

Opsonophagocytosis of *Chlamydia pneumoniae* by human monocytes and neutrophils

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1 **Opsonophagocytosis of *Chlamydia pneumoniae* by human**
 2 **monocytes and neutrophils**

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28 **ABSTRACT**

29 The human respiratory tract pathogen *Chlamydia pneumoniae* (*C. pneumoniae*) causing mild to severe
30 infections have been associated with the development of chronic inflammatory diseases. To understand
31 the biology of *C. pneumoniae* infections several studies have investigated the interaction between *C.*
32 *pneumoniae* and professional phagocytes. However, these studies have been conducted under non-
33 opsonizing conditions making the role of opsonization in *C. pneumoniae* infections elusive. Thus, we
34 analyzed complement and antibody opsonization of *C. pneumoniae* and evaluated how opsonization
35 affects chlamydial infectivity and phagocytosis in human monocytes and neutrophils.

36 We demonstrated that IgG antibodies and activation products of complement C3 and C4 are deposited
37 on the surface of *C. pneumoniae* elementary bodies when incubated in human serum. Complement
38 activation limits *C. pneumoniae* infectivity *in vitro* and have the potential to induce bacterial lysis by
39 formation of the membrane attack complex. Co-culture of *C. pneumoniae* and freshly isolated human
40 leukocytes showed that complement opsonization is superior to IgG opsonization for efficient
41 opsonophagocytosis of *C. pneumoniae* in monocytes and neutrophils. Neutrophil-mediated
42 phagocytosis of *C. pneumoniae* was crucially dependent on opsonization while monocytes retain minor
43 phagocytic potential under non-opsonizing conditions. Complement opsonization significantly
44 enhanced the intracellular neutralization of *C. pneumoniae* in peripheral blood mononuclear cells and
45 neutrophils and almost abrogated the infectious potential of *C. pneumoniae*.⁴

46 In conclusion, we demonstrated that complement limits *C. pneumoniae* infection *in vitro* by interfering
47 with *C. pneumoniae* entry into permissive cells, by direct complement-induced lysis and by tagging
48 bacteria for efficient phagocytosis in both monocytes and neutrophils.

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50 INTRODUCTION

51 *Chlamydia pneumoniae* (*C. pneumoniae*) is a human pathogen frequently causing mild respiratory
52 symptoms during infection that usually resolves spontaneously but can cause severe, long-lasting
53 atypical pneumonia and has been associated with the development of several chronic disease conditions
54 including atherosclerosis and asthma (1, 2). *C. pneumoniae* is an obligate intracellular Gram-negative
55 bacterium with a unique biphasic developmental cycle. The bacterium alternates between the
56 extracellular infectious elementary body (EB) and the intracellular non-infectious reticular body (RB).
57 During infection, *C. pneumoniae* EBs engage airway epithelium and induce their own uptake by
58 secreting pre-formed effectors into the host cell cytoplasm by the type-III secretion system (3).
59 Intracellularly, the EBs transform into RBs and start replicating in a modified vacuole called an
60 inclusion. After 48-72 hours RBs start to divide asynchronously and EBs are formed and released from
61 the host cell by cell lysis or by membrane extrusion (4).

62 Upon infection, *C. pneumoniae* EBs first encounter airway epithelial cells and alveolar macrophages
63 which respond to infection by secreting inflammatory mediators stimulating vascular endothelium
64 activation and immune cell chemotaxis (5). Neutrophil granulocytes are the first immune cells to
65 transmigrate from the blood stream into the alveolar space and subsequently signal for mononuclear
66 cell recruitment and infiltration (6, 7). Thus, neutrophils and monocytes comprise an early cellular
67 defense against *C. pneumoniae* and the interaction between extracellular chlamydial EBs and these
68 phagocytes likely determines the course of the infection. Multiple studies have proposed that *C.*
69 *pneumoniae* can evade intracellular killing mechanisms in both monocytes and neutrophils and thereby
70 use these cells as cellular vectors for extrapulmonary dissemination potentially causing chronic disease
71 (8–10). However, these pro-survival mechanisms may be altered by the route of bacterial ingestion

72 since engagement with different phagocytic receptors activates different intracellular signaling and
73 trafficking pathways.

74 It was previously shown that phagocytic uptake of the intracellular bacterium *Mycobacterium*
75 *tuberculosis* (*M. tuberculosis*) through complement receptors or the mannose receptor protects the
76 bacterium from intracellular killing by delaying phagosome maturation (11, 12). On the contrary,
77 uptake of antibody opsonized *M. tuberculosis* through Fc γ -receptors causes rapid phagosome maturation
78 and phagolysosomal fusion leading to reduced infection outcome. Thus, opsonin-directed phagocytosis
79 is a critical factor determining the intracellular fate of *M. tuberculosis* and similar mechanisms could be
80 involved in *C. pneumoniae* infections and pathogenesis, despite these pathogens being very different in
81 nature.

82 The complement system consists of more than 30 soluble and membrane-bound proteins that works in
83 a cascade-like manner to induce anti-microbial effector functions including opsonization, chemotaxis
84 and cell lysis. The cascade process is initiated within minutes of engagement (13) and proceeds through
85 three separate activation pathways: Classical- (recognition of antibody complexes), lectin- (recognition
86 of carbohydrate moieties), and alternative pathway (spontaneous hydrolysis of C3) (14). Following
87 activation, complement C4 and C3 are proteolytically cleaved into small soluble C4a/C3a fragments (9
88 kDa each) and large C4b/C3b fragments (75 kDa and 110 kDa, respectively) that attach covalently to
89 the activating surface and opsonize the microbe for phagocyte detection. C3b can be further cleaved
90 into iC3b, C3dg or C3d, which are all potent opsonins recognized by different complement receptors
91 on phagocytic cells (14). The final stage of the complement cascade includes cleavage of complement
92 C5 and generation of a multimeric membrane-spanning C5b-9 complex (membrane attack complex,
93 MAC) that induces pore formation and cell lysis (14).

94 During primary infection, *C. pneumoniae* engages the complement system in the alveolar space.
95 Although alveolar complement concentration and activity is reduced compared to concentrations and
96 activity seen in serum, the system is sufficiently functional to opsonize *M. tuberculosis* with
97 complement C3 activation products (15, 16). Primary infection leads to *C. pneumoniae*-specific IgG
98 production, but these antibodies offers limited protection against *C. pneumoniae* since reinfections are
99 frequently observed (17). Thus, during secondary infections both antibodies and complement are
100 present in the alveolar space, which have the potential to modulate the intracellular fate of *C.*
101 *pneumoniae* in phagocytes and hence the outcome of the infection.

102 To further characterize the pathogenesis of *C. pneumoniae* during primary and secondary infections we
103 investigated how complement- and antibody opsonization affect phagocytosis and intracellular survival
104 of *C. pneumoniae* in human monocytes and neutrophils.

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114 **RESULTS**

115 **Complement- and antibody opsonization of *C. pneumoniae***

116 To examine the role of opsonization and phagocytosis during *C. pneumoniae* infections, we first
117 screened the serum from 10 healthy donors for IgG antibodies against *C. pneumoniae* and found that
118 nine out of ten donors were positive for IgG against *C. pneumoniae*. Serum from one seropositive
119 individual and serum from the only identified seronegative individual were used in all further
120 experiments. These sera are called immune serum and non-immune serum, respectively, throughout
121 this paper.

122 Different cleavage fragments of complement C3 and C4 are known to bind and opsonize bacteria for
123 phagocytic recognition. C3 deposition on *C. pneumoniae* has only been investigated by flow cytometry
124 using an antibody recognizing various C3 cleavage forms (18). Thus, the exact C3 opsonins that bind
125 the *C. pneumoniae* surface remain unknown. In addition, complement C4 deposition has never been
126 investigated on *C. pneumoniae*. We therefore analyzed the deposition of these complement components
127 on purified *C. pneumoniae* EBs using immunoelectron microscopy (IEM) and western blotting.

128 Figure 1A demonstrates that complement C3 is abundantly deposited on the chlamydial surface when
129 incubated in non-immune serum (NHS-). Very limited C3 deposition was observed in heat-inactivated
130 serum (HIHS-) (Fig. 1B) suggesting that C3 deposition was due to activation of the complement system
131 and not due to unspecific antibody binding. This observation was confirmed by quantification of gold
132 particles showing a median gold-binding ratio of 21.8 in NHS- (12.1-20.9) vs. 1.9 in HIHS- (1.2-2.6)
133 (Fig. 1C).

134 These observations were confirmed by western blot analysis demonstrating solid deposition of C3 in
135 NHS-, but not in HIHS- (Fig. 1D). Several high molecular bands (above 110 kDa) are seen on the
136 western blot, indicating cleavage of the alpha chain of native C3 and covalent attachment of C3
137 fragments to chlamydial surface structures (Fig. 1D). The 40 kDa fragment shown in Fig. 1D
138 corresponds to the $\alpha'2$ chain of C3, demonstrating deposition of the potent opsonin iC3b.

139 Interestingly, we also demonstrated deposition of C4 on the chlamydial surface when incubated in
140 NHS- (Fig. 1E), but not in HIHS- (Fig. 1F). Compared to C3, C4 was more sporadically deposited on
141 the chlamydial surface indicated by a lower bacteria-to-background ratio for C4 (Fig. 1E+G). The
142 observations were confirmed by western blot analysis which demonstrated deposition of the C4b
143 opsonin. C4b deposition is indicated by the emergence of high molecular bands suggesting cleavage
144 and exposure of the thioester group in the alpha chain of C4 along with the presence of the C4 beta- (70
145 kDa) and gamma chains (35 kDa) (Fig. 1H). These observations suggest involvement of the lectin-
146 mediated pathway since the non-immune serum tested negative for both anti-*C. pneumoniae* IgG and
147 IgM making classical-mediated C4 activation unlikely.

148 To evaluate opsonization during secondary infection we incubated *C. pneumoniae* EBs in immune-
149 serum and investigated both antibody- and complement opsonization. Immunofluorescence microscopy
150 demonstrated that immune serum reacted with individual chlamydial organisms located in perinuclear
151 inclusions in infected HeLa cells (Fig. 2A). The IgG antibodies reacted with the surface of RBs
152 indicated by the ring-shaped structures surrounding a DNA core (Fig. 2A). As expected, non-immune
153 serum did not react with the chlamydial inclusions (Fig. 2B). These observations were made on fixed
154 and permeabilized cells and may not accurately reflect native surface labeling.

155 Thus, to investigate native antibody labeling and to determine if antibodies in immune-serum also bind
156 *C. pneumoniae* EBs, we incubated purified *C. pneumoniae* EBs with immune-serum and evaluated IgG
157 binding to the surface by IEM. Gold-conjugated anti-human IgG antibodies bind to the surface of
158 chlamydial EBs when incubated in immune-serum (Fig. 2C), while only few gold particles were
159 associated with chlamydial EBs when incubated in non-immune serum (Fig. 2D). Quantitative analysis
160 demonstrated a median gold-binding ratio of 27.1 (23.6-39.1) in HIHS+ vs. 1.1 (0.6-2.5) in HIHS-,
161 showing that the limited bacterial gold deposition observed in non-immune serum equals the
162 background gold deposition (Fig. 2E). Thus, human *C. pneumoniae* IgG antibodies present in serum
163 interact with epitopes located both on fixed RBs (Fig. 2A) and native EBs (Fig. 2C+E).

164 Next, we wanted to investigate if anti-*C. pneumoniae* IgG affects complement activation and
165 deposition on the chlamydial surface. Complement C3 was heavily deposited when *C. pneumoniae*
166 were incubated in NHS+, but not in HIHS+ (Fig. 2F-H). Similar levels of C3 deposition between
167 immune serum and non-immune serum were observed (median ratios: NHS-: 21.8 vs. NHS+: 23.8)
168 suggesting a negligible role of IgG in complement C3 deposition (Fig. 1A+C and Fig 2F+H).

169 As expected, complement C4 was deposited on *C. pneumoniae* incubated in NHS+, but not in HIHS+
170 (Fig. 2J-L). More complement C4 deposition was observed in immune serum, in the presence of IgG,
171 compared to non-immune serum (median ratios: NHS-: 3.5 vs. NHS+: 27.5) suggesting increased
172 classical complement activation.

173 Increased complement C4 deposition in immune serum compared to non-immune serum was also
174 indicated by western blot analysis demonstrating more intense C4 bands when bacteria were incubated
175 in immune-serum (Fig. 2M vs. Fig. 1H). Several high molecular bands were observed suggesting
176 cleavage of the alpha chain of C4 and covalent attachment of C4b to the bacterial surface. In addition,

177 the cleaved alpha chain (α' chain) was observed around 80 kDa suggesting non-covalent attachment of
178 C4b (Fig. 2M).

179 In summary, *C. pneumoniae* is opsonized by iC3b and C4b in the absence of anti-*C. pneumoniae* IgG.
180 *C. pneumoniae* IgG is able to bind both EBs and RBs leading to increased C4b deposition on purified
181 EBs.

182

183 **Complement-mediated bacterial lysis**

184 We wanted to investigate if the initial binding of complement and antibodies alone could interfere with
185 reproductive infection of *C. pneumoniae*, since antibody binding and complement activation precede
186 immune cell infiltration.

187 Electron microscopy revealed that most chlamydial organisms were intact after 30 minutes of
188 incubation in serum, demonstrating no signs of bacterial lysis (Fig. 3A). However, few bacteria showed
189 signs of complement-mediated lysis indicated by disruption of normal bacterial morphology together
190 with pore-forming structures (Fig. 3B, arrowheads). Thus, we aimed to investigate if the complement
191 cascade proceeds through the terminal complement pathway leading to MAC-induced bacterial lysis in
192 these cases. Chlamydial organisms were incubated in non-immune- and immune serum and
193 immunogold-labeled using a monoclonal antibody, recognizing a neoepitope of the C5b-9 complex. As
194 shown in Fig. 3C, C5b-9 is asymmetrically deposited on disintegrated chlamydial organisms and is
195 located in close proximity to 10 nm pore-like structures indicating MAC formation and MAC-induced
196 lysis. Bacteria incubated in heat-inactivated serum showed no signs of bacterial lysis (Fig. 3D). MAC
197 formation was observed in both immune- and non-immune serum, but more gold was observed in

198 NHS+ samples (median ratio: 34.5 (12.0-72.4)) compared to NHS- samples (median ratio: 12.1 (6.0-
199 36.2)), suggesting that serum IgG promotes formation of C5b-9 complexes. Interestingly, most gold-
200 labeled bacteria appeared healthy, with normal morphological features, and with no signs of bacterial
201 lysis. These observations suggest that non-lytic C5b-9 complexes are formed on the chlamydial
202 surface. Chlamydial organisms with altered morphology and signs of bacterial lysis appeared generally
203 larger than unaffected chlamydiae possibly representing intermediate- or reticular bodies inevitably
204 located within the EB fraction during the EB purification process. These observations imply that lytic
205 C5b-9 formation occurs primarily on intermediate- or reticular bodies but leaving EBs unaffected.
206 Thus, some *C. pneumoniae* organisms are sensitive to complement-mediated lysis in both immune and
207 non-immune serum.

208 Our observations show that complement fragments and antibodies deposit on almost all *C. pneumoniae*
209 EBs while only few bacteria are directly killed by complement-mediated lysis. We therefore asked if
210 opsonin deposition on apparently intact *Chlamydia* EB had any effect on chlamydial infectivity. To
211 answer this question, we tested the ability of immune- and non-immune serum to neutralize chlamydial
212 infection in HeLa cells by enumerating inclusion forming units (IFU) in each experimental condition.
213 Figure 4 shows that complement had a significant impact on chlamydial infectivity. Only $0.38\% \pm 0.2$
214 (mean \pm SD) and $1.19\% \pm 0.72$ of the initial inoculum was recovered after incubation in immune-
215 (NHS+) and non-immune serum (NHS-), respectively. These observations indicate that the
216 combination of complement and antibodies more efficiently reduces chlamydial infectivity than
217 complement alone, but the difference was not statistically significant. When serum was heat-inactivated
218 $19.1\% \pm 6.7$ and $44.7\% \pm 5.8$ (Fig. 4) of inoculum was recovered demonstrating that complement
219 significantly inhibits *C. pneumoniae* infectivity. The difference between heat-inactivated immune-

(HIHS+) and non-immune serum (HIHS-) further suggests that antibodies alone can reduce *C. pneumoniae* infectivity. To confirm this observation, we supplemented heat-inactivated non-immune serum (HIHS-) with the IgG fraction from a seropositive donor and evaluated chlamydial infectivity. As shown in Figure 4, supplementing non-immune serum with anti-*C. pneumoniae* IgG (HIHS-+IgG) significantly reduced chlamydial infectivity compared to non-immune serum alone confirming that anti-*C. pneumoniae* IgG reduces infectivity of *C. pneumoniae*. These data demonstrate that both antibody-, but especially complement opsonization have profound impact on *C. pneumoniae* infectivity.

Complement-mediated phagocytosis of *C. pneumoniae*

As demonstrated above, neither complement- nor antibody opsonization completely abrogates chlamydial infectivity. We therefore aimed to investigate how phagocytes participate in the clearance of opsonized *C. pneumoniae*. To quantitatively evaluate the role of opsonization in phagocytic uptake during primary and secondary infections, we analyzed the uptake of opsonized and non-opsonized *C. pneumoniae* EB in monocytes and neutrophils by flow cytometry. Intracellular staining of cells for flow cytometry is troublesome as it requires cell permeabilization which causes considerable changes to physical characteristics of the cells. To avoid permeabilization *C. pneumoniae* organisms were labeled by FITC and uptake of FITC-labeled bacteria and FITC-labeling specificity were evaluated by confocal microscopy. Figure S1 shows that FITC-labeled organisms appear small (around 0.5 μm) and round in accordance with the morphology of chlamydial EBs. To confirm the intracellular location of these FITC-signals,

monocytes were stained for the cytoplasmic protein S100A8 to visualize the cell cytoplasm (Fig. S1B). As demonstrated in Fig. S1C all FITC-positive organisms are located inside the cell cytoplasm. FITC-labeling is a rather unspecific fluorescent labeling technique since the isothiocyanate group reacts with primary amine groups. Thus, to evaluate whether the FITC-signal in Fig. S1 originates from chlamydial organisms and not contaminants like cell debris from the chlamydial isolation procedure, we stained FITC-labeled chlamydiae with a monoclonal antibody against chlamydial LPS. As demonstrated in Fig. S1D+F all FITC-positive structures reacted with the anti-*Chlamydia* LPS antibody confirming that all FITC-positive structures are chlamydial organisms. Thus, FITC-labeling of *C. pneumoniae* is efficient and specific and the FITC-labeled bacteria locate inside the cells enabling the bacteria to be used in a flow cytometric assay.

To analyze the effect of opsonization on phagocytosis of *C. pneumoniae*, isolated leukocytes were incubated with FITC-labeled *C. pneumoniae* in 30 min under different opsonizing conditions and subjected to flow cytometry analysis. To quantify the uptake, monocyte and neutrophil cell populations were gated as demonstrated in Fig. S2. Both cell types were gated based on forward- and side-scatter characteristics and monocytes were additionally gated based on CD14 surface expression.

We first analyzed the role of complement-mediated opsonophagocytosis to experimentally mimic the infection conditions during primary infection. Thus, phagocytic uptake was quantified in medium containing either normal non-immune serum (NHS-) or heat-inactivated non-immune serum (HIHS-). As depicted in Fig. 5A+B, phagocytosis of *C. pneumoniae* in both monocytes and neutrophils is much more efficient in NHS- compared to HIHS- suggesting an important role for complement opsonization in phagocytosis of *C. pneumoniae*. Interestingly, neutrophil-mediated phagocytosis was almost absent under non-opsonizing conditions (Fig 5B, HIHS-), suggesting that opsonization is paramount for

neutrophil phagocytosis of *C. pneumoniae* while monocytes exploit opsonin-independent phagocytic pathways.

Phagocytosis in the presence of anti-chlamydial antibodies

We demonstrated that monocyte- and especially neutrophil-mediated phagocytosis of *C. pneumoniae* were critically dependent on complement opsonization. Complement activation and activity in the alveolar space is reduced compared to serum and is primarily dependent on classical complement activation (15, 16). Thus, we sought to investigate whether anti-*C. pneumoniae* antibodies could mediate efficient phagocytosis or potentiate complement-mediated phagocytosis as both mechanisms may be important to understand the biology of *C. pneumoniae* reinfections.

By comparing the heat-inactivated immune serum (Fig 5C+D, HIHS+) with heat-inactivated non-immune serum (Fig 5A+B, HIHS-) it is evident that antibody opsonization increased phagocytic uptake of *C. pneumoniae* in both monocytes (3-fold increase) and especially in neutrophils (17-fold increase), however this effect was significantly lower compared to complement alone. To confirm these observations, we purified the IgG-fraction from immune serum and supplemented HIHS- with purified IgG. As demonstrated in Fig. 5E+F, supplementing non-immune serum with the IgG-fraction from immune serum increased the phagocytic uptake in both monocytes and neutrophils, confirming that the observed differences are due to anti-*C. pneumoniae* antibodies. We could not assess whether the presence of IgG potentiated complement-mediated phagocytosis since both monocyte and neutrophil cell populations were saturated with bacteria when complement competent serum was used (Fig. 5A-D, NHS+/-). We therefore re-analyzed these samples using *C. pneumoniae* at MOI=1 and showed that

284 monocytes and neutrophils more efficiently ingest *C. pneumoniae* when both complement and IgG
285 antibodies are present (Fig. 5G+H).

286

287 **Intracellular survival of ingested bacteria**

288 *C. pneumoniae* is ingested through both complement- and antibody dependent mechanisms. Evidence
289 from other intracellular bacteria shows that the intracellular fate of ingested bacteria is highly
290 dependent on the route of uptake, and it is therefore important to study the intracellular fate of *C.*
291 *pneumoniae* under the opsonizing conditions we have demonstrated here.

292 To evaluate the intracellular fate of ingested bacteria, PBMCs and neutrophils containing opsonized
293 and non-opsonized bacteria were lysed by ultrasonication and liberated bacteria were used to infect
294 HeLa cell monolayers.

295 Statistically significantly less IFU were recovered when bacteria were ingested by PBMCs in the
296 presence of immune serum (NHS+: 45 ±19 IFU/μl) and non-immune serum (NHS-: 70 ±16 IFU/μl)
297 compared to bacteria ingested in media supplemented with heat-inactivated immune serum (HIHS+:
298 315 ±49 IFU/μl) and non-immune serum (HIHS-: 452 ±25 IFU/μl) (Fig. 6A). Similar results were
299 obtained for neutrophils with 99 ±4 IFU/μl and 141 ± 32 IFU/μl recovered IFU in immune- and non-
300 immune serum, respectively (Fig. 6B). Serum heat-inactivation caused a statistically significant
301 increase in IFU recovery from both immune serum (Fig. 6B, HIHS+: 452 ±139 IFU/μl) and non-
302 immune serum (Fig. 6B, HIHS-: 1151 ±259 IFU/μl). Although more bacteria are present
303 intracellularly in cells incubated in NHS compared to HIHS (Figure 5A-D) more reproductive bacterial
304 organisms can be recovered from cells in HIHS. These data suggest that complement not only facilitate

305 rapid and efficient uptake of *C. pneumoniae*, but also mediates effective intracellular neutralization.
306 We also observed statistically significant less IFU recovered from bacteria ingested in the presence of
307 heat-inactivated immune serum (HIHS+) compared to heat-inactivated non-immune (HIHS-) in both
308 cell types (Fig. 6A+B) suggesting that antibodies also play a role in bactericidal activity against *C.*
309 *pneumoniae*.

310 Studies performed on *M. tuberculosis* suggest that bacterial uptake by complement receptors delays
311 phagosomal maturation and phagolysosomal fusion thereby facilitating intracellular bacterial survival.
312 Our results suggest the opposite; that complement-mediated uptake leads to rapid intracellular
313 neutralization of *C. pneumoniae*. We therefore tested whether *C. pneumoniae* were targeted to
314 lysosomal compartments in monocytes after complement-mediated ingestion. Figure 6C-E shows that
315 *C. pneumoniae* localizes within LAMP1-positive vesicular structures in monocytes after 30 min
316 incubation in media supplemented with non-immune serum (NHS-). Staining of mock infected cells
317 demonstrates that FITC-signal in LAMP1-positive structures originates from chlamydial organisms
318 (Fig. 6F). Thus, complement-mediated uptake of *C. pneumoniae* in monocytes directs ingested bacteria
319 to lysosomal compartments.

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326 DISCUSSION

327 In the present study, we demonstrated that *C. pneumoniae* EBs are opsonized with both IgG and the
328 complement opsonins iC3b and C4b when incubated in human serum. We showed that complement
329 activation limits *C. pneumoniae* infection in multiple ways by interfering with *C. pneumoniae* entry
330 into permissive cells, by direct MAC-induced lysis and by tagging bacteria for efficient phagocytosis
331 by both monocytes and neutrophils.

332 The activation and deposition of complement C3 and the terminal complement complex on *C.*
333 *pneumoniae* demonstrated in this study were previously shown by Cortes and colleagues (18).
334 Interestingly, using electron microscopy, we observed C5b-9 deposition without pore-formation and
335 bacterial lysis. C5b-9 complexes associated with pore-like structures and altered bacterial morphology
336 were primarily observed on reticular- and intermediate bodies. Thus, our observations suggest that
337 extracellular EBs may be resistant to MAC-induced lysis. It is likely, that the cysteine-rich proteins in
338 the highly cross-linked chlamydia outer membrane of EBs provide a physical barrier that hinders
339 components of the terminal complement complex to interact with the outer membrane lipid bilayer.
340 Alternatively, MAC formation could be inhibited by recruitment of host-derived complement
341 regulators to the EB surface. Thus, it was previously demonstrated that *C. trachomatis* binds the MAC-
342 inhibitor vitronectin, however, it was not investigated if vitronectin modulates complement deposition
343 on the chlamydial surface (19).

344 Cortes et al. concluded that properdin and alternative complement activation was indispensable for C3b
345 deposition and complement-mediated neutralization. Interestingly, we found that complement C4 is
346 deposited on the surface of *C. pneumoniae*. Deposition of complement C4 in both immune- and
347 nonimmune serum suggests that also the lectin-mediated activation pathway is involved in *C.*

348 *pneumoniae* induced complement activation. This observation is supported by previous studies
349 demonstrating that different lectins can bind the surface of *C. pneumoniae* (20, 21). Moreover,
350 quantification of gold-labeling and semi-quantitative western blot analysis suggested that more C4 was
351 deposited in immune serum compared to non-immune serum, indicating activation of the classical
352 pathway. This is supported by the finding that IgG1 is the predominant IgG subclass raised against *C.*
353 *pneumoniae* and to a lesser extent IgG3 and both IgG1 and IgG3 are efficient complement activators
354 (22). The IgG subclasses raised against *C. pneumoniae* may also explain why we observe an IgG-
355 dependent inhibition of *C. pneumoniae* infectivity in HeLa cells (Fig. 4). Studies on *C. trachomatis*
356 showed that HeLa cells express FcγRIII and that Fc-mediated endocytosis through this receptor
357 promotes reproductive infection of *C. trachomatis* (23, 24). It is likely that *C. trachomatis* is more
358 prone to FcγRIII-mediated uptake compared to *C. pneumoniae* since IgG3 is the predominant IgG
359 subclass raised against *C. trachomatis* (22). Thus, the IgG subclass distribution seems important for the
360 infectivity and reproductive outcome of *Chlamydia spp. in vitro*.

361 A possible explanation for the discrepancies between our results and the findings by Cortes et al. could
362 be that classical- and/or lectin-mediated activation pathways are abrogated at the C4 level leading to
363 pathway termination before C3 cleavage. C4 binding protein (C4bp), a fluid-phase negative
364 complement regulator, can terminate complement activation at the C4 level by inducing C4b cleavage
365 and C2a dissociation from the classical C3 convertase (25). Several bacterial pathogens are able to
366 recruit and bind C4bp leading to complement resistance, but this has not yet been demonstrated for
367 chlamydial organisms (26, 27).

368 The alternative activation pathway seems important for *C. pneumoniae*-induced complement activation
369 *in vitro* but the significance of this pathway during *in vivo* lung infections is still elusive. In the lungs,

370 complement activation proceeds primarily through the classical pathway and alternative complement
371 activation fails to activate and deposit C3 on both mycobacteria and streptococci in bronchoalveolar
372 lavage fluid (15, 28). It remains uncertain whether *C. pneumoniae* is opsonized in the alveolar space of
373 the lungs, but the classical complement activation is functional, and during secondary infection
374 complement activating IgG is present in the alveolar space (15).

375 We show that complement efficiently facilitates phagocytosis of *C. pneumoniae* in both monocytes and
376 neutrophils and that most opsonized bacteria are unable to cause reproductive infection when liberated
377 from phagocytes. Complement receptor 3 (CR3, CD11b/CD18) recognizes different C3 cleavage
378 fragments including iC3b identified on *C. pneumoniae* EBs here. CR3 is, together with Fcγ-receptors,
379 the most important phagocytic receptor and is widely expressed on both monocytes and neutrophils. It
380 is, therefore, likely that complement-mediated phagocytosis takes place via CR3 leading to intracellular
381 destruction. This is different from CR-mediated uptake of other intracellular bacteria. CR-mediated
382 uptake of *M. tuberculosis* into human monocytes and macrophages allow safe entrance by delaying
383 phagosomal maturation and phagolysosomal fusion (11). We observed that *C. pneumoniae* is rapidly
384 targeted to LAMP1-positive intracellular compartments showing efficient phagosomal trafficking
385 facilitating intracellular neutralization of the chlamydial reproductive potential. Thus, safe entrance into
386 host cells through CR-mediated phagocytosis is not a conserved mechanism for all intracellular
387 bacteria. This is supported by the observation that the facultative intracellular bacterium *Francisella*
388 *tularensis* (*F. tularensis*) fails to escape phagosomal trafficking in murine bone-marrow macrophages
389 when opsonized with either complement or IgG (29). Knockout of CD11b, a subunit of the
390 heterodimeric CR3, leads to phagosomal escape with cytosolic localization of *F. tularensis*, supporting
391 that CR3 plays opposing roles during experimental infections with different intracellular bacteria (29).

392 Direct interaction between opsonized bacteria and phagocytic opsonin-receptors provides one
393 explanation for the increased phagocytic uptake and intracellular neutralization observed here.
394 Phagocytic priming by the complement anaphylatoxins C3a and C5a may provide another likely
395 explanation. In an *in vivo* *C. psittaci* lung infection model C5aR^{-/-} mice displayed slightly worsened
396 clinical score compared to WT during early stage infection (30). The same authors later demonstrated
397 that C3a and its receptor C3aR are critically involved in anti-chlamydial immunity in the same *C.*
398 *psittaci* lung infection model (31). Both receptors are expressed on monocytes and neutrophils and can
399 potentiate phagocytic functions by inducing both an increased surface expression of complement
400 receptors and induce a hostile intracellular environment (32, 33). C5a induces increased surface
401 expression of CD11b and increased phagocytosis of *Escherichia coli* (*E. coli*) in neutrophils (34) and
402 can also potentiate intracellular killing of *E. coli* by increasing the oxidative burst in a whole blood
403 assay (35).
404 In addition, priming neutrophils with C5a leads to translocation of CR1 containing secretory vesicles to
405 the cell surface (36, 37). CR1 recognizes the activation products C3b and C4b together with several
406 other complement-related proteins (38). We demonstrate for the first time, that C4b is deposited on the
407 surface of *C. pneumoniae* and hence, C5a-induced translocation of CR1 to the plasma membrane may
408 induce increased bacterial phagocytosis. However, the role of C4b in opsonophagocytosis remains
409 elusive.
410 We demonstrated that *C. pneumoniae* ingested in the presence of complement-competent serum had
411 impaired ability to cause reproductive infection in HeLa cells compared to bacteria ingested under non-
412 opsonizing conditions. Intracellular survival and replication of *C. pneumoniae* within phagocytes have
413 previously been evaluated in several *in vitro* studies; however, these have been conducted under

414 various experimental conditions. Both *C. pneumoniae* and *C. trachomatis* are able to replicate in
415 human neutrophils when ingested under non-opsonizing conditions (10, 39). Rajeeve et al. showed that
416 *C. trachomatis* uses the chlamydial protease-like activating factor (CPAF) to inhibit neutrophil
417 activation and cell death thereby creating a replicative environment (39). Since CPAF is highly
418 conserved among chlamydiae, *C. pneumoniae* may use a similar CPAF-dependent mechanism to
419 survive and replicate in neutrophils.

420 Similarly, co-incubating monocytes and *C. pneumoniae* under non-opsonizing conditions leads to
421 recovery of chlamydial progeny up until 48 hours post infection (9, 40) suggesting that opsonin-
422 independent uptake also facilitates *C. pneumoniae* survival in monocytes. This idea was further
423 supported by the observation that differentiated macrophages support intracellular replication of *C.*
424 *pneumoniae*. Differentiated macrophages, but not peripheral blood monocytes, express the mannose
425 receptor (MR) which facilitate intracellular survival of *M. tuberculosis*, and MR has also been linked to
426 phagocytic uptake of chlamydial organisms (12, 41, 42). Others used commercially available human
427 serum from AB blood type donors in the infection medium combined with centrifugation. These
428 studies were unable to recover chlamydial progeny after 6 and 48 hours, but detected 16S rRNA
429 suggesting metabolic, but not replicative activity (8, 43). However, neither complement functional
430 activity nor serostatus were evaluated for these sera making it difficult to draw any conclusions on the
431 role of opsonization. We demonstrated that both complement- and IgG-opsonization potentiates
432 phagocytosis and intracellular killing of *C. pneumoniae* suggesting that the uptake mechanism is
433 important for the intracellular fate of *C. pneumoniae* previously demonstrated for other chlamydial
434 organisms (44).

435 Here we demonstrated that complement opsonization and to a lesser extend IgG opsonization inhibits
436 *C. pneumoniae* progeny infection and facilitates *C. pneumoniae* uptake in human monocytes and
437 neutrophils. Ingested bacteria are rapidly trafficked to destructive intracellular compartments and
438 eliminated showing that opsonization and phagocytosis are efficient means of controlling extracellular
439 *C. pneumoniae* organisms. Thus, opsonization is a critical factor to include in future *in vitro* studies
440 exploring the interaction between *C. pneumoniae* and human phagocytes.

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454 **MATERIALS AND METHODS**

455 **Antibodies and reagents**

456 The following primary antibodies were used in this study: PE anti-human CD14 (Clone: M5E2)
457 (BioLegend, CA, USA), MAb 15.2.3 against chlamydial LPS (45), PAb198 against *C. pneumoniae*
458 outer membrane (46), Anti-MRP8 (S100A8) (Abcam, UK), Anti-LAMP1 (Sino Biological, China),
459 Polyclonal Rabbit Anti-Human C3c (Agilent Technologies, Glostrup, Denmark), Polyclonal Rabbit
460 Anti-Human C4c (Agilent) and Monoclonal Mouse Anti-Human C5b-9 (clone aE11, Agilent).
461 The following secondary antibodies were used in this study: Goat anti human IgG-, Goat anti rabbit
462 and Goat anti mouse antibody conjugated to 10 nm colloidal gold (British BioCell, Cardiff, UK) was
463 used for immunoelectron microscopy. Horseradish peroxidase conjugated Affinipure Goat Anti-Human
464 Fcγ-specific IgG was used for ELISA. Anti-Rabbit IgG conjugated to alkaline phosphatase was used
465 for western blotting (Sigma Aldrich, MO, USA). FITC-conjugated Goat Anti-Human IgG, Fcγ
466 fragment specific (Jackson ImmunoResearch, Cambridge, UK), Alexa Fluor® (AF) 555 goat anti-
467 rabbit, AF555 goat anti-mouse and AF647 goat anti-rabbit (Invitrogen, Thermo Fisher Scientific, MA,
468 USA) were used for immunofluorescence staining.
469 TMB/ONE (3,3',5, 5'-tetramethylbenzidine) and BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/
470 Nitro Blue Tetrazolium) were purchased from Kementec (Kementec, Taastrup, Denmark) and used for
471 ELISA and western blotting, respectively.

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475 **Cell lines and culture**

476 BHK- and HeLa cell lines were purchased from the American Type Culture Collection (ATCC, VA,
477 USA) and cultured in complete medium consisting of RPMI 1640 supplemented with 5% fetal calf
478 serum (FCS), and 0.01 mg/ml gentamicin and maintained at 37°C and 5% CO₂. Cell lines were tested
479 free of mycoplasma by Hoechst (33258) staining.

480

481 **Bacterial organism and propagation**

482 *C. pneumoniae* (CDC/CWL-029/VR1310) was purchased from ATCC and cultivated in Baby Hamster
483 kidney (BHK) cells in complete medium added 2 µg/ml cycloheximide and 0.1% glucose. The infected
484 cells were cultured at 37°C and 5% CO₂ for 72 hours. *C. pneumoniae* EBs were purified by density
485 gradient centrifugation as previously described (46). Mock inoculum was prepared by processing
486 uninfected BHK cells in parallel with infected cells.

487

488 **Serum samples**

489 Serum was obtained from 10 healthy volunteers at Aalborg University, Denmark. Blood was drawn by
490 venipuncture and collected in S-Monovette® serum tubes (Sarstedt, Nümbrecht, Germany) and serum
491 was isolated immediately according to manufactures instructions. Isolated serum was immediately
492 stored on ice before freezing at -80 °C for later use. Serum heat inactivation was done for 30 min at
493 56°C. Serum containing anti-*C. pneumoniae* IgG is denoted “immune serum” and serum without anti-
494 *C. pneumoniae* IgG is denoted “non-immune serum”. Throughout this paper the following

495 abbreviations will be used described the different sera: NHS+: Immune serum, NHS-: Non-immune
496 serum, HIHS+: heat-inactivated immune serum and HIHS-: heat-inactivated non-immune serum.
497 All protocols were approved by the Regional ethics committee of Region Nordjylland (N-20150073)
498 and all experiments were carried according to the Declaration of Helsinki. All participants gave written
499 informed consent to participate in the study.

500

501 **ELISA**

502 *C. pneumoniae* specific IgG was measured using *Chlamydia pneumoniae*-IgG ELISA Medac plus
503 (Medac, Wedel, Germany) using *C. pneumoniae* outer membrane complex as antigen. Absorbance
504 were measured at 450 nm on a Sunrise™ microplate reader (Tecan, Mannedorf, Switzerland).

505

506 **Immunofluorescence validation of serum reactivity**

507 HeLa cell monolayers, seeded on coverslips, were infected with 5×10^5 inclusion forming units (IFU) *C.*
508 *pneumoniae* by centrifugation at 1000 x g for 30 min at 37°C followed by 30 min incubation at 37°C
509 and 5% CO₂. Medium containing extracellular bacteria was removed and cells were incubated in
510 complete medium added 2 µg/ml cycloheximide and 0.1% glucose for 48 hours and subsequently fixed
511 in 3.7% formaldehyde for 20 min at 4 °C. Cells were permeabilized in ice cold methanol for 10 min,
512 blocked in 0.5% bovine serum albumin (BSA) for 15 min at 37°C and incubated with serial dilutions of
513 immune- and non-immune serum for 30 min at 37°C. Cells were washed three times in PBS and
514 incubated with FITC-conjugated anti-human IgG secondary antibody diluted 1:200 in 0.1% BSA in
515 PBS for 30 min at 37°C. Cells were washed three times in PBS and counter stained with using 2 µM

516 To-Pro-3 Iodide (Invitrogen, Thermo Fisher Scientific) for 20 min at room temperature. Cells were
517 imaged using a Leica TCS SP5 confocal laser scanning microscope with a HCX PL Apo 63x/1.40 and
518 CX PL Apo 100x/1.47 objective (Leica Microsystems, Wetzlar, Germany).

519

520 **Immunogold-labeling and transmission electron microscopy (TEM)**

521 Purified *C. pneumoniae* EBs were incubated in 50% NHS or HIHS for 30 min at 37°C and washed
522 three times in PBS with centrifugation at 15.000 x g, for 15 min at 4°C between each wash. Processing
523 and immunogold-labeling of bacteria for TEM was performed as previously described (47). Briefly,
524 serum coated (NHS or HIHS) *C. pneumoniae* EBs were added to carbon coated 400-mesh glow-
525 discharged nickel grids and washed three times in PBS before blocking in 1% ovalbumin (Sigma-
526 Aldrich) in PBS (pH 6.5). To determine binding of human antibodies to the chlamydial surface, grids
527 were incubated for 30 min at 37°C with Goat anti human IgG conjugated to 10 nm colloidal gold
528 diluted 1:25 in 0.5% ovalbumin in PBS. To determine complement binding, samples were incubated
529 with primary antibodies (anti-C3c, 1:200; anti-C4c, 1:200; anti-C5b-9, 1:20) diluted in 0.5% ovalbumin
530 in PBS for 30 min at 37°C. Grids were washed in three drops of PBS and subsequently incubated for
531 30 min at 37°C with secondary antibodies (Goat anti rabbit or Goat anti mouse antibody conjugated to
532 10 nm colloidal gold) diluted 1:25 in 0.5% ovalbumin in PBS. The grids were washed in three drops
533 PBS, incubated in three drops of 0.5% cold fish gelatin in PBS and negatively stained with 0.5%
534 phosphotungstic acid (PTA). Grids were blotted dry on filter paper and investigated using a Jeol 1010
535 transmission electron microscope (Jeol, Tokyo, Japan). Gold-deposition was quantified as previously
536 described (47). Briefly, a minimum of seven random fields were imaged for each grid containing at
537 least 12 chlamydial organisms in total. For each chlamydial organism, gold particles on the bacteria

538 and in the background were counted and the density of deposition was calculated for both (gold per
539 area). From these numbers, a bacteria-to-background ratio was calculated to quantitatively describe
540 bacterial gold deposition relative to background deposition. From these ratios the median and
541 interquartile ranges (IQR) were calculated for each sample.

542

543 **Western blotting**

544 SDS-PAGE and western blotting were performed essentially according to Lausen et al. (47). Briefly,
545 purified *C. pneumoniae* EBs were incubated in 50% serum for 30 minutes at 37°C and unbound
546 complement was removed by washing three times in PBS with centrifugation at 15,000 x g for 15 min
547 between each wash. The chlamydial pellet was lysed in SDS Sample buffer + 5% β -mercaptoethanol
548 and proteins were separated on an 8 % polyacrylamide gel. Proteins were blotted on to a nitrocellulose
549 membrane (GE Healthcare Life Sciences IL, USA) in Tris-Glycine buffer (25 mM Tris, 192mM
550 glycine) + 20% methanol for two hours at 100 V using a TE22 Mighty Small Transfer Tank (Hoefer,
551 Inc., Holliston, MA).

552 Membranes were blocked for 30 min at 37°C in 3% gelatin in Tris-buffered saline (TBS). Antibodies
553 diluted in TBS + 0.05% Tween-20 (TBST) + 0.2% gelatin were added to the membranes and left for
554 incubation for one hour at 37°C. Blots were washed three times in TBST and incubated with alkaline
555 phosphatase-conjugated secondary antibody for one hour at 37°C. Antigen-antibody complexes were
556 visualized by adding BCIP/NBT (Kementec) alkaline phosphatase substrate.

557

558

559 **Serum neutralization assay**

560 *C. pneumoniae* EBs were incubated in RPMI 1640 supplemented with 10% human serum for 30 min at
561 37°C. Opsonized bacteria (10.8×10^4 IFU for NHS samples and 1.2×10^4 IFU for HIHS samples) were
562 used to infect HeLa cell monolayers as described above. Cells were processed for immunofluorescence
563 microscopy as described above with minor modifications. PAb198 (1:400) and FITC-conjugated goat
564 anti-rabbit (1:200) were used as primary and secondary antibodies, respectively, and cell nuclei were
565 stained with 2 µg/ml DAPI.

566 The cells were inspected using a Leica LSM550 fluorescence microscope and images were captured
567 from seven random fields in each sample. All samples were analyzed in duplicates and repeated in
568 three independent experiments. The number of IFU was enumerated and expressed as percentage of
569 initial inoculum. Data from the three experiments are expressed as mean ± standard deviation (SD).

570

571 **FITC-labelling of bacteria**

572 *C. pneumoniae* inoculum was suspended in 0.1 M NaHCO₃ (pH = 9) and centrifuged for 15 min at
573 15.000 x g. Fluorescein isothiocyanate isomer 1 (FITC) (Sigma, MO, USA) was added at a
574 concentration of 0.1 mg/ml in 0.1 M NaHCO₃ buffer and bacteria were incubated for one hour
575 protected from light. The labeled bacteria were washed three times in PBS to remove unbound FITC
576 and the final bacteria pellet was suspended in sucrose-phosphate buffer (2SP), aliquoted, and stored at -
577 80°C.

578

579

580 **Immune cell isolation and culture**

581 Peripheral blood was obtained from two donors (one seropositive and one seronegative) and collected in
582 S-Monovette (Sarstedt) EDTA tubes. Blood was layered on Polymorphprep™ density gradient medium
583 (Axis-Shield, Dundee, UK) and centrifuged for 40 min at 300 x g at 20°C. Layers with peripheral
584 blood mononuclear cells (PBMCs) and polymorphnuclear cells were harvested and collected in the
585 same tube when used for flow cytometry. The two cell populations were kept separate when used for
586 immunofluorescence microscopy. Platelets was removed by centrifugation at 120 x g for 10 min at
587 20°C. Cells were resuspended in complete RPMI 1640 medium.

588

589 **Immunofluorescence staining and microscopy of immune cells**

590 For immunofluorescence staining, PBMCs were seeded in 8-well Nunc® Lab-Tek® Chamber™ slides
591 with a density of 9×10^5 cells/well. Cells were left to adhere for 90 min and non-adherent cells were
592 removed by washing twice in PBS. Bacteria were added at a multiplicity of infection (MOI) 10 in
593 RPMI 1640 + 10% human serum.
594 Non-phagocytized bacteria were removed by washing three times in PBS and cells were processed for
595 immunofluorescence staining as described above. PAb198 (1:400), anti-LAMP1 (1:400), anti-S100A8
596 (1:300) and MAb 15.2.3 (1:20) were used as primary antibodies and AF555-conjugated goat anti-
597 mouse, AF555-conjugated goat anti-rabbit and AF647-conjugated goat anti-rabbit were used as
598 secondary antibodies in an 1:200 dilution. Cell nuclei were stained using 2 μ M To-Pro-3 Iodide
599 (Invitrogen, Thermo Fisher Scientific). Cells were imaged using a Leica TCS SP5 confocal laser

600 scanning microscope with a CX PL Apo 100x/1,47 objective (Leica Microsystems).

601

602 **Phagocytosis assay**

603 FITC-labeled *C. pneumoniae* EBs were opsonized in RPMI 1640 + 10% human serum for 15 min at
604 37°C. Bacteria (1×10^7 or 1×10^6 bacteria/tube) and immune cells (1×10^6 cells/tube) were mixed in 5 ml
605 polypropylene tubes (MOI=10 and MOI=1, respectively) and co-incubated for 30 min at 37°C and cells
606 were subsequently processed for flow cytometry.

607

608 **Flow cytometry**

609 Cells were washed in cold PBS + 0.05% sodium azide to remove non-phagocytized bacteria. Fc-
610 receptors were blocked using 20 µg/ml human IgG (Sigma) for 15 min at 4°C. Primary fluorochrome-
611 conjugated antibodies, diluted in PBS + 0.05% sodium azide + 0.01% BSA, were added and cells were
612 stained for 30 min at room temperature. Unbound antibody was removed by washing in PBS and cell
613 viability was assessed using eBioscience™ Fixable Viability dye eFluor™ 450 according to
614 manufacturers's instructions. The cells were washed twice in PBS and fixed in 1% formaldehyde for 20
615 min at 4°C and analyzed on a CytoFLEX flow cytometer (Beckman Coulter, CA, USA).

616

617 **IgG purification**

618 Serum from one seropositive donor was mixed 1:1 with binding buffer (1 M glycine, 150 mM NaCl,
619 pH 8.5) and the serum IgG fraction was isolated on an affinity chromatography column packed with

620 GammaBind Plus protein G Sepharose (GE Healthcare). Bound IgG was eluted from the column using
621 elution buffer containing 100 mM glycine-HCl pH 2.7 and collected in 25 fractions in tubes containing
622 1 M Tris-HCl pH 9 to restore neutral pH. The protein concentration in each fraction was determined
623 using PierceTM BCA Protein Assay Kit (Thermo Scientific) according to manufacturer's instruction.

624

625 **Intracellular survival assay**

626 PBMCs and neutrophils were co-incubated with bacteria as described above. Cells were washed three
627 times in PBS to remove non-phagocytized bacteria. The cell pellet was suspended in 2SP buffer and
628 cells were lysed by ultrasonication to liberate ingested bacteria. The cell lysates were used to infect
629 HeLa cells and IFU were quantified from duplicate samples in three independent experiments as
630 described above.

631

632 **Data analysis**

633 Flow cytometry data were analyzed using FlowLogicTM v.7.2.1 (Inivai Technologies, Mentone
634 Victoria, Australia). One-way ANOVA with Tukey's post hoc or Welch ANOVA with Games-Howell
635 multiple comparison test was used to investigate differences between multiple means. Differences
636 between means from two independent groups were investigated using student's independent t-test or
637 Welch's t-test. All statistical analyses were performed in SPSS Statistics 25 (IBM, Armonk, NY,
638 USA).

639

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644 Denmark)

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646 **CONFLICT OF INTEREST**

647 The authors declare no conflict of interest.

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816 **FIGURE LEGENDS**

817 **Figure 1**

818 Complement opsonization of *C. pneumoniae* in non-immune serum. Purified *C. pneumoniae* EBs were incubated
819 in A) non-immune serum (NHS-) or B) heat-inactivated non-immune serum (HIHS-) and analyzed by
820 immunoelectron microscopy (IEM) using anti-C3c and anti-rabbit 10 nm colloidal gold as primary and
821 secondary antibodies, respectively. C) Gold deposition on each bacterium was quantified relative to the
822 background gold level for each condition (NHS- and HIHS-). D) Purified *C. pneumoniae* EBs were subjected to
823 western blot analysis and labeled against C3c. *C. pneumoniae* EBs were incubated in E) NHS- or F) HIHS- and
824 subjected to IEM using anti-C4c as primary antibody. G) Complement C4 deposition was quantified as described
825 for panel C). H) Western blot analysis of complement C4 deposition on *C. pneumoniae* incubated in NHS- or
826 HIHS-. Western blot analysis was repeated three times and representative blots are shown. IEM images were
827 captured from at least 8 random fields for each sample in one experiment. For each bacterium, a bacterium-to-
828 background gold deposition ratio was calculated and plotted with each dot representing one chlamydial
829 organism. The median ratio is presented as black lines. Scale bars indicate 200 nm.

830

831 **Figure 2**

832 Antibody- and complement opsonization of *C. pneumoniae* in immune serum. *C. pneumoniae* (5×10^5 IFU/well)
833 were grown in HeLa cells for 48 hours and chlamydial inclusions were immunofluorescently stained using
834 two-fold serial dilutions of A) immune serum or B) non-immune serum as primary antibody. Purified *C.*
835 *pneumoniae* EBs incubated in C) heat-inactivated immune serum (HIHS+) or D) heat-inactivated non-immune
836 serum (HIHS-) were immuno-labeled with anti-human IgG 10 nm colloidal gold and subjected to immuno-
837 electron microscopy (IEM). E) From IEM images, chlamydial gold deposition, for each bacterium, was
838 quantified relative to the background gold level. Purified *C. pneumoniae* EBs were incubated in F) immune

839 serum (NHS+) or G) heat-inactivated immune serum (HIHS+) and analyzed by IEM using anti-C3c and anti-
840 rabbit 10 nm colloidal gold as primary and secondary antibodies, respectively. H) Gold deposition in IEM
841 images was quantified as described for panel E. I) Purified *C. pneumoniae* EBs were subjected to western blot
842 analysis and labeled against C3c. J-M) *C. pneumoniae* were treated as described in panel F-I except bacteria
843 were labeled using an anti-C4c antibody. For immunofluorescence microcopy, each serum was tested in four
844 different dilutions and images were captured from five random fields for each dilution. The experiment was
845 repeated twice. In IEM, a bacterium-to-background gold deposition ratio was calculated for each bacterium and
846 plotted, with each dot representing one chlamydial organism. The median ratio is presented as black lines. IEM
847 images were captured from at least 8 random fields for each sample in one experiment. Western blot analysis
848 was repeated three times and representative blots are shown. Scale bars indicate 10 μ m (A,B) and 200 nm
849 (C,D,F,G,J,K).

850

851 **Figure 3**

852 Formation of membrane attack complex (MAC) on *C. pneumoniae*. Purified *C. pneumoniae* EBs were incubated
853 in immune- and non-immune serum and processed for IEM. Images show A) *C. pneumoniae* incubated in
854 immune-serum (NHS+) and negatively stained with PTA. B) *C. pneumoniae* incubated in immune-serum
855 (NHS+) and negatively stained with PTA and C) *C. pneumoniae* incubated in non-immune serum (NHS-) and
856 immuno-gold labeled against C5b-9. Area with disintegrated bacterial morphology was enlarged (hatched boxes)
857 demonstrating 10 nm pore-like structures (yellow arrowheads) (B+C) in close proximity to gold particles (C). D)
858 *C. pneumoniae* incubated in heat-inactivated immune-serum (HIHS+) and immuno-gold labeled against C5b-9.
859 Anti-mouse 10 nm colloidal gold was used as secondary antibody.
860 IEM images were used to quantify gold deposition on *C. pneumoniae* incubated in E) non-immune serum and F)
861 immune serum. For each chlamydial organism, the gold particle deposition was quantified by calculating a

862 bacteria-to-background ratio and the ratios were used to create scatter plots. Each dot represents the ratio from
863 one chlamydial organism and the black lines show the median ratio. Scalebars indicate 200 nm. HIHS-: heat-
864 inactivated non-immune serum.

865

866 **Figure 4**

867 Serum neutralization of *C. pneumoniae* EBs. *C. pneumoniae* inoculum were incubated in immune-serum
868 (NHS+), non-immune serum (NHS-), heat-inactivated immune-serum (HIHS+), heat-inactivated non-immune
869 serum (HIHS-) or HIHS- supplemented with 1.5 mg/ml IgG from immune-serum (HIHS-+IgG) for 30 min and
870 used to infect HeLa cells. HeLa cells were inoculated with 10.8×10^4 inclusion forming units (IFU) of NHS-
871 opsonized *C. pneumoniae* and 1.2×10^4 IFU of HIHS-opsonized *C. pneumoniae*. IFU were quantified by
872 immunofluorescence staining of chlamydial inclusions. Images were captured from seven random fields in each
873 sample using a 16X objective. Data were obtained from duplicate samples from three independent experiments.
874 All data are presented as mean \pm SD. Differences between means were analyzed by Welch's ANOVA with
875 Games-Howell multiple comparisons test. P-values < 0.05 were considered statistically significant and denoted
876 with an asterisk (*).

877

878 **Figure 5**

879 Opsonophagocytosis of *C. pneumoniae* in monocytes and neutrophils. The percentage of *C. pneumoniae*-positive
880 A) monocytes and B) neutrophils incubated with *C. pneumoniae* at MOI=10 in non-immune serum was
881 quantified by flow cytometry. The combined role of complement and anti-*C. pneumoniae* IgG in C) monocyte
882 and D) neutrophil phagocytosis was tested using immune serum. Heat-inactivated non-immune serum and heat-
883 inactivated non-immune serum supplemented with the IgG fraction from immune serum was used as control to

determine the role of IgG in phagocytosis in E) monocytes and F) neutrophils. The percentage of *C. pneumoniae*-positive G) monocytes and H) neutrophils incubated with *C. pneumoniae* at MOI=1 in immune serum and non-immune serum. Data were obtained from duplicate samples from three independent experiments except for E) + F) which was obtained from duplicate samples from two independent experiments. A minimum of 8,000 gated events were obtained from each sample. All data are presented as mean \pm SD. One-way ANOVA with Tukey's post hoc test, Welch's ANOVA with Games-Howell multiple comparison test or Welch's t-test were used to compare column means. P-values < 0.05 were considered statistically significant and denoted with an asterix (*).

NHS+: Immune serum, NHS-: Non-immune serum, HIHS+: heat-inactivated immune serum, HIHS-: heat-inactivated non-immune serum, HIHS+ IgG: heat-inactivated non-immune serum supplemented with IgG fraction from immune serum.

Figure 6

Intracellular neutralization of opsonized *C. pneumoniae* in PBMCs and neutrophils. *C. pneumoniae* (MOI=10) were incubated with A) PBMCs and B) neutrophils under different culture conditions for 30 min and cell lysates were prepared by ultrasonication. Lysates were used to infect HeLa cell monolayers and inclusion forming units (IFU) was enumerated after 48 hours by immunofluorescence microscopy. Intracellular localization of *C. pneumoniae* in monocytes after 30 min was investigated by immunofluorescence staining and confocal microscopy of C) *C. pneumoniae* and D) LAMP1. Frame E) shows an overlay image. F) Monocyte incubated with mock control. Confocal microscopy was repeated twice for all four (NHS+/NHS-/HIHS+/HIHS-) conditions and images were captured from five random fields in each sample. Quantitative data were obtained from duplicate samples from three independent experiments. Data are presented as mean \pm SD. Differences between means were analyzed by Welch's ANOVA with Games-Howell multiple comparisons test. P-values < 0.05 were considered statistically significant and denoted with an asterix (*). Scale

908 bar indicates 5 μm . NHS+: Immune serum, NHS-: Non-immune serum, HIHS+: heat-inactivated

909 immune serum, HIHS-: heat-inactivated non-immune serum

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