5 Discussion

5.1 *C. pneumoniae* and respiratory diseases

Chlamydophila pneumoniae, an obligate intracellular bacterium, is a widespread respiratory pathogen causing serious airway infection (bronchitis, pneumonia, etc) with subsequent persistent or recurrent severe immunopathologic effects [2, 4-6]. Little is kown about pathomechanisms of the immunobiology of pulmonary chlamydial diseases or chlamydia-induced epithelial cell alterations and *C. pneumoniae*-mediated interactions among all cell types involved in orchestrating airway inflammation (e.g. lymphocytes, macrophages, granulocytes).

Following airway infection bronchial epithelial cells are the first line of defense getting in contact with *Chlamydophila pneumoniae*. Several recent studies have suggested that, in the acute inflammatory reaction mediated by pathogens like chlamydia, the airway epithelium itself plays a prominent and active role [127, 128].

We could demonstrate that *C. pneumoniae* was able to induced a productive infection in freshly isolated human (tracheal) airway epithelial cells [***5 K***], small airway epithelial cell (SAEC [***17 K***]), freshly isolated (rat) type II pneumocytes [***10 K***] and the epithelial cell lines BEAS-2 (bronchial epithelial cells) and A549 (alveolar epithelial cells) [***5 K***].

Following airway infection *C. pneumoniae* induced a profound proinflammatory phenotype in epithelial cells, a crucial step during acute or chronic respiratory diseases:

1) After initial attachment Chlamydiae are internalized via endocytosis; 3-6 h postinfection, *C. pneumoniae* could be localized in early endosomes (EEA1- and Rab5-positive). After prolonged infection (12 h), intracellular bacteria were transported to a perinuclear location near the late endosomes (Rab7- and M6PR-positive); CLSM-experiments, however, demonstrated, that chlamydia did not enter these compartments, neither lysosomal nor lamellar body markers could be identified close to the bacteria [***10 K***]. Interaction with this secretory pathway therefore appears to provide a pathogenic mechanism that allows chlamydiae to establish themselves in a site, that is not destined to fuse with lysosomes, enabling prolonged intracellular survival.

2) Epithelial cell activation including cytokine release (IL-8, GM-CSF) as well as upregulation of adhesion molecules (ICAM-1) followed by an enhanced interaction of leukocytes (PMN, lymphocytes) with activated epithelium was noted [***5 K***, ***17 K***]. The

recruitment of leukocytes might augment a subsequent systemical spread of *C. pneumoniae* as recently demonstrated [132].

3) Impairment of surfactant homeostasis with changes in the metabolism of surfactant by type II cells is a hallmark during severe pneumoniae. We could demonstrate, that *C. pneumoniae* infection of type II pneumocytes was associated with an increase in intracellular surfactant protein-A and lipid accumulation in early endosomes (EE), as well as an inhibition of surfactant recycling from EE back to plasma due to chlamydia-mediated-rearrangements of β-tubulin and F-actin. Therefore, a chlamydia-mediated alteration in the surfactant regulation might be another crucial pathomechanism during respiratory infections with *Chlamydophila pneumoniae*.

Our studies, aimed to identify possible intracellular signaling steps involved, indicated that a $[Ca^{2+}]_i$- and p38 MAPK-dependent NF-κB-activation/translocation occurred within minutes after the addition of chlamydiae to epithelial cells, indicating that *C. pneumoniae* triggers immediate events of cell activation suggesting that attachment of the pathogen is sufficient to initiate an epithelial response and that bacterial uptake might not be required for this process [***5 K***, ***14 K***, ***17 K***].

Recent work suggested that chlamydial heat shock protein hsp60 is a possible mediator of target cell activation [75, 77, 84, 171]. Using recombinant GroEL-1 from *Chlamydophila pneumoniae*, we now could demonstrate that the purified protein dose-dependently induced expression of GM-CSF in epithelial cells [***17 K***]. The protein was almost as effective as viable or UV-inactivate whole bacteria. Heat inactivation of GroEL-1 reduced GM-CSF-expression almost completely. Additional studies using a monoclonal antibody against GroEL-1 are necessary the specify the importance of this protein during target cell activation by whole bacteria. In addition, using a commercially available antibody against chlamydial outer membrane protein-A we showed, that this membrane component may also have some effects for target cell activation, GM-CSF expression was significantly reduced in cells incubated with pretreated chlamydia. TW183-preincubation with polymyxin B in order to inactivate chlamydial lipopolysaccharide had no effects on epithelial MAPK-phosphorylation or GM-CSF expression. Heated bacteria did not induce a GM-CSF-expression. Since LPS is thermostable, this also speaks against the role of LPS in this response. Additional studies, however, using purified Omp-A or LPS from *Chlamydophila pneumoniae* as well as monoclonal antibodies against chlamydial LPS may help to clarify the role of these virulence factors during target cell activation [***5 K***, ***17 K***].

Several studies have demonstrated that airway epithelial cells may also dampen a proinflammatory situation thereby playing a double role during infection and inflammation [135, 141, 242]. PGE_2 is the most important prostaglandin secreted by epithelial cells and has been suggested to modulate overwhelming inflammatory reactions especially during neutrophil-epithelial-cell interactions [135, 242]. Prostaglandins are synthesized by cyclooxigenases (COX)-1 and -2 [138]. COX-1 is expressed constitutively whereas enhanced expression of inducible COX-2 in epithelial cells has been associated with (cytokine-mediated) inflammatory processes [143, 144, 243, 244] or neoplasia [245-247]. While in our study COX-1 expression remained unchanged (data not shown) *C. pneumoniae* induced a marked upregulation of COX-2 mRNA and COX-2- activity with subsequently enhanced release of PGE_2 after stimulation of epithelial cells with cyclooxigenase substrate arachidonic acid [***5 K***].

Taken together, we could demonstrate, that *Chlamydophila pneumoniae* is able to infected and activated human epithelial cells. The data suggested that during an acute infection with *C. pneumoniae* the airway epithelium is not only victim, but also culprit in infection and plays a prominent and active role. These informations may improve our understanding of the pathogenesis of *C. pneumoniae*-mediated inflammatory airway diseases.

5.2 *C. pneumoniae* and vascular diseases

The endothelium lines the inner surface of the vessel wall and is involved during almost all local or systemic inflammatory reactions. In several recent studies from our own group, different pathogens (e.g. *Listeria monocytogenes*, *Bartonella hensellae, Porphyromonas gingivalis*), pathogen-derived products/virulence factors (*E. coli* hemolysin, *Staph. aureus* α-toxin, *L. monocytogenes* hemolysin and phospholipases, *B. henselae* outer membrane proteins, *Salmonella abortus equii* lipopolysaccharide), as well as agents of the activated host defense (e.g. PAF, TNFα, IL-1β, IL-8, paracrine activation) have been identified to activate preformed, stereotypic signal transduction pathways and proinflammatory mechanisms in endothelial cells [***1 K***, ***2 K, 3 K, 6 K, 7 K, 11 K***].

Chronic inflammatory reactions of the endothelium and the vascular intima as well as proliferation of intimal smooth muscle cells and fibroblasts are key pathogenic mechanisms for the development of atherosclerotic lesions (“response to injury”-theory [169]). Although (chronic) infections with several pathogens like CMV, HSV, *Helicobacter pylori*, or different periodontal-derived pathogens have been suggested

as a trigger and promoter of inflammation which may cause vascular lesions and atherosclerosis, until now no clear cut evidence has been elaborated to hold up this theory. In addition, none of the recently analysed endothelotropic pathogens (*L. monocytogenens*, *B. henselae*) has ever been demonstrated in atherosclerotic plaques.

The story is different with respect to *Chlamydia pneumoniae*. There is an almost consistent chain of evidence that a repetitive/chronic persistent systemic infection with *C. pneumoniae* might be associated with development of atherosclerotic lesions (for details see also chapter 2.4.2, [9, 10, 31, 150, 151]). Using more analytical (in vitro) approaches, we could show, that *C. pneumoniae* infected human umbilical vein and arterial endothelial cells, subsequently activated a profound proinflammatory phenotype in these cells and induced atherosclerosis-relevant pathomechanisms like expression of endothelial adhesion molecules (E-selectin, ICAM-1, VCAM-1), cytokines (IL-6, IL-8, RANTES, MCP-1) as well as an enhanced interaction (rolling, adhesion and transmigration) of monocytes and infected endothelial cells [***4 K, 12 K***].

Moreover, we demonstrated, that effects on target cells involved in the development of cardio-vascular diseases were specific for *Chlamydophila 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 proartherogenic 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 affect 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 [***12 K***]. 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 well able to infect human monocytes, but did not induce monocyte activation and adhesion to endothelial cells, indicating a unique activation pathway for *C. pneumoniae* [248].

Although the genome of *C. pneumoniae* has been sequenced completely (REF Stephens) it still remains unclear which bacterial structures (virulence factors) or which intracellular mechanisms/signal transduction pathways activated upon infection determine the specificity of the “chlamydia-atherosclerosis hypotheses”. As an intracellular pathogen, *C. pneumoniae* has the ability to survive within a host cell for several days (months/years ??), either by the induction of a persistent infection or by

inhibition of host cell apoptosis. Several recent studies suggested, that a chronic target cell activation by intracellular persistent chlamydia via yet unknown mechanisms might play the key role for perpetuating a chronic (pro-)inflammatory phenotype of infected target cells (endothelial cells, monocytes, lymphocytes) involved in the process of atherogenesis. Different models of persistent *C. pneumoniae* infection in host cells have been established [106, 240, 241], but until now, there is still only limited knowledge on the mechanisms of intracellular survival [190]. Further studies are now required to determine relationship between distinct steps of this chlamydial development cycle, development of persistent infection, importance of different chlamydial virulence factors and initiation of host cell signaling pathways because there is some evidence, that primary infection of vascular smooth muscle cells and blood monocytes with *C. pneumoniae* resembles rather a persistent than an active/productive infection.

5.3 Mechanisms of target cell infection and activation

Incubation of endothelial cells with *C. pneumoniae* activated different signal transduction pathways, an overview is summarized in figure 4 (s.a.)

5.3.1 Dead or Alive ?

Our studies aimed to identify early intracellular signalling steps demonstrated, that increase of $[Ca^{2+}]_i$ as well as phosphorylation and activation of all three MAPK pathways (ERK1/2, p38, and JNK) occurred within minutes of chlamydial contact with target cells. This immediate cell activation suggests that chlamydial attachment is sufficient to initiate a cellular response and that bacterial uptake may not be required [***12 K***, ***14 K***]. Moreover, chlamydial heat- or UV-inactivation did not reduce MAPK phosphorylation markedly suggesting that viable bacteria may only in part be required for initial cell activation, heat-stable as well as heat-labile factors might be responsible for this initial target cell activation.

Heat-killed chlamydiae, however, were not able to infect endothelial cells as demonstrated by CLSM. A profound and prolonged endothelial cell activation with subsequent expression of a proinflammatory phenotype (release of cytokines, expression of adhesion molecules) therefore could only be initiated by viable chlamydiae infecting the endothelial cells suggesting that 1) a sole MAPK activation - initiated also by heat resistant chlamydial membrane compounds - is insufficient for this response and 2) additional intracellular immune receptors might be involved. Importance of this receptors could be elaborated in further studies (see below).

5.3.2 Importance of chlamydial virulence factors

Although the genome of *C. pneumoniae* has been sequenced completely, little is know about the importance of different chlamydial virulence factors for activation of signal transduction pathways in target cells.

Work in this field is difficult since chlamydiae are obligate intracellular pathogens with a unique development cycle and are not accessible for genetically modification to create selective mutants (e.g. dominant negative knockouts). All studies concerning the importance of different virulence factors, therefore have to make use of either (monoclonal) antibodies (mab) against these factors or of isolated, recombinant expressed proteins. Until now, purified mab against chlamydial LPS, outer membrane protein-A (Omp-A) and heat shock protein 60, GroEL-1 have been described [75, 84, 90, 191], not all of them are commercially available.

In order to characterize impact of potential chlamydial virulence factors for initial target cell activation we made use of heat-inactivated chlamydiae. In an “in vitro” model of target cell activation we could demonstrate, that heat-inactivated pathogens were unable to induce a profound proinflammatory phenotype in endothelial or epithelial cells (no release of cytokines, no expression of adhesion molecules). This suggests, that heat labile chlamydial membrane compounds like outer membrane proteins or heat shock proteins or other - not yet identified - virulence factors are possible candidates for target cell activation, heat resistant structures like chlamydial LPS (cLPS) are not very likely. In addition, chlamydial-preincubation with polymyxin B in order to inactivate chlamydial lipopolysaccharide (LPS) had no effects on MAPK-phosphorylation or cytokine expression, this also speaks against a dominating role of LPS in this response [***12 K***, ***17 K***, ***18 K***].

Several recent studies have suggested that chlamydial heat shock protein-60 may act as a possible mediator of target cell activation [75, 77, 84, 171]. In first studies, using recombinant GroEL-1 from *Chlamydophila pneumoniae*, we could demonstrate that the purified protein dose-dependently induced expression of GM-CSF in epithelial cells and of IL-8 in endothelial cells. The protein was almost as effective as viable or UV-inactivate whole bacteria. Heat inactivation of GroEL-1 reduced cytokine-expression almost completely. Additional studies using a monoclonal antibody against GroEL-1 are necessary the specify the importance of this protein during target cell activation by whole bacteria. In addition, using a commercially available antibody against chlamydial outer membrane protein-A we were able to demonstrate that this membrane component may also have some effects for target cell activation, GM-

CSF or IL-8 expression was significantly reduced in cells incubated with pretreated chlamydia [***17 K***, ***18 K***].

Additional studies involving purified Omp-A or LPS from *Chlamydophila pneumoniae*, monoclonal antibodies against chlamydial LPS or genetically modified (dominant negative knockout) strains may help to clarify the role of different virulence factors during target cell activation. Until now, theses tools are not available.

5.3.3 Importance of target cell receptors

The innate immune system relies on surveillance proteins to recognize pathogens by sensing pathogen-associated molecular patterns. Several recent studies demonstrated the involvement of Toll-like receptor-2 (TLR2) and –4 (TLR4) in initiation of innate immune cell activation by *C. pneumoniae* or chlamydial components ([75, 77, 79, 83, 187, 201-203], ***15 K***). Moreover, we were able to demonstrate, that the recently identified nucleotide-binding oligomerization domain (Nod) proteins might play a key role for profound and prolonged endothelial cell activation [***15 K***].

Viable, but not heat-inactivated chlamydia were able to infect endothelial cells and to induce a prolonged IL-8 expression in human umbilical vein (HUVEC) and arterial (HAEC) endothelial cells. This raised the question if additional 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. Moreover, 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 (***12 K***, ***15 K, 16 K***). 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 Nod1- 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 [209, 210].

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 [211-214]. Although recent studies suggest a functional peptidoglycan pathway in chlamydia [69, 70], a clear cut biochemical evidence for the synthesis of peptidoglycans in chlamydia is missing [71, 72]. Chlamydia, however, are sensitive to antibiotics that inhibit pepti-

doglycan synthesis [73]. 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 contains indeed peptidoglycan or peptidoglycan-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 [74]. 2) Nod proteins act as receptors for molecules other than peptidoglycans. Proteins such as heat-shock proteins or outer membran-proteins (OMP’s) could be involved in Nod activation since the minimal motif recognized by Nod1 is a dipeptide containing diaminopimelic acid [211], 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 GroEL-1 by Nod proteins in HEK293 cells (unpublished data).

TLR2 was suggested to be more important than TLR4 for recognition by and activation of innate immune cells by chlamydia [77, 202]. 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 [201]. 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 subsecuently activate Nod proteins. Several recent studies have demonstrated that chlamydia have a type III secretory apparatus [109, 249]. This may facilitate transport of potential virulence factors such as chlamydial

proteasome/protease-like activity factor (CPAF), Omp-A or GroEL-1 to the host cytoplasm [103]. Moreover, several other authors were able to demonstrate that even in the persistence phase, chlamydia are able to produce and secret proteins into the cytoplasm [51, 103, 106]. 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. In addition, the recently established Nod1 and Nod2 knock-out mice will now be of outstanding value for our future studies [211, 212, 250].

5.4 Future research and therapeutic strategies

The data presented suggest that *Chlamydophila 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. Additional 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 signaling pathways that could lead to target cell damage and inflammation which in turn may result in acute diseases like bronchitis or (community-acquired) pneumonia or may promote chronic diseases like COPD, bronchial asthma or atherosclerosis. New techniques of biochemical and genetical analysis (genomics, proteomics, epigenetics), are now available to improve our understanding about the specificity of *C. pneumoniae*-induced pathomechanisms of infection and inflammation and offer unprecedented opportunities to address many fundamental questions regarding chlamydial interactions with the host cells. These results subsequently will direct new research avenues in terms of diagnostics, therapeutics as well as vaccination strategies. Especially chronic persistent infections with metabolically aberrant chlamydiae that are refractory to current antimicrobial treatment schemes are a challenge for the development of new therapeutic strategies. In addition, vaccination against *Chlamydophila pneumoniae* (e.g. via DNA immunization as recently demonstrated [230]) could be a beneficial approach for either preventing or controlling infection by this human respiratory pathogen.