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Innate immune responses to *Chlamydia pneumoniae* infection: Role of TLRs, NLRs, and the Inflammasome

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Abstract

Chlamydiae are important human pathogens that are responsible for a wide range of diseases with a significant impact on public health. In this review article we highlight how recent studies have increased our knowledge of *Chlamydia pneumoniae* pathogenesis and mechanisms of innate immunity directed host defense against *Chlamydia pneumoniae* infection.

Keywords

Chlamydia pneumoniae; TLR; NLR; inflammasome

1. Introduction

Chlamydia pneumoniae is an obligate intracellular bacterium that infects the respiratory tract of many vertebrates, including humans. *C. pneumoniae* infection causes both asymptomatic and acute pneumonia and bronchitis, and has been associated with the development of and/or exacerbation of chronic respiratory diseases like obstructive pulmonary disease and asthma, and pathogenesis of infection has been associated with diverse diseases such as atherosclerosis, Alzheimer's disease and multiple sclerosis [1-4]. *C. pneumoniae* specific antibodies can be detected in the serum of up to 80% of healthy adults implicating that most individuals will eventually have contact with these organisms [5]. Recently, two reports have demonstrated that *Chlamydia* specific IgE is associated with the prevalence *C. pneumoniae* and severity of asthma or other respiratory symptom in children [6, 7]. *C. pneumoniae* pathogenesis is a complex process that depends on the cell population invaded. *Chlamydiae* undergo a distinct developmental cycle, alternating between morphologically and functionally discrete forms, the infectious elementary body (EB) and the non-infectious metabolically active reticulate body (RB). Consequently, the immune response and its regulation during infection that contribute to host resistance or susceptibility have not been fully elucidated. In this review article, we focus on recent progress in understanding the role

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of TLRs, NLRs, the inflammasome, and autophagy in directing the innate immune response to *C. pneumoniae* infection

2. Toll-Like Receptors in sensing *C. pneumoniae* infection

Toll-like receptor (TLR) family members are pattern-recognition receptors (PRR) [8] that collectively recognize lipid, carbohydrate, peptide, and nucleic acid structures that are broadly expressed by microorganisms. Currently, 10 TLRs in humans (TLR1-10) and 12 TLRs in mice (TLR1-9 and TLR 11-13) have been identified that respond to microbial and endogenous antagonists i.e. lipopolysaccharide (LPS) for TLR4, triacylated lipopeptides for TLR1/TLR2, diacylated lipopeptides and lipoteichoic acid (LTA) for TLR2/TLR6, OxLDL and amyloid- β fibrils for TLR4/TLR6, dsRNA for TLR3, Flagellin for TLR5, ssRNA for TLR7 and TLR8, as well as unmethylated CpG DNA and hemozoin for TLR9. *C. pneumoniae* is a gram-negative bacteria, suggesting that LPS is the major surface antigen of Chlamydia, against which antibodies are raised in all types of chlamydial infections, and can drive innate immune responses. However, in 293HEK cells, *C. pneumoniae* activates NF- κ B in a TLR2 dependent manner [9]. Additionally, TLR2 predominantly mediates cytokine production in dendritic cells (DC) and macrophages [9]. One study found that in bone marrow derived macrophage (BMDM) chlamydial entry is dispensable for TLR2 recognition and induction of TNF- α . *C. pneumoniae* production [10]. Indeed, TLR2 is critical for bacterial clearance and survival during *C. pneumoniae* infection *in vivo* [11, 12]. TLR4 deficient mice can respond normally to *C. pneumoniae* lung infection, although TLR2 and TLR4 doubly deficient mice displayed much higher mortality than *Tlr2* or *Tlr4* single KO [11]. However, TLR4 recognition in response to chlamydial TLR4 ligands, i.e. chlamydial LPS and heat shock protein 60 (HSP60), mediates DC maturation and macrophage foam cell formation during *C. pneumoniae* infection [13, 14]. One study using a human monocytic cell line found that TLR4 neutralizing antibody abrogated *C. pneumoniae* induced human β defensin-2 secretion [15]. The study by Rodriguez et al demonstrated that TLR2 is required for the production of chemokines MIP-2 and MIP-1 during the early phase of infection *in vivo*, however, the chemokine responses are independent of TLR2 signaling during the later stages of infection [11]. Thus, it is most likely that TLR2 and TLR4 are both important to orchestrate cytokine and chemokine responses and host defense against *C. pneumoniae* infection *in vivo*, with TLR2 playing the greater role (Figure 1). Rothfuchs et al have reported that TLR4 is required for enhanced IFN- α mRNA in BMDM [16]. In this study they have also described that only *Tlr4*^{-/-} BMDM showed increased Chlamydial progeny concomitant with the IFN- α expression but not *Tlr2*^{-/-}, *Tlr6*^{-/-}, and *Tlr9*^{-/-} BMDM [16]. TLR9 recognizes bacterial DNA containing unmethylated CpG motifs and TLR9 mRNA is upregulated in DC during intranasal Chlamydia infection [17], however, direct contribution of TLR9 during Chlamydia infection has not yet been reported. In fact, TLR9 was not necessary for *C. pneumoniae* induced TNF- α production in BMDM [10].

MyD88-independent TLR4 and TLR3 signaling adaptor molecule (TLR2, TLR4, TLR5, TLR7-9), is crucial for the initiation of effective host defenses induced by recognition of *C. pneumoniae* by TLRs and bridges innate immunity to downstream Th1 responses [18]. MyD88 mediates not only the TLR signaling pathway but also the IL-1 receptor family signaling pathway, suggesting that MyD88 may have a redundant role during Chlamydia infection. However, we will focus on the IL-1 family signaling pathway later in this review. Additionally, TRIF, the MyD88-independent TLR4 and TLR3 signaling adaptor molecule, can mediate *C. pneumoniae*-induced macrophage foam cell formation [13] as well as MyD88, although the role of TRIF is still unclear in *C. pneumoniae* infection *in vivo*.

In summary, during *C. pneumoniae* respiratory infection, it is likely that both TLR2 and TLR4 signaling via MyD88 induce early cytokine and chemokine production and control

infection. The absence of TLR2 or MyD88 results in enhanced infection that drives greater chronic inflammatory responses in the lungs.

3. NOD-like receptors in sensing *C. pneumoniae* infection

The existence of a TLR independent pathway in *C. pneumoniae* recognition was implicated by the fact that TLR4 and MyD88 doubly deficient macrophages could still produce IFN- α / γ in response to by *C. pneumoniae* infection [16]. Nucleotide-binding oligomerization domain (NOD) proteins and NOD-like receptors (NLR) have been identified as intracellular PRRs that recognize peptidoglycan (PGN) components and other danger signals. A recent study found that Nod1 siRNA reduced IFN- β expression in HeLa cells infected with *Chlamydia muridarum* [19]. However, while biochemical evidence of PGN in *C. pneumoniae* is still missing [20, 21], Chlamydiae do possess peptidoglycan (PGN) synthesis machinery and Chlamydiae is sensitive to Penicillin, a PGN synthesis inhibitor [22, 23]. Opitz et al reported that overexpression of NOD1 and NOD2 induced NF- κ B activation in HEK293 cells in response to *C. pneumoniae* (Figure 1)[24]. These data suggest that Chlamydia possesses PGN or a PGN-like structure on its cell wall that is recognized by NOD1 and NOD2 despite the lack of biochemical evidence. Perhaps the reason for this paradox could be due to low quantities of the chlamydia cell wall. Thus a biochemical approach to identify PGN with the small amount of chlamydial cell wall may prevent PGN detection. Another level of complexity to this problem could be the developmental cycle of *C. pneumoniae*, i.e. the switching between the infectious EB to the metabolic RB [25]. Penicillin treatment altered the morphology of EB and inhibited chlamydial development, indicating that Penicillin sensitive structures are newly synthesized during maturation from EB to RB [26, 27]. However, intracellular delivery of killed *C. pneumoniae* also activates both NOD1 and NOD2 dependent NF- κ B in HEK293 cells [24]. One explanation for these results could be that the Chlamydia preparation in these studies might contain noninfectious RB, and artificial intracellular delivery of noninfectious RB can signal via NOD1 and NOD2 in the cytosol. While the exact ligand structure of *C. pneumoniae* detected by the Nods have not yet been identified, NOD1 and NOD2 do play a role in KC (CXCL1) production and NO synthesis in macrophages, which are required for clearance of *C. pneumoniae* in the lung during infection [28]. A recent study found that a polymorphism of Nod1, G796A, is associated with *C. pneumoniae* infection in stroke patients [29]. Importantly, deficiency of the receptor interacting protein kinase 2 (RIP2), the NOD1 and NOD2 signaling adaptor, results in impaired NO and KC production, and delayed neutrophil recruitment, thus resulting in greater susceptibility to *C. pneumoniae* lung infection [28]. Interestingly, a *Rip2* polymorphism is also associated with severity of asthma [30]. While TLRs may be important for initial activation upon Chlamydial contact, it is likely that NODs play a role in the sequential and intracellularly triggered prolonged activation of target cells by intracellular Chlamydia.

Bronchial epithelial cells are a first line of defense during *C. pneumoniae* airway infection. Epithelial cells at the mucosal surface are capable of secreting chemoattractants and proinflammatory cytokines in response to bacterial infection, both of which are important mediators in both lung defense and inflammation. However, little information exists about which cells harbor *C. pneumoniae* during airway infection. Reservoir cells are mostly macrophages and neutrophils rather than epithelial cells during *C. pneumoniae* infection [28, 31]. Consistent with this localization, bone marrow chimera experiments demonstrated that RIP2 in BM-derived cells rather than non-hematopoietic stromal cells played a key role in host responses in the lungs and clearance of *C. pneumoniae* [28].

In summary, both Nod1 and Nod2, via Rip2 signaling, play important roles during *C. pneumoniae* infection. Importantly, the divergent pattern recognition receptors that are

expressed in distinct compartments (surface versus cytosol) can nevertheless direct cooperative responses that successfully combat invasion by common pathogens such as *C. pneumoniae*. Coordinated and sequential activation of TLRs and NODs signaling pathways may be necessary for efficient immune responses and host defenses against *C. pneumoniae*. While TLRs might be important for initial activation upon *C. pneumoniae* contact, it is likely that NOD proteins play a role in the sequential and intracellularly triggered prolonged activation of target cells by intracellular Chlamydia.

4. NLRP3 inflammasome in sensing cellular stress caused by *C. pneumoniae* infection and role of mitochondria in host responses

The NLR family in particular is involved in the recognition of host derived ‘danger’-associated molecules that are produced under conditions of cellular stress or injury. Activation of these receptors leads to assembly of high-molecular-mass complexes called inflammasome, which in turn leads to the generation of active Caspase-1, a requirement for the production of mature IL-1 β and IL-18. *Caspase-1*^{-/-} mice displayed significantly increased mortality and greater bacterial burden in the lung during *C. pneumoniae* infection [10], and during milder infections, results in an increase in fibrosis [32]. Blocking IL-1 β by administration of IL-1 receptor antagonist into wild-type mice produces a similar phenotype as observed in *Caspase-1*^{-/-} mice infected with *C. pneumoniae* [10]. On the other hand, IL-1 β administration rescued the increased mortality and bacterial burden observed in infected *Caspase-1*^{-/-} mice [10]. This was especially important during the early stages of the *C. pneumoniae* infection. These data clearly demonstrated that Caspase-1 dependent IL-1 β signaling is crucial for host defense against *C. pneumoniae* infection and that NLRP3 inflammasome is a key player in this process.

The inflammasome is a multiprotein oligomer consisting of caspase-1, ASC, and NLRs that regulates maturation of IL-1 β and IL-18. Inflammasome activation is required for many inflammatory processes and its activation requires two separate signals. NF- κ B activation, resulting from signaling such as TLRs, induces the immature form of IL-1 β and IL-18, called pro-IL-1 β and pro-IL-18 respectively, and is called ‘signal 1’. Live *C. pneumoniae* induces IL-1 β and IL-18 secretion in macrophages [33], however, the mechanism for this was unclear. In macrophages, *C. pneumoniae*-induced inflammasome is dependent on NLRP3, ASC and Caspase-1 in coordination with TLR2 and MyD88 as the 1st signal [10, 32]. Previously, NLRP3 (also known as NALP3, Cryopyrin, CIAS1) was believed to recognize pathogen-associated specific molecules through its Leucine-rich repeats (LRRs) domain. However, new data suggest that NLRP3 plays a role in sensing danger-associated molecules, not pathogen-associated specific molecules, because in addition to these pathogen-associated stimuli, a number of endogenous stress signals are reported to activate the NLRP3 inflammasome. However, the molecular mechanism for NLRP3 activation was not identified until recently. Three potential mechanisms of NLRP3 inflammasome activation were initially proposed: reactive oxygen species (ROS) generation [34], lysosomal damage [35], and cytosolic K⁺ efflux [36]. Reports on their relative contributions to NLRP3 activation have often been contradictory and have not yielded an integrated, unified model. We recently linked K⁺ efflux and mitochondrial ROS production with the generation of oxidized mitochondrial DNA (mtDNA) and found that subsequent release of oxidized mtDNA in the cytosol may directly act as a NLRP3 agonist to promote inflammasome assembly [37]. These observations are consistent with two recent reports demonstrating that mitochondria are central to NLRP3 activation [38, 39] and unveiled the mechanism of NLRP3 activation. Indeed, this is in line with the observation that *C. pneumoniae* infection leads to mitochondrial damages in alveolar macrophages [10]. These data demonstrated that experimental *C. pneumoniae* infection causes apoptosis via the mitochondrial metabolic pathway, and when it is concomitant with the first signal (NF- κ B),

IL-1 β is secreted as an alarming danger signal during apoptosis, but before cell death (Figure 2). This can be interpreted that IL-1 β is a dying message. However, it is still unclear how *C. pneumoniae* causes mitochondrial damages in macrophages. In contrast, Chlamydiae can actively inhibit both chemically and spontaneously induced apoptosis in various cell types at different time intervals [40-42]. However, most of these studies examined inhibition of apoptosis *in vitro* using a low infectious dose. Indeed, macrophages infected with higher doses of Chlamydia release large amounts of LDH, indicating significant cell death [43]. Perhaps a low dose Chlamydia provides a situation that benefits bacterial persistence and evasion of host defenses. These data suggest that upon infection of a macrophage, a battle is waged between the host and Chlamydia, where the macrophage's response would be apoptosis (and prevention of CP replication), inflammasome induction, and propagation of pro-inflammation via IL-1 β , while Chlamydia attempt to subvert and repress the apoptotic machinery, thus allowing replication and prevention of IL-1 β secretion.

Physical association of mitochondria with Chlamydiae inclusions has long been observed, but a functional link was lacking [44]. *C. pneumoniae* EB's Type III secretion system (T3SS) is a putative candidate [45, 46], however, direct studies have been hindered by a lack of genetic manipulation in Chlamydiae and the type III secretion inhibitors available have off-target effects, thus alternative strategies are required to determine the exact mechanism. Interestingly, Chlamydial effector protein associating with death domain (CADD) mimics the host TNF receptor death domain and can interact with it, inducing apoptosis upon exogenous gene expression in HEK293 cells [47], however the functional relevance of this is unknown. Low dose chloramphenicol inhibited *C. pneumoniae* induced NLRP3 inflammasome [10], suggesting that chlamydial neo protein synthesis and subsequent chlamydial metabolic change to RB may be associated with these events, however, bacterial protein synthesis and cell entry are not necessary for signal 1 through the TLR2/MyD88 [10]. Another potential reason may be that *Chlamydia pneumoniae* hijacks mitochondrial metabolic nutrition, e.g. glucose (mitochondrial glucose metabolism) [48]. Importantly, *C. trachomatis* is known to hijack cardiolipin (mitochondrial inner membrane) [49], indicating that Chlamydiae in general potentially have the ability to induce mitochondrial stress. Additionally, Chlamydia inclusion membrane (Inc) are likely candidates for host nutrient acquisition. Consistent with this idea, *C. pneumoniae* Inc Cpn0585, interacts with the endosomal trafficking molecules Rab 1, 10 and 11 [50], suggesting that Rab GTPase might be involved in Chlamydial development. *C. trachomatis* is known to interact with the motor protein dynein to facilitate transportation of the inclusion along microtubules to a perinuclear region near the microtubule-organizing center (MTOC) [51] and mitochondria also interacts with dynein to change cellular distribution [52]. *C. trachomatis* sphingolipids derived from the Golgi apparatus [53] can also affect mitochondrial sphingolipid metabolism and apoptosis [54]. Additionally, obligate intracellular bacteria have nucleotide transporters and import ATP from the cytosol of host cells, potentially making the host cell energy poor, suggesting that the traditional relationship between mitochondrion and host has been subverted by intracellular obligate bacteria [55]. The *C. pneumoniae* genome encodes an ATPase-Na⁺ pump that imports K⁺ [56], which could affect both intracellular and mitochondrial K⁺ exchanges. Iron transport is an indispensable element during *C. pneumoniae* development as well as mitochondrial biology [57]. Bafilomycin A, a potent and specific inhibitor of vacuolar H⁺ ATPase (V-ATPase), which is required for lysosomal acidification during both phagosome maturation and autophagy, inhibits *C. pneumoniae* development [58]. However, it is known that Bafilomycin A also interrupts iron transport and releases chelatable iron from endosomes/lysosomes, which is taken up by mitochondria, resulting in mitochondria damage [59]. Thus the effects of Bafilomycin A on *C. pneumoniae* growth are not clear. A recent study found that the small GTPase Rac1 regulates *C. pneumoniae* induced IL-1 β without affecting bacterial entry [60]. These observations may indicate that mitochondrial Rac1, not cellular membrane associated Rac1 is important in this

process. Mitochondrial Rac1 can import electron transfer from cytochrome c, leading to mitochondrial H₂O₂ production and modulating oxidative stress in alveolar macrophages that may be linked to pulmonary fibrosis [61].

During NLRP3 inflammasome activation, autophagy is induced in parallel [38]. Activation of autophagy inhibits the activation of the NLRP3 inflammasome, presumably through the prevention of apoptosis, and the clearance of damaged mitochondria [62]. In reality, many autophagy inducers also concomitantly induce apoptosis although this is dose, cell type, and time point dependent. Indeed, *Chlamydiae* also induces autophagy [63]. This suggests that autophagy and Apoptosis/NLRP3 inflammasome act in a ‘Yin and Yang’ manner with respect to mitochondrial damage or mitochondrial metabolic arrest (Figure 2), and that the interaction of this “Stressome” would theoretically measure the degree of stress and would determine the fate the host response and homeostasis. Taken together, these data all indicate a critical involvement of the host cellular machinery with *C. pneumoniae* infection and underscores the complexity of these mechanisms and the need for further studies.

5. *C. pneumoniae* infection and vascular diseases

The first evidence that *C. pneumoniae* infection was associated with atherosclerosis and coronary heart disease dates back to 1986 [64]. This association has now been shown by seroepidemiology, immunochemistry, PCR, electron microscopy, and tissue culture. Animal models and of atherosclerosis have also been studied to investigate the potential role of *C. pneumoniae* infection in atherosclerosis and based on these studies, antibiotic treatment was proposed for atherosclerosis [65]. While larger clinical trials failed to demonstrate any effect of macrolide antibiotics on subsequent coronary events [66], researchers still believe *Chlamydia pneumoniae* plays a role in the development of heart disease.

Chlamydia pneumoniae may induce early damage to the blood vessels, resulting in antibiotic inefficacy for this intracellular organism. Therefore, a vaccine against *Chlamydia pneumoniae* might be a more efficient and practical way to be used as an alternative to long term antibiotics to prevent coronary events if indeed this pathogen leads to accelerated atherosclerosis. The presence of *C. pneumoniae* in atherosclerotic plaques was shown by electron microscopy, PCR, and positive cultures. Furthermore, a large number of studies in hypercholesterolemic experimental models have also shown a clear acceleration of atherosclerotic lesion development following chronic respiratory infection with *C. pneumoniae* [67]. The mechanisms for this infection-induced acceleration of atherosclerosis have also been studied. *C. pneumoniae*-induced acceleration of atherosclerosis in hypercholesterolemic mouse models is significantly attenuated in the absence of TLR2, TLR4, MyD88, p55 TNF- α receptor or IL-17A [68, 69]. However, a few studies have also reported no acceleration of lesion following *C. pneumoniae* infection [70]. It is unclear why these studies did not observe the accelerated atherosclerosis, but the differences are most likely due to differences between strains of *C. pneumoniae* used as well as different study designs. Perhaps, *C. pneumoniae* infection itself is not a trigger for the disease, but instead can significantly accelerate the pathogenesis of atherosclerosis in the presence of hyperlipidemia. This is also supported by the fact that other pathogens including *Porphyromonas gingivalis*, *Helicobacter pylori*, influenza A virus, hepatitis C virus, cytomegalovirus, and human immunodeficiency virus are also considered to contribute to the acceleration of atherosclerosis [67]. In Summary, while there is a great deal of evidence for *C. pneumoniae* infection playing a role in acceleration of atherosclerosis in the presence of hyperlipidemia, there is also some evidence to the contrary. Thus there is a clear need for further well-designed experimental studies to determine the impact of *C. pneumoniae* infection on atherosclerosis and potentially other human diseases.

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Figure 1. *Chlamydia pneumoniae* sensing by TLRs and NLRs
 TLR2 and TLR4 recognize *C. pneumoniae* EB on the cell surface. NOD1 and NOD2 recognize *C. pneumoniae* RB intracellularly.

Figure 2. Cross-talk between “Chlamydia vs mitochondria”, and between “Autophagy vs Apoptosis/NLRP3 inflammasome”

C. pneumoniae EB-RB injects the effector(s) that induce mtROS and leads to apoptosis. The *C. pneumoniae* RB acquires nutrition, lipids, and energy that triggers mtROS and Autophagy in parallel. Autophagy/mitophagy inhibits apoptosis and NLRP3 inflammasome activation but if autophagic regulation is not sufficient to remove the damaged mitochondria and stabilize intracellular homeostasis, Apoptosis will occur. In the presence of signal 1, released oxidized mtDNA binds to NLRP3, resulting in inflammasome activation and IL-1 β secretion.