Chlamydophila pneumoniae
Mechanisms of target cell infection and activation

Matthias Krüll1, Matthias Maass2, Norbert Suttorp1, Jan Rupp3

1Department of Internal Medicine/Infectious Diseases, Charité, Universitätsmedizin Berlin, Berlin, Germany
2SALK Labor, Salzburger Landeskliniken, Salzburg, Austria
3Institute of Medical Microbiology and Hygiene, University of Lübeck, Lübeck, Germany

Summary
Chlamydophila (Chlamydia) pneumoniae, a gram-negative obligate intracellular bacterium, is a widespread respiratory pathogen. Chronic C. pneumoniae infection has been suggested as a trigger/promoter of inflammation that may result in vascular lesions. Although the genome of C. pneumoniae has been sequenced completely this information has not yet led to an understanding of the mechanisms of acute infection and target cell activation nor to the identification of potential chlamydial virulence factors. Intriguingly, current antibiotic treatment options for acute chlamydial infection were proven to be ineffective with respect to clinical outcome in different groups of atherosclerotic patients. The reason might be that primary infection of vascular smooth muscle cells and blood monocytes with C. pneumoniae resembles rather a persistent, antibiotic-resistant, than an active infection. In this review we will focus on the importance of putative host cell receptors for C. pneumoniae and subsequently activated signal transduction pathways.

Keywords
Atherosclerosis, endothelial cells, infection / bacterial, viral, signal transduction, bacteria

Chlamydophila pneumoniae and vascular diseases
Chlamydophila (Chlamydia) pneumoniae, a gram-negative obligate intracellular bacterium, is a widespread respiratory pathogen causing diseases ranging from minor sinusitis to severe pneumonia (a lymphocyte dominated alveolitis, (1–4)). Up to the age of 20, almost 70% of all adults are seropositive for C. pneumoniae, reinfections, however, are common. Chronic-persistent or recurrent Chlamydophila pneumoniae infections may be a trigger and promoter of inflammation which may cause vascular lesions and atherosclerosis (5–9). The field is troubled by the “hen vs. egg” problem and causative proof is difficult because apart from different anti-chlamydial isotypes of antibodies there are no good markers to differentiate between new versus old (IgM vs. IgG) as well as acute versus chronic persistent (IgM vs. IgA) C. pneumoniae infection. The theory, however, is supported by

– a serological association between C. pneumoniae infection and coronary heart disease as well as other vascular diseases (arterial occlusive disease, carotid artery stenosis and stroke (5, 10–12)),

– the demonstration of C. pneumoniae in atherosclerotic plaques by electronmicroscopy, immunocytochemistry, PCR, and isolation of viable chlamydia (indicating a productive chlamydial infection, (7, 8, 13–17)), and

– different animal models, demonstrating that intranasal infection of mice and rabbit with C. pneumoniae leads to pneumonia, perimyocarditis, septic circulatory dysregulation and – more delayed – systemic spread of chlamydia in spleen, lymphnodes, peritoneum and atherosclerotic plaques of arterial blood vessels. New Zealand White rabbits repetitively infected intratrachealy with low doses of chlamydia developed significantly more atherosclerotic alterations in different arterial vessels (aorta, carotids, coronary arteries) than sham infected animals (using sterile saline solution) or rabbits infected with Mycoplasma pneumoniae (which results in similar pathological changes in the lung) (18–23).

Current antibiotic treatment options for acute chlamydial infection, however, were proven to be ineffective with respect to clinical outcome in different groups of atherosclerotic patients (WIZARD, AZACS, ACES or PROVE-IT study (24–30)). Interpre-
tation of these clinical trials are challenging as definite markers of chronic vascular *C. pneumoniae* infection are still missing and serologic testing seems to be inaccurate in patient populations with high chlamydial IgG seroprevalence. Moreover, the persistent state of chlamydial infection, as observed in blood monocytes, shows high resistance against macrolides, tetracyclines and rifampicin (31–33) and aggravates the prediction of pathogen eradication from (persistently) infected patients. "Chlamydial persistence" has been described as a long-term association between chlamydiae and their host cells in which these microorganisms remain in a viable but culture-negative state with a notably reduced metabolism. Because of the reduced or negative ribosomal cell activities, these bacteria have no adequate targets for the known chlamydia-targeting antibiotics.

Atherosclerotic lesions develop upon chronic inflammatory reactions of the endothelial cells and the vascular intima ("response to injury"-theory (34)). The role of *C. pneumoniae* in atheroma formation has not been studied in detail. Although chlamydiae may reside and replicate in different cell types involved (monocytes, macrophages, smooth muscle cells, fibroblast, and endothelial cells (35–38)) and induce a chronic immune activation, little is known about the mechanisms of *C. pneumoniae*-induced target-cell alteration.

**Role of receptors for chlamydial infection of target cells**

Airway derived organisms may be able to spread systemically via at least two different ways: (I) by direct access to the blood stream following a severe pulmonary infection and causing a short interval of chlamydial bacteremia or (II) carried within recirculating monocytes, macrophages and/or lymphocytes from the respiratory tract (23, 39, 40). Using a model of intranasally infected White New Zealand rabbits, Gieffers et al. were recently able to identify alveolar-macrophages as carriers for the systemic spread. AM transported the pathogen to the peribronchial lymphatic tissue, and subsequently *C. pneumoniae* entered the spleen and the aorta via systemic dissemination by peripheral blood monocytes (23). M. Maass et al. isolated different *C. pneumoniae* strains from endarterectomy and bypass samples of patients with severe coronary heart disease (8). Subsequently, *C. pneumoniae* are able to infect different target cells involved during development of atherosclerotic plaques. Infection or invasion is an active process requiring the existence of viable chlamydia, heat- or UV-inactivated bacteria are not able to invade target cells. However, there is still limited knowledge of the mechanisms of chlamydiae entry into host cells. The chlamydial growth cycle is initiated when an infectious elementary body (EB) attaches to a susceptible target cell, promoting entry into a host cell-derived phagocytic vesicle.

EB are internalized, dissociate from the endocytotic pathway by actively modifying the vacuole to become fusogenic with exocytic vesicles (31, 41). Coombs and Mahony suggested a receptor-mediated induction of specific cell signalling by chlamydiae as an essential step in *C. pneumoniae* invasion of epithelial cells (42). They could show that MEK-dependent phosphorylation and activation of ERK1/2, followed by PI 3-kinase-de-
Importance of specific receptors for activation of target cells

Toll-like receptors
The innate immune system relies on surveillance proteins to recognize pathogens by sensing pathogen-associated molecular patterns. A well-studied group of pattern-recognition receptors are the Toll-like receptors (TLRs), which are mainly expressed on the surface of a broad diversity of cells. Several recent studies demonstrated the involvement of Toll-like receptor-2 (TLR2) and -4 (TLR4) in initiation of innate immune cell activation by *Chlamydophila pneumoniae* or chlamydial components (56–62). Prebeck et al. demonstrated that *C. pneumoniae*-mediated secretion of cytokines as well as translocation of nuclear factor-κB (NF-κB) in dendritic cells was dependent on the presence of TLR2 and independent from TLR4 with the exception of IL-12p40 secretion (58). These results were supported by Netea et al. demonstrating a *C. pneumoniae*- (sonicated bacteria) induced expression of pro-inflammatory cytokines TNFα and IL-1β in PBMC through TLR2, but not TLR4 or CD14 (57). Using a TLR4-antagonist, Sasu et al., however, suggested, that *C. pneumoniae* and isolated chlamydial heat shock protein 60 are potent inducers of human vascular smooth muscle cell (VSMC) proliferation via a rapid TLR4-mediated activation of ERK1/2 (59). These results were supported by Haralambieva et al. demonstrating that an anti-TLR4 antibody was able to abolish *C. pneumoniae*-induced ERK1/2 activation in human fibroblasts, while an anti-TLR2 antibody had no effect in their system (63). Moreover, the situation is complicated by different reports demonstrating a TLR independent target cell activation by *C. pneumoniae* (62, 64). In addition, little is known about possible chlamydial virulence factors activating the TLR. Miethke and coworkers found that purified recombinant heat-shock protein 60 (chsp60) from *C. pneumoniae* stimulated bone marrow derived dendritic cells (BMDC) in a TLR2– and TLR4-dependent fashion similar to the whole microorganism and suggested that chsp60 might act as an important mediator of inflammatory responses (61, 65). These results were supported by Bulut et al. (56). Interestingly, Erridge et al. could demonstrate that isolated lipopolysaccharide from *Chlamydia trachomatis* (strain LGV-1) also induced a TLR2-mediated NF-κB-activation (66). TLR2 therefore seems to play a predominant role in TLR-mediated target cell activation by chlamydia or isolated chlamydial virulence factors. Endothelial cells predominantly express TLR4, expression of TLR2 is still controversially discussed (67). They are, however, highly susceptible for infection and activation by *Chlamydia pneumoniae* or isolated chlamydial heat-shock protein 60 (Krůll et al., unpublished results). Moreover, additional own studies demonstrated that chlamydia were able to infect and substantially activate TLR2 or -4 negative cells (e.g. HEK293-cells) suggesting the existence of additional receptors.

Nod-proteins
The recently identified nucleotide-binding oligomerization domain (Nod) proteins, also called caspase-recruitment-domain (CARD)-containing proteins, are molecules that have been implicated in intracellular pattern recognition (68, 69). More than 20 proteins that are homologues to Nod1 have been identified in the human genome, but only a few members of this growing family are functionally characterized (69). Via a functionally active CARD-domain, Nod1 has been described to mediate the activation of NF-κB induced by peptidoglycans containing mesodiaminopimelate acid found mainly in gram-negative bacteria (70, 71), whereas Nod2 (CARD15) mediates responsiveness to the muramyldipeptide MurNAc-L-Ala-D-Isogln conserved in peptidoglycans of basically all bacteria (72, 73). In contrast to Nod1 which could be detected in a multitude of tissues including endothelial cells, Nod2 has only been demonstrated in dendritic and epithelial cells (74, 75). Little is known about the Nod-dependent signalling cascade activated by ligand-binding. There is evidence, that oligomerization of Nod1 (and -2) induces the recruitment of its interacting partner Rip2 kinase (RICK or CARDIAK). Subsequent activation of NF-κB therefore relies on activation of downstream effectors of RICK like the inhibitor of NF-κB kinase (IKK) complex (74, 76, 77). In a recent study, we were able to demonstrate, that in endothelial cells (EC), Nod1 played a dominant role in triggering a *Chlamydia pneumoniae*-mediated inflammatory process (78). Viable, but not heat- or UV-inactivated chlamydia were able to infect endothelial cells and to induce a prolonged IL-8 expression in human umbilical vein (HUVEC) human arterial endothelial cells (HAEC) up to 96 h post infection. Preincubation of *C. pneumoniae* with polymyxin B in order to inactivate lipopolysaccharides did not significantly reduce IL-8 expression. The fact that intracellular infection of endothelial cells appeared to be essential for IL-8 induction raised the question if intracellular immune receptors might be involved. In line with this hypothesis, we were able to detect Nod1-mRNA in HUVEC and HAEC by RT-PCR. In addition, analyzing different epithelial, monocytic and lymphocytic cell lines, we found, that human endothelial cells seemed to express the highest levels of Nod1. Nod2-mRNA could hardly be found in HUVEC (78, 79). Nod1 gene silencing by siRNA blocked the IL-8 production induced by *C. pneumoniae* in HUVEC and HAEC. In addition, we demonstrated that *C. pneumoniae* activated a Nod–1 and 2-mediated signal transduction pathway in HEK293 cells involving Rip2, but not MyD88. Since no differences in the downstream signalling of Nod1 and Nod2 were observed so far, both receptors might substitute for each other in some cases for intracellular recognition of bacteria in varying tissues (68, 69).

In our system, heat stable chlamydial components were responsible for the demonstrated effects. However, it remained unclear, which pathogen-associated molecular patterns (PAMP) of the chlamydial surface interfere with the Nod-proteins. Nod proteins have so far been associated with recognition of different types of peptidoglycans (70–73). Although recent studies suggest a functional peptidoglycan pathway in chlamydia (80, 81), a clear cut biochemical evidence for the synthesis of peptidoglycans in chlamydia is missing (82, 83). Chlamydia, however, are sensitive to antibiotics that inhibit peptidoglycan synthesis (84). This phenomenon has been referred to the “chlamydial anomaly”. Our finding that *C. pneumoniae*-induced a Nod-mediated endothelial cell activation via heat-stable components could be interpreted in at least two different ways: 1) the chlamydial cell wall does indeed contain peptidoglycan or peptidogly-
can-like structures. This hypothesis is in accordance with several studies suggesting the expression of peptidoglycan-like structures not on the surface of elementary bodies but—after invasion of the target cells—on the subsequently developed reticular bodies (85). 2) Nod proteins act as receptors for molecules other than peptidoglycans. Proteins such as heat-shock proteins or major outer membrane-proteins (MOMP’s) could be involved in Nod activation since the minimal motif recognized by Nod1 is a dipeptide containing diaminopimelic acid (70), and chlamydia could possibly synthesize this dipeptide in a peptidoglycan-independent way. In line with this hypothesis are preliminary data suggesting the recognition of recombinant chsp60 by Nod proteins in HEK293 cells (Krüll et al., unpublished data).

TLR2 was suggested to be more important than TLR4 for recognition by and activation of innate immune cells by chlamydia (57, 58). In a system of TLR2-overexpressing HEK293 cells, we could demonstrate that viable and heat-inactivated C. pneumoniae were able to induce NF-κB-activation upon cell contact, when “applied” from the extracellular side. Heat-killed chlamydia, however, failed to activate NF-κB in Nod1 or Nod2-overexpressing HEK293 cells upon extracellular challenge, indicating that Nod proteins serve as intracellular receptors. These considerations are in line with our observation, that viable, but not heat-inactivated chlamydia were able to induce a marked release of IL-8 from infected endothelial cells, since HUVEC express Nod1 but hardly TLR2 (67). Thus, Nod proteins rather than TLR2 appear to contribute to C. pneumoniae-mediated endothelial cell activation.

It still remains unclear how chlamydia, intracellularly located in endosomal inclusion bodies, can activate Nod proteins. The Nod proteins belong to the family of cytoplasmatic pattern recognition receptors. Until now, however, there are no studies using e.g. confocal laser scanning or electron microscopy demonstrating a precise location of the Nod proteins in the cytoplasm or in a possible association with certain intracellular organelles like chlamydia-containing vacuoles. One might speculate about chlamydial cell wall components or other virulence factors released into the cytoplasm to get in contact to subsequently activate Nod proteins. Several recent studies have demonstrated that Chlamydiae have a type III secretory apparatus (86, 87). This may facilitate transport of potential virulence factors such as “chlamydial proteasome/protease-like activity factor” (CPAF), MOMP-1 or chsp60 to the host cytoplasm (88). Moreover, several other authors were able to demonstrate that even in the persistence phase, chlamydia are able to produce and secrete proteins into the cytoplasm (31, 89). Further studies are now required to determine the relationship between distinct steps of the chlamydial development cycle, importance of different chlamydial virulence factors in different phases of chlamydial infection (acute, chronic, persistent) and initiation of host cell signaling pathways via different (extra- and intracellularly located) receptors to develop (chronic) inflammatory processes (e.g. atherosclerosis) in the endothelium. The recently established Nod1 and Nod2 knock-out mice will now be of outstanding value for future studies (70, 71, 90).

**C. pneumoniae-mediated signal transduction**

Incubation of endothelial cells with C. pneumoniae activated different signal transduction pathways, an overview is summarized in Figure 1.

Members of the mitogen activated protein kinase (MAPK) family are ubiquitously expressed and activated in response to a variety of stimuli. They have been demonstrated to be key players mediating a proinflammatory and prothrombotic phenotype (p38 MAPK, p38-MAPK phosphorylation and activation of all three MAPK pathways (ERK1/2, p38, and JNK) occurred within 10–15 min of chlamydial contact with endothelial cells. This immediate cell activation suggests that chlamydial attachment is sufficient to initiate an endothelial response and that bacterial uptake may not be required. Endothelial cell activation by C. pneumoniae was followed by enhanced expression of a multitude of (pro-) inflammatory mediators (e.g. adhesion molecules, cytokines, chemokines, growth factors (79, 91, 95–101)) including IL-8 and ICAM-1 in endothelial cells. Inhibition of the MAPK-pathway with specific inhibitors suggested that chlamydial-stimulation of p38-MAPK and to a minor degree ERK1/2, but not JNK appeared to be of particular importance for IL-8 secretion. The importance of p38-MAPK for C. pneumoniae-mediated IL-8 expression could be substantiated by demonstrating that overexpression of upstream located p38-MAPK-activating MAP kinase kinases-6 (MKK6, (79)) induced a more sustained release of IL-8 from C. pneumoniae-stimulated endothelial cells.

![Figure 1: Scheme of the supposed signalling cascades induced by C. pneumoniae infection in human cells (TyrKi, tyrosin kinase).](image_url)
Interestingly, in endothelial cells expression of intercellular adhesion molecule-1 (ICAM-1) could only slightly be reduced by p38-MAPK or ERK1/2-inhibition, suggesting that additional signal transduction pathways distal to MAPKs or two or more parallel signalling pathways are operative in C. pneumoniae infected HUVEC (79). These data are in accordance with results from Vielma et al. They were able to demonstrate that inhibition of the classic MAPK pathway (p38-MAPK, ERK1/2, SAPK/JNK) did not suppress C. pneumoniae-induced ICAM-1 expression on human arterial endothelial cells (HAEC). Moreover, they showed, that PKC is activated in HAECs on infection with C. pneumoniae. Activation of PKC leaded to NF-κB activation, and that, in turn, to an increased transcription of the ICAM-1 gene (102).

Intracellular infection of target cells induced (a more delayed) activation of the IκB kinase complex (IKK) with degradation of IκBz and activation and translocation of NF-κB into the nucleus followed by expression of different NF-κB-dependent (pro-) inflammatory mediators (e.g. adhesion molecules, IL-6, IL-8, MCP-1, RANTES (91, 96, 100, 103)). This C. pneumoniae-induced NF-κB-translocation was dependent on activation of p38-MAPK or ERK1/2 but not SAPK/JNK (79).

Further upstream from the MAP kinase signalling cascades, C. pneumoniae has been found to stimulate cell-membrane associated Rac1 and RhoA from the class of small G-proteins (101). C. pneumoniae infection induced prenylation of Rac1 and RhoA in coronary artery smooth muscle cells over 48 hours, with subsequent NF-κB activation and enhanced RANTES and MCP-1 mRNA expression (101). Inhibition of the C. pneumoniae induced pro-atherosclerotic signalling in vascular cells was obtained by pre-treatment with statins, a class of lipid lowering drugs with proven immunomodulatory capacity. Pre-incubation of the cells with cerivastatin not only reduced Rho family GTPase activation, but also blocked RANTES and MCP-1 protein secretion from infected cells (101). Moreover, efficient inhibition of NF-κB mediated signalling by statins has also been observed in C. pneumoniae infected macrophages and vascular endothelial cells (97).

Likewise intensive analysis of the C. pneumoniae induced proliferation of smooth muscle cells and the signalling cascades involved has not been performed so far. Miller et al. could previously show that direct infection of vascular smooth muscle cells with C. pneumoniae resulted in cell proliferation and activation of NF-κB and AP-1 (104). An initial approach to investigate the pathway for C. pneumoniae-induced cell proliferation was made by Sasu et al., who demonstrated the TLR4 mediated activation of the ERK1/2 as a central step in this process (59). We could recently identify the induction of the immediate early gene Egr-1 via ERK1/2 as crucial for the enhanced proliferative activity of C. pneumoniae infected VSMCs (105). Pre-treatment of VSMCs with siRNA against Egr-1 not only blocked Egr-1 mRNA expression but also reduced proliferation of infected VSMCs. The induction of Egr-1 in arterial vasculature through Chlamydiae was confirmed in a rat aortic ring model and in a mouse model using C. pneumoniae infected blood monocytes as a vector (105).

Investigations on C. pneumoniae induced signalling in vascular cells have predominantly been limited to the acute phase of the infection, comprising 48 hours after infection. As an intracellular pathogen, C. pneumoniae has the ability to survive within a host cell for several days, either by the induction of a persistent infection (characterized by an aberrant intracellular morphology and low metabolism) or by inhibition of host cell apoptosis. Different models of persistent C. pneumoniae infection in host cells have been established, using IFN-γ, penicilline treatment, or iron depletion (89, 106, 107), but less is known about the induction of pro-atherosclerotic signalling cascades in persistent chlamydial infection. Further studies are urgently needed, because there is some evidence that primary infection of vascular smooth muscle cells and blood monocytes with C. pneumoniae resembles a persistent rather than an active infection (33, 108). This has to be considered in clinical studies aiming to eradicate vascular chlamydial infection, as the persistent state of chlamydial infection of blood monocytes can not be eradicated by antibiotics normally used to treat replicative C. pneumoniae infection of lower respiratory tract infections (33).

A second strategy of Chlamydiae to prolong intracellular survival is to inhibit pro-apoptotic pathways of the infected host cells. It is thought that C. pneumoniae can protect infected cells by inhibiting the release of cytochrome c from mitochondria and upregulate the expression of the anti-apoptotic mediators IAP and MCL-1. Thus, protection against apoptosis may be a strategy that the chlamydiae use to maintain a persistent, chronic infection. For a more extended view of the mechanisms involved in Chlamydia interference with apoptosis signalling in host cells please refer to the review by Byrne et al. (109).

Effects on target cells involved in the development of cardiovascular diseases were specific for Chlamydothila pneumoniae since infection of e.g. endothelial cells or smooth muscle cells with Chlamydia trachomatis, serovar K, did not induce phosphorylation and activation of MAPK or expression of proinflammatory and proatherogenic marker (no release of endothelial IL-8, no upregulation of ICAM-1). Infection of HUVEC with C. trachomatis leads to the development of many small atypical serovar K inclusions. This “suboptimal” infection procedure could effect the results and may be due to the fact, that endothelial cells are not primary target cells and that therefore serovar K – although inducing a productive infection – might not grow very well in HUVEC (79). However preliminary data demonstrated that C. trachomatis serovar E and L2 induced similar results. In addition May et al. could demonstrate that C. trachomatis, serovar L2, was also very capable of infecting human monocytes, but did not induce monocyte activation and adhesion to endothelial cells, indicating a unique activation pathway for C. pneumoniae (110).

Perspective

Overall, the data presented suggest that Chlamydothila pneumoniae are able to infect a multitude of target cells and subsequently to activate and trigger a cascade of early and prolonged signal transduction events that may relate to the development of atherosclerosis. After initial attachment chlamydiae are internalized; they dissociate themselves from the endocytic pathway by actively modifying the vacuole to become fusogenic with exocytic vesicles. Interaction with this secretory pathway ap-
pears to provide a pathogenic mechanism that allows chlamydiae to establish themselves in a site that is not destined to fuse with lysosomes. This persistent infection that is refractory to current antimicrobial treatment schemes triggers a plethora of pathological signalling events, which have been highlighted recently. Further studies are required to determine the relationship between distinct steps of initial attachment, the chlamydial development cycle, importance of different chlamydial virulence factors and initiation of host cell signalling pathways that could lead to target cell damage and inflammation which in turn may result in or may promote chronic diseases like atherosclerosis.

Acknowledgements
The authors apologize for not citing more original manuscripts due to space limitations and hope that the cited reviews will provide more detail. This work was in part supported by the Deutsche Forschungsgemeinschaft to M.K., N.S. (Kr 2197/1–2) and M.M. (Ma 2070/4–2) as well as by the Bundesministerium für Bildung und Forschung (BMBF) to N.S. (BMBF-NBL3 and -CAPNETZ).

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