveteau, 2013; Bucur *et al.*, 2018). Especially poor storage conditions of food cause outbreaks from time to time underlined by current cases in South Africa (Thomas *et al.*, 2020).

1.1.3. Infection in mammals/humans

The foodborne bacterium is not only resistant to its environment, but also highly adapted to its hosts. This becomes evident by the fact that upon entry into host cells at 37 °C the PrfA master regulon activity switches on (Vázquez-Boland *et al.*, 2001). This tightly regulated component directly and indirectly controls the expression of hundreds of the most important virulence genes (Ermolaeva *et al.*, 2004; Portman *et al.*, 2017). Thus, because of its host adaption *Listeria monocytogenes* became one of the most life threatening human and zoonotic pathogens (Vivant, Garmyn and Piveteau, 2013).

Listeria cause one of the most severe bacterial diseases called listeriosis with a mortality rate of up to 30 % (Hamon, Bierne and Cossart, 2006; Bonazzi, Lecuit and Cossart, 2009). This is based on

the fact that listeriosis is a rare disease often remaining under-diagnosed (Bonazzi, Lecuit and Cossart, 2009). The predicted incubation time for maternal-fetal listeriosis lasts from 19 to 28 days (Charlier, Disson and Lecuit, 2020). Most healthy immunocompetent individuals suffer from none to mild symptoms including spontaneously dissolving gastroenteritis or flu-like symptoms (Radoshevich and Cossart, 2017). However, in elderly people, pregnant women, fetuses and immunocompromised humans (e.g. due to HIV infection) listeria infection can lead to sepsis, meningitis or encephalitis (Hamon, Bierne and Cossart, 2006). In case of pregnancy, severe consequences such as pregnancy complications due to neonatal infection, preterm labor or death of the fetus can occur (Lamond and Freitag, 2018).

Once orally ingested via contaminated food, such as non-pasteurized cheese or other ready to eat meals/products, they affect epithelial cells of the intestinal tract (Lecuit, 2020). Via interaction of Lm with apical intestinal villi and intracellular transfer through goblet cells they cross the intestine (Nikitas *et al.*, 2011; Drolia and Bhunia, 2019). After passing the intestinal barrier they infect the mesenteric lymph nodes and spread via portal vein and blood to the liver and the spleen (Bonazzi, Lecuit and Cossart, 2009). There they are either eliminated by phagocytes (e.g. neutrophils or Kupffer cells) or non-phagocytes (e.g epithelial cells) in case of a healthy organism, or they infect other organs like the brain or in case of pregnancy the placenta (Radoshevich and Cossart, 2017). This way, they can cross the three major barriers in humans: 1. the intestinal, 2. the blood-brain, and 3. the placental barrier (Figure 1) (Gessain *et al.*, 2015).

The latter plays a crucial part in the complex maternal fetal interface (1.2).

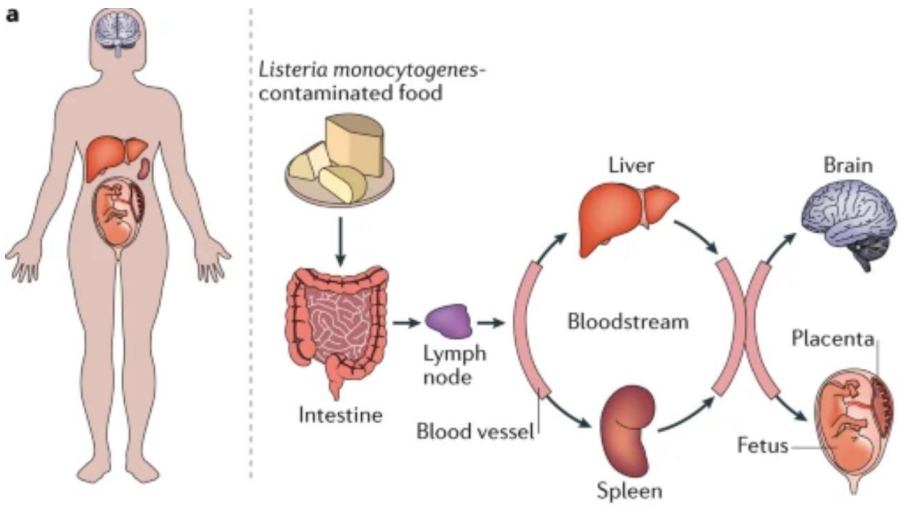


Figure 1. Natural infection cycle of *Listeria monocytogenes* (Lm) in the human host.
Once orally ingested, Lm infects the intestine. In case of crossing the gastro-intestinal barrier, transfer to the liver and spleen occurs. Either phagocytes (polymorphonuclear leukocytes (PMNs), macrophages, dendritic cells) or non-phagocytes (enterocytes, hepatocytes) eliminate the bacteria, or they disseminate to other organs e.g. the placenta, where they are able to overcome the maternal-fetal barrier. In case of listeriosis during pregnancy spontaneous abortion, stillbirth, and preterm labor can occur. Figure obtained from (Radoshevich and Cossart, 2017).

1.2. Maternal fetal interface: a unique environment that still is an immunological enigma

Protecting the fetus against pathogens, but also avoiding rejection of a genetically distinct (hemi allogeneic) embryo (allograft), is a huge challenge for the maternal immune system during pregnancy (Ander, Diamond and Coyne, 2019). Disbalances of this system can cause pregnancy complications like unexplained misscarriage, intrauterine growth retardation, preeclampsia, placenta accreta and in the worst case maternal or fetal death (Silva and Serakides, 2016; Knöfler *et al.*, 2019).

The exceptional immunological condition of pregnancy was often compared to a classical organ transplantation. However, the immunological paradox of pregnancy is more complex than 'just' an organ transplantation (Moffett and Loke, 2006). To be precise, it is not just the immunological interplay between mother and fetus itself, as often described. It is the interaction and exchange between the maternal and fetal part (Moffett and Loke, 2006). For that purpose, maternal immune cell responses need to be restricted. The composition and functions of maternal leukocytes during pregnancy differ (PrabhuDas *et al.*, 2016). Within the decidua, 70 % of immune cells are decidual NK cells (dNK cells), about 20 % decidual macrophages and less than 10 % are T-cells (Lamond and Freitag, 2018). These leukocytes also show a different phenotype compared to the non-pregnant state (Ander, Diamond and Coyne, 2019). They seem to be immunologically suppressed. For example, dNKs facilitate remodeling of the spiral arteries and sense interferon gamma (IFNγ) during that process, which dampens the activation of non-dNKs in the environment. Also cytokines like interleukin 10 (IL-10) are produced by dNKs, which cause differentiation and silencing of decidual macrophages (Lamond and Freitag, 2018). In addition, the amount of regulatory T-cells (Treg cells) increases to support fetal tolerance (PrabhuDas *et al.*, 2016).

Furthermore, the fetal system contributes to that unique immunological environment. The trophoblast cells are of high relevance in creating the special immunological conditions during pregnancy (PrabhuDas *et al.*, 2016). Trophoblasts inactivate the paternal X chromosome and express endogenous retroviral products and oncofetal proteins (e.g. carcinoembryonic antigens) (Moffett and Loke, 2006). They secrete hormones to change the metabolism of the mother and e.g. mobilize nutrients for fetal supply (Knöfler *et al.*, 2019). Major histocompatibility complex (MHC) class II antigen expression and instead of this a variable MHC class I expression (Moffett and Loke, 2006). Those are predicted to be the ligands for receptors on the dNK cells and seem to be also the pivotal regulator in allowing the coexistence of mother and fetus (Moffett and Loke, 2006).

1.3. Placental Barrier

The placenta has an important role during pregnancy, because it represents the major barrier between the maternal and fetal part with essential functions enabling the successful development and birth of the embryo. It is a chimeric organ, because it consists of both, maternal and fetal cells (Figure 2) (Maltepe, Bakardjiev and Fisher, 2010). The formation of the placenta (placentation) and development of the uterine wall-associated maternal decidua (decidualization), is the initial step to ensure fetal health (Knöfler *et al.*, 2019).

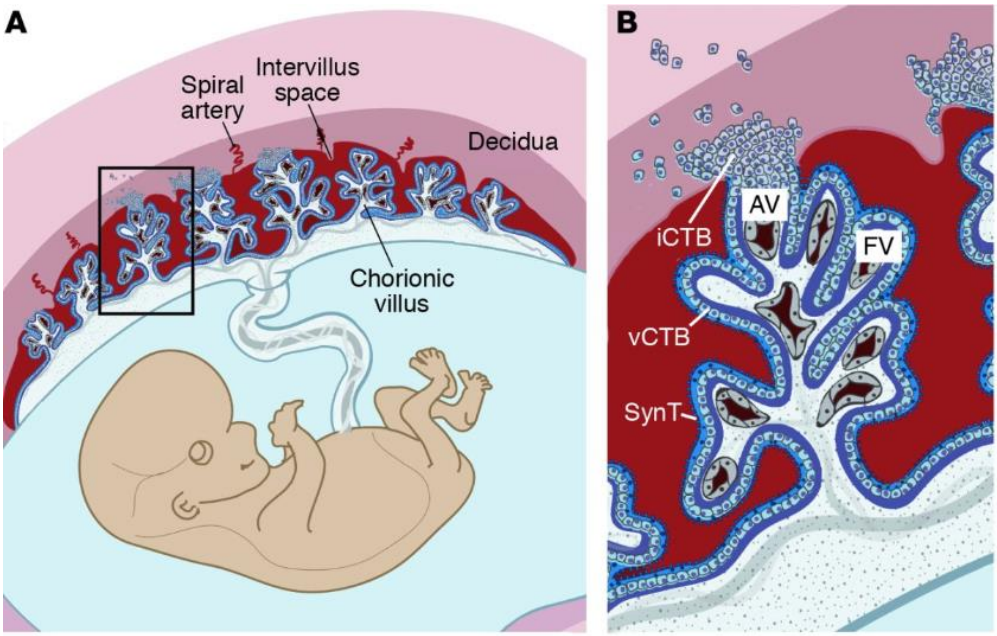


Figure 2. Placental barrier of humans.
A. Orientation of the embryo in the maternal uterus. The decidua lines the uterine wall during pregnancy and presents the maternal part of the barrier. Maternal blood reaches the placental structures via spiral arteries (Robbins *et al.*, 2010). B. Box of placental area of A is enlarged showing detailed placental cell types of the hemochorial placenta with the fetal part in blue and the maternal side in red. Invasive extravillous trophoblasts (iCTB) anchor villi (AV) into the decidua. Floating villi (FV) are covered by the outermost syncytiotrophoblast (SynT), villous cytotrophoblast (vCTB) and a basement membrane (Robbins *et al.*, 2010). Figure obtained from (Emin Maltepe, Anna I. Bakardjiev and Susan J. Fisher, 2010).

1.3.1. Decidualization

The development of the decidua, is called decidualization and originates from ther uterine mucosa (the endometrium) (Moffett and Loke, 2006). Spiral arteries of maternal and fetal origin are remodeled so that placental tissue is bathed in maternal blood. In case of successful implantation, endothelial lining of the vessels is gradually replaced by fetal trophoblasts (Ander, Diamond and Coyne, 2019). Decidualization, which exclusively occurs during hemochorial placentation, is associated with e.g. the migration of a special subtype of NK cells, the dNK cells ($CD56^{hi}CD16^{-}$) into the placenta (Moffett and Loke, 2006). Maternal leukocytes are in the placenta are involved in

spiral artery dilatation which increases blood flow to the placenta (Ander, Diamond and Coyne, 2019).

1.3.2. Placentation

The development of the placenta is called placentation. After implantation, the outer layer (trophoectoderm) of the blastocyst gives rise to trophoblast cells which form the placenta and build the barrier between mother and fetus (Moffett and Loke, 2006). The trophoblasts attach the blastocyst into the decidua during implantation (Ander, Diamond and Coyne, 2019).

After the third week of human pregnancy, the definitive structure of the placenta is formed and consists of two types of villi – the floating and anchoring villi (Figure 2). The trophoblast layer that covers the villous tree and is in direct contact with the maternal blood is called syncytiotrophoblast (SYN) (Ander, Diamond and Coyne, 2019). It is a special multinucleated single cell layer (syncytium) providing exchange of nutrients and waste products as well as oxygen and carbon dioxide between the maternal and the fetal circulation (PrabhuDas *et al.*, 2016). The SYN is a very tight cell layer without cellular junctions and contains a dense actin cytoskeleton to prevent e.g. pathogens of breaching the barrier (Ander, Diamond and Coyne, 2019). Furthermore, the SYN is lined with mononucleated cytotrophoblasts (CTB) and a basement membrane (Knöfler *et al.*, 2019).

CTBs are undifferentiated trophoblasts that can give rise to the SYN by fusion, or they differentiate into another mononucleated trophoblast cell type called extravillous cytotrophoblasts (EVT) (Ander, Diamond and Coyne, 2019). Maternal blood starts to float and directly bath the intervillous space (IVS) after EVT anchor the villous tree (localized at the tips) into the decidua and remodel the lumen of spiral arteries interacting with decidual leukocytes. This happens at the end of the first trimester of gestation (Ander, Diamond and Coyne, 2019; Knöfler *et al.*, 2019). EVTs release factors like proteases and cytokines, because they also need to degrade extracellular matrix (ECM) proteins to facilitate invasion into the decidua. The decidua itself regulates migration via expression of inhibitory proteins (Silva and Serakides, 2016).

Maternal blood in the IVS replaces a fluid that contains uterine gland secretions originating from the endometrium and internalized by the SYN to be used as a nutrient reservoir for the embryo (Ander, Diamond and Coyne, 2019). With floating of the IVS with maternal blood the supply with nutrients and other factors increases to support fetal development, while also the possible contact of fetal tissues with pathogens or other life threatening factors rises (Ander, Diamond and Coyne, 2019).

1.4. Comparison of the human and murine placenta

As humans and mice both have hemochorial placentas (humans show higher invasiveness), mice are chosen as laboratory animals for placental investigations (PrabhuDas *et al.*, 2016). But despite similarities, many differences exist that need to be taken into account (Georgiades, Fergyson-Smith and Burton, 2002). Humans have a hemomonochorial villous placenta whereas mice have a hemotrichorial labyrinth placenta (Figure 3 A,B) (Lamond and Freitag, 2018). In humans, the invasive cytotrophoblasts (iCTB) and the EVTs anchor the villous tree into the decidua and floating villi are bathed in maternal blood. Three layers cover the villous trees. The outer most is the SYN underlined by CTB and a basement membrane (Figure 3 B) (Lamond and Freitag, 2018). In mice, two layers of SYN are located between the maternal and fetal blood, covered outside with a discontinuos mononucleated trophoblast layer (MNT) and underneath with fetal endothelial cells (Lecuit *et al.*, 2004; Lamond and Freitag, 2018). No EVTs anchor the labyrinth shaped placenta into the decidua. In mice trophoblast giant cells (TGC) and spongiotrophoblasts (SpT) fulfill that function (Figure 3 E) (Lamond and Freitag, 2018). In comparison to the human system, where only the SYN is in direct contact with the maternal blood, in the murine system it is the discontinuous MNT and SYN (Lamond and Freitag, 2018).

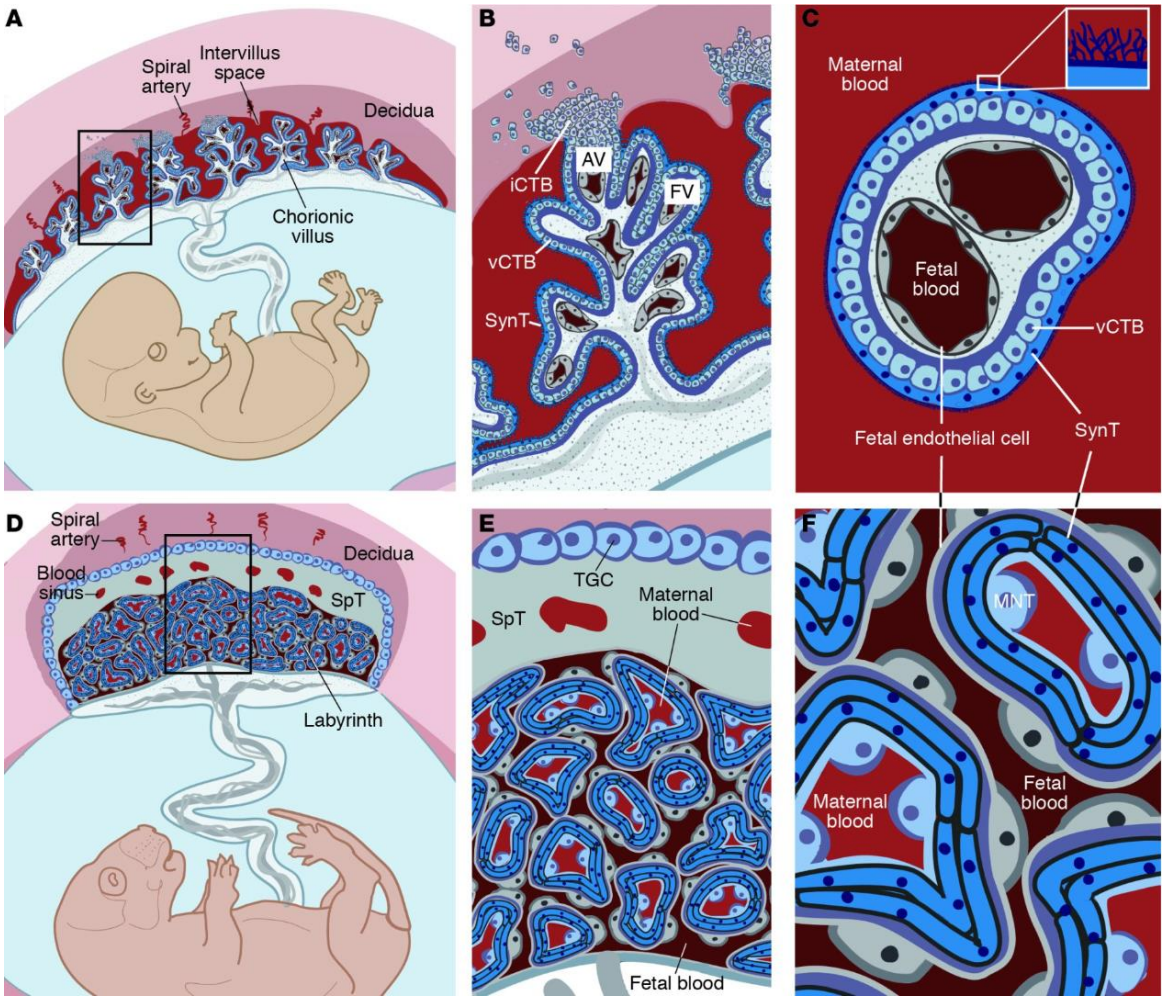


Figure 3. Direct comparison of human (A,B,C) and murine (D,E,F) placental anatomy.
Orientation of the embryo and the structure of the hemochorial placentas with the fetal part in blue and the maternal side in red (Robbins *et al.*, 2010). A,D.The decidua lines the uterine wall during pregnancy . From spiral artieries in the decidua maternal blood reaches the fetal structures and gets in direct contact (Robbins *et al.*, 2010). Boxes of placental areas of A and B are enlarged showing detailed placental cell types. B. Invasive extravillous trophoblasts (iCTB) anchor villi (AV) into the decidua. Floating villi (FV) are covered by the outermost syncytiotrophoblast (SYN), villous cytotrophoblast (vCTB) and a basement membrane. C. This layer touches the stromal cells, which are in contact with the fetal endothelial cells and blood . E. In mice, trophoblast giant cells (TGC) and spongiotrophoblasts (SpT) are the counterpart of the human EVTs and anchor the labyrinth shaped placenta into the decidua. F.Two layers of SYN and one discontinuos mononucleated trophoblast layer (MNT) are bathed with maternal blood (Lamond and Freitag, 2018). Figure obtained from (Emin Maltepe, Anna I. Bakardjiev and Susan J. Fisher, 2010).

1.5. Two controversial hypotheses by which Listeria can traffic and infect the placenta

Although the placenta is a functional highly evolved gatekeeper, which developed plenty of defense mechanisms as described above, some pathogens possess subtle mechanisms to overcome this barrier, one of them is *Listeria monocytogenes* (Lamond and Freitag, 2018).

Currently, there exist two controversial hypotheses how Lm can infect the fetus through the transplacental route (Robbins *et al.*, 2010). On the one hand, Bakardjiev et al. argues that transplacental infection of the fetus happens via cell-to-cell spread from the decidua or infected maternal cells getting in contact with the EVT (Bakardjiev *et al.*, 2004). On the other hand, in the

same year Lecuit et al. published that this happens via the direct invasion of free floating bacteria using their surface proteins and the respective receptor on the SYN (Lecuit *et al.*, 2004).

Thus, the two major hypotheses how listeria traffic to and infect the placenta are:

1. Lm infected shuttle entering through EVT (1.5.1 and Figure 4) and
2. Free Lm entering through SYN (1.5.2 and Figure 4)

1.5.1. Hypothesis 1: Lm infected shuttle entering through EVT

The first hypothesis of infected shuttle to EVT is shown in Figure 4 a. Here, EVTs are supposed to be the major entry point for Listeria originating from infected maternal immune cells. Many different pathogens like cytomegalovirus (CMV), human immunodeficiency virus, parasites (*Toxoplasma gondii*) and of course, the bacterium *Listeria monocytogenes* are capable of breaching the placental barrier. They have one thing in common: their intracellular life cycle (Bakardjiev *et al.*, 2004). This predisposes these pathogens (with placental tropism) to reach and infect the placenta through an infected host cell of the circulating blood (Robbins *et al.*, 2010).

The EVT is a special trophoblast layer that grows at the end of the first trimester during invasion and at this point gets more accessible for maternal cells (Knöfler *et al.*, 2019). It is reported that Listeria can traffic to the placenta without leaving the protective intracellular environment (Bakardjiev, Theriot and Portnoy, 2006). Other pathogens like *Plasmodium faclicparum* also use a shuttle (erythrocytes) to reach the placenta, supporting the shuttle hypothesis (Lecuit *et al.*, 2004). It has been reported that EVTs recruit maternal immune cells to the placenta in an active manner. For example, T-cells and monocytes are attracted via the chemokine CXCL16 (Huang *et al.*, 2008). NK cells ($CD56^{high}$) and monocytes have also been recruited via sensing of monocyte inflammatory protein (MIP) 1 alpha which initiates migration (Drake *et al.*, 2001).

Infection of first trimester human placental organ cultures with Listeria or infected human cells (artificially differentiated macrophages) *in vitro* showed a resistence of the SYN but a premissiveness of EVTs (Robbins *et al.*, 2010). The phenomen observed with infected human cells was absent when using a listerial mutant harbouring a cell-to-cell spreading defect. In both conditions, infection with wt or mutant Listeria, SYN itself restricted spreading (Robbins *et al.*, 2010). The dense cytoskeleton of the SYN could be an explanation for restriction by inhibiting listerial actin assembly and formation of protrusions (Robbins *et al.*, 2010).

EVTs are harder to reach by Listeria, but also more vulnerable to infection possibly due to its special immunological status (Cao and Mysorekar, 2014). EVTs also serve as a bottleneck, because some subpopulations of invasive trophoblasts are able to restrict bacterial growth (Zeldovich *et*

al., 2011). Nevertheless, one single bacterium could be sufficient to cause clonal expansion in the EVT (Bakardjiev, Theriot and Portnoy, 2006).

Listeria are predicted to traffic in maternal immune cells to the EVT and infect it via cell-to-cell spread or via lysis of the maternal cell at the EVT and Lm placental passages in an InlA/E-Cad dependent manner. This might be supported by the fact that EVTs show high expression of E-Cad *in vivo* (Robbins *et al.*, 2010).

1.5.2. Hypothesis 2: Free Lm entering through SYN

The second hypothesis of free Listeria to SYN is shown in Figure 4 b. Here, SYNs are assumed to be the major entry point for Listeria, because most reported cases of listeriosis are during the late phase of pregnancy (Charlier, Disson and Lecuit, 2020).

SYNs express two important receptors to which Listeria can bind to (Lecuit, 2020). These receptors on SYNs are E-Cadherin (E-Cad) and c-Met, the latter is also referred to as hepatocyte growth factor receptor. They are engaged by their respective ligands Internalin A (InlA) and Internalin B (InlB) on Lm (Disson *et al.*, 2008). The transmembrane receptor E-Cad generates adherent-junctions between cell layers like epithelial cells or trophoblasts (Gessain *et al.*, 2015). Lecuit et al. have shown that placental infection occurs via InlA-/E-Cad interaction in e.g. trophoblast cell lines (BeWo) and human placental explants (Lecuit *et al.*, 2004). However, it was also demonstrated in an *in vivo* pregnant guinea pig model, which is the natural host of *Listeria monocytogenes*, that there was no pivotal role of InlA in placental infection and that E-Cad was also absent on SYN surfaces (Bakardjiev *et al.*, 2004). Furthermore, Lecuit et al. showed via immunohistochemistry of placenta sections from women with pregnancy-associated listeriosis that Listeria are located primarily at the surface of SYNs and CYTs where they also found expression of E-Cad (Lecuit *et al.*, 2004). Disson et al. showed no infection of gerbil placenta *in vivo* using InlA/B deletion mutants for infection. In contrast, the bacterial wt strain was detectable at the area of the SYN (Disson *et al.*, 2008).

InlB is important for crossing the placental barrier, because InlB is necessary for phosphoinositide 3-kinase (PI3-K) activation that is constitutively expressed by enterocytes but not by trophoblastic cells. Via InlB/c-Met interaction PI3-K is activated, phosphoinositide-4,5-bisphosphate (PIP2) gets phosphorylated into phosphoinositide-3,4,5-trisphosphate (PIP3) resulting in membrane ruffling and cell wall disruption which makes it possible for Lm to cross the barrier (Gessain *et al.*, 2015). Thus, InlA and InlB could act in a zipper-like mechanism in a conjugated manner (Lecuit, 2020).

Interaction of listerial InlA with E-Cad may indicate a general mechanism how listeria overcome the major barriers in the body, because beside the intestinal epithelium and SYNs of the placenta also cells of the blood-brain barrier express that receptor (Lecuit *et al.*, 2004; Nikitas *et al.*, 2011).

Besides these two major routes described here, plenty of other possible invasion pathways exist that could influence the mode of infection at the placental barrier and need to be considered. For example, it is published by Gessain et al. that InlB is essential for entry into SYNs (Gessain *et al.*, 2015). Another group observed in human placental explants, that InlA is indispensable in EVT invasion and that Listeria rather invade EVT than SYN (Robbins *et al.*, 2010). Furthermore, the same group considers another variable in this scenario, namely the developmental stage of the placenta, which could be of high relevance for the route of invasion Listeria will take. Faralla et al. identified a novel internalin with a high placental tropism and responsibility for adequate intracellular spreading called InlP. They hypothesized that InlP is the essential factor for Listeria to avoid growth restricting mechanisms of EVTs (Faralla *et al.*, 2016).

Since these controversial details complicate deciphering of how Lm traffic to the placenta, this study aims to investigate this process with focus on the two major putative infection routes described here.

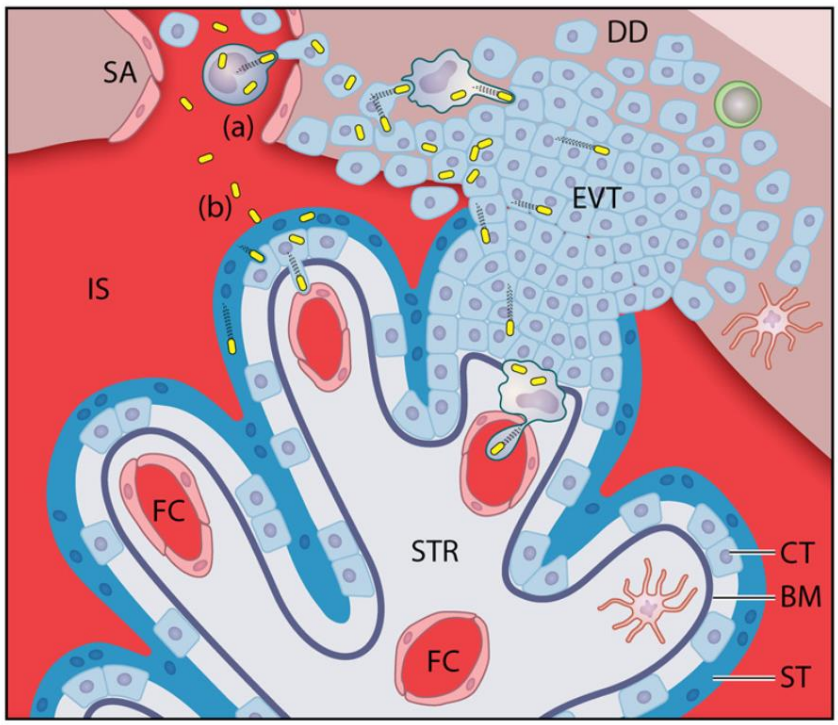


Figure 4. Two major routes of vertical transmission by which *Listeria monocytogenes* (yellow rods) can invade the placenta.
(a) Lm infected maternal leukocytes infect the decidua (DD) and thus the extravillous cytotrophoblast (EVT) via cell-to-cell spread. Lm then breaches the underlying basement membrane (BM) and reaches the fetal capillaries (FC). (b) Free floating Lm in the intravillous space (IS) directly infect the syncitiotrophoblast (ST) in a receptor-mediated mechanism.

After infection of the ST, Lm transfers via cell-to-cell spreas to the cytotrophoblast (CT), breaches the underlying BM and reaches the FC. Figure obtained from (Vázquez-Boland, Krypotou and Scortti, 2017).

1.6. Intracellular life cycle of *Listeria monocytogenes*

As shown for the second hypothesis of free Lm entering through SYN in Figure 4 a, Figure 4 b, Lm are capable of directly infecting cells. This is not just the case for trophoblasts, but also for non-phagocytic cells like epithelial cells,endothelial cells and phagocytes (Vázquez-Boland *et al.*, 2001). The intracellular life cycle of Lm consists of six steps shown in Figure 5, which will be explained in this and the following chapter.

1.6.1. Adhesion is a critical step (1)

Adhesion is the initial step in the pathogenesis of Lm. As already mentioned, Lm are capable to infect phagocytes and non-phagocytes. Infection of non-phagocytic cells requires the attachment of the bacterium onto the host cell surface, which is facilitated by several Lm surface molecules (Vázquez-Boland *et al.*, 2001). Since a huge diversity of virulence factors exists and consistently new ones are investigated, the most classical and prominent molecules will be introduced.

The facultative intracellular pathogen *Listeria monocytogenes* uses virulence factors to infect the host cells (Disson *et al.*, 2008). Two relevant factors are the internalins InlA and InlB, which, as bacterial surface proteins, bind to their respective receptors E-Cad and c-Met on the surface of the host cell. Internalins will be described in more detail in chapter 1.7.

1.6.2. Bacterial escape (2)

After adherence to non-phagocytic cells and internalization due to listerial virulence determinants, the intracellular life cycle is promoted by further key virulence factors. Their expression is regulated by the transcriptional master regulator PrfA. Vacular escape listerolysin O (LLO), phospholipase PlcA and PlcB cause lysis of the host vacuole by pore formation, supported by the metalloprotease Mpl (Le Monnier *et al.*, 2007; Rolhion and Cossart, 2017). Disruption of the membrane initiated by hemolysin starts 30 min after engulfment of Listeria, of which half of them is predicted to be in the cytoplasm one to two hours later (Vázquez-Boland *et al.*, 2001). A more detailed description of LLO will be depicted in chapter 1.8.

1.6.3. Interference with host cell function (3)

After vacuolar escape, Listeria enter the cytoplasm and start to multiply (reproduction cycle 1 h) (Vázquez-Boland *et al.*, 2001). Both, multiplication as well as protection from the host immune system is facilitated by factors like the secreted InlC, a sugar uptake system called hexose

phosphate translocase (Hpt), a peptidoglycan N-deacetylase (PgdA), as well as the O-acetyl-transferase A (OatA) (Rolhion and Cossart, 2017).

1.6.4. Intracellular movement (4)

Listerial surface protein Actin assembly-inducing protein (ActA) initiates host actin polymerization for comet tail formation via Actin Related Protein 2/3 complex (Arp2/3 complex) recruitment. Through this process actin networks are formed and drive Lm propulsion to reach the host cell membrane (Rolhion and Cossart, 2017). ActA will be described in more detail in chapter 1.9.

1.6.5. Dissemination by cell-to-cell spread (5)

Together with the action of ActA, the membrane secreted virulence protein InlC assists membrane protrusion formation (Dussurget, Pizarro-Cerda and Cossart, 2004). This leads to direct infection of the neighbor cell without the necessity to disrupt their intracellular proliferation (Tilney and Portnoy, 1989). This mechanism allows Listeria to perform the so-called 'cell-to-cell spread' (Rolhion and Cossart, 2017). Lm actin tails are formed about two hours after infection and can grow up to a size of 40 µm. They move the bacterium through the cytoplasm with a speed of 0.3 µm/s (Vázquez-Boland *et al.*, 2001). For infection of the neighboring cell, they form protrusions at the tip of Lm and penetrate the adjacent uninfected cell (Dussurget, Pizarro-Cerda and Cossart, 2004). Those protrusions are again phagocytozed resulting in a phagosome covered by two membranes: one inner from the donor cell and the other outer membrane from the newly infected host cell (Figure 5) (Vázquez-Boland *et al.*, 2001).

1.6.6. Establishment after cell-to-cell spread (6)

After successfully entering the neighboring host cell, LLO, PlcA and PlcB initiate the escape from the two-membrane vacuole into the cytoplasm after five min and the same cycle can start again (Vázquez-Boland *et al.*, 2001; Rolhion and Cossart, 2017).

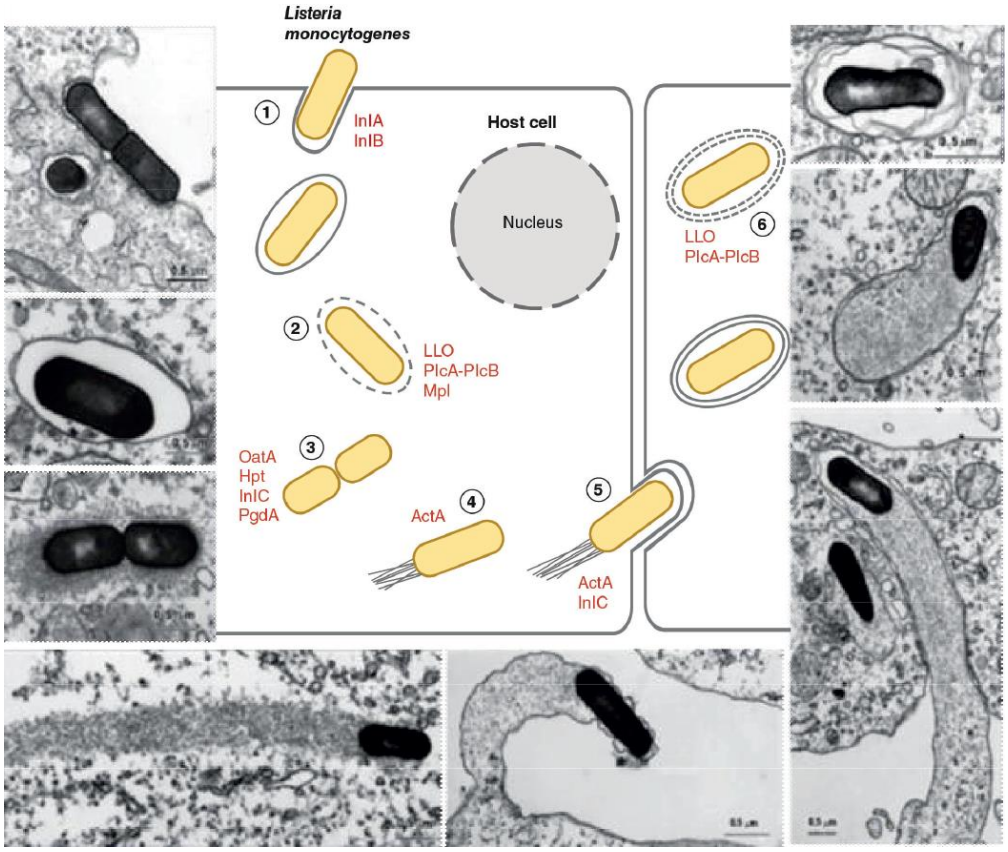


Figure 5. Intracellular infection cycle of *Listeria monocytogenes*.
Lm adhesion to the surface of the host cell (1), bacterial vacuolar escape into the cytoplasm (2), multiplication in the cytoplasm of the host cell (3), intracellular movement of the bacteria via actin polymerization (4), cell-to-cell spread (5), and escape from the two-membrane vacuole of the neighbouring host cell (6). Electron micrographs show the different steps of the infection cycle that is described before.Figure obtained from (Rolhion and Cossart, 2017).

1.7. Internalins

As described in chapter 1.6.1 internalins are bacterial surface proteins and involved in the first step of the Lm life cycle, the adhesion (Figure 5). In general, bacterial surface proteins directly bind to receptors on the host cell membranes and thereby initiate internalization into the target cell (Faralla *et al.*, 2016). Internalins (Inl) are a group reported to be indispensable for this step (Bécavin *et al.*, 2014) The internalin family consists of 25 members with leucine-rich repeats (LRRs) for protein-protein interaction (Faralla *et al.*, 2018). Eleven of them are cell-wall bound surface proteins (e.g. InlA/B), others are secreted (e.g. InlC, InlP) (Rolhion and Cossart, 2017).

1.7.1. InlA/B - how the entry into host cells occurs

Essential and well investigated internalins are InlA and InlB, which bind their respective receptors E-Cad and c-Met (Le Monnier *et al.*, 2007).

E-Cad is a cell adhesion molecule next to adherens junctions, acting as a connector to the cortical actin cytoskeleton via catenin interactions. Those transmembrane glycoproteins are responsible

for cell-to-cell spread in a calcium-dependent manner (Cossart and Lecuit, 1998). Besides c-Met, InlB also binds to glycosaminoglycans and a receptor of complement component C1q (gC1qR) (Gessain *et al.*, 2015). Binding of InlB to its main receptor, c-Met, occurs non-covalently (Dussurget, Pizarro-Cerda and Cossart, 2004). The genes of both internalins are located in the same operon (Dussurget, Pizarro-Cerda and Cossart, 2004). InlA is important for crossing the intestinal barrier. For breaching the placental barrier also the InB is necessary (Disson *et al.*, 2008; Lamond *et al.*, 2021).

1.7.2. Species specifity of InlA and InlB

The interaction between InlA-and InlB with its respective receptors E-Cad and c-Met occurs in a species-specific manner. InlA binds to the human, guinea pig and gerbil E-Cad (permissive species), but does not interact with mouse and rat E-Cad (non-permissive species) (Disson *et al.*, 2008). The reason for the different permissiveness between species is a variation in a single amino acid located at position 16 of the first extracellular domain (EC1) of E-cad (Dussurget, Pizarro-Cerda and Cossart, 2004). In permissive species, it is a proline, and in non-permissive species, it is a glutamic acid. In contrast to InlA, InlB interacts with human, gerbil and mouse c-Met, but does not bind the c-Met of guinea pig and rabbit (Charlier, Disson and Lecuit, 2020). Consequently, humans and gerbils are natural hosts of Listeria (Disson *et al.*, 2008).

To circumvent this problem in mice, we used in this project a humanized mouse model constructed by the Lecuit Lab in Paris. By investing the gerbil E-Cad receptor, they identified the proline at position 16 in the amino acid (aa) sequence to be responsible for permissiveness to listerial InlA (Disson *et al.*, 2008). Hence, they replaced the glutamic acid at position 16 by proline and generated a humanized, ubiquitously expressed E-Cad receptor. This humanized E-Cad gene was inserted into the mouse E-Cad (knock-in). The generated mouse line was named KiE16P and using this mouse line the natural infection cycle of listeria can also take place in mice (Charlier, Disson and Lecuit, 2020).

1.7.3. InlC- a secreted internalin preventing, but also supporting innate immune responses

InlC is mostly involved in the third and mainly fifth step of the Lm life cycle, the dissemination within cells (cell-to-cell spread) (Figure 5). It is a membrane secreted virulence protein and (like InlP) reported to bind to cytoplasmatic host cell components (Faralla *et al.*, 2018**).** It is under control of the PrfA regulon (Gouin *et al.*, 2019). Within the Lm life cycle, it assists membrane protrusion formation together with the action of ActA. Another function of InlC is that it restricts innate host immune responses via blocking the translocation of NFkB into the nucleus through

preventing phosphorylation of IkB-alpha and thereby transcription of NFkB-regulated genes (Faralla *et al.*, 2016; Gouin *et al.*, 2019). Furthermore it was reported that InlC has scaffolding functions by binding Tuba and thus blocking its interaction with N-WASP to form a complex that decreases the tension of cell-cell junctions and thus facilitates listerial cell-to-cell spread (Faralla *et al.*, 2018; Gouin *et al.*, 2019).

Interestingly, InlC can also restrict infection via host cell-mediated monoubiquitination of InlC at K^{224} (Gouin *et al.*, 2019). This posttranslational modification of InlC enables interaction with an intracellular alarmin that is constitutively expressed in neutrophils as well as in other myeloid cells like monocytes after stimulation by bacterial products (Gouin *et al.*, 2019). This alarmin, called S100A9, is a calcium binding protein of the S100 family (Pruenster *et al.*, 2015). Together with S100A8 it forms heterodimers (calprotectin) and mediates various neutrophil effector functions (Pruenster *et al.*, 2015).Interestingly, Lm bound to intracellular calprotectin stabilizes calprotectin and induces reactive oxygen species production (Gouin *et al.*, 2019).

1.7.4. InlP- a new member of the internalin family with high placental tropism

In a genomic screen of pregnant guinea pigs in 2016, Bakardjiev et al. identified a new virulence factor with (high) placental tropism (Faralla *et al.*, 2016). This gene was termed *inlP*, a new member of the internalin family encoding for a secreted protein. That secreted protein is thought to facilitate infection of placental host cells *in vivo* and transcytosis of bacteria from epithelial cells to basement membrane. Transcytosis occurs via interaction with (the cytoplasmatic site of) cell-cell junctions associated afadin *in vitro* (Faralla *et al.*, 2016, 2018).

1.8. LLO - the pore forming listeriolysin

As described in Figure 5 LLO is involved in the second and the last step of the Lm life cycle, the vacuolar escape. After phagocytosis or invasion of non-phagocytic cells Listeria escape from the first and second phagolysosome by producing the pore-forming cytolysin called listeriolysin (LLO) (Vázquez-Boland *et al.*, 2001). It is a secreted cytolysin interacting with in the vascular membrane (Dussurget, Pizarro-Cerda and Cossart, 2004). LLO shows adaption of Listeria to the host, because this toxin is active at a low pH. Activity peaks at pH 5.5 – 6.0, which resembles the exact pH value of the early phagosome (Dussurget, Pizarro-Cerda and Cossart, 2004).

For fetal infection LLO is a prerequisite, because Listeria need to replicate in the trophoblasts before fetal infection can occur and this requires phagosomal escape into the host cytoplasm (Le Monnier *et al.*, 2007).

1.9. ActA for cell-to-cell spread

ActA is involved in the fourth and fifth step of the Lm life cycle, the intracellular movement and the dissemination within cells (cell-to-cell spread) (Figure 5). The surface-exposed bacterial molecule ActA initiates actin cytoskeleton rearrangements as well as polymerization and thus facilitates bacterial movement and spreading in the cytosol (Vázquez-Boland *et al.*, 2001). It has its signal sequence at the amino-terminus and binds with it to the Arp2/3 protein in the host cell initiating actin polymerization. With its central repeat domain, ActA guides the spead and direction of the bacterial movement via binding to enabled/vasodilator-stimulated phosphoproteins (Ena/VASP). The carboxy-terminal region of the ActA protein anchors the protein to the bacterial cell wall (Dussurget, Pizarro-Cerda and Cossart, 2004). Thus, this virulence factor is indispensible to cross and overcome the feto-placental barrier (Le Monnier *et al.*, 2007). This is underlined by murine *in vivo* experiments, in which ActA deletion mutants were not capable of performing cell-to-cell spread into the fetal tissue (Le Monnier *et al.*, 2007).

1.10. Phagocytosis

Lm are not just capable of infecting non-phagocytes, they can be also internalized by phagocytes, which occurs in an internalin independent manner. Phagocytes are extremely important for systemic clearance of the bacteria from the blood compartment, but might also been involved to spread Lm into various organs including the placenta and fetus.

Phagocytosis was first described by Elie Metchnikoff in 1884, who received the Nobel Prize for his investigation in 1908 (Metchnikoff, 1884). It is defined as a receptor-mediated process in which particles (≥ 0.5 µm) like bacteria or apoptotic cells are ingested for pathogen elimination as well as tissue homeostasis (Rosales and Uribe-Querol, 2017). This first line of defense by professional phagocytes, e.g. neutrophils, leads to clearance of infectious foci or antigen presentation to lymphocytes and thus is an indispensible mechanism of the innate and in consequence adaptive immune system (Flannagan, Jaumouillé and Grinstein, 2012).

Phagocytosis is a coordinated event that consist of four steps:

1. Particle recognition by phagocytes
2. Particle internalization via membrane (lipid) ruffling and cytoskeleton remodeling
3. Formation of the phagosome
4. Maturation of phagolysosome and elimination of ingested particle

A variety of different receptor types can recognize specific pathogens. Those infectious particles can be either internalized directly or indirectly by opsonization (Rosales and Uribe-Querol, 2017).

1.10.1. Opsonic receptors – Fc receptors (FcR) and complement receptors (CR)

Host-derived molecules called opsonins can cover foreign particles like Lm. Important opsonins are e.g. complement components and antibodies (Rosales and Uribe-Querol, 2017). These opsonins function as a label and are recognized by opsonic receptors and subsequently ingested (Flannagan, Jaumouillé and Grinstein, 2012). Highly relevant receptors are Fc receptors (FcR) and complement receptors (CR) (Rosales and Uribe-Querol, 2013).

CR – front line defense of innate immunity

First defenders of the innate immune system manage quick reactions after contact with pathogens. An important player beside antimicrobial peptides is the complement system (Rosales and Uribe-Querol, 2013).

CRs bind particles of the complement cascade that tag the surface of pathogens (Rosales and Uribe-Querol, 2017). The family of complement receptors can be devided into: the short consensus repeat (SCR) modules (CR1 & CR2), the β_2 integrins (CR3 & CR4) and the immunoglobulin superfamily (CRIg) (Rosales and Uribe-Querol, 2017).

A well-studied CR is CR3 ($\alpha_M\beta_2$ integrin; Mac-1), which, after complement activation, binds to iC3b marked particles. During CR3-mediated phagocytosis, Rho kinase (RhoA) initiates cytoskleletal rearrangements (Flannagan, Jaumouillé and Grinstein, 2012).

FcR – the bridge between innate and adaptive immunity

Immunoglobulins (Ig) bind antigens with their antigen binding fragment (Fab) and signal through their Fc domain via engagement of respective FcRs (Bournazos *et al.*, 2017). After receptor-clustering, intracellular signaling (e.g. FcγR phosphorylation by Scr-kinases) causes cellular responses (Flannagan, Jaumouillé and Grinstein, 2012). One of the best investigated FcR is the FcγR, a glycoprotein found on leukocytes (Rosales and Uribe-Querol, 2013). This phagocytic receptor recognizes IgG-opsonized pathogens, induces signaling via receptor clustering and internalizes, thus clearing the particels in a zipper like mechanism including cytoskeletal (actin) rearrangements and pseudopod extension (Flannagan, Jaumouillé and Grinstein, 2012). FcγRI (CD64), FcγRII (CD32), and FcγRIII(CD16) are expressed on human cells (Uribe-Querol and Rosales, 2020).

1.10.2. Non-opsonic receptors - pattern recognition receptors

Pathogen-associated molecular patterns (PAMPs) can be directly bound by phagocytic receptors called PRRs. The group of non-opsonic receptors include scavenger receptors, lectin-like molecules and Toll-like receptors (TLRs) (Rosales and Uribe-Querol, 2017).

TLRs can recognize molecular patterns on e.g. bacteria like Lm, but they do not belong to the group of phagocytic receptors (Uribe-Querol and Rosales, 2020). Nevertheless they are involved, because they enhance phagocytosis via interaction and co-activation with PRRs or induction of phagocytotic genes (Doyle *et al.*, 2004; Rosales and Uribe-Querol, 2017). Furthermore, they increase intergrin ligand binding affinity by facilitating inside-out signals (Pruenster *et al.*, 2015).

1.11. Opsonization of Listeria

In the murine system it was shown that uptake of Lm by PMNs was enhanced after opsonization with serum (Pitts, Combs and D'Orazio, 2018). Murine inflammatory macrophages phagocytose Listeria via the complement system (CR3) in which C3 is bound to peptidoglycans of the cell wall. The process is supposed to occur via the alternative complement pathway (Drevets and Campbell, 1991).

In the human system, internalization of Lm (serotype 4b) by human PMNs is increased in the presence of (adult) serum (Bortolussi, Issekutz and Faulkner, 1986). Comparison of neonatal and adult serum showed that neonatal serum had no listerial killing capacity due to the absence of IgM and low (classical) complement activity. (Bortolussi, Issekutz and Faulkner, 1986). Another group provided evidence that human plasma enhances the uptake of Listeria by monocyte-derived dendritic cells (MoDC) via opsonization with immunglobulins. Furthermore, they claim that uptake of opsonized Listeria (EGD) might be facilitated by FcR and that the bacterial p60 protein could be a key player in that process (Kolb-Mäurer *et al.*, 2001).

Heat inactivation of human serum did not influence bacterial uptake, which is why they argued it is a complement system-independent process. Whereas antibody treatment against CD16 decreases listerial uptake, which indicates a FcγRIII receptor-mediated internalization by MoDCs (Kolb-Mäurer *et al.*, 2001).

1.12. Leukocyte recruitment cascade

For efficient clearance of Lm in the human organism via phagocytosis, professional phagocytes need to be recruited to the site of bacterial infection or inflammation. Professional phagocytes like monocytes and neutrophils, are present in the circulation and first defenders in case of infection or inflammation in humans (Schmidt, Moser and Sperandio, 2013; Margraf, Ley and Zarbock, 2019). Leukocyte recruitment is a well-defined process, which normally successfully eliminates local infection or inflammation (Mitroulis *et al.*, 2015).

The classical leukocyte recruitment cascade starts with capturing of neutrophils from the blood stream to the inflamed vessel wall (Nourshargh and Alon, 2014). This is followed by neutrophil rolling. Both, capture and rolling are mediated via the interaction of receptors on neutrophils (P-selectin glycoprotein ligand-1 (PSGL-1), L-selectin & CD44 that interact with E-and P-selectin on the inflamed endothelium (Figure 6). Rolling via P-selectin and E-selectin can be reduced in velocity by additional activation of β2 integrins (Schmidt, Moser and Sperandio, 2013; Margraf, Ley and Zarbock, 2019). Additional signals deposited on the inflamed endothelium such as chemokines trigger firm arrest of neutrophils. Following adhesion, neutrophils start crawling along the inflamed endothelium to appropriate sites where they finally transmigrate into the inflamed tissue (Németh, Sperandio and Mócsai, 2020).

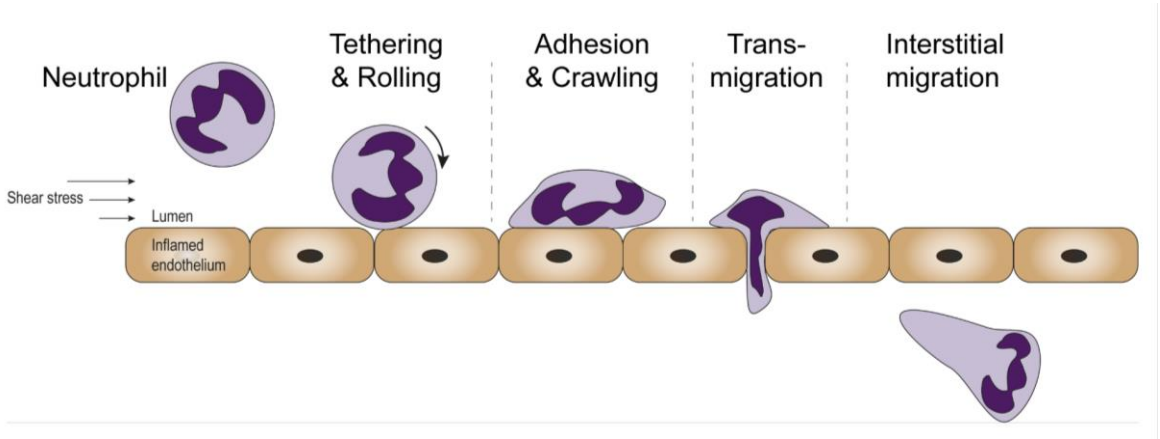


Figure 6. Leukocyte recruitment cascade.
Leukocyte recruitment is a well-defined process consisting of tethering, rolling, adhesion, intraluminal crawling, transmigration and interstitial migration to the site of inflammation. Figure obtained from (Németh, Sperandio and Mócsai, 2020).

1.13. Adhesion

An important step in the cascade is the β2 integrin-mediated adhesion. Beside the fact that leukocytes (neutrophils) need the step of adhesion to reach the site of inflammation, adhesion is also facilitating leukocyte-covered pathogens to enter target cells (e.g. epithelial cells), or to cross barriers (e.g. blood-brain barrier) and in case of Listeria to theoretically breach the placental barrier.

1.13.1. β2 integrines

Adhesion is facilitated by β2 integrins, which are heterodimeric transmembrane molecules that comprise an α- and a β-subunit. The combination of 18 α and eight β subunits results in a final number of 24 different heterodimers in mammals (Schmidt, Moser and Sperandio, 2013).

In the family of β2-integrins, the β-subunit (CD18) can associate with four different α-subunits. The two most essential ones for adhesion during neutrophil recruitment are:

1. αLβ2 lymphocyte function-associated antigen 1 (LFA-1) (CD11a/CD18)
2. αMβ2 macrophage-1 antigen (MAC-1) (CD11b/CD18) also known as complement receptor CR3

LFA-1 and Mac-1 both bind to intracellular cell adhesion molecule 1 (ICAM-1) on endothelial cells.

1.13.2. CEACAM family

The carcinoembryonic antigen (CEA) family contains two subgroups. On the one hand the pregnancy specific glycoproteins (PSG) and on the other hand the carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) (Vićovac *et al.*, 2007). CEACAMs are a family of mammalian immunoglobulin-like molecules composed of 12 glycoprotein members (CEACAM-1, CEACAM-2, CEACAM-3, CEACAM-4, CEACAM-5, CEACAM-6, CEACAM-7, CEACAM-8, CEACA-M16, CEACAM-18, CEACAM-19, CEACAM-20 and CEACAM-21) (Han *et al.*, 2020). CEACAMs are widely expressed in different kinds of tissues, e.g. epithelial cells or leukocytes, and fulfill a plethora of different functions such as modulating cellular processes and immune responses, supporting cell-cell recognition and serving as important receptors for host specific pathogens (Kuespert, Pils and Hauck, 2006; Zimmermann, 2019). On granulocytes, CEACAM-1, -3, -6, and -8 are present (Behrens *et al.*, 2020).

CEACAM-8 (CD66b)

CEACAM-8, also known as CD66b is an exclusive (activation) marker found on human granulocytes (Kuespert, Pils and Hauck, 2006; Singer *et al.*, 2014). It is a single chain glycoprotein with a GPI-anchor (Yoon, Terada and Kita, 2019). CEACAM-8 is stored in specific vesicles and mobilized or secreted upon stimulation with Phorbol-12-myristate-13-acetate (PMA) as well as bacterial DNA. Importantly, in rodents no CEACAM-8 homolog is identified so far (Singer *et al.*, 2014). Soluble CEACAM-8 binds with its Fc part to CEACAM-1 that is expressed on epithelial or endothelial cells (Singer *et al.*, 2014). This is important for signaling, because in contrast to CEACAM-8, CEACAM-1 is a transmembrane receptor with kinase activity through its intracellular domain (Singer *et al.*, 2014). Nothing specific is known about intracellular signaling in neutrophils after CEACAM mobilization. Transduction of the signal leading to adhesion could occur via tyrosine kinases, since it was discovered that CEACAM-8,-6 and -1 are associated with the kinases Lyn and Hck and CEACAM-1 additionally with Src kinase (Skubitz and Skubitz, 2008). CEACAM-8 is expected to form dimers with CEACAM-1, CEACAM-3 or CEACAM-6 (Skubitz and Skubitz, 2008).

The roles of CEACAMs in human neutrophil function are not completely understood. Upon stimulation, CEACAMs are exocytosed to the surface of neutrophils (Skubitz and Skubitz, 2008). This is accompanied with calcium fluxes and oxidative bursts resulting in activation of β2 integrins and leading to neutrophil adhesion on monolayers like endothelial cells or in case of pregnancy on EVTs (Lund-Johansen *et al.*, 1993). EVTs express ICAM-1 and CEACAM-1 (Vićovac *et al.*, 2007). Thus, CEACAM mobilization and in consequence β2 integrin activation on human neutrophils might support adhesion to an inflammed monolayer (endothelial cells or trophoblasts).

Not only PMA, but also pathogens function as strong induces for CEACAM-8 mobilization on human neutrophils. Another potent activator beside lipotheichoic acid is PGN, a part of the bacterial cell wall and more prominent in Gram-positive bacteria like *Staphylococcus aureus* (Schmidt *et al.*, 2015). Thus, Gram-positive bacteria (e.g. *S. aureus*) are able to initiate CEACAM-8 expression, neutrophil aggregation and an increase in adhesion (Schmidt *et al.*, 2012). .

1.14. Aim of the thesis

Over the last decades *Listeria monocytogenes* became one of the most intensively studied pathogens and a model organism for obtaining new insight into host pathogen interactions (Hamon, Bierne and Cossart, 2006; Faralla *et al.*, 2016). Despite this intensive research, the initial mechanism of overcoming the placental barrier and infect the unborn fetus still remains unclear. Thus, in this project, it is the aim on the one hand to image the initial invasion step at the maternal-fetal barrier *in vivo*. On the other hand, suggested hypotheses of how Lm is able to migrate through the trophoblast layer should be investigated using trophoblast cell lines, which reflect the specific cell layer of the placenta. Thereby the aim was to elucidate the exact mechanisms of placental and fetal infection hypothesising that Lm need a blood cell carrier as a niche to escape from immune recognition and infect the placenta and fetus.

2. Materials

2.1. Bacterial strains

Listeria monocytogenes strains used in this study are EGDe and EGD of which the latter has a point-mutated PrfA virulence regulon (PrfA*) causing constitutive expression of relevant virulence genes (Bécavin *et al.*, 2014).

All bacterial strains harbor a chromosomal integrated plasmid (pAD plasmid with pPL2 backbone) with a fluorescent protein (EGDe_GFP, EGD-e_tomato, EGD_GFP) and a cassette for antibiotic (chloramphenicol) resistance (Balestrino *et al.*, 2010). All human experiments were conducted using the EGD strain, whereas for murine studies exclusively EGDe was used for infection.

2.2. Throphoblastic cells

HTR-8/SVneo cells (ATCC CRL-3271) and JEG-3 cells (ATCC HTB-36) were used for *in vitro* studies. JEG-3 cells were kindly provided by Udo Jeschke (Department of Obstetrics and Gynecology, University Hospital, LMU Munich).

2.3. Human blood samples

Human blood samples donated from healthy pregnant and non-pregnant female volunteers were taken based on the approvement by the ethical committee from the Ludwig-Maximilians-Universität München, Munich, Germany (Az.611-15).

2.4. Animals

KiE16P mice were kindly provided by the Lecuit lab at Institut Pasteur, Paris, France, and have been already described elsewhere (Disson *et al.*, 2008). C57BL/6 (Nomenclature C57BL/6NCrl) mice from Charles River served as WT controls. For intravital microscopy KiE16P mice were crossed with Ly-6A (Sca1) GFP transgenic mice to mark the hematopoetic system (Ma *et al.*, 2002). Animals were maintained at the Walter Brendel Center for Experimental Medicine, LMU, Munich, or at the Biomedical Center, LMU, Planegg-Martinsried and included in experiments at an age of 7-25 weeks. All experiments were approved by the government of Oberbayern, Germany, AZ 55.2-1-54-2531-122/12, -229/15, ROB-55.2-2532.Vet_02-18-26 and AZ 50-8791-14.835.2259.

2.5. Consumables

Table 1. Used consumables.

Item	Supplier	Ref
The Big Easy Magnet	StemCell	18001
Cell Scraper	Sarstedt	83.1830
Cell SpreaderSigma	Sigma	HS8151
Flask	Corning	430641U
Incubator	New Brunswick	
Monovette	Sarstedt	03.1524
Neubauer chamber	Optik Labor	
Petri-dish	Merck	CLS430165
Syringe (Blood)	BD Biosciences	300928
Syringe (Insuline)	Braun	9161619
6 well cell culture plate	Corning Incorporated	3516
12 well ibidi chamber	ibidi	80601

2.6. Substances

Table 2. Used substances.

Substance	Company/Supplier	Ref
Accutase solutation	Sigma-Aldrich	A694-100ML
Agar	Sigma-Aldrich	05040-250G
BD FACS Lysing Solution	BD Biosciences	237500
BHI	BD Biosciences	256120
BSA	PAA Laboratories	K41-001-100
Cascade Blue (Dextran)	Thermo Fisher	D1976
CellTrace Cell Proliferation Kit	Invitrogen	C34544
Chloramphenicol	VWR	0219032105
Cobra Venom Factor (CVF)	Quidel	
DAPI	Invitrogen	D21490
EasySep Direct Human Neutrophil Isolation Kit	Stem cell	19666
FCS	Sigma	F7524
Gelatine	Life technologies	D-12054

Gentamicin	Sigma	G1914
Hanks Salt Solution	Biochrom AG	L2045
Heparin	Rotexmedica	ETI3L318-16
PAM3CSK4	InvivoGen	Tlrl-pms
PBS	Invitrogen	70011-051
PFA	Merck	P6148
ProLong Diamond antifade mounting medium	Invitrogen	P36965
Polymorphprep	Axis-shield	1114683
Lymphoprep	Axis-shield	1114545
Lysing solution	BD	349202
RPMI	Sigma	R0883
TNF-α	ImmunoTools	11343013
Triton X	AppliChem	A49750500
Tetramethylrhodamineisothiocyanate	Sigma	T1287
Türksche Solution	Merck	1.09277.0100
Zombie Yellow Fixable Viability Kit	Biolegend	423103

2.7. Software

Table 3. Used software.

Name	Distributor
Graph Pad Prism 7	Graphpad software
FlowJo (10.4)	Treestar
Kaluza (1.5)	Backman Coulter
Affinity Designer	Serif
ImSpector software	LaVision Biotech

2.8. Antibodies

Table 4. Used antibodies.
APC: allophycocyanin, BV: brilliant violet, FITC: fluorescein isothiocyanate, PE: phycoerythrin, PerCP: Peridinin-Chlorophyll-Protein.

Antigen	Dye	Reactivity	Clone	Company
IgG	purified	isotype	polyclonal	BioLegend
IgG1	purified	isotype	polyclonal	R&D

IgG1,k	APC	isotype	MOPC-21	Biolegend
IgG1,k	purified	isotype	MOPC-21	Biolegend
IgG1,k	PE	isotype	P3.6.2.8.1.	Invitrogen
IgG2a,k	APC	isotype	MOP-173	Biolegend
CD3 (T-cells)	purified	Mouse anti human	OKT3	C.Kemper
CD3 (T-cells)	BV 510	Mouse anti human	HCDM	Biolegend
CD11a (LFA-1)	Purified	Rat anti human	TIB217	INVIVO
CD11b	Purified	Rat anti human	M1/70	Biolegend
CD11b/CD18 (MAC-1)	purified	Rat anti mouse	TIB128	INVIVO
CD14 (Monocytes)	purified	Mouse anti human	My4	Beckman Coulter
CD 14 (Monocytes)	PerCP	Mouse anti human	HCD14	Biolegend
CD18	purified	Mouse anti human	TS1/18	Biolegend
CD19 (B-cells)	APC	Mouse anti human	LT19	ImmunoTools
CD31 (PECAM-1)	Purified	Mouse anti human	9G11	R&D
CD41 (Platelets)	APC/Cy7	Mouse anti mouse	HIP8	Biolegend
CD54 (ICAM-1)	purified	Mouse anti human	84H10	Serotec
CD56 (Natural killer cells)	PE	Mouse anti human	HCD56	Biolegend
CD62E (E-Selectin)	APC	Mouse anti human	HAE-1	Biolegend
CD62P (P-Selectin)	PE	Mouse anti human	Psel.KO2.3	eBioscience
CD66b (Neutrophils)	PE-Cy7	Mouse anti human	G10F5	Biolegend
CD88	Purified	Mouse anti human	S5/1	Biolegend
CD106 (VCAM-1)	Purified	Mouse human	BBIG-V(4B2)	R&D
CD182 (CXCR2)	purified	Mouse human	6C6	Pharmingen
CD281 (TLR1)	purified	Mouse anti human	TLR1.136	Biolegend
CD282 (TLR2)	APC	Mouse anti human	TLR2.1	Biolegend
c-Met	purified	Goat human	Polyclonal	Thermo Scientific
c-Met	purified	Rabbit human	monoclonal	Abcam
FcγR	purified	human		Biolegend
E-Cadherin	purified	Mouse anti human	Polyclonal	Cell Signalling
L. monocytogenes	Biotin	Rabbit anti Listeria	Polyclonal	Abcam

IgG	Brilliant Violet 510	Donkey anti rabbit	Biolegend
IgG1	APC	Rat anti mouse	Biolegend
IgG	Alexa Fluor 488	Goat anti mouse	Invitrogen
IgG	Alexa Fluor 488	Donkey anti rabbit	Invitrogen
IgG	Alexa Fluor 546	Goat anti mouse	Invitrogen

3. Methods

3.1. Bacteria

3.1.1. Bacterial cultivation

Listeria were grown in Bacto brain heart infusion (BHI) medium at 37 °C and 180 rpm. For antibiotic selection a final concentration of 7 µg/ml chloramphenicol (CM) was added (Balestrino *et al.*, 2010). Overnight (ON) cultures were grown to an optical density ($OD_{600\,nm}$) of 0.05-0.5.

3.1.2. Bacterial growth conditions

To get an insight into the growth behavior of different Lm strains, cultures were inoculated to 0.05 $OD_{600\,nm}$ with the ON culture. Only precultures were supplemented with 7 µg/ml CM for plasmid stabilization and to exclude influence of antibiotics in mice for *in vivo* experiments. For the growth curve the optical density ($OD_{600\,nm}$) was constantly measured (every 20 min) and different dilutions of the culture were plated on BHI agar plates to count the colony forming units (CFU) on the following day.

3.1.3. CFSE staining

Listeria were stained with CellTrace CFSE Cell proliferation Kit (C34554) from Invitrogen to guarantee a 100 % fluorescent population of bacteria. Therefore, Listeria were grown to an optical density of 0.05-0.5 resembling the exponential growth phase. 5 x 10^8 Lm were centrifuged and resuspended in 1 ml PBS + 10 % BHI medium. 5 µM CFSE were added and incubated for 30 min in the dark at 37 °C. Listeria were washed twice with PBS. 100 % purity was checked using FACS analysis.

3.1.4. Bacterial infection. Listeria were grown to mid-logarithmic phase, pelleted by centrifugation at 300 xg, washed, resuspended and diluted in PBS for respective application. For murine *in vivo* experiments, main culture was done without antibiotic selection to prevent any influence on living organisms.

3.2. Human cells

3.2.1. Trophoblastic cell cultivation

HTR-8/SVneo cells (ATCC CRL-3271) and JEG-3 cells (ATCC HTB-36) were cultured in RPMI 1640 growth medium supplemented with 10 % FCS, penicillin and streptomycin (both 100 U/ml) at 37 °C in 5 % CO_2. JEG-3 cells were kindly provided by Udo Jeschke (Department of Obstetrics and Gynecology, University Hospital, LMU Munich). Before each experiment involving Listeria infection, culture medium was changed the day before the experiment to RPMI 1640 growth

medium supplemented with 1 % FCS and without antibiotics to exclude any influence on bacteria and to force the trophoblasts to better absorb extracellular substances/particles.

3.2.2. Splitting of trophoblast cell lines

Adherent cells were grown to 80 % conflueny. Accutase was added for 5 min after washing with PBS, and cells splitted 1:10 and further cultured in RPMI 1640 growth medium supplemented with 10 % FCS, penicillin and streptomycin (both 100 U/ml) at 37 °C in 5 % CO_2.

3.2.3. Isolation of human neutrophils

Blood was donated from healthy pregnant and non-pregnant female volunteers of similar age and BMI. For neutrophil isolation, two approaches were used.

For uninfected blood samples, a density centrifugation was conducted. Therefore, whole blood was added on a layer of Polymorphprep. After centrifugation at 500 xg for 30 min at room temperature (RT) the cell layer containing neutrophils was collected in a Falcon tube, washed with PBS and resuspended in HBSS.

For human blood samples that were harvested before Lm infection, the EasySep Direct Human Neutrophil Isolation Kit from Stem Cell was used to circumvent density gradient centrifugation. After blood harvest, 1 mM EDTA was added to the sample and negative selection of neutrophils was performed according to the manufacturer's instructions.

3.2.4. Isolation of human lymphocytes

For density centrifugation, whole blood was diluted 1:1 with PBS and added on a layer of Lymphoprep, centrifuged at 800 xg for 20 min at RT. The layer containing lymphocytes was collected in a Falcon tube, washed with PBS and resuspended in HBSS.

3.2.5. Identification of surface markers relevant for leukocyte recruitment

Expression levels of surface molecules relevant for leukocyte recruitment were analyzed by flow cytometry. Therefore, 5 x 10^6 HTR-8/SVneo cells or JEG-3 cells were seeded into flasks and grown overnight. Cells were stimulated with PBS as control, 10 ng/ml TNF-α, Listeria with a MOI8, or TNF-α and Listeria in combination. After incubation with TNF-α for 6 h and Lm for 2 h cells were washed with PBS, scraped from the bottom, washed again and collected into FACS tubes for staining. Cells were stained with antibodies against CD62E (E-Selectin, clone HAE-1), CD62P (P-Selectin, clone Psel.KO2.3), CD106 (VCAM-1, clone BBIG-V (4B2)), CD54 (ICAM-1, clone 84H10), CD31 (PECAM, clone 9G11), CD182 (CXCR2), E-Cad (polyclonal), and c-Met (monoclonal) for 20 minutes at RT. All primary antibodies were used in a final concentration of 5 µg/ml, secondary antibodies were

diluted 1:400. Samples were fixed with 2 % PFA and analyzed with a Beckman Coulter Gallios flow cytometer and Kaluza Flow analysis Software.

3.2.6. Heat inactivation of human serum

To investigate whether factors in the serum are responsible for Listeria internalization whole blood from female donors was taken. One part of it was used for density centrifugation to isolate neutrophils. The other part was used to extract serum by centrifugation of the samples for 10 min at 2000 xg. For heat inactivation serum was treated 30 min at 56 °C and 450 rpm (Drevets and Campbell, 1991). Isolated PMNs were infected with CFSE stained Listeria with a MOI8 for 30 min that were opsonized with heat inactivated serum, untreated serum and HBSS as a control for 30 min prior to infection. Extracellular Listeria were killed with gentamicin (100 µg/ml) for 30 min at 37 °C and 120 rpm and washed with PBS. Samples were fixed with 1.5 % PFA and intracellular Listeria were quantified with a CytoFLEX S cytometer and FlowJo analysis Software.

3.2.7. Inactivation of complement system with Cobra Venom Factor

To decomplement human serum prior to Listeria opsonization, 12 µg of CVF were added to 500 µl of extracted serum and incubated for 1 h at 37 °C (Haihua *et al.*, 2018). Isolated PMNs were infected with CFSE stained Listeria with a MOI8 for 30 min that were opsonized with CVF treated serum, untreated serum and HBSS as a control for 30 min prior to infection. Extracellular Listeria were eliminated with gentamicin (100 µg/ml) for 30 min at 37 °C and 120 rpm and washed with PBS. Samples were fixed with 1.5% PFA and intracellular Listeria were quantified with CytoFLEX S cytometer and FlowJo analysis Software.

3.2.8. Blocking of c-Met receptor and Fc-gamma receptor

To block the c-Met receptor and FCγR on human neutrophils, isolated PMNs were treated with respective antibodies for 30 min at 37 °C and 120 rpm, respectively.

The following treatment was used: PBS as control, non-blocking c-Met ab (monoclonal), blocking c-Met ab (polyclonal), FcγR, non-blocking c-Met ab (monoclonal) in combination with FcγR, and blocking c-Met ab (polyclonal) in combination with FcγR. Antibodies were used at a final concentration of 5 µg/ml. Isolated pre-treated PMNs were infected with CFSE stained Listeria with a MOI8 for 30 min that were opsonized with untreated serum for 30 min prior to infection. Extracellular Listeria were eliminated with gentamicin (100 µg/ml) for 30 min at 37 °C and 120 rpm and washed with PBS. Samples were fixed with 1.5 % PFA and intracellular Listeria were quantified with a CytoFLEX S flow cytometer and FlowJo analysis Software.

3.2.9. Blocking of complement receptor 3 and C5aR

To block CR3 and C5aR on human neutrophils, isolated PMNs were treated with appropiate antibodies for 30 min at 37 °C and 120 rpm, respectively.

The following treatments were used: PBS as control, CD11b (M1/70), CD11b (M1/70) in combination with CD18 (TS1/18), and CD88 (S5/1) antibodies were used in a final concentration of 5 µg/ml. Isolated pre-treated PMNs were infected with CFSE-stained Lm with a MOI8 for 30 min that were opsonized with untreated serum for 30 min prior to infection. Extracellular Listeria were eliminated with gentamicin (100 µg/ml) for 30 min at 37 °C and 120 rpm and washed with PBS. Samples were fixed with 1.5 % PFA and intracellular Lm were quantified with a CytoFLEX S flow cytometer and FlowJo analysis Software.

3.2.10. Phagocytosis of Listeria by human leukocytes

Human PMNs and PBMCs were isolated using respective density gradient centrifugation. Listeria of the EDG strain were used to infect the cells using a MOI8 for 1 h at 37 °C and 120 rpm. 12-well Ibidi chambers were coated with 0.1 % poly-L-lysine. For attachement, infected cells were added to the coated object slides for 20 min at 37 °C. After fixation with 2 % PFA for 10 min, cells were blocked and permeabilized with 2 % PBS/BSA and 0.1 % TritonX for 1 h at RT. Cells were stained ON at 4 °C with antibodies targeting CD66b (Neutrophils, clone G10F5), CD14 (Monocytes, clone My4), CD19 (B-cells, clone LT19) and CD3 (T-cells, clone OKT3) with a final concentration of 5 µg/ml. An Alexa546-conjugated goat anti-mouse antibody (5 µg/ml) was added for 1 h at RT and nuclei were stained with DAPI for 5 min at RT. All samples were embedded in ProLong Diamond antifade mounting medium. For imaging a Leica SP8X WLL microscope equipped with a HC PL APO 40x /1.30NA oil immersion objective at the Core facility Bioimaging of the Biomedical Center was used. ImageJ was applied for analysis of the images.

3.2.11. Shuttle screening

To screen for a potential shuttle of the Listeria to the placenta in the human system, leukocyte populations were infected with Lm. Three different approaches were performed.

First, whole blood of human female donors was infected with CFSE-stained Listeria using a MOI1 for 1 h at 37 °C and 120 rpm. Infected blood was treated with gentamicin (100 µg/ml) for 30 min at 37 °C and 120 rpm to eliminate all extracellular Lm. Blood samples were washed with PBS and stained with antibodies against CD3 (T-cells, clone HCDM), CD14 (Monocytes, clone HCD14), CD19 (B-cells, clone LT19), CD41 (Platelets, clone HIP8), CD56 (Natural killer cells, clone HCD56) and CD66b (Neutrophils, clone G10F5). All primary antibodies were used in a final concentration of 5

µg/ml. Samples were fixed with 1.5 % PFA and erythrocytes were lysed using BD FACS Lysing Solution. Amounts of intracellular *Listeria* were analyzed with a Beckman Coulter Gallios flow cytometer and Kaluza Flow analysis Software.

Second, PMNs and PBMCs of female donors were isolated using respective density centrifugation methods. Isolated blood cell populations were then infected with Listeria of MOI8 to guarantee similar intracellular bacterial burden of infected blood cell populations compared to whole blood infections. The same staining procedure as in the first approach was performed. To check for viability of Listeria after internalization by leukocytes, cells were plated on BHI agar dishes supplemented with CM (7 µg/ml) and CFUs were counted on the next day.

Third, whole blood of female donors was taken and infected with Listeria using a MOI8 for 1 h at 37 °C and 120 rpm. Infected blood was treated with gentamicin (100 µg/ml) for 30 min at 37 °C and 120 rpm to eliminate all extracellular bacteria. PMNs and PBMCs were isolated using repective density centrifugation methods and the same staining procedure as in the first approach was performed. To test the viability of Listeria within leukocytes leukocytes were plated on BHI agar dishes supplemented with CM (7 µg/ml) and CFUs were counted on the next day.

3.2.12. Viability check of leukocytes

To check for viability of cells after Listeria infection or gentamicin treatment Zombie Yellow Fixable Viability Kit from Biolegend was used. This amine-reactive dye selectively enters mammalian cells with a disrupted membrane resulting in a bright fluorescent signal. For application, 1-10 x 10^6 cells were diluted in 100 µl Zombie solution and incubated in the dark for 15 min at RT. After incubation, stained cells were washed with 2 ml PBS/BSA and analyzed via flow cytometry.

3.2.13. Viability of Listeria in human neutrophils *in vitro*

To investigate the life span of Listeria in human neutrophils, PMNs were isolated using Polymorphprep, resuspended in HBSS and infected with bacteria (MOI 1) at 37 °C and 120 rpm. At different time points after infection (0 min, 30 min, 1 h, 2 h, 4 h) blood samples were treated with 100 µg/ml gentamicin for 30 min at 37 °C to kill all extracellular bacteria. Cells were washed with PBS and lysed in deionized water, plated on agar dishes in a defined cell number (1 x 10^5 PMNs/plate) for adequate comparison and colonies were counted the day after.

3.2.14. Toll like receptor 1/2 expression on human trophoblasts

To test for the expression of TLR1 and TLR2 on HTR-8/SVneo and JEG-3 trophoblast cell lines were fixed then in 4 % PFA solution for 10 min on ice, scraped and stained with antibodies against CD281 (TLR1, mouse anti human, clone TLR1.136), IgG1,κ (mouse isotype, clone MOPC-21), CD282 (TLR2,

mouse anti human, clone TLR2.1) and IgG2a,κ (mouse isotype, clone MOPC-173). All primary antibodies were used in a final concentration of 5 µg/ml. An Alexa488-conjugated goat anti-mouse antibody was diluted 1:400 and used as secondary antibody. Samples were analyzed with a CytoFLEX S flow cytometer and FlowJo analysis Software.

3.2.15. E-Cadherin and c-Met expression on human trophoblast cells

To test for E-Cad and c-Met expression on human trophoblast cells, HTR-8/SVneo cells and JEG-3 cells were seeded on gelatine coated coverslips and grown ON. After fixation with 2 % PFA for 10 min, cells were blocked and permeabilized with 2 % PBS/BSA and 0.1 % TritonX for 1 h at RT. Cells were stained ON at 4 °C with antibodies against E-Cad (polyclonal), and c-Met (monoclonal) with a final concentration of 5 µg/ml. An Alexa488-conjugated donkey anti-rabbit antibody (5 µg/ml) was added for 1 h at RT and nuclei were stained with DAPI for 5 min at RT. All samples were embedded in ProLong Diamond antifade mounting medium. For imaging a Leica SP8X WLL microscope equipped with a HC PL APO 40x /1.30NA oil immersion objective at the Core facility Bioimaging of the Biomedical Center was used. ImageJ was applied for analysis of the images.

3.2.16. Invasion assay

To identify a potential stimulus that makes the trophoblast layer more susceptible to Listeria infection cells were treated with different substances. Therefore, HTR-8/SVneo cells and JEG-3 cells were seeded in gelatin-coated 6-well plates using a concentration of 1 x 10^6 cells/well and grown ON. On the next day, medium was refreshed and cells were stimulated for 6 h with 10 ng/ml TNF-α, 10 ng/ml LPS, 100 ng/ml IL-8, 20 pg/ml IL-1α, 5 ng/ml IL-1β, 100 U IFNγ and PBS as a control. Listeria of the EGD strain were added to the different conditions with a MOI0.0008 on JEG-3 cells and with a MOI8 on HTR8 cells. After 1 h cells were washed twice with PBS and treated with gentamicin (100 µg/ml) for 1 h at 37 °C to kill all extracellular bacteria. Infected cells were washed with PBS three times and incubated with 1 ml H_2O for 10 min to lyse the trophoblasts. After removal of the adherent cells by scraping, different cell solutions were plated on BHI agar dishes supplemented with CM (7 µg/ml), CFUs were counted on the next day to check for viability of Listeria.

3.2.17. Shuttle infection of human trophoblasts

The day before the experiment human trophoblast cell lines (HTR8/ SVneo cells and JEG3 cells) were seeded into coated 6-well plates with a density of 1 x 10^6 cells per well. On the next day, cells were stimulated with the TLR1/2 Agonist PAM3CSK4 or PBS as control for 6 h.

Whole blood of human female donors was infected with CFSE stained Listeria using a MOI8 for 1 h at 37 °C and 120 rpm. Infected blood was treated with gentamicin (100 µg/ml) for 30 min at 37 °C and 120 rpm to eliminate all extracellular bacteria. Blood samples were washed with PBS. For isolation of infected neutrophils, the EasySep Direct Human Neutrophil Isolation Kit was used to circumvent density gradient centrifugation. After taking the blood 1 mM EDTA was added to the sample and negative selection of neutrophils was performed according to the manufacturer's instructions. Amounts of isolated cells were counted. One half of isolated infected neutrophils was lysed in deionized water to have a free, but comparable amount of Listeria to the other half of infected neutrophils that was kept untreated and thus functional in HBSS.

Prior to gentamicin treatment 5 ml of the infected blood was used to generate platelet rich plasma.

With a MOI6, free Listeria that were exposed to neutrophils but lysed with water to release Listeria, Listeria infected neutrophils, Listeria infected platelets and untreated Listeria were added on stimulated or unstimulated trophoblasts, respectively. Infection of the cells occurred for 1 h at 37 °C before gentamicin (100 µg/ml) was added for 30 min to kill all extracellular bacteria. Each well was washed twice with PBS and trophoblasts were scraped in PBS to generate cell solutions. Those solutions were used to plate a defined amount on agar dishes to check for the viability by counting CFUs on the day after. The other half of each sample was fixed with 1.5 % PFA and stained with antibodies against CD54 (ICAM-1, clone 84H10) and CD66b (Neutrophils, clone G10F5) for 20 min at RT. All primary antibodies were used in a final concentration of 5 µg/ml and intracellular Listeria were quantified with a BD LSR Fortessa (5 laser) flow cytometer and FlowJo analysis Software.

3.3. Animals

3.3.1. Animal handling

One day after mating, pregnancy of KiE16P or C57BL/6 mice was validated by checking for a copulation plug (embryonic day 0.5). Mice were included in experiments between embryonic day 13.5-15.5.

3.3.2. Identification of murine association partners of Lm

To obtain an overview about blood components Lm bind to, time course experiments (1 & 5 min) were conducted before systemic clearance of Lm in mice occurs (Broadley *et al.*, 2016). To do this, defined listerial inocula were injected into the Arteria carotis and whole blood was taken after defined time points. In this setting pregnant and non-pregnant KiE16P mice were compared.

3.3.3. Viability of Listeria in murine blood cells *in vivo*

KiE16P mice were infected i.v. with 4 x 10 ^{6} Listeria via tail vein. At different time points after infection (30 min, 1 h, 4 h) animals were sacrificed, blood was harvested and treated with either PBS or 100 µg/ml gentamicin for 30 min at 37 °C and 120 rpm to kill all extracellular bacteria to distinguish between free floating and intracellular bacteria. Cells were washed with PBS and lysed in deionized water, plated on agar dishes and colonies were counted the day after.

3.3.4. Infection model

Depletion of neutrophils. Mice were treated i.v. with Ly6G (1A8, 100 µg per injection) antibody 24 h and 4 h before the experiment started having a concentration of 100 µg per injection. Depletion of neutrophil population was confirmed via FACS analysis of peripheral blood samples.

MAC-1/LFA-1 blocking. To block neutrophil adhesion in the placenta *in vivo*, antibodies against CD11b/CD18 (MAC-1, clone TIB128, 100 µg) and CD11a (LFA-1, clone TIB217, 30 µg) were injected i.p. 2 h before bacterial infection.

*Role of E-Cadherin***.** To test a functional role of the humanized E-Cadherin receptor in the murine placenta during Lm infection KiE16P mice were infected with an EGDe strain lacking the Internalin A.

Quantification of bacterial burden in placental fetal unit (PFU). Pregnant KiE16P or C57BL/6 mice were infected i.v. with 4 x 10^6 Listeria of the EGDe strain in 100 µl PBS after neutrophil depletion, blocking neutrophil adhesion or in the control group without pretreatment. 8 h after Lm injection, mice were sacrificed, maternal liver (control organ), placentas and fetus were obtained, washed and homogenized. To remove extracellular bacteria, gentamicin (100 µg/ml) was added for 30 min. Samples were washed twice with PBS and incubated with deionized water for 10 min for cell lysis of the respective organ.

Evaluation of susceptibility to Lm during pregnany was conducted via culturing dilutions of smashed organs on BHI agar dishes. Colony-forming units (CFUs) were counted the days after the experiment.

3.3.5. IVM Placenta Model

We used two-photon microscopy (2PM) to study the unique immunological environment of the placenta *in vivo*. This intravital microscopy technique enables increased penetration depth into tissue, as well as excitation of fluorescent dyes.

To combine this imaging tools with the central theme of this project, we generated a genetic mouse model to distinguish the maternal and the fetal circulation during microscopy. To do this

we crossed female mice (KiE16P) with transgene males (Sca1-GFP) in which a GFP-reporter gene is inserted in the first exon of the Ly6A gene. Consequently, cells and tissues of the fetus were marked with the green fluorescent protein. To visualize the maternal blood circulation a fluorescent dye (Blue dextran or Tetramethylrhodamine (200 µl TRITC, in a 1:100 dilution) was injected. To be able to track Listeria directly after infection a strain that constitutively expresses a fluorescent protein (EGDe_GFP or EGDe_tomato) was chosen.

Anesthetized mice were intubated and a carotid artery catheter introduced to sample blood, administer substances, and inject bacteria. A tail vein catheter for dye application was also inserted. Furthermore, the placenta was gently prepared and mounted on a self-customized stage for imaging and together with the embryo covered with ultrasound gel for protection. For stabilization a vacuum suction pump with a cover slip and viewing window was exerted to be able to image the placenta at the border of the maternal and fetal part.

To image the first events after Listeria arrival at the maternal vessels of the placenta, Listeria were injected carefully into the carotic artery catheter after starting the imaging process.

Imaging was conducted using LaVision BioTec's TriM Scope II Series. For measurements 16x ojective and 3D time lapse was chosen to be able to image over hours. Images were recorded using a step size of 2 microns, an image size of 512 x 512 micrometer and filter settings with excitations of 800 nm (TISA) and 1100 nm (OPO). Data were acquired with ImSpector software.

3.4. Statistical Analaysis

For analysis and editing of all data GraphPad Prism 7 software was used. All data were depicted as either mean±SEM, cumulative frequencies, median or representative images and plots. Depending on the number of groups that were compared respective statistical test were applied. Unpaired or paired student's t-test were used to compare two groups. In case of more than two groups, a 1-way or 2-way analysis of variance (ANOVA) with either Dunnett's (comparison of experimental groups against control) or Turkey's (comparison of all groups with each other) were conducted. Statistical significance was assessed as follows: *p <0.05; **p <0.01; ***p<0.001.

4. Results

4.1. Growth conditions of bacterial strains

In a first set of experiments, growth conditions for the strain EGD and EGDe were established. Furthermore, a growth curve experiment was performed to determine the colony forming units (CFU) at respective OD_{600} (3.1.2). The OD (Figure 7 A,B) was measured constantly and different dilutions of the culture were plated on BHI agar plates to count the CFUs on the following day (Figure 7 C,D). OD_{600}=1 corresponds to 2 x 10^8 cells and was chosen as a defined inoculum to be injected in mice, because bacteria are in their exponential phase and growth is constant.

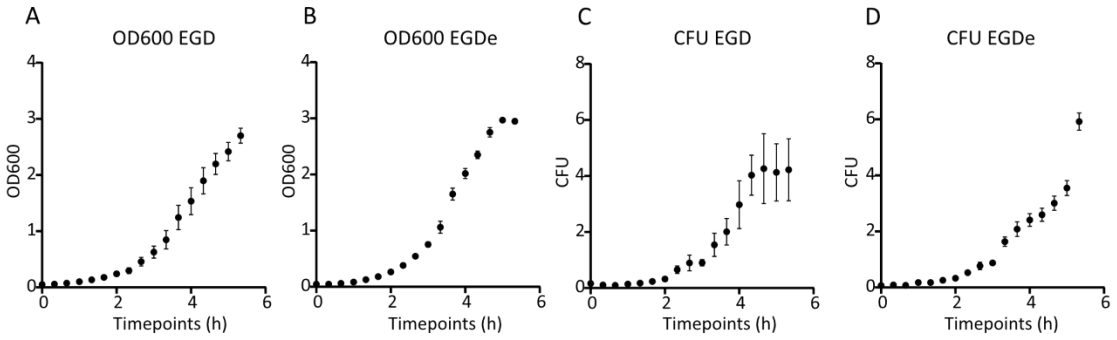


Figure *7*. Growth curve experiment of Lm strain EGD and EGDe.
A, B Constant measurement (every 20 min) of the OD_{600} till bacteria reach the stationary phase. C, D Counted CFUs on the next morning of samples taken every 20 min.

4.2. IVM Placenta model

To study the first steps of bacterial invasion of the placenta, *in vivo* 2PM was chosen. Pregnant KiE16P mice crossed with Sca1-GFP mice, were put on a special stage and the placenta was prepared (Figure 8 A and 3.3.5). A suction device was placed onto the placenta (Figure 8 A). A fluorescent dye, to visualize maternal circulation and distinguish from the fetal part, was injected via the maternal tail vein. After recording the placental microcirculation under control conditions via 2PLSM without bacteria, fluorescent Listeria were injected via carotid artery catheter during recording, to be able to track the initial steps of placental invasion.

After injection of 2 x 10^8 cells, Listeria reached the placenta rapidly, moved through the maternal vessels (blue), and were almost completely cleared after 18 sec (Figure 8 B). In rare cases bacteria had not been cleared from the blood flow after a few seconds, Lm slowly moved in the maternal blood (red) and seemed to accumulate at some areas (Figure 8 C). No transfer to fetal vessels of the bacteria could be observed in the first 1.5 h.

Because clearance of systemic Lm was very quick, which was previously shown by Broadley and colleagues, and a transfer from the maternal to the fetal part could not be observed within almost

2 h, Listeria might need to be associated or taken up by a cell-carrier to be protected from being cleared and transferred over the placental barrier (Broadley *et al.*, 2016). Overall, 2PLSM experiments strongly suggest that overcoming the placental barrier by Lm and infect the fetus is a rare event. Imaging this event turned out not to be a suitable approach to uncover the molecular mechanisms of Lm invasion of the placenta and fetus.

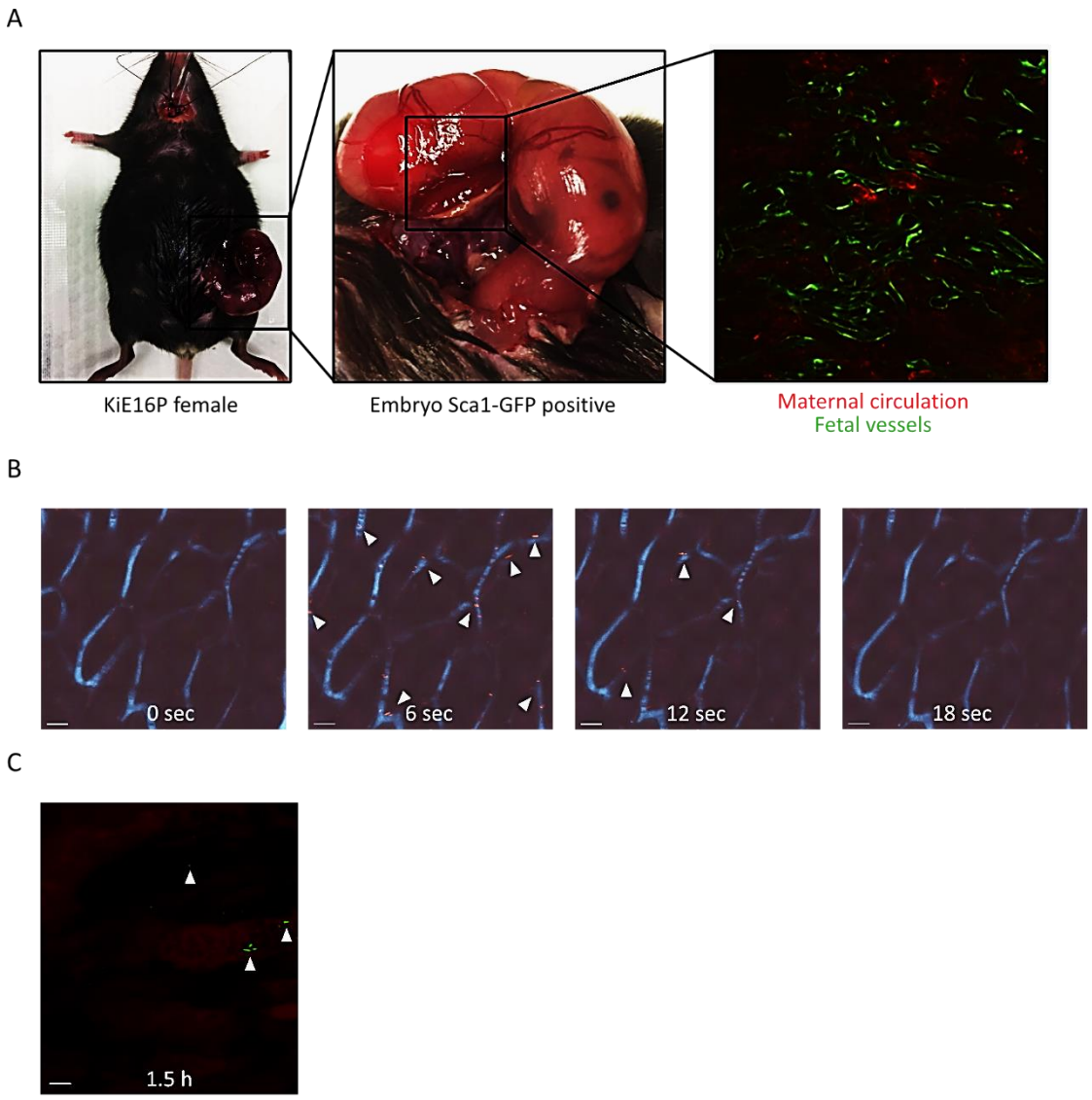


Figure 8. IVM Placenta model.
A. KiE16P mice were crossed with Sca1-GFP positive males, so that in consequence Sca1+ cells harbor a green fluorescent protein. Pregnant mice were intubated, the carotid artery cannulated, and one placenta of a pregnant mouse gently prepared. A suction device was applied onto the placenta to be able to image the organ at the border of the maternal fetal part. For the visualization of the maternal blood stream a red fluorescent dye was admistered via the carotid artery catheter. B. To image the first events after Lm of the EGDe_tomato strain, arriving at the maternal vessels of the placenta, Lm were injected carefully into the carotic artery catheter after starting the imaging process. For the visualization of the maternal blood flow a blue fluorescent dye was applied (KiE16P E12.5). Imaging was conducted using LaVision BioTec's TriM Scope II Series. For measurements 16x ojective and 3D time lapse was chosen to be able to image over hours. Images were recorded using a step size of 2 microns, an image size of 512 x 512 micrometer and filter settings with excitations of 800 nm (TISA) and 1100 nm (OPO). Data were acquired with ImSpector software. Still images of a movie 0 sec, 6 sec, 12 sec, and 18 sec after infection with Lm (white arrows) in the placenta are shown. (Scale bar: 8 µm). C. EGDe-GFP strain was injected into the carotic artery. For the visualization of the maternal blood flow a red

fluorescent dye was applied (KiE16P E15.5). Imaging was processed as described in B. A representative still image of a movie 1.5 h after infection with Lm (white arrows) in the placenta is shown. (Scale bar: 8 µm).

As intravital microscopy did not lead to the desired results, the approach was changed and a potential carrier for Lm within the circulation was investigated. Earlier studies had shown that clearance of free Lm from blood occurs within a few minutes (Broadley et al. 2016). Therefore, we focused on blood cells as potential carrier for Lm after entering the blood circulation.

Because neutrophils as well as platelets are known to participate in the first defense against bacterial infections, these cell types were investigated concerning interaction with Lm (Broadley *et al.*, 2016; Witter, Okunnu and Berg, 2016). In the first experiments, whole blood was harvested via the carotic artery catheter one minute after Listeria infection. Blood of pregnant (E14.5-17.5) and non-pregnant KiE16P mice was compared regarding binding affinities of Lm with neutrophil granulocytes and platelets. The same set of experiments were performed after five min of infection (3.3.2).

Figure 9 shows the results of FACS experiments of pregnant mice. Whole blood was analyzed for platelets and neutrophils associated with Lm. As a negative control, uninfected blood was analyzed. After five minutes of Listeria infection 40 % of neutrophils of non-pregnant mice and almost 20 % of neutrophils of pregnant mice were associated with bacteria (Figure 9 A).

A similar pattern could be observed for Listeria-associated platelets (Figure 9 B). After Lm infection for five minutes about 40 % of platelets of non-pregnant KiE16P mice interacted with Lm, which was significantly reduced to almost 10 % in pregnant mice (Figure 9 B).

For neutrophils and platelets no difference between pregnant and non-pregnant mice was oberserved after one minute of infection, although four-fold more platelets were associated with the Lm than neutrophils, however platelets have a 100-fold higher count than neutrophils (Figure 9).

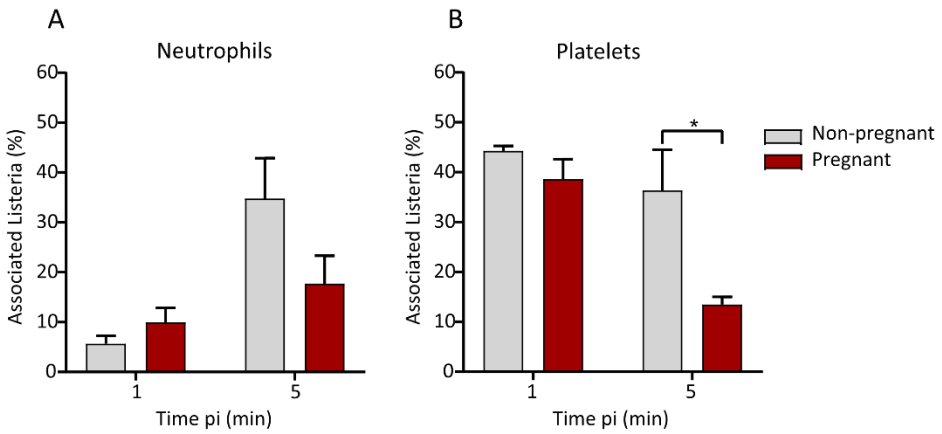


Figure 9. Interaction of Lm with neutrophils and platelets of pregnant and non-pregnant KiE16P mice in vivo.
A. Amounts of neutrophils associated with Lm after one and five minutes infection in pregnant and non-pregnant mice (mean±SEM, n=3-5 mice per group, 2-way repeated measurements ANOVA, repeated Sidak's multiple comparison). B. Amounts of Lm associated platelets after one and five minutes infection in pregnant and non-pregnant mice (mean±SEM, n=3-5 mice per group, 2-way repeated measurements ANOVA, repeated Sidak's multiple comparison).

4.3. Human leukocytes interact with Listeria *in vitro*

After obtaining a first hint on the ability of Lm to associate with neutrophils and platelets *in vivo* in mice, the possibility of blood cells to shuttle Lm to the placenta and thereby protect them against clearance, was then tested in human blood samples. In a first approach, human polymorphonuclear cells (PMNs) and peripheral blood mononuclear cells (PBMCs) were isolated and infected with Lm using a MOI8 for 1 h at 37 °C and stained in 12-well Ibidi chambers with antibodies against CD66b (Neutrophils), CD14 (Monocytes), CD19 (B-cells) and CD3 (T-cells), as well as DAPI to visualize cell nuclei and Lm. For imaging a Leica SP8X WLL microscope equipped with a HC PL APO 40x /1.30NA oil immersion objective was used and ImageJ was applied for analysis of the images (3.2.10). Cells were then investigated by confocal microscopy.

Confocal images of show that human neutrophils, monocytes, B-cells and T-cells associate with Lm (Figure 10), after one hour of incubation. Whether Lm are intracellularly or phagocytozed, or stick extracellularly to the cells can not be distingushed with this assay, because it is a static approach and infected cells only were washed with PBS. However, since human leukocytes associate with Lm they could function as a shuttle to the placenta and protect bacteria from clearance.

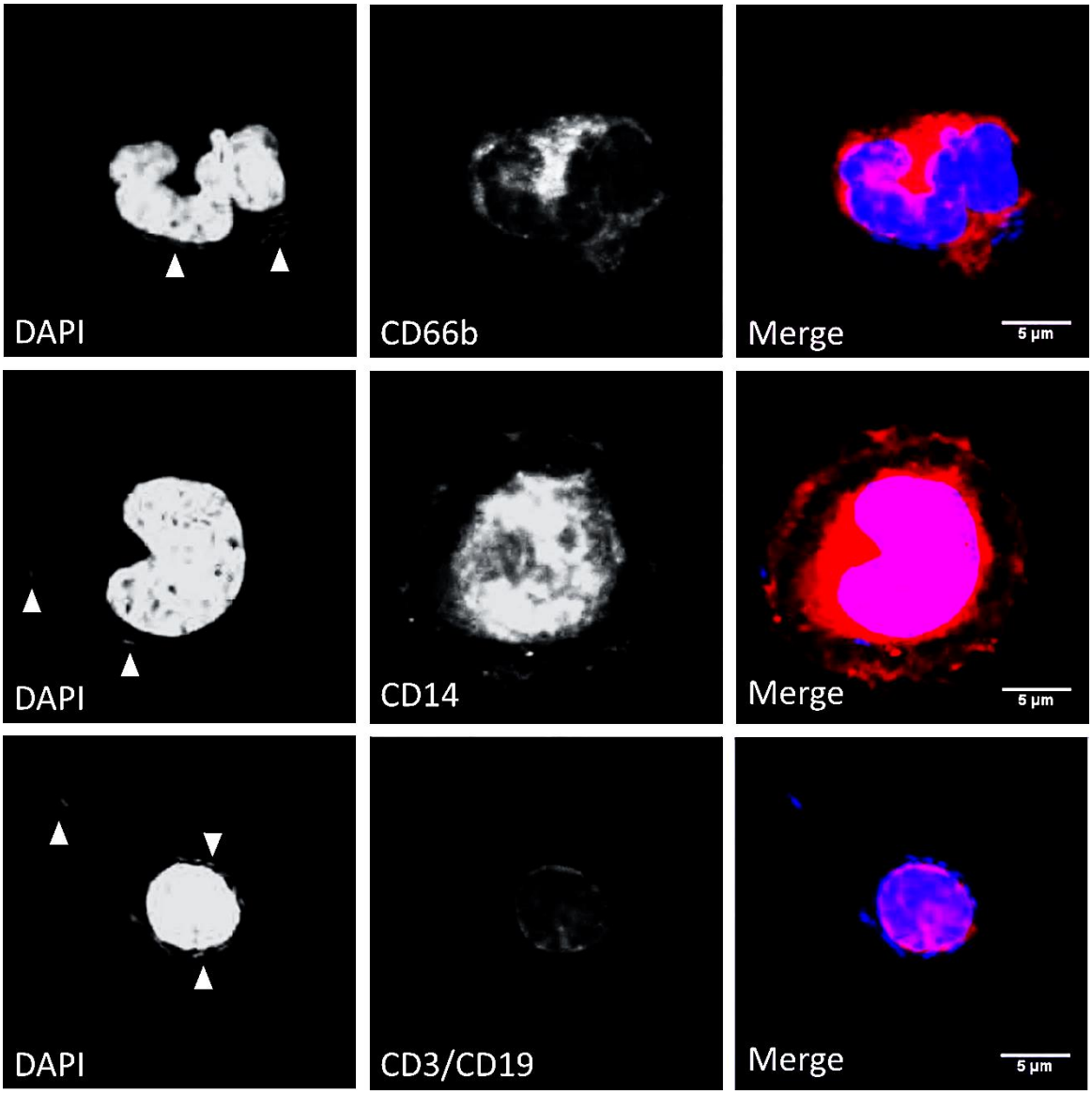


Figure 10. Human leukocytes associate with Lm in vitro.
Confocal microscopy of isolated human PMNs and PBMs stained for neutrophils (CD66b), monocytes (CD14) and lymphocytes, B-cells and T-cells,(CD3,CD19) respectively. Cell nuclei and Lm (white arrows) were stained with DAPI. (Representative confocal images are shown, n=3 independent experiments, scale bar: 5µm).

4.4. Neutrophils act as a survival niche and viability factor for Listeria in the intravascular compartment

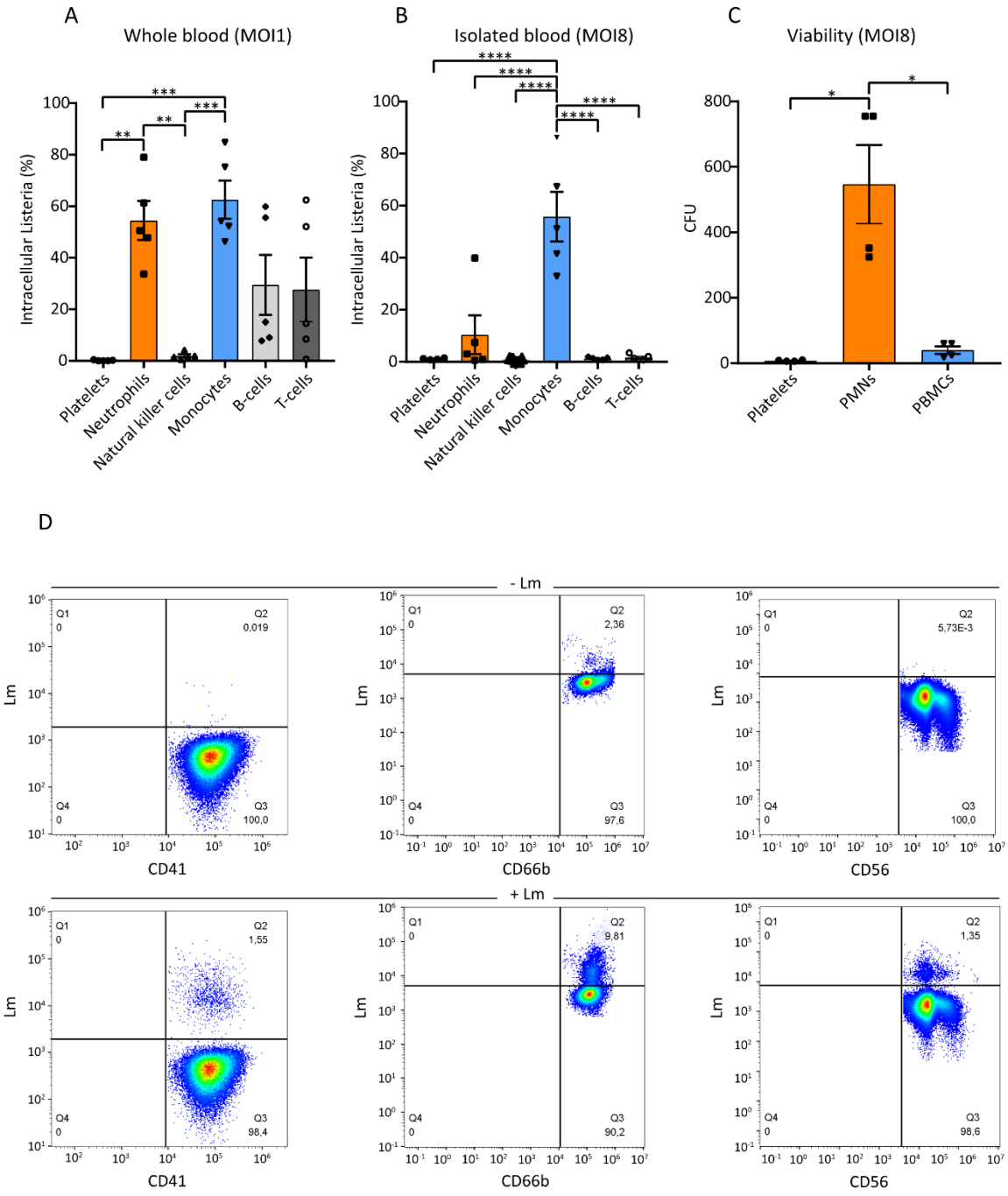
Next, whole blood of human female donors was infected with CFSE stained Listeria using a MOI1 for 1 h at 37 °C and 120 rpm. Infected blood was treated with gentamicin (100 µg/ml), an antibiotic drug that eliminates all extracellular Lm, but does not enter cells leaving intracellular Lm unaffected. Therefore, this treatment defines the location of Lm out or inside the cell. Blood samples were stained with antibodies against CD3 (T-cells), CD14 (Monocytes), CD19 (B-cells), CD41 (Platelets), CD56 (Natural killer cells) and CD66b (Neutrophils). Platelets were used as a negative control, because it is known that Lm stick to them extracellularly (Broadley *et al.*, 2016).

Intracellular amounts of Lm in peripheral blood cells were then investigated using FACS analysis. Figure 11 A shows that neutrophils and monocytes have almost 60 % of intracellular Lm and thus are candidates to function as a shuttle.

Next, PMNs and PBMCs of female donors were first isolated and then infected with Listeria of MOI8. The same staining procedure and analysis method as in the first approach were used (3.2.11). Figure 11 B shows the amounts of intracellular Lm for the various blood cell populations. Like in Figure 11 A neutrophils and monocytes were the two candidates with the highest intracellular burden. As for monocytes still 60 % of intracellular Lm were observed, the amount of intracellular Lm in neutrophils was decreased to about 10 %. This decrease might be due to factors in the serum that are important for Lm uptake into neutrophils (4.7).

Next, PMNs and PBMCs were isolated and platelet rich plasma generated to investigate, whether Lm are still living within the respective cell populations after uptake. It is not just important to identify the potential shuttle, but also prove viable Lm in Lm+ cell, because otherwise no infection of the placenta can occur. To check for viability of Listeria after internalization by leukocytes, cells were plated on BHI agar dishes supplemented with CM (7 µg/ml) and CFUs counted on the next day (3.2.11).

Figure 11 C shows the amount of viable Lm one day after infection in PMNs compared to PBMCs and platelests as a negative control. Although neutrophils had about four-fold less intracellular Listeria than monocytes (Figure 11 B), PMNs carried significantly more viable Lm (about 10-fold) compared to PBMCs. Thus, neutrophils are a potent candidate to function as a shuttle, survival niche and viability factor for Lm in the intravascular compartment.


A
Whole blood (MOI1)
Intracellular Listeria (%)
Platelets
Neutrophils
Natural killer cells
Monocytes
B-cells
T-cells
B
Isolated blood (MOI8)
Intracellular Listeria (%)
C
Viability (MOI8)
CFU
Platelets
PMNs
PBMCs
D
- Lm
+ Lm
Lm
CD41
CD66b
CD56
Q1 0
Q2 0,019
Q4 0
Q3 100,0
Q2 2,36
Q3 97,6
Q2 5,73E-3
Q3 100,0
Q2 1,55
Q3 98,4
Q2 9,81
Q3 90,2
Q2 1,35
Q3 98,6

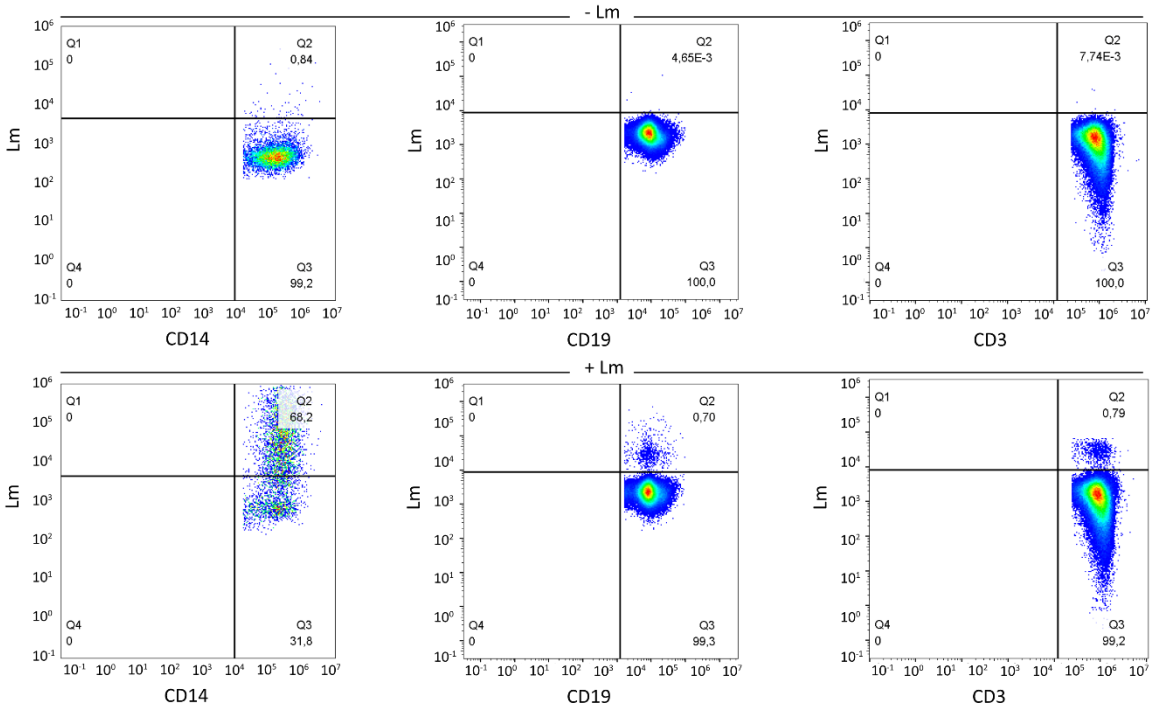


Figure 11. Human neutrophils are a potentional intravascular shuttle of Lm.
A. Amounts of intracellular Lm in peripheral blood cells in whole blood samples of non-pregnant adults (mean±SEM, n=5 independent experiments, 1-way ANOVA, Tukey's multiple comparison). B. Amounts of intracellular Lm in peripheral isolated PMNs, PBMCs and platelet rich plasma (mean±SEM, n=5 independent experiments, 1-way ANOVA, Tukey's multiple comparison). C. CFUs of intracellular Lm of isolated PMNs, PBMCs and platelet rich plasma (mean±SEM, n=4 independent experiments, 1-way ANOVA, Tukey's multiple comparison). D. Gating strategy to identify platelets (CD41), neutrophils (CD66b), NKs (CD56), monocytes (CD14), B-cells (CD19) and T-cells (CD3) before and after infection with Lm. Cell populations that are positive for Lm marker and the respective marker of the cell population were included in the analysis (representative FACS plots are shown).

4.5. Human neutrophils serve as a survival niche and viability factor for Listeria and could play a critical role during pregnancy

To further test whether human neutrophils serve as a relevant factor for placental infection, investigations of Lm infected neutrophils of pregnant donors were continued and bacterial burden of neutrophils of pregnant compared to non-pregnant donors studied. To prove the same pattern of infected leukocytes and consequently the same candidates that could function as a shuttle to the placenta the same assay as in chapter 4.4. was conducted (3.2.11).

Figure 12 A shows the amount of intracellular Lm in isolated human PMNs, PBMCs and platelet rich plasma. Like in Figure 11 B neutrophils and monocytes are the two candidates, with the highest intracellular burden with similar amounts of infected monocytes (60 % of intracellular Lm in monocytes). The number of infected neutrophils was 30 %.

When checking for the viability of Lm one day after infection in PMNs compared to PBMCs and platelests via plating cells on BHI agar dishes supplemented with CM (7 µg/ml), the same pattern as in Figure 11 C was found (Figure 12 B). Again, PMNs carried significantly more viable Lm (about

10-fold) compared to PBMCs although FACS analysis proved about two-fold less infection. The amount of CFUs on the next day increased to a value of over 600 compared to infected PMNs of non-pregnant donors (Figure 11 C).

Figure 12 C shows the comparison of the amount of Lm in neutrophils from non-pregnant and pregnant donors indicating an increase of Lm+ neutrophils from pregnant donors.

To sum up, these results show that neutrophils could function as a shuttle, survival niche and viability factor for Lm in the intravascular compartment in the blood circulation of pregnant women and therefore might play a critical role for Lm infection during pregnancy.

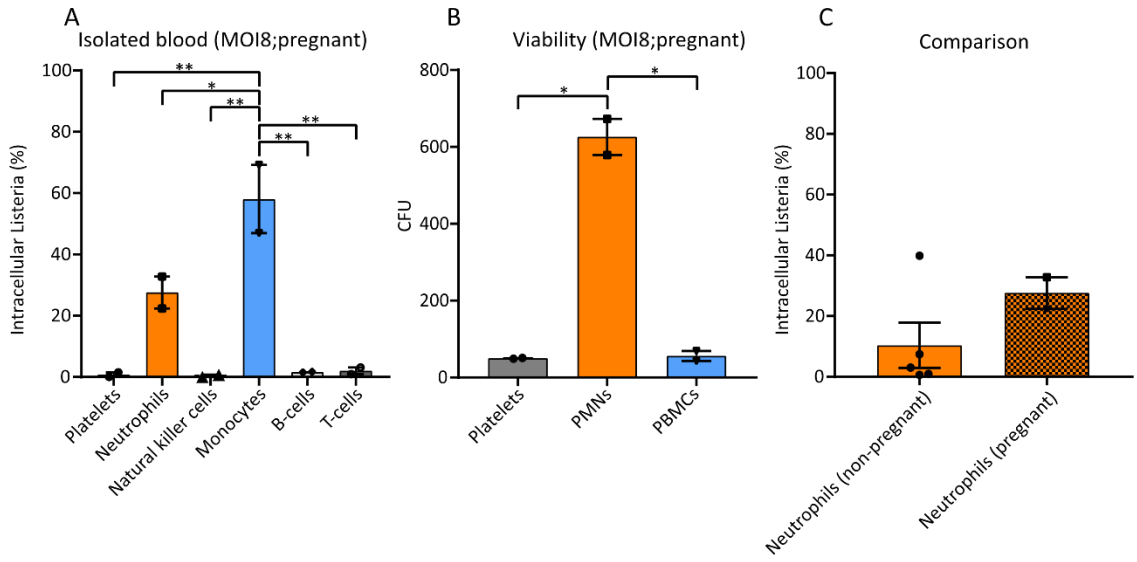


Figure 12. Human neutrophils are a potent carrier of Lm during the state of pregnancy.
A. Amounts of intracellular Lm in isolated peripheral blood PMNs, PBMCs and platelet rich plasma of pregnant donors (mean±SEM, n=2 independent experiments, 1-way ANOVA, Tukey's multiple comparison). B. CFUs of intracellular Lm of isolated PMNs, PBMCs and platelet rich plasma of pregnant donors (mean±SEM, n=2 independent experiments, 1-way ANOVA, Tukey's multiple comparison). C. Comparison of the amount of intracellular Lm of neutrophils from non-pregnant and pregnant donors (mean±SEM, n=2-5 independent experiments, 1-way ANOVA, Tukey's multiple comparison).

4.6. CD66b mobilization significantly increases during pregnancy

To elucidate, which factor could be involved for higher bacterial burden in neutrophils of pregnant compared to neutrophils of non-pregnant women, first WBCs and second mobilization of CD66b was analyzed. CD66b is an activation marker found on human granulocytes (Kuespert, Pils and Hauck, 2006; Singer *et al.*, 2014). CD66b, also known as CEACAM-8, is stored in specific vesicles and mobilized upon stimulation with PMA or PGN, a known part of the cell wall of Gram-positive bacteria (Schmidt *et al.*, 2015). It has been reported that bacteria directly bind to CEACAMs to enter target cells (Behrens *et al.*, 2020). Lm could directly bind to CEACAM-8, or to CEACAM-1, CEACAM-3 or CEACAM-6, which are known to form dimers with CEACAM-8 (Skubitz and Skubitz,

2008). Since Lm are Gram-positive bacteria, it is necessary to test, whether Lm initiate strong activation impulses resulting in CEACAM-8 overexpression on neutrophils of pregnant donors, which could lead to a potential increase in neutrophil adhesion on the placenta (Schmidt *et al.*, 2012).

Figure 13 A shows the comparison oft the WBC of non-pregnant and pregnant donors with a significant increase during pregnancy. Differential counts of monocytes (Figure 13 B) and neutrophils (Figure 13 C) show no difference in the monocyte count, but a significant increase in the neutrophil count of pregnant donors.

To investigate and compare CD66b mobilization on neutrophils from non-pregnant and pregnant women, amounts of CD66b surface expression before and after Lm infection in non-pregnants and pregnant women were analyzed (3.2.11).

We found a significant increase of CD66b mobilization after Lm stimulation in whole blood samples of pregnant donors compared to non-pregnant donors (Figure 13 D) but no difference in samples of isolated PMNs without Lm stimulation (Figure 13 E). The decrease of CD66b mobilization of isolated PMNs compared to CD66b mobilization of whole blood samples after Lm infection, shown in Figure 13 F, might be due to factors in the serum that are important for Lm uptake by neutrophils. This is further investigated in 4.7 and goes along with results shown in Figure 11, where Lm infection of neutrophils was significantly enhanced in the presence of serum.

In collaboration with the working group of Prof. Dr. Rainer Haas from the Max von Pettenkofer-Institut, LMU Munich, binding of Lm to CEACAM-1 and CEACAM-3, as well as direct binding of Lm to CEACAM-8 were investigated using a FACS-based pulldown assay. The results showed no interaction of LM with CEACAM-1, CEACAM3 and CEACAM-8 (data not shown).

To sum up, we found that CD66b surface expression on neutrophils after Lm stimulation is signifantly increased in blood samples of pregnant women compared to samples of non-pregnant donors.

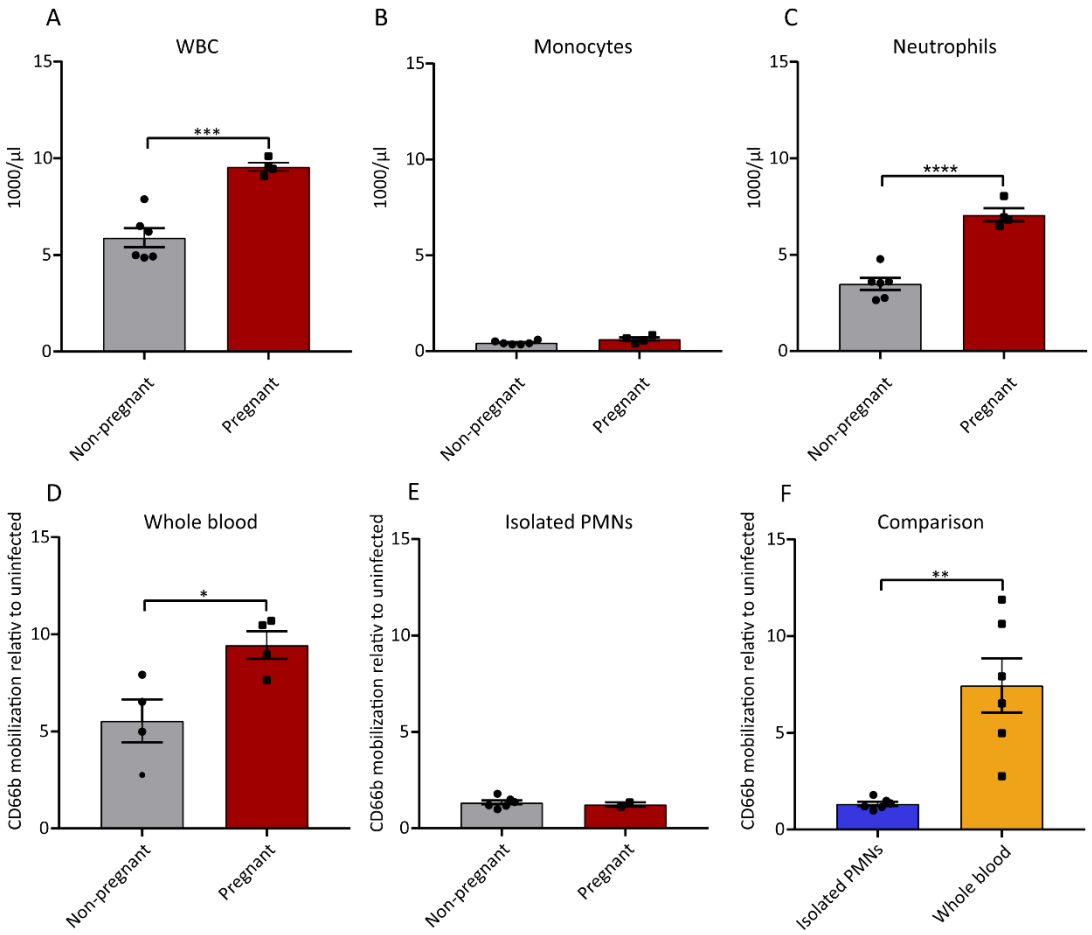


Figure 13. Increase of CD66b mobilization in whole blood of pregnant women.
A,B,C. Comparison of WBC, monocyte count and neutrophil count of pregnant and non-pregnant donors (mean±SEM, n=3-6 independent experiments, unpaired student's t-test). D,E Comparison of CD66b mobilization between non-pregnant and pregnant samples of whole blood (D) and isolated PMNs (mean±SEM, n=4-6 independent experiments, unpaired student's t-test). F. Comparison of CD66b mobilization after Lm stimulation between isolated and whole blood PMNs of non-pregnant and pregnant women (mean±SEM, n=6 independent experiments, unpaired student's t-test).

4.7. Complement factor C3 increases the uptake of Listeria by human neutrophils

Because CEACAMs do not seem to be involved in the entry for Lm into human neutrophils, further molecules need to be taken into account. As already mentioned in chapter 4.4 and 4.6 factors in the human serum seem to facilitate neutrophil infection with Lm.

Earlier work already showed that internalization of Lm (serotype 4b) by human PMNs is increased in the presence of (adult) serum (Bortolussi, Issekutz and Faulkner, 1986). Another group provided evidence that human plasma enhances the uptake of Listeria by monocyte derived dendritic cells (MoDC) via opsonization with immunoglobulins (Kolb-Mäurer *et al.*, 2001).

Furthermore, Kolb-Mäurer and colleagues showed that plasma enhances the uptake of Listeria MoDCs via opsonization with immunoglobulins. Heat inactivation of human serum had no

influence on Lm uptake in MoDcs, whereas antibody treatment against CD16 did, indicating FcγRIII receptor-mediated internalization by MoDCs (Kolb-Mäurer *et al.*, 2001).

One of the potential binding sites for Lm on neutrophils for invasion could be c-Met, which binds to InlB (Glodde *et al.*, 2017). c-Met is also expressed on several other target cells, like placental trophoblasts (Lecuit, 2020). To test a role of c-Met and FcγR on human neutrophils as potential facilitater of Lm entry into neutrophils, c-Met and FcγR were blocked using different antibody combinations for 30 min at 37 °C, respectively, prior to Lm infection (3.2.8). Isolated antibody pre-treated PMNs were then infected with CFSE stained pre-opsonized Lm. Extracellular Lm were eliminated with gentamicin (100 µg/ml) and intracellular amounts of Listeria were quantified using FACS analysis. Figure 14 A shows no influence of c-Met and FcγR on neutrophil infection by Lm.

One potential other factor in the serum responsible for uptake of Lm by murine inflammatory macrophages has recently been described to be CR3, a component of the complement system and also known as MAC-1 (αMβ2; CD11b/CD18) (Drevets and Campbell, 1991). To check whether CR3 and also its downstream effector C5aR could be a responsible for Lm uptake into human neutrophils both receptors were blocked in a next set of experiments. To do this, isolated PMNs were treated with different antibody conditions for 30 min at 37 °C, respectively (3.2.9). Isolated and antibody pre-treated PMNs were infected with CFSE-stained pre-opsonized Lm. Extracellular Lm were eliminated with gentamicin (100 µg/ml) and intracellular amounts of Listeria were quantified using FACS analysis. Results of the FACS analysis are depicted in Figure 14 B showing a slight but not significant reduction of intracellular Lm if CR3 was blocked.

To further investigate a role of complement factors in the serum to be responsible for Lm internalization, serum was heat inactivated. In addition Cobra Venom Factor (CVF) was added to extracted serum to deplete complement protein through continously activating and consuming it (Haihua *et al.*, 2018). Isolated PMNs were infected with CFSE stained Listeria that were opsonized with heat inactivated serum, CVF treated serum, untreated serum and HBSS prior to infection. Extracellular Lm were eliminated with gentamicin (100 µg/ml) and intracellular amounts of Listeria were quantified using FACS analysis (3.2.6 and 3.2.7). Compared to normal serum-treatment, CVF or heat inactivation reduced Lm uptake by PMNs (Figure 14). Thus, the complement system is facilitating uptake of Lm into human neutrophils.

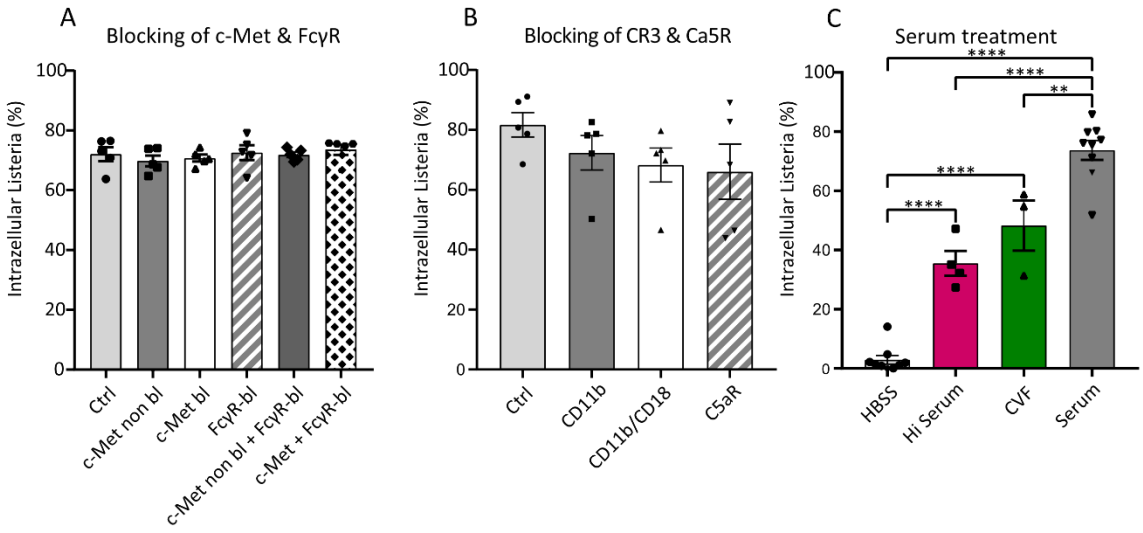


Figure 14. Opsonization via C3 increases Lm uptake into neutrophils.
A. Amount of intracellular Lm into human neutrophils after blocking c-Met and FcγR. (mean±SEM, n=5 independent experiments, 1-way ANOVA, Tukey's multiple comparison). B. Amount of intracellular Lm of human neutrophils after blocking of complement receptors CR3 and Ca5R (mean±SEM, n=5 independent experiments, 1-way ANOVA, Tukey's multiple comparison). C. Serum treatment via heat inactivation (Hi) and Cobra venom factor (CVF) of human neutrophils and Hanks' Balanced Salt Solution (HBSS) as a control occured before isolated neutrophils were infected with Lm (mean±SEM, n=3-11 independent experiments, 1-way ANOVA, Tukey's multiple comparison).

4.8. Characterization of trophoblast cell line HTR8

Since neutrophils are identified and analyzed as the potential carrier of Lm to the placenta, we then moved to the placenta and investigated the role of trophoblast cell lines in placental Lm infection. On the one hand, HTR8 trophoblasts that depict the EVT and should be used to mimic hypothesis 1: Lm infected shuttle to EVT (1.5.1). On the other hand, JEG3 should be tested for hypothesis 2: Free Lm entering through SYN (1.5.2). Thus, the two cell lines were characterized regarding their expression of surface molecules that are relevant for neutrophil recruitment as well as expression of the two receptors E-Cad and c-Met that are important for binding of free Listeria. This was investigated during unstimulated and Listeria and TNF-α stimulated conditions, to include an inflamed environment.

First, the HTR8 cells were characterized. For that purpose, expression levels of surface molecules relevant for leukocyte recruitment were analyzed by flow cytometry. HTR8 cells were seeded into flasks and grown overnight. Cells were stimulated with PBS as control, 10 ng/ml TNF-α, Listeria with a MOI 8, or TNF-α and Listeria in combination. After incubation withTNF-α for 6 h and Lm for 2 h, cells were washed and stained with antibodies against CD62E (E-Selectin,), CD62P (P-Selectin), CD106 (VCAM-1), CD54 (ICAM-1), CD31(PECAM), CD182(CXCR2), E-Cad, and c-Met (3.2.5).

Figure 15 A, B, C and D present the expression levels for the four different conditions relative to isotype control. We found a strong increase of ICAM-1, a slight increase of VCAM-1, PECAM-1, E-

Selectin and no detectable expression of P-Selectin, E-Cadherin and c-Met relative to isotype control. Only for VCAM-1 a significant increase of expression could be observed after TNF-α and Listeria stimulation compared to the unstimulated status (Figure 15 E). High levels of ICAM-1 as well as a significant increase of ICAM-1 after stimulation indicate a potential ability of HTR8 cells to recruit neutrophils, which would favor hypothesis 1: Lm infected shuttle entering through EVT (1.5.1).

Although published in literature, no expression of E-Cad nor substantial expression of c-Met could be detected using FACS analysis (Abou-Kheir *et al.*, 2017). Confocal microscopy was chosen to confirm this. HTR-8 cells were seeded on gelatine-coated coverslips and grown ON. After fixation, blocking and permeabilizing with cells were stained ON at 4 °C with antibodies against E-Cad, and c-Met as well as nuclei were stained with DAPI (3.2.15.).Interestingly, confocal images showed c-Met expression, but only faint E-Cadherin expression, similar to literature reports (Figure 15 F) (Abou-Kheir *et al.*, 2017).

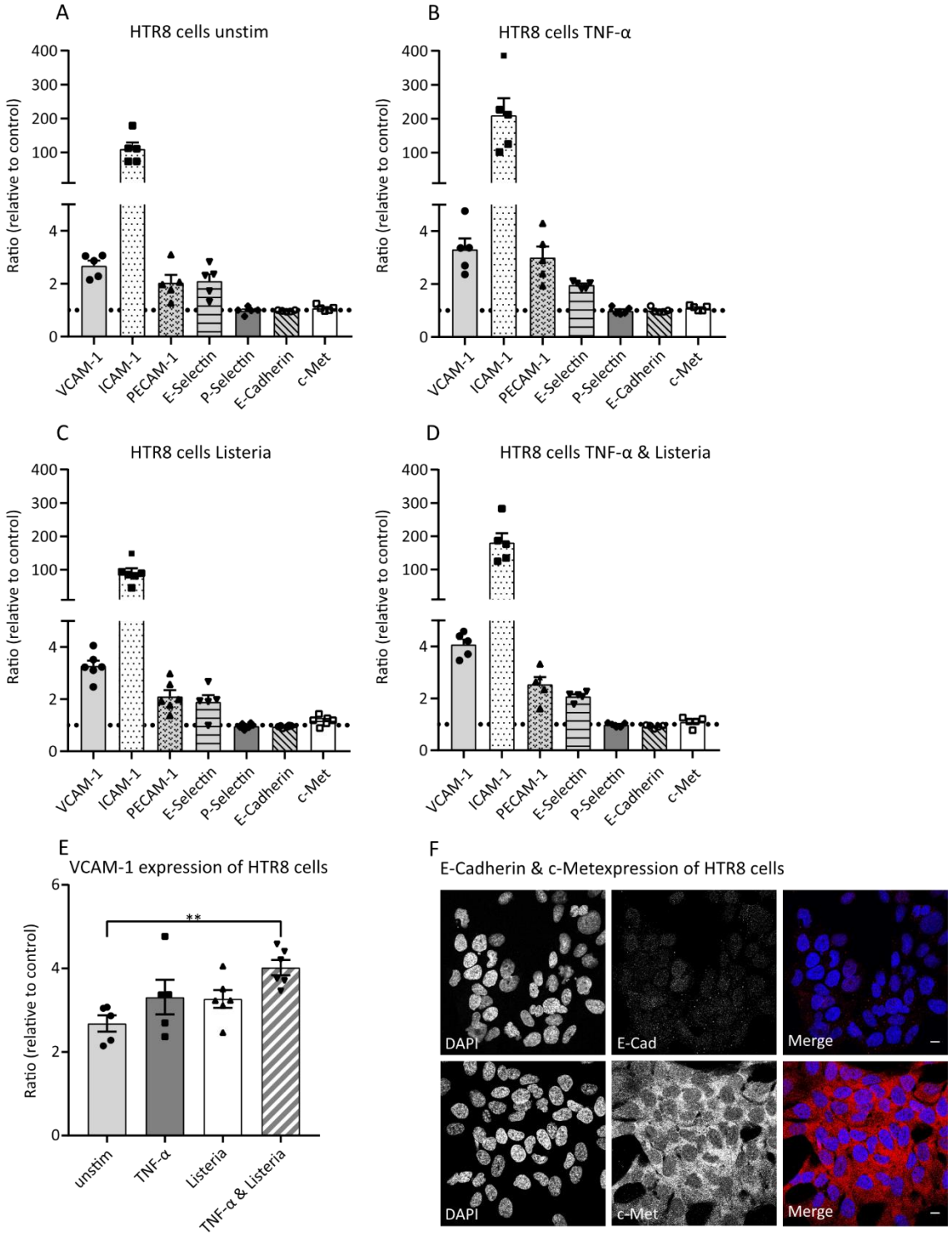


Figure 15. Expression of surface molecules, relevant for leukocyte recruitment and Lm infection on HTR8 trophoblasts. A,B,C,D. Expression of surface molecules relevant for leukocyte recruitment on HTR8 cells during different conditions. (mean±SEM, n=5-6 independent experiments, 1-way ANOVA, Tukey's multiple comparison). E. VCAM-1 expression of unstimulated, TNF-α, Lm and TNF-α stimulated HTR8-cells (mean±SEM, n=5-6 independent experiments, 1-way ANOVA, Tukey's multiple comparison). F. Confocal microscopy of E-cad and c-Met expression of HTR8 cells (Representative confocal images are shown, n=3 independent experiments, scale bar: 10µm).

4.9. Characterization of trophoblast cell line JEG3

Next, JEG3 cells were characterized, relevant for hypothesis 2: Free Lm entering through SYN (1.5.2). Expression levels of surface molecules relevant for leukocyte recruitment were analyzed by flow cytometry. JEG3 cells were seeded into flasks and grown overnight. Cells were stimulated with PBS as control, 10 ng/ml TNF-α, Listeria with a MOI 8, or TNF-α and Listeria in combination. After incubation withTNF-α for 6 h and Lm for 2 h cells were washed and stained with antibodies against CD62E (E-Selectin), CD62P (P-Selectin), CD106 (VCAM-1), CD54 (ICAM-1), CD31 (PECAM-1), CD182 (CXCR2), E-Cad, and c-Met (3.2.5). Figure 16 A,B,C and D present levels of surface molecule expression for the four different conditions relative to isotype control. Similar expression profiles are shown, with an increase of ICAM-1, a slight increase of VCAM-1, PECAM-1, E-Selectin and no detectable expression of P-Selectin, E-Cadherin and c-Met relative to isotype control. Of note, expression of ICAM-1 was about 16-fold decreased in JEG3 cells compared to HTR8 cells (Figure 15 A,B,C,D). In contrast to HTR8 cells no significant difference in expression of individual molecules existed between the different stimulation conditions. Like for HTR8 cells, E-Cad and c-Met expressions in JEG3 cells were tested via confocal microscopy. JEG3 cells were seeded on gelatine-coated coverslips and grown ON. After fixation, blocking and permeabilizing, cells were stained ON at 4 °C with antibodies against E-Cad, and c-Met as well as with DAPI (3.2.15). Confocal images of Figure 16 E show both, E-Cadherin and c-Met expression, as reported earlier (Abou-Kheir *et al.*, 2017).

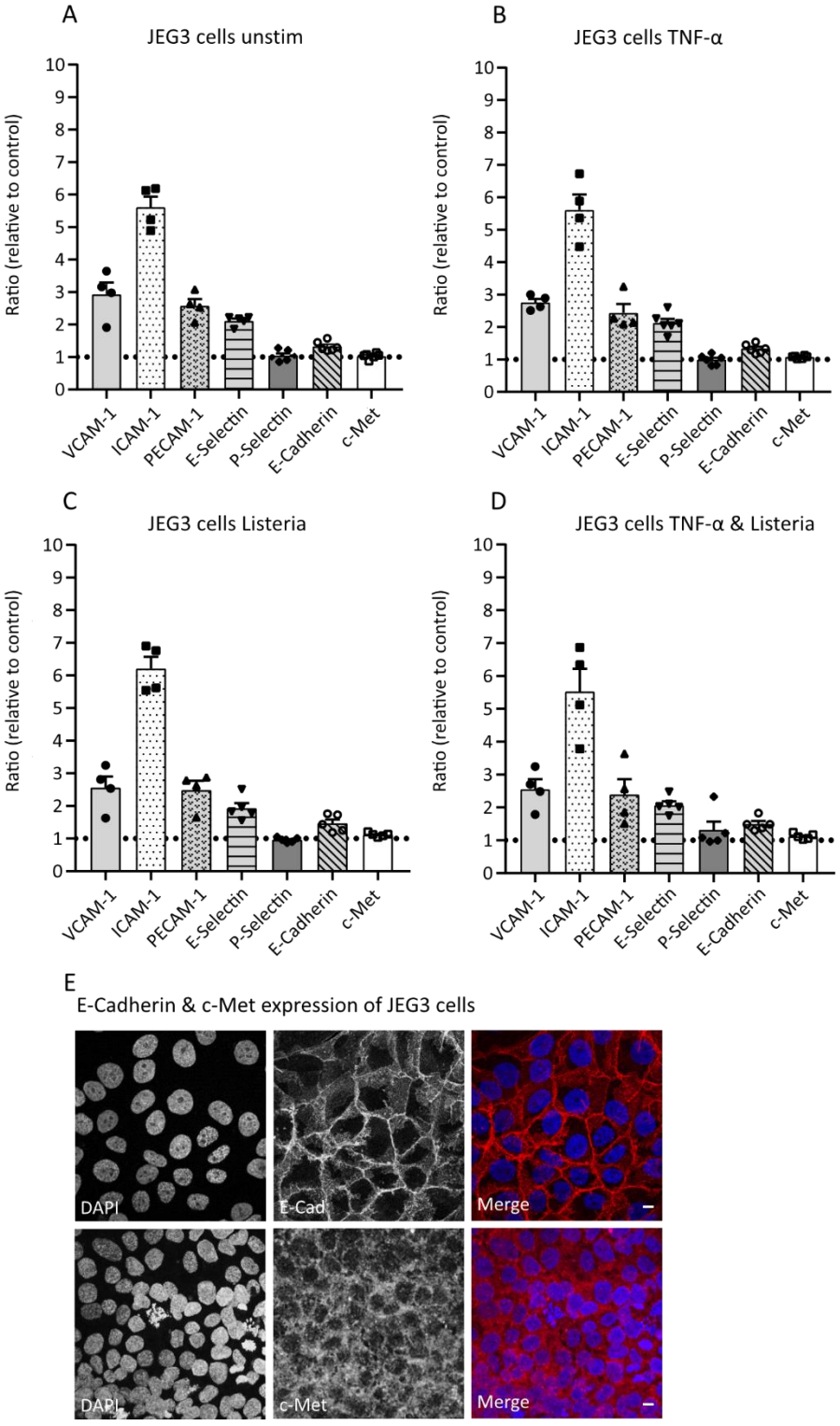


Figure 16. Expression of surface molecules, relevant for leukocyte recruitment and Lm infection on JEG3 trophoblasts. A, B, C, D. Expression of surface molecules relevant for leukocyte recruitment on JEG3 cells for different conditions. (mean±SEM, n=4 independent experiments, 1-way ANOVA, Tukey's multiple comparison).E. Confocal microscopy of E-cad and c-Met expression on JEG3 cells (Representative confocal images are shown, n=3 independent experiments, scale bar: 10µm).

4.10. Invasion capacity of Listeria into pre-stimulated trophoblasts

The significant increase of VCAM-1 expression on HTR8 cells after stimulation with TNF-α and Lm could be a hint that trophoblast cells of the placenta need to be prestimulated to facilitate Lm infection (Figure 15 F). Furthermore, ICAM-1 and E-Selectin levels could not be high enough for neutrophil adhesion without stimulation (Figure 15 A, B, C, D and Figure 16 A, B, C, D). In addition, different cytokines and substances could activate the trophoblast making it more suceptible to pathogens (Abou-Bacar *et al.*, 2004; Vásquez, Segura and Blair, 2013).

Several candidates were tested regarding their potential of priming the two different trophoblast cell lines for higher Lm invasion. Therefore, HTR-8 cells or JEG-3 cells were seeded in coated 6-well and grown ON. On the next day, cells were stimulated for 6 h with 10 ng/ml TNF-α, 10 ng/ml LPS, 100 ng/ml CXCL1, 20 pg/ml IL-1α, 5 ηg/ml IL-1β, 100 U IFNγ and PBS as a control. Listeria were added to the different conditions with a MOI0.0008 on JEG-3 cells and with a MOI8 on HTR8 cells to have comparable results. After 1 h of incubation with Lm cells were treated with gentamicin (100 µg/ml) for 1 h at 37 °C to kill all extracellular bacteria. Infected cell solutions were plated on BHI agar dishes and CFUs were counted the next day to check for Lm viability (3.2.16).

Figure 17 A and B shows no difference of Lm burden in HTR8 cells and JEG3 cells after trophoblast stimulation compared to control, although there is a slight tendency of infection of HTR8 trophoblastrs after LPS and CXCL1 stimulation. Furthermore, regarding the MOI, it is important to note that JEG3 cells are four-fold more prone to infection in general, although none of the substances had a specific influence (Figure 17 B). The minimal increase of Lm infection of HTR8 cells after LPS and CXCL1 stimulation could be an indication that an infected shuttle to EVTs and Lm invasion into placenta is more effective, if target cells are pre-stimulated.

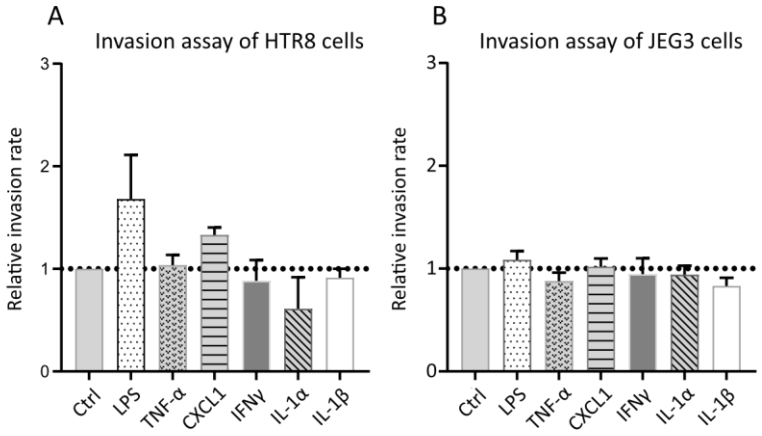


Figure 17. Invasion of Lm into pre-stimulated trophoblasts.
A. Relative invasion rate of Lm into HTR8 cells after stimulation compared to unstimulated cells as control (mean±SEM, n=3 independent experiments, 1-way ANOVA, Tukey's multiple comparison). B. Relative invasion rate of Lm into JEG3 cells after stimulation compared to unstimulated cells as control (mean±SEM, n=4 independent experiments, 1-way ANOVA, Tukey's multiple comparison).

4.11. Invasion of Listeria into trophoblast cells

4.11.1. Neutrophils and the invasion of Listeria into trophoblast cells

Since Lm alone are rather insufficient to invade the two different trophoblast cell lines (Figure 17) and neutrophils seem to function as a shuttle, survival niche and viability factor for Lm in the intravascular compartment (Figure 11), we wanted to further clarify how neutrophils facilitate Lm transfer into the two trophoblast cell lines.

Therefore, an experiment was designed in which the susceptibility of trophoblasts to Lm alone, platelet associated Lm and Lm-infected neutrophils was compared (Figure 18 A). Before starting the experiment, it was important to figure out, whether Lm taken up by neutrophils, or uncovered Lm that were treated with deionized water to lyse neutrophils, possess the same viability.

Therefore, whole blood of human female donors was infected with CFSE-stained Listeria using a MOI8 for 1 h at 37 °C. Infected blood was treated with gentamicin (100 µg/ml) to eliminate all extracellular bacteria. For isolation of infected neutrophils, the EasySep Direct Human Neutrophil Isolation Kit was used according to the manufacturer's instructions. Numbers of isolated cells were counted. One half of isolated infected neutrophils was lysed in deionized water to have a free, but comparable amount of Listeria to the other half of infected neutrophils that was kept untreated and thus functional in HBSS. Dilutions of both samples were plated on agar dishes and CFU counted on the day after (3.2.17). Figure 18 B indicates the same amount of viability of Lm for both samples, showing that free Lm grow the same way as Lm contained in neutrophils plated on agar dishes.

Although vability of Lm after treatment with deionized water is the same as for neutrophil covered Lm, it needed to be tested whether invasion capacity was influenced during the water treatment. For that purspose, Lm that were exposed to neutrophils and treated with deionized water to remove the neutrophils and Lm never exposed to neutrophils were compared regarding their invasion capacity and viability.

To test this, at MOI6 free Listeria that were exposed to neutrophils but lysed with water and an estimated amount of unexposed Lm added on trophoblasts was tested. Infection of the cells occurred for 1 h at 37 °C before gentamicin (100 µg/ml) was applied to kill all extracellular bacteria. Each well was washed twice with PBS and trophoblasts were scraped in PBS to generate cell solutions. Those solutions were used to plate a defined amount on agar dishes to check for the viability by counting CFUs next day. The other half of each sample was stained with antibodies against CD54 (ICAM-1) and analyzed via FACS (Figure 18 A) (3.2.17). Results shown in Figure 18 C indicate that amounts of intracellular Lm in HTR8 trophoblasts is the same. Despite the estimated amount of non-exposed was higher, which is visible in Figure 18 D, this does not impact invasion capacity of HTR8 cells.

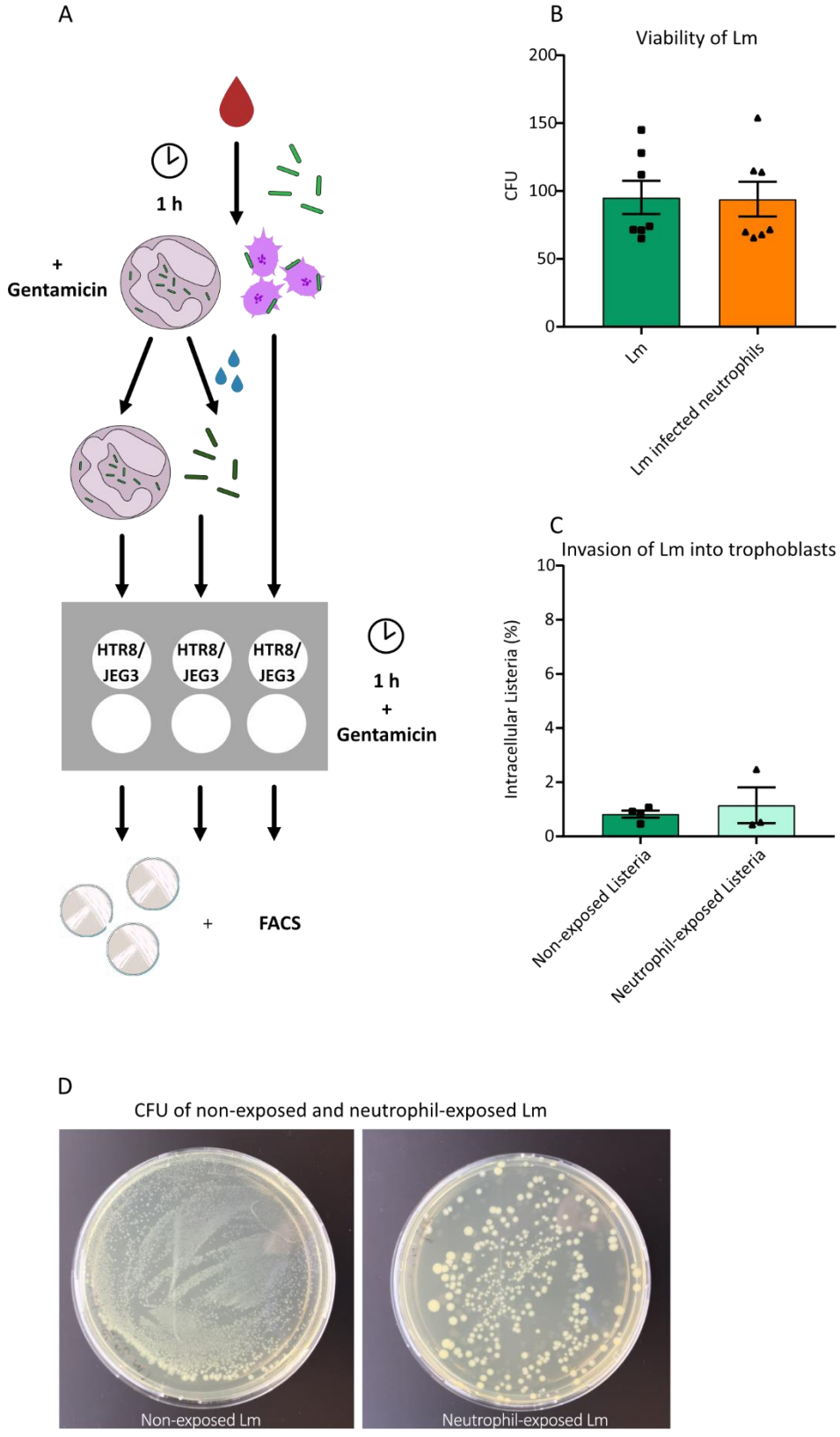


Figure 18. Experimental setup for investigating the ability of Lm infected neutrophils to invade trophoblasts.
A. Experimental procedure of HTR8 and JEG3 cell infection by 1.) neutrophils infiltrated by Lm, 2.) Lm alone and 3). platelets associated with Lm. Neutrophils were infected with Lm for one h before one half of neutrophils was treated with deionized water to lyse neutrophils and generate free Lm. Infection of trophoblasts occured for one hour before cells were treated with gentamicin to kill all extracellular Lm. Samples were analyzed via FACSs and cell dilutions plated on agar dishes to check for viability of intracellular Lm of trophoblasts. B. Viability of Lm after lysis of neutrophils with

water compared to Lm still within neutrophils. Dilutions were plated on agar dishes and CFU counted on the day after (mean±SEM, n=7 independent experiments, unpaired student's t-test). C. FACS results of intracellular amounts of non-exposed, neutrophil-exposed, and water treated Lm of HTR8 cells after 1 h infection and gentamicin treatment (mean±SEM, n=3-4 independent experiments, unpaired student's t-test). D. Viability of non-exposed, neutrophil-exposed and water treated Lm of HTR8 cells after 1 h infection and gentamicin treatment (Representative images are shown, n=3-4 independent experiments).

4.11.2. Gating strategy of HTR8 cells

After setting up and conducting the experiment as decribed in chapter 4.11.1, a gating strategy needed to be established to identify the amount of intracellular Lm that were capable to enter HTR8 trophoblasts.

With a MOI6 free Lm that were engulfed by neutrophils before and lysed with deionized water, Lm infected neutrophils and Lm infected platelets were added on stimulated or unstimulated trophoblasts, respectively. Infection of the cells occurred for 1 h at 37 °C before gentamicin (100 µg/ml) was added for 30 min to kill all extracellular bacteria. Each well was washed with PBS and trophoblasts were scraped in PBS to generate cell suspensions. Cell suspensions were stained with antibodies against CD54 (ICAM-1) and CD66b (Neutrophils) Samples were analyzed using a flow cytometer (3.2.17). Figure 19 presents the gating strategy for HTR8 trophoblast infection without stimulation (Figure 19 A) and after stimulation with the TLR1/2 agonist Pam3CSK4 (Figure 19 B), which will be introduced in the next chapter (4.11.3).

In the first FACS plot trophoblasts were identified based on the scatter parameters namely site scatter (SS) and forward scatter (FS). Further in the second plot discrimination of the target population occurred via selection of ICAM-1 positive cells, ICAM-1 served as identification marker for trophoblasts. The third plot indicates the amount of intracellular Lm (CFSE-labeled) in ICAM-1 positive cells. Plot interpretation and analysis are depicted in the next chapter (4.11.3).

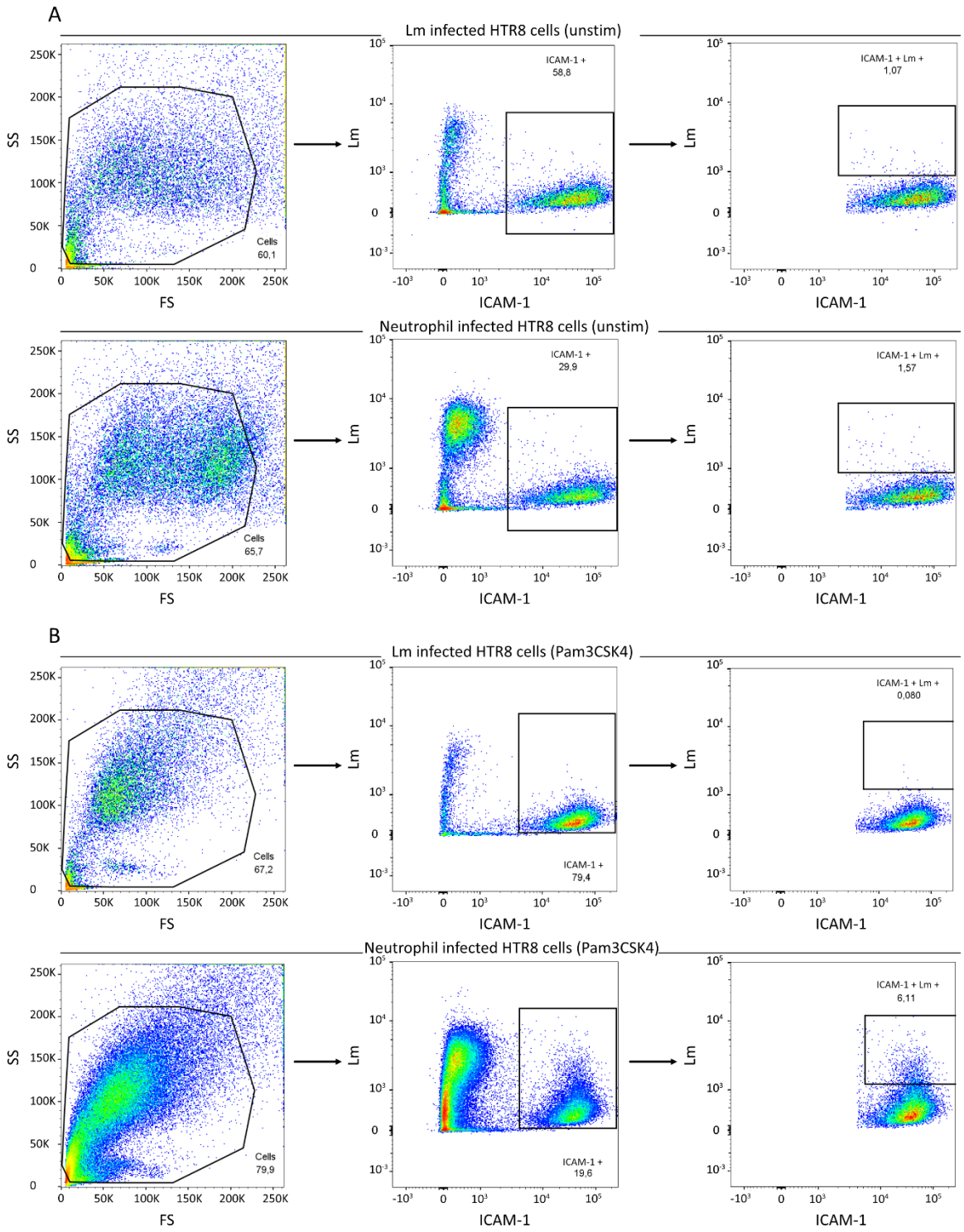


Figure 19. Gating strategy for free Lm infected and Lm-neutrophil infected HTR8 trophoblasts.
A. Infection of unstimulated HTR8 trophoblasts. Trophoblasts double positive for Lm and ICAM-1 were selected for analysis of percentage of intracellular Lm (Representative FACS plots are shown). B. Infection of Pam3CSK4 stimulated HTR8 cells. Trophoblasts double positive for Lm and ICAM-1 were selected for analysis of percentage of intracellular Lm (Representative FACS plots are shown).

4.11.3. Neutrophils mediate the invasion of Listeria into HTR8 trophoblast cells

To identify the amount of intracellular Lm that were capable to enter HTR8 trophoblasts, experimental procedure, gating strategy and analysis were applied as described in the previous section (3.2.17 and 4.11.1-3.).

In a first set of experiments unstimulated HTR8 trophoblasts were analyzed regarding their susceptibility to infection by free Lm, Lm associated with platelets or Lm-infected neutrophils. The FACS results show no differences of HTR8-intracellular Lm for all three conditions compared to uninfected trophoblasts as control (Figure 20 A). These results are in line with the viability investigations via CFU counting, showing the same low levels of CFU (Figure 20 B).

Earlier reports illustrated a role for TLR1/2 stimulation in the placenta to make the organ more susceptible for Lm infection (Chung *et al.*, 2014). Furthermore, it had been reported that after stimulation with the TLR1/2 agonist Pam3CSK4 leukocyte slow rolling and adhesion are induced via β2 integrins (Chung *et al.*, 2014).Thus, a potential role of TLR2 on Lm invasion was tested using Pam3CSK4.

Figure 20 B depicts the percentage of intracellular Lm after infection of Pam3CSK4 stimulated HTR8 trophoblasts. The amount of intracellular Lm in HTR8 cells significantly increased (about four-fold) after Lm-infected neutrophils were applied onto the trophoblasts compared to uninfected, Lm-infected and Lm-platelet infected HTR8 cells. The same effect could be confirmed and was even more dominant in the viability assay (Figure 20 D). About 80-fold more viable bacteria were counted on the dishes were Lm-infected neutrophils were applied compared to Lm-infected HTR8 cells. A slight increase of viable Lm after infection with Lm-associated platelets is also visible, which could be explained by the fact that the amount of platelets is so huge and numbers are difficult to count precisely.

To sum up, neutrophils mediate the invasion of Lm into HTR8 cells after stimulation with Pam3CSK4. These results fit to the hypothesis 1: Lm infected shuttle entering through EVTs, because the infected shuttle (neutrophil) mediates a significant increase of invasion of EVTs (HTR8 cells), compared to Lm or Lm infected platelets.

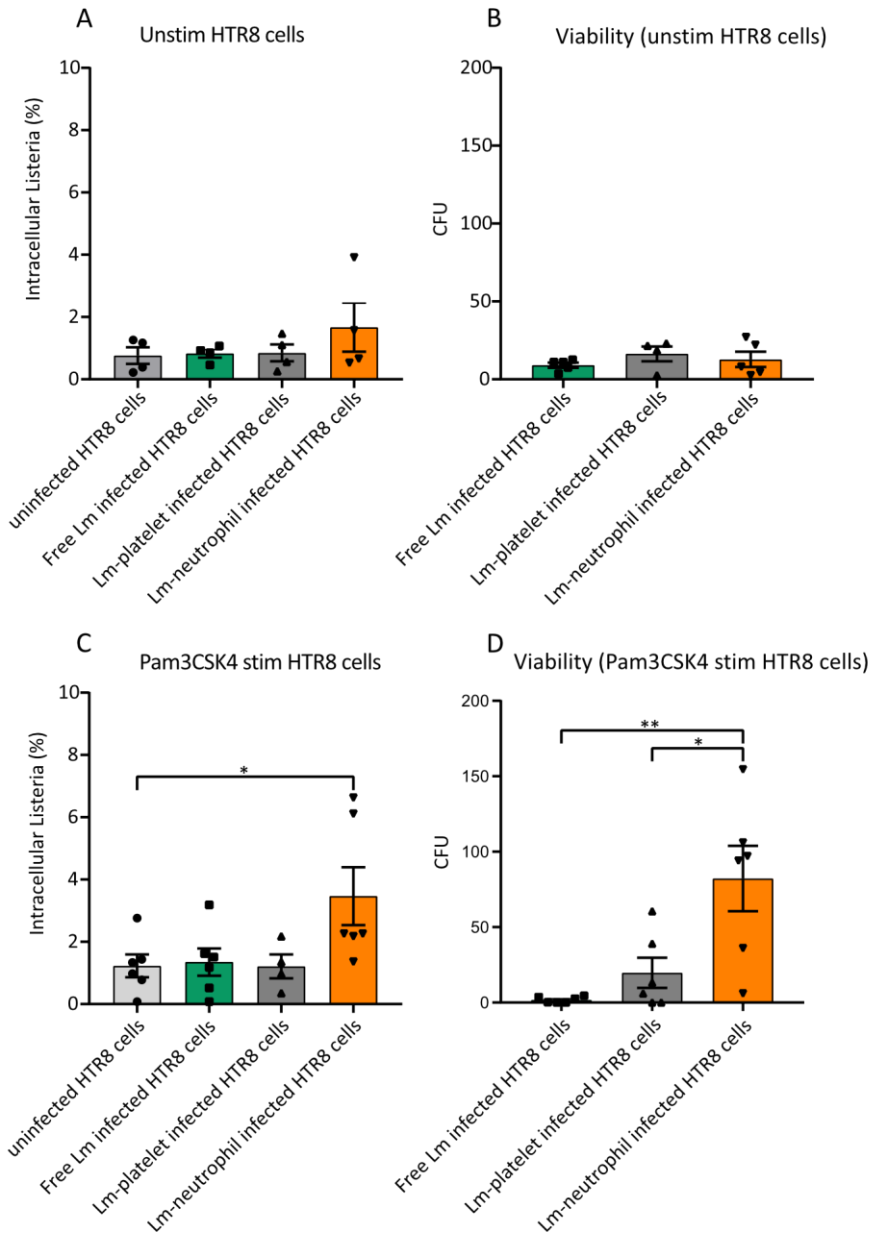


Figure 20. Invasion of HTR8 trophoblasts via free Lm, Lm-associated platelets and Lm-infected neutrophils. .
A. FACS analysis of infection of unstimulated HTR8 cells (mean±SEM, n=4 independent experiments, 1-way ANOVA, Tukey's multiple comparison). B. Check for viability of Lm in unstimulated HTR8 cells after infection (mean±SEM, n=4 independent experiments, 1-way ANOVA, Tukey's multiple comparison). C. FACS analysis of infection of Pam3CSK4 stimulated HTR8 cells (mean±SEM, n=5 independent experiments, 1-way ANOVA, Tukey's multiple comparison). D. Check for viability of Lm in Pam3CSK4 stimulated HTR8 cells after infection (mean±SEM, n=4 independent experiments, 1-way ANOVA, Tukey's multiple comparison).

4.11.4. Gating strategy of JEG3 cells

Next, we investigated JEG3 trophoblasts to identify the amount of intracellular Lm that were capable to invade JEG3 cells.

With a MOI6 free Lm that were taken up by neutrophils and the lysis obtained by deionized water, Lm infected neutrophils and Lm-associated platelets were added on stimulated or unstimulated

trophoblasts, respectively. Infection of the cells occurred for 1 h at 37 °C before gentamicin (100 µg/ml) was added for 30 min to kill all extracellular bacteria. Each well was washed with PBS and trophoblasts were scraped and cells stained with antibodies against CD54 (ICAM-1) and CD66b (neutrophils). Samples were then analyzed using a flow cytometer (3.2.17).

Figure 21 presents the gating strategy for JEG3 trophoblast infection without stimulation (Figure 21 A) and after stimulation with Pam3CSK4 (Figure 21 B). As in chapter 4.11.2 the JEG3 trophoblast population was identified based on the site scatter (SS) and forward scatter (FS) (plots not shown). The target population was identified via selection of ICAM-1 positive cells (plots not shown). Plots in Figure 21 depict the amount of cells double positive for Lm (CFSE) and ICAM-1 reflecting Lm that were capable of entering JEG3 cells.

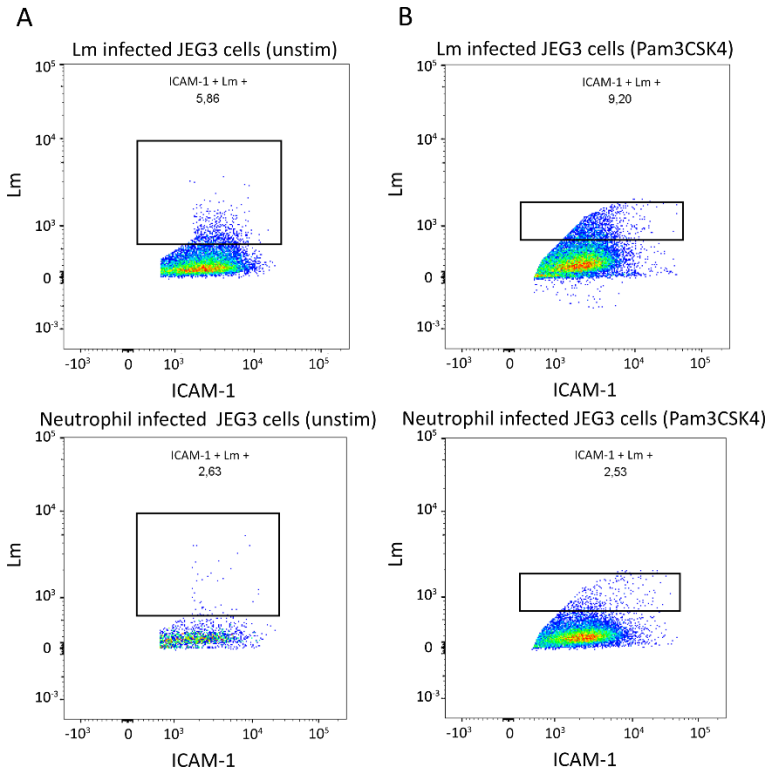


Figure 21. Gating strategy for free Lm infected and Lm-neutrophil infected JEG3 trophoblasts.
A. Infection of unstimulated JEG3 trophoblasts. Trophoblasts double positive for Lm and ICAM-1 were selected for analysis of percentage of intracellular Lm (Representative FACS plots are shown). B. Infection of Pam3CSK4 stimulated JEG3 cells. Trophoblasts double positive for Lm and ICAM-1 were selected for analysis of percentage of intracellular Lm (Representative FACS plots are shown).

4.11.5. Neutrophils do not mediate the invasion of Listeria into JEG3 trophoblast cells

After having established the gating strategy and handling of cells for this assay, we analyzed Lm uptake by JEG3 cells. In a first approach, unstimulated JEG3 trophoblasts were analyzed regarding their susceptibility to infection by Lm, Lm-associated platelets, or Lm-infected neutrophils. FACS plots in Figure 22 A indicate that free Lm were taken up by JEG3 cells. In addition, it was also found

that almost no Lm were entering JEG3 cells when Lm-associated platelets and Lm-infected neutrophils were co-incubated with JEG3 cells. Interestingly, viability investigations via CFU counting showed low numbers of CFUs suggesting that JEG3 cells are able to kill Lm and growth restrict Lm expansion and/or spreading (Figure 22 B.).

Furthermore, the same experiments were repeated with Pam3CSK4 stimulated JEG3 cells. Figure 22 B depicts the percentage of intracellular Lm after infection of Pam3CSK4 stimulated JEG3 cells. Lm infection of JEG3 cells again occurred and was comparable to unstimulated JEG3 cells for all groups. Similar to unstimulated JEG3 cells, no significant changes could be obersved in the viability assay, except for a slight increase of viable bacteria when free Lm had been applied (Figure 22 D).

To sum up, invasion of Lm into JEG3 cells is stronger than seen for HTR8 cells and, interestingly, independent of stimulation with Pam3CSK4. This is in line with hypothesis 2: Free Lm entering through SYNs, because infection of uncovered, free Lm of SYN (JEG3 cells) is significanly stronger than with Lm-associated platelets and Lm-infected neutrophils. However, when infected, JEG3 cells are more effective in killing Lm intracellularly therefore restricting Lm growth and spread.

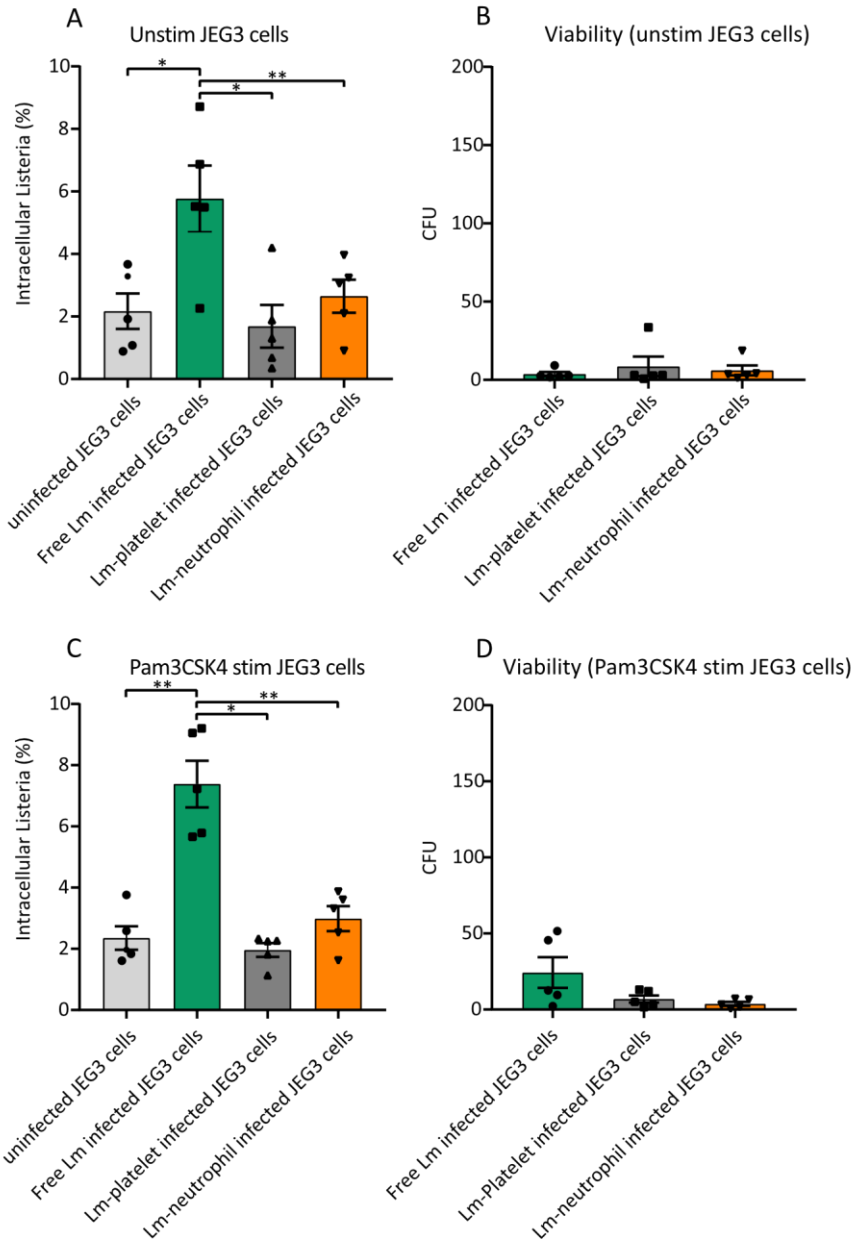


Figure 22. Invasion of JEG3 trophoblasts via Lm, Lm-associated platelets and Lm-infected neutrophils.
A. FACS analysis of infection of unstimulated JEG3 cells (mean±SEM, n=5 independent experiments, 1-way ANOVA, Tukey's multiple comparison). B. Viability check of Lm in unstimulated JEG3 cells after infection (mean±SEM, n=5 independent experiments, 1-way ANOVA, Tukey's multiple comparison). C. FACS analysis of Lm-infection of Pam3CSK4 stimulated JEG3 cells (mean±SEM, n=5 independent experiments, 1-way ANOVA, Tukey's multiple comparison). D. Viability check of Lm in Pam3CSK4-stimulated JEG3 cells after infection (mean±SEM, n=4 independent experiments, 1-way ANOVA, Tukey's multiple comparison).

4.11.6. Toll-like receptor 1/2 Expression on trophoblast cells

Because neutrophils mediate the invasion of Lm into HTR8 cells after stimulation with the TLR1/2 agonist Pam3CSK4, both trophoblast cell lines are characterized regarding their TLR1/2 expression.

Expression of TLR1 and TLR2 on HTR-8 cells and JEG-3 cells were investigated via FACS analysis. Trophoblasts were stained with antibodies against CD281 (TLR1), IgG1κ (isotype control), CD282 (TLR2) and IgG2aκ (isotype control) (3.2.14).

Low TLR1 and TLR2 receptor expression was found in both cell lines, suggesting that the observed difference in the effects of TLR1/2 stimulation on Lm infection and viability between HTR8 cells and JEG3 cells cannot be explained by TLR1/2 expression differences (Figure 23).

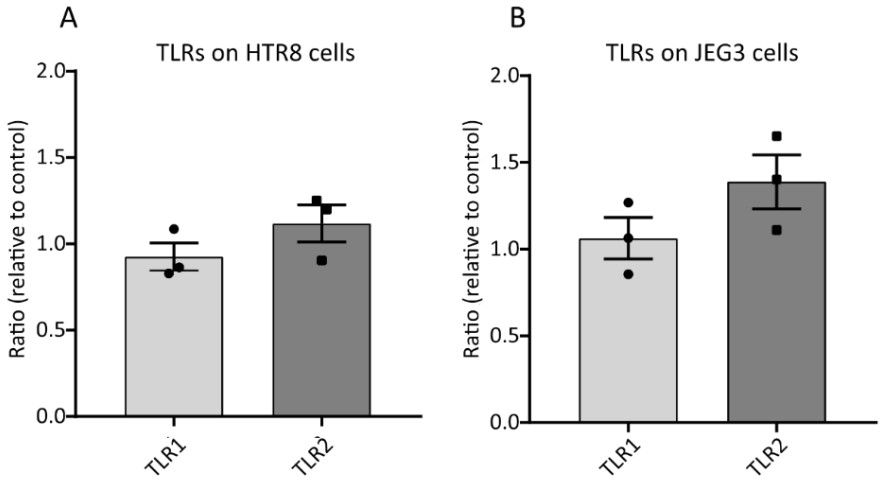


Figure 23. TLR1/2 receptor expression of trophoblasts.
A. FACS analysis of the expression of TLR1/2 on HTR8 cells (mean±SEM, n=3 independent experiments, unpaired student's t-test). B. FACS analysis of the expression of TLR1/2 on JEG3 cells (mean±SEM, n=3 independent experiments, unpaired student's t-test).

4.12. Listeria and host cell viability over time

So far, the *in vitro* data obtained in the mouse and human system indicate that neutrophils:

- provide a niche for Lm in the intravascular compartment
- facilitate uptake of Lm in the presence of C3 (serum)
- mediate the invasion of Lm into HTR8 trophoblast cells

Therefore, I conducted *in vivo* assays to further investigate a role of neutrophils during Lm-infection of placenta and fetus.

First, viability of cells was checked after Lm infection and gentamicin treatment using the Zombie Yellow Fixable Viability Kit. This amine-reactive dye selectively enters mammalian cells with a disrupted membrane resulting in a bright fluorescent signal. Cells were incubated with Zombie in the dark and analyzed via flow cytometry (3.2.12). FACS results of Figure 24 A show that over time no significant increase of dead PMNs could be observed. Furthermore, there was no influence of

gentamicin on human PMNs after four hours of infection (Figure 24 B). These results demonstrate that treatment and experimental conditions do not influence viability of human PMNs.

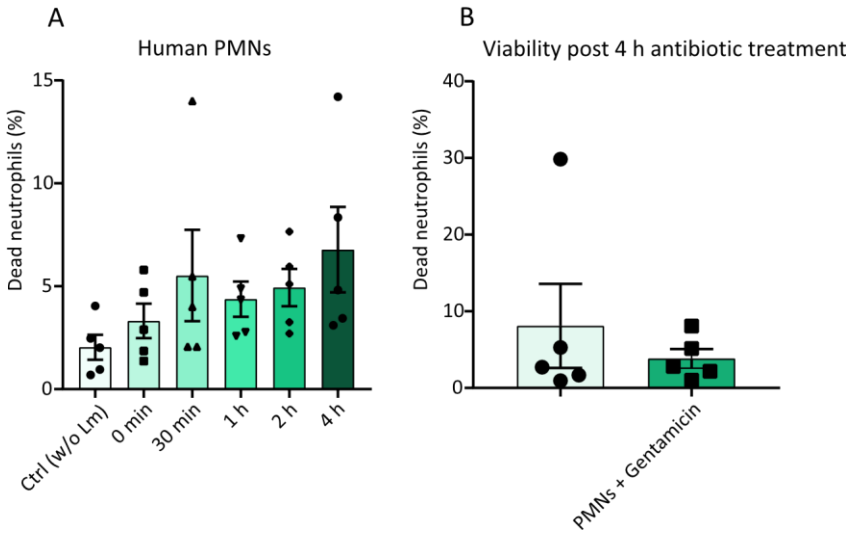


Figure 24. Viability of Lm and host cells over time.
A Viability of human PMNs during four hours infection with Lm (mean±SEM, n=5 independent experiments, 1-way ANOVA, Tukey's multiple comparison). B. Influence of four hours gentamicin treatment on viability of human PMNs (mean±SEM, n=5 independent experiments, unpaired student's t-test).

4.13. Neutrophils are critical for placental and fetal infection with Listeria

As Lm can successfully invade neutrophils for a limited time, we next tested whether neutrophil depletion in the pregnant mouse *in vivo* would influence placental and fetal Lm infection. To do this mice were treated i.v. with Ly6G (1A8, 100 µg/injection) antibody 24 h and 4 h before the experiment started. In another approach pregnant mice were treated with antibodies against CD11b/CD18 (MAC-1, clone TIB128, 100 µg) and CD11a (LFA-1, clone TIB217, 30 µg) injected i.p. 2 h before Lm-infection to block neutrophil adhesion (Figure 25). In both approaches, pregnant KiE16P or C57BL/6 mice were infected i.v. with 4 x 10^6 Listeria of the EGDe strain in 100 µl PBS after neutrophil depletion and blocking neutrophil adhesion has been performed. After 8 h, mice were sacrificed and maternal liver (control organ), all placentas and fetuses were taken out, washed and homogenized. To remove extracellular bacteria, gentamicin (100 µg/ml) treatment was conducted. Therefore, cell suspensions of smashed organs were plated on agar dishes. CFUs were evaluated the day after the experiment (3.3.4).

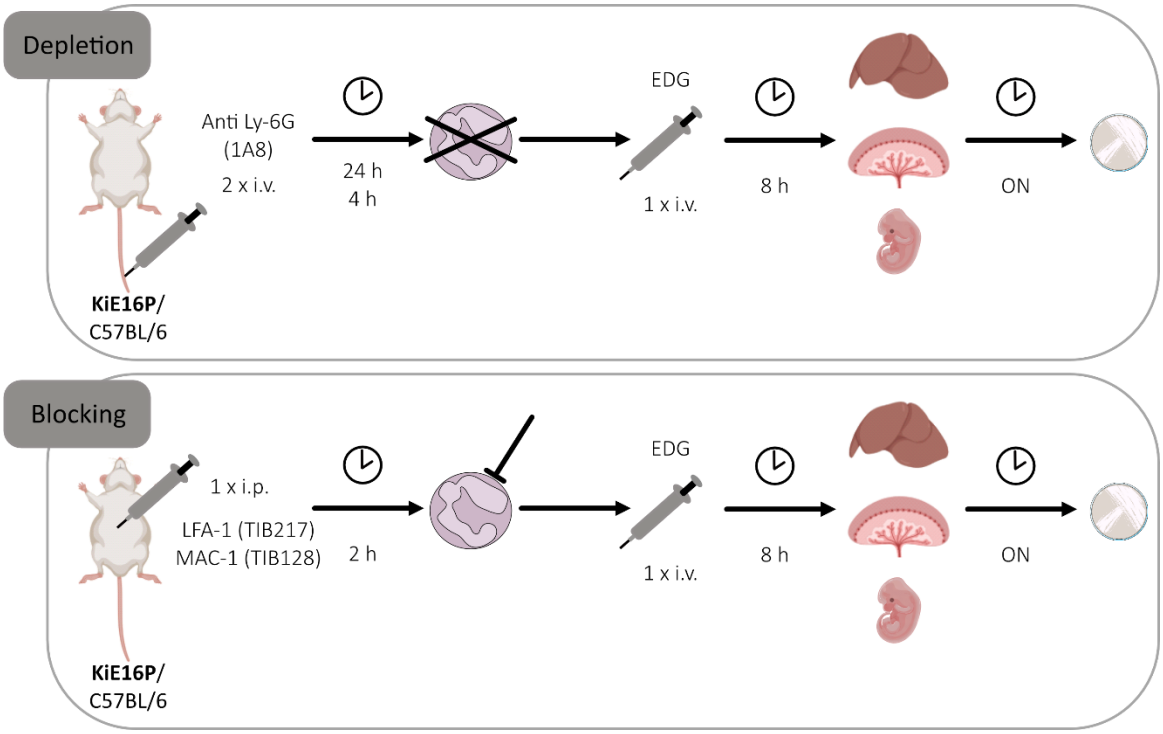


Figure 25. Experimental design to gain insight into neutrophil function during fetal and placental infection with Lm. A. Neutrophil depletion in vivo in KiE16P or C57BL/6 mice. B. Blocking of neutrophil adhesion in vivo in KiE16P or C57BL/6 mice.

For depletion experiments, depletion of neutrophils was confirmed via FACS analysis of peripheral blood samples. Figure 26 A depict the gating strategy to identify neutrophil populations before and after depletion. Leukocytes were identified via CD45 indicated in the first plot of A from which in the second plot CD11b positive cells were selected. Discrimination of neutrophils, inflammatory monocytes, non-inflammatory monocytes as well as Gr1 intermediate cells occurred through additional markers of Ly6C/G (Gr1) and CD15. After injection of depleting antibodies slight shifts of the populations were visible. Percentages of neutrophils were analyzed before and after depletion. Neutrophil markers decreased by about 70 % in C57BL76 mice (Figure 26 B). In KiE16P mice an almost 60 % reduction was observed (Figure 26 C).

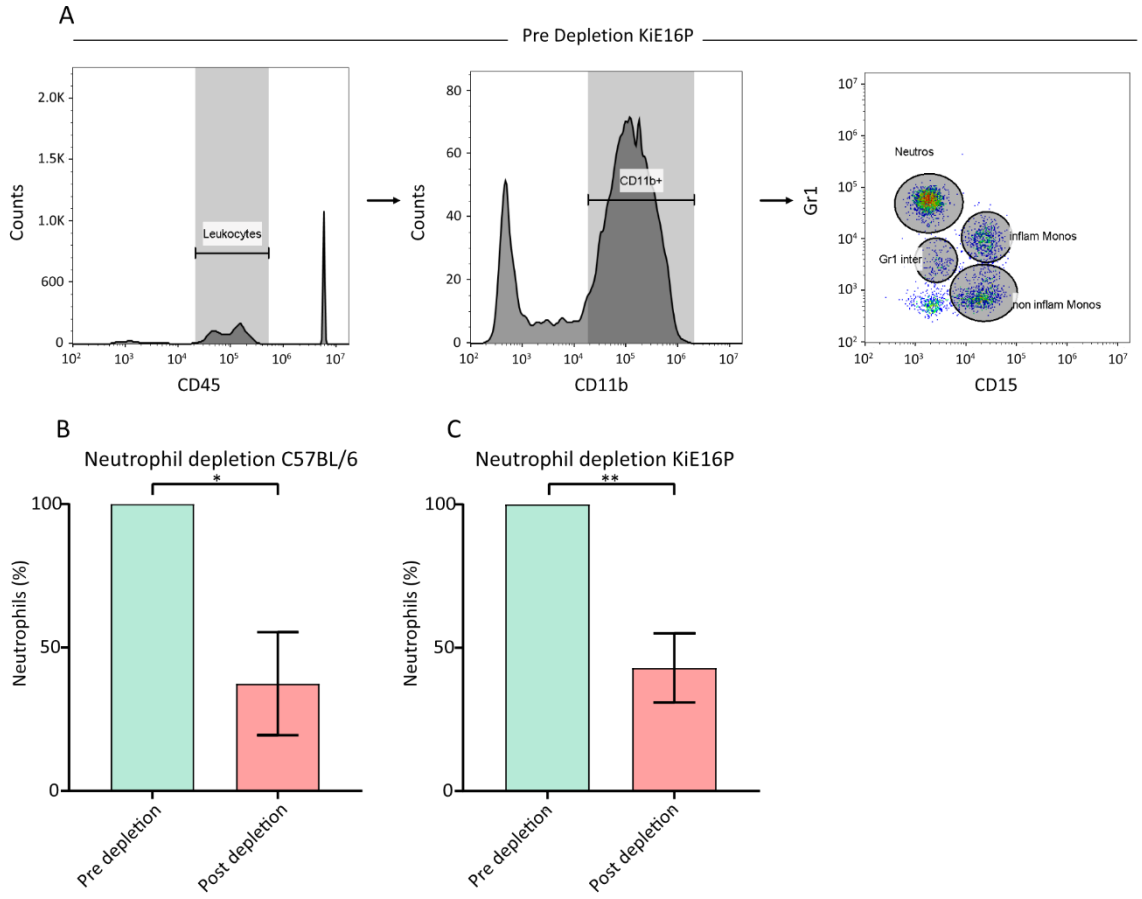


Figure 26. Neutrophil depletion in KiE16P and C57BL/6 mice.
A. Gating strategy to identify neutrophils in blood samples of KiE16P mice before depletion. B. FACS analysis of percentage of neutrophils before and after depletion in C57BL76 mice as control (n=5 mice). C. FACS analysis of percentage of neutrophils before and after depletion in KiE16P mice (n=5 mice).

4.13.1. Blocking adhesion of neutrophils or their depletion impairs placental and fetal infection.

Lm infection in mice *in vivo* were conducted with C57BL/6 and KiE16P mice. To test the hypothesis that neutrophils are a viability niche in the intravascular compartment und function as a shuttle to the placenta and thus assist Lm to overcome the placental barrier, infection events of C57BL/6 and KiE16P mice were expected to be the same. Thus, after depletion of neutrophils less or no infection of the placenta and fetus were expected. In both mice, two conditions were compared:

1. Lm infection of untreated mice
2. Lm infection after neutrophil depletion

Therefore, C57BL/6 and KiE16P mice were infected with Lm and after 8 h, mice were sacrificed and maternal liver (control organ), placentas and fetuses were taken out, washed and homogenized and treated with gentamicin, before cell suspensions of the respective smashed organ were plated on agar dishes. CFUs were evaluated the day after the experiment (3.3.4).

In the next set of experiments the same procedure was conducted, but after neutrophil depletion. Figure 27 shows the amount of viable Lm in the placentas (Figure 27 A) and the bacterial burden in fetuses (Figure 27 B). The first bars in Figure 27 A and B show the results of Lm infection in untreated and neutrophil depleted in C57BL/6 and KiE16P mice. Neutrophil depletion did not influence the bacterial burden after depletion in C57BL/6 mice, but in KiE16P mice. Neutrophil depletion significantly decreased listerial burden in placentas of KiE16P mice (Figure 27 A). In general, the amount of viable Lm was significantly increased in untreated KiE16P mice compared to WT mice. This points to a functional role of humanized E-Cadherin receptor, which is the only difference between the two mouse lines.

A similar pattern could be observed for listerial burden in fetuses (Figure 27 B.). The results are depicted as overall percental ratio of infected fetuses and not as amount of CFUs per mouse, as in Figure 27 A. The fetal infection of untreated KiE16P mice went down from 30 % to around 10 % after neutrophil depletion in KiE16P mice. Surprisingly, 0 % of fetuses were infected when neutrophils were depleted in C57BL/6 mice, although placentas were infected after neutrophil depletion in C57BL/6 mice, which was similar to untreated C57BL/6 mice.

Next, neutrophil adhesion blockng antibodies were injected into KiE16P mice before Lm infection occured. For blocking neutrophil adhesion, the same effects as for neutrophil depletion was observed. Placental burden was significantly reduced compared to untreated KiE16P mice (Figure 27 A) and amount of infected fetuses dropped to about 15 % (Figure 27 B). These results demonstrate that blocking adhesion of neutrophils or their depletion impairs placental and fetal infection with Lm.

To test for a functional role of humanized E-Cadherin, KiE16P mice were infected with an EGDe strain lacking Internalin A, which binds to human E-Cad (3.3.4).

Bacterial burden of the InlA deficient mutant strain were significantly reduced in placentas of KiE16P mice, and similar to experiments where neutrophils were depleted or neutrophil adhesion blocked (Figure 27 A). Also, listeral burden in fetuses was decreases in KiE16P mice (Figure 27 B).

To conclude, human E-Cad expressed in the mouse is critical for Lm infection of placenta and fetus.

For all conditions also bacterial burden of maternal liver was investigated. No significant difference was observed for the different treatment groups (data not shown).

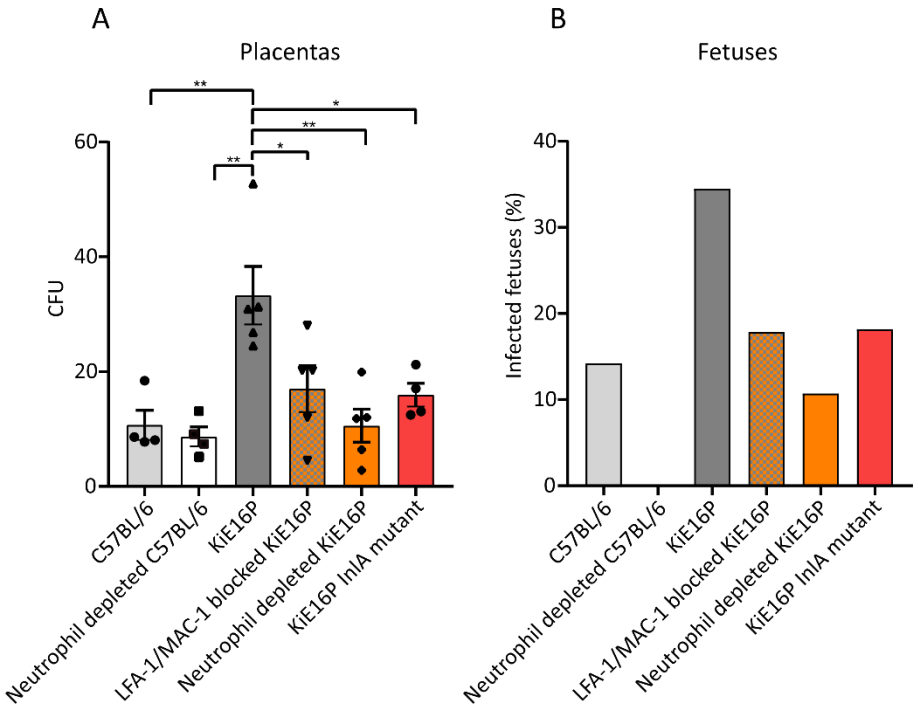


Figure 27. Bacterial burden of placentas and fetuses.
A. Bacterial burden of placentas depicted in amount of CFUs per mice (mean±SEM, n=4-5 mice per condition, 1-way ANOVA, Tukey's multiple comparison). B. Overall percentage of infected fetuses per condition (n=4-5 mice per condition).

5. Discussion

In this work, my aim was to elucidate the molecular mechanisms of placental and fetal Lm-infection hypothesizing that Lm need a cell carrier as a niche to escape from immune recognition and infect the placenta and fetus. Conducted experiments revealed that neutrophils provide a niche for Lm in the intravascular compartment and mediate the invasion of Lm into stimulated HTR8 trophoblast cells *in vitro*. Furthermore, our *in vivo* experiments uncover a crucial role of neutrophils for placental and fetal infection with Lm, because blocking adhesion of neutrophils or their depletion impairs placental and fetal infection with Lm.

5.1. Neutrophils act as a shuttle, survival niche and viability factor for Listeria in the intravascular compartment

5.1.1. Role of neutrophils as a shuttle for Listeria

Neutrophils provide a niche for Lm in the intravascular compartment and serve as a shuttle for Lm to the placenta. That conclusion is one of the major statements in this work and of high relevance, but was an unexpected finding, as monocytes were considered to serve as a predominant shuttle and survival niche of Lm. Interestingly, neutrophil depletion studies indicated a crucial role for neutrophils but not monocytes in murine Lm infected liver (Witter, Okunnu and Berg, 2016). Furthermore, surprisingly it was shown for Lm infected monocytes in the gut that they are the cell type, which gets infected most, but does not serve as a growth niche or viability factor (Jones and D'Orazio, 2017). The majority of Lm was internalized by monocytes, but only a few Lm reached the cytosol (Jones and D'Orazio, 2017). Consequently, Lm cannot replicate and stay viable in that cell type. Those results were confirmed in this thesis work for peripheral monocytes. Confocal images indicate high amounts of Lm associated with monocytes, which was in line with the results of our shuttle screening and following viability check. Although the highest amount of monocytes was infected with Lm, significantly less Lm stayed viable in PBMCs, which was the opposite for neutrophils and thus identified neutrophils as the shuttle and viability factor for Lm. Ongoing experiments in cooperation with Dr. Bastian Popper from the Core Facility Tiermodelle of the Biomedical Center Munich using electron microscopy (EM), will help to demonstrate that Lm are really located in vacuoles or the cytoplasm of neutrophils and monocytes. A

5.1.2. Neutrophils act as a survival niche for Listeria

We showed in the human system that neutrophils serve as a survival niche and viability factor in the intravascular compartement. That finding leads to the next question, which needs to be assessed in the future: How do Lm survive in the neutrophils?

Plenty of virulence factors are known that help Lm to survive, like LLO, which helps Lm to escape from host vacuoles into cytoplasm (Vázquez-Boland *et al.*, 2001). Survival of Lm in phagocytes could be due to its adaption to the pH of the host cell, because LLO is active at a low pH, pH 5.5 – 6.0, which resembles the exact pH value of the early phagosome (Dussurget, Pizarro-Cerda and Cossart, 2004). Furthermore, Lm produces the enzymes catalase and superoxide dismutase to stay resistent against oxidative mechanism and thus ROS produced by host cells (Pitts, Combs and D'Orazio, 2018). However, survival in phagocytic cells like neutrophils is in general untypical. An interesting and further possible explanation could be e.g. the circumvention of inflammasome activation. A mechanism that was identified for the bacterium *Salmonella typhimurium* in human macrophages. It was shown that Salmonella was capable of evading NLRC4 and NLRP3 inflammasome responses via its virulence factor Salmonella pathogenicity island-2 (SPI2) and thus preventing pyroptosis (Bierschenk *et al.*, 2019). For *Burkholderia thailandensis*-infected neutrophils it was shown that NLRC4 activation alone is not able to clear cytosolic bacteria, but caspase-11 does (Kovacs *et al.*, 2020). Because the majority of bactericidal properties is restricted to neutrophil vacuoles, Lm could evade inflammasome activation and use neutrophils as a niche. At least this could be true for a certain time window till inflammasome via caspase-11 is activated (Kovacs *et al.*, 2020). It would be of interest, whether a similar mechanism is true for Lm in human neutrophils, which could be investigated via detection of caspase-1/11 cleavage or IL-1β release after Lm infection.

5.2. Neutrophils mediate the transfer of Listeria into trophoblast cells (HTR8)

5.2.1. EVTs are the major entry – Verification of hypothesis 1: Lm infected shuttle entering through EVTs

My thesis work showed that the syncytiotrophoblast cell line JEG3 was susceptible to infection with free Lm, independent of stimulation, whereas Lm-infected neutrophils mediate the invasion of Lm into the extravillous trophoblast cell line HTR8. Conducted viability experiments revealed that JEG3 cells are effective in intracellular Lm killing, because viability of Lm always was very low. In contrast to that, the amount of viable intracellular Lm in HTR8 cells significantly increased after Lm-infected neutrophils were layed over the trophoblasts.

Our results are in line with the literature, where it could be shown that SYN was immense resistent against bacterial spreading, although the SYN shows the biggest area that is in direct contact with the maternal blood. The opposite was observed for the EVT (Robbins *et al.*, 2010). During development and growth of the placenta, E-Cad is downregulated at the apical surface, which enhances the selection pressure for Lm to take an E-Cad independent infection mechanism of the

placenta (Robbins *et al.*, 2010). In the early state of pregnancy, the entry point for Lm could be the EVT and in the late trimester the SYN (Charlier, Disson and Lecuit, 2020). Thus, both hypotheses, hypothesis 1: Lm infected shuttle entering through EVT, and hypothesis 2: Free Lm entering through SYN, might be true. Interestingly, the possibility of two ways of infection by Lm was already shown for endothelial cells long time ago (Drevets *et al.*, 1995). However, our finding is that both ways of Lm infections are possible, but the SYN, in this work reflected by the JEG3 trophoblast cell line, restricts the bacterial growth and consequently this pathway is a 'cul-de-sac' for Lm. Furthermore, not just only hypothesis 1 could be verified, but also an unexpected function of neutrophils was identified: Lm-infected neutrophils mediate the invasion of Lm into stimulated HTR8 trophoblasts.

InlP binds to afadin, which is a cell-cell junction associated protein with scaffolding functions. Upon binding cortical tension is disrupted, which initiates transcytosis of Lm at the basal site of cell monolayers and in consequence facilitates cell-to-cell spread across the basement membrane (Faralla *et al.*, 2018). SYNs are highly resistent to Listeria because of their architecture (Ander, Diamond and Coyne, 2019). They do not possess e.g. cell-cell junctions and thus afadin and in consequence, InlP would be unimportant. In contrast to the SYN, the EVT fulfill all of the mentioned criteria (Robbins *et al.*, 2010). This is of importance, because it would explain why growth of Lm was restricted in our experiments. It would also give the explanation, how Lm overcome the basement membrane and infect the fetal system (Faralla *et al.*, 2018). Thus, the infection route of Lm could be: 1.) infection of EVTs, 2.) cell-to-cell spread of the underlying CTs, 3.) spread to the basement membrane, 4.) infection of fetal stromal cells and 5.) entry into fetal capillaries and thus dissemination in the fetal circulation.

5.2.2. Lm entry into the trophoblast

For the entry of Lm into trophoblast cells Listeria might traffic in maternal neutrophils to the EVT and infect it via cell-to-cell spread or via local release and extracellular/direct invasion of Lm (Robbins *et al.*, 2010).

Although we do not know how Lm enter EVTs (cell-to-cell spread or direct invasion), pre-stimulation of EVTs helped to infect EVTs with Lm. This might explain why certain women get infected with Lm during pregnancy and others not and why certain host cells get infected and others not, which is one of the major question in infection biology (Eisenreich *et al.*, 2017). Small lesions in the placenta due to disruptions, other pre-infections or activated placental neutrophils could create spots that support the entry for bacteria into host cells (Chung *et al.*, 2014; Giaglis *et al.*, 2016; Dudeck *et al.*, 2019). During those szenarios, different cytokines and substances could

cause that pre-stimulation or activation of trophoblast (Abou-Bacar *et al.*, 2004; Vásquez, Segura and Blair, 2013).

I had performed pre-stimulation experiments of the two different trophoblast cell lines to investigate which stimulus makes trophoblasts more suceptible to Lm infection. Trophoblast stimulation with Pam3CSK4 facilitated the transfer of Lm from neutrophils into HTR8 cells, but not into JEG3 trophoblasts.

Several mechanisms of how intracellular pathogens exit host cells are known. This can be lytic or non-lytic and destroy the host cell or not (Hybiske and Stephens, 2015). Pyroptosis was initially identified as defense mechanism of immune cells, but additionally serves as an exit mechanism from host cells for bacteria like Francisella, Salmonella, Shigella, Legionella and also Listeria (Flieger *et al.*, 2018). Since it is already known for macrophages and endothelial cells that Lm uses pyroptosis as exit stratgy, might also be the case for neutrophils (Hybiske and Stephens, 2015). Lm induces pyroptosis in macrophages and endothelial cells via three ways when already located in the cytoplasm:

1. Listerial Flagellin activates canonical NLRC4 inflammasome
2. DNA of listerial origin activates absent-in-melanoma-2 (AIM2) inflammasome
3. LLO activates NLRP3 inflammasome

All three ways cause caspase-1 activation, and IL-1β and IL-18 secretion and finally host cell lysis via gasdermin D pores (Hybiske and Stephens, 2015).

5.3. Neutrophils are crucial for placental and fetal infection with Listeria

Our murine *in vivo* experiments with KiE16P and C57BL/6 mice pointed to a crucial role of neutrophils for placental and fetal Lm infection. Blocking adhesion of neutrophils or their depletion impaired placental and fetal infection and human E-Cad, expressed in the KiE16P mouse, was critical for Lm infection of placenta and fetus. To further decipher the mechanism of how neutrophils deliver Lm to the placenta and whether Lm directly invade the trophoblast via cell-to-cell, without leaving the neutrophil, a listerial ActA mutant could be used (Bakardjiev, Stacy and Portnoy, 2005). In case Lm infection at the placenta occurs via cell-to-cell spread, no infection of the placenta would proceed.

5.3.1. The role of CD66b on neutrophils during Listeria infection

Our experiments demonstrate that CD66b (CEACAM-8) is a suitable activation marker for neutrophils after Lm infection and intensity of CD66b mobilization reflected amounts of listerial burden of neutrophils.

During pregnancy immunological conditions changes, which is reflected in this work by a significant increase of the WBC caused by a significant increase of neutrophils but not monocytes. The identification of neutrophils as a shuttle and growth niche I could also show in pregnant women, although the number of experiments was limited due to restricted availability of pregnant blood donors. Comparison of CD66b mobilization of neutrophils of pregnant and non-pregnant donors indicated differences in the intensity of Lm infection. This is an interesting finding of this work, because this marker could be involved in mediating Lm infection during pregnancy, since CD66b overexpression was found on decidual PMNs in the placenta (Giaglis *et al.*, 2016).

It was already shown that PMA or Gram-positive bacteria, like *Staphylococcus aureus*, induce CD66b mobilization (Schmidt *et al.*, 2015). This is in line with our results of CD66b mobilization on neutrophils after Lm stimulation,which may favor neutrophil adhesion via CD11/CD18 (Skubitz and Skubitz, 2008; Schmidt *et al.*, 2012).

Of note, direct binding of Lm to CEACAM-1, CEACAM-3, and CEACAM-8 could not be shown in this thesis work. Binding of Lm to other receptors, like TLR2, can cause co-localization with CEACAMs (Singer *et al.*, 2014). This would also make sense, since it was shown that CEACAM-1 strongly is expressed by EVTS at the site of implantation as well as by primary invasive EVT cultures (Vićovac *et al.*, 2007). One could speculate that Lm take the infection route via the EVT and adherence of neutrophils to the placenta is supported by CEACAMs. A lot of bacterial pathogenes, like *Neisseria gonorrhoeae*, enhance adhesion to the host cell via CEACAM stimulation (Muenzner *et al.*, 2005). Furthermore, *Helicobacter pylori* manipulates human neutrophils via interaction with CEACAMs and thus facilitates e.g. translocation, phagocytosis and bacterial survival in the phagosome (Behrens *et al.*, 2020). The missing CEACAM-8 homolog, together with the absent E-Cad receptor in rodents, could give reasons for the species specifity of Lm infection in humans versus mice (Singer *et al.*, 2014).

5.4. Clinical relevance of the results

A huge problem concerning placental infections with Lm and potentially the loss of pregnancy is the estimated number of unreported cases. Pregnant women often do not know that they are infected, because of missing symptoms. Treatment prophylaxis to prevent listeriosis during

pregnancy is difficult, and currently not available. An interesting new concept could be to take probiotics. Recently, it could be shown that bioengineered *Lactobacillus casei* probiotic (BLP) expressing Listeria adhesion protein (LAP) impedes vertical transmission of the placenta as well as pro-inflammatory immune response of the mother in pregnant guinea pigs (Ryan *et al.*, 2021).

5.5. Conclusion

This work reports for the first time a trojan horse mechanism of Lm hijacking neutrophils enabling infection of the placenta and fetus and proceeding as follows (summarized in Figure 28):

I. Uptake of Lm by human neutrophils, which is facilitated by complement factor C3.
II. Following Lm uptake, neutrophils provide a survival niche for Lm in the intravascular compartment and function as a shuttle to the placenta.
III. Lm infection results in CEACAM-8 mobilization on human neutrophils.
IV. Neutrophils mediate the invasion of Lm into HTR8 trophoblasts after PamCSK4 stimulation *in vitro* and are crucial for placental and fetal infection with Lm. Blocking adhesion of neutrophils or their depletion impairs placental and fetal infection with Lm *in vivo*.

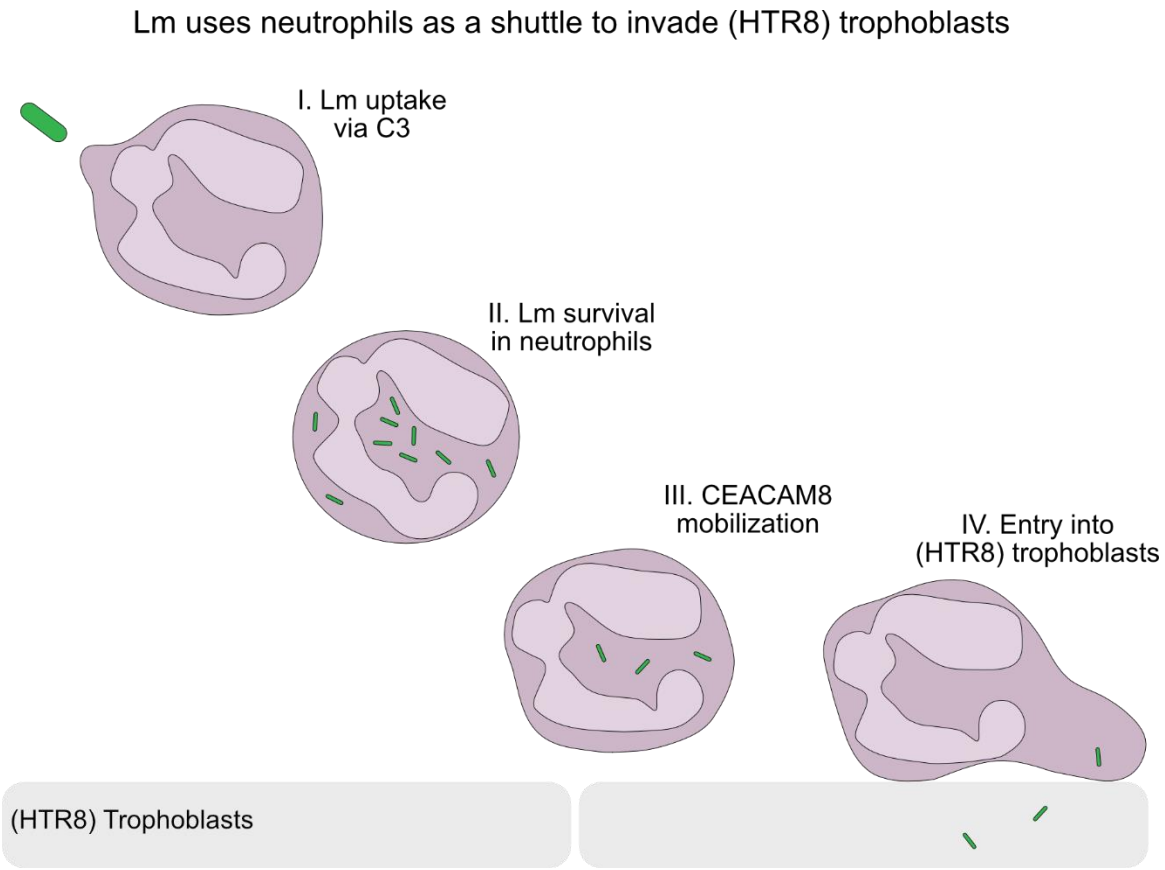


Figure 28. Trojan horse mechanism of Lm of trafficking into neutrophils to the placenta and infecting the placenta *in vivo* or HTR8 trophoblasts *in vitro*.

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List of Publications

Knapp A, **Ripphahn M**, Volkenborn K, Skoczinski P, Jaeger KE. J.: Activity-independent screening of secreted proteins using split GFP, *Biotechnol.*, 2017 Sep 20; 258:110-116.

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Rohwedder I, Heinig K, Wackerbarth L, Florian A, Nussbaum C, Altstätter J, **Ripphahn M**, Salvermoser M, Straub T, Gunzer M, Klein-Hitpass L, Schmidt-Supprian M, Schulz C, Ma A, Walzog B, Heinig M, Sperandio M: A20 and the non-canonical NF-κB pathway are key regulators of neutrophil recruitment during fetal ontogeny (in preparation).

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