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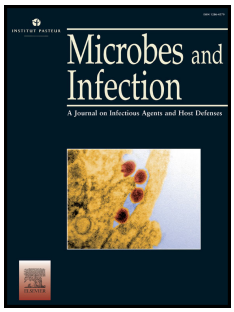

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Complement C3 opsonization of Chlamydia trachomatis facilitates uptake in human monocytes

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Abstract

Chlamydia trachomatis is an obligate intracellular bacterium that causes severe infections, which can lead to infertility and ectopic pregnancy. Although both innate and adaptive immune responses are elicited during chlamydial infection the bacterium succeeds to evade host defense mechanisms establishing chronic infections. Thus, studying the host-pathogen interaction during chlamydial infection is of importance to understand how *C. trachomatis* can cause chronic infections. Both the complement system and monocytes play essential roles in anti-bacterial defense, and, therefore, we investigated the interaction between the complement system and the human pathogens *C. trachomatis* D and L2.

Complement competent serum facilitated rapid uptake of both chlamydial serovars into monocytes. Using immunoelectron microscopy, we showed that products of complement C3 were loosely deposited on the bacterial surface in complement competent serum and further characterization demonstrated that the deposited C3 product was the opsonin iC3b. Using C3-depleted serum we confirmed that complement C3 facilitates rapid uptake of chlamydiae into monocytes in complement competent serum. Complement facilitated uptake did not influence intracellular survival of *C. trachomatis* or *C. trachomatis*-induced cytokine secretion. Hence, *C. trachomatis* D and L2 activate the complement system leading to chlamydial opsonization by iC3b and subsequent phagocytosis, activation and bacterial elimination by human monocytes.

Keywords

monocytes; Chlamydia trachomatis; complement C3

1. Introduction

Chlamydia trachomatis is estimated to infect 100 million people annually causing chronic genital and ocular infections [1]. The course of genital infection is mostly asymptomatic leaving the infection undiagnosed and untreated. Untreated genital chlamydial infection can cause severe tissue damage and lead to pelvic inflammatory disease, ectopic pregnancy, and infertility [2].

C. trachomatis is an obligate intracellular Gram-negative bacterium with a unique biphasic developmental cycle. The infectious, but metabolic inactive elementary body (EB) infects epithelial cells in the genital mucosa. Intracellularly, the EB transforms to a larger non-infectious but metabolic active reticulate body (RB) [3]. During entry, *C. trachomatis* inhibits phagosome-lysosome fusion and resides in a modified vacuole called an inclusion, which provides a niche for bacterial replication [4].

Chlamydial infections tend to be chronic even though both humoral and cell-mediated immunity are elicited [5]. Monocytes and macrophages play essential roles in anti-bacterial immunity in general, but little is known about the exact role of monocytes during *C. trachomatis* infections. During infection, epithelial cells respond by secreting several cytokines and chemokines creating a local inflammatory condition that recruits monocytes to the site of infection [6]. In vitro studies show that several *C. trachomatis* serovars infect human monocytes inducing cellular activation with secretion of inflammatory cytokines, such as IL-1β, IL-6, and IL-8 [7]. Chlamydial uptake into host cells is supposedly carried out by phagocytosis or by receptor-mediated endocytosis, but the exact mechanisms and the receptors involved remain elusive [8]. An involvement of plasma membrane lipid rafts and the mannose receptor have been suggested, but also complement receptors could be involved since these receptors facilitate uptake of other intracellular bacteria such as *Mycobacterium tuberculosis* and *Legionella pneumophila* [9–12].

The complement system consists of more than 30 different proteins comprising both soluble factors and cell surface receptors [13]. Complement activation initiates a cascade of proteolytic cleavages leading to both direct and indirect anti-microbial effects. The direct anti-microbicidal functions are carried out by the membrane attack complex (MAC), a pore-formed structure consisting of repetitive membrane-spanning complement factors that causes membrane permeability and cellular lysis. Another function of the complement system is mediated by the so-called opsonins, which bind to the surface of pathogens tagging them for uptake and degradation in professional phagocytes.

The complement system is mainly activated through three distinct pathways which are triggered by different structural motifs and involve different intermediate complement products, but they all converge at the common downstream effector C3 convertase. C3 convertase cleaves complement factor C3 into the anaphylatoxin C3a and the opsonin C3b. C3b may be further cleaved into additional opsonins called iC3b and C3dg. C3b, iC3b, and C3dg are all recognized by surface receptors expressed on different host immune cells and opsonin-receptor engagement leads to receptor-mediated phagocytosis of the opsonized organism. It has been shown that C3b and iC3b are recognized by complement receptor (CR) 1 and CR3, respectively, and both receptors are ubiquitously expressed on monocytes and macrophages and are important for mononuclear phagocytosis of infectious bacteria [14].

C. trachomatis is able to activate the complement system and it has been demonstrated that *C. trachomatis* induced complement activation leads to binding of C3 to the bacterium [15].

To further explore the interaction between the complement system and *C. trachomatis*, we investigated how complement deposition on *C. trachomatis* affects the uptake of chlamydial EBs into human monocytes and how complement modulates the intracellular fate of *C. trachomatis* in monocytes. Uncovering new aspects of the interaction between complement,

monocytes, and *C. trachomatis* are important to understand how chlamydial infections are controlled by the innate immune system.

2. Materials and methods

2.1. Antibodies

The following primary antibodies were used in this study: Anti-human CD11b (MEM-174) (ImmunoTools GmbH, Friesoythe, Germany), Polyclonal Rabbit Anti-Human C3c Complement (Agilent Technologies, Glostrup, Denmark), Mab32.3 against *C. trachomatis* MOMP [16], and PAb17 against *C. trachomatis* outer membrane [17]. FITC-, Alexa Flour® 488-, and rhodamine-conjugated secondary antibodies were purchased from Jackson ImmunoResarch (Jackson ImmunoResearch, PA, USA). Anti-Rabbit IgG Alkaline Phosphatase was purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). Goat anti-rabbit antibody conjugated with 10 nm colloidal gold (British BioCell, Cardiff, UK) was used for immunoelectron microscopy.

2.2. Bacteria strains and culture

C. trachomatis D/UW-3/cx and L2/434/Bu were obtained from the American Type Culture Collection (ATCC, VA, USA) and propagated in McCoy cells according to Ripa and Mårdh [18]. *Chlamydia* were tested free of mycoplasma by PCR according to Huniche et al. [19]. McCoy cells were obtained from ATCC and tested free of mycoplasma by Hoechst 33342 staining and PCR according to[19].

C. trachomatis D and L2 EB were purified by density gradient centrifugation essentially according to Caldwell et al. 1981 [20] and purity was estimated using negative staining and transmission electron microscopy (TEM), (see 2.7)

2.3. Cell isolation and culture

Blood samples were obtained from *C. trachomatis* seronegative donors at Aalborg University (Approved by The Ethics Committee of Region Nordjylland, case no. N-20150073). Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by density gradient centrifugation on LymphoPrep™ (STEMCELL Technologies™, Vancouver, Canada) according to Carlsen et al. [21]. The cells were seeded in 8 well Lab-Tek® Chamber Slide™ Permanox slides (Thermo Scientific, MA, USA) at a density of $5x10^5$ cells/well and cultured in standard medium containing RPMI 1640 supplemented with 10% heat inactivated fetal calf serum (FCS), and 0.01 mg/ml gentamicin. Cells were allowed to adhere for 90 minutes at 37 °C and 5% CO_2 and non-adherent cells were subsequently removed by washing the cells twice in PBS.

2.4. Monocyte infection

C. trachomatis was suspended in RPMI 1640 (Biowest, Nuaillé, France) containing either 10% human autologous serum (NHS) or 10% heat-inactivated human autologous serum (HIHS) and added immediately to adherent monocytes. Serum was heat-inactivated by incubating serum for 30 minutes at 56 °C. Infection was carried out for 1, 4 or 24. For 24 hours infection, extracellular bacteria were removed after 4 hours and infection medium was replaced by standard medium for the remaining incubation period. In some experiments, medium was supplemented with 10% C3-depleted human serum alone or added 5 µg purified C3 (Sigma Aldrich) to a final concentration of 20 µg/ml.

For some experiments lipopolysaccharide (LPS) from *Escherichia coli* (026:B6, Sigma-Aldrich) was used as positive controls at a concentration of 1 µg/ml.

2.5. Immunofluorescence microscopy

Immunofluorescence staining was carried out essentially according to Carlsen et al. [21]. Extracellular bacteria and surface bound CD11b were stained prior to fixation by incubating cells for 30 minutes at 37 °C with Pab17 (1:200) or anti-CD11b (5 µg/ml) diluted in PBS containing 0.1% bovine serum albumin (BSA) and 0.05% sodium azide. Cells were washed twice in PBS and fixed for 20 minutes in 3.7% formaldehyde at 4 °C. Cells were permeabilized for 7 minutes in 0.2% Triton-X 100 at room temperature and blocked in 0.1% BSA for 15 minutes at 37 °C. Primary antibodies were diluted in antibody buffer containing 0.1% BSA in PBS (Mab32.3: 5 µg/ml) and cells were incubated with primary antibody for 30 minutes at 37 °C. Cells were washed three times in antibody buffer and incubated with secondary antibodies diluted 1:200 in antibody buffer. Cells were washed three times in antibody buffer and counter-stained with either 2 µM To-Pro-3 Iodide or 2 µM DAPI for 10 minutes at room temperature. Finally, mounting medium was added to each well and slides were mounted with cover slips.

Cells were visualized and imaged using a Leica SP5 confocal microscope or a Leica DM 5500 B fluorescence microscope.

2.6. Immunoelectron microscopy

Purified EBs were mixed with 1/10 volume of NHS or HIHS. Five microL purified EB was added to the surface of carbon-coated glow discharged 400 mesh nickel grids as described (20). The grids were washed on three drops of PBS (pH 6.5) and blocked on one drop of 1% ovalbumin (Sigma-Aldrich) in PBS. The grids were then incubated for 30 min at 37 °C with 1/200 rabbit anti-C3c antibody (Agilent Technologies) diluted in ovalbumin. The grids were then washed on three drops PBS and incubated for 30 min at 37 °C in goat anti-rabbit antibodies conjugated with 10 nm colloidal gold (1:25) in ovalbumin. Following this, the grids were washed on three drops of PBS, incubated on three drops 0.5% cold fish gelatin

(Sigma-Aldrich) in PBS (10 min each), washed on three drops of PBS, one drop of H_2O and stained with one drop of 0.5% phosphotungstic acid and blotted dry on filter paper. Electron microscopy was done at 60 keV on a JEOL 1010 transmission electron microscope (Jeol, Tokyo, Japan). Images were obtained using a KeenView digital camera (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

2.7. SDS-PAGE and immunoblotting

Purified EBs from *C. trachomatis* D and L2 were incubated with an equal volume of either NHS or HIHS for 30 minutes at 37 °C. EBs were washed twice in PBS with centrifugation at 20000 x g for 15 minutes between each wash. Samples were boiled in RunBlue LDS Sample Buffer (Expedeon, CA, USA) containing 5% v/v β-mercaptoethanol and proteins were separated on a 7,5% SDS polyacrylamide gel according to Laemmli (Laemmli 1970). Proteins were blotted on a nitrocellulose membrane according to Drasbek et al. (Drasbek 2004). The membrane was blocked in Tris buffered saline (TBS) with 3% gelatin. Polyclonal Rabbit Anti-Human C3c Complement (Agilent Technologies) (1:1000) was used as primary antibody and Anti-Rabbit IgG Alkaline Phosphatase (Sigma-Aldrich) (1:20,000) was used as secondary antibody. Protein bands were developed by adding BCIP/NBT alkaline phosphatase substrate (Kem-En-Tec Diagnostics, Taastrup, Denmark).

2.8. Reinfection assay

Monocytes were cultured and infected according to section 2.1 except PBMCs were seeded in 24-well plates at a density of $2x10^6$ cells/well. After 4 and 24 hours, adherent monocytes were washed thoroughly three times in PBS and detached by scrabing of cells in 2SP buffer (0.2 M sucrose, 0.02 M phosphate, pH = 7.2). Monocytes were lysed by ultrasonication and lysates from two wells were pooled and diluted 1:2 in standard medium (see 2.3) and added to

196 confluent McCoy cells. McCoy cells were incubated for 1 hour at 37 °C and 5% CO_2 and
197 subsequently washed three times in PBS and cultured for additional 23 hours in standard
198 medium containing 2 µg/ml cyclohexamide.
199 Cells were processed for immunofluorescence staining as described in 2.5.
200
201 *2.9. Enzyme-linked immunosorbent assay (ELISA)*
202 IL-6 and IL-8 ELISA kits were purchased from ImmunoTools GmbH and the analyses were
203 performed according to manufacture's protocol with minor changes. Briefly, MaxiSorp plates
204 (NUNC) were coated with capture antibody diluted in PBS over night at 4 °C. Excess binding
205 was blocked with 1% BSA in PBS for one hour at room temperature. Monocyte culture
206 supernatants were diluted in 0.1% BSA + 0.05% Tween-20 in PBS and added to the wells and
207 left for incubation for one hour at room temperature. Captured IL-6 and IL-8 were detected
208 using a biotinylated detector antibody and subsequently streptavidin conjugated to horseradish
209 peroxidase (HRP). The enzymatic reaction was initiated by adding the HRP substrate TMB-
210 ONE (Kem-En-Tec Diagnostics) and stopped after 30 minutes by adding 1M HCl.
211
212 *2.10. Statistics*
213 Statistical differences between two independent groups were calculated using Student's *t*-test.
214 Multiple comparisons were analyzed by One-way ANOVA with Tukey's multiple
215 comparison test. All statistical analyses were performed in GraphPad Prism 7 (GraphPad
216 Software Inc., CA, USA). P-values < 0.05 were considered statistically significant.
217

218 ## 3. Results

219 *3.1. Investigating the role of complement components in C. trachomatis uptake*

We aimed to investigate whether complement components affect the uptake of *C. trachomatis* into monocytes. First, dilutions of *C. trachomatis* D and L2 were titrated to obtain an average *Chlamydia*-to-monocyte ratio of 1. Intracellular chlamydiae were visualized using a monoclonal antibody against chlamydial MOMP (Fig. 1A). The intracellular localization in monocytes were confirmed both by membrane staining against CD11b and by differential staining of intracellular and extracellular bacteria. Monocytes were infected in media containing either normal autologous serum (NHS) or heat-inactivated autologous serum (HIHS). Heat-inactivation of serum was done to denature complement factors, abrogating a functional complement system. Cells were fixed after 1 hour of infection and chlamydial uptake was quantified by counting the percentage of infected cells.

Fig. 1B shows that the percentage of infected cells was statistically significantly higher for both serovars after 1 hour of infection in NHS samples compared to HIHS samples. Fig. 1B also shows that there was no difference between uptake efficiency between serovars. These findings suggest that *C. trachomatis* D and L2 are taken up by monocytes with the same efficiency and that complement-competent serum facilitates rapid uptake of *C. trachomatis* D and L2 into human monocytes.

3.2. Complement deposition on C. trachomatis D and L2

Our observations suggest that complement opsonization of *C. trachomatis* D and L2 facilitates uptake into monocytes. Monocytes express different receptors recognizing the C3 opsonins, C3b and iC3b, and it was previously demonstrated that these complement proteins bind to *C. trachomatis* L2 [15]. We therefore used a polyclonal antibody against C3c to visualize possible opsonizing complement by immuno-gold electron microscopy, since C3c is a common component found in both C3b and iC3b. Purified *C. trachomatis* D and L2 EBs were incubated with NHS and subsequently stained against C3c and with gold-conjugated

IgG as secondary antibody [22]. Fig. 2A+E show that C3 complement fragments were deposited in patchy areas on the surface of both serovars when incubated in NHS. In contrast, when chlamydial EBs were incubated with HIHS, only few gold particles were observed on the EB surface (Fig. 2B, F). No gold was observed on the EB surface when EBs were incubated with NHS and anti-C3c was omitted (Fig. 2C, G). To quantify complement deposition, bacteria associated gold particles and gold particles associated with the background were enumerated and these numbers were expressed as a ratio. Fig. 2D+H show that more gold particles are deposited on the bacterial surface when incubated with NHS compared to HIHS. Thus, complement factors containing the C3c domain bind to the surface of *C. trachomatis* D and L2 in the presence of NHS, but not HIHS, and this may account for the observed differences in uptake efficiency.

3.3. Investigation of Chlamydia-bound C3

By immune-gold electron microscopy we confirmed that complement C3 fragments bind to the surface of *C. trachomatis* D and L2 EBs. However, since C3c is a common structure found in different C3 fragments we could not elucidate exactly which fragments were bound to the EBs or if activation of the cleavage cascade had occurred.

We therefore conducted an immunoblot analysis of purified chlamydial EBs incubated in either NHS or HIHS. Western blotting was performed three times using different sera with similar results and a representative blot is shown in Fig. 3A. Fig. 3A shows that uncleaved α and β chains of C3 (119 and 74 kDa, respectively) were present on EB after incubation with either NHS or HIHS, though much stronger when incubated with NHS. Several other C3 protein bands were bound to both *C. trachomatis* D and L2 when incubated in NHS. The protein band observed around 45 kDa corresponds to the α'2 fragment of C3 which is only found in complement iC3b (Fig. 3B). These findings showed that the complement cascade is

only activated in NHS leading to production and binding of iC3b to both *C. trachomatis* D and L2. In addition to the protein bands just described, two protein bands were present in the high molecular area (165 and 250 kDa, respectively) in the lanes in which EB were incubated with NHS (Fig. 3A). These bands represent fragments of either the α' chain of C3b or the α´1 chain of iC3b covalently linked to unidentified proteins. Both chains contain an exposed thioester site that allows covalent interactions between C3b/iC3b and target proteins (Fig. 3B).

Thus, iC3b binds to the surface of both *C. trachomatis* D and L2 and may be involved in covalent interactions with chlamydial surface proteins.

3.4. Complement C3 facilitated uptake of C. trachomatis into monocytes

The above results suggest that C3 opsonization of *C. trachomatis* could explain the differential uptake efficiency observed using complement-competent serum and heat-inactivated serum, respectively. To elucidate whether C3 in fact facilitates uptake into monocytes, we investigated the monocyte uptake of *C. trachomatis* L2 in the presence C3-depleted human serum after one hour of incubation Fig. 4. shows that using C3-depleted serum reduces the uptake of chlamydia into monocytes compared to bacteria incubated in the presence of NHS. Adding purified human C3 to the C3-depleted serum restored the monocyte uptake efficiency, demonstrating that complement C3 facilitates uptake of C. trachomatis into monocytes.

3.4. Intracellular fate of C. trachomatis after complement-mediated monocyte ingestion

As early uptake of *C. trachomatis* in monocytes is facilitated by complement C3 opsonization, we analyzed the fate of *C. trachomatis* when ingested by monocytes to elucidate the biological significance of the rapid uptake.

We have previously observed that both serovar D and L2 detection diminishes over time in monocytes (data not shown), suggesting that both serovars are eradicated in monocytes. One previous study demonstrated that *C. trachomatis* D and L2 can survive intracellularly in monocytes for up three days post infection [23]. These results conflict with our initial observations, and we speculated whether the observed differences may be due to the presence/absence of functional complement. To test this, we evaluated the viability and growth potential of complement-opsonized and non-opsonized *C. trachomatis* L2 using a reinfection assay. Monocytes containing *C. trachomatis* L2 were lysed by ultrasonication and the lysates were applied to confluent McCoy cells. The viability of ingested bacteria was evaluated by quantifying the percentage of McCoy cells containing mature inclusions (Fig. 5A, right image). As demonstrated in Fig 5A (table), only few McCoy cells contained mature inclusions when *C. trachomatis* was incubated with monocytes for 4 hours. No differences in chlamydial viability were observed between NHS and HIHS, suggesting that complement-mediated uptake of *C. trachomatis* does not affect intracellular degradation of *C. trachomatis* in monocytes. When *C. trachomatis* was incubated within monocytes for 24 hours no mature inclusions were observed in either condition demonstrating that *C. trachomatis* is efficiently killed in monocytes independently of complement.

3.6. Complement modulation of C. trachomatis induced cytokine production

We showed that complement C3 potentiates the chlamydial uptake, and that uptake leads to efficient intracellular killing of the bacteria, which is one of the primary roles of monocytes during infection. Another key role of monocytes during infection is to produce and secrete inflammatory cytokines potentiating anti-microbial immune mechanisms. Thus, to further extend our understanding of the functional consequences of bacterial opsonization we

explored if complement affects secretion of IL-6 and IL-8 in monocytes incubated with *C. trachomatis*.

Monocytes were cultured for 4 hours with *C. trachomatis* L2 in either NHS or HIHS, LPS or media alone. After 4 hours of incubation cells were washed and new medium was added and cells were incubated for further 20 hours. The conditioned monocyte medium was harvested and the concentration of IL-6 and IL-8 was determined by ELISA.

As shown in Fig. 5B. *C. trachomatis* induces the secretion of both IL-6 and IL-8 as reported previously [7,24]. Neither IL-6 or IL-8 secretion were significantly affected by the presence of functional complement since monocytes incubated in NHS and HIHS demonstrates similar concentrations of the cytokine. However, for both cytokines a small non-significant difference was observed between NHS and HIHS with a higher concentration in monocytes incubated with HIHS. Thus, complement opsonization does not affect *C. trachomatis* induced secretion of IL-6 and IL-8 in monocytes.

4. Discussion

We demonstrated that purified EBs of the two serovars, D and L2, of *C. trachomatis* activated the complement system leading to deposition of C3 fragments on the chlamydial surface and that complement C3 facilitates rapid chlamydial uptake into human primary monocytes leading to bacterial elimination and cytokine production.

Complement activation and complement-mediated phagocytosis of bacterial agents by monocytes and macrophages have been demonstrated for different intracellular bacteria such as *M. tuberculosis* and *Listeria monocytogenes* [25,26]. Other studies have demonstrated the ability of *C. trachomatis* to activate the proteolytic complement cascade leading to activation of both the C3- and C5-convertase [15,27].

To our knowledge, we are the first to demonstrate direct involvement of complement C3 in monocyte ingestion of *C. trachomatis* and that complement C3 is deposited on the surface of *C. trachomatis* D EBs. Additionally, using immunoelectron microscopy, we directly visualized complement deposition on serovar L2 previously reported by Hall et al. [15]. We found that complement was activated generating iC3b which was bound to the surface of both serovar D and L2. iC3b is a potent opsonin that has been involved in opsonization and phagocytosis of other intracellular bacteria such as *M. tuberculosis* [25].

iC3b is recognized by complement receptor 3 (CR3), a heterodimeric integrin consisting of CD11b and CD18 that is ubiquitously expressed on the surface of monocytes [28]. CR3 participates in phagocytosis of other intracellular bacteria, such as *Mycobacteria spp*. and Peyron et al. [29] showed that CR3 is involved in lipid raft-dependent internalization of *M. kanasii*. In line with these findings, it has been demonstrated that the integrity of lipid rafts is important for host cell entry of several *C. trachomatis* serovars [9,30]. Additionally, binding and internalization of *Borellia burgdoferi* was shown to be dependent on complement C3 and CD14-dependent recruitment of CR3 to lipid rafts, suggesting that CD14 may also be involved in the enhanced uptake, since CD14 is widely expressed on monocytes [31,32]. Thus, it seems likely that the enhanced uptake of *C. trachomatis* observed in NHS is due to iC3b-mediated phagocytosis by CR3 engagement. However, iC3b is not exclusively recognized by CR3. CR1 and CR4 can also bind iC3b leading to iC3b-mediated phagocytosis [33].

An important parameter to discuss in this context is the involvement of the complement anaphylatoxins C3a and C5a, which are generated by proteolytic cleavage of C3 and C5 during complement activation. These inflammatory mediators were not investigated in the current study, but we demonstrate C3 cleavage and, therefore, we know that C3a is generated. Although some degree of C3b inactivation was observed, it is likely that the complement

cascade proceeds to C5 cleavage. This was previously shown by Megran and colleagues who demonstrated that *C. trachomatis* L2 induced cleavage of C5 to C5a [27]. Both anaphylatoxins are recognized by G-protein coupled receptors expressed on monocytes. These mediators could likely contribute to the increased monocytic phagocytosis, since C5aR antagonists were shown to reduce phagocytosis of heat-killed *Staphylococcus aureus* in monocytes [34]. Supporting this observation, it was demonstrated that both C3a and C5a upregulates CD11b surface expression in neutrophils and monocytes [35]. The contribution of the anaphylatoxins was not addressed in our study, but literature suggests that anaphylatoxins likely contribute to the observed effects presented in this study [27,34,35]

A unique feature of C3 opsonins is their ability to covalently attach to target structures through a thioester site located in the α' chain of C3b and in the α'1 chain of iC3b (Fig. 3B). Our data suggest that iC3b is covalently attached to protein structures on both serovars since several high molecular protein bands are observed under both denaturing and reducing conditions. Under reducing conditions iC3b will split into three protein fragments: α'1 (63 kDa), α'2 (39 kDa), and β (75 kDa) [36]. We observe the latter two, but not the α'1 fragment. The α'1 fragment is likely located in the observed high molecular bands covalently attached to other proteins. It was previously proposed that C3 fragments interact with MOMP on the chlamydial surface, but it was not conclusively determined due to antibody cross-reactivity [15]. We observed anti-C3c reactive protein bands migrating approximately at 165 and 250 kDa, which does not correspond to the summed molecular mass of MOMP and the α'1 fragment (40 kDa + 110 kDa, respectively). The protein bands observed around 250 kDa suggest that iC3b interacts with high molecular weight surface structures. Potential high molecular candidates to interact with C3 are the polymorphic membrane proteins, which ranges in size from 95 kDa to 187 kDa, however this was not further investigated.

We used immunoelectron microscopy to directly visualize protein deposition on the EB surface. Interestingly, we observed that the C3 fragments were loosely bound to the chlamydial surface and this observation does not fit with the idea that C3 is covalently linked to chlamydial outer membrane proteins, however, several chlamydial-complement bindings may be involved. It has been demonstrated that LPS is loosely bound in the chlamydial outer membrane and we observed a very similar gold-labelling pattern that could suggest that C3 also interacts with non-protein structures like LPS, which has been demonstrated for other bacteria previously [22,37].

The loosely attachment of complement to the bacterial surface may be advantageous to the bacterium allowing some degree of complement shedding which can reduce the rapid recognition and ingestion by phagocytes. Thus, complement binding to *C. trachomatis* EBs may involve interactions with both protein structures and LPS on the bacterial surface.

We showed that uptake of *C. trachomatis* was accompanied by rapid inactivation and elimination of the bacteria inside monocytes. In our experiments, no viable chlamydiae were recovered after 24 hours inside monocytes even though chlamydia could still be detected by immunofluorescence staining against MOMP at this time (data not shown). This demonstrates the limitation of antibodies as a detection tool when questions regarding bacterial viability is addressed. Under these circumstances, it is important to include functional assays or include analyses of bacterial metabolites that can highlight important differences in bacterial viability. There is some ambiguity related to the fate of *C. trachomatis* in monocytes and macrophages. In murine macrophages *C. trachomatis* L2 is rapidly directed to destructive intracellular compartments including both lysosomes and autophagosomes [38]. However, a study using primary human monocytes showed that *C. trachomatis* can remain viable and infectious after 48 hours in monocytes [23]. In addition, the authors found no reduction in the number of

infected cells over a 72-hour period which conflicts with our observations. This discrepancy may be explained by the infection method, since Datta et al. [23] used centrifugation for monocyte infection. It was previously demonstrated that centrifugation of *C. psittaci* on McCoy cells reduced the bacterial association with cell lysosomes compared to static infection [39]. Thus, using centrifugation instead of static infection the normal endo-lysosomal pathway may be omitted leading to increased chlamydial survival and growth. This remains to be demonstrated in monocytes, but it is well-known that the mechanisms and receptors involved in the uptake process influence the subsequent intracellular fate of the ingested organism in monocytes and macrophages. Beside altered intracellular trafficking induced by complement receptor signaling also complement anaphylatoxins may affect the intracellular fate of *C. trachomatis* in monocytes. Anaphylatoxins can modulate the production of reactive oxygen species (ROS) in monocytes, which have been proposed to be important for intracellular degradation of *C. trachomatis* [35,40]. Mollnes et al. showed that an antibody directed against C5a was able to inhibit *E.coli*-induced ROS production in both monocytes and neutrophils [35]. Therefore, it is important to consider possible effects of anaphylatoxins when looking at intracellular survival of *C. trachomatis* in monocyte cultures supplemented with fresh serum. These observations, together with our results, emphasizes the need to carefully revise the methods used for cell-chlamydia culture/infection used in many *in vitro* studies on host-chlamydial interactions, since method parameters such as centrifugation and culture supplements have important implications for the observed biological effects.

In this study, we did not observe any statistically significant effect of complement on chlamydia-induced cytokine secretion. Several studies, however, suggest that monocyte cytokine secretion can be triggered and/or potentiated by the presence of complement. Both C3a and C5a was demonstrated to induce IL-8 secretion in human neutrophils and that

specific antibodies against these anaphylatoxins reduced PAMP-induced cytokine secretion (Vecchiarelli 1998). Similar effects were later observed in monocytes when Cheng et al. showed that complement C5a potentiates *Candida albicans*-induced cytokine production in human PBMCs [41]. Asgari and colleagues [42] further demonstrated that C5a directly induces IL-6 secretion and that C3a receptor ligation potentiates LPS-induced IL-1β production in human primary monocytes. Thus, both anaphylatoxins may influence the cytokine profiles observed in this study.

Both macrophages and complement are present in the genital mucosal lining, and during infection-induced inflammation additional circulating monocytes are recruited [6,43,44]. Thus, complement activation and complement-directed phagocytosis by monocytes may provide an important innate mechanism to restrict chlamydial infection. This is further supported by in vivo studies using knock-out mice infection models. $C3^{-/-}$ mice displayed decreased survival following intranasal infection with different chlamydial species compared to wild-type mice and this reduced survival was not attributed to differences in antibody titers [44,45]. Thus, our findings could provide a mechanistic explanation for the observed differences between $C3^{-/-}$ and wild-type mice, but generally it is difficult to translate complement-mediated effector functions demonstrated in vitro to the complex in vivo environment. This was highlighted by a study by Yang et al. who demonstrated that Chlamydia-induced pathology were C5-dependent, but occurred independently of C3 [46]. This observation conflicts with the normal paradigm of complement activation where C5 functions downstream of C3 activation and cleavage. Thus, several in vivo factors can modulate complement functions, and these were not addressed in our “clean” in vitro system. These factors could include other cell-types expressing complement receptors or soluble factors, such as coagulation factors neither of which were included in our experimental setup.

Thus, during initial infection, before adaptive immunity is developed, complement opsonization with C3 and subsequent monocytic phagocytosis may be a key process for controlling bacterial dissemination until adaptive immunity is developed.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgements

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641 ## Figure legends

642 **Fig. 1.** Immunofluorescence assay of *C. trachomatis* uptake into human monocytes**.** Primary
643 human monocytes were incubated with *C. trachomatis* D and L2 in media supplemented with
644 either normal autologous human serum (NHS) or heat-inactivated autologous human serum
645 (HIHS) for 1 hour. Cells were fixed, immunestained using a chlamydial MOMP antibody and
646 the number of infected cells was quantified. A-D) Confocal microscopy of monocytes fixed
647 after 1 hour post infection and stained against MOMP (green). E) Intracellular location of EBs
648 (green) in monocytes was confirmed by CD11b surface staining (red). F) Extracellular and
649 intracellular bacteria were distinguished by successive staining of extracellular and
650 intracellular bacteria (red and green, respectively). G) Percentage of infected cells at 1 hour
651 post infection. Data were from three independent experiments with duplicate samples in each.
652 All data are presented as means ± SEM. * indicates $P < 0.05$. Scale bars indicate 10 µm.

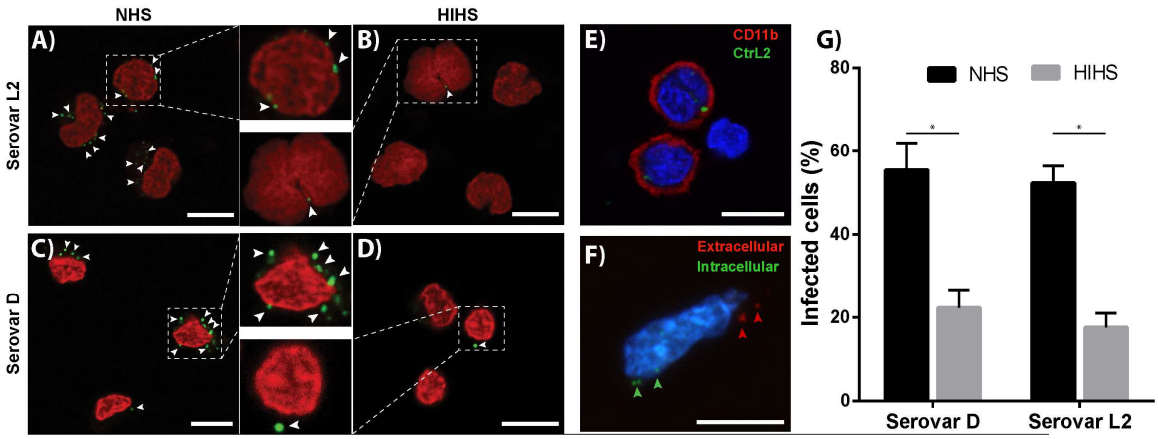
653
654 **Fig. 2.** Transmission electron microscopy of immunostained purified *C. trachomatis* serovar
655 D and L2 EBs. Primary antibody: rabbit anti C3c and secondary antibody: goat anti rabbit IgG
656 conjugated with 10 nm colloidal gold. A) Serovar D EB incubated with NHS subsequently
657 stained for C3c. B) Serovar D EB incubated with HIHS and thereafter stained for C3c. C)
658 Serovar D EB incubated with NHS and thereafter secondary colloidal gold conjugated
659 antibody. D) Serovar L2 EB incubated with NHS and thereafter stained for C3c. E) Serovar
660 L2 EB incubated with HIHS and thereafter stained for C3c. F) Serovar L2 EB incubated with
661 NHS and thereafter secondary colloidal gold conjugated antibody. G+H) Chlamydia-
662 associated gold particles were counted from three chlamydial EBs from two independent
663 experiments (6 cells for each condition). Gold particles per area was estimated for the bacteria
664 and the background, respectively, and a ratio of these numbers was used as quantitative
665 measure of gold particle deposition. The data are represented as means ± SEM. Scale bar
666 indicates 200 nm.

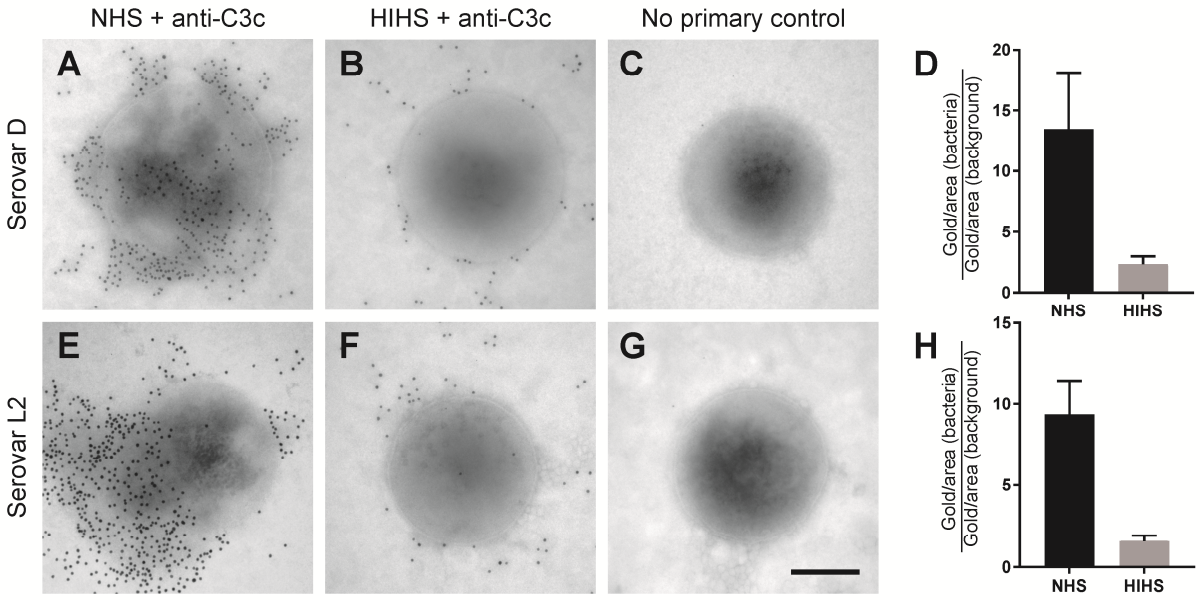
Fig. 3. Immunoblot analysis of complement C3 deposition on *C. trachomatis* EBs. *C. trachomatis* D and L2 were incubated in either NHS and HIHS, washed and proteins were separated under reduced conditions on a 7.5% SDS gel, transferred to a nitrocellulose membrane and stained with anti-C3c. A) Different fragments of complement C3 are deposited on chlamydial EBs when incubated in NHS. The blot shows C3 (119 kDa and 74 kDa), iC3b (74 kDa and 45 kDa) depositions, and in addition bands of higher molecular size (165 and 250 kDa) were seen after incubation with NHS. B) Diagram showing the consecutive cleavage of C3 with theoretical molecular sizes of the cleavage products.

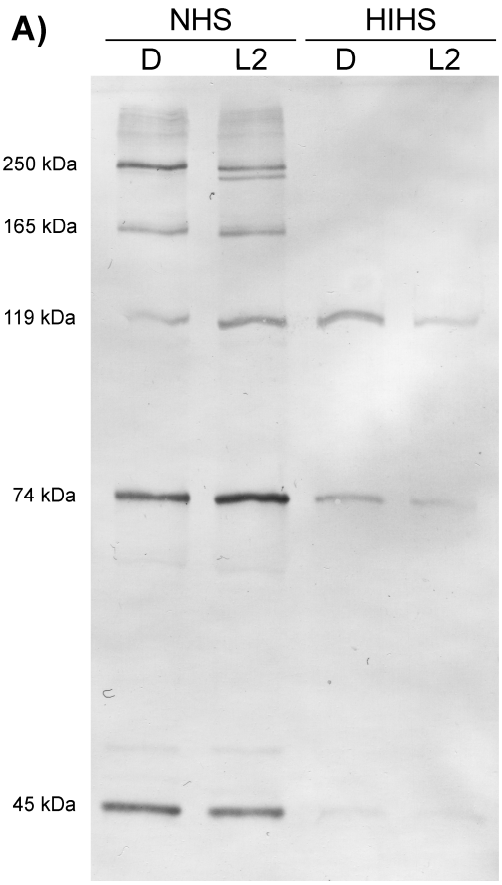
Fig. 4. Effect of complement C3 on chlamydial uptake into monocytes. Monocytes were incubated with *C. trachomatis* L2 for 1 hour in the presence of either NHS, HIHS, C3-depleted serum (ΔC3) or C3-depleted serum + purified human C3 (ΔC3+C3). The cells were fixed after 1 hour, stained against chlamydial MOMP, and the percentage of infected cells were quantified. Statistically significantly more cells were infected in presence of NHS compared to C3-depleted serum. Adding C3 to C3-depleted serum causes a statistical significant increase in percentage of infected cells. Data were from four biologically independent experiments with duplicate samples in each. All data are presented as means ± SEM. * indicates $P < 0.05$. n.s.: non-significant difference.

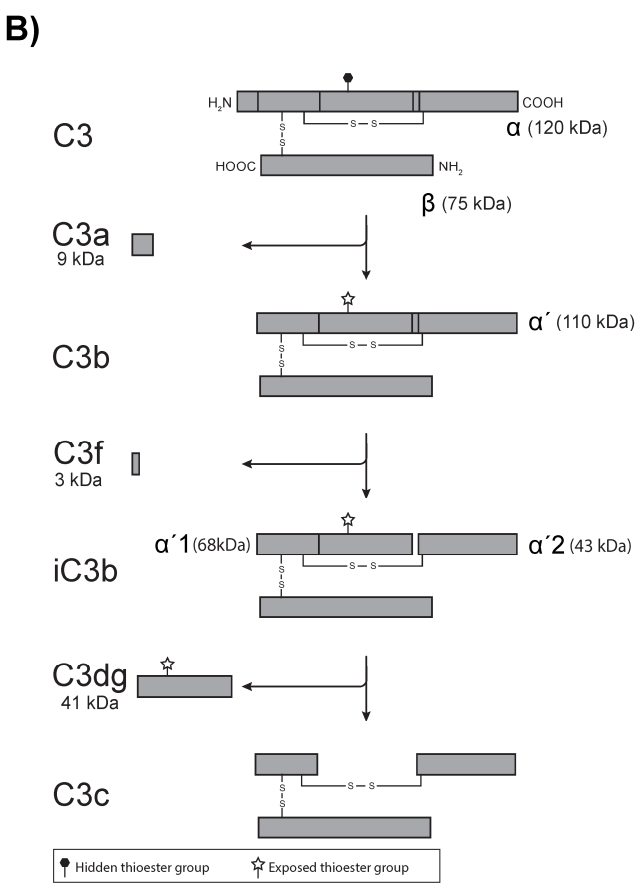
Fig. 5. Functional consequences of complement-mediated uptake of *C. trachomatis* into monocytes. A) Intracellular survival of *C. trachomatis* L2 in monocytes. Monocytes were incubated with *C. trachomatis* L2 for 4 or 24 hours in media containing either NHS or HIHS and subsequently lysed by ultrasonication. Monocyte lysates were added to confluent McCoy cells for one hour and McCoy cells were incubated for additional 23 hours. Chlamydial inclusions were identified by immunofluorescence staining against MOMP. Left image:

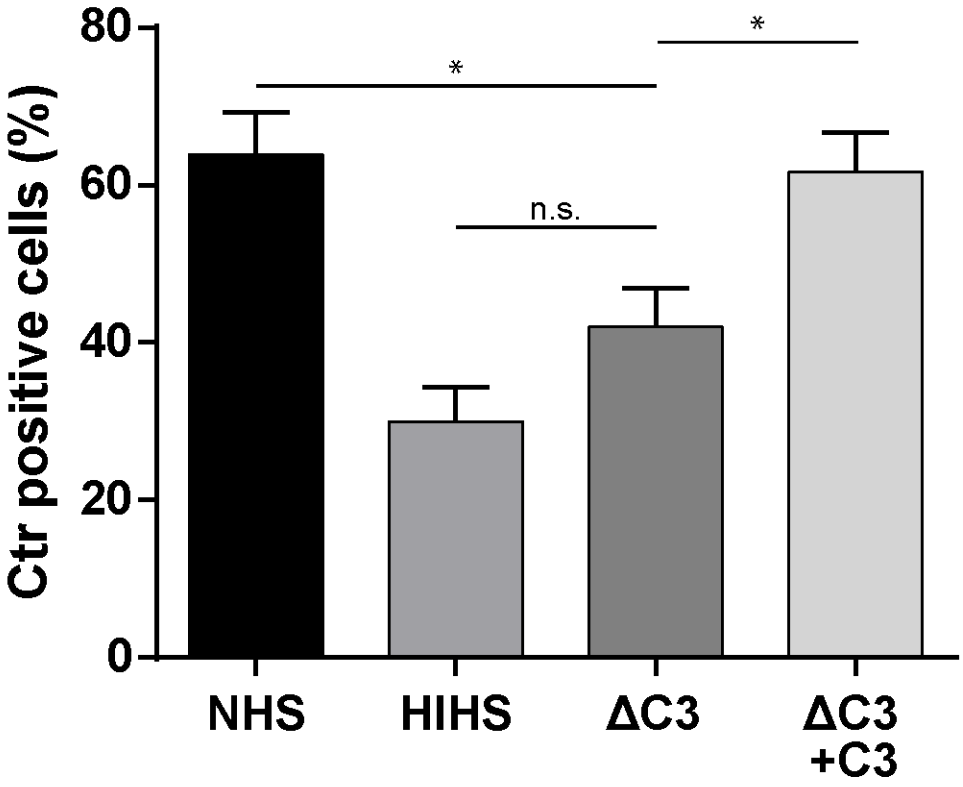
693 McCoy cells with EB that had not developed to an inclusion (arrowhead). Right image:
694 McCoy cell with an inclusion (green) from monocytes incubated in HIHS. Table: Mean
695 percentage (± SEM) of McCoy cells containing mature inclusions quantified from duplicate
696 samples from three biological independent experiments. Scalebars indicate 10 µm.
697 B) IL-6 and IL-8 concentrations in media from monocytes cultured with *C. trachomatis* L2.
698 Monocytes were incubated with *C. trachomatis* L2 for 4 hours in media supplemented with
699 either NHS or HIHS. After 4 hours, extracellular bacteria were removed and the monocytes
700 were incubated for further 20 hours. The culture supernatants were harvested and used for
701 ELISA. Standard medium and standard medium supplemented with 1 µg/ml LPS were used
702 as negative and positive controls, respectively. No statistically significant differences were
703 observed between and NHS and HIHS groups. Each condition was analyzed in triplicates and
704 three biologically independent experiments were performed. Data are presented as means ±
705 SEM.
706



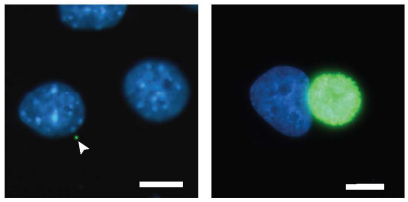








A)



Percentage of McCoy cells containing mature *C. trachomatis* inclusions

	NHS	HIHS
4 h	0.17 (±0.08)	0.15 (±0.02)
24 h	0	0

B)