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Trojan Horses and Achilles' Heel

Listeria monocytogenes use blood monocytes to travel to the decidua
and infect the placenta

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Abstract

Listeria monocytogenes are Gram-positive facultative intracellular bacteria and are a well-known threat for women and fetuses during pregnancy. Recent findings suggest that the primary vulnerability of the placenta is in the implantation site, specifically at the level of extravillous cytotrophoblasts (EVTs). However, EVT's are not accessible to the bacteria from the blood where they are found. Here, we used human primary blood cells and human placental explants to explore the possible pathways used by *L. monocytogenes* to travel from blood to placenta and then to spread to the fetus. We first showed that blood monocytes are specifically susceptible to listerial infection and are a good candidate as carrier of the bacteria through the bloodstream. After blood monocyte infection, *L. monocytogenes* would have to colonize the Decidua in order to access the placenta. We then characterized the infection of decidual cells and observed that decidual fibroblasts, macrophages and NK cells are very sensitive to infection. Decidual Nk cells actually undergo a rapid induction of apoptosis following the infection with *L. monocytogenes*. Finally, we showed that these infected leukocytes can interact with the human placenta, and that EVT's are much more susceptible to infection than the syncytium. *L. monocytogenes* can then travel from infected leukocytes to EVT's and thus infect the placenta, and later the fetus.

Key words: *Listeria monocytogenes*, blood leukocytes, monocytes, neutrophils, decidua, placental explants, cell-to-cell spread, extravillous cytotrophoblasts, syncytium.

Introduction

Listeria monocytogenes, a Gram-positive facultative intracellular bacterium, are a well-known foodborne pathogen of humans and other mammals. Human's exposure to *L. monocytogenes*, through contaminated food, can occur many times per year but is mostly asymptomatic (Notermans *et al.* 1998). Cases of listeriosis are associated with ingestion of high doses of bacteria (up to 10^6 bacteria/g of food) (Lamont *et al.* 2011) or for immunocompromised individuals. However, during pregnancy, prevalence of Listeriosis is estimated to be up to 20 times higher compared to non-pregnant population (Janakiraman 2008, Lamont *et al.* 2011). In the US, among 1600 cases per year, 16.9% to 27% of cases are associated with pregnancy (Janakiraman 2008, Jackson *et al.* 2010). This suggests either a specific sensitivity to *L. monocytogenes* during pregnancy or a specific tropism of the bacteria. The severity of the infection is variable but pregnancy-associated listeriosis can be a real threat to the fetus. Complications can lead to stillbirth, neonatal diseases, such as meningitis, or spontaneous abortions. The mechanisms by which *L. monocytogenes* cross the maternal-fetal barrier and infect the placenta are still not completely understood and remain controversial.

The placenta is a chimeric organ at the interface between fetal and maternal blood. It is the organ responsible of both the nutrition and the defense against infections of the fetus. It is then the major barrier for *L. monocytogenes* to access the fetus. The human placenta (Suppl. 1) forms a villous tree bathed in maternal blood and is composed of differentiated fetal cells from a common origin: the fetal trophoblasts. Fetal blood capillaries are surrounded by a stroma made of fibroblast and by trophoblasts (Suppl. 1c); at the interface with maternal blood, these trophoblasts differentiate in a multinucleated syncytium (SYN) (Benirschke *et al.*

2006). The SYN represents most of the interface between mother and fetus. Most villi of this villous tree are floating freely in the maternal blood (Floating villi=FV) and some villi anchor the placenta in the maternal endometrium by invading the uterine lining (Anchoring villi=AV). At the tip of these AV, fetal trophoblasts, called Extra Villous Trophoblasts (EVTs), invade and modify the endometrium and form a novel immunologic organ, the decidua (Red-Horse *et al.* 2004). The decidua is responsible for the defense and the tolerance of the placenta. Depending on the gestational age, 50 to 70% of the decidual cells are leukocytes, mainly NK cells and macrophages (Benirschke *et al.* 2006). The rest of the tissue is composed of fibroblasts, EVT's and blood vascular cells.

It has been shown that infection of the placenta depends on the ability of *L. monocytogenes* to travel intracellularly from cell to cell (Bakardjiev *et al.* 2005, Le Monnier *et al.* 2007). Three different pathways have been described for the intracellular infection by *L. monocytogenes*. Firstly, *L. monocytogenes* can induce its own internalization. Surface proteins, such as the Internalins A or B (InlA, InlB), interact with specific cellular receptors, i.e. E-cadherin and c-Met respectively, and induce bacterial internalization into a vacuole. The bacteria can then escape to the cytoplasm of the host cell and multiply. Secondly, *L. monocytogenes* can be phagocytosed by a professional phagocyte, survive and escape the phagosome to reach the cytoplasm. Finally, once in the cytoplasm, *L. monocytogenes* use actin-based motility, notably depending on the actin nucleator ActA, and can drive itself in an adjacent cell through both plasmic membranes. *L. monocytogenes* can then enter cells by induced phagocytosis, natural phagocytosis or by cell-to-cell spread from a previously infected cell.

The transmission of *L. monocytogenes* from mother to fetus can occur at two sites of direct contact between maternal cells and fetal trophoblasts in the human placenta: It has been proposed that free bacteria travelling in maternal blood could infect directly the SYN in an

Internalin-dependent manner (Lecuit *et al.* 2004). But this direct pathway is debated since Internalins have been shown to have only a modest effect in placental infection *in vivo* (Bakardjiev *et al.* 2005, Wollert *et al.* 2007, Disson *et al.* 2008). However, recent findings suggest that the primary vulnerability is in the implantation site, specifically EVT. For example Redline and Lu have shown that infection of pregnant mice with *L. monocytogenes* leads to infection of the decidua first, followed by infection of the placenta and fetus (Redline *et al.* 1988, Redline *et al.* 1988). Recently, using human placental explants we have shown that EVTs are much more susceptible to infection than SYN (Robbins *et al.* 2009). *L. monocytogenes* can infect EVTs directly, in an Internalin A-mediated invasion as well as through intercellular spread from a previously infected human macrophage-like U937 cell line (Sundstrom *et al.* 1976).

The main obstacle to validate this hypothesis was that EVTs are not directly accessible from the bloodstream where bacteria are found. *L. monocytogenes* would first have to reach EVTs into the decidua. However a direct crossing of blood vessels has never been described and is then unlikely. Previous reports using guinea pig and mice models have shown that *L. monocytogenes* can travel through the bloodstream inside blood cells (Bakardjiev *et al.* 2006). In fact, in mice, *L. monocytogenes* are found inside of “inflammatory” Ly-6C^{high} monocytes (Drevets *et al.* 2001, Join-Lambert *et al.* 2005). Such cells are known to travel all around the body and to be recruited in different organs. We therefore explored the hypothesis that *L. monocytogenes* infect and use blood leukocytes to travel to the decidua where bacteria infect local cells as well as fetal trophoblasts. Species-specificities of the Internalin-A/E-cadherin interaction (Lecuit *et al.* 1999, Tsai *et al.* 2013) as well as structural differences of the placenta between human and mice (Maltepe *et al.* 2010) suggest that the mouse model of listeriosis is not adequate to study the human pathology. For these reasons, we used exclusively primary human cells and tissues as well as human placental explants in this work.

We explored each step of this process using a complete human cells and organs model, from the infection of blood cells and of decidual cells and the transmission of this infection to the placenta itself. We infected primary human blood leukocytes and showed that monocytes are the best vehicle for *L. monocytogenes* because of their phagocytic capacity associated with a poor ability to kill the bacteria. In a second time, we purified of decidual monocytes, NK cells and fibroblasts and characterized their infection. *L. monocytogenes* display a good ability to infect, survive and multiply inside decidual monocytes but cannot efficiently infect fibroblasts. Surprisingly, *L. monocytogenes* were also capable to infect, survive and multiply in decidual NK cells. However, infection of NK cells rapidly induces the apoptosis and death of these key elements of the local immune system. Finally, we have shown that *L. monocytogenes* can secondary infect human placental explants from previously infected blood or decidual leukocyte *in vitro*. This infection occurs especially at EVT. These results highlight the steps of a novel pathway of infection to the placenta and support the hypothesis that, despite the local immune system, the implantation site is the Achilles' heel of the placenta and the entry door to the fetus.

Results

Susceptibility of primary human peripheral blood stem cells to infection with L. monocytogenes

Previous findings in mice (Drevets *et al.* 2001, Join-Lambert *et al.* 2005) and in the pregnant guinea pig model of listeriosis (Bakardjiev *et al.* 2006) suggest that *L. monocytogenes* traffic to the decidua inside of maternal blood leukocytes *in vivo*. It was shown that *L. monocytogenes* infect and disseminate in murine bone marrow derived mononuclear cells (Join-Lambert *et al.* 2005). In this study, it was proposed that *L. monocytogenes* specifically infect myeloid precursors in the bone marrow (CD34^{high} stem cells). We decided to answer this hypothesis and to decipher, in human peripheral blood cells, what are the cells capable to internalize *L. monocytogenes*, allow intracellular bacterial growth and transmit bacteria to in primary human fetal trophoblasts.

In order to identify the cells involved and to test the possible role of CD34^{high} monocytes precursor, we used primary human peripheral blood enriched in bone marrow myeloid precursors (Peripheral Blood Stem Cells, PBSC). PBSC subpopulations were characterized by flow cytometry with antibodies directed against the surface markers CD14, CD16 and CD34 (Fig. 1a). Six subpopulations were characterized using these three markers. Two subpopulations of CD14-expressing monocytes were discerned based on differential expression of CD16 (Geissmann *et al.* 2003): (1) “inflammatory” monocytes CD14^{high}CD16^{low}, the human equivalent to murine Ly-6C^{high} monocytes, and (2) “resident” monocytes CD14^{high}CD16^{high}. Neutrophils (3) were identified by their large size (FSC), irregular shape (SSC; data not shown) and low expression of CD14 and CD16 (CD14^{low}CD16^{low}), whereas circulating Natural killer cells (4; NK) were CD14^{low}CD16^{high}. The

remaining cells that did not have any significant CD14 and CD16 expression were lymphocytes (6) or belonged to the subpopulation of monocyte precursor CD34-expressing stem cells (5).

PBSC in suspension were infected with GFP-expressing *L. monocytogenes*, gentamicin was added 1 hour post infection (p.i.) to eliminate extracellular bacteria, and the percentage of infected leukocytes at 2 hours p.i. was determined for each cell type by flow cytometry (Fig. 1b). This procedure has allowed to identify cells that internalized as little as one GFP-expressing bacteria (not shown). Approximately 30% of all leukocytes were positive for GFP (Fig. 1c). Strikingly, only the phagocytic subpopulations of leukocytes had a significant percentage of GFP-positive cells: 51-56% and 44% of monocytes and granulocytes, respectively. None of the other leukocyte subpopulation, including the CD34-expressing myeloid precursors, had more than 4% of GFP-positive cells, suggesting that *L. monocytogenes* uptake is mediated exclusively through natural phagocytosis.

Interestingly, the analysis of the PBSC population after infection showed the progressive disappearance of the monocyte CD14^{high}CD16^{high} (Suppl. 2). After 5 hours of infection, almost no remaining CD14^{high}CD16^{high} monocytes were observable (Suppl. 2a). We decided to determine whether these cells were killed by *L. monocytogenes* or whether they were losing the expression of the CD16 marker. We used fluorescence-activated cell sorting (FACS) to purify the two subpopulations of monocytes using the CD14^{high} phenotype (not shown). The separation of these subpopulations depending on the size and scatter (FSC, SSC subset) showed two populations A and B (Suppl. 2b). The population A was composed exclusively with CD14^{high}CD16^{low} monocytes while the population B was composed for 60% with CD14^{high}CD16^{low} monocytes and for 40% with CD14^{high}CD16^{high} monocytes. These cells were then infected for 2 hours as described above. After infection, there was the same ratio 90:10 between the population A and B showing there was no specific disappearance of cells in the B

population (Suppl. 2d). However the analysis of the B population clearly showed a 100% of CD14^{high}CD16^{low} monocytes. This demonstrated that the monocytes CD14^{high}CD16^{high} were not dead, but were indeed losing the expression of CD16. This result suggests that these monocytes might be ongoing a specific differentiation after infection.

The experiments done on PBSC cells were repeated with classic PBMC (without CD34^{high} precursor). Results were identical (data not shown) and because of the limited number of cells purified from PBMC compared to PBSC, we decided to keep working with PBSC. In conclusion, the high percentage of infected monocytes and granulocytes suggests that these cell types could act as carriers of *L. monocytogenes* in the peripheral blood.

Listeria monocytogenes survive and multiply in primary human monocytes.

Next we aimed to determine whether *L. monocytogenes* survive and grow in primary phagocytic cells. Therefore, we used FACS to purify the two subpopulations of monocytes and neutrophils (Fig. 2a), and to determine intracellular bacterial entry and growth in these cells. Primary human monocytes (CD14^{high}CD16^{low} and CD14^{high}CD16^{high}) and neutrophils (CD14^{low}CD16^{low}) were grown on glass coverslips and infected with wild type *L. monocytogenes* at an MOI of 1:1. Gentamicin was added to the culture medium at 1 hour p.i. to kill extracellular bacteria. At different time after infection, cells were lysed and bacteria enumerated by CFU counting. Consistent with infection of primary human PBSC in suspension (Fig. 1c), adherent primary neutrophils and monocytes were easily infected at 2 hours p.i. (Fig. 2b). Both monocyte subpopulations internalized *L. monocytogenes* with similar efficiency at 2 hours p.i., whereas, infection of neutrophils was approximately 2-fold lower. Subsequently, the number of intracellular bacteria in neutrophils decreased (Fig. 2c). At 8 hours p.i., it was rare to detect intracellular bacteria in neutrophils and if present there was usually only a single bacterium per cell (Fig. 2d), indicating that neutrophils eliminate

intracellular bacteria and are therefore less likely to contribute to their systemic hematogenous dissemination.

In contrast, net intracellular bacterial growth was observed in primary human monocytes (Fig. 2c). The net bacterial increase between 2 and 8 hours p.i. was 8- and 40-fold in CD14^{high}CD16^{low} and CD14^{high}CD16^{high} monocytes respectively. Multiple bacteria, typically up to six per cell, were seen in both monocyte subpopulations (Fig. 2d). We also infected differentiated human U937 cells (human monocytic cell line) with *L. monocytogenes* under the same conditions. The comparison between these cells revealed that bacterial uptake and intracellular growth were much higher in the U937 cell line than in primary monocytes. The entry was up to 100-fold more efficient (Fig. 2b) and the net bacterial increase between 2 and 8 hours p.i. was 4,6 higher in U937 cells compared to the CD14^{high}CD16^{high} monocytes and 48-fold compared to the CD14^{high}CD16^{low} monocytes (Fig. 2c). We observed up to 3.3-fold more bacteria per cell in U937 cells at 24 hours p.i. (Fig. 2d).

These differences in the susceptibility to infection between U937 and primary human monocytes highlight the relevance to study the behavior of pathogens in primary cells and tissues. Overall, we conclude from these findings that monocytes are more likely than neutrophils to facilitate dissemination of *L. monocytogenes* to the placenta *in vivo*, because of the better ability of *L. monocytogenes* to enter and grow in these cells. Interestingly, these results in human primary cells correspond and explain results observed in mice: among the three only populations capable to internalize *L. monocytogenes*, Neutrophils are efficiently killing phagocytosed bugs while CD14^{high}CD16^{high} monocytes change their markers' pattern to resemble CD14^{high}CD16^{low} monocytes. Eventually, the inflammatory monocytes are the only infected cells found in mice blood.

Susceptibility of primary human decidual leukocytes to infection with *Listeria monocytogenes*

Multiple lines of evidence suggest that placental infection begins in the decidua, the uterine implantation site (reviewed in (Robbins *et al.* 2012)). Abundant maternal leukocytes are recruited to the decidua (Gomez-Lopez *et al.* 2010) where they are juxtaposed to fetal trophoblasts. Decidual leukocytes consist predominantly of macrophages and NK-cells, and the local cytokine profile differs from peripheral blood, which may influence the susceptibility and course of infection in these cells and tissues. Therefore, we characterized infection of decidual leukocytes with *L. monocytogenes*.

We purified CD45^{high} leukocytes from human decidua, infected these cells in suspension with GFP-expressing *L. monocytogenes*, and analyzed the percentage of infected leukocyte subpopulations by flow cytometry at 2 hours p.i. The two main leukocyte subpopulations identified were CD14^{low} macrophages and CD56^{high} NK cells (Fig. 3a). About 20% of decidual leukocytes internalized *L. monocytogenes* (Fig. 3a-b). Similarly to our findings with blood monocytes (Fig. 1b-c), about 40% of decidual macrophages were infected. Surprisingly, approximately 20% of decidual NK cells were also GFP positive (Fig. 3a-b). This finding seemed to be specific to decidual NK cells because NK cells from peripheral blood were only marginally GFP positive (Fig. 1b-c). To our knowledge infection of NK cells by *L. monocytogenes* has not been reported previously. Furthermore, it is unknown how NK internalized *L. monocytogenes*, since these cells have no phagocytic activity or E-cadherin expression reported. Above results show that decidual macrophages and NK cells are both capable to internalize *L. monocytogenes*.

***L. monocytogenes* grow in decidual leukocytes and quickly induce apoptosis in NK cells**

It is likely that decidual leukocytes contribute to the defense against pathogens as well as tolerance of the fetal allograft. Thus, we tested whether these cells eliminate intracellular *L.*

monocytogenes, or are hospitable to intracellular bacterial growth. The quantity of primary decidual leukocytes that was available from each donor was limited and insufficient for a classic CFU based assay. Therefore, we analyzed the entry and the intracellular growth of *L. monocytogenes* by manual quantification with immunofluorescence microscopy. Since this quantification involve adherent cells rather than cells in suspension and possibly complete different results, and in order to compare the results obtained with the previous method, we also infected U937 cells under the same conditions. Cells were purified using anti-CD14 and anti-CD56 coated magnetic beads and cultured on glass coverslips as described previously. After infection, cells were fixed and stained with DAPI and anti-*L. monocytogenes* antibody. The percentage of infected cells was determined and the number of bacteria per cell was enumerated (Fig. 4).

Primary decidual macrophages were incubated with *L. monocytogenes* at an MOI of 1:1. This resulted in the infection of 8.1% of cells at 2 hours p.i. (Fig. 4b). The percentage of infected cells did not vary significantly between 2 and 8 hours p.i., but the number of bacteria per cell increased, proving intracellular bacterial multiplication. At 24 hours p.i., the percentage of infected cells increased ~2-fold, suggesting bacterial dissemination by cell-to-cell spread. Infection of U937 cells with a MOI of 1:1 led to similar results (Fig. 4a) between 2 and 8 hours. p.i. This result suggest that decidual macrophages are as efficient as U937 cells, therefore more efficient than blood monocytes or neutrophile for ingesting *L. monocytogenes*. At 24 hours p.i. 50% of U937 cells were infected, and 30% were infected with multiple bacteria, suggesting higher rates of cell-to-cell spread and intracellular growth in comparison to decidual macrophages.

In contrast, 2% of decidual NK cells were infected at 2 hours p.i. but the number of bacteria per NK cell was rarely more than 1 bacterium and the percentage of infected cells decreased after 5 hours p.i. (Fig. 4c). This decrease could be due to, either the death of the bacteria once

ingested, or the death of the infected cells. Therefore, we tested whether *L. monocytogenes* infection induces cell death of NK cells as well. We used a dual staining with AnnexinV (marker for early apoptosis) and 7-AAD (marker for dead cells) of infected cells versus uninfected cells. In uninfected samples of CD45^{high} decidual leukocytes, the percentage of dead cells (apoptosis + necrosis) was around 28.4% of decidual macrophages and 12.3% of NK cells respectively (Fig. 4d). After infection, the cell population was separated into two groups: GFP+ cells that internalized bacteria, and GFP- cells that did not. The percentage of infected decidual macrophages (GFP+) that were dead or dying, increased slightly over time: 30% after 2 hours, 35% after 5 hours and 40% after 8 hours. The percentage of dead or dying cells was constant (around 30%) in the population of GFP- macrophage. This shows that *L. monocytogenes* induce a slow apoptosis in decidual macrophages. In contrast, *L. monocytogenes* infection significantly and quickly increased the percentage of apoptotic NK cells in comparison to uninfected controls: 58.7% versus 25.4% two hours post-infection, and up to 73.2% versus 28.6% 8 hours post-infection.

Our results show that *L. monocytogenes* can enter, survive and multiply in decidual macrophages. Surprisingly, *L. monocytogenes* can enter decidual NK cells as well, which differs from our findings in NK cells purified from peripheral blood. However, *L. monocytogenes* induced a rapid apoptosis of decidual NK cells. It is tempting to speculate that *L. monocytogenes*-induced apoptosis of NK cells play a key role in limiting the immune response of the decidua to infection.

***L. monocytogenes* infection and multiplication in primary decidual fibroblasts**

Depending on the gestational age, 30 to 50% of the decidual cells are non-leukocytes cells. The CD45-negative decidual cells are fibroblasts and EVT. In fact, infection of primary human EVT has been reported previously (Bakardjiev *et al.* 2004, Zeldovich *et al.* 2011), and

although EVT can become infected by direct internalin-mediated invasion and cell-to-cell spread, these cells have innate resistance mechanisms and are inhospitable to intracellular growth of *L. monocytogenes*. In contrast, *L. monocytogenes* grow well in primary human placental fibroblasts as well as in the choriocarcinoma cell line BeWo that are fetal in origin (Zeldovich *et al.* 2011). We assessed the ability of *L. monocytogenes* to infect and proliferate into decidual fibroblasts of maternal origin. In these experiments we used BeWo as a control because of their previously characterized properties and because they express the InlA receptor E-cadherin.

We purified decidual fibroblasts according to their adhesive properties. Adherent decidual cells were cultured for 2 weeks, and the phenotype and purity of cells were confirmed by immunofluorescence microscopy using antibodies against desmin (marker for decidualization), cytokeratin 7 (CK7, marker for trophoblast) and CD45. More than 95% of cells were negative for all markers and thus identified as fibroblasts (Fig. 5a).

Since no phagocytic activity was expected from decidual cells and fibroblasts, we tested the role of InlA and InlB in the infection. We infected BeWo cells and primary decidual fibroblast cultured on glass coverslip at a MOI of 1:1 and enumerated the CFU at different time p.i.. The initial entry in fibroblasts was very low, about 200 times lower than entry in BeWo cells, and corresponded to the entry of the double mutant $\Delta inlA/B$ in BeWo (figure 5b). This initial entry into fibroblasts was then not dependent of InlA nor InlB.

Next we determined whether *L. monocytogenes* survive and grow in fibroblasts cells compared to BeWo cells. Primary fibroblasts were grown on glass coverslips and infected with wild type *L. monocytogenes* at an MOI of 1:1. Gentamicin was added to the culture medium at 1hour p.i. to kill extracellular bacteria. Net intracellular bacterial growth was measured (figure 5c). The net bacterial increase between 2 and 5 hours p.i. was 10 to 15-fold

in BeWo cells and around 20-fold in fibroblasts. Between 2 and 8 hours p.i., net bacterial increase was 15 to 25-fold for BeWo and above 70-fold for fibroblasts. Picture shows the type of infected cells observable when fibroblasts were infected (Fig 5d). Unlike in EVT, once in the cytoplasm of fibroblasts, *L. monocytogenes* multiplied without restriction and a large number of bacteria per cell was observed and measured. Thus, the decidual fibroblasts might act as reservoir for listerial growth inside of the decidua.

Differential ability of leukocytes to adhere and transmit bacteria to EVT

Two major corollaries of our model are: first that infected leukocytes interact with placental trophoblasts and second that the interaction is followed by cell-to-cell spread of *L. monocytogenes* from leukocytes to trophoblasts. We have previously shown that extracellular as well as intracellular *L. monocytogenes* infect EVT of human placental explants. For this study we used infected U937 cells (Robbins *et al.* 2009). We used our previous model to characterize the ability of primary human leukocytes from peripheral blood and the decidua to adhere and transmit *L. monocytogenes* to placental explants.

Leukocytes, stained with CellTracker™ green, were infected with a MOI that was adjusted for each cell type to result in infection of 50% of cells. For each leukocyte type described above, including U937, infected stained cells were incubated with primary human placental explants in the presence of gentamicin for 24 h. Gentamicin kills extra-cellular bacteria, thus bacteria could only travel to placental explants by gentamicin-protected cell-to-cell spread. First we determined the density of leukocytes per explant, and further analyzed the density of leukocytes per EVT compared to SYN (Fig. 6a and b). For all the leukocytes, between 10 to 20 cells/mm² adhered to placental explants. This slight variation in density between leukocyte subpopulations did not reach statistical significance (Fig. 6b). The structure of actin differs between EVT and SYN (Robbins *et al.* 2010, Robbins *et al.* 2012) (Fig. 6a), which enabled us to determine the density of leukocytes per EVT versus SYN on the whole placental explants.

The density of peripheral blood CD14^{high}CD16^{low} monocytes and decidual NK cells per EVT was ~2-fold higher in comparison to the density of these cell types per SYN. This difference, comparable to the density of U937 cells to EVT versus SYN, was statistically significant (($p < 0.05$ and $p < 0.005$ by t-test). The same tendency was observed for blood neutrophils, CD14^{high}CD16^{high} monocytes and decidual macrophages but the difference was less pronounced and did not reach statistical significance. Thus, all leukocyte types can interact with the placental explants and leukocytes tend to interact preferentially with the EVT.

Next, we enumerated the number of bacteria transmitted per explant. Infected placenta were frozen and sectioned, with 7-8 μ m thick sections, for an immunohistochemistry analysis. Placental sections were stained with anti-HCG antibody to delineate the SYN (Fig. 7a). Interestingly, we found a difference between the ability of different leukocyte subpopulations to transmit *L. monocytogenes* to placental explants (Fig. 7b). The total number of bacteria per explant was 3 to 4 times higher for U937, decidual macrophages and NK cells in comparison to peripheral blood mononuclear cells. Consistent with our previous findings (Robbins *et al.* 2009) EVT were preferentially infected, whereas the intact SYN was much less infected (Fig 7c). This specificity of site of infection was found whatever the cell type considered. Taken together, these data showed that *L. monocytogenes* infected leukocytes can interact with placental explants and that *L. monocytogenes* can spread from primary human leukocytes to trophoblasts, specifically EVT. Bacterial cell-to-cell spread to EVT is more efficient from decidual leukocytes in comparison to blood leukocytes.

Discussion

L. monocytogenes have the rare ability to cross the most resistant human cellular barriers described: the intestinal barrier, the blood brain barrier and the maternal-fetal barrier. Crossing the blood brain barrier is somehow considered as an accident, because of the rarity of the event. However, crossing the intestinal barrier is a part of the life cycle of *L. monocytogenes* since the bacteria developed specific tools, i.e. InlA, to interact specifically with epithelial cells (Lecuit *et al.* 1999). It is not yet clear whether infection of the placenta is a part of the life cycle of *L. monocytogenes*, as a mean of spreading for example, or if it is the unfortunate consequence of the normal cycle of the bacteria. In the first hypothesis, it would be possible to find bacterial components or mechanisms specifically involved in placental or fetal infection. In this study, in regard with previous study on animals, we highlighted evidence suggesting that placental infection by *L. monocytogenes* might be more than an accident. Indeed, a surprising succession of events leads to the infection of the placenta: from the intestinal tractus, *L. monocytogenes* first access the blood where bacteria enter and survive inside of blood monocytes capable to travel through the body. These cells would then be recruited into the decidua. Inside this immunological organ, *L. monocytogenes* spread to other cell-type, multiply efficiently and specifically induces apoptosis of NK cells, killing a key component of the immune system. Eventually *L. monocytogenes* travel from cell to cell to EVT's and then through trophoblast to fetal blood (Robbins *et al.* 2009).

It has been proposed that *L. monocytogenes* can cross the placental barrier directly from maternal blood through the SYN in an InlA/InlB dependent manner (Lecuit *et al.* 2004, Disson *et al.* 2008). The parsimony principle suggests that this direct pathway would be the most likely considering that this is the largest zone of contact between mother's blood and

placenta: about 20% of maternal blood bath the placenta and the size of the surface of the SYN varies from 3000cm² during the first trimester to 125.000cm² at term (Benirschke *et al.* 2006). However, *in vivo* experiments showed that the participation of both Internalins is very low (Bakardjiev *et al.* 2005, Wollert *et al.* 2007, Disson *et al.* 2008), suggesting that another mechanism might be involved. In addition, our *in vitro* experiment using human placental explants determined that the SYN is highly resistant to *L. monocytogenes* infection by both InlA-mediated invasion and cell-to-cell spread from macrophages (here and Robbins *et al.* 2009). Instead, the small subpopulation of EVT₁s, that is not accessible from the maternal blood, serves as the primary portal of entry for *L. monocytogenes*. Interestingly, the fact that the SYN is a very resistant barrier against infection and that EVT₁s might be the Achilles' heel of the placenta could be a general property. It has been recently shown that another pathogen, *Toxoplasma gondii*, probably uses the same pathway to infect the placenta (Robbins *et al.* 2012, Robbins *et al.* 2012).

The main remaining question was to understand how *L. monocytogenes* could reach EVT₁s from maternal blood. It has been shown that *L. monocytogenes* can use blood monocytes to cross the blood brain barrier (Drevets *et al.* 2001, Drevets *et al.* 2004, Join-Lambert *et al.* 2005). We postulated that such infected monocytes could as well travel from blood to decidua. O. Join-Lambert and co. (2005) as well as D. Drevets and co. (2004) have shown that *L. monocytogenes* travel in mice blood inside of monocytes identified as Ly-6C^{high} monocytes (corresponding to human inflammatory monocytes CD14^{high}CD16^{low}). The proposed hypothesis explaining this tropism was that *L. monocytogenes* would travel to bone marrow where it would infect monocytes CD34^{high} precursors. Here we showed that CD34^{high} monocyte precursors are actually resistant to direct infection by *L. monocytogenes*, suggesting that these cells are probably not the initial infected cells. However, in the architectural context of the bone marrow, bacteria could be internalized by resident phagocytic cells and

subsequently infect myeloid precursors by cell-to-cell spread. Thus, we cannot fully exclude the possibility that infected hematopoietic precursors contribute to the systemic hematogenous dissemination of *L. monocytogenes*. Here, we evidenced that the specificity of infection can be explained by the properties of monocytes. Indeed, these cells are the only cells tested allowing a good rate of phagocytosis associated to a loose ability to kill intracellular *L. monocytogenes*. We showed that neutrophils phagocytosis was less efficient and that they killed more efficiently *L. monocytogenes in vitro*. The same property has been shown for dendritic cells (Westcott *et al.* 2007). Even if neutrophils or dendritic cells cannot be crossed out as carrier candidates of *L. monocytogenes*, it is more likely that *L. monocytogenes* infect monocytes in blood, or in bone marrow, and travel inside of these cells to the decidua and to the brain. We studied the infection of two populations of monocytes: monocytes CD14^{high}CD16^{low} and monocytes CD14^{high}CD16^{high}. Overall it seems that, in spite of a slightly better ability of *L. monocytogenes* to multiply into CD14^{high}CD16^{low}, both type of monocytes present comparable characteristics in our study. Of note, we showed that the monocytes CD14^{high}CD16^{high} were losing the expression of CD16 after the infection *in vitro*. This result suggests that these monocytes might be ongoing differentiation after infection, thus explaining why they are not found infected in animal model studies. Eventually, the inflammatory monocytes are the only infected cells found in mice blood.

The decidua is an immunological organ very rich in immune cells. It is especially responsible for the allograft tolerance as well as for the defense of the placenta against infections. However, infecting decidua seems to be the first step to infect the placenta and eventually the fetus (Redline *et al.* 1988). NK cells are a key component of the innate immune system; in particular, they are responsible of the recruitment and activation of macrophages through the secretion of IFN- γ during infection by *L. monocytogenes* (Lieberman *et al.* 2002, Berg *et al.* 2005). Decidual NK cells have specific local properties compared to other NK

cells but keep their cytotoxic ability (Manaster *et al.* 2010). Here we show that *L. monocytogenes* have the unique ability to invade decidual NK cells, but not blood NK cells, and induces apoptosis in these infected cells, thus leading to their death. Until now, the only other bacteria proved to be internalized into NK cells and triggers their apoptosis was *Pseudomonas aeruginosa* (Chung *et al.* 2009). Such mechanism, if confirmed *in vivo*, could be key to understand the infection of the decidua. The death of NK cells would slow considerably the immune response to *L. monocytogenes*. Moreover, we showed that *L. monocytogenes* infect and multiply very efficiently into the decidual macrophages. From these cells, the transfer of bacteria to EVT is very efficient as well. Despite a low ability to directly infect fibroblasts, the multiplication inside of fibroblast is impressive and a large number of bacteria are observable in the cytoplasm of cells. Previous works from our group showed that infection of EVTs is not always followed by a propagation of the infection to deeper tissues (Robbins *et al.* 2009). Indeed, trophoblasts have the ability to restrain *L. monocytogenes* escape from the initial phagosomal vacuole (Zeldovich *et al.* 2011). One hypothesis is that a strong inoculum of bacteria could bypass this EVT barrier. Thus, the decidua could be an obligatory step to infect the placenta because bacteria can multiply without any restriction and reach an inoculum threshold necessary to efficiently cross EVTs.

Finally, we showed that even by cell-to-cell spread, *L. monocytogenes* have a tropism for EVTs. A part of this tropism *in vitro* depends on a better interaction between leukocytes and EVTs compared to SYN. But, since the difference of density of cells/EVT compared to cells/SYN is not significant for all leukocyte types, there might be an additional explanation for the tropism of *L. monocytogenes* for EVT. Two hypotheses could be that cell-to-cell spreading is more efficient from leukocyte to trophoblast compared to leukocytes to SYN or that bacterial growth could be restrained into SYN like it is in trophoblasts. Interestingly, the total number of bacteria per explant was higher when spreading from infected decidual

leukocytes compared to blood leukocytes, highlighting once more that unique properties of decidual cells could explain why pregnancy is such a sensitive state to Listeriosis.

In conclusion, our *in vitro* characterization of the infection of human primary blood leukocytes and decidual cells correspond and explain the situation described in animal models of listeriolysis. Quickly following the infection of blood by *L. monocytogenes*, bacteria are found into blood inflammatory monocytes in mice (Drevets *et al.* 2001, Join-Lambert *et al.* 2005). In pregnant guinea pig, *L. monocytogenes* can travel through the bloodstream to the placenta inside a protected cellular compartment (Bakardjiev *et al.* 2006). In the decidua, where bacteria will be found into trophoblasts (Le Monnier *et al.* 2007), the progression of *L. monocytogenes* to the placenta depends on cell-to-cell spread in both models (Bakardjiev *et al.* 2005, Le Monnier *et al.* 2007). In addition, in this study, we demonstrated that *L. monocytogenes* can infect human placental explants from pre-infected primary leukocytes. It is well known that during pregnancy blood leukocytes are recruited to the decidua and placenta (Gomez-Lopez *et al.* 2010), however, there is no evidence on leukocytes behavior during an ongoing infection. In particular, there is no proof that infected blood leukocytes actually migrate from blood to the decidua and that *L. monocytogenes* could then spread to local cells. This would have to be investigated in future studies.

Material and Methods

Ethics statement

This study was conducted according to the principles expressed in the Declaration of Helsinki. The study was approved by the Institutional Review Board at the University of California, San Francisco (IRB#: 11-05530 and 10-01998). All patients provided written informed consent for the collection of samples and subsequent analysis.

Pathogen strains and growth conditions

The wild type strain of *L. monocytogenes* used in this study is 10403S (Bishop *et al.* 1987). Mutant strains included Δ *InlAB* (DPL4455) (Bakardjiev *et al.* 2004) and sGFP-expressing 10403S *L. monocytogenes* (DH-L1039) (Shen *et al.* 2005). Bacteria were cultured using brain heart infusion (BHI) broth or agar (Becton Dickinson Company, Sparks, MD).

Cell Lines

Human macrophage-like U937 cells (ATCC 1593.2 (Sundstrom *et al.* 1976)) were grown in RPMI-1640 containing 4500 mg/L glucose, 10% FBS, and 1% penicillin/streptomycin. Forty-eight hours prior to infection, cells were differentiated by addition of phorbol 12-myristate 13-acetate (PMA; concentration 18 nM; Sigma) to the medium. The choriocarcinoma cell line BeWo (ATCC CCL-98) was cultured in Ham's F12 medium with 10% FBS, 1% L-glutamine, 0.15% sodium bicarbonate, and 1% penicillin/streptomycin. For growth curves and immunofluorescence microscopy, cells were plated on glass coverslips in 24-well plates at $2 \cdot 10^5$ cells/well density.

Primary cells and organ cultures

All primary cells and tissues were cultured in RPMI-1640 (UCSF Cell Culture Facility) containing 4500 mg/L glucose, 10% FBS (Fisher Scientific), 1% penicillin/streptomycin/amphotericin (Invitrogen), and 50 μ g/mL gentamicin.

Peripheral Blood Stem Cells (PBSC) were provided by the Blood and Bone Marrow transplant laboratory at the University of California, San Francisco. PBSC were obtained from peripheral blood of human donors whose bone marrow myeloid precursors were mobilized by treatment with granulocyte colony stimulating factor (G-CSF). Cells were collected using standard clinical procedures and purified over a Ficoll-Hypaque gradient (Ficoll-Paque Plus, GE Healthcare).

Placentas and decidual tissues from elective terminations of pregnancy (gestational age 4 to 8 weeks) were collected. Placenta were collected and prepared as described elsewhere (Genbacev *et al.* 1992). Briefly, villous tree were dissected into 1 to 3 mm pieces (1 to 5 villi per explant) and cultured on Matrigel (BD Biosciences, San Jose, CA) coated Transwell filters (Millipore, Bedford, MA, 30-mm diameter, 0.4 μm pore size) over night to allow invasion of the Matrigel by EVT's (Extra Villous Trophoblasts). The day after, Dulbecco's modified Eagle's medium-F12 medium (DMEM-F12; 1:1, vol/vol) supplemented with 20% fetal bovine serum (FBS, Fisher Scientific), 1% L-glutamine and 1% penicillin/streptomycin/amphotericin (Invitrogen, Carlsbad, CA) and 50 $\mu\text{g}/\text{mL}$ Gentamicin was added.

Decidual tissues were dissected into 1-5 mm pieces and digested in PBS containing 0.1% Collagenase A (Roche), 0.1% Hyaluronidase I (Sigma-Aldrich), 5 U/mL DNase for 1 h. Cell suspensions were filtered through Gauze Sponge (Kendall, Tyco Healthcare) and 70 μm cell strainers (BD Falcon). To purify decidual leukocytes, the decidual cell suspension was incubated with CD45, CD56, or CD14 magnetic MicroBeads (MACS, Miltenyi Biotec) according to the manufacturer's protocol. Briefly, cells were re-suspended in cold MACS buffer, and incubated with MicroBeads for 15 min. Cells were washed in buffer, passed through MACS LS separation columns (Miltenyi Biotec), fixed to the quatro MACS separation system (MACS, Miltenyi Biotec), eluted and re-suspended in media. To purify

decidual fibroblasts, digested decidual cell suspensions were incubated in cell culture flask for 2 hours. Non-adherent cells were washed with fresh medium. Adherent cells were fibroblast and cultured for up to two weeks before use. For growth curves and immunofluorescence microscopy, cells were plated on glass coverslips in 24-well plates at 2.10^5 cells/well density.

Flow cytometry and fluorescence-activated cell sorting of human cells

The following fluorophore-conjugated antibodies and reagents were purchased from BD Biosciences or eBioscience and used for flow cytometry on PFA fixed cells or FACS on live cells: allophycocyanin (APC)-conjugated anti-human CD14, APC-H7-conjugated anti-human CD16, phycoerythrin (PE)-conjugated anti-human CD34, APC-H7-conjugated anti-human CD45, PE-conjugated anti-human CD56, 7-aminoactinomycin D (7-AAD), APC-conjugated Annexin V. All analyses were performed using 4-color flow cytometer (FACS ARIA) and Flow-JO software. All analyses and cell sorting were performed at the platform of cell cytometry of UCSF.

Enumeration of intracellular bacteria in primary cells and cell lines

For bacterial entry assays all cells were cultured in antibiotic free medium 2 hours prior to infection. Primary human monocytes ($CD14^{high}CD16^{low}$ and $CD14^{high}CD16^{high}$), neutrophils ($CD14^{low}CD16^{low}$), decidual macrophages ($CD14^{low}$), decidual NK cells ($CD56^{high}$), and cell lines U937 and BeWo, were grown on glass coverslips in a 24 wells culture plate (Costar, Corning Incorporated) o a density estimated to 2.10^5 cell/well. Cells were infected with *L. monocytogenes* at an MOI of 1:1. One hour after infection, Gentamicin ($50 \mu\text{g/ml}$) was added to the culture medium to kill extracellular bacteria. Two hours after the start of the infection, cells were lysed with distilled sterile water and subjected to serial dilutions and plated on BHI agar plates to enumerate colony-forming units (CFU) per well.

To determine the net intracellular bacterial growth, cells were plated at a density of 10^5 cells/well in 48-well cell culture plates (Costar, Corning Incorporated). Above protocol was

then followed. At specified times after infection (2, 5, 8 and 24hrs) cells were lysed in distilled sterile water and bacteria enumerated by CFU counting. CFU were normalized to the average number of cells as determined by microscopy. The ratio of CFU compared to the initial time point (2hrs p.i.) was considered the net intracellular bacterial growth.

Immunofluorescence microscopy of primary cells and cell lines

Cells were plated at a density of $2 \cdot 10^5$ cell/well on glass coverslips in 24-well tissue culture plates and infected as described above. At specified times after infection, cells were fixed in PFA, exposed to primary antibodies, washed with PBS followed by secondary antibodies at the indicated concentrations (see below) and washed again. Coverslips were mounted in Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA). Primary antibodies: polyclonal rabbit *Listeria* O antiserum (1:1000 Becton, Dickinson), monoclonal mouse anti-human cytokeratin 7 (1X, Clone OV-TL, Dako, Carpinteria, CA), polyclonal rabbit anti-human desmin (1/100; Thermo Fisher scientific). Secondary antibodies from Invitrogen: Alexa Fluor 488 goat anti-rabbit IgG (1:1000) and Alexa Fluor 594 goat anti-mouse IgG (1:500). DAPI stained cells were photographed on a Leica MZ16F stereomicroscope (Leica Microsystems, Wetzlar, Germany) using an Axiocam MR monochrome camera (Carl Zeiss, Munich, Germany). The average number of cells per field was established by counting the number of cells in at least three different fields per experiment.

Infection of explants by cell-to-cell spread

On the day of infection, leukocytes were labeled with CellTracker™ green CMFDA (Invitrogen) for 30 min before infection and washed twice with RPMI. U937 cells were infected with GFP expressing *L. monocytogenes* for 1 h at an MOI of 1:1. All other primary cells were infected at an MOI of 10:1. Cells were washed in RPMI medium, detached from the tissue culture plate by incubation in ice cold PBS without calcium and magnesium (UCSF

Cell Culture Facility) for 7.5 minutes followed by mechanical scrapping. Cells were re-suspended in explants medium containing 50 $\mu\text{g/ml}$ gentamicin, and 10^5 cells per Transwell were added to the placental explants. At 24 h p.i. the number of leukocytes per EVT versus SYN, and the percentage of infected placental cells were determined by immunofluorescence microscopy.

Immunofluorescence microscopy of placental explants

For whole mount examination, after infection, placental explants were incubated in PFA for 5 h at 4°C, washed 3 times in PBS and incubated in DAPI (1:1000) and Alexa Fluor 594 phalloidin (1:100, Invitrogen) over night. Explants were washed 3 times in PBS and mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA), photographed on a Leica MZ16F stereomicroscope (Leica Microsystems, Wetzlar, Germany) using an Axiocam MR monochrome camera (Carl Zeiss, Munich, Germany). The actin structure was used to distinguish between EVT and SYN and their surface area was measured using ImageJ software (NIH, Bethesda, MD). The number of cells per surface area of EVT versus SYN was calculated. For examination of placental sections, explants were removed from the matrigel and placed into vinyl cryomolds (Ted Pella, Redding, CA), then covered with optimal cutting temperature (OCT) media (Ted Pella) and flash frozen in dry ice cold ethanol. Histological slicing was performed using a Hacker-Slee cryostat. Sections coated on glass slides were incubated 5 min in acetone, dried, and stained with primary antibodies: polyclonal rabbit anti-*Listeria* O antiserum (1:1000 Becton, Dickinson) and monoclonal mouse anti-human bHCG (1:500, clone SPM105, Neomarkers, Fremont, CA). Secondary antibodies from Invitrogen: Alexa Fluor 594 goat anti-mouse IgG (1:500) and Alexa Fluor 488 goat anti-rabbit IgG (1:1000). Coverslips were affixed over Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA). Slides were viewed using an inverted TE2000-E microscope (Nikon, Tokyo, Japan) equipped with a 12-bit cooled CCD camera (Q Imaging, Surrey,

Canada). Images were collected using Simple PCI software (Hamamatsu, Sewickley, PA). Count of *L. monocytogenes* and determination of their location was made by tallying every infected cell in each section at 40X magnification.

Image processing

Images were prepared using Photoshop and Illustrator (Adobe, San Jose, CA). RGB color hues were linearly adjusted for better CMYK printing but no non-linear alterations were performed.

Statement of the author and acknowledgments

This work was performed solely by the author in 2010 and 2011 in A. Bakardjiev's laboratory at the Department of Pediatric of the University of California of San Francisco (UCSF). Because of personal disagreements, and in spite of her valuable advising contribution, A. Bakardjiev has opposed the publication of this work in a peer-reviewed scientific journal. This is why the author decided to share this study with the scientific community through this free medium. Reader has to be conscious that this work has not been through regular peer-reviewed critics and that the author alone takes responsibility for what has been written here.

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