

Theme Issue Article

Chlamydia pneumoniae adversely modulates vascular cell properties by direct interaction with signalling cascades

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Summary

Due to its dependence on intracellular development *Chlamydia pneumoniae* has developed numerous strategies to create an adequate environment within its host cells ensuring both chlamydial reproduction and target cell survival. The bacterium that has been related to atherogenesis due to its presence in vascular tissue is able to enter a persistent state of chronic infection in the vasculature that escapes antibiotic targeting. Ingestion of the bacterium results in severe modifications and reprogramming of signalling pathways and the metabolism of the host cell. Processes range from the prevention of direct lysosomal destruction of chlamydial inclusions to the inhibition of host cell apop-

toxis and an enhanced cellular glucose uptake to maintain energy-consuming mechanisms. Furthermore, infection regularly causes the development of a proinflammatory and proproliferative phenotype in the host cell *in vitro*, *ex vivo* and *in vivo* and own new findings suggest a detrimental proliferative loop within vascular cells upon a modified endothelin-I axis demonstrating a potential for proatherosclerotic processes in early and progressed atherosclerosis. This review displays crucial mechanisms of *Chlamydia pneumoniae*-induced interactions with vascular host cell signalling cascades with an emphasis on mitogenic and inflammatory processes as well as target cell activation.

Keywords

Chlamydia pneumoniae, signal transduction, angiogenesis, inflammation, atherosclerosis

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Introduction

The obligate intracellular bacterium *Chlamydia pneumoniae* (*Cp*) is a respiratory pathogen that causes acute pneumonia and other infections of the respiratory tract ranging from mild pharyngeal symptoms to severe infections. According to epidemiological studies the seroprevalence of *Cp* is up to 80% in the adult population and it is maintained by numerous reinfections during lifetime (1). Since the bacterium was frequently isolated from atherosclerotic tissue but not from healthy vasculature *Cp* may either be related to atherosclerotic development or be a coloniser of preinjured vascular tissue.

The unique intracellular life cycle of chlamydiae includes two different phenotypes: the infectious but non-replicative elementary bodies (EBs), which after ingestion turn into the non-infectious but replicative reticulate bodies (RBs). Apart from this productive physiological development *Cp* is able to enter a non-replicative persistent state within the host cell. Chronic persist-

ent *Cp* form morphologically aberrant inclusions and are completely refractory to common antibiotic treatment schemes, which makes chlamydial eradication difficult if not impossible. Due to its intracellular niche within a non-lysosomal vacuole the pathogen has developed numerous strategies to ensure its survival in the host cell. Chlamydiae are able to fundamentally modulate host cell properties and signalling cascades in order to provide an adequate intracellular environment, effects that are thought to be mediated by secreted chlamydial effector proteins. Type-III-apparatus-secreted chlamydial proteins were also shown to be crucial for intracellular chlamydial development (2). A novel finding is for example that the bacteria are able to effectively interfere with complex host cell structures and might induce fragmentation of the Golgi apparatus, an effect that provides lipid transport to and propagation of the intracellular bacteria (3). Furthermore, *Cp* bypasses cellular defence mechanisms by inhibition of cellular destruction or apoptosis (4–6). These processes maintain a *Cp*-friendly environment but alter

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the host cell phenotype towards inflammation or proliferation. Own recent studies have shown that the *Cp* adversely interferes with the potent mitogenic Endothelin-1 (ET-1) axis *in vitro* and *ex vivo* eliciting a detrimental proliferative loop in vascular endothelial cells (7).

Even under hypoxic conditions, a pathological hallmark in inflammatory tissue, *Cp* is able to survive and to take advantage of host cell properties. By direct interaction with the transcription factor hypoxia-inducible factor-1-alpha (HIF-1 α) *Cp* takes control of the cellular oxygen sensing mechanisms and activates the glucose uptake in the early phase of its development, which is thought to be needed for efficient intracellular replication and differentiation (8). Here we give a brief overview on recent molecular findings in chlamydia-induced vascular damage.

Chlamydia pneumoniae and atherosclerosis

The comprehension of the pathogenesis of atherosclerosis has changed fundamentally during the past 20 years. Models suggesting a static system of atherogenesis including macrophage accumulation and thrombocyte aggregation at the vessel wall were extended to a more dynamic progression pattern. Today three key pathomechanisms concerning atherogenesis can be named: endothelial dysfunction, proliferation of vascular cells and inflammation of the vascular wall, the latter being present in all stages of atherosclerosis.

In 1988 a seroepidemiologic study could relate *Cp* to atherosclerotic processes (9). Patients with coronary artery disease and myocardial infarction showed significantly enhanced anti-*Cp* antibodies compared to a control group. Encouraged by this finding, to date more than 50 clinical studies of variable design followed and were able to detect chlamydial infection in atherosclerosis (10–12). The bacterium is present in atherosclerotic lesions as indicated by immunohistochemical staining of aortic lesions, electron microscopy and PCR detection (13–15). Recultivation of *Cp* from these lesions made clear that the bacterium is not only present antigenetically in the vasculature but also as a viable organism that chronically interacts with injured vasculature. However, the pathogen was not retrieved from unaffected vasculature. These findings might indicate a tropism of *Cp* to pre-injured tissue – a fact that is highly important discussing a causative role of the bacterium in atherogenesis assuming the impairment of pre-existing atherosclerotic lesions by *Cp*. Primarily known as a respiratory pathogen *Cp* can be found in epithelial and lymphoepithelial tissue of the lung where it interacts with peripheral blood mononuclear cells (PBMC). Infected PBMC seem to act as a vehicle for the bacterium to disseminate towards peripheral vascular tissue eventually causing the infection of vascular endothelial cells and smooth muscle cells (16–19). *Cp* infection almost invariably causes an inflammatory phenotype within its host cells via enhanced expression of various inflammatory chemokines and cytokines (Table 1). Vascular endothelial cells and smooth muscle cells, which under *in vitro* conditions are highly susceptible to *Cp*, develop a proatherosclerotic phenotype upon chlamydial infection underlining a possible role of the bacterium in atherogenesis.

Atherosclerosis is obviously a multifactorial disease on base of pathological signalling within the vasculature. Beyond the

common risk factors such as hypertension, hyperlipidaemia, diabetes mellitus and smoking infectious agents like cytomegalovirus, herpes simplex virus or *Helicobacter pylori* have been implicated in causing proatherosclerotic processes. However, *Cp* remains the only pathogen that could be recovered reproducibly and viable from atherosclerotic lesions and that has already been shown to modulate infected vascular cells in a angioproliferative and proinflammatory manner.

Chronic infection with *Chlamydia pneumoniae* and antibiotic treatment

Due to their unique replication cycle that involves different phenotypes and requires viable host cells, chlamydiae were able to develop and undergo evolution in a separated intracellular niche. The physiological replication cycle results in acute infection and cell death as seen in *Cp*-pneumonia. However, *Cp* may also enter a persistent state. Hallmarks of this chronic *Chlamydia* infection are an atypical ultrastructural morphology indicated by aberrant inclusions with few but enlarged bacterial structures (20, 21). Persistent *Cp* are characterised by a modified protein and gene expression profile and reduced metabolic activity (22). However, the type III secretion apparatus of the bacterium remains fully functional and chlamydial effector proteins keep being released into the host cell cytoplasm (23) establishing an adequate and *Cp*-customised environment by interaction with their human target proteins. An *ex vivo* study of chronically infected PBMC demonstrated *Cp* to be completely refractory to the otherwise highly potent drugs azithromycin or rifampin (24). First-choice anti-chlamydial antibiotics may even induce chlamydial persistence (17). Failure of a benefit of anti-chlamydial treatment in vascular *Cp*-infection could also be shown *in vitro*: Hyperlipidaemic ApoE knock-out mice which were treated with azithromycin for six weeks showed no amelioration in *Cp*-induced acceleration of atherosclerotic lesion development (25). Interestingly, statins, while exerting no discernible antichlamydial effect on replicating or persistent chlamydiae, were able to suppress the proinflammatory response in *Cp*-infected human vascular smooth muscle cells by blocking Rac1 and RhoA prenylation (26). The intractable persistent state of *Cp* apparently present in the vascular wall may explain the complete failure of large clinical trials as ACADEMIC, WIZARD and ACES in showing any clear amelioration of progredient coronary artery disease under treatment with anti-chlamydial drugs (27–30). The inherent problem with these trials is that they were initiated without addressing the ability of *Cp* to enter a chronic persistent state of infection aside from its physiological development cycle.

Angiogenic effects of *Chlamydia pneumoniae*-infection in vascular cells

Cp strongly interacts with the tissue infected and modulates host cell properties leading to promitogenic and proinflammatory signalling in vascular cells. Rupp et al. were able to demonstrate that *Cp* activates the early growth response gene-1 (Egr-1), a zinc finger transcription factor that is linked closely to mitogenic pro-

cesses (18). Egr-1 activates chemokines like tissue factor or FGF-2, which stimulate the proliferation and migration of vascular cells crucial to atherogenesis (31, 32). Chlamydial infection caused an enhanced expression of Egr-1 at early timepoints and subsequent nuclear translocation of the transcription factor *in vitro* and *in vivo*. In contrast to previous studies, which also described an involvement of the p38 mitogen-activated protein kinase (MAP kinase) in Egr-1 regulation, effects here were exclusively mediated via the p44/42 MAP kinase. *Cp*-infected human coronary artery smooth muscle cells (CASMC) could be shown to proliferate in an Egr-1-dependent manner and transfection of CASMC with siEgr-1 significantly abrogated mitogenic effects. To verify Egr-1-inducing effects through *Cp in vitro* Egr-1 enhancement was additionally confirmed *ex vivo* in a rat aortic ring model system. Rat vasculature acquired focal chlamydial infection and Egr-1 mRNA was measured by RT-PCR in homogenised aortic tissue within 2 hours. Coincubation with heat-killed *Cp* caused no enhanced Egr-1 mRNA expression indicating that viable bacteria are needed for this effect.

As PBMCs are supposed to act as a vehicle to disseminate *Cp* from the lung to the vasculature a more dynamic mouse aorta *in vivo* model was established. Chronically infected and non-infected PBMC from C57BL/6 mice were intravenously reinjected into the animals and investigation of total aortal tissue showed a distinct increase of Egr-1 mRNA in *Cp*-infected but not in control mice. This might also indicate that not only direct bacterial contact leads to an enhanced Egr-1 expression in the vasculature but also indirect mechanisms via complex cell-to-cell interactions between infected PBMC and vascular cells.

We have recently shown a new aspect in *Cp*-mediated reprogramming of proliferative signalling cascades apart from the NF κ B-dependent Egr-1 pathway (7). The bacterium was found to effectively interact with the mitogenic endothelin-1 (ET-1) axis *in vitro* and *ex vivo* causing a detrimental proliferative loop in vascular cells. The potent vasoconstrictor ET-1, physiologically originating from the endothelial layer, is a major regulator of the vascular tone. It maintains its effects via the two receptors endothelin-A/-B-receptor (ETAR, ETBR), which show a typical distribution within the vasculature: ETAR, predominantly located on smooth muscle cells mediates strong vasoconstrictive effects and has mitogenic properties. ETBR, however, which is mainly expressed on endothelial cells clears ET-1 from the blood and transduces vasodilatation. *In vitro* studies revealed *Cp*-infected CASMC to become a significant source of ET-1 in a p38 MAP kinase-dependent manner, whereas infected HCAEC remained unaffected. Furthermore, we could show *Cp* to cause a switch in the ET-1 receptor distribution. *Cp*-infected HCAEC but not CASMC significantly overexpressed mitogenic ETAR, an effect that could be abrogated by transfection with siETAR. *Cp*-infected and hence ETAR-expressing HCAEC were highly susceptible to an ET-1 stimulus and recombinant ET-1 caused an ETAR-dependent HCAEC proliferation that was again abrogated by transfection with siETAR. The mitogenic properties of *Cp* mediated via enhanced ET-1 susceptibility in ETAR-expressing HCAEC could completely be abolished by the selective ETAR-antagonist BQ-123, which is already in use in clinical trials on atherosclerotic treatment (33). *Ex vivo* experiments using a mouse aortic tissue infection model showed comparable

results as *Cp*-infection of total mouse aortic tissue caused a disarrangement of the ET-1 axis resulting in a distinctly enhanced ET-1 expression and a switch of the ET-1 receptor expression with enhanced ETAR mRNA. However, ETBR remained unaffected upon the infectious stimulus. In conclusion, *Cp*, already suggested to trigger proatherosclerotic events, was able to effectively dysregulate the ET-1 axis in order to develop a proproliferative loop in infected vascular cells, which may be of immense consequence when present *in vivo*.

Sasu et al. (34) could show that the chlamydial heat shock protein 60 (cHSP60) alone caused proliferation of human vascular smooth muscle cells in a p44/42 MAP kinase-dependent manner *in vitro*. In their experiments an active infection was not obligatory as even *Cp* inactivated through UV radiation or heat (56°C) led to significant saphenous vein smooth muscle cell proliferation. Yet heat-killed chlamydiae (100°C) only caused a slight mitogenic activity, ruling out a major role of the chlamydial LPS, which is heat resistant. In addition to chlamydial LPS and the major outer membrane protein (MOMP) cHSP60 is known to stimulate vascular cells and macrophages and to contribute to the oxidation of low-density lipoproteins (LDL) (35) by mononuclear cells exposed to *Cp*. Furthermore, the possible role of cHSP60 in atherosclerotic development is underlined by its stimulating effect on inflammatory chemokines like TNF- α , adhesion molecules and matrix-degrading metalloproteases in macrophages related to atherosclerotic sites (36), proatherosclerotic processes, which may in part be regulated by TLR4-mediated pathways.

However, not only chlamydial but also human HSP60 might be involved in *Cp*-mediated cellular proliferation. Hirono et al. (37) demonstrated that vascular smooth muscle cells (vSMC) showed an enhanced expression of hHSP60 after infection with *Cp*. Overexpression of endogenous hHSP60 that co-localised with TLR2/4 in neointimal lesions (38), subsequently caused proliferation in venous smooth muscle cells, results that oppose Sasu's findings as cHSP60 alone had no such effect here when added exogenously. This might be due to the cytotoxic potency of cHSP60. However, the complex connection between chlamydial effectors and target cell mediators is far from being completely understood as both cHSP60 and hHSP60 have been found to co-localise within human atherosclerotic lesions (36) and have since long been suspected of being involved in atherogenesis (39, 40).

Due to the complex cell-to-cell communication within the vasculature, more physiological experimental settings, which focus on several cell populations interacting in one system, need to be established. Experiments by Coombes and Mahony (41) revealed several aspects of the interaction between vascular endothelial cells (vEC) and vSMC. They reported the supernatant of *Cp*-infected endothelial cells to contain one or more soluble factors triggering vSMC proliferation in a time- and dose-dependent manner. Viable chlamydiae were not required for this effect as both heat-inactivated and chloramphenicol-treated *Cp* led to the proliferative response. Putative mitogenic stimuli in this context might be PDGF, FGF, IP-10 or MCP-1 all of which have strong proatherosclerotic effects. Indeed the chemoattractant and mitogen PDGF was later found to be significantly enhanced in *Cp*-infected vEC (42).

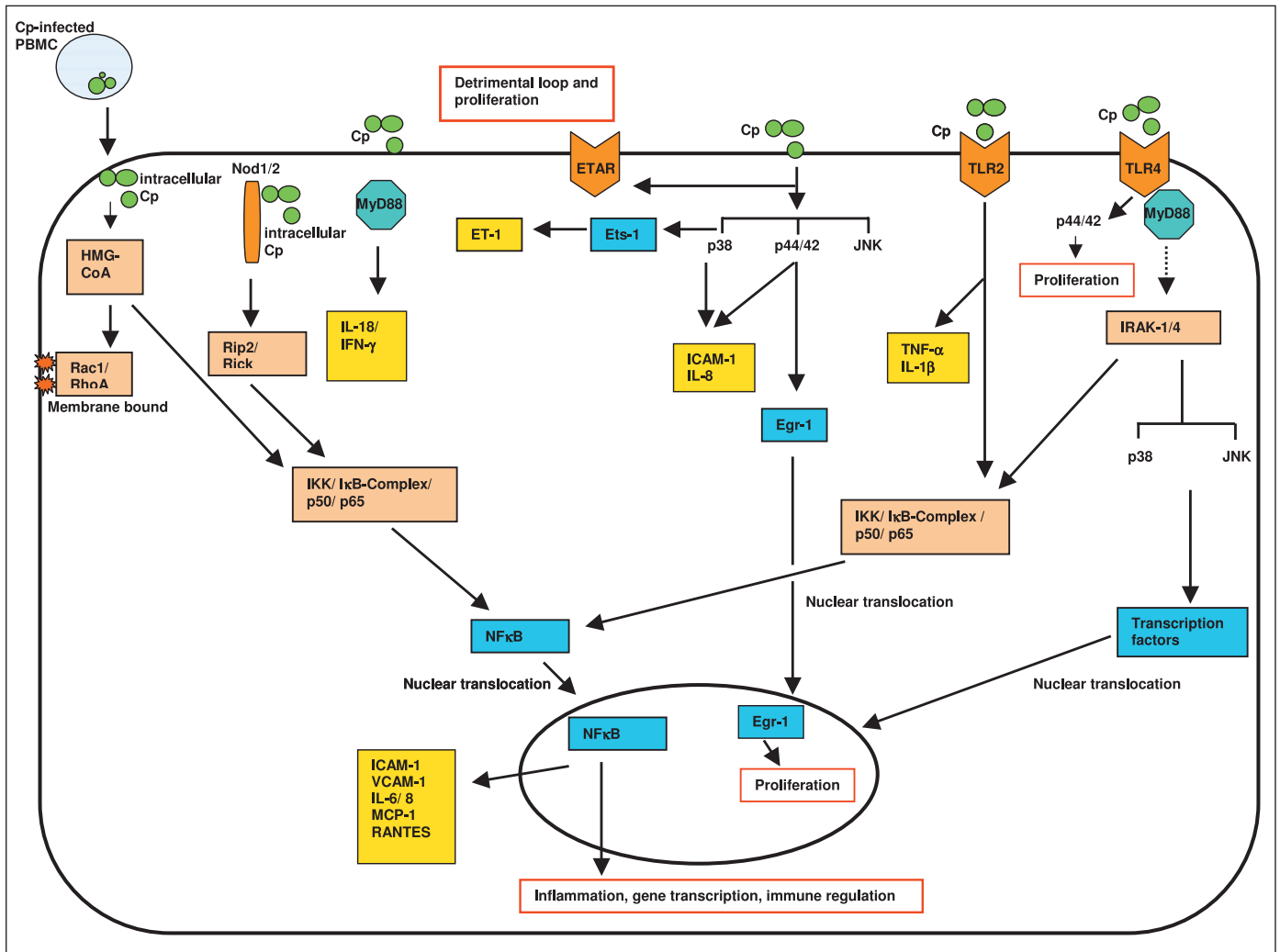


Figure 1: Synopsis of supposed interactions with human target cell structures by *Chlamydia pneumoniae* (→ activation pathway).

Concerning their *in vitro* results Coombes et al. were able to depict a detrimental loop within the vascular wall in an animal model system. Normocholesterinaemic rabbits infected with *Cp* developed a significant thickening of the arterial intima compared to non-infected animals as measured by histological examination. Furthermore, arterial lesions of *Cp*-infected rabbits overexpressed PDGF-B suggesting a model of infected vEC secreting PDGF-B as a mitogenic stimulus for underlying smooth muscle cells with subsequent thickening of the arterial intima and final remodeling of the vessel wall. Candidates for chlamydial components eliciting PDGF-B are cLPS, cHSP60 or even fragments of the MOMP.

Target cell activation through *Chlamydia pneumoniae*

Mitogen-activated protein kinases

Activating and reprogramming the target cell is a complex mechanism that is essential for the intracellular survival of chlamydiae, and that requires numerous signalling cascades and recep-

tor systems. *Cp* could be shown to increase the expression of miscellaneous MAP kinases, which are ubiquitously expressed and activated in response to a broad variety of stimuli: they act as key mediators in proinflammatory processes (p38 MAPK), cell propagation (ERK 1/2, p44/42 MAPK) and stress (cJNK/SAPK). Krüll et al. demonstrated endothelial infection with *Cp* to cause the stimulation of c-Jun kinase (JNK), p38 and p44/42 MAPK, the latter being partly involved in *Cp*-internalisation (43). MAP kinases were already activated at early timepoints indicating that already surface contact to *Cp* causes EC activation and that internalisation is not necessarily required for this process.

Chlamydial structures that represent a possible trigger to activate signalling cascades might again be cHSP60 or components of the MOMP as LPS-inactivation by polymyxin B did not diminish cellular activation. Endothelial *Cp*-infection resulted in a proinflammatory phenotype characterised by stimulation of IL-8 in a p38 MAP kinase-dependent manner and of ICAM-1, which was regulated by p44/42 and p38 MAPK. Furthermore, upon chlamydial infection the transcription factor NF-κB is inducible in HUVEC and translocates to the nucleus activating numerous

Cell population	Target cell activation via	Enhanced expression of effectors and mediators through <i>Cp</i> -infection
Vascular endothelial cells (human)	c-Jun kinase, p38 and p44/42 MAPK (43) NF- κ B, Nod1 (47)	ICAM-1, VCAM-1, IL-6, IL-8, PAI-1, tissue factor (54, 55) MCP-1, RANTES (6, 44)
Vascular smooth muscle cells (human)	GTPases Rac1 and RhoA, NF- κ B (26) TLR4/ p44/42 MAPK (34)	IL-6, RANTES (CCL5), MCP-1, FGF-2, Egr-1 (18, 26, 41)
Peripheral blood mononuclear cells (PBMC) (human)	TLR-2 (53), MyD88 (53)	IL-1 β , IL-6, IL-8, IL-12 MCP-1, TNF- α , MMP-1, -2, -7, -9, PGE2, IL-18/ IFN- γ (12, 36, 53, 56–59)
Mast cells (human)		IL-8, MCP-1, TNF- α (60)
Lung epithelial cells (human)		IL-8, IFN- γ , TNF- α (61)
Glia cells (murine)		IL-10, IL-12, TNF- α , MMP-9 (62)
Human embryonic kidney cells (HEK 293)	Nod1 and Nod2 / Rip2, NF- κ B (47)	
Dendritic cells (murine)	TLR 2 (49)	

Table 1: Direct interaction with signalling cascades of the host cell by *Cp* infection.

proinflammatory effectors. Translocation of active NF- κ B is mediated via activation of the I κ B kinase complex (IKK) and subsequent degradation of I κ B α followed by expression of proinflammatory mediators like IL-6, IL-8, MCP-1 and RANTES (CCL5) and adhesion molecules as ICAM-1 and VCAM-1 (6, 44) (Fig. 1, Table 1). However, *Cp* may also be able to inhibit IL-17A receptor-dependent NF- κ B activation by direct interaction with the activator protein Act1 indicating a protective component in *Cp*-infected cells (45) by delaying host defence mechanisms.

Upstream of the MAPK pathways *Cp* induces the small GTPases Rac1 and RhoA, which both mediate complex processes including cellular migration, cytoskeletal arrangement and inflammation. Activation could significantly be abrogated by statins, which were able to inhibit vSMC activation through NF- κ B, ROS-formations or stimulation of proinflammatory chemokines like MCP-1, RANTES (CCL5) or IL-6 and IL-8 and thus exerted anti-inflammatory properties (26). This study also discussed a far reaching protective effect of statins as they prevented vSMC from PBMC-mediated acquisition of *Cp*-infection.

Another finding in *Cp*-dependent target cell activation describes the control of transcription factors and the gene expression machinery by acetylation/ phosphorylation of histone complexes (46). Schmeck et al. could show that in human endothelial cells *Cp* but not *Chlamydia trachomatis* causes Rac1-dependent modulations of histones, a process which subsequently led to an enhanced expression of inflammatory mediators. Effects were significantly abrogated by statins indicating a regulating role of HMG-CoA.

Proteins of the Nod-family

Proteins of the nucleotide-binding oligomerisation domain (Nod) family, known as intracellular pattern recognition receptors, could recently be implicated in *Cp*-induced cell activation. Nod1, which seems to be predominantly expressed in endothelial cells was found to be considerably involved in *Cp*-induced

IL-8 activation (6, 47). Furthermore, viable as well as heat-inactivated *Cp* were determined to cause NF κ B stimulation in EC in a Nod1- and Nod2-dependent fashion (Fig. 1, Table 1). However, activation of the transcription factor by this pathway was dependent on intracellular availability of *Cp*. This finding is in contrast to recognition pathways through Toll-like receptor 2 (TLR2), which is a surface receptor for *Cp*-induced target cell activation. Activation of Nod is closely connected to the recruitment of the serine-threonine kinase receptor-interacting protein 2 (Rip 2), an important downstream mediator in Nod signalling. *Cp*-induced activation of NF κ B and recruitment of inflammatory chemokines seems to be critically dependent on the interaction of Nod1/2 and its downstream Rip 2 concerning both intracellular pathogen recognition and subsequent arrangement of inflammatory processes (48). Nevertheless, it has not been well defined, which chlamydial structure is involved in Nod activation. Nod proteins have been associated with the recognition of different types of peptidoglycans; however, there is no clear evidence for peptidoglycan synthesis in *Cp*. Members of the chlamydial MOMP or the chaperone GroEL might represent possible ligands for Nod-binding properties.

Toll-like receptors

Toll-like receptors (TLRs) are crucial for recognition pattern mechanisms and cell activation pathways. TLR2, but not TLR4, mediated NF κ B activation in dendritic cells (49), and the production of TNF- α and IL-1 β in PBMC (50). Possible chlamydial ligands for a TLR interaction are cLPS or cHSP60 (51). Although most TLR-mediated cellular activation pathways seem to be regulated via TLR2, the role of TLR4 must not be underestimated. Sasu et al. (34) suggest a rapid TLR4-mediated activation of the p44/42 MAPK in vascular smooth muscle cells upon stimulation with *Cp*, leading to cellular proliferation. Another study revealed that inhibition of TLR4 by specific antibodies (52) abolished *Cp*-induced p44/42 MAPK activation in human fibroblasts, while antagonising TLR2 had no such effects. Obviously this is not the end of the list of target genes that can be ac-

tivated by *Cp*. Netea et al. discussed a TLR-independent target cell activation showing IFN- γ activation via MyD88-dependent IL-18 release through *Cp* (53) (Fig. 1, Table 1).

Conclusion and outlook

Since its description in 1986, *Chlamydia pneumoniae* has remained one of the most enigmatic pathogens and its pathogenesis is a complex process, which we only begin to understand. The bacterium is highly seroprevalent in the population and is able to infect a multitude of respiratory and vascular target cells. It can switch between a proliferative and a persistent, non-replicative state, where it exists viable in aberrant inclusions and remains able to release effector proteins into the host cell cytoplasm that effectively reprogram the host cell to support chlamydial survival. Currently, we do not even have convenient diagnostic tools to distinguish persistent from previous infection. The key to the pathobiology and effective therapy of *Cp*-infection is in understanding this treatment-refractory persistent state. The host cell repertoire to react towards noxious stimuli is limited. Other persisting injuries may result in very similar responses as the ones seen in chlamydial infection. The vascular host cell responds to *Cp*-infection by inflammation and proliferation in a

way that is also seen in atherosclerosis, which leaves the hypothesis of a bacterial contribution to atherosclerosis at least plausible – but the extent to which chlamydiae may promote atherogenesis remains to be established. A perspective for research on involvement of *Cp* in atherosclerosis and chlamydial pathogenesis in general lies in the functional analysis of the complex interaction of bacterial gene products and regulatory pathways of the human host cell on a genome wide level in the bacterium and the target organism. These approaches are currently followed upon in several research networks funded within the European Union ERA-NET PathoGenoMics program that correlate the chlamydial genomics, transcriptomics, and proteomics with the human interacting target proteins.

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