1 Introduction

Chlamydophila (Chlamydia) pneumoniae, a Gram-negative obligate intracellular bacterium, is a widespread respiratory pathogen causing sinusitis, pharyngitis, bronchitis and pneumonia. Repetitive or chronic persistent infections as a trigger/promoter of inflammation have been associated with an increased risk for asthma, chronic obstructive pulmonary disease (COPD) or vascular lesions. Although the genome of *C. pneumoniae* has been sequenced completely this information has not led yet to an understanding of the mechanisms of infection and target cell activation nor to the identification of potential chlamydial virulence factors. In airway infection bronchial epithelial cells are the first line of defense getting in contact with *C. pneumoniae.*

First objective of the present work, therefore, was to clearly assess *C. pneumonia's* capability to infect and subsequently activate different types of airway epithelial cells. In different studies, key mechanisms of epithelial cell activation could be identified. The data demonstrated that during an acute infection with *C. pneumoniae* the airway epithelium itself plays a prominent and active role by releasing pro- and anti-inflammatory mediators.

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 bacteriaemia or (II) carried within recirculating monocytes, macrophages and/or lymphocytes from the respiratory tract. Subsequent chronic or persistent intracellular infection of endothelial cells with *C. pneumoniae* has been associated with development of cardiovascular diseases.

Second objective of this work was to elaborate the mechanisms of endothelial cell infection with special respect to the importance of different chlamydial virulence factors. In addition, we examined the importance of cytosolic immune receptors in this context and provide evidence that Nod1 might act as a potent innate immune receptor for *C. pneumoniae* in endothelial cells. Nod-proteins may therefore be the key for mediating chlamydia-induced chronic inflammatory processes in the endothelium.

Our results improve the understanding of the pathogenesis of *C. pneumoniae*-mediated respiratory and vascular diseases and may help in the establishment of innovative therapeutic strategies.

2 *Chlamydophila pneumoniae* mediated diseases

2.1 Epidemiology, diagnosis and clinical manifestation

Chlamydophila pneumoniae, a Gram-negative obligate intracellular bacterium, is a widespread respiratory pathogen causing sinusitis, pharyngitis, bronchitis and pneumonia [1-3]. The majority of *C. pneumoniae* infections are subclinical, but severe pulmonary infection and profound lymphocytic alveolitis are observed [4]. In addition, chronic-persistent or recurrent infections may be an important risk factor for adult-onset asthma [5], chronic obstructive pulmonary disease (COPD, [6]) and development of vascular lesions [7-11]. Regarding vascular diseases, the field, however, is troubled by the "hen vs. egg" problem and causative proof is difficult because besides different anti-chlamydial isotypes of antibodies there are no good markers to differentiate among new vs. old (IgM vs. IgG) as well as acute vs. chronic persistent (IgM vs. IgA) *C. pneumoniae* infection. Several recent studies have suggested a chronic/persistent *C. pneumoniae* infection as a possible risk factor for central nervous system diseases like Alzheimer's disease or Multiple Sclerosis, data, however, are poor and clear evidence is still missing [12, 13].

2.1.1 Epidemiology

C. pneumoniae was first isolated 1965 from the conjunctiva of a Taiwanese child (strain TW183, [14]). The first respiratory strain (AR 39) could be isolated 1983. Both strains had a DNA-homology of > 99,5% and were established in 1986 as a new (third) human-pathogenic chlamydia species "*Chlamydia pneumoniae"*, synonym TWAR [1]. Until now about 50 strains have been isolated worldwide, DNA-homology between these strains is 94-100%.

Chlamydiae have been placed in their own order, *Chlamydiales*, with one family, *Chlamydiaceae*. Molecular evaluation of rRNA sequences confirms that chlamydiae are bacteria, but with only very distant relationship to other bacterial divisions [15, 16]. Although it has been proposed that *Chlamydia trachomatis* and *Chlamydia pneumoniae* represent different genera [17], their gene content and genome organization are extremely similar [18] as are their structure and biology [19]. The newly proposed nomenclature -as used throughout this manuscript- "*Chlamydophila pneumoniae"*, however, has not been generally accepted [20].

Chlamydophila pneumoniae is found worldwide. Seroepidemiologic surveys have demonstrated, that more than 70% of all adults have been exposed to this organism during their lifetimes [21]. Age-specific prevalence rates start to rise early in childhood, although there is only little disease associated with these early time points of

infection. The most rapid rise (in the Western world) in age-specific prevalence occurs during the age of 5- to 20-years.

In spite of a high percentage of *C. pneumoniae*-seropositive adults reinfections are common. According to these seroepidemiologic studies, *C. pneumoniae* infection seems to be both endemic and epidemic with frequent reinfection during a lifetime. Currently available data suggest that *C. pneumoniae* is primarily transmitted from human to human without any animal reservoir. Transmission seems to be inefficient, although household outbreaks with high transmission rates are reported [22, 23].

2.1.2 Diagnosis

For differential diagnosis of an acute, repetitive or chronic/persistent *C. pneumoniae* infection the kinetics of the antibody expression has to be taken into consideration. In patients with primary infection IgM antibodies appear about 2-3 weeks post infection and remain elevated/detectable up to 2-6 month. IgG antibodies may not reach high titers until 6-8 weeks after onset of clinical symptoms. *C. pneumoniae* does not induce a persistent protective immunity and reinfections are common. In case of reinfection, the level of IgG antibodies increase rapidly within 1-2 weeks while IgM antibodies may not appear again [22, 23]. Until now there is no reference test for validating a chronic/persistent infection by means of serological testing. Persistently elevated IgG or the presence of IgA antibodies has been suggested as potential serological marker. Several studies have proposed that high IgA titers might be a better marker for a chronic infection than IgG titers because serum IgA has a half life of about 5-7 days, whereas IgG has a half life of weeks to months [7, 9]. Further evaluation, however, and confirmation by other test have to be awaited.

A lack of validated and standardized diagnostic techniques in diagnosis of acute, recurrent or chronic persistant infection with *C. pneumoniae* has made interpretation of published data difficult. Recommendations for standardized approaches were recently published by the Center for Disease Control and Prevention (CDC, Atlanta) and the Laboratory Centre for Disease Control (Ottawa, Canada) in cooperation with members of the *C. pneumoniae*-study group [24]. Diagnostic approaches include serological testing, isolation/culture, nucleic acid-based amplification techniques such as PCR, and tissue diagnostics like immunofluorescence, immunohistochemistry (IHC) or in situ hybridization.

2.1.2.1 Serological testing

The microimmunofluorescence (MIF) test, developed by Wang et al. in the 1970s is currently the serological testing method of choice for diagnosis of acute *C. pneumoniae* infection [25]. This test allows the quantitative determination of antibody reactivity to formalinized elementary bodies from *C. pneumoniae*, *C. trachomatis* and *C. psittaci* fixed as dots on a single glass slide. Dilution of sera are placed over the antigen dots and incubated. Use of the MIF test allows definition of criteria for serologic evidence of acute infection (defined by a 4-fold rise in IgG between acute and convalescent samples or an acute IgM titer ≥1:16) or past exposure (indicated by an IgG titer ≥1:16). Kits based on the MIF format are commercially available, however, it should be noted that the quality of commercially available MIF kits varies and interpretation of the results is subjective [26]. Due to the lack of species specificity or sensitivity, other serological tests like complement fixation (CF), whole fluorescence, ELSIA and EIA cannot currently be endorsed [26-28].

2.1.2.2 Culture

Chlamydophila pneumoniae is an obligate intracellular pathogen and must be cultivated on specific eukaryotic host cells. All currently established culture techniques involve inoculation of specimens onto a human cell line via centrifugation, incubation for up to 72h and subsequent staining with fluorescent-labeled species-specific antibodies to visualize intracellular inclusion bodies ([10, 29, 30], figure1). Sensitivity is low due to the complexicity of the assay. Specificity is dependent on the ability of the lab staff to distinguish between true chlamydia inclusions and artifacts.

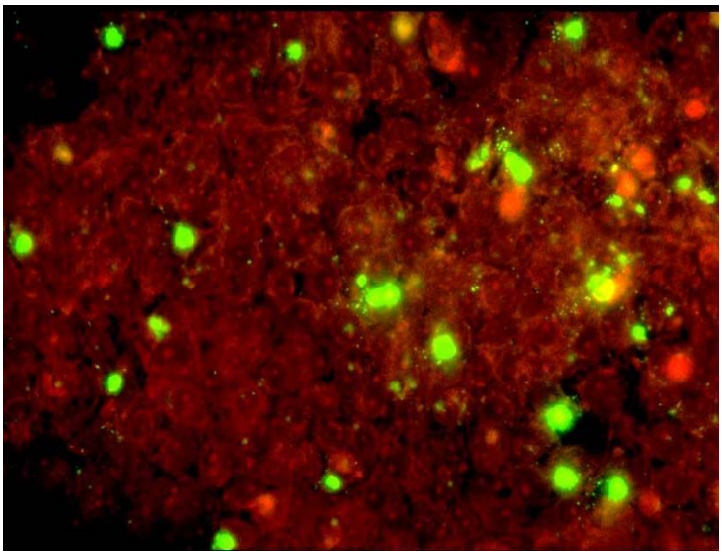

Figure 1, *C. pneumoniae* inclusion bodies in human airway epithelial cells (IF x400)

2.1.2.3 PCR

Although many inhouse PCR techniques for detection of *C. pneumoniae* have been published [31-34], sensitivity and specificity remain almost unknown. Due to a broad variety of methods for specimen-collection and –processing, primer design, nucleic acid extraction, amplification product detection and identification of false-positive and – negative results, no commercially standardized tests have been approved by the FDA.

2.1.2.4 Tissue diagnostics/immunohistochemistry (IHC)

Tissue diagnostic methods offers the advantage of preserving tissue morphology and permitting localization of the infectious agent to specific areas and cells. IHC has been the most frequently used in published studies [35, 36]. Detection rates are higher than those of PCR. This attributes to a faster degradation of DNA, difficulties of extracting DNA from tissues and the presence of PCR inhibitors. The most widely used IHC-technique is the avidin-biotinylated immune-complex method. However, interpretation of IHC-results is a critical challenge due to difficulties to distinguish between true- and false-positive results of the staining.

Specific recommendations for standardized culture/isolation or detection tests (e. g. specimen-collection, -transport and -processing conditions) have been suggested elsewhere (s.a. [24]).

Taken together, diagnosis of *C. pneumoniae* infection is difficult because cell culture techniques are not available for routine clinical use and nonculture techniques using antigen detection methods (IHC) or DNA probes have not been developed for commercial use. The MIF test is currently the serological testing method of choice for diagnosis of acute *C. pneumoniae* infection (“gold standard”), although the test is technically complex, requiring experience in fluorescence microscopy, interpretation is therefore subjective [24].

2.2 Cell biology

Monocytes, macrophages, smooth muscle cells, endothelial cells, human airway epithelial cells, BEAS-2B (bronchoepithelial cell line), HEp-2 and Hela-229 cells have all been shown to be susceptible for *C. pneumoniae* infection ([37-45] ***4 K***, ***5 K***, ***10 K***, ***12 K, 17 K***). Following inhalation, bronchial epithelial cells, however, are the first line of defense getting in contact with *C. pneumoniae* and respiratory epithelium has been identified as the primary target of infection ([37, 38, 46] ***5 K***, ***17 K***). Little is known about Chlamydiae-induced epithelial cell alterations and *C. pneumoniae*-mediated interactions among all cell types involved in orchestrating airway inflammation (e.g.

lymphocytes, macrophages, granulocytes). In addition, there is still limited knowledge of the mechanisms of Chlamydiae entry into host cells. With the exception of a possible role for the estrogen receptor complex [47], however, until now, no specific host cell receptor to which chlamydia bind has been identified. Wuppermann et al. showed, that heparan sulfate-like glycosaminoglycans (GAG) might act as possible chlamydial receptors on the surface of epithelial cells (HEp-2 cells [48]). Heparin and heparan sulfate were found to inhibit the attachment of *C. pneumoniae*. Enzymatic removal of heparan sulfate moieties from the host cell surface led to a marked decrease in *C. pneumoniae* infectivity. These data were supported by Beswick et al. who demonstrated, that cleavage of GAGs from host cells or elementary bodies (EBs) decreased infection rates with *C. pneumoniae* and *C. trachomatis* (serovar L2) in (bronchial) epithelial cells, but not in human (umbilical vein) endothelial cells [49]. In a recent paper, Puolakkainen et al. demonstrated, that *C. pneumoniae* uses the mannose 6-phosphate/insulin-like growth factor 2 receptor for infection of endothelial cells, additional studies, however are necessary to confirm these results [50].
Chlamydiae have a unique development cycle with two functionally and morphologically distinct forms [51-53], the condensed, “spore-like” infectious but metabolically inactive elementary body (EB, 0.3 µm, figure 2a) and the labile, noninfectious metabolically active reticulate body (RB, 0.9 µm, figure 2b). Infection or invasion is an

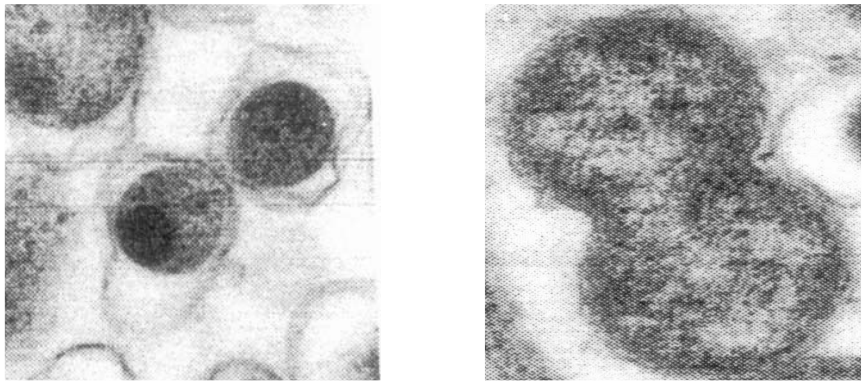

Figure 2, *C. pneumoniae* elementary (a) and reticulate bodies (b) (TEM x100.000)

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 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. Interaction with this secretory pathway appears to provide a pathogenic mechanism that allows chlamydia to establish themselves in a site that is not destined to fuse with lysosomes [51, 52]. Coombes and Mahony suggested a receptor-mediated induction of specific cell signaling by Chlamydiae as an essential step in *C. pneumoniae* invasion of epithelial cells [54]. After internalization EB then develop into reticulate bodies, a process which could be detected metabolically within 15 min and microscopically 12-15 h after addition of Chlamydiae to HEp-2 and Hela-229 cells [37, 38]. RBs are first observed dividing by binary fission after about 12 h. As the RBs multiply, the inclusion membrane expands to accommodate the increasing numbers of bacteria. After about 18-24 hours postinfection, the first RBs begin differentiating back into EB which accumulate in the lumen of the inclusion as the remainder of the RBs continue to multiply. 48 to 72 h after completing the development cycle -depending primarily on the infecting species- infectious EB are released either by lysis of the host cell or by fusion of the inclusion membrane with the plasma membrane to release the content of the inclusion into the environment (for review see [53, 55]).

Under some conditions however, such as in the presence of IFNγ, or penicillin or depletion of essential nutrients (iron, tryptophan) Chlamydiae can achieve a state of intracellular chronic, persistent infection in which they remain viable but metabolically quiescent and do not replicate [51, 56, 57]. Because of the reduced or negative ribosomal cell activities, these bacteria have no adequate targets for the known chlamydia-targeting antibiotics [58, 59]. Persistant Chlamydiae fail to complete development from RBs into EBs, are enlarged and morphologically aberrant and form small inclusions. Moreover, they exhibit a characteristic gene and protein expression profiles showing reduced levels of outer membrane proteins like the outer membrane proteins OmpA or OmcB and substantially increased levels of Heat shock protein 60 (Hsp 60, [60]). Chlamydiae may be reactivated from persistence by removal of the inducing stimulus.

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 another strategy that the Chlamydiae use to maintain a persistent, chronic infection. For a more extend view of the mechanisms in-

volved in Chlamydia interference with apoptosis signaling in host cells please refer to the review of Byrne et al. [61].

2.3 Virulence factors

Although the genome of *C. pneumoniae* has been sequenced completely in 1999 (1.23 x 10^6 nt encoding for approx. 1052 proteins, Gen-Bank No.: AE001363, [18]), until now little is known about structures on the chlamydial surface (proteins, glycolipids) initiating and mediating bacterial contact to target cells and inducing subsequent target cell activation. A multitude of the identified sequences from the chlamydial genome encode for proteins of bacterial metabolism. Other sequences demonstrated homologies to known proteins and virulence factors of other bacterial pathogens. Most of these factors of *C. pneumoniae* and *C. trachomatis* have now been identified by proteom-analysis of both species [62, 63]. Proteins of the “outer membrane complex” (OmpA/B, Omp3, OmcB, POMP), chlamydial lipopolysaccharide (cLPS), chlamydial heat-shock-proteins (e.g. cHsp60/GroEL-1), a type III secretion apparatus, the “chlamydial protease- or proteasome-like activity factor” (CPAF) or peptidoglycans and peptidoglycan-like structures are likely candidates as possible virulence factors.

2.3.1 Chlamydial envelope/outer membrane complex

The chlamydial outer membrane complex is composed primarily of three proteins specifically the outer membrane protein A, OmpA, formerly referred to as major outer membrane protein (MOMP) and two cysteine-rich proteins, the outer membrane complex B protein (OmcB) and the outer membrane complex A protein (OmcA) [19]. The gene that encodes MOMP, *ompA* [64] exhibits extensive DNA sequence variations that is confined mainly to four variable segments or domains (VS or VD 1 to VD 4) that contain subspecies- and serovar-specific antigenic determinants [65]. Until recently, OmpA was the only protein unequivocally shown to be expressed on the surface of all chlamydia. The protein is the most important immunodominant structure in all chlamydial strains except *C. pneumoniae* [66, 67]. Until now, this lack of antigenicity has not bee resolved. It might be possible, that either the exposed variable regions of *C. pneumoniae* OmpA are not recognized by human or animal immune systems or that surface exposure of the OmpA is somehow masked, perhaps by one o the polymorphic outer membrane proteins (POMP) as suggested by Christiansen et al. [67]. OmpA of the chlamydial elementary body is the main component for chlamydial protection against the environment outside the host, defense against the host immune response, and attachment to host cells [53].

OmcB, encoded by *omcB*, does not appear to be surface exposed but is thought to form a supramolecular lattice in the periplasm. Another important difference is that OmcB is extremely highly conserved (for review see [19]). Stephens et al. could demonstrate that the 60 kDa cystein-rich outer chlamydial membrane complex protein OmcB is also able to bind heparin [68]. This protein therefore might be the link mediating chlamydial attachment and initial steps of invasion into target cells.

Although recent studies indicated a functional peptidoglycan (PG) 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 peptidoglycan synthesis [73]. This phenomenon has been referred to the "chlamydial anomaly". Recent studies, using genomics or proteomics suggested the expression of peptidoglycan-like structures not on the surface of elementary bodies but –after invasion of the target cells- during cell division on the subsequently developed reticular bodies [70, 74]. Elucidating the existence of PG in *Chlamydia* is of significance for the development of novel antibiotics targeting the chlamydial cell wall.

2.3.2 Chlamydial heat shock protein 60 (cHsp60, GroEL-1)

Heat shock proteins (Hsp) belong to a family of evolutionarily highly conserved proteins, which are produced by eucaryotic and procaryotic cells during a variety of conditions such as heat shock, nutrient deprivation, infections, and inflammatory reactions, functioning to stabilize cellular proteins.

Several studies using neutralizing monoclonal antibodies or purified recombinant chlamydial heat-shock protein 60 (cHsp60, GroEL-1), however suggested, that this protein can act as an extracellular agonist and might be a key player in activation of different intracellular signal transduction pathways with a subsequently expression of a profound and prolong proinflammatory phenotype in treated cells [75-77]. Moreover, it has recently been demonstrated, that GroEL-1 is highly expressed in interferon-γ-induced persistent infections of tissue culture cells [51, 78]. Sasu et al. demonstrated that GroEL-1 is a potent inducer of human vascular smooth muscle cell proliferation and that this effect is mediated by rapid TLR4-mediated activation of ERK1/2 [79]. We were able to show, that purified recombinant GroEL-1 induced a rapid phosphorylation of ERK1/2 and p38-MAPK with subsequent enhanced release of IL-8 from human umbilical vein endothelial cells [***18 K***] and of GM-CSF from human bronchial epithelial cells [***17 K***]. Moreover, GroEL-1 protein has been shown to stimulate a hyperinflammatory response in animal models [80-82]. The responses

were mediated via a TLR2- and TLR4-dependent fashion similar to the whole microorganism and differed markedly from responses induced by endotoxin or CpG oligonucleotides [83, 84].

Several groups have demonstrated that elevated IgA antibody-titers of chlamydial heat shock proteins are predictors of chronic chlamydial infections like bronchial asthma, COPD, arteriosclerosis or pelvic inflammatory disease (PID) [76, 81, 85, 86]. The molecular mechanisms, however, by which *C. pneumoniae* might contribute to development of chronic diseases remain unclear. Due to the close structural homology between human and bacterial Hsps and their highly immunogenic nature, Hsps - especially Hsp60- have been proposed as key antigens in the development of autoimmune diseases [87]. Bachmaier et al. showed that a peptide from the murine heart muscle-specific α-myosin heavy chain, that has sequence homology to the Hsp60 of *C. pneumoniae*, *C. psittaci*, and *C. trachomatis*, was shown to induce autoimmune inflammatory heart disease in mice suggesting that Chlamydia-mediated heart disease is induced by antigenic mimicry of a heart muscle-specific protein [88]. In addition, Kol et al. demonstrated that cHsp60, produced in large amounts during chronic chlamydial infections, colocalizes within plaque macrophages with human Hsp60 [76]. Human Hsp60, when expressed by heat-shocked endothelial cells, can provoke an autoimmune reaction mediating endothelial cytotoxicity [89]. Chlamydial Hsp60 might therefore augment atherosclerosis and/or stimulate humoral and cellular immunity in atheroma.

2.3.3 Chlamydial LPS

Although chlamydia are Gram-negative bacteria, the common LPS group antigen of all chlamydial species (cLPS) differs significantly from LPS of other Gram-negative pathogens. In 1998 a first monoclonal antibody was isolated which recognizes LPS and neutralizes the infectivity of *C. pneumoniae* strain TW183 [90]. This antibody, however, does not neutralize other strains of *C. pneumoniae* suggesting the presences of more than a genus-specific epitope on cLPS. Until now, at least two important differences between cLPS and LPS from of e.g. enteric bacteria have been demonstrated: 1. the core trisaccharide 3-deoxy-D-manno-octulosonic acid (KDO) structure of chlamydial LPS contains a 1-8 linkage, a genus specific epitope as well as a 1-4 linkage similar to that of other bacteria, encoded by a single multifunctional KDO transferase [91-93], and 2. the chlamydial LPS has low endotoxic activity, although inducing some cytokines [94, 95]. Immunogold studies suggested that surface

exposure of LPS is greater on RBs than on EBs [96, 97]. Moreover, using different antibodies which recognized either RB or EB Birkelund et al. suggested that epitope exposure or the chemical structure of LPS might differ during the development cycle [98]. cLPS can be released from intracellular, intrainclusion Chlamydiae to the inclusion membrane, to the host cell cytoplasm and surface, and to surrounding infected cells [99, 100, 101]. Although this release might have an impact on the pathogenesis of chlamydial infections and the host's immune disposition of infected cells [102], several studies have demonstrated, that cLPS plays only a minor role for target cell activation [77, 93].

2.3.4 Chlamydial protease- or proteasome-like activity factor (CPAF)

2001 Zhong et al. demonstrated for the first time that a chlamydial species, *C. pneumoniae*, secreted a protease into the cytoplasm of infected target cells [103]. This protease, "chlamydial protease- or proteasome-like activity factor" (CPAF) splitted host cell transcription factors necessary for MHC-I (RFX5) and –II ("upstream stimulatory factor 1", USF-1) antigen presentation. Shaw et al. and Dong et al. showed that CPAF is secreted by different chlamydial species [104, 105]. In addition, Heuer et al. suggested that CPAF could be located in the inclusion lumen or associated with bacteria during the first 48 h of an acute infection [106]. Seventy-two hours and later, CPAF was present predominantly in the cytoplasm of the infected cells. Translocation of CPAF into cytoplasm correlated in time with degradation of the transcription factor RFX5 [106]. CPAF does not preexist in chlamydial organisms, synthesis requires organism replication in cells. Moreover, mice inoculated with viable chlamydial organisms produced a strong antibody response to CPAF. Moreover, sera from women diagnosed with *C. trachomatis* cervicitis displayed higher levels of antibodies to CPAF than to either chlamydial major outer membrane protein or heat shock protein 60. This sera neutralized the proteolytic activity of CPAF in vitro, suggesting that CPAF is both produced and immunogenic during human chlamydial infection [107, 108].

2.3.5 Type III secretion system (TTS)

The first genetic evidence that Chlamydia might have a type III secretion (TTS) system was presented by Hsia et al. in 1997 when they described four genes homologous to structural and regulatory components of a contact-dependent or TTS apparatus share high homology with TTS systems of other bacterial pathogens [109]. Subsequently, these results could be confirmed by genome and proteom analysis as well as by microscopic observations [18, 62, 110, 111]. Since TTS systems have

been shown to play a major role in the pathogen-host interaction in several other pathogens like e.g. *Shigella* or *Salmonella*, the TTS may also act as a key virulence mechanism of chlamydia [112].

One can speculate about a role for TTS both in the initial stages of infection where Chlamydia first comes into contact with the host cell as well as in the intracellular phase of chlamydial development using the structure of the TTS system to translocate different effectors into the host cell, depending on the phase of the developmental cycle. TTS genes expressed in the mid- to late stage of the developmental cycle appear to be down-regulated by IFN-γ treatment [113]. This suppression may play a role in maintaining *C. pneumoniae* in a persistent or altered state within the host cell. It will therefore be important to determine what structures are present in *C. pneumoniae* and what role each of them plays in the development and possible persistence of Chlamydia.

2.4 Clinical manifestation

The following chapter will give an overview about *Chlamydophila pneumoniae* mediated respiratory and vascular diseases. It will especially focus on the latest experimental data concerning mechanisms of target cell infection and activation.

2.4.1 *C. pneumoniae* and respiratory diseases

2.4.1.1 Clinical approach

Chlamydophila pneumoniae causes acute respiratory diseases and is responsible for approximately 5% of bronchitis and sinusitis cases and 5-10% of community acquired pneumonia (CAP) cases in adults worldwide ([114-117], figure 3). However, a brought geographic diversity and periodicity with higher incidence rates of *C. pneumoniae*-mediated CAP-cases for 2 or 3 years followed by 4 or 5 years with low incidence has been suggested [21]. A recently submitted study from the German “community acquired pneumonia-network, CAPNETZ”-study group, including more than 4000 CAP-patients in Germany demonstrated, that between 2001 and 2004 using recommended standardized diagnosis protocols (MIF test, PCR from BAL-fluid) *C. pneumoniae* could be identified as causative agent in < 1 % of all CAP-cases (personal communication N. Suttorp, speaker of CAPNETZ, [118]).

C. pneumoniae infection has also been implicated in the pathogenesis of asthma in both adults and children. This hypothesis is based on clinical studies and on the evidence of specific IgE production, direct epithelial damage, induction of T-cell immunopathologic diseases, and vascular smooth cell infection. Moreover, asthma patients, especially those with moderate asthma, had significantly higher serum IgA

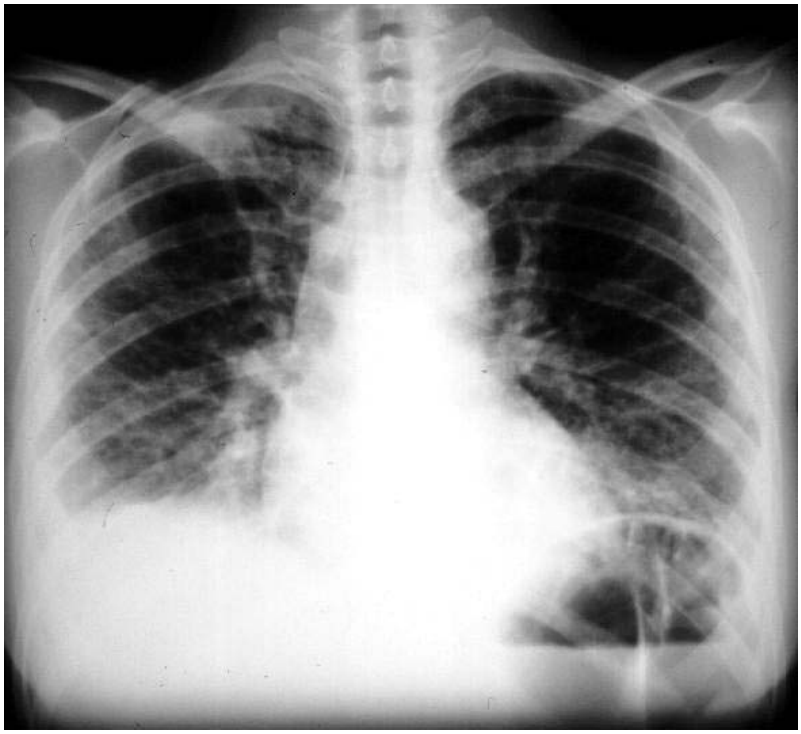

Figure 3, *C. pneumoniae* induced community acquired pneumonia, X-ray radiograph.

antibody levels to chlamydial heat shock protein 60 (cHsp60) than healthy controls [5, 86, 119-121]. Moreover, recurrent or chronic persistent *C. pneumoniae* infection seems to be common in patients with chronic bronchitis whether exacerbated or not, and is characterized by a strong humoral immune response to this intracellular pathogen, which is present in the majority of patients with severe chronic bronchitis. Increased antichlamydial IgG and IgA indicated acute exacerbations with *C. pneumoniae* in COPD patients [5, 122-124]. In addition, some authors suggest a possible role of this organism in the etiology of lung cancer, future studies, using measures of chronic *C. pneumoniae* status, however are necessary, to substantiate these data [125, 126].

2.4.1.2 Experimental approach

Little is kown about pathmechanisms of the immunobiology of pulmonary chlamydial diseases. Experimental animal models -especially the use of genetically different

mice-strains- have been established as useful tools. The histopathology of *Chlamydophila pneumoniae* lung infections in mice is a characteristic pneumonitis. *C. pneumoniae* induces prolonged lung infection. Polymorphonuclear neutrophils (PMN) dominate the early stages of the inflammatory infiltrates mononuclear cells the later stages of infection [127, 128]. The chlamydial target cells within the lung in acute and chronic infection, however, have not been described though they are crucial to the resulting pathology. Several recent studies suggested bronchial epithelial cells (BEC), alveolar (type II) epithelial cells (AEC), and endothelial cells as preferred target cells for acute chlamydial infection ([41, 129, 130], ***5 K***, ***10 K***) and demonstrated a systemic spread of *C. pneumoniae* from the respiratory tract to the vasculature via mononuclear cells after chronic/repetitive infection [131, 132].

Following airway infection bronchial epithelial cells are the first line of defense getting in contact with *Chlamydophila pneumoniae*. However, little is known about chlamydia-induced epithelial cell alterations and *C. pneumoniae*-mediated interactions among all cell types involved in orchestrating airway inflammation (e.g. lymphocytes, macrophages, granulocytes). 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. Although the mechanisms controlling the inflammation are complex and poorly understood, the release of pro- and antiinflammatory mediators plays a central role in initiation, perpetuation, and limitation of this process [133-136]. Epithelial cells have been shown to generate mediators such as interleukin (IL)-8, 15-hydroxyeicosatetraenoic acid (15-HETE), tumor necrosis factor- α (TNF-α), granulocyte macrophage colony-stimulating factor (GM-CSF), and nitric oxide (NO, [137-142]). Activation dependent expression of epithelial cyclooxigenase-2 (COX-2) has been demonstrated to be important for the regulation of pulmonary prostaglandin - especially prostaglandin E_2 (PGE_2) – synthesis [143, 144]. Additionally, bronchial epithelial cells are also target of a variety of mediators released by neighboring cells (NO, TNF-α, etc. [137, 139-141]).

Moreover, lung-derived mononuclear cells from infected mice display IFN-γ responses upon in vitro restimulation with inactivated *C. pneumoniae*, whereas cells isolated from naive mice do not [145]. In a murine model, IFN-γ induced the in vivo gene expression of inducible nitric oxide synthase (iNOS), indoleamine 2,3-dioxygenase (IDO) and gp 91 NADPH oxidase, all likely to be involved in the destruction of *C. pneumoniae* [146].

In addition, Geng et al. reported the increased production of IL-12, IFN-γ, tumor necrosis factor-alpha (TNF-α) and IL-10, but not IL-4 in the lungs of chronically infected wild-type BALB/c mice [147]. In a recent study, Naiki et al could demonstrate that the common TLR adaptor molecule MyD88 is essential to recognize chlamydial infection and to initiate a prompt and effective immune host response [148].
Objectives of own studies were to elaborate early mechanisms of epithelial cell activation following acute infection with *C. pneumoniae*, for details please see also chapter 4.1 [***5 K, 10 K, 14 K, 17 K***].

2.4.2 *C. pneumoniae* and vascular diseases

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 bacteriaemia or (II) carried within recirculating monocytes, macrophages and/or lymphocytes from the respiratory tract [45, 131, 132, 149]. Using a model of intranasally infected White New Zealand rabbits, Gieffers et al. were recently able to identify alveolar-macrophages as carrier for the systemic spread. AM transported the pathogen to the peribronchiolar lymphatic tissue, and subsequently *C. pneumoniae* entered the spleen and the aorta via systemic dissemination by peripheral blood monocytes [132]. Subsequently, *C. pneumoniae* are able to infect different target cells involved during development of atherosclerotic plaques. Different *C. pneumoniae* strains could be isolated from endarterectomy and bypass samples of patients with severe coronary heart disease [10]. 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 [***4 K***, ***12 K***].

2.4.2.1 Clinical approach

Chronic-persistent or recurrent *Chlamydophila pneumoniae* infections may be a trigger and promoter of inflammation which may cause vascular lesions and atherosclerosis [9, 10, 31, 150, 151]. The theory, is supported by
1) a serological association between *C. pneumoniae* infection and coronary heart disease (CHD) as well as other vascular diseases (arterial occlusive disease (AOD), carotid artery stenosis (CAS) and stroke [7, 9, 152, 153]),
2) the demonstration of *C. pneumoniae* in atherosclerotic plaques by electronmicroscopy, immunocytochemistry, PCR, and isolation of viable chlamydia (indicating a productive chlamydial infection [8, 10, 31, 33, 36, 154, 155]), and

3) 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) [11, 132, 155-159].

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) [157, 160-167]. Interpretation 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 and probably more important, *C. pneumoniae* in the persistent state, as observed in blood monocytes, shows high resistance against macrolides, tetracyclines and rifampicin [51, 56, 58, 168] This observation may explain the ineffectiveness of all interventional studies in atherosclerotic 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 notedly reduced metabolism. Because of the reduced or negative ribosomal cell activities, these bacteria have no adequate targets for the known chlamydia-targeting antibiotics.

2.4.2.2 Experimental approach

Chronic inflammatory reactions of the endothelial cells 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]). The role of *Chlamydophila 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 [39-45] ***4 K***, ***12 K***) and may induce a chronic immune activation via direct (bacteria-host cell contact) or indirect, paracrine (release of pro-inflammatory/-

proliferative mediators) effects, little is known about the mechanisms of *C. pneumoniae*-induced target-cell alteration.

Several studies have demonstrated, that (chronic) infection of susceptible target cells as well as direct activation of endothelial cells may initiate and perpetuate a local inflammation by inducing cytokine or growth factor release (e.g. IL-6, IL-8, TNFα, MCP-1, MMP-3, PDGF, TGFβ ([42, 76, 170-176], ***4 K***, ***12 K, 15 K***, ***16 K***), increased expression of adhesion molecules (P-/E-selectin, ICAM-1, VCAM-1 ([171, 177-179], ***4 K***, ***12 K***) and subsequent adhesiveness of the endothelium for leukocytes or platelets ([180], ***4 K***, ***12 K***), pathogenic mechanisms activated during different stages atherogenesis in humans [181, 182]. Whole viable bacteria as well as isolated chlamydial heat-shock protein 60 induced a proliferation of vascular smooth muscle cells, presumably by activation of the ERK1/2 pathway, resulting in enhanced transcriptional activity of the early-growth response gene-1 (Egr-1, [79, 183, 184]). In addition, induction of smooth muscle cell proliferation and intimal thickening via increased paracrine expression of PDGF-B in *C. pneumoniae* infected endothelial cells has previously been demonstrated in vivo in a rabbit atherosclerosis model [174, 183]. Besides induction of vascular inflammation and smooth muscle cell proliferation, *C. pneumoniae* infection has also been shown to mediate cellular lipid metabolism by stimulating oxidation of LDL [185] and inducing foam cell formation in macrophages [102, 173, 180, 186]. Furthermore, *C. pneumoniae* infection of macrophages as well as endothelial and smooth muscle cells resulted in a time-dependent expression of procoagulatory PAI and tissue factor, which plays an essential role in thrombus formation after plaque rupture [170, 187].

Many of the inflammatory immune responses in vascular cells caused by chlamydia are thereby mediated via activation of the nuclear factor κB (NF-κB, [170, 171, 188, 189], ***4 K***, ***15 K***, ***16 K***). In addition, for regulation of atherosclerosis-associated factors, the early-growth response gene-1 turns out to be another central signal transduction factor, involved in the formation of direct (vascular smooth muscle cell proliferation) and indirect (release of tissue factor) pro-atherosclerotic phenotype [184, 187, 190].

2.5 Innate and adaptive immunity

Little is know about a humoral or cell-mediated immunity (“CMI”) induced during acute or chronic/persistent infection with *Chlamydophila pneumoniae*. Most of the data have been elaborated using other Chlamydiae-species (*C. trachomatis*, *C. psit-*

taci). As intracellular bacteria, Chlamydiae pose an extra challenge for the defense mechanisms of the host. While importance of anti-chlamydial antibodies still is controversially discussed [191], cell-mediated immune responses are decisive, at least in mice. CMI against *C. pneumoniae*, however, is weak since recurrent infections as well as persistency of viable pathogens in different target cells are common phenomena.

2.5.1 Innate immunity

C. pneumoniae is internalized by a multitude of cells of the innate immune system like monocytes, macrophages, dendritic cells, lymphocytes or granulocytes where it survives and replicates [41-44, 58, 192-195]. Subsequently, different intracellular signal transduction pathways are activated to induce a proinflammatory phenotype ([54, 170, 189], ***16 K***). Moreover, in these cells IFNγ synergizes with bacterial products to activate various bactericidal or bacteriostatic mechanisms [146]. IFNγ is a strong activator of indoleamine 2,3-dioxygenase (IDO), limiting the availability of L-tryptophan to intracellular microorganisms [196]. Induction of IDO has been demonstrated to inhibit chlamydial growth in vitro [197, 198]. IFNγ can also activate inducible nitric oxide synthase (iNOS), which catalyzes production of NO from L-arginine. Inhibition of chlamydial growth through induction of iNOS has also been reported [199]. Moreover, stimulation of neutrophils or monocytes with IFNγ induced transcription of the gp91 component of NADPH oxidase (ox) mRNA with subsequent enhanced respiratory burst of phagocytic cells [146]. In addition, Rottenberg et al. could demonstrate that IFNγ-receptor-double knock-out mice (IFNγ-$R^{-/-}$) showed a dramatically increased susceptibility to *C. pneumoniae*, mediated via a reduced iNOS-mRNA accumulation, independent of diminished levels of specific antibodies. An increased susceptibility of $iNOS^{-/-}$ mice substantiated the protective role of this enzyme-activity during infection with *C. pneumoniae* [200]. These data suggested a relevant protective role of IFNγ–dependent innate mechanisms of protection.

2.5.1.1 Importance of specific receptors for activation of target cells

Until now, there is only limited knowledge about specific receptors on the surface of target cells like e.g. cells of the innate immune system. Heparan sulfate-like glycosaminoglycans (GAG), Toll-like receptors (TLRs) and the recently identified nucleotide-binding oligomerization domain (Nod) proteins a possible candidates for (primary) attachment and subsequent target cell activation.

2.5.1.1.1 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, 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 *C. pneumoniae* or chlamydial components ([75, 77, 79, 83, 187, 201-203], ***15 K***). 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 [202]. 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 [77]. Using an 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 [79]. 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 [204]. Moreover, the situation is complicated by different reports demonstrating a TLR independent target cell activation by *C. pneumoniae* [203, 205]. In addition, little is known about possible chlamydial virulence factors activating the TLR. Purified recombinant heat-shock protein 60 (chsp60) from *C. pneumoniae* could stimulate bone marrow derived dendritic cells (BMDDC) in a TLR2- and TLR4-dependent fashion similar to the whole microorganism and might act as an important mediator of inflammatory responses [83, 84]. These results were supported by Bulut et al. [75]. Interestingly, Erridge et al. could demonstrate that isolated lipopolysaccharide from *Chlamydia trachomatis* (strain LGV-1) also induced a TLR2-mediated NF-κB-activation [206]. 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 still is controversially discussed [201]. They are, however, highly susceptible for infection and activation by *Chlamydophila pneumoniae* or isolated chlamydial heat-shock protein 60 as demonstrated in our own studies [207, 208]. Moreover, additional own studies dem-

onstrated that chlamydia were able to infect and substantially activate TLR2 or- 4 negative cells (e.g. HEK293-cells) suggesting the existence of additional receptors.

2.5.1.1.2 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 [209, 210]. 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 [210]. Via a functionally active CARD-domain, Nod1 has been described to mediate the activation of NF-κB induced by peptidoglycans containing *meso*-diaminopimelate acid found mainly in gram-negative bacteria [211, 212], whereas Nod2 (CARD15) mediates responsiveness to the muramyldipeptide MurNAc-L-Ala-D-isoGln conserved in peptidoglycans of basically all bacteria [213, 214]. In contrast to Nod1 which could be detected in a multitude of tissues including endothelial cells, Nod2 has only been demostrated in dendritic and epithelial cells [215, 216]. 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 [215, 217, 218]. In a recent study, we were able to demonstrate, that in human aortic endothelial cells and human umbilival vein endothelial cells (HUVEC), Nod1 played a dominant role in triggering a *Chlamydophila pneumoniae*-mediated inflammatory process, for detail please refer to chapter 4.2.2 [***15 K***, ***16 K***].

2.5.2 Adaptive immunity

In *C. trachomatis* infection models, both $CD4^+$ and $CD8^+$ cells have been shown to confer protection, although the former are considered of major importance [219, 220]. In *C. psittaci* infection, $CD8^+$ rather than $CD4^+$ cells have been reported to confer protection in mice [221]. Since the overall DNA homology between *C. pneumoniae* and *C. trachomatis* or *C. psittaci* is less than 5 or 10%, respectively [18] the parameters of infection identified with the later two cannot be directly extrapolated to *C. pneumoniae*. Most data have been acquired using mouse models for *C. pneumoniae*-mediated pneumonia [127, 222]. Situation, however, is complicated due to mouse strain-specific differences studying mechanisms of adaptive immunity. In addition,

importance for a protective role of T-cell subspecies during primary infection and reinfection still is controversially discussed.

2.5.2.1 Cell mediated adaptive immunity

Using C57BL/6J mice genomically lacking T cell coreceptors or cytokine receptors, Rottenberg et al. demonstrated that $CD4^+$ T cells played a dual role, deleterious, promoting bacterial growth and disease early after infection, but participating in the control of bacterial growth at later time points as well as in protection against reinfection. The early damaging effect of $CD4^+$ cells in the absence of $CD8^+$ cells was associated with enhanced IL-4 and interleukin10 (IL-10) mRNA levels and delayed IFN-γ mRNA accumulation in the lungs of mice [200].

$CD8^+$ T cells inhibited the $CD4^+$ activity. The $CD8^+$ T cell mediated protective immunity during early stages of primary infection was perforin independent and associated with an altered cytokine balance as indicated by increased IL-4 and IL-10 and delayed accumulation of IFNγ mRNA in $CD8^{-/-}$ mice. The early higher susceptibility of CD8-deficient mice correlated with an immune deviation from a normal Th1 response to a Th2 cytokine pattern [200]. These results were confirmed by demonstrating the importance of peptide-specific $CD8^+$ T cell lines in local and systemic compartments after primary (intranasal) infection with *C. pneumoniae* [223]. These CTL lines suppressed chlamydial growth in vitro by direct lysis of infected target cells and by secretion of IFNγ. In addition they were able to identify eighteen H-2(b) binding peptides representing sequences from 12 *C. pneumoniae* antigens. The importance of $CD8^+$ CTL during reinfection has been demonstrated by several groups. In a BALB/c nude mice model with absence of all T cells, the overall clearance kinetic after primary *C. pneumoniae* infection was not dependent on either $CD4^+$ or $CD8^+$ cells alone [224], but, after reinfection, acquired immunity was strongly CD8 dependent with an enhanced IFNγ-production and an increased local lymphoid reaction in the lungs [145, 224].

Analyzing lymphocytes from male patients with respiratory tract infection Halme et al. demonstrated a *C. pneumoniae*-specific CMI response during acute, primary infection early after onset of disease symptoms and simultaneously with a humoral response (increase of *C. pneumoniae*-specific IgM-antibodies). *C. pneumoniae*-induced lymphocyte activation involved $CD8^+$ T-cells in the early phase of infection and $CD4^+$ cells in the later stage [225].

The mechanism underlying the protection mediated by the $CD8^+$ cells in *C. pneumoniae* infection is unclear. CTL specific for *C. trachomatis* have been demonstrated in *C. trachomatis*-infected mice [226, 227]. $CD8^+$ cells may function by secreting cytokines such as IFNγ. Rottenberg et al. showed that C57BL/6 mice produce IFNγ in response to *C. pneumoniae* primary infection and suggested a IFNγ-mediated protection mechanisms [146]. This was supported by demonstrating an altered bacterial load in anti-IFNγ-treated C57BL/6 mice with markedly exacerbated signs of pulmonary inflammation [228]. In BALB/c mice an IFNγ-independent cellular response was suggested [145, 228]. Interestingly although BALB/c mice appear not to be dependent on IFNγ during primary infection, they do not develop a typical Th2 type response either [228]. During reinfection, neutralization of IFNγ exacerbated the infection in both strains [145, 146]. $CD8^+$ cells may therefore at least partially function through IFNγ production, however actively IFNγ-producing cells have also been demonstrated in CD8-depleted mice, in which the acquired immunity seen during reinfection is abolished [224]. Thus, IFNγ is an important, but not the only effector mechanism in acquired immunity.

Overall, different mouse model of *C. pneumoniae*-infection demonstrated that immunity is critically dependent on $CD8^+$ CTL. Recently, a first successfully immunization of C57BL/6 mice with a $CD8^+$ T cell heptaepitope based DNA vaccine to induce a protective immunity against *C. pneumoniae* has been described [229]. These results were confirmed [230], suggesting, that DNA immunization is a promising possibility for developing much wanted vaccines against important chlamydial pathogens. Further studies are now required to elaborate the optimal design of (multicomponent) anti-*C. pneumoniae* vaccines for humans.

2.5.2.2 Humoral immunity

Infection with *C. pneumoniae* induces a strong serum response. Little is know about the immunogenic antigens of *C. pneumoniae*. The 40 kDa major outer membrane protein (MOMP) is the most important immunodominant structure during *C. trachomatis*-infection, during infection with *C. pneumoniae* it is a relatively immunorecessive antigen [66, 67]. This lack of antigenicity has not been resolved yet. Among the different antigens of *C. pneumoniae* the 60 kDa heat-shock protein 60 has been suggested to be a key player during *C. pneumoniae*-induced humoral immunity, further studies, however, are required to identify the importance of this antigen during acute and chronic infection (for details please refer also to chapter 2.3.2 [66, 85], ***17***

K, ***18 K***). Antibodies to different structures on the *C. pneumoniae* elementary body can neutralize the organism in cell culture, however, the epitope specificity of these neutralizing monoclonal antibodies to specific *C. pneumoniae* proteins remains undefined at a molecular level ([3, 67] ***17 K***). Moreover, in vivo, protective effects of antibodies seem to be weak since reinfections or chronic chlamydial infections are common despite high antibody titers [21, 22]. Under some circumstances, antibody response during *C. pneumoniae*-infection might be immunopathological, for details please see chapter “virulence factors”.

2.6 *C. pneumoniae*-mediated signal transduction in target cells

Incubation of target cells with *C. pneumoniae* activated a multitude of different signal transduction pathways, an overview is summarized in figure 4 [***16 K***].

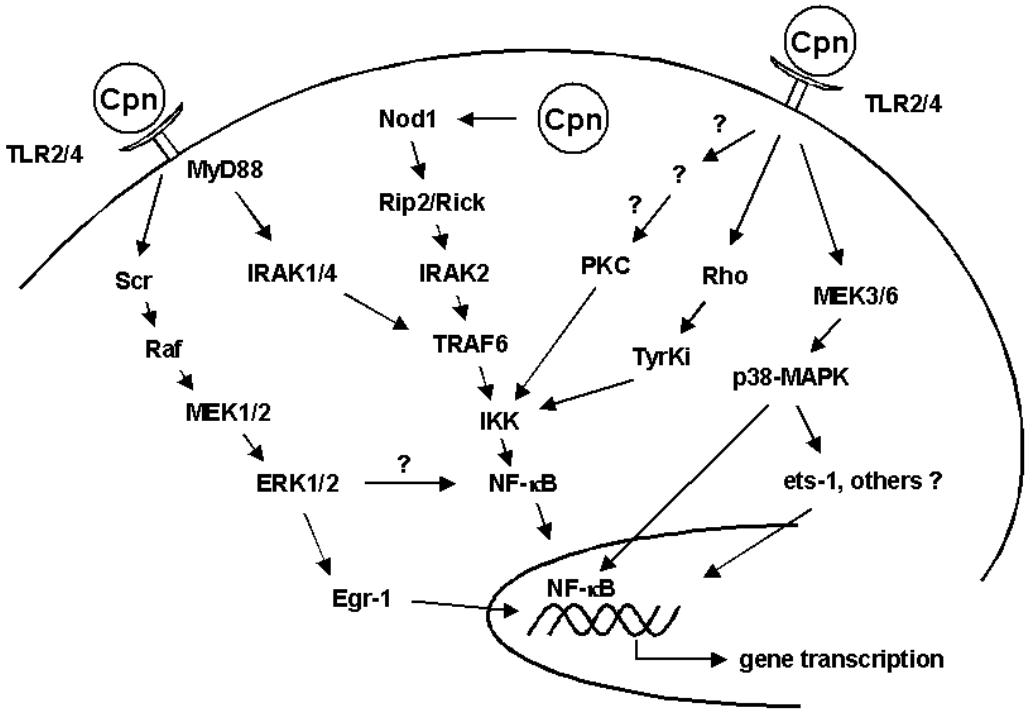

Figure 4, Signal transduction pathways in *C. pneumoniae*-activated target cells.

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 a key players mediating a proinflammatory and prothrombotic phenotype (p38 MAPkinase, [231]), differentiation and cell growth (extracellular receptor kinase [ERK]) and stress responses (c-Jun-NH2 kinase [JNK/SAPK], for review see [232, 233]). Within minutes upon contact of chlamydial elementary bodies with endo-

thelial cells, a significant increase in total protein tyrosine phosphorylation as well as phosphorylation and activation of p38-MAPK, ERK1/2 and JNK/SAPK is noted [***5 K***, ***12 K***]. This MAPK-activation in target cells was dependent on a chlamydia-mediated increase of intracellular calcium ($[Ca^{2+}]_i$) [234-236]. Moreover, Coombes and Mahony could demonstrate that activation of the MEK-ERK1/2 pathway is important for *C. pneumoniae* invasion of target cells [54].

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, s.a.) including IL-8 and ICAM-1 in endothelial cells ([170, 175-178], ***5 K***, ***12 K***). 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 [***12 K***]. 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) induced a more sustained release of IL-8 from *C. pneumoniae*-stimulated endothelial cells. Additional studies using different types of airway epithelial cells (small airway epithelial cells, SAEC; BEAS-2B bronchoepithelial cells; type II pneumocytes) confirmed the role of MAP-kinases for signal transduction in target cells [***10 K***, ***14 K***, ***17 K***]. Interestingly chlamydia-induced p38-MAPK activity seemed to be critical for NF-κB-stimulation. Using dominant negative mutants of the four until now identified p38 MAP kinase isoforms, p38α, $-\beta_2$, -γ and -δ [237], we could show that all isoforms significantly reduced chlamydia-dependent NF-κB activation [***17 K***].

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 [***12 K***]. 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. Moreover, they showed, that PKC is activated in HAECs upon 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 [178].

Intracellular infection of target cells induced (a more delayed) activation of the IκB kinase complex (IKK) with degradation of IκBα 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 [170, 188, 189, 207]). This *C. pneumoniae*-induced NF-κB-translocation was dependent on activation of p38-MAPK or ERK1/2 but not SAPK/JNK [***10 K***, ***14 K***].

Further upstream of 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 [170]. *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. 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 [170]. Moreover, efficient inhibition of NF-κB mediated signalling by statins has also been observed in *C. pneumoniae* infected macrophages and vascular endothelial cells [238].

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 [239]. A first 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 [79]. Rupp et al. 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 VSMC [184]. Pretreatment of VSMC with siRNA against Egr-1 not only blocked Egr-1 mRNA expression but also reduced proliferation of infected VSMC. 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 [184].

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 [106, 240, 241], but less is known about the induction of pro-atherosclerotic signalling cascades in persistent chlamydial infection [190]. 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 rather a persistent than an active infection. 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 [51, 56, 58, 168].