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Trafficking of *Estrella lausannensis* in Human Macrophages

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Running Title: Trafficking of *E. lausannensis* in Macrophages

Summary

Estrella lausannensis is a new member of the *Chlamydiales* order. Like other *Chlamydia*-related bacteria it is able to replicate in amoebae and in fish cell lines. Compared to other *Chlamydiales,* little is known about the pathogenic potential of *E. lausannensis.* A single study suggested the role of *E. lausannensis* in pneumonia in children. In the present work we thus investigated the ability of *E. lausannensis* to grow in human macrophages and its intracellular trafficking. The replication in macrophages was very efficient and was associated with a significant cytopathic effect. The intracellular trafficking of members of the *Chlamydiales* order varies considerably. We therefore analyzed the interaction of the *E. lausannensis* inclusion with various endocytic pathway markers as well as host organelles. The *E. lausannensis* inclusion escapes the endocytic pathway rapidly avoiding maturation into a phagolysosome by preventing EEA-1 and LAMP-1 accumulation. Like members of the *Chlamydiaceae* family, it causes Golgi fragmentation, but without associating with the fragmented Golgi. The recruitment of mitochondria and endoplasmic reticulum is not as efficient as for *Waddlia chondrophila*, another member of the *Chlamydiales* order. In conclusion, *E. lausannensis* is rapidly growing in human macrophages, but has a restricted growth when compared to *W. chondrophila*, likely due to its reduced capacity to control programmed cell death.

Introduction

Estrella lausannensis is a new member of the *Chlamydiales* order (Lienard *et al.*, 2011b), first isolated from a Spanish river sample by amoebal co-culture (Corsaro *et al.*, 2009). With *Criblamydia sequanensis* that was recovered by amoebal co-culture from the Seine river in Paris (Thomas *et al.*, 2006), *E. lausannensis* is one of the two species assigned to the *Criblamydiaceae* familiy (Thomas *et al.*, 2006, Lienard *et al.*, 2011b). The number of members in the *Chlamydiales* order has been constantly growing in the recent years (Everett *et al.*, 1999, Greub, 2010). Many more have been detected in environmental samples, but were not isolated in culture, showing the high biodiversity and widespread occurrence (Lienard *et al.*, 2011b)of this clade of strict intracellular bacteria (Pizzetti *et al.*, 2012, Lagkouvardos *et al.*, 2013).

Criblamydiaceae exhibit the same replicative cycle with infectious elementary bodies (EBs) and replicative reticulate bodies (RBs) than other members of the *Chlamydiales* order. *Chlamydiales* reside within a vacuole, called inclusion, throughout the infection. The nature of the inclusion and the organelles that interact with each bacterial species vary between members of the *Chlamydiales* order (Kebbi-Beghdadi *et al.*, 2012). Thus, *Chlamydia trachomatis* inclusion associates with Golgi fragments (Hackstadt *et al.*, 1995), *Waddlia chondrophila* with the endoplasmic reticulum (Croxatto *et al.*, 2010), whereas *Parachlamydia acanthamoebae* remains in the endocytic pathway somehow inhibiting the acidification of the inclusion and the acquisition of lysosomal hydrolases, such as cathepsin (Greub *et al.*, 2005). However, the intracellular trafficking of *E. lausannensis* was yet unknown.

Upon an initial report on the growth of *E. lausannensis* in several amoebal hosts (Lienard *et al.*, 2011b), Kebbi et al., showed that *E. lausannensis* also replicates within fish cell lines (Kebbi-Beghdadi *et al.*, 2011), as well as endometrial cells and pneumocytes (Kebbi-Beghdadi, personal communication). The wide range of amoebae that could be infected by *E. lausannensis* proved the high versatility of this bacterium to adapt to different hosts. Interestingly, it was shown that its growth extent within amoebae is temperature dependent, increasing at higher temperatures (Lienard *et al.*, 2011b). This observation suggested that *E.*

lausannensis could also replicate within phagocytic human cells, such as macrophages and could represent an emerging pathogen.

Moreover, presence of *E. lausannensis* or a related *Criblamydiaceae* in human samples was shown in a study on nasopharyngeal swabs from children with pneumonia (Lienard *et al.*, 2011a). We therefore decided to investigate the replication of *E. lausannensis* in human macrophages, since they are major innate immune cells in the lung. We could show that human macrophages are permissive to *E. lausannensis* and therefore further investigated its intracellular trafficking.

Results

E. lausannensis replicates within macrophages

PLB-985 monocytes were differentiated into macrophage-like adherent cells. The macrophages were infected with *E. lausannensis* with a bacterial load of 2-3 bacteria per cell and an infection rate above 50%. *E. lausannensis* readily re-differentiated into reticulate bodies (RBs) four hours post infection. The bacteria started to replicate shortly after and at 12 hours post-infection larger inclusions were visible (Figure 1A). At 16 hours post infection (p.i.) some of the bacteria were already re-differentiating into elementary bodies (EBs). The growth was more than one log (24 fold increase) in 24 hours (Figure 1B). *Waddlia chondrophila*, another member of the *Chlamydiales* order, which had previously been shown to multiply in macrophages (Croxatto *et al.*, 2010), which was used as a control, was confirmed to replicate also in this cell line (Figure 1A). During the same period of time, the growth of *W. chondrophila* was about one log higher than the growth of *E. lausannensis* with the same infection rate and a bacterial load of 4 bacteria per cell (Figure 1B). At four and six hours p.i., a significant fraction of *E. lausannensis* infected cells showed a fragmented nucleus, while this was not observed at the same time points in non-infected cells or in cells infected with *W. chondrophila* (Figure 2A-B). We therefore determined the cytopathogenicity

of *E. lausannensis* by measuring the lactate dehydrogenase (LDH) release. Only cells infected with live *E. lausannensis* caused a significant release of LDH (Figure 2C). Infections with *W. chondrophila* or with formol-inactivated bacteria did not result in an increase in cell death.

E. lausannensis escapes endosomal compartments

E. lausannensis containing inclusions did not accumulate the early endosomal marker EEA1 while it is the case for *W. chondrophila*-containing vacuoles (Figure 3A). The percentage of EEA1 positive *E. lausannensis* bacteria-containing-vacuoles (BCV) rose from 5 to 10% in the first 30 min post-infection and subsequently dropped again at one hour post infection. Conversely, *W. chondrophila* BCV showed, as previously described, (Croxatto *et al.*, 2010) an EEA1 staining that accumulated around 15 minutes post infection, but that was also rapidly lost. We could not analyze the trafficking of *E. lausannensis* inactivated with various chemical and physical procedures, since the inactivated bacteria were rapidly recycled back outside the macrophages or degraded.

Even though the *E. lausannensis* BCV did not strongly accumulate EEA1, we investigated if it might acquire lysosomal markers later in the infection. Up to six hours post infection, when the bacteria were already re-differentiated into RBs, we did not observe any increase in LAMP1 staining. Only about 10% of *E. lausannensis* BCVs showed a LAMP1 staining and the percentage remained stable throughout time (Figure 3C). To determine if the *E. lausannensis* BCV was completely disconnected from the endosomal network we used the solvochromic FM4-64 in live cells. If *E. lausannensis* BCV could still fuse with endosomal compartments it would acquire FM4-64 that enters the cell through the endosomal pathway upon integration into the plasma membrane (Vieira *et al.*, 2003). Since FM4-64 can also integrate into the bacterial membrane, staining was performed without permeabilization in order to distinguish between the extra- and the intracellular bacteria. We observed a strong accumulation of FM4-64 in the BCV. The majority of the inclusions was marked by FM4-64 for up to one hour post infection (Figure 3E). Even though the EEA1 staining started to

decrease already after 30 min, *E. lausannensis* BCV was still interacting with the endosomal network, since the FM4-64 staining was still strong at one hour post infection. To determine if *E. lausannensis* BCV could fuse with pre-existing lysosomes instead of maturating into a lysosome, we performed a pulse and chase with FM4-64. The cells were incubated with FM4-64 for one hour and then chased for three hours prior to infection. Upon infection by spinoculation, cells were fixed and bacteria were stained without permeabilization of the cells. None of the bacteria were surrounded by FM4-64 positive membranes at one hour post-infection, suggesting an absence of fusion between preformed lysosomal compartments and the nascent BCV.

E. lausannensis causes fragmentation of the Golgi network

Once we determined that *E. lausannensis* escapes the endosomal route early on, we analyzed the interaction of the BCV with several host organelles. For *E. lausannensis* we observed a significant Golgi fragmentation at four hours post-infection (Figure 4A). However, the fragmented Golgi did not accumulate around the BCV, contrarily to what is known for *Chlamydiaceae* (Ying et al., 2008). Since, *E. lausannensis* caused nuclei fragmentation at the same stage of infection, we only analyzed infected cells with intact nuclei to exclude that Golgi fragmentation is a consequence of induced cell death. Interestingly, we also observed a Golgi degradation in macrophages infected with *W. chondrophila*, although not to the same extent (Figure 4A). Here as well the BCV did not accumulate Golgi fragments.

E. lausannensis is less associated with the endoplasmic reticulum and mitochondria than W. chondrophila

Another important source of membranes and proteins is the endoplasmic reticulum. For *E. lausannensis* BCV we observed less than 20% of the inclusions with a PDI staining, compared to up to 60% for *W. chondrophila* inclusions at eight hours p.i.

Then we determined the co-localization of the *E. lausannensis* inclusions with mitochondria. The recruitment of mitochondria was much less pronounced in *E. lausannensis* and

occurred at later stages (Figure 4C) then what is observed with *W. chondrophila*. The recruitment is observed mainly at six hours post *E. lausannensis* infection, whereas *W. chondrophila* (used as a positive control) already recruits mitochondria two hours post infection. Moreover, the number of mitochondria found around the BCV is lower for *E. lausannensis* than for *W. chondrophila*.

The role of the cytoskeleton in replication

The interaction of the BCV with the different cellular organelles and compartments is regulated by the cytoskeleton. Either the inclusion is transported to a given location or organelles are transported to the inclusion. For the *Chlamydiales* several components of the cytoskeleton have been shown to be relevant. *Chlamydiaceae* use microtubuli to reach the microtubule organization center (MTOC) (Grieshaber *et al.*, 2003). For *W. chondrophila,* single disruption of microtubuli or actin is not enough to prevent mitochondria recruitment, the recruitment being abolished only when both cytoskeletal elements are perturbed (Croxatto *et al.*, 2010). Microtubuli can be disrupted by the drug nocodazole and actin filaments by cytochalasinD. Since the mitochondrial recruitment was less pronounced in *E. lausannensis,* we analyzed the progression of infection by looking at the size of the inclusions at 12 hours post-infection after incubation with one or both drugs. Small inclusions contain 5 to 10 replicating bacteria, medium sized inclusions have 10 to 20 bacteria, and large inclusions have between 20 and 50 bacteria. When cells were treated with nocodazole, the number of inclusions was not significantly altered compared to untreated cells (Figure 5A). Only for 0-2h there were significantly more small inclusions compared to untreated cells ($p<0.041$). There were also significantly less large inclusions with 2-12h nocodazole compared to untreated cells ($p<0.048$). On the other hand, early treatment with cytochalasinD significantly reduced growth of *E. lausannensis*, even if the cells were only exposed to the drugs during two hours (Figure 5B). Later exposures to these drugs did not affect growth of *E. lausannensis*. Combination of the two drugs also blocked infection when applied early in infection and further blocked progression of infection when applied at later

time points, since significantly less large inclusions were observed for all conditions except 4-8h and 8-12h incubation times (Figure 5C). Interestingly, although both filaments were disrupted, the growth of *E. lausannensis* was only reduced and not completely abolished.

Discussion

In this work, we showed that *E: lausannensis* replicates in human macrophages and escapes early the endocytic pathway. Although, the growth of *E. lausannensis* is not as efficient as the one of *W. chondrophila* in the same macrophage cell line, their kinetics are similar. The difference might be due to the lack of cell death control by *E. lausannensis* compared to *W. chondrophila*. Indeed, *E. lausannensis* induces a strong cytopathic effect early on during infection preventing the completion of the replication cycle. Only a fraction of infected cells survive, therefore limiting the pool of cells that will sustain bacterial growth. Lack of apoptosis inhibition has also been observed with members of the *Parachlamydiaceae* family (Sixt et al., 2012). *P. acanthamoebae* for example replicate only to a very limited extent within macrophages and cause a rapid cell death (Greub *et al.*, 2003, Greub *et al.*, 2005, Roger *et al.*, 2010). When apoptosis is blocked with a pan-caspase inhibitor the bacterial growth is greatly improved (Sixt *et al.*, 2012). Conversely, *C. trachomatis* and *C. pneumoniae* can both prevent the activation of apoptosis through external stimuli (reviewed in Ying, 2007). This is achieved by degrading the pro-apoptotic BH3-only proteins. Moreover, each species has developed additional pathways to prevent apoptosis at early stages of infection. Since protozoa do not encode caspases, the programmed cell death (PCD) mechanisms differ greatly from those of metazoan. Nevertheless, PCD has been described in several protozoan parasites with similar morphological characteristics to PCD in metazoan (Proto *et al.*, 2013). In amoebae cell death is thought to occur mainly through autophagy (Calvo-Garrido *et al.*, 2010). These differences in PCD might be responsible for the difference in growth ability within macrophages observed between the different *Chlamydia*-related bacteria. *W. chondrophila* is rapidly growing within human macrophages without early cytopathic effect (Goy *et al.*,

2008), whereas *P. acanthamoebae* causes a rapid cytopathic effect limiting its replication. By comparison, *E. lausannensis* displays an intermediary growth phenotype in human macrophages with rapid growth limited by macrophage PCD. More detailed studies on the lack of PCD control by *E. lausannensis* will allow to determine its importance in growth restriction. A reduced metabolic synthesis is an alternative hypothesis explaining this reduced growth (Kebbi-Beghdadi *et al.*, 2011), supported by the genomic data that demonstrates a significantly reduced number of metabolic pathways in the genome of *E. lausannensis* (Bertelli *et al.,* unpublished) compared to those encoded by the *Waddlia* genome (Bertelli *et al.*, 2010).

E. lausannensis is leaving the endocytic pathway early upon infection. Escaping the endosomal network early is a strategy developed by many intracellular bacteria to avoid degradation. *Mycobacterium tuberculosis* prevents EEA1 accumulation in the vacuole, therefore preventing further maturation into phagolysosomes (Fratti *et al.*, 2001). Only about 10% of the mycobacterial vacuoles are EEA1 positive compared to 30% with latex beads at ten minutes post-infection (Fratti *et al.*, 2001). These results are very similar to the ones obtained with *W. chondrophila* and *E. lausannensis* at 15 minutes post-infection. We hypothesize that *E. lausannensis,* like *M. tuberculosis,* escapes the endosomal route very early. The mechanisms allowing early endosomal escape might be quite different between *M. tuberculosis* and *E. lausannensis*, since for *M. tuberculosis* the species-specific mannose-lipoarabinomannans have been involved in blocking EEA1 recruitment. However, the lipid alone only reduces EEA1 recruitment by 40% implicating additional control mechanisms (Fratti *et al.*, 2001). The recruitment of EEA1 is dependent on Rab5 and interaction of the FYVE domain of EEA1 with phosphatidylinositol 3-phosphates (PtdIns(3)P) on the phagosomal membrane (Christoforidis *et al.*, 1999). Additional studies with Phosphoinositol-3 Kinase (PI3K) inhibitors will allow to determine if the underlying mechanisms of endosomal maturation blockage are the same for *E. lausannensis*. However, PI3K inhibitors have to be used with caution, since wortmannin (PI3K inhibitor) is known to also block other lipid-kinases (Carnero, 2009).

Interestingly, for *C. trachomatis* trafficking, very different results are obtained depending on the cell line used for infection. In epithelial cells the bacterial inclusions do not recruit early endosomal markers such as EEA1 and Rab5 (Heinzen *et al.*, 1996) and prevent the acquisition of lysosomal and fluid phase markers (Wolf *et al.*, 2001, Grieshaber *et al.*, 2002). *Chlamydia pneumonia* inclusions as well do not acquire lysosomal and fluid phase markers in epithelial cells (Al-Younes *et al.*, 1999). However, in macrophages, *C. trachomatis* localizes rapidly in Rab7 positive compartments with increased acidity. Moreover, *C. trachomatis* (serovar L2) has only a very limited replication in macrophages (Sun *et al.*, 2012). A similar behavior is observed for *Parachlamydia acanthamoebae* in macrophages, with LAMP-1 accumulation and Lysotracker staining in the early steps of infection (Greub *et al.*, 2005). *P. acanthamoebae* has like *C. trachomatis* only a limited replication within macrophages (Greub *et al.*, 2005). Even though, *E. lausannensis* does not recruit early endosomal markers, it escapes the fusion with lysosomes, since there is no accumulation of LAMP-1, (unlike what is observed for *C. trachomatis* (Sun *et al.*, 2012) or *P. acanthamoebae* (Greub *et al.*, 2005))**.** We therefore hypothesize that the relatively reduced replication of *E. lausannensis* (as compared to *W.* chondrophila) is not due to a targeting of the bacteria to lysosomal compartments (like *C. trachomatis* or *P. acanthamoebae* in macrophages) but rather, as mentioned above, to a lack of control of programmed cell death.

The interaction of the maturating chlamydial inclusion with several host organelles differs greatly between the different species, but depends also on the cell type used for infection. Golgi fragmentation has been observed for *Chlamydiaceae* in epithelial cells with recruitment of Golgi fragments to the chlamydial inclusion (Hackstadt *et al.*, 1995). However, macrophages infected with *C. trachomatis* (serovar L2) did not display Golgi fragmentation nor interaction with the Golgi (Sun *et al.*, 2012). For *E. lausannensis*, the Golgi was fragmented, but not recruited to the inclusion. Although, the chlamydial protease or protease-like activity factor (CPAF), a ubiquitous protein found in all *Chlamydiales,* has been linked to Golgi degradation (Christian *et al.*, 2011), a more recent publication questions the relevance of CPAF in Golgi and other substrates degradation (Chen *et al.*, 2012). It is

therefore still open to debate, which chlamydial protein causes Golgi fragmentation. CPAF is present in *E. lausannensis* genome (Bertelli et al., unpublished), as well as in those of *W. chondrophila* (Bertelli *et al.*, 2010) and *P. acanthamoebae* (Greub *et al.*, 2009), but in those two latter species Golgi fragmentation was not observed or only to a lesser extent.

The role of endoplasmic reticulum (ER) in *C. trachomatis* infection of HeLa was recently elucidated (Dumoux *et al.*, 2012). Several ER markers accumulate in patches within the inclusion and calreticulin marks the inclusion membrane. Since PDI, the ER marker used in our study, does not co-localize with the *C. trachomatis* inclusion, but rather forms patches within the inclusion, more detailed studies with Z-stacks of *E. lausannensis* would be useful to determine if the same phenotype is observed for *E. lausannensis.* In contrast, for *W. chondrophila* the PDI and calnexin staining showed a co-localization with the inclusion (Croxatto *et al.*, 2010). Noteworthy, the publication by Dumoux *et al.,* 2012 clearly shows that depending on the ER protein used, the interaction with the inclusion varies considerably. Other ER-markers, like calreticulin or calnexin could be used to further assess the role of ER in *E. lausannensis* inclusion biogenesis.

Requirement for mitochondria at the inclusion varies greatly between members of the *Chlamydiales* order. Inclusions of *Chlamydia psittaci,* but not *C. trachomatis* or *C. pneumoniae* are surrounded by mitochondria (Matsumoto *et al.*, 1991). The same observation was made for *W. chondrophila* (Croxatto *et al.*, 2010)**.** Moreover, *Chlamydia caviae* replication was dependent on the presence of the Tim-Tom import complex found in mitochondria (Derre *et al.*, 2007). For *E. lausannensis* mitochondrial recruitment is not as pronounced as for *W. chondrophila*. The same observation was made in endometrial cells and pneumocytes infected with *E. lausannensis* (Kebbi-Beghdadi, personal communication).

Phospholipids and cholesterol must be acquired from the host, since the abilities for lipid metabolism are limited in *Chlamydiales*. *E. lausannensis* has a slightly increased lipid metabolic capacity compared to *C. trachomatis* (Bertelli *et al.,* unpublished), but still less than *W. chondrophila*. Croxatto *et al.,* 2010 hypothesized that mitochondria could be a source of energy and lipids for the replicating *Waddlia*. Since, *E. lausannensis* does not

associate with Golgi fragments, like *C. trachomatis,* nor mitochondria, like *W. chondrophila,* the lipid source for the replicating bacteria is still unknown. Alternative lipid sources for *C. trachomatis* have been described (see below) and might play a role in *E. lausannensis* replication. For example lipid droplets have been shown to accumulate at *C. trachomatis* inclusions in HeLa cells and even enter within the inclusions (Cocchiaro *et al.*, 2008). Another source of lipids and cholesterol are multivesicular bodies (MVB) that originate in late endocytes by invagination of the endocytic membrane. The MVB have been associated with *C. trachomatis* inclusions in HEp-2 cells and are believed to be the main source of cholesterol during replication (Beatty, 2008). Since the lipid composition of the EBs and RBs of *E. lausannensis* is not known, it is difficult to assess which organelles might provide the lipids required for replication.

To recruit and interact with the host organelles, the bacterial inclusion needs to either promote its own locomotion within the cytoplasm or act on the cytoskeleton to attract the organelles of interest. For *C. trachomatis* the vacuole is known to migrate to the microtubule organization center (MTOC) near the nucleus (Grieshaber *et al.*, 2003). The movement is mediated by a modified dynein-dynactin complex (Grieshaber *et al.*, 2003). Since, *E. lausannensis* replication is not affected by the presence of the microtubule disrupting drug nocodazole, the reorganization of the cell by *E. lausannensis* must occur through a different mechanism (than for *C. trachomatis)*. The sensitivity of *E. lausannensis* to cytochalasinD treatment reveals the importance of actin in *E. lausannensis* replication. So far, actin has been involved in entry and exit of *C. trachomatis* (Carabeo *et al.*, 2002, Chin *et al.*, 2012, Jiwani *et al.*, 2012, Jiwani *et al.*, 2013) and mitochondria recruitment (Croxatto *et al.*, 2010). For *E. lausannensis* the reduced replication is not due to a decreased entry, since the actin depolymerization was only induced upon internalization. Interestingly, the effect of cytochalasinD was not reversible, since removal of the drug after two hours did not allow complete recovery of replication. Noteworthy, in contrast to what was observed for *W. chondrophila* (Croxatto *et al.*, 2010), *E. lausannensis* replication was only decreased and could not be completely abolished, even when cytochalasinD was combined with

nocodazole,. Either *E. lausannensis* is able to use intermediary filaments to recruit and transport the nutriments required for replication, or the bacteria can find everything within the replicative vacuole. Since, the replication of *E. lausannensis* is not very efficient, a suboptimal growth might be possible even without an intact cytoskeleton.

Finally, we conclude that macrophages are probably not the primary target cell for *E. lausannensis* replication. The inability of *E. lausannensis* to control cell death in the infected macrophages and the limited growth within suggest a lack of adaptation of this strict intracellular bacteria to the macrophage. Nevertheless, the replication in macrophages observed with *E. lausannensis* is significantly more pronounced than with *P. acanthamoebae.* The different degrees of replication within macrophages of *W. chondrophila, P. acanthamoebae*, and *E. lausannensis* make them interesting tools for comparative studies to determine bacterial and host factors that determine replication and infection outcome, and to precise how the different members of the *Chlamydiales* order have evolved specific mechanisms to adapt to different eukaryotic hosts.

Experimental Procedures

Cell differentiation and bacterial strains

The human cell line PLB-985 was kindly provided by M. Grez (Frankfurt, Germany). Cells were cultured in RPMI (Gibco-BRL Life Technologies, Basel, Switzerland), 10% FCS (Biochrom, Berlin, Germany) at 37°C with 5% CO_2. To differentiate monocytes into macrophages cells were exposed to 50nM PMA for 48h. The *Chlamydia*-related bacteria *Estrella lausannensis* (CRIB30) and *Waddlia chondrophila* (ATCC VR-1470) were cultivated in *Acanthamoeba castellanii* (ATCC30010) as previously described (Bertelli *et al.*, 2010).

Antibody staining

Coverslips were blocked in blocking solution (0.3% Triton X-100 and 5% FCS in PBS) for 1h at room temperature. For early endosomes (EEA1), late endosomes (LAMP-1), Golgi (RCAS1), and ER (PDI) staining, the organelle localization kit (Cell Signaling Technology Inc.) was used according to manufacturer's instructions with overnight incubation at 4°C with primary antibody. Coverslips were washed three times in PBS and incubated with in-house mouse polyclonal antibodies against *W. chondrophila* (1:1000) or *E. lausannensis* (1:1000) for 1 hour at room temperature. Coverslips were washed again three times in PBS and incubated for 1h at room temperature with DAPI (Life Technologies, Paisley, Scotland,) and secondary antibodies Alexa Fluor 488 goat anti-mouse (1:1000) and Alexa Fluor 594 donkey anti-rabbit (1:1000)(Molecular Probes Leiden, Netherlands). Coverslips were washed again three times in PBS and mounted with MOWIOL (Sigma-Aldrich) on glass slides.

For tubulin (Abcam Inc, Cambridge, UK) staining the same procedure was used except that primary antibody (1:200) was incubated with the polyclonal anti-*Waddlia* or anti-*Estrella* for 1 hour. at room temperature. Actin filaments were directly stained with Phalloidin CF594 (Biotium, Hayward, USA) together with the polyclonal anti-*Waddlia* or anti-*Estrella* antibody at room temperature during 1 hour. All subsequent steps were performed as described above. For mitochondrial staining, cells were incubated with 250nM of MitoTracker Red CMXRos (Life Technologies) 30 min prior to fixation with acetone. After 1 hour blocking in

triton-free blocking solution coverslips were incubated as described above with anti-*Waddlia* or anti-*Estrella* antibodies.

Infection and fixation

Differentiated macrophages grown on glass coverslips were infected with *E. lausannensis* or *W. chondrophila* by spinoculation. Cells were centrifuged for 15 min at 1790g and further incubated 15 min at 37°C, 5% CO_2, in order to allow bacterial internalization. Cells were then washed to eliminate all non-internalized bacteria and fresh medium was added. At different time points post infection, cells were fixed using a protocol depending on the subsequent antibody staining. For actin and tubulin staining, cells were fixed in -20°C methanol for 10 min. For MitoTracker Red CMXRos, cells were incubated during 10 min in -20°C acetone. For LAMP-1 (Cell Signaling Technology Inc., Beverly, USA) staining, cells were fixed in a 1:1 acetone/methanol solution for 10 min at -20°C. For all other organelle markers, cells were fixed with 4% paraformaldehyde at room temperature for 15 min. For PDI staining an additional methanol permeabilization (10 min, -20° C) was performed. All fixed samples were washed three times with PBS.

DNA extraction and qPCR

Genomic DNA of *W. chondrophila* and *E. lausannensis* was extracted using the tissue extraction protocol from Wizard ® SV Genomic DNA kit (Promega, Madison, USA). Samples were quantified by qPCR as published before (Goy *et al.*, 2009, Lienard *et al.*, 2011b).

Microtubules and actin disruption

20μM nocodazole (Sigma-Aldrich) and or 10 μM cytochalasinD (Sigma-Aldrich) were used to disrupt the cytoskeleton of the infected macrophages. The drugs were added at different time points p.i.. The cells were fixed with methanol 12 hours p.i.. The fixed coverslips were blocked and the bacteria were stained with the mouse anti-*Estrella* antibody and the secondary Alexa488 anti-mouse antibody as described above. The mounted coverslips were

analyzed with an automated Axiovision (Zeiss, Feldbach, Switzerland). Four images of eight by eight tiles with a 20% overlap were taken with a 40x magnification. Images were acquired in the FITC and DAPI channels. The acquired images were then further processed with ImageJ to split the channels, define the cell outline and the size of the inclusions. The inclusions were divided into large, medium and small and normalized by the number of infected cells. The number of inclusions were represented with GraphPadPrism normalized to 100 infected cells. t-tests were performed with GraphPadPrism.

Confocal microscopy and co-localization analysis

Images were acquired with a confocal microscope Zeiss LSM510 Meta or Zeiss LSM 710 and analyzed with Zen 2009 Light edition software. For Golgi fragmentation cells were analyzed with an epifluorescent microscope (Leitz Diaplan, Leica, Switzerland). Co-localization of bacteria containing vacuole and organelles was assessed on 100 infected cells in three independent experiments. Statistical analyses of the acquired data were performed with GraphPadPrism v6.0 (GraphPad, LaJolla, USA).

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Figure 1 Growth of *E. lausannensis* in human macrophages. (**A**) Growth of *E. lausannensis* and *W. chondrophila* follows the same timeline. Bacteria re-differentiate into reticulate bodies between 3 and 6 hours post-infection. Replication occurs rapidly with large inclusions already visible at 12 hours post-infection. At 16 hours post-infection the bacteria have differentiated back into EBs and start to be released by lysis. At 24 hours post-infection most of the inclusions have burst and a second infection cycle has started. Bacteria are stained with in-house polyclonal rabbit or mouse antibodies (green). Cells are marked with ConcanavalinA (red) and DAPI (blue). Scale bar 10μm. Pictures taken at 1000x magnification. (**B**) Growth of the bacteria was confirmed by specific qPCR. Data of at least three independent experiments performed in duplicates with SEM.

Figure 2 Cytopathic effect of *E. lausannensis*. (**A**) Infected cells showed fragmented (white arrow) or condensed nuclei already prior to bacterial replication. (**B**) *E. lausannensis* causes a significant increase in cells with fragmented nuclei at six hours of infection compared to *W. chondrophila* and uninfected cells. The percentage of infected cells with fragmented nuclei was quantified in three independent experiments for 100 infected cells each. (**C**) Cytopathic effect was confirmed by monitoring of LDH release. Data of two independent experiments in duplicates with SEM. The area under the curve (AUC) was quantified for each condition to determine the extent of the cytopathic effect. The cytopathic effect was absent in cells infected with *W. chondrophila* or infected with formol-inactivated bacteria.

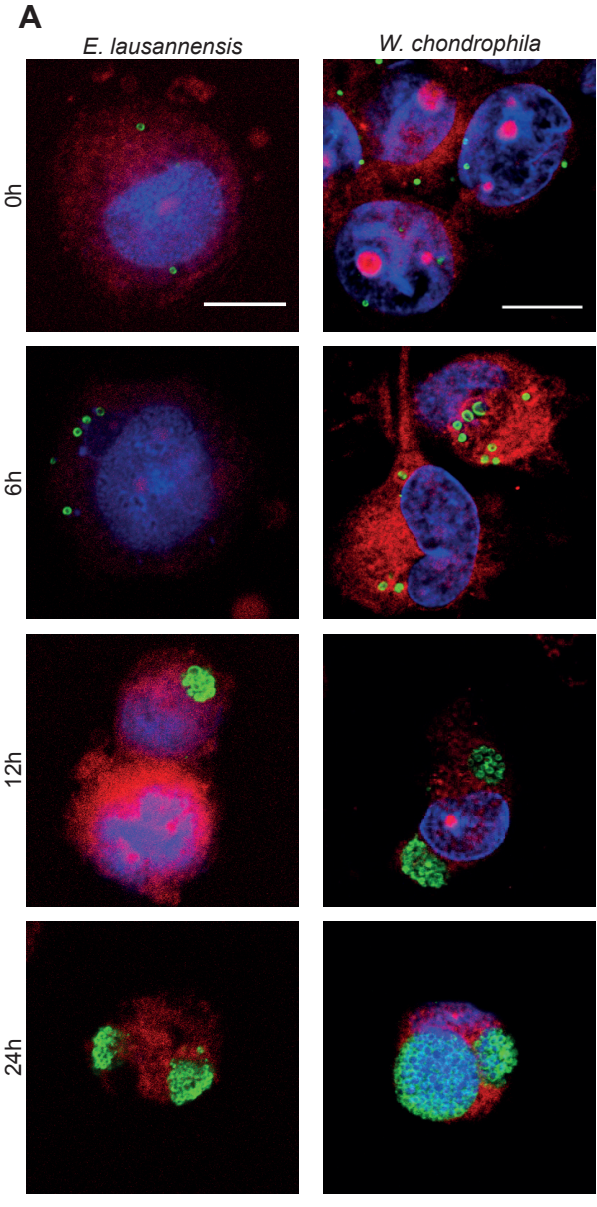
Figure 3 Early intracellular trafficking of *E. lausannensis.* (**A**) Infected cells were fixed at early time points to determine EEA1 (red) localization at chlamydial inclusions. *W. chondrophila* (green, Wch) significantly co-localizes with EEA1 at 15 minutes post-infection. *E. lausannensis* (green, Ela) does not. (**B**) LAMP-1 (red) staining was determined in *E. lausannensis* (green, Ela) vacuoles. No accumulation was observed for up to six hours. (**C**) Acquisition of FM4-64 by *E. lausannensis* inclusions was followed for up to two hours. The

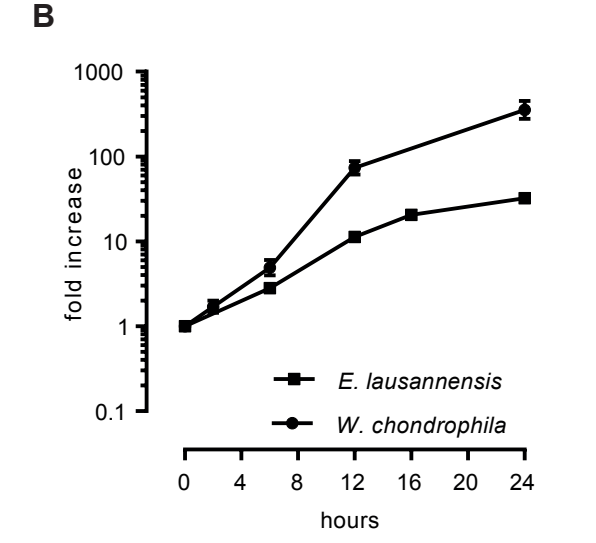
internalized bacteria acquire a strong FM4-64 staining (red). Extracellular bacteria were stained with polyclonal mouse anti-*Estrella* antibody (green. Ela). Internalized bacteria were visualized with Hoechst staining (blue). All experiments were performed in triplicates on 100 infected cells. Results are presented with SEM. Images were acquired at a 630x magnification (scale bar 10μm).

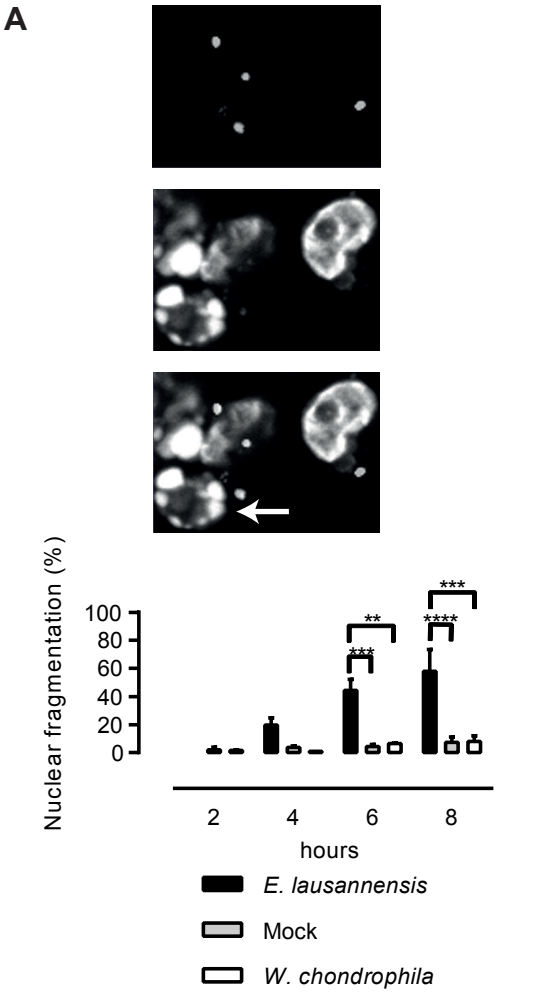
Figure 4 Interaction of *E. lausannensis* inclusion with host organelles. (**A**) Infection with *E. lausannensis* (green, Ela) causes Golgi (red) fragmentation without recruitment of the Golgi fragments to the inclusion. Partial Golgi fragmentation is also observed in macrophages infected with *W. chondrophila*. (**B**) Co-localization of endoplasmic reticulum marker PDI (red) was not observed for *E. lausannensis* inclusions (green, Ela). For *W. chondrophila* inclusion the co-localization increased up to 60% at eight hours p.i. (**C**) Mitochondria (MitoTracker Red CMXRos) are recruited to a lesser extent by *E. lausannensis* inclusions (green) compared to *W. chondrophila.* All experiments were performed in triplicates on 100 infected cells. Results are presented with SEM. Images were acquired at a 630x magnification. Nuclei are marked with DAPI (blue).

Figure 5 Role of the cytoskeleton in the replication of *E. lausannensis*. (**A**) Treatment with nocodazole alone does not significantly reduce *E. lausannensis* replication. Infected cells were treated with 10 μM nocodazole for different time spans. (**B**) Cells were treated with cytochalasinD 20 μM for different time intervals. Treatment at early time points significantly reduced growth of *E. lausannensis* (orange bars). (**C**) Treatment of infected cells with nocodazole and cytochalasinD for different time spans. Drug treatment only affected replication when applied at early time points and persisted upon removal. Drug treatments: start at 0h p.i. orange, start at 2h p.i. green, start at 4h p.i. blue, start at 8h p.i. dark grey, 0.1% DMSO for 12h light grey, 0.2% DMSO for 12h white, untreated black. Cells were fixed 12 hours post infection and number of inclusions was quantified. Images of fixed cells were

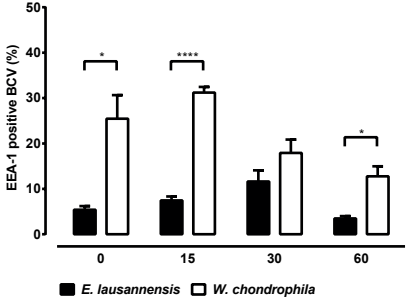
taken at 400x with tiling. Data of at least three independent experiments in quadruplicates with SEM. Student's *t*-test was performed to determine significance.

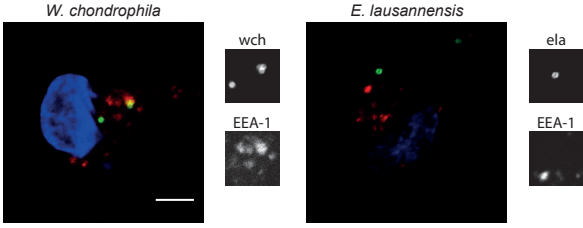

A
E. lausannensis
W. chondrophila
0h
6h
12h
24h


B
1000
100
10
1
0.1
fold increase
E. lausannensis
W. chondrophila
0
4
8
12
16
20
24
hours

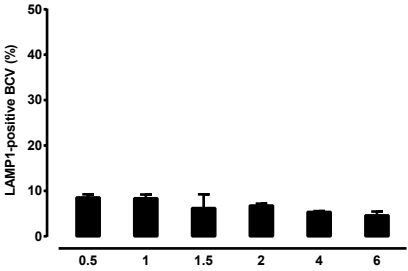

A
Nuclear fragmentation (%)
100
80
60
40
20
0
**
***
***
****
2
4
6
8
hours
E. lausannensis
Mock
W. chondrophila
B
LDH release (%)
100
80
60
40
20
0
0
3
6
8
12
16
hours
E. lausannensis
inactivated E. lausannensis
W. chondrophila
inactivated W. chondrophila
Mock
AUC
500
400
300
200
100
0
***
Mock
E. lausannensis
inactivated E. lausannensis
W. chondrophila
inactivated W. chondrophila

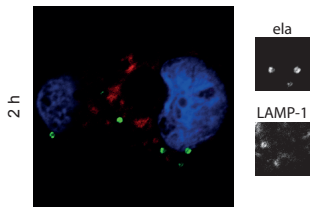
A


EEA-1 positive BCV (%)
50
40
30
20
10
0
*
****
*
0
15
30
60
E. lausannensis
W. chondrophila

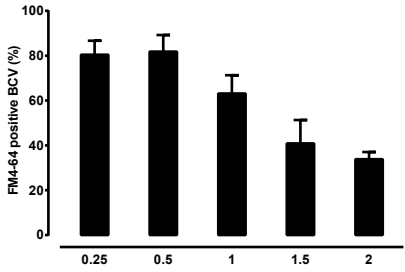

W. chondrophila
E. lausannensis
15 min
wch
EEA-1
ela
EEA-1

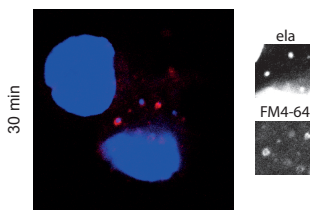
B


LAMP1-positive BCV (%)
50
40
30
20
10
0
0.5
1
1.5
2
4
6

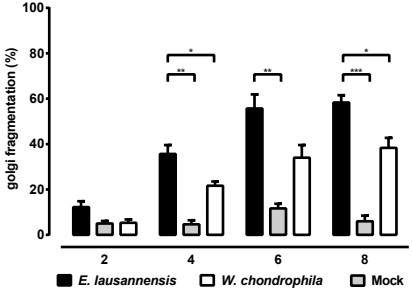

2 h
ela
LAMP-1

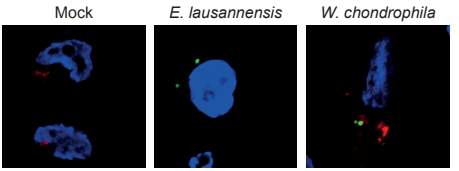
C


FM4-64 positive BCV (%)
100
80
60
40
20
0
0.25
0.5
1
1.5
2

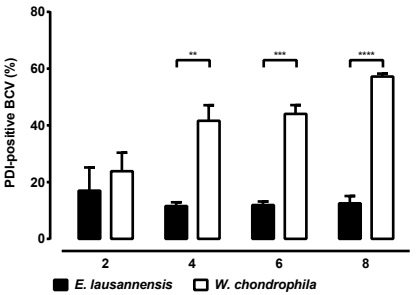

30 min
ela
FM4-64

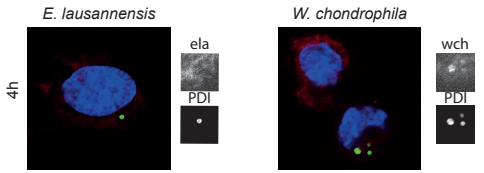
A


golgi fragmentation (%)
100
80
60
40
20
0
*
**
**
*
***
2
4
6
8
E. lausannensis
W. chondrophila
Mock

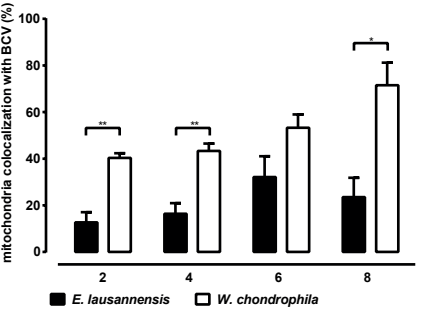

Mock
E. lausannensis
W. chondrophila
4h

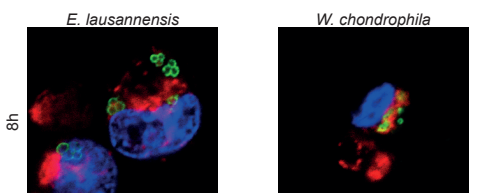
B

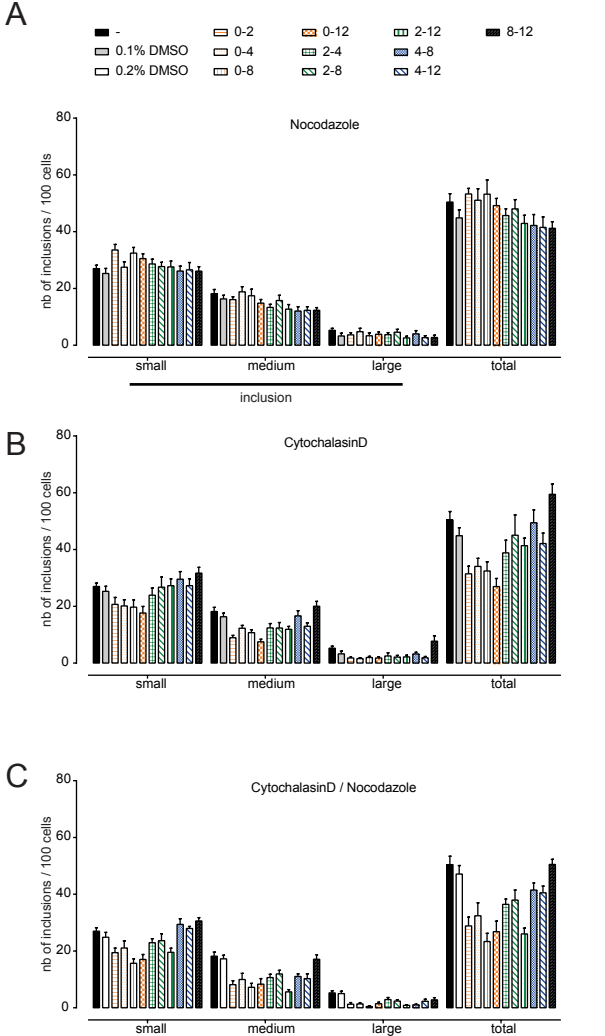

PDI-positive BCV (%)
80
60
40
20
0
**
***
****
2
4
6
8
E. lausannensis
W. chondrophila


E. lausannensis
W. chondrophila
4h
ela
PDI
wch
PDI

C


mitochondria colocalization with BCV (%)
100
80
60
40
20
0
**
**
*
2
4
6
8
E. lausannensis
W. chondrophila


E. lausannensis
W. chondrophila
8h


A
-
0.1% DMSO
0.2% DMSO
0-2
0-4
0-8
0-12
2-4
2-8
2-12
4-8
4-12
8-12
Nocodazole
nb of inclusions / 100 cells
80
60
40
20
0
small
medium
large
total
inclusion
B
CytochalasinD
nb of inclusions / 100 cells
80
60
40
20
0
small
medium
large
total
C
CytochalasinD / Nocodazole
nb of inclusions / 100 cells
80
60
40
20
0
small
medium
large
total