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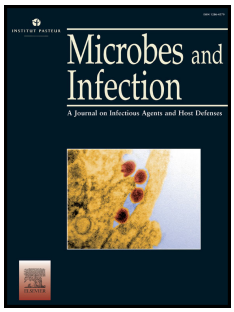
Published in:
Microbes and Infection

DOI (link to publication from Publisher):
10.1016/j.micinf.2018.10.007

Publication date:
2019

Document Version
Accepted author manuscript, peer reviewed version

Accepted Manuscript



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PII: S1286-4579(18)30192-8

DOI: https://doi.org/10.1016/j.micinf.2018.10.007

Reference: MICINF 4614

To appear in: *Microbes and Infection*

Received Date: 30 July 2018

Revised Date: 11 October 2018

Accepted Date: 11 October 2018

Please cite this article as: M. Lausen, G. Christiansen, T.B. Guldbæk Poulsen, S. Birkelund, Immunobiology of monocytes and macrophages during Chlamydia trachomatis infection, *Microbes and Infection*, https://doi.org/10.1016/j.micinf.2018.10.007.

Immunobiology of monocytes and macrophages during Chlamydia trachomatis infection

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Abstract

Infections caused by the intracellular bacterium *Chlamydia trachomatis* are a global health burden affecting more than 100 million people annually causing damaging long-lasting infections. In this review, we will present and discuss important aspects of the interaction between *C. trachomatis* and monocytes/macrophages.

Keywords: Monocytes; macrophages; *Chlamydia trachomatis*

1 Introduction

Chlamydia trachomatis (*C. trachomatis*) is a small intracellular Gram-negative human pathogenic bacterium, which comprises a range of serovars based on variations in the major outer membrane protein (MOMP). These serovars are genetically similar, but cause different pathological manifestations. Serovar A-C cause the blinding eye condition, trachoma; D-K cause sexually transmitted genital infection, which can lead to pelvic inflammatory disease, ectopic pregnancy, and infertility. Finally, serovar L1-L3 can spread from the genital tract to the lymphatic system causing more disseminated infections.

Chlamydiae are obligate intracellular bacteria with a unique biphasic developmental cycle. Initially, the small (0.3 μm) infectious but metabolic inactive elementary body (EB) infects the epithelial host cell. Intracellularly, the EB transforms to a larger (1 μm) and metabolic active reticular body (RB) and the RB starts to replicate.

C. trachomatis serovars preferably infect mucosal epithelium, but can also infect a range of other cells including fibroblasts and cells of the immune system [1].

Monocytes and macrophages are recruited to the genital tract during experimental genital *Chlamydia* infection and the initial engagement between macrophages and *C. trachomatis* may determine the overall outcome of the infection [2,3]. Efficient phagocytosis and intracellular killing can limit ascension of the infection and provide antigenic material for activating CD4+ T-cells towards a Th1-mediated immune response - the most critical immune response to eradicate *C. trachomatis* infections [4]. Different murine infection models have demonstrated the importance of these mechanisms in controlling *Chlamydia* infections.[5,6]. However, if intracellular elimination in macrophages fails, macrophages may be used as Trojan horses for bacterial dissemination to the lymphatic system with bacterial replication in the draining lymph nodes. Especially the L-biovars have been linked to intracellular survival and dissemination [7]. Lastly, monocytes and macrophages also play important roles in the

immunopathology of *C. trachomatis* infections by secreting proinflammatory cytokines causing collateral tissue damage [3]. Thus, understanding the interaction between macrophages and *C. trachomatis* is critical to understand how protective immunity develops and how the immunological response causes pathology.

A proposed role for *C. trachomatis*-infected monocytes in the pathogenesis of reactive arthritis prompted a number of studies in the late 1980's trying to understand the interaction between monocytes/macrophages and *C. trachomatis*. Since these initial studies, several efforts have been made to understand monocyte/macrophage functions in *Chlamydia*-induced inflammation and to understand why *C. trachomatis* infections tend to be chronic.

Clearly, the intracellular fate of *C. trachomatis* in macrophages is completely distinct from the normal developmental cycle observed in epithelial cells. Thus, before discussing the immunobiology of macrophages during chlamydial infection, we will begin with a concise presentation of current knowledge about the developmental cycle in epithelial cells to set the scene for discussions.

2 The developmental cycle of *C. trachomatis* in epithelial cells

The developmental cycle of *C. trachomatis* in epithelial cells has been studied in decades and is now rather well characterized. Depending on the serovar, *C. trachomatis* EBs engage epithelial cells in the eye or in the genital mucosa where they attach to host cell surface components namely heparan sulfate proteoglycans. Upon attachment, *C. trachomatis* induces its own uptake by secreting pre-formed effector proteins into the host cell cytosol through a type III secretion system. One of these effectors is translocated actin-recruiting phosphoprotein (TarP), which is tyrosin phosphorylated by host cell kinases when translocated [8,9]. TarP is an actin modifying protein inducing rearrangement of the actin cytoskeleton and uptake of *C. trachomatis* into a membrane-enclosed vesicle [10]. Each chlamydial EB is taken up in an independent vesicle, which is transported to the microtubule-

organizing center in the perinuclear area of the cell. This process is facilitated by interaction with microtubules and the motor protein dynein [11]. At the microtubule organizing center, the independent *Chlamydia*-containing vesicles undergo homotypic fusion thereby establishing a single large membrane enclosed vacuole called an inclusion [12].

The stability and unique physiology of this replicative niche is established by inserting translocated secreted inclusion membrane proteins (Incs) into the inclusion membrane. Inc proteins face the cytoplasmic site of the inclusion membrane and interact with different membrane-sorting proteins including numerous Ras-related protein Rab (Rab) GTPases. These interactions inhibit fusion with destructive vesicular compartments, e.g. lysosomes while promoting fusion with nutrient-rich compartments such as lipid-rich Golgi-derived vesicles [13].

During inclusion formation, the infectious EBs differentiate into metabolically active RBs that start replicating by binary fission or polarized cell division leading to growth of the inclusion [14]. After 48-72 hours, the end of the developmental cycle is reached when RBs have transformed back to EBs. Burst of the cell or membrane extrusion liberates infectious EBs ready for new rounds of infection. Generally, the underlying mechanisms mediating host cell exit remain poorly described. However, it was recently shown that chlamydial membrane extrusion is mediated by interaction with inclusion membrane proteins and host Ca^{2+}-channels reducing myosin motor activity necessary for extrusion formation [15].

3 Macrophage encounter of *C. trachomatis*

The first encounter between *Chlamydia* and mononuclear phagocytes takes place in the genital tract mucosa. The genital mucosa contains tissue-resident macrophages and monocytes which engage *Chlamydia* EBs once liberated from lysed epithelial cells after completion of the developmental cycle [16]. In early infectious stages, epithelial cells secrete

several chemokines and proinflammatory cytokines leading to local inflammation and leukocyte recruitment [17,18]. Using mouse models of genital *C. trachomatis* infection it was demonstrated that CD11b-positive cells (monocytes/macrophages) infiltrate the mucosa during infection [2]. This recruitment is likely induced by secretion of chemokines including CCL2 and macrophage inflammatory protein-1α known to attract monocytes to the site of infection [19,20]. Thus, both resident macrophages and monocyte-derived macrophages recruited from the bloodstream engage invading *C. trachomatis* in the genital mucosa. The encountered *Chlamydia* organisms, liberated from the epithelial cells, consist of both EBs and RBs. Both forms can trigger the inflammatory response and provide antigenic material as discussed in the following sections.

4 Macrophage sensing of *C. trachomatis*

At the site of infection, macrophages recognize the bacteria directly through different innate immune receptors. Abundant evidence shows that *C. trachomatis* recognition activates MyD88- and P38/ERK-dependent signaling pathways, suggesting a role for pattern recognition receptors (PRRs) in chlamydial sensing [21–23].

Monocytes and macrophages are equipped with numerous PRRs, which detect a variety of conserved structural motifs known as pathogen associated molecular patterns (PAMPs). *C. trachomatis* contains several PAMPs; the most well-studied being LPS and Heat Shock Protein (HSP) 60. Furthermore, HSP70, pORF5, lipoproteins, and macrophage infectivity potentiator (MIP) have been confirmed to activate host macrophages through PRRs [21,23,24].

Using photo-chemically inactivated *C. trachomatis* EBs, Bas et al. show a prominent cell activation of monocytes and macrophages [24]. In addition, macrophages stimulated with viable or inactivated *C. trachomatis* display different cytokine profiles [25–27]. Collectively,

these observations suggest that both surface and intracellular receptors detect and respond to chlamydial infection presumably activating different downstream signaling pathways. Particularly, members of the toll-like receptor (TLR) family and the nucleotide-binding oligomerization domain (NOD) like receptor family have been implicated in chlamydial recognition.

The macrophage receptors involved in *C. trachomatis* recognition and the subsequent intracellular events are illustrated in Fig. 1.

4.1 Toll-like receptors in *C. trachomatis* recognition

Like other Gram-negative bacteria *C. trachomatis* contains LPS in the outer membrane, a potent ligand for TLR4 and the co-receptor CD14. Therefore, it is rational to expect an important role of TLR4 in *C. trachomatis* recognition. Using CD14 and TLR4 transfected cell lines, early studies did indeed discover a role for these receptors in recognition of chlamydial LPS [28,29]. In support, Heine et al. showed that preincubating human peripheral blood mononuclear cells with a CD14-blocking antibody completely abrogated cellular activation by chlamydial LPS confirming the *Chlamydia*-sensing role of CD14 [29]. More recent studies, however, suggest that the contribution of TLR4 in chlamydial recognition by monocytes may be limited [23,24,30]. Instead, several reports suggest that *C. trachomatis* induced activation of monocytes is TLR2 dependent. These observations originate from studies using different strategies including cell lines transfected with different TLRs, primary cells treated with receptor-blocking antibodies, and primary cells from TLR-deficient mice [21,23,24,30–32]. Collectively, these studies suggest that TLR2 recognizes live *C. trachomatis* EBs together with several PAMPs such as LPS, pORF, lipoproteins, and MIP. Interestingly, Agrawal et al. found that both TLR2 and TLR4 are involved in *C. trachomatis* recognition in human cervical monocytes with a time-dependent contribution of each receptor[16]. Thus, early detection was TLR4-dependent, but switched to TLR2-dependent

recognition at later time points. In addition, activation through TLR4, but not TLR2, induced interleukin(IL)-12 production [16]. These observations outline the necessity of careful interpretation of studies investigating chlamydial activation of host cell receptors when considering experimental design.

An interesting study by Nagarajan et al. found that neither TLR2 nor TLR4 are involved in *C. trachomatis* induced interferon (IFN)-β production. Instead they showed the induction of IFN-β was dependent on endosome acidification and the adaptor molecule MyD88 [26]. The authors did not identify the involved receptors, but suggested that the recognition could be mediated by intracellular TLRs, including TLR7, -8, and -9 [26]. However, using macrophages from TLR7- and TLR9 KO mice, the same authors demonstrated that these receptors are dispensable for IFN-β production [32]. Applying macrophages generated from human induced pluripotent stem cells, Yeung and colleagues demonstrated an important role for interferon regulatory factor 5 (IRF5) in intracellular survival of *C. trachomatis* in macrophages [33]. IRF5 is activated downstream of TLR7 and TLR8, suggesting a possible role for these receptors in chlamydia recognition by human macrophages.

Lastly, also TLR1 and TLR6 have been shown to participate in chlamydial recognition by inducing cell activation in response to chlamydial MIP and the lipopeptide PamCSK4 [24]. Yet, blocking these receptors does not have the same effect as blocking TLR2. Thus, TLR2 seems to be the predominating TLR used for macrophage recognition of *C. trachomatis* while *Chlamydia*-induced type I interferon response is TLR-independent highlighting the importance of other PRRs outside the TLR family.

4.2 NOD-like receptors

TLR-deficiency or TLR-blockage does not abrogate cellular activation completely, proposing a redundancy in TLR-based *C. trachomatis* recognition. NOD-like receptors are cytosolic receptors playing an important role in microbial sensing and innate defense. The NOD-like

receptor family consists of 23 members of which two have been reported in *C. trachomatis* sensing: NOD1 and nucleotide-binding domain, leucine-rich repeat family, pyrin domain containing 3 (NLRP3). The involvement of NOD1 in *Chlamydia* recognition was established using expression and gene knockdown studies in HeLa cells [32,34,35]. At present, no direct evidence for NOD-based recognition in macrophages exists, although NOD contribution has been confirmed for other intracellular bacteria and may also be involved in macrophage recognition of *C. trachomatis* [36]. Nonetheless, the contribution of NOD1 has been obscure since these receptors recognize and ligate peptidoglycan fragments from the bacterial cell wall [37]. Until recently, peptidoglycan has not been directly detected in *C. trachomatis*, even though the *C. trachomatis* genome contains all necessary genes for peptidoglycan assembly and is sensitive to beta-lactam antibiotics [38]. In 2014, the Maurelli group, however, directly detected peptidoglycan in *C. trachomatis* using a novel metabolic cell wall labeling approach [39] and later confirmed the presence of muropeptides using mass spectrometry [40]. Finally, it has been demonstrated that NOD2 expression is upregulated in *C. trachomatis*-infected macrophages, suggesting that NOD2 may also participate in macrophage recognition of *C. trachomatis* [41].

NLRP3 is another NOD-like receptor which senses molecules associated with cell damage including adenosine triphosphate (ATP) and uric acid [37]. It constitutes the pattern recognition moiety of a large multiprotein complex known as the inflammasome. PAMP mediated inflammasome activation leads to caspase-1 activation and subsequently cleavage and secretion of IL-1β and IL-18. Chlamydial infection of monocytes activates the inflammasome in a NLRP3, AIM2 and MyD88-dependent manner [27,42,43]. Whether NLRP3 directly recognizes chlamydial PAMPs or if the activation results from endogenous danger-associated molecular patterns (DAMPs) induced by *C. trachomatis* is not fully

understood, but a role for reactive oxygen species (ROS) [43] and autocrine cytokine signaling (please see the section below) [27] have been proposed.

4.3 Cytosolic DNA receptors

Finally, the cytosolic DNA sensors stimulator of interferon genes (STING) and the absent in melanoma 2 (AIM2) might also participate in *C. trachomatis* recognition by sensing chlamydial nucleic acids (Fig. 1). STING detects cytosolic double-stranded DNA and plays an important role during both bacterial and viral infections. It was previously demonstrated that STING mediates IFN-β induction in *Chlamydia* infected HeLa cells and that *C. muridarum* induced IFN-β production in J774 macrophages was cyclic GMA-AMP synthase (cGAS)-dependent. cGAS is a cytosolic DNA-sensing enzyme that detects foreign DNA converting it to cyclic nucleic acids which is recognized by STING [32,44]. Direct STING-mediated recognition of *Chlamydia* by macrophages was shown recently by Webster and colleagues [27]. They demonstrated that STING recognizes cyclic di-AMP from metabolic active *C. trachomatis* in murine macrophages leading to IFN-β secretion and autocrine IFN-β dependent inflammasome activation and IL-1β secretion [27]. However, this observation awaits confirmation in human primary macrophages. Translating this conclusion directly to human conditions is controversial due to the debatable metabolic state of *C. trachomatis* in human primary macrophages.

AIM2 is another cytosolic receptor sensing double-stranded DNA and like NLRP3 involved in inflammasome activation. A recent study showed that *C. trachomatis*-induced inflammasome activation in murine macrophages was AIM2 dependent implying that AIM2 might detect chlamydial DNA [27,42].

4.4 Cellular activation and cytokine production

Although the exact mechanisms mediating macrophage recognition of *C. trachomatis* are not fully comprehended, macrophage engagement with *C. trachomatis* elicits a potent cell activation inducing the expression of several cytokines, chemokines, and growth factors that are summarized in Table 1.

5 *C. trachomatis* entry into macrophages

Several *C. trachomatis* serovars are internalized into both murine and human primary macrophages and into different cell lines. However, the involved receptors and molecular mechanisms mediating chlamydial entry into host immune cells have not been determined yet [1,43,45,46]. The entry mechanisms are supposedly carried out by phagocytosis or by receptor-mediated endocytosis [46–48] and the involved receptors might be located to lipid rafts in the plasma membrane [49].

Comparing chlamydial infection rates in cell types with different surface receptor profiles could highlight the involvement of receptors and receptor families. Since *C. trachomatis* infects many different cell types the receptors involved may be ubiquitously expressed or involve multiple entry mechanisms working with essentially equal efficiency [1,50,51]. This theory is supported by the findings by Sun et al. who observed a similar infection rate between HeLa cells and murine RAW macrophages [52]. In contrast, others find that *C. trachomatis* entry occurs much less efficiently in monocytes compared to epithelial cells indicating involvement of cell-specific receptors [53]. However, this study, among others, evaluated the entry efficiency by enumerating inclusions two days post infection. Thus, the data presented in this study may not reflect the actual entry efficiency, since inclusion numbers after two days also depend on bacterial survival and replication.

Glycosylated chlamydial surface proteins may provide a moiety for host cell attachment and entry. Kuo et al demonstrated that *C. trachomatis* entry into macrophages was significantly reduced in macrophages deficient in the mannose receptor [54]. The chlamydial ligand attaching to the mannose receptor has not been identified, but it has been suggested that chlamydial MOMP is glycosylated by mannose [55] and might therefore serve as ligand for the mannose receptor facilitating chlamydial entry. The mannose receptor is used by *Mycobacterium tuberculosis* to enter macrophages and entry through this receptor is beneficial for intracellular survival [56].

Another receptor involved in *Mycobacterium tuberculosis* entry is the complement receptor CR3 [57]. Complement receptors are also likely involved in chlamydial entry because *C. trachomatis* is opsonized by the complement C3 fragment iC3b which is recognized by CR3 expressed on monocytes and macrophages [58,59]. We recently demonstrated that complement C3 facilitates rapid uptake of *C. trachomatis* in human monocytes supporting the role for CR3 in chlamydial uptake, [59].

Lastly, chlamydial recognition and uptake may be dependent on how *Chlamydia* are liberated from infected epithelial cells after completing the development cycle. *C. trachomatis* liberated by membrane extrusion is engulfed by murine macrophages through an actin-dependent mechanism involving extrusion membrane phosphatidylserine (PS) [60].). PS is normally exposed in the membrane of apoptotic cells and is recognized by apoptotic receptors on phagocytes. However, blocking PS-receptor interaction by annexin V only partially inhibit macrophage uptake of *Chlamydia* containing extrusions, indicating involvement of other receptor-ligand interactions [60].

6 The intracellular fate of *C. trachomatis* in macrophages

Studies exploring the intracellular fate of *C. trachomatis* in macrophages have been carried out since the 80's, but despite more than 30 years of research there is still no clear understanding of the intracellular trafficking and fate of *C. trachomatis* in macrophages. Early studies indicated that *C. trachomatis* can persist in monocytes for more than 7 days [61–63], while others, more recent studies, show that *C. trachomatis* is rapidly degraded in macrophages [52]. One thing is however certain; the intracellular fate of *C. trachomatis* in monocytes and macrophages differs drastically from the normal developmental cycle seen in epithelial cells as demonstrated in Fig. 2.

After macrophage entry *C. trachomatis* can induce a state of persistency, where the bacterium is viable and metabolic active, but does not replicate [1,22,63]. This phenomenon has been demonstrated for several serovars including Ba, D, K, and L2. Although viable and metabolic active, the different serovars cannot maintain the developmental cycle, except for serovar L2 [22,64,65]. It appears that serovar L2 can maintain its infectious potency during monocyte infection, because lysates from L2-infected monocytes induce inclusion formation in HeLa cells [53,65]. Nonetheless, we recently demonstrated that *C. trachomatis* L2 were unable to maintain its infectious and growth potential after 24 hours of incubation within monocytes [59]. Different infection/incubation protocols are likely to cause these discrepancies. Table II provides an overview of studies investigating the intracellular fate of *C. trachomatis* in monocytes and macrophages as well as the main findings. Collectively, these findings indicate that monocytes may respond differently to different serovars; that serovar-specific survival mechanisms exist; that infection protocols may affect the chlamydial outcome and/or different macrophage cell types respond differently to *C. trachomatis* infection.

6.1 Macrophage strategies to restrict *C. trachomatis* growth

Why is the development of *C. trachomatis* infection successful in epithelial cells but not in macrophages? Following entry into the epithelial cell, *C. trachomatis* forms a membrane-bound vacuole; the inclusion, as previously described in section 1. Yet, *C. trachomatis* fails to form a mature inclusion in macrophages and this failure is likely due to several mechanisms involving phagosome-lysosome fusion, autophagy, and nutrient starvation.

6.1.1 Targeting *C. trachomatis* for lysosomal degradation

Lysosomal degradation of engulfed bacteria is an important mechanism for bacteria elimination. Usually, a coordinated procedure involving sequential trafficking to vesicles of increased acidity target endocytosed or phagocytosed bacteria to lysosomes. . Recruitment of the proton pump vacuolar H+ ATPase (V-ATPase) mediates the acidification and the sequential trafficking is coordinated by a set of GTP-binding proteins including the Rab GTPases. Of these, Rab5 and Rab7 target vesicles for early endosomes and late endosomes, respectively [66].

Several studies propose that *C. trachomatis* fails to inhibit phagosome-lysosome fusion in macrophages. Shortly after entry into murine macrophages, chlamydial EBs locate to Rab7-positive compartments, a late endosome marker, and subsequently associate with the lysosome marker lysosomal-associated membrane protein 1 (Lamp1) [52,67]. Reducing lysosome acidification by inhibiting V-ATPase supports chlamydial growth in macrophages and suggests that *C. trachomatis* EBs are trafficked through the conventional phagosome/lysosome pathway in macrophages [52,67,68]. This is completely different from epithelial cells where Rab GTPases, different from Rab5 and Rab7, are recruited and target the *Chlamydia*-containing vesicles to non-destructive vesicular compartments.

6.1.2 Anti-chlamydial defense by autophagy

Autophagy is another means of targeting bacteria to lysosomes. Autophagy induction by *C. trachomatis* was first described by Pachikara et al. in HeLa cells [69] and accumulating evidence suggests that autophagy also plays a substantial role in macrophage clearance of *C. trachomatis* [52,67].

Autophagy is a ubiquitous mechanism used to degrade and sequester cytosolic protein and organelles to maintain cell homeostasis [70]. During autophagy, a double membrane structure assembles which surrounds the protein/organelle/pathogen thereby creating a vesicular structure called an autophagosome. The autophagosome is directed to lysosomes and after fusion, the autophagosomal content is degraded [70]. The autophagic pathway is illustrated in Fig. 3.

Upon entry into macrophages, *C. trachomatis* associates with the autophagosomal marker LC3 and is observed in large doubled membrane structures resembling autophagosomes [52,68]. In accordance, functional experiments show that autophagic activity is elicited in infected macrophages, but not in infected epithelial cells [52].Knockdown of autophagy protein 5 (ATG5), a key regulator of autophagy, increases *C. trachomatis* progeny numbers in THP-1 cells [67]. The autophagic potency of macrophages can be enhanced by IFN-γ stimulation mediated by IFN-inducible proteins called guanylate-binding proteins. During IFN-γ cell activation, these proteins co-localize with chlamydial EBs and direct them for lysosomal fusion through an autophagy-dependent pathway [67]. External ATP stimulation can induce chlamydial vacuole fusion with lysosomes in addition to IFN-γ activation,, but whether this process occurs through autophagy has not been determined [71]. The entry and intracellular trafficking of *C. trachomatis* into macrophages is illustrated in Fig. 3.

6.1.3 Direct interaction by perforin-2

Perforin-2 is a phylogenetic conserved pore-forming protein containing a domain, which is also found in other vital immunological proteins such as complement C9 and perforin-1 [72]. Varying expression of perforin-2 during *C. trachomatis* infection may account for the different infection outcome between macrophages and epithelial cells [73]. Monocytes and macrophages constitutively express perforin-2, and IFN-γ stimulation induce expression in epithelial cells. Unfortunately, this induction is inhibited by chlamydial proteins [73]. Perforin-2 expression increases in macrophages, but not in epithelial cells, during *C. trachomatis* infection indicating that perforin-2 expression may be regulated by gene regulatory factors acting downstream of immune receptors. The local cytokine milieu generated by *C. trachomatis* infected epithelial cells increases perforin-2 expression in either resident macrophages or invading monocytes, potentially boosting perforin-2 expression before direct contact with the bacterium [74].

Inducing perforin-2 knock down by small interfering RNA in macrophages leads to maturation of *C. trachomatis* inclusions and the growth pattern resembles that of epithelial cells. In addition, chlamydial growth is restricted in perforin-2 expressing epithelial cells. The anti-chlamydial defense mechanism responsible for these observations is mediated through direct contact with the bacterium [73]. Thus, macrophages synthesize perforin-2 in response to *C. trachomatis* and prevent chlamydial-induced perforin-2 degradation by limiting chlamydial de novo protein synthesis. This provides an efficient chlamydial killing mechanism involving direct contact with the bacterium.

6.1.4 Induction of reactive oxygen and nitrogen species

Production of reactive oxygen species and reactive nitrogen species (ROS and RNS) are important microbicidal mechanisms against various pathogens [75]. Inducible nitric oxide

synthase (iNOS) is produced during *C. trachomatis* infection in macrophages and leads to nitric oxide production [16,64], which is strongly correlated with chlamydial clearance [76]. The mechanisms leading to iNOS induction involve a ROS- and cathepsin-dependent mechanism acting downstream of TLR2 activation [77]. In addition, *C. trachomatis,* but not *C. pneumoniae*, induces ROS production in macrophages. The differential induction of ROS could explain why *C. trachomatis* is killed earlier than *C. pneumoniae* in macrophages [64]. Indeed, macrophages deficient in NADPH oxidase, a ROS generating enzyme, support intracellular survival and replication of *C. trachomatis* [27]. Finally, ROS has also been implicated in inflammasome activation since adding an antioxidant to *C. trachomatis* infected macrophages reduces caspase-1 activation [43].

6.1.5 Limiting access to host cell nutrients

C. trachomatis exploits a parasitic nature relying on host cell components for maintaining metabolism and survival. Hence, restricting chlamydial access to host cell nutrients inhibits bacterial growth.

Tryptophan is an essential amino acid required for chlamydial growth and survival. An essential anti-chlamydial defense mechanism is IFN-γ induced expression of indoleamine 2,3-dioxygenase (IDO). IDO catabolizes tryptophan to L-kynurenine leading to depletion of cytosolic tryptophan and chlamydial growth restriction [78]. Macrophages induce IDO expression in response to *C. trachomatis* infection by different serovars, which may contribute to the growth restriction observed in macrophages [22,79].

Acquisition of host cell lipids to the inclusion membrane is regarded an essential step in chlamydial inclusion maturation and reproduction [80]. This process involves Golgi-disruption and acquisition of lipid-containing Golgi-vesicles. By preventing cleavage of golgin84, macrophages prevent Golgi-disruption during infection thereby preventing

inclusion maturation [52]. In epithelial cells however, golgin84 is cleaved leading to Golgi disruption and acquisition of lipid-rich Golgi-vesicles to the growing inclusion [52].

Another approach of restricting chlamydial growth by nutrient starvation is by reducing intracellular iron levels [81]. Increasing intracellular iron levels by reducing surface-expressed ferroportin in macrophages increases the fraction of large *C. trachomatis* inclusions [82]. Thus, chlamydial growth is dependent on host-cell iron metabolism. Modulation of these pathways could provide a defense mechanism against *C. trachomatis*. Expression of ferritin heavy chain is increased during *C. trachomatis* infection of monocytes [79]. Ferritin could be anti-chlamydial by binding intracellular iron thereby decreasing the concentration of free iron available for *C. trachomatis* in the infected cell.

7 Antigen-presentation of *C. trachomatis* infected macrophages

The primary role for monocytes and macrophages in anti-bacterial immunity is mediated by phagocytosis and secretion of proinflammatory cytokines. However, monocytes and especially macrophages contain major histocompatibility complex (MHC) class I and MHC class II molecules making them competent inducers of adaptive immunity. Possible antigen-presentation pathways in *C. trachomatis* infected macrophages are illustrated in Fig. 4.

7.1 Macrophages and CD4+ T-cells in *C. trachomatis* infection

Th1 responses are the predominant adaptive immunological response to control and eliminate *C. trachomatis* infection like most other intracellular bacteria [83]. Activated Th1 cells secrete IFN-γ and TNF-α, which potentiate microbicidal mechanisms in macrophages and inhibit chlamydial growth in infected epithelial cells as previously described.

How do monocytes and macrophages contribute to Th1 immunity during chlamydial infection? Activation of naïve CD4+ T-cells requires T-cell recognition of chlamydial

antigens presented in MHC class II molecules together with co-receptor ligation and an appropriate cytokine signal. Several *C. trachomatis* proteins have been shown to contain MHC class II epitopes including HSP60, MOMP and PMP [84]. During infection with *C. trachomatis* monocytes upregulate the expression of MHC class II molecules and the co-stimulatory receptors CD40, CD80 and CD86 [16,41,74,79]. IFN-γ and IL-12 drive T-cell polarization in the Th1 direction. Several studies have shown that *C. trachomatis* leads to IFN-γ and IL-12 expression and secretion from infected macrophages (Table I) [16,41,85,86]. Hence, macrophages infected with *C. trachomatis* seem to direct the adaptive response towards Th1 immunity.

Although Th1 mediated immunity is pivotal for infection control and resolution, the macrophage induced T-cell response is not directed solely against Th1 activation. Some investigations suggest that *C. trachomatis* infected monocytes might also drive a Th2 mediated response or modulate the effector functions of activated T-cells [87–89]. Lu et al. showed that murine macrophages pulsed *ex vivo* with UV-inactivated *C. muridarum* failed to induce a Th1 dominant response when adoptively transferred. Instead, mice immunized with *ex vivo* pulsed macrophages had high titers of IgG1 *Chlamydia*-specific antibodies suggesting an IL-4 mediated Th2 response [88]. The authors did not evaluate whether macrophages in fact induced IL-4 secretion in response to *C. trachomatis* pulsing. In fact, macrophage secretion of IL-4 have not yet been established, but micro array analysis have shown that IL-4 mRNA is upregulated in human monocytes early after infection [90].

7.2 Macrophages and CD8+ T-cells in *C. trachomatis* infection

Besides the Th1- response, cell-mediated immunity against *Chlamydia* may also involve CD8+ T-cells. When activated, these cells differentiate into cytotoxic T-cells, which possess efficient killing mechanisms targeted against host cells infected with intracellular pathogens. The relevance and importance of CD8+ mediated immunity during chlamydial infections has

not yet been fully established. Different studies have shown that *Chlamydia*-specific CD8+ T-cells are generated during *C. trachomatis* infection and that they participate actively in anti-chlamydial immunity [91].

CD8+ T-cells recognize small peptides loaded on MHC class I molecules. Therefore, pathogen-derived antigens need to be proteolytically processed before loading onto MHC class I happens. Enzymatic processing of MHC class I antigens is mediated by the ubiquitin/proteasome system located in the cytosol. Thus, only pathogens/antigens accessing the cytosol are targets for MHC class I antigen presentation and CD8+ T-cell activation. The process of presenting exogenously acquired antigens on MHC class I is known as antigen cross-presentation and this immunological mechanism is restricted to professional antigen-presenting cells, such as dendritic cells and macrophages [92]. Accordingly, *C. trachomatis* is only a potential target for antigen cross-presentation if chlamydial antigens enter the cytosol. In epithelial cells, *C. trachomatis* secretes different proteins into the host cell cytosol. If these proteins are secreted in macrophages too, entering MHC class I processing is possible[9,93–95]. However, these proteins are important for inclusion formation and may not be secreted in macrophages since *C. trachomatis* fail to induce inclusion maturation in macrophages. Interestingly though, Prantner et al. demonstrated that the translocon protein sec61 locates to the chlamydial inclusion in macrophages [32]. Sec61 has recently been demonstrated to facilitate antigen translocation from an endosomal compartment into the cytosol [96]. Thus, when *C. trachomatis* EBs or RBs are degraded in macrophages, chlamydial proteins may escape the vesicular compartment entering the cytosol and may be tagged for MHC class I presentation. This process is potentially facilitated by increased expression of MHC class I and transporter associated with antigen processing (TAP1) in macrophages activated by conditioned medium from *C. trachomatis* infected epithelial cells [74]. TAP is a

transmembrane protein that facilitates transport of antigenic peptides from the cytosol to the MHC class I loading compartment in the ER.

7.3 Modulation of T-cell responses

Although chlamydial infection initiates both CD4+ and CD8+ cell-mediated immune responses, eradiction of the infection does not occur. The insufficiency of chlamydial clearance mechanisms may be due to chlamydial-induced attenuation of T-cell immunity. Jendro and colleagues demonstrated that culture supernatants from *C. trachomatis* infected monocytes induced apoptosis of T-cells by a TNF-α dependent mechanism [97,98]. Another way of regulating T-cell immunity is by attenuating T-cell effector functions. It has been demonstrated that chlamydial-infected macrophages reduce IFN-γ release from co-cultured T-cells [99].

8 Summary

Chlamydial growth in monocytes and macrophages is limited and differs drastically from the classical growth pattern seen in epithelial cells. The restricted growth pattern is mediated by several mechanisms including lysosome trafficking, perforin-2 interaction, production of reactive species, and nutrient starvation. The receptors and mechanisms mediating chlamydial recognition and entry are poorly understood and need further investigation. Additionally, there is still dissension on the intracellular trafficking of *C. trachomatis* in macrophages. Confirmation of current observations in human primary cells remains.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgements

TBGP was supported by a grant from the Sino-Danish Center. SB is supported by grants from the Birthe Meyers Foundation, the Beckett Foundation and, the Hertha Christensen Foundation. [100,101,110–112,102–109]

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Figure legends

Figure 1. Macrophage receptors involved in recognition of *C. trachomatis*. TLRs expressed at the cell surface recognize several chlamydial PAMPs such as LPS, HSP60, lipoproteins, MIP, pORF5, and probably many others. Ligation of the different TLRs initiate

a signaling cascade that ultimately leads to nuclear translocation of transcription factors and expression of genes encoding proinflammatory cytokines. After entry, *C. trachomatis* is engaged by another set of receptors in the intracellular compartment. Induction of IFN-β is dependent on endosomal acidification and MyD88, suggesting that intracellular TLRs may participate in IFN-β induction. The cytosolic NOD-like receptor NOD1 recognizes peptidoglycan and ligation leads to activation of IKKs (IκB kinases) and NF-κB. NLRP3, another NOD-like receptor, recruits the adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD) and P-Casp1 (pro-caspase 1) during infection forming a multiprotein complex known as the inflammasome. Inflammasome assembly leads to caspase-1 activation and caspase-1-dependent cleavage and secretion of IL-1β. The NLRP3 activating compound has not been identified yet, but several endogenous molecules including ATP and ROS (reactive oxygen species) can activate NLRP3. Also the DNA-sensing receptor AIM2 is involved in *Chlamydia*-induced inflammsome activation. Finally, chlamydial DNA can be recognized by the ER-associated receptor STING (stimulator of interferon genes). STING ligation leads to translocation of IRF3 (Interferon Regulatory Factor 3) and transcription of type I interferons.

Figure 2. *C. trachomatis* infection in epithelial cell and monocyte.
Both cell types have been cultured with *C. trachomatis* L2 for 24 hours. In HeLa cells (left), *C. trachomatis* replicate and form a large inclusion at 24 hours, but it fails to do so in monocytes (right).

Figure 3. Entry and intracellular trafficking of *C. trachomatis* in macrophages. *C. trachomatis* entry into macrophages is facilitated by both ubiquitous and cell type-specific surface receptors. The entry mechanisms are supposedly carried out by receptor-mediated

endocytosis and phagocytosis, involving the mannose receptor, complement receptors and possibly receptors recognizing phosphatidylserine (PS) in *Chlamydia*-containing extrusions. Upon entry, *C. trachomatis* EBs are localized to Rab7-positive compartments indicative of late endosomes. Rab7 traffics *Chlamydia*-containing vesicles to lysosomes, where the bacteria are killed by the acidic pH and lysozymes.

Autophagy is another mechanism that targets *C. trachomatis* to lysosomes. Here, several ATG proteins facilitate the formation of a double-membrane structure that surrounds the bacteria creating an autophagosome. Interferon-inducible GBPs (guanylate binding proteins) modifies the autophagosomal membrane and facilitates fusion with lysosomes creating autolysosomes that leads to chlamydial killing. Finally, perforin-2 leads to *C. trachomatis* growth restriction by targeting EB directly or by modulating the compartment in which the bacteria reside.

Figure 4. Antigen-presentation in *Chlamydia*-infected macrophages. Both CD4+ T-cells and CD8+ T-cells are activated during *C. trachomatis* infection. Both MHC class II and the co-stimulatory molecules CD80/CD86 are upregulated in macrophages during chlamydial infection. In addition, both IL-12 and IFN-γ are secreted from activated macrophages directing the CD4+ T-cell differentiation in a Th1 direction. Activated Th1 cells secrete TNF-α and IFN-γ, which potentiate the microbicidal potency of macrophages. In addition, also Th2 immunity is elicited and the Th2-differentiation is mediated by IL-4, which is transcriptionally upregulated in response to infection.

The mechanisms involved in *Chlamydia*-induced CD8+ T-cell activation have not been elucidated, but it may occur through antigen cross-presentation. In this pathway, chlamydial proteins may escape the endosomal compartment leading to enzymatically processing by the proteasome. *Chlamydia*-derived peptides are trafficked to the ER or endosomal structures via

868 TAP (Transporter associated with antigen processing) where they are loaded onto MHC class

869 I molecules.

Table 1. Cytokines and chemokines induced by *Chlamydia* in macrophages

Study	Cytokine/chemokine	Species	Cells	*Chlamydia* spp.	Reference
Abdul-Sater et al.	IL-1β	Human	THP-1	*C. trachomatis* L2	[37]
Agrawal et al.	IL-1β, IL-6, IL-12, IFN-γ	Human	Cervical monocytes	*C. trachomatis**	[9]
Bas et al.	IL-1β, IL-6, IL-8, TNF-α	Human	Monocytes	*C. trachomatis* L2	[18]
Darville et al.	IL-6, TNF-α	Mouse	Peritoneal macrophages	*C. muridarum*	[39]
Datta et al	IL-1β, IL-10, TNF-α	Human	Monocytes	*C. trachomatis* Ba, D, and L2.	[16]
Hui et al.	IL-1β, IL-8, TNF-α	Human	THP-1	pORF5 from *C.trachomatis*	[15]
Jendro et al.	TNF- α	Human	Monocytes	*C. trachomatis* K	[40]
Kol et al.	IL-6	Human	Monocytes	HSP60 from *C. trachomatis*	[41]
Krausse-Opatz et al.	IL-8	Human	Monocytes	*C. trachomatis* K	[42]
Lausen et al.	IL-6, IL8	Human	Monocytes	*C. trachomatis L2*	[43]
Manor et al.	TNF- α	Human	Monocyte-derived macrophages	*C. trachomatis* K and L2	[44]
Marangoni et al.	IFN-γ, TNF- α	Human	Monocytes	*C. trachomatis* D	[45]
Mpiga et al.	IL-1 β, IL-6, IL-8, IL-12	Human	THP-1 (human)	*C. trachomatis* L2	[46]
Nagajaran et al.	IFNa, IFNb, IP10, TNF-α	Mouse	Peritoneal macrophages	*C. trachomatis* Nigg.	[20]
Rothermel et al.	IL-1α, IL-1β	Human	Monocytes	*C. trachomatis* L2	[47]
Schrader et al	IL-1α, IL-1β, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-11, IL-15, IL-16, IL-17, IL-18, IFN-γ, TGF- β1, TGF- β2, TNF- α	Human	Monocytes	*C. trachomatis* K	[48]
Yilma et al.	IL-6, IL-8, TNF- α	Mouse	J774 macrophages	*C. muridarum*	[19]
Yilma et al.	IL-1α, IL-1β, IL-6, IL-9, IL-12, IL-15, GM-CSF, G-CSF, CCL2, CXCL1, CXCL5, CXCL10.	Mouse	J774 macrophages	*C. muridarum*	[35]
Wang et al.	MIP-2	Mouse	Monocyte-derived macrophages	Recombinant predicted lipoproteins from *C. trachomatis D.*	[17]

*Serovar not specified

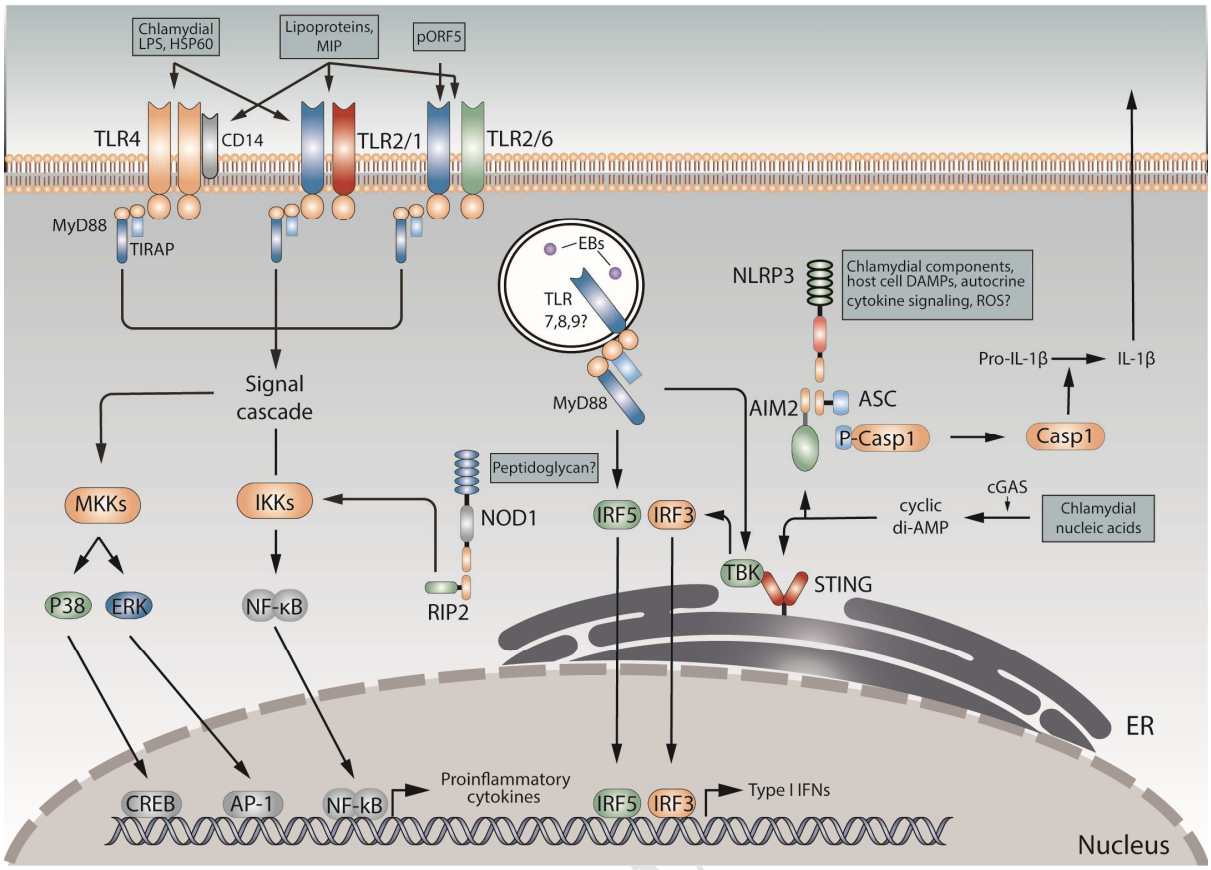
Table 2. Intracellular survival of *C. trachomatis* in monocytes/macrophages

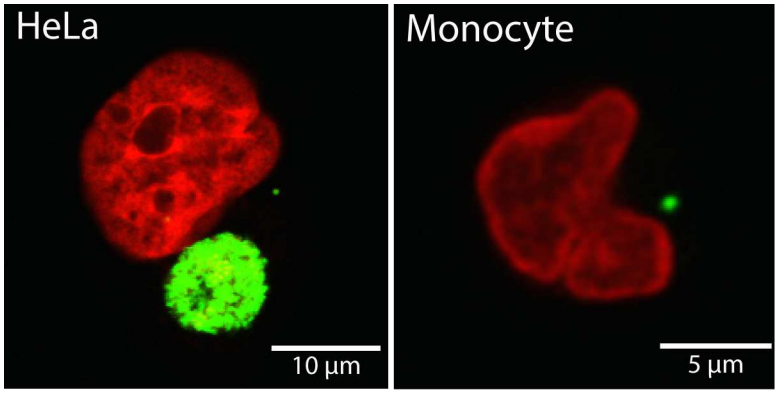
Study	*Chlamydia* spp.	Species	Cells	Method	Results	Ref
Lausen et al. 2018	*C. trachomatis* L2	Human	Peripheral blood monocytes	IFU[1] on McCoy cells	No IFUs 24h p.i.[2]	[59]
Nagarajan et al. 2018	*C. muridarum*	Mouse	Peritoneal macrophages	IFU on L929 cells	200% of initial IFUs are recovered 24h p.i.	[100]
Webster et al. 2017	*C. trachomatis* ?*	Mouse	BMDM[3]	Quantification of LPS and qPCR on 16S RNA	LPS can be detected 24h p.i.	[27]
Yeung et al. 2017	*C. trachomatis* F	Human	iPSdM[4] and monocyte-derived macrophages	Quantification of GFP and IFU assay on McCoy cells	Bacteria replicates in iPSdM for 48h p.i.	[33]
Zuck et al. 2017	*C. trachomatis* L2	Mouse	BMDM	IFU assay on HeLa cells	No IFUs 4h and 8h p.i.	[60]
Zuck et al. 2016	*C. trachomatis* B, D, L2 and *C. muridarum*	Mouse	BMDM	Direct detection by fluorescence	All serovars except serovar B are detected 6h p.i.	[101]
Finethy et al. 2015	*C. muridarum*	Mouse	BMDM	qPCR on 16S RNA	Survives 24h p.i.	[42]
Rajaram et al. 2015	*C. muridarum*	Mouse	RAW264.7 cells	IFU assay on McCoy cells	Reproductive infection is observed 24h p.i.and is MOI[5] dependent	[77]
Datta et al. 2014	*C. trachomatis* Ba, D and L2	Human	Peripheral blood monocytes	IFU assay on HeLa cells	Serovar L2, but not Ba and D survives for 2 days p.i.	[53]
Marangoni et al. 2014	*C. trachomatis* D	Human	Peripheral blood monocytes	IFU assay on LLC-MK2 cells	No detectable IFUs 24h p.i.	[64]
Chen et al. 2013	*C. trachomatis* G	Human	Peripheral blood monocytes	IFU assay on Hep-2 cells	IFUs are detected 48h p.i.	[102]
Fields et al. 2013	*C. trachomatis* B, D, L2 and *C. muridarum*	Mouse	BV2 macrophages + RAW 264.7 cells	IFU assay on HeLa cells	L2 IFUs are detected 24h p.i	[73]
Sun et al. 2012	*C. trachomatis* L2	Mouse	RAW 264.7 cells	IFU assay on HeLa cells	IFUs are detected 24h p.i.	[52]
Azenabor et al. 2011	*C. trachomatis* ?*	Human	THP-1 cells	IFU assay on Hep-2 cells	Few detectable IFUs 72h	[99]
Yasir et al. 2011	*C. trachomatis* L2 and *C. muridarum*	Mouse	RAW 264.7 cells	IFU assay on HeLa cells	Four times as many IFUs are recovered from muridarum compared to L2 24h p.i	[68]

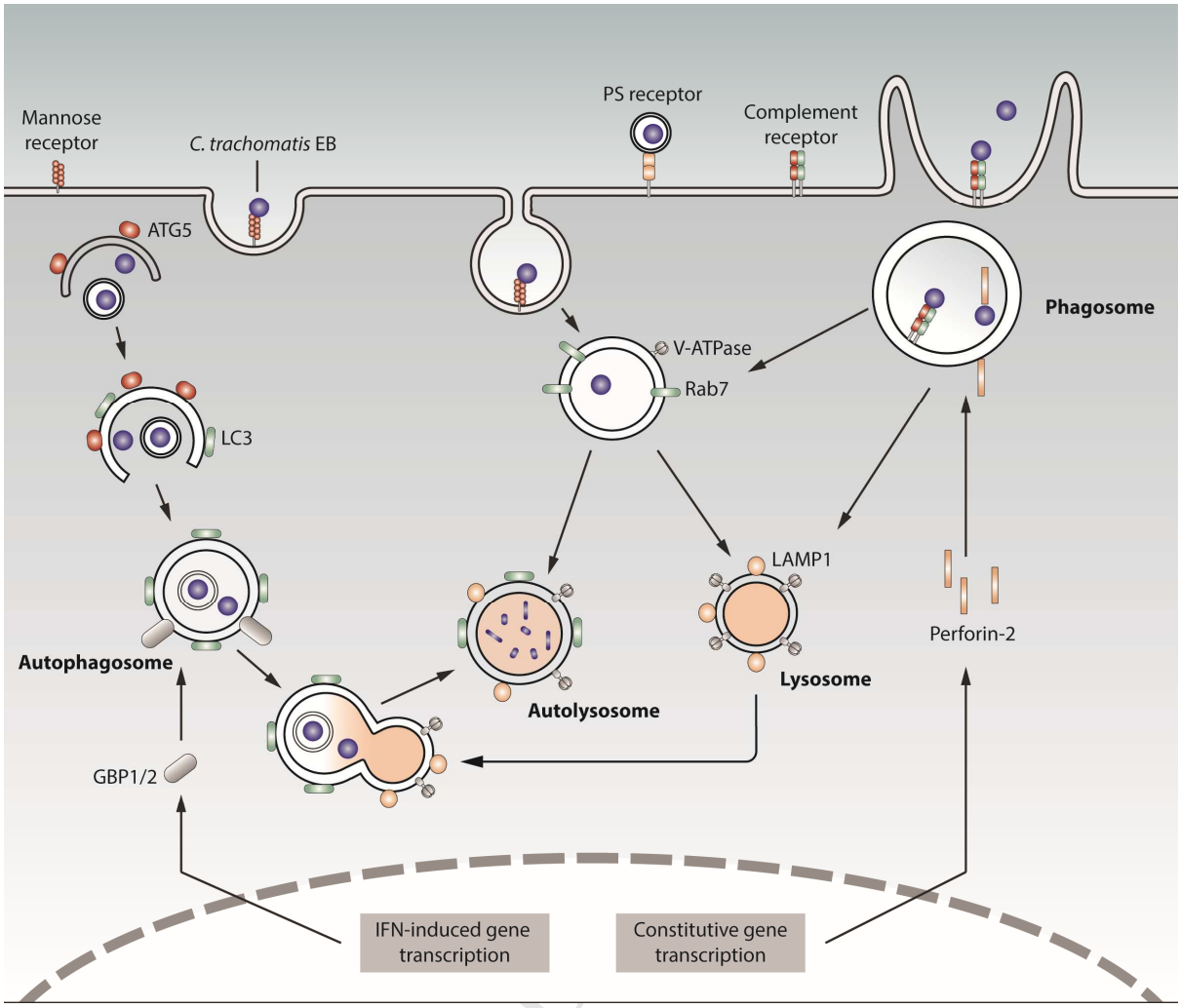
Paradkar et al. 2008	*C. trachomatis* ?*	Mouse	BMDM	Direct inclusion visualization	10% cells contain large inclusions 24h p.i.	[82]
Schnitger et al. 2006	*C. trachomatis* K	Human	Peripheral blood monocytes	qPCR on ompA, euo and groEL1	Expression of all genes are observed after 7 days in monocytes	[103]
Gerard et al. 2002	*C. trachomatis* K	Human	Peripheral blood monocytes	qPCR on chlamydial rRNA and metabolic enzymes	Most mRNAs are detected after 2 days and rRNA after 5 days p.i.	[104]
Gerard et al. 1998	*C. trachomatis* K	Human	Peripheral blood monocytes	qPCR on chlamydial mRNA	Transcripts of glyQs, gseA, hsp60 and omp2 are observed 10 days p.i.	[105]
Nettelnbreker et al.1998	*C. trachomatis* K	Human	U937 cells	Direct inclusion visualization and IFU assay on Hep-2 cells	Chlamydial replication is observed from day 1 to 10 p.i.	[106]
Chen et al. 1996	*C. trachomatis* D	Mouse	RAW 264.7 cells	Direct inclusion visualization and IFU assay on McCoy cells	IFU recovery increases from 24h to 48h and IFUs are recovered 6 days p.i.	[76]
Koehler et al. 1996	*C. trachomatis* K	Human	Peripheral blood monocytes	TEM[6] visualization of inclusions	Atypical inclusions are observed for up to 10 days p.i.	[63]
Numazaki et al. 1995	*C. trachomatis* L2	Human	U937 cells	PCR on chlamydial DNA	DNA is detected 90 days p.i.	[62]
Schmitz et al. 1993	*C. trachomatis* K	Human	Peripheral blood monocytes	IFU assay on Hep-2 cells	3-5% of original inoculum is detected after 2h and few inclusions after 24h. MOMP is detected up until 14 days p.i.	[61]
Zhong et al. 1988	*C. trachomatis* L1	Mouse	Peritoneal macrophages	In vivo infection followed by IFU assay on HeLa cells	IFUs are recovered 60h p.i.	[107]
Bard et al. 1987	*C. trachomatis* L2	Human	HL-60 cells	Direct inclusion visualization and IFU assay on McCoy cells	IFUs are recovered 72h p.i	[108]
Yong et al. 1987	*C. trachomatis* B, C, I, L1 and L2	Human	Peripheral blood monocytes	Direct inclusion visualization and IFU assay on HeLa cells	LGV biovars survive and replicate for 48h, but only in cells incubated for 8-9 days. Trachoma biovars do not survive.	[109]
Bard et al. 1986	*C. trachomatis* L2	Human	Peripheral blood monocytes	Direct inclusion visualization and IFU assay on McCoy cells	<0,5% IFU yield compared to initial inoculum 32h p.i.	[110]
Manor et al. 1986	*C. trachomatis* L2	Human	Peripheral blood monocytes and monocyte-derived macrophages	TEM visualization of inclusions + IFU assay on MA-104 cells	Recovery of IFUs from monocytes at all tested time points up until 120h p.i. IFUs from macrophages decrease at 24h and increase at 72h.	[111]

					Macrophages contain typical inclusions.	
[Kuo et al. 1978	*C. trachomatis* B and L2	Mouse	Peritoneal macrophages	IFU assay on HeLa cells	Both serovars form inclusions at 48h. 4% of serovar B is recovered after 48h while 50% of serovar L2 is recovered. B is detected for 4 days and L2 for 9 days p.i..	[112]

[1]IFU: Inclusion forming units; [2]p.i.: post inoculation; [3]BMDM: Bone marrow-derived macrophages; [4]iPSdM: Induced pluripotent stem cell-derived macrophages; [5]MOI: Multiplicity of infection, [6]TEM: Transmission electron microscopy.
*Serovar not specified






Mannose receptor
C. trachomatis EB
PS receptor
Complement receptor
ATG5
Phagosome
V-ATPase
Rab7
LC3
LAMP1
Perforin-2
Autophagosome
Autolysosome
Lysosome
GBP1/2
IFN-induced gene transcription
Constitutive gene transcription