Transcriptional analysis of persistent *Chlamydia pneumoniae* infection *in vitro*

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ABBREVIATIONS

AB	aberrant RB-forms
bp	base pairs
cDNA	complementaty DNA
CF	complement fixation test
CPAF	chlamydial proteasome-like activity factor
COPD	chronic obstructive pulmonary disease
cRNA	complementary RNA
DAM	deferoxamine mesylate
DNA	deoxyribonucleic acid
EB	elementary body
EIA	enzyme immunoassay
ECM	extracellular matrix
EM	electron microscopy
hai	hours after inoculation
hGBP	human guanylate binding proteins
IDO	indoleamine 2,3-dioxygenase
IF	intermediate filament
IFN-γ	gamma interferon
Ig	immunoglobulin
Inc	inclusion membrane protein
INPs	small molecule inhibitors of the Yersinia type III secretion system
LPS	lipopolysaccharide
MHC	major histocompatibility complex
MIF	microimmunofluorescence test
MOMP	major outer membrane protein

OMC	outer membrane complex
omp 2	outer membrane protein 2
omp 3	outer membrane protein 3
PCR	polymerase chain reaction
PBMC	peripheral blood mononuclear cells
pmp	polymorphic membrane protein
qPCR	quantitative PCR
RB	reticulate body
RNA	ribonucleic acid
RNAi	RNA interference
RT-PCR	reverse transcriptase PCR
TARP	translocated acting recruiting phosphoprotein
TNF-α	tumor necrosis factor alpha
TRE	typical respiratory epithelium
T1-6S	type I-VI secretion system
T3S	type III secretion
USF-1	upstream stimulatory factor-1

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles, which are referred to in the text by their Roman numerals. In addition, some unpublished data are presented.

I **Mannonen L**, Kamping E, Penttilä T, Puolakkainen M. (2004) IFN-γ induced persistent Chlamydia pneumoniae infection in HL and Mono Mac 6 cells: characterization by real-time quantitative PCR and culture. Microb Pathog 36:41-50.

II **Mannonen L**, Nikula T, Haveri A, Reinikainen A, Vuola JM, Lahesmaa R, Puolakkainen M. (2007) Up-regulation of host cell genes during interferon-gamma-induced persistent Chlamydia pneumoniae infection in HL cells. J Infect Dis 195:212-9.

III **Mannonen L**, Markkula E, Puolakkainen M**.** (2011) Analysis of Chlamydia pneumoniae infection in mononuclear cells by reverse transcription-PCR targeted to chlamydial gene transcripts. Med Microbiol Immunol Jan 30. (Epub ahead of print) DOI 10.1007/s00430-011-0184-3

SUMMARY

Chlamydia pneumoniae can cause acute respiratory infections including pneumonia. Repeated and persistent *Chlamydia* infections occur and persistent *C. pneumoniae* infection may have a role in the pathogenesis of atherosclerosis and coronary heart disease and may also contribute to the development of chronic inflammatory lung diseases like chronic obstructive pulmonary disease (COPD) and asthma. In this thesis *in vitro* models for persistent *C. pneumoniae* infection were established in epithelial and monocyte/macrophage cell lines. Expression of host cell genes in the persistent *C. pneumoniae* infection model of epithelial cells was studied by microarray and RT-PCR. In the monocyte/macrophage infection model expression of selected *C. pneumoniae* genes were studied by RT-PCR and immunofluorescence microscopy.

Chlamydia is able to modulate host cell gene expression and apoptosis of host cells, which may assist *Chlamydia* to evade the host cells' immune responses. This, in turn, may lead to extended survival of the organism inside epithelial cells and promote the development of persistent infection. To simulate persistent *C. pneumoniae* infection *in vivo*, we set up a persistent infection model exposing the HL cell cultures to IFN-γ. When HL cell cultures were treated with moderate concentration of IFN-γ, the replication of *C. pneumoniae* DNA was unaffected while differentiation into infectious elementary bodies (EB) was strongly inhibited. By transmission electron microscopy small atypical inclusions were identified in IFN-γ treated cultures. No second cycle of infection was observed in cells exposed to IFN-γ , whereas *C. pneumoniae* was able to undergo a second cycle of infection in unexposed HL cells. Although monocytic cells can naturally restrict chlamydial growth, IFN-γ further reduced production of infectious *C. pneumoniae* in Mono Mac 6 cells. Under both studied conditions no second cycle of infection could be detected in monocytic cell line suggesting persistent infection in these cells.

As a step toward understanding the role of host genes in the development and pathogenesis of persistent *C. pneumoniae* infection, modulation of host cell gene expression during IFN-γ induced persistent infection was examined and compared to that seen during active *C. pneumoniae* infection or IFN-γ treatment. Total RNA was collected at 6 to 150 h after infection of an epithelial cell line (HL) and analyzed by a cDNA array (available at that time) representing approximately 4000 human transcripts. In initial analysis 250 of the 4000 genes were identified as differentially expressed upon active and persistent chlamydial infection and IFN-γ treatment. In persistent infection more potent up-regulation of many genes was observed in IFN-γ induced persistent infection than in active infection or in IFN-γ treated cell cultures. Also sustained up-regulation was observed for some genes. In addition, we could identify nine host cell genes whose transcription was specifically altered during the IFN-γ induced persistent *C. pneumoniae* infection. Strongest up-regulation in persistent infection in relation to controls was

identified for insulin like growth factor binding protein 6, interferon-stimulated protein 15 kDa, cyclin D1 and interleukin 7 receptor. These results suggest that during persistent infection, *C. pneumoniae* reprograms the host transcriptional machinery regulating a variety of cellular processes including adhesion, cell cycle regulation, growth and inflammatory response, all of which may play important roles in the pathogenesis of persistent *C. pneumoniae* infection.

C. pneumoniae DNA can be detected in peripheral blood mononuclear cells indicating that the bacterium can also infect monocytic cells *in vivo* and thereby monocytes can assist the spread of infection from the lungs to other anatomical sites. Persistent infection established at these sites could promote inflammation and enhance pathology. Thus, the mononuclear cells are in a strategic position in the development of persistent infection. To investigate the intracellular replication and fate of *C. pneumoniae* in mononuclear cells we analyzed the transcription of 11 C. pneumoniae genes in Mono Mac 6 cells during infection by real time RT-PCR. Our results suggest that the transcriptional profile of the studied genes in monocytes is different from that seen in epithelial cells and that IFN-γ has a less significant effect on *C. pneumoniae* transcription in monocytes. Furthermore, our study shows that type III secretion system (T3SS) related genes are transcribed and that *Chlamydia* possesses a functional T3SS during infection in monocytes. Since *C. pneumoniae* infection in monocytes has been implicated to have reduced antibiotic susceptibility, this creates opportunities for novel therapeutics targeting T3SS in the management of chlamydial infection in monocytes.

1. INTRODUCTION

Chlamydiales are obligate intracellular bacteria that infect a broad spectrum of multicellular and unicellular organisms. Chlamydiae share a unique biphasic developmental cycle where the bacteria multiply inside a phagosomal vesicle called inclusion. During the developmental cycle, chlamydiae alternate between infectious, elementary body (EB) that has been considered metabolically inactive (Haider *et al.*, 2010), and noninfectious, metabolically active reticulate body (RB). Chlamydiae are among the most successful bacterial pathogens infecting humans and may be responsible for a wide range of acute and chronic diseases of significant clinical and public health importance. *Chlamydia trachomatis* causes ocular infections which can lead to blindness (trachoma) and genital infections which can affect female reproductive health (Grayston and Wang 1975, Cates and Wasserheit 1991, Paavonen and Lehtinen, 1996). *Chlamydia pneumoniae* causes acute respiratory infections including pneumonia. Repeated and persistent *Chlamydia* infections occur and persistent *C. pneumoniae* infection may have a role in the pathogenesis of atherosclerosis and coronary heart disease (Saikku *et al.*, 1988, Saikku *et al.*, 1992b, Melnick *et al.*, 1993) and may also contribute to the development of chronic inflammatory lung diseases like chronic obstructive pulmonary disease (COPD) and asthma (Hahn *et al.*, 1991, von Hertzen *et al.*, 1999, von Hertzen *et al.*, 2002). *C. pneumoniae* can infect immune cells including monocyte/macrophages *in vitro* and *C. pneumoniae* DNA has been detected in peripheral blood mononuclear cells from patients with cardiovascular disease and from blood donors (Boman *et al.*, 1998, Maass *et al.*, 2000b, Beagley *et al.*, 2009). It is likely that *C. pneumoniae* uses monocytes as a means of transport from the initial site of infection to the site of chronic inflammatory disease.

Chlamydial persistence is defined as a viable but noncultivable growth stage resulting in a long-term relationship with the infected host cell (Beatty *et al.*, 1994a). *In vitro* persistent chlamydial infection can be induced by nutrient and iron depletion or by exposure to certain antibiotics or cytokines (Clark *et al.*, 1982, Coles *et al.*, 1993, Beatty *et al.*, 1993, Al-Younes *et al.*, 2001). Spontaneous development of a persistent infection in cell culture without external induction has also been described (Moulder *et al.*, 1980, Kutlin *et al.*, 1999). Furthermore, chlamydial infection in monocyte/macrophage cultures has the appearance of a persistent infection (Beagley *et al.*, 2009) and circulating monocytes carrying *C. pneumoniae* may have reduced antibiotic susceptibility (Gieffers *et al.*, 2001). Although the molecular and cellular mechanisms allowing the development of persistent infections are not yet fully understood, genome sequencing and new methods such as quantitative PCR, microarray and proteomic profiling have significantly increased our knowledge of *Chlamydia* host cell interactions in recent years, revealing potential markers of persistent chlamydial infection and molecules for putative new drug targets.

2. CHLAMYDIAE

The *Chlamydiae* are a group of obligate intracellular bacteria with a broad host range that share a developmental cycle characteristic for all *Chlamydiae*. To accommodate newly discovered chlamydia-like organisms new families have been introduced into the phylum. In addition to the family *Chlamydiaceae*, three new families Parachlamydiaceae, Simkaniaceae and Waddliaceae have been introduced and at the present systematics order *Chlamydiales* contains four families, 6 genera and 13 species (Everett *et al.*, 1999, Rurangirwa *et al.*, 1999). In the original classification order *Chlamydiales* contained one family *Chlamydiaceae* and one genus *Chlamydia*. Based on differences in 16S and 23S rRNA gene sequences, in 1999 Everett *et al.* proposed splitting of the family *Chlamydiaceae* into two genera: *Chlamydia* and *Chlamydophila*. The genus *Chlamydia* ("trachomatis group") consists of 3 species and *Chlamydophila* ("psittaci group") consist of 6 species including 3 new species derived from *C. psittaci*: *C. abortus, C. caviae* and *C. felis* (Everett *et al.*, 1999). The new taxonomy and especially the reorganization of *Chalmydiaceae* into two genera is not universally accepted (Schachter *et al.*, 2001, Stephens *et al.*, 2009). Since new knowledge of chlamydial genomes and shared biological properties between proposed *Chlamydia* and *Chlamydophila* genera are in favor of a single genus model, in this thesis the one-genus, multiple species nomenclature proposed by Stephens in 2009 is used (Stephens *et al.*, 2009) (Table 1.). The best known human and animal pathogens are in the family *Chlamydiaceae*. *C. trachomatis* is an important pathogen causing sexually transmitted diseases worldwide and trachoma in developing countries while *C. pneumoniae* is a wide spread respiratory pathogen, also implicated in some chronic diseases. *C. psittaci* causes avian chlamydiosis and may occasionally spread to human causing psittacosis. The other *Chlamydia* species are mainly veterinary pathogens infecting birds, cats, sheep, cattle, pigs, koalas, mice, and guinea pigs (Table 1.).

2.1 Chlamydia pneumoniae

Originally chlamydiae were thought to be viruses or intermediate organisims with properties from both viruses and bacteria. Based on e.g. cell division, cell wall structure, DNA-composition, folate synthesis and the presence of ribosomes with antibiotic susceptibilities characteristic of procaryotic ribosomes, Moulder demonstrated the definitive bacterial nature of chlamydiae in 1966 (Moulder, 1966) and shortly after, the genus *Chlamydia* was established (Page, 1966) and then divided into two species: *Chlamydia trachomatis* and *Chlamydia psittaci* (Page, 1968). *C. pneumoniae* was first isolated in 1965 from the conjunctiva of a Taiwanese child (TW-183 strain) participating in trachoma study (Kuo *et al.*, 1986), and at that time the isolate was thought to be a strain of *C. trachomatis*. In 1971, when cell culture methods were available the organism (T183) was observed by inclusion morphology to resemble more *C. psittaci* than *C. trachomatis*

(Kuo *et al.*, 1995). The TW-183 strain was associated to pneumonia by serologic evidence (Saikku *et al.*, 1985) and this lead to isolation of the first respiratory isolate (AR-39) in Seattle 1983 (Grayston *et al.*, 1986). Derived from the first conjunctival and respiratory isolates (TW-183 and AR-39) the strain was named TWAR. In 1989, the establishment of a third species in the genus *Chlamydia*, called *Chlamydia pneumoniae* was proposed (Grayston *et al.*, 1989).

2.1.1. Structure

Chlamydia is classified as gram-negative bacteria and the structure of the chlamydial cell wall resembles that of other gram-negative bacteria. A chlamydial cell has two distinctive forms: the smaller (∅ ~0,3 µm) "more tightly" packed elementary body (EB) adapted for extracellular survival and larger (∅ ~1 µm) vegetative intracellular reticulate body (RB), which is believed to divide by binary fission. Both forms have inner and outer membranes with a periplasmic space in between (Figure 1.). The extracellular EB form of *Chlamydia* has been thought to be metabolically inactive. However, in a recent study some protein production was shown for extracellular EB when incubated in growth media providing nutrients (Haider *et al.*, 2010). Unlike the other gram-negative bacteria, *Chlamydia* seems to lack detectable amounts of peptidoglycan in the periplasm (Fox *et al.*, 1990). Still *Chlamydia* has genes for peptidoglycan synthesis and is at least partially sensitive to penicillin (Moulder, 1993, Rockey *et al.*, 2000). Peptidoglycan usually provides rigidity to bacterial cell walls, but in chlamydial EB the cell wall rigidity has been proposed to be due to cross-linked disulfide bonds between the proteins of outer membrane complex (OMC) (Hatch, 1996). The OMC consist mainly of major outer membrane protein (MOMP), outer membrane protein 2 (omp 2), outer membrane protein 3 (omp 3), and polymorphic membrane proteins (pmp). Typically gram-negative bacteria have lipopolysaccharide (LPS) on the outer membrane that functions as an endotoxin. Chlamydial LPS is similar to the rough forms of enterobacterial LPS and consists of the lipid A and core polysaccharide moieties. Also in *Chlamydia* the LPS is localized on the surface (Birkelund *et al.*, 1989), yet the endotoxin activity of chlamydial LPS is much lower than that of enterobacteria (Nurminen *et al.*, 1983, Ingalls *et al.*, 1995, Heine *et al.*, 2003). This reduced activity may be due to the unusual structure of chlamydial lipid A, which contains a reduced number of fatty acids and an enlarged hydrophobic domain (Kosma, 1999). It has recently been suggested that the lower potency of chlamydial LPS may be due to its lower binding affinity to LPS recognizing molecules such as LPS binding protein and CD14 (Tsutsumi-Ishii *et al.*, 2008).

Table 1. Taxonomy of *Chlamydiae*, modified from Beagley *et al*., 2009

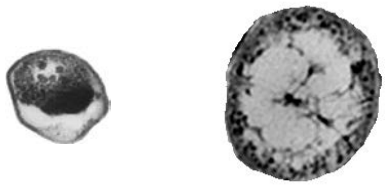
Systematics	Natural host	Pathogenicity
Order *Chlamydiales*		
Family I: *Chlamydiaceae*		
Genus I: *Chlamydia*		
C. trachomatis	Humans	Ocular, urogenital infections
C. pneumoniae	Humans, horses, koalas	Respiratory, ocular, urogenital infections
C. muridarum	Rodents	Respiratory, ocular, urogenital infections
C. suis	Swine	Enteritis
C. psittaci	Birds	Avian chlamydiosis
C. abortus	Ruminants	Abortion
C. pecorum	Cattle, sheep, koalas	Enteritis, abortion, polyarthritis, ocular, urogenital infections
C. felis	Cats	Ocular, respiratory infections
C. caviae	Guinea pigs	Ocular, urogenital infections
Family II: *Parachlamydiaceae*		
Genus I: *Parachlamydia*		
P. acanthamoebae	Acanthamoeba	Endosymbiont
Genus II: *Neochlamydia*		
N. hartmannellae	Hartmannella	Parasite
Family III: *Simkaniaceae*		
Genus I: *Simkania*		
S. negevensis	Humans (?)	Respiratory infections (?)
Genus II: *Fritschea*		
F. bemisiae	Insects	
F. eriococci	Insects	
Genus III: *Rhabdochlamydia*		
R. crassificans[a]	Woodlice	Hepatopancreas
R. porcellionis[b]	Cockroach	Abdominal swelling
Family IV: *Waddliaceae*		
Genus I: *Waddlia*		
W. chrondrophila	Cattle (?), fruit bats (?)	Abortion (?)

a Kostanjsek *et al*., 2004

b Corsaro *et al*., 2007

2.1.2 Genome

The chlamydial nucleoid apparent in the EB form of *Chlamydia* (Figure 1.) consists of the chromosome and two eukaryotic histone-like proteins Hc1 and Hc2. The Hc-proteins are involved in the nucleoid condensation and in the regulation of chlamydial gene expression (Hackstadt *et al.*, 1991, Hackstadt *et al.*, 1993, Pedersen *et al.*, 1994, Pedersen *et al.*, 1996). *C. pneumoniae* has a small genome of ~1.23 x 10^6 base pairs (bp). The small genome results probably from reductive evolution during which *Chlamydia* has gradually dispensed the unessential genes in the process of adapting to intracellular environment (Bavoil *et al.*, 2000). The genome of *C. pneumoniae* is slightly larger than that of *C. trachomatis*. When compared to the *C. trachomatis* genome, *C. pneumoniae* has additional 214 coding sequences. Only few of the additional coding sequences are homologous to genes from other organisms. The major functionally known addition to *C. pneumoniae* genome is a large expansion of genes coding pmps. *C. pneumoniae* genome contains genes for 21 pmps while *C. trachomatis* has only 9. There are also a few genes that *C. trachomatis* codes but that are missing from the genome of *C. pneumoniae*. The most important of these are perhaps the genes of the tryptophan biosynthesis operon, which may affect the ability of *Chlamydia* to persist in their hosts (Kalman *et al.*, 1999). *C. pneumoniae* differs also from *C. trachomatis* in respect of extra chromosal DNA elements: *C. pneumoniae* does not harbor a plasmid like *C. trachomatis* does, but a single stranded DNA bacteriophage ϕCpn1 has been found in some *C. pneumoniae* strains and all strains may be susceptible for ϕCpn1 infection (Read *et al.*, 2000, Karunakaran *et al.*, 2002, Rupp *et al.*, 2007). Up to date genome sequences of at least five *C. pneumoniae* strains have been completed including human isolates AR39, CWL029, TW183, and J138 (GenBank accession numbers: AE002161, AE001363, AE009440 and BA000008, respectively) and a koala isolate LPCoLN (GenBank accession number CP001713). Only slight differences have been found between the different human *C. pneumoniae* strains (Shirai *et al.*, 2000a, 2000b, Vandahl *et al.*, 2004). However, the koala *C. pneumoniae*, which has a slightly larger genome than the human strains, seems to represent an "older" *C. pneumoniae* strain, retaining some functions that have been lost or fragmented during evolution in human strains (Myers *et al.*, 2009, Mitchell *et al.*, 2010).



EB RB

Figure 1. Electron microscopic image of *Chlamydia pneumoniae* EB and RB. Note the electron dense (black) nucleoid apparent in the EB.

The lack of suitable methods for genetic manipulation of the *Chlamydia* genome has made it very difficult to study the functions of individual genes. Even though some progress has been made in recent years, the ability to manipulate the chlamydial genome remains a key challenge. However, there is hope that the identified bacteriophages capable to infect *C. pneumoniae* offer a vector for delivering foreign DNA into *C. pneumoniae* in the future.

2.1.3. Developmental cycle

The *C. pneumoniae* infection is initiated by attachment of EB to the cell surface and the EB are rapidly internalized by the host cell. *Chlamydia* uses several mechanisms to enter the host cell, including receptor-mediated endocytosis in clathrin coated pits, pinocytosis in non-clathrin coated pits and phagocytosis. There are probably also multiple mechanisms for attachment and internalization and these mechanisms are likely chlamydia species and/or host cell type dependent (Wyrick, 2000, Puolakkainen *et al.*, 2005). Association of *Chlamydia* with lipid rafts and/or actin-rich pedestrials have been described to facilitate chlamydial entry and a putative T3S-effector protein TARP (translocated acting recruiting phosphoprotein) homolog has been shown to recruit actin to the entry site (Clifton *et al.*, 2004, Dautry-Varsat *et al.*, 2005, Jewett *et al.*, 2010). Several bacterial molecules, such as glucosaminoglycan, MOMP, Omp2, Hsp70, and Pmps have been proposed as ligands or adhesins mediating the attachment (Zhang and Stephens, 1992, Raulston *et al.*, 1993, Su *et al.*, 1996, Wehrl *et al.*, 2004a, Fadel *et al.*, 2007, Mölleken *et al.*, 2010) and for cellular receptors of EB such molecules as heparan sulfate, mannose receptor, mannose 6-phosphate/insulin like growth factor 2 receptor and estrogen receptor have been suggested (Kuo *et al.*, 2002, Davis *et al.*, 2002, Puolakkainen *et al.*, 2005). In addition, recent microarray and RNAi studies have revealed several additional host cell molecules important in chlamydial entry (Elwell *et al.*, 2008, Wang *et al.*, 2010).

Transcription and translation are initiated rapidly after infection. The trigger for the initiation of transcription is unknown, but it may happen already upon attachment. The internalized EB containing phagosome resists fusion with lysosomes, which is dependent on chlamydial gene transcription and translation (Scidmore *et al.*, 1996). Inside the phagosome, EB transform rapidly into RB which start to replicate their genomes inside the phagosomal inclusion. The inclusion exits soon from the endocytic pathway, enters into the exocytic pathway and the inclusion is transported to perinuclear region. To accommodate multiplying bacteria the developing inclusion must expand and the inclusion membrane grows by acquisition of lipids derived from the host cell (Hackstadt *et al.*, 1995, Hackstadt *et al.*, 1996, Wylie *et al.*, 1997). One source of the lipids is the Golgi apparatus, which is abundant in the perinuclear region (Hacksadt *et al.*, 1996, Heuer *et al.*, 2009). A network of host cell cytoskeletal structures primarily composed of F-actin and intermediate filaments (IFs) provide structural stability for the inclusion during development (Kumar and Valdivia, 2008). During active productive infection, RB-forms

are thought to divide by binary fission, whereas it is typical for a persistent infection that this stage is impaired. In persistent infection the replication of the DNA seems apparently normal, but division of the bacteria is inhibited resulting in aberrant RB-forms (AB) observed by electron microscopy (Beatty *et al.*, 1993, Byrne *et al.*, 2001). In active infection, after multiplication, RB transform to EB and exit the cell either by cell lysis or inclusion extrusion ready to initiate new cycle of infection (Hybiske and Stephens, 2007), while in persistent infection the immature inclusions persists (Figure 2.).

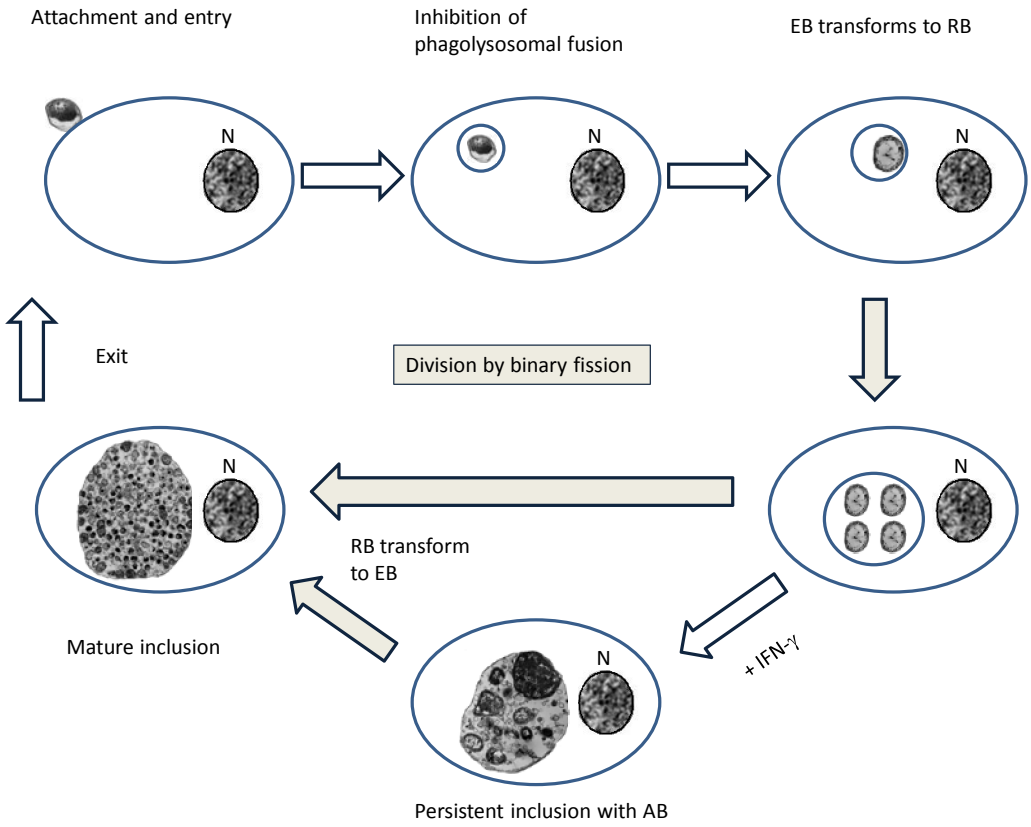


Figure 2. Schematic presentation of the chlamydial developmental cycle. N= nucleus, AB= aberrant body.

2.1.4. Regulation of gene expression

In order to survive the bacterium needs to monitor its surroundings and adjust gene expression accordingly. The gene expression can be regulated at a transcriptional level, post-transcriptional level, translational level or post-translational level. Bacterial development is largely regulated at the level of transcription, mediated by both activators and repressors. In bacterial chromosomes a transcriptional unit consists of a regulatory region, a transcription start site, one or more open reading frames, and a transcription termination site. The regulatory region contains such elements as the promoter, where the RNA polymerase initially binds, and transcription factors binding sites, where transcription factors bind to modulate the binding of the RNA polymerase. Transcription initiation in bacteria requires proteins known as sigma factors (σ), which are essential for proper promoter recognition by RNA polymerase. Bacteria can have multiple σ factors enabling transcription initiation from different promoters (Balleza *et al.*, 2009). In addition to regulation of initiation of transcription by different promoters, sigma factors and transcription factors, also DNA accessibility affects transcription. Compactness of the DNA might represent a physical constraint to transcription initiation and the packing of some chromosomal regions depends on the activity of several proteins such as integration host factor (DNA bending protein), histone like proteins, DNA isomerases, DNA chaperones, and accessory proteins (Balleza *et al.*, 2009). Also alternate mRNA folding and mRNA stability affects whether or not the RNA molecule is translated. Recently small regulatory non-coding RNAs have been reported to attenuate transcription. In addition, mRNA can act as a direct sensor of small molecules and binding of the molecule directs the RNA to fold differently, preventing translation or the bound molecule may trigger cleavage of the RNA molecule by ribozyme activity (Brantl, 2004).

Large-scale analyses of chlamydial gene expression have showed that the genes are transcribed coordinately in a temporal fashion (Shaw *et al.*, 2000, Belland *et al.*, 2003a, Nicholson *et al.*, 2003, Ouellette *et al.*, 2006, Mäurer *et al.*, 2007). Three stages of transcription have been identified: 1) The early expressed genes initiate macromolecular synthesis and potentially modify the bacterial phagosome enabling it to escape fusion with lysosymes. Among the early expressed genes are *euo* and inclusion membrane proteins (Wichlan and Hatch, 1993, Shaw *et al.*, 2000, Belland *et al.*, 2003a). 2) The mid cycle expressed genes represent the metabolically most active stage when chlamydial genomes replicate and cells divide. Indeed, at this stage Belland *et al.* noticed that almost all genes were transcribed, thus highlighting the small facultative capacity of the chlamydial genome (Belland *et al.*, 2003a). 3) The genes expressed at late cycle represent transition of RB to EB and accordingly, cystein-rich membrane proteins omp2 and omp3 involved in EB maturation and histone like proteins Hc1 and Hc2 mediating the condensation of the chromosome are up regulated at this stage (Belland *et al.*, 2003a, Nicholson *et al.*, 2003, Mäurer *et al.*, 2003). Based on the transcription profiles also the type III secretion apparatus seems to be assembled late in the developmental cycle of *Chlamydia* and likely

arms the forming EB to confront the new host cell (Nicholson *et al.*, 2003, Mäurer *et al.*, 2007). In addition to early, mid and late cycle genes Mäurer *et al.* identified a fourth temporal class, which they named tardy. Tardy genes are expressed very late at the developmental cycle and the gene transcripts are suggested to represent mRNA packed in the EB (Mäurer *et al.*, 2007).

It seems that the temporal expression of the chlamydial genes is well coordinated and the genes are transcribed only at the time they are needed. However, relatively little is known about the regulation of transcription. Genomic sequencing has shown that *Chlamydia* has three different sigma factors σ^{28}, σ^{54}, and σ^{66} (Stephens *et al.*, 1998, Kalman *et al.*, 1999) and they are expressed in a temporal fashion (Mathews *et al.*, 1999). The σ^{66} is the major sigma factor while σ^{54} and σ^{28} allow alternate promoter identification. The σ^{28} have been proposed to be a stage specific regulator of late genes (Yu *et al.*, 2006), but not all genes under σ^{28} regulation have late gene profile (Mäurer *et al.*, 2007) and some of the late genes are under σ^{66} regulation (Fahr *et al.*, 1995), supporting earlier observation that it is unlikely that the major role of chlamydial sigma factors is to recognize the promoters of stage-specific genes (Douglas and Hatch, 2000).

Schaumburg and Tan have suggested that chlamydial promoters may be intrinsically weak to allow transcription factors to regulate the complexity of chlamydial growth and development (Schaumburg and Tan, 2003). Several transcription factors have been identified for *Chlamydia* including both activators (Zhong *et al.*, 2001b, Koo *et al.*, 2006, Hickey *et al.*, 2011) and repressors (Wyllie and Raulston, 2001, Wilson and Tan, 2002, Wood *et al.*, 2003). Also the DNA structure plays a role in the regulation of chlamydial gene transcription, and integration host factor and histone like proteins are likely involved in the accessibility of a gene or its promoter for transcription (Mathews and Stephens 1999, Niehus *et al.*, 2008). Recently, Niehus *et al.* demonstrated that individual chlamydial promoters show a differential response to changes in DNA supercoiling that correlates with the temporal expression pattern. This suggests a role for DNA structure in the regulation of the global patterns of temporal gene expression (Niehus *et al.*, 2008). Like in other bacteria, some recent reports indicate a role for small non-coding RNAs in the regulation of chlamydial transcription (Grieshaber *et al.*, 2006a, 2006b, AbdelRahman *et al.*, 2010, Albrecht *et al.*, 2010).

2.1.5. Interaction with the host cell

In order to efficiently infect and colonize their eukaryotic hosts, bacteria have evolved specialized secretion mechanisms to transport their proteins to the extracellular space or to the host cell compartments. Gram-negative bacteria possess several different secretion systems (Table 2.).

Table 2. Protein export, secretion and translocation systems in gram-negative bacteria, systems indicated in *Chlamydiae* are in bold, adapted from Beeckman and Vanrompay, 2010

Name	Process	Sec/Tat dependence	Remarks
Sec and Tat	Export[a]	NA	
T1SS	Secretion[b]	No	
T2SS	Secretion	Yes	
T3SS	Translocation[c]	No	
Flagellum	Secretion	No	Equivalent to T3SS
T4SS	Translocation	Variable	Conjugation system
T4P	Secretion	Yes	Equivalent to T2SS
T5SS	Secretion	Yes	
T6SS	Translocation	No	
C/U	Secretion	Yes	Assembly of pili and fimbriae

[a] Export refers to transport of unfolded and/or folded proteins to the periplasmic space

[b] Secretion refers to transport of proteins to or across the bacterial surface

[c] Translocation refers to the transport of proteins directly across the eukaryotic cell membrane into the host cell cytoplasm

Sec = Sec pathway
Tat = Twin-arginine translocation pathway

T1-6SS = Type 1-6 secretion system

T4P = Type IV pili

C/U = Chaperone/usher pathway

In *Chlamydia* Sec-dependent, Type III and Type V (autotransporter pathway) associated secretion has been identified (Subtil *et al.*, 2001, Jorgensen and Valdivia, 2008, Chen *et al.*, 2010). In addition, *Chlamydia* possesses significant, but incomplete number of type II core proteins (Peabody *et al.*, 2003) and active type II secretion has been implicated (Betts *et al.*, 2009, Zhong *et al.*, 2010). Many pathogenic gram-negative bacteria modulate host cell responses via type III secretion system by injecting specific effector molecules to the host cell cytoplasm. Over 20 years ago "surface projections" resembling morphologically the *Salmonella* typhimurium type III apparatus were identified on *Chlamydia* (Matsumoto *et al*, 1976, Matsumoto, 1981, Nichols *et al.*, 1985) and 10 years later genes for type III secretion system were revealed in chlamydial genome (Hsia *et al.*, 1997, Stephens *et al.*, 1998). However, the genome sequence data demonstrated that *Chlamydiae* encode a reduced collection of proteins involved in the secretory machine (Rockey *et al.*, 2000). Several putative *C. pneumoniae* effector proteins secreted into the host cell cytosol and nucleus have been identified (Table 3.) and these effectors are likely important virulence factors for *Chlamydia*. *Chlamydiae* also produce proteins that are inserted into the inclusion membrane and at least some of them are T3S secreted (Table 3.). Inclusion membrane protein (Inc) A was the first protein demonstrated to be localized to the inclusion membrane (Rockey at al., 1995). And since then over 40 candidates of Inc proteins have been identified in the chlamydiae genome. The Inc proteins share little sequence homology, but they share a unique bilobed hydrophobic domain suggesting structural homology (Bannantine *et al.*, 2000). Based on their location in the inclusion membrane Incs are likely to play important role in the interaction between *Chlamydia* and the host cell. IncA has been shown to be required for homotypic fusion in *C. trachomatis* inclusions and *C. trachomatis* Inc G has been implicated to interact with host cell signaling pathways (Hackstadt *et al.*, 1999, Scidmore and Hackstadt, 2001). An inclusion membrane protein of *C. pneumoniae* (Cpn0585) has been suggested to interact with host Rab GTP:ases, which are involved in the generation, transport, docking, and fusion of vesicles, as well as in many other membrane trafficking functions. Interestingly, overexpression of Cpn0585 in HEp-2 cells led to restricted *C. pneumoniae* infection resembling persistent infection (Cortes *et al.*, 2007).

The Th1 type immune response and production of gamma interferon (IFN-γ) are critical for eradication of chlamydial infection (Johansson *et al.*, 1997, Penttilä *et al.*, 1998). Given the importance of the Th1-biased cellular immune response for protection, it is significant that *Chlamydia* seem to have developed specific immunoevasion strategies. *Chlamydia* can inhibit the expression of major histocompatibility complex (MHC) II antigen by degradation of the upstream stimulatory factor-1 (USF-1) and the expression of MHC class I antigen by degradation of the downstream transcription factor RFX5 (Zhong *et al.*, 2000). A secreted chlamydial proteasome-like activity factor (CPAF) is responsible for RFX5 and USF-1 degradation (Zhong *et al.*, 2001a, Fan *et al.*, 2002). CAPF is secreted across the inner membrane in a Sec-dependent manner and is further transported across the outer membrane with an unknown mechanism (Chen *et al.*, 2010). In addition to cleaving host cell transcription factors, CPAF is also capable of cleaving host cell IF-

proteins cytokeratin 8, cytokeratin 18, and vimentin possibly contributing to cytoskeletal arrangements seen during chlamydial infections (Kumar and Valdivia 2008, Dong *et al.*, 2004, Heuer *et al.*, 2003). Another important chlamydial immunoavoidance mechanism targets major innate immunity signaling pathways, particularly NFκB. *Chlamydia* has been implicated to reduce cell surface exposure of tumor necrosis factor receptor 1 (Paland *et al.*, 2008), to modulate other cytokine receptors (Shirey and Carlin 2006, Wolf *et al.*, 2009), to inhibit IkBa degradation (Le Negrate *et al.*, 2008) and to specifically degrade p65 subunit of NFκB by chlamydial tail-specific protease (Lad *et al.*, 2007a, 2007b)

Chlamydia has been reported to both inhibit and induce programmed cell death, apoptosis (Fan *et al.*, 1998, Ojcius *et al.*, 1998, Fischer *et al.*, 2001, Airenne *et al.*, 2002, Dean and Powers, 2001, Perfettini *et al.*, 2002, Stenner-Liewen *et al.*, 2002, Dumrese *et al.*, 2005). *Chlamydia* prevents apoptosis by inhibiting cytochrome *c* release from mitochondria. Release of cytochrome c is required for activation of caspase 3 and *Chlamydia* cannot resist apoptotic stimuli once caspase 3 has been activated (Fan *et al.*, 1998, Fischer *et al.*, 2001). Infection of monocytes has been reported to activate NF-κB, which may at least in part be responsible of apoptosis inhibition in this cell type (Wahl *et al.*, 2001). Persistent *Chlamydia* infection has been shown also to resist apoptotic stimuli (Dean and Powers, 2001, Airenne *et al.*, 2002). Airenne *et al.*, showed that during persistent *C. pneumoniae* infection the inhibition of apoptosis was still observed at the end of the follow-up period at five days post infection and was restricted to the cells carrying chlamydial inclusions (Airenne *et al.*, 2002). Evasion of host cell apoptosis may be an important mechanism for *C. pneumoniae* to establish persistent infection, since active maintenance of host-cell viability and longevity would allow a long term relationship between *Chlamydia* and the host cell. On the other hand at the end of the developmental cycle of active infection *Chlamydia* must exit the host cell and induction of apotosis may facilitate the exit, although the cell death at the end of developmental cycle may be *Chlamydia* induced, but nonapoptotic (Paschen *et al.*, 2008). A Chlamydial effector protein CADD has been reported to induce apoptosis in a variety of mammalian cells via binding to the death domains of TNF family receptors (Stenner-Liewen *et al.*, 2002).

Table 3. Overview of putative T3S effector proteins of *Chlamydia pneumoniae*

Protein	Localization (evidence from)	Function/interactions (evidence from)	T3S (evidence from)	Remarks	References
CADD/Cpn0761	Host cell cytocol, in the vicinity of the inclusion (Ctr)	Modulates host cell apoptosis via binding to the death domains of TNF family receptors (Ctr).	Presumed T3S	Late cycle expression	Stenner-Liewen *et al*., 2002, Schwarzenbacher *et al*., 2004
CopN/LcrE/Cpn0324	Surface of chlamydia (Ctr, Cpn), inclusion membrane (Ctr, Cpn)	Probable gating protein (tip of the T3SS needle). Overexpression disrupts microtubules in host cells and delays cell cycle progression (Cpn). Small molecule inhibitors of CopN inhibits Cpn, but not Ctr replication.	Secreted via heterologous T3SS (Ctr)	Late cycle expression and detected late in the inclusion membrane (Cpn)	Fields and Hackstadt, 2000, Slepenkin *et al*., 2003, Ho and Starnbach 2005, Herrmann *et al*., 2006, Huang *et al*., 2008
IncA/Cpn0186	Inclusion membrane	Homotypic inclusion fusion in Ctr (Cpn inclusions do not fuse) and recruitment of host SNARE proteins (Ctr).	Secreted via heterologous T3SS (Cpn)	Detectable in the Cpn inclusion membrane at 12 h after infection	Hackstadt *et al*., 1999, Subtil *et al*., 2001, Delevoye *et al*., 2008, Paumet *et al*., 2009
Inc B/Cpn0291	Inclusion membrane (Chp, Ctr, Cpn), in inclusion body (Cpn)		Secreted via heterologous T3SS (Cpn)		Subtil *et al*., 2001, Lugert *et al*., 2004
Inc C/Cpn0292	Inclusion membrane (Chp, Ctr, Cpn), in inclusion body (Cpn)		Secreted via heterologous T3SS (Cpn)		Subtil *et al*., 2001, Lugert *et al*., 2004
Mip/Cpn0661	Surface of chlamydia (Ctr, Cca), inclusion membrane (Cpn)	Suggested to be important in the infection initiation, since infection is reduced after inhibiting Mip associatedpeptidyl prolyl cis–trans isomerase activity with FK506 (Ctr, Cpn). However, FK506 affects also many other functions in the cells, which may be resposble for the effects on chlamydia infection.	Presumed T3S (may also be Sec-dependent)	Homologous to the macrophage infectivity potentiator (Mip) gene of *L. pneumophila*. Constitutive expression in *C. pneumoniae*.	Lundemose *et al*., 1993, Rockey *et al*., 1996, Montigiani *et al*., 2002, Herrmann *et al*., 2006
NUE/Cpn0708	Host cell nucleus	Associated to host chromatin, possess histone methyltransferase activity that targets host cell histones but does not modify bacterial histone-like proteins (Ctr).	Secreted via heterologous T3SS (Ctr, Cca, Cpn)	Late cycle expression	Pennini *et al*., 2010
PknD/Cpn0095	Host cell cytocol and inclusion membrane				Huang *et al*., 2008
Pkn5/Cpn0703	Inclusion membrane (Cpn)		Secreted via heterologous T3SS (Ctr)	Mid cycle expression (Cpn)	Ho and Starnbach, 2005
SctP/Cpn0705	Host cell cytocol (Cca)		Secreted vie heterologous T3SS (Cca)		Subtil *et al*., 2005
TARP/Cpn0572	Host cell cytocol, present in EB (Ctr)	Phosphorylateted on tyrosine residues, involved in recruitment and nucleation of actin to facilitate entry of EBs into host cells. Interaction with host SHC1, which regulates apoptosis- and growth-related genes (Ctr).	Secreted via heterologous T3SS (Ctr)		Clifton *et al*., 2004, Mehlitz *et al*., 2010
Cpn0146	Inclusion membrane (Cpn)		Presumed T3S	Unique to Cpn, detectable in the inclusion membrane 24 h after infection	Luo *et al*., 2007b

Cpn0147	Inclusion membrane (Cpn)		Presumed T3S	Unique to Cpn, detectable in the inclusion membrane 6 h after infection	Luo *et al* ., 2007b
Cpn0516	Inclusion membrane (Cpn)	Interacts with NFκB activator 1 (Act1) leading to inhibiton of IL-17R signalling pathway (Cpn).	Presumed T3S		Wolf *et al* ., 2009
Cpn0585	Inclusion membrane (Cpn)	Interacts with Rabs 1, 10, and 11, overexpression leads to restricted Cpn infection resembling persistent infection (Cpn).	Presumed T3S	Similar to Cps incA, late cycle transcription and early cycle protein expression reported (Cpn), up-regulation of transcription in IFN-γ induced persistent infection reported	Cortes *et al* ., 2007, Luo *et al* ., 2007a, Mathews *et al* ., 2001
Cpn0709	Inclusion membrane (Cpn)		Presumed T3S		Herrmann *et al* ., 2006
Cpn0712	Inclusion membrane and host cell cytocol (Cpn)	Colocalizes with actin.	Presumed T3S		Herrmann *et al* ., 2006, Müller *et al* ., 2008
Cpn0725			Secreted via heterologous T3SS (Cca)		Subtil *et al* ., 2005
Cpn0726	Host cell cytocol, and nucleus (ctr)		Presumed T3S	Secreted late in the developmental cycle	Hobolt-Pedersen *et al* ., 2009
Cpn0796	Host cell cytocol		Presumed T3S		Dong *et al* ., 2006
Cpn0797	Host cell cytocol		Presumed T3S		Dong *et al* ., 2006
Cpn0809	Host cell cytosol and inclusion body (Cpn)	Colocalizes with ER marker calnexin (Cpn).	Presumed T3S	Late cycle expression (Cpn)	Lugert *et al* ., 2004, Müller *et al* ., 2008
Cpn0859			Secreted via heterologous T3SS (Cca)		Subtil *et al* ., 2005
Cpn0872	Inclusion membrane		Presumed T3S		Herrmann *et al* ., 2006
Cpn1004	Host cell cytosol	Interacts with mammalian Grap2 cyclin D-interacting protein (GCIP), ubiquitously expressed protein affecting eucariotic cell cycle that is degraded during chlamydial infection (Ctr).	Secreted via heterologous T3SS (Ctr)	Mid cycle expression	Chellas-Géry *et al* ., 2007
Cpn1005			Secreted via heterologous T3SS (Cca)		
Cpn1020	Host cell cytosol	Colocalizes with ER marker calnexin (Cpn).	Presumed T3S	Low level constitutive expression (Cpn)	Lugert *et al* ., 2004, Huang *et al* ., 2008, Müller *et al* ., 2008
Cpn1027	Inclusion membrane (Cpn)		Presumed T3S	Unique to Cpn, detectable in the Cpn inclusion membrane at 12 h after infection	Flores *et al* ., 2007

Cpn = *C. pneumoniae* , Ctr = *C. trachomatis* , Cps = *C. psittaci* , Cca= *C. caviae* , T3S = Type III secretion, T3SS = Type III secretion system

2.1.6. *Chlamydia pneumoniae* diseases

C. pneumoniae is a respiratory pathogen and it has been estimated to cover ~10 % of community acquired pneumonia and ~5% acute bronchitis and sinuitis cases. However, more often *C. pneumoniae* infection causes common cold or the infection is asymptomatic. Cough is common and often prolonged. Incubation period of *C. pneumoniae* infection can be several weeks, which is exceptionally long. Epidemics have been reported, and their course is usually long. The organism is thought to spread from person to person via respiratory droplets and the spread of infection seems to be relatively inefficient (Ekman *et al.*1993, Kauppinen *et al*, 1995). *C. pneumoniae* infection appears to be most common with children over 5 years of age. The prevalence of *C. pneumoniae* antibodies increases most from 5 to 14 years of age, and by age 20 approximately 50% are seropositive, and the seropositivity reaches approximately 75-80% among the elderly (Saikku, 1992a, Kuo *et al.*, 1995). Reinfection with *C. pneumoniae* appears to be common and the acute phase can be followed by a persistent or chronic *C. pneumoniae* infection (Grayston *et al.*, 1990, Saikku, 1992a). Also co-infections occur with other respiratory tract pathogens especially with *Streptococcus pneumoniae* and *Mycoplasma pneumoniae* (Kauppinen *et al.*, 1995, 1996, Heiskanen-Kosma *et al.*, 1999, Monno *et al.*, 2002).

Persistent *C. pneumoniae* infection may be a major contributor to several common human diseases, including atherosclerosis, asthma and even lung cancer. The ability of *C. pneumoniae* to persist and therefore chronically stimulate the immune system with bacterial antigens is likely an important biological property of the bacteria contributing to the chronic inflammatory process of atherosclerosis (Mahony and Coombes, 2001). Atherosclerosis is characterized by persistent inflammation at the vessel wall, overgrowth of smooth muscle cells, formation of fibrous scar like-tissue, lipid deposition and intimal thickening leading to narrowing of the vessel lumen (Ross, 1999). The first association of *C. pneumoniae* with atherosclerosis was based on the serological land mark study by Saikku and his colleagues at 1988 (Saikku *et al.*, 1988). This association is supported by several pathological studies. *C. pneumoniae* genome, proteins and even viable bacteria have been identified in atherosclerotic tissues (for more detail see chapter 2.1.7. Cell tropism). In mouse and rabbit experimental infection models, repeated intranasal infection with *C. pneumoniae* results in increased aortic lipid accumulation, induction of early atherosclerotic lesions or accelerated progression of atherosclerosis (Fong *et al.*, 1997, Laitinen *et al.*, 1997, Campbell *et al.*, 2000, Erkkilä *et al.*, 2004, Törmäkangas *et al.*, 2005a). Recently, Campbell *et al.*, showed in an experimental infection model that the expression of scavenger receptor for oxidized low density lipoprotein and proatherogenic factors in the lung and aorta of mice are induced by repeated *C. pneumoniae* infections (Campbell *et al.*, 2010). In addition, some data from animal models indicate that antibiotics given in the early course of the infection prevent induction or reduce the progression of atherosclerosis by *C. pneumoniae* (Muhlestein *et al.*, 1998, Fong *et al.*, 2002). However, if the antibiotic is given later, there is much less or no reduction in the

progression of atherosclerosis by *C. pneumoniae* (Rothstein *et al.*, 2001, Fong *et al.*, 2002). Antibiotic treatment for the prevention of cardiac events in humans has produced varied results. However, a number of preliminary clinical trials of antibiotic therapy directed at atherosclerotic changes in the carotid artery, peripheral arteries, and the aorta have reported favorable effect on the progression of disease. The large clinical trials (WIZARD, PROVE-IT, and ACES) studying antibiotic monotherapy for the prevention of cardiac events in humans have shown that antibiotic administration cannot be recommended for the treatment of chronic coronary heart disease. Yet, antibiotic administration may be beneficial for prevention of disease development (Campbell and Kuo, 2004, Grayston *et al.*, 2005).

Asthma is a chronic inflammatory disease of the airways and it is characterized by bronchial construction, wheezing, breathlessness, chest tightness, and coughing. Along with some viruses and *M. pneumoniae*, *C. pnemoniae* is associated with initiation, exacerbation and promotion of asthma (Hahn, 1996). The pathogenesis of virus associated asthma exacerbations has been postulated to involve several mechanisms including epithelial damage, release of inflammatory mediators, and generation of virus-specific IgE-antibodies (Hahn, 1999). Hahn suggests that some of the mechanisms of virus-associated asthma also apply to *Chlamydia* associated asthma: 1) *C. pneumoniae* infects the human bronchial tree causing ciliary dysfunction and epithelial damage. 2) *Chlamydia* generates inflammatory cytokines both *in vitro* and *in vivo* 3) *C. pneumoniae*-specific IgE has been associated to asthma in culture positive children and in patients with adult-onset asthma. Additional potential mechanisms include *C. pneumoniae*-induced macrophage and/or smooth muscle cell dysregulation and LPS-induced bronchial hyperreactivity (Hahn, 1999).

2.1.7. Cell tropism

In addition to a broad host range, *C. pneumoniae* seems to be able to infect a large variety of human cells *in vitro*. In addition to alveolar epithelial cells, which are primary target cells of *C. pneumoniae* (Rupp *et al.*, 2004), association of *C. pneumoniae* to numerous chronic diseases has lead to setting up experimental infection models and *in vitro* infection models of relevant cell types for the associated diseases (Campbell and Kuo 2004, Dreses-Werringloer *et al.*, 2006, Beagley *et al.*, 2009).

Typical respiratory epithelium (TRE) consists of ciliated pseudostratified columnar epithelial cells with mucus-producing goblet cells scattered throughout. TRE is the characteristic lining epithelium from the nose to the small bronchioles. The alveoli are lined by squamous type I epithelium (Type I pneumocytes). Type II pneumocytes are typically found at the alveolar-septal junctions and they produce and secrete surfactant, a

lipid rich material that coats the inside of the alveoli. Type II pneumocytes have also been suggested to significantly contribute to pulmonary inflammation response when exposed to endotoxin (Sunil *et al.*, 2002). Also macrophages are present in the alveoli and during infection their amount increases greatly as monocytes migrate to the lungs and differentiate into macrophages. In an *ex vivo* model of pulmonary infection, *C. pneumoniae* was detected in similar percentages in type I and type II pneumocytes (~4% of cells) by immunohistochemical staining and *in situ* hybridization (Rupp *et al.*, 2004). In contrast, in pulmonary tissues from COPD patients *C. pneumoniae* was mainly observed in type II cells (Rupp *et al.*, 2004). *C. pneumoniae* was only sporadically detected in TRE cells, whereas it was present in ~30% of alveolar macrophages (Rupp *et al.*, 2004).

Monocytes are thought to be the carriers of *C. pneumoniae* from the initial site of infection to the site of inflammatory disease. Indeed, *C. pneumoniae* can infect peripheral blood mononuclear cells (PBMC) and monocyte/macrophage cell lines such as U-973, THP-1 and Mono Mac 6 (Beagley *et al.*, 2009). In addition, *C. pneumoniae* DNA has been detected in peripheral blood mononuclear cells from patients with cardiovascular disease and from blood donors (Beagley *et al.*, 2009, Boman *et al.*, 1998, Maass *et al.*, 2000b) suggesting that mononuclear cells are susceptible to *C. pneumoniae* infection also *in vivo*. Consistently in infection studies with monocyte/macrophages, chlamydial replication is limited and it seems that macrophages are more susceptible to chlamydial infection than monocytes (Beagley *et al.*, 2009). However, usually while infectious EB cannot be isolated from monocyte/macrophages, the bacteria still remains viable inside the cell as demonstrated by the presence of bacterial RNA transcripts. In addition to means of transport from initial site of infection, infection of macrophages with *C. pneumoniae* seems to enhance adherence and migration of macrophages to endothelial cells (Molestina *et al.*, 1999, Kalayoglu *et al.*, 2001, May *et al.*, 2003). *C. pneumoniae* has also been shown to induce low density lipoprotein oxidation and increased uptake of cholesterol by macrophages and subsequent foam cell formation (Kalayoglu *et al.*, 1999, Kalayoglu and Byrne, 1998). Recently, Bobryshev *et al.*, demonstrated in atherosclerotic tissue samples the presence of chlamydial elementary, reticulate, and aberrant body like stuctures in the cytoplasm of macrophage foam cells (Bobryshev *et al.*, 2008).

C. pneumoniae can infect also other immune cells *in vitro* including dendritic cells, neutrophils and T-lymphocytes (Haranaga *et al.*, 2001, van Zandbergen *et al.*, 2004, Wittkop *et al.*, 2006), and is capable to at least survive and induce inflammatory response in mast cells (Oksaharju *et al.*, 2009). Several studies have shown *C. pneumoniae* antigen or DNA in atheromatous plaques (Campbell *et al.*, 1995, Muhlestein *et al.*, 1996, Maass *et al.*, 1998b, Bartles *et al.*, 1999) and viable *Chlamydia* from atherosclerotic coronary and caroitid arteries (Ramirez, 1996, Jackson *et al.*, 1997, Maass *et al.*, 1998a). *In vitro C. pneumoniae* has been shown to be capable to infect human endothelial cells, smooth muscle cells, and fibroblasts (Kaukoranta-Tolvanen *et al.*, 1994, Godzik *et al.*, 1995, Gaydos *et al.*, 1996, Maass *et al.*, 2000a). *C. pneumoniae* has been identified within

astrocytes and microglia in the brain of late-onset alzheimer disease patients (Balin *et al.*, 1998, Arking *et al.*, 1999) and *C. pneumoniae* has been isolated from brain samples of late-onset alzheimer disease patients (Dreses-Werringloer *et al.*, 2009). In addition, *C. pneumoniae* is capable of productive infection in human astrocytoma and microglioma cell lines (Dreses-Werringloer *et al.*, 2006). The capability of *C. pneumoniae* to infect a large variety of cell types is likely an important contributor to its virulence because the capacity to induce disease is dependent on the cell and tissue infected.

2.1.8. Immunopathology

Immunopathology is a disease process that has an immunologic cause e.g. injury induced by antibodies or other products of an immune response. Tissue damage and scarring seen in chlamydial infections (e.g. blinding trachoma and tubal factor infertility associated to *C. trachomatis* infection) are caused by inflammation-based pathology and inflammation is a key pathologic feature of chronic *C. pneumoniae* disease associations. Chlamydial infection, especially reinfection, evokes an inflammatory response in the host and the response aims at the clearance of the infection, but it may also lead to immunopathology. The immune response to chlamydial infection involves both innate and adaptive immunity (Puolakkainen, 2009). The innate immunity offers the first line of defense against microbial infections. The innate immune response involves pattern recognition receptors (PRR) on the surfaces of immune and non-immune cells. With the aid of these molecules, cells sense the invading micro-organism by recognizing specific structures (pathogen associated molecular patterns, PAMP) of the invading micro-organism (Puolakkainen, 2009). In connection to chlamydial infection, toll like receptors 2 (TLR2) and 4 (TLR4) are the most studied PRR and several putative PAMP on *Chlamydia* have been identified including LPS and Hsp60 (Puolakkainen, 2009). PRR recognition of PAMP leads to the activation of phagocytes and signaling cascades that activate host transcription factors NF-κB and AP-1 leading to expression of proinflammatory cytokines such as IL-1β, TNF-α and IL-6 (Darville, 2006, Puolakkainen, 2009). Although immune cells such as macrophages and neutrophils are the key elements of the innate immune system, the epithelial cells are the first line of defence against the *Chlamydia* infection. Rasmussen *et al.* were the first to show that epithelial cells secrete proinflammatory cytokines in response to chlamydial infection (Rasmussen *et al.*, 1997). Since then others have shown the production of inflammatory mediators from non-immune host cells during chlamydial infection suggesting that the inflammatory response to *Chlamydia* is initiated and sustained by infected non-immune host cells (Darville, 2006). Persistent *C. pneumoniae* infection may result in constant activation of host cell signaling pathways leading to persistent inflammation, and thus contributes to the pathogenesis of a chronic inflammatory disease. In contradiction, *Chlamydia* has also been proposed to disrupt NF-κB signal transduction and thus the subsequent inflammatory response with the aid of chlamydial tail-specific protease. However, it was suggested that it may allow for persistent *Chlamydia* infection to establish in humans and prevent the formation of a long-

lasting immunity against the infection (Lad *et al.*, 2007a, 2007b), and on the other hand it has been suggested that once persistent infection has been established the cells would no longer be protected from NF-κB stimulation (Betts *et al.*, 2009).

The cytokines secreted by epithelial cells may also have a role in directing the adaptive immune response. The adaptive immune system consists of two arms, humoral immunity (B lymphocytes) and cell mediated immunity (T lymphocytes). In adaptive immunity, a strong T-cell response is required to eradicate infected cells, whereas humoral immunity associated antibody response may neutralize the EB in the extracellular environment. However, only partial protection is achieved during natural infection and reinfections are common. Two T helper cell subsets are recognized based on their cytokine secretion profiles, T helper 1 (Th1) and T helper 2 (Th2). Th1 cells secrete e.g. IFN-γ, IL-2, and lymphotoxin, and Th2 cells secrete e.g. IL-4, IL-5, IL-6, and IL-13 (Cherwinski *et al.*, 1987, Mosmann and Coffman, 1989). Also Th0 pattern, which includes both Th1 and Th2 cytokines, has been described (Caccamo *et al.*, 2005). The Th1 response and production of IFN-γ are critical for eradication of chlamydial infection (Johansson *et al.*, 1997, Penttilä *et al.*, 1998), while Th2 response with production of IL-4 and IL-10 may promote persistence and pathology (Yang *et al.*, 1996). Indeed, patients with severe trachomatous scarring have been shown to elicit Th2 type cytokines (Holland *et al.*, 1996).

There are numerous factors that probably influence the susceptibility to and the severity of *Chlamydia* infection, including environmental and epidemiological factors, co-infections and host's genetic background, including HLA haplotypes and polymorphisms in inflammatory response genes. *Chlamydia* infections with increased or decreased risk of sequelae have been associated to genetic polymorphisms of hosts by several reports (reviewed in Puolakkainen 2009).

2.1.9. Diagnostics

Serology is commonly used to diagnose acute *C. pneumoniae* infections. According to the current recommendations from 2001 for *C. pneumoniae* serology, microimmunofluorescence test (MIF) (Wang and Grayston, 1970) is considered the only acceptable approach (Dowell *et al.*, 2001). This approach uses purified EB as an antigen. The method is species-specific and it is capable of measuring IgA, IgG and IgM antibodies. Separation between antibody classes is important in order to distinguish recent from past infection as well as primary from reinfection (Kuo *et al.* 1995). Interpretation of the results is not straightforward and requires an experienced microscopist. According to current recommendations, acute infection is defined by a 4-fold rise in IgG or an IgM titer of ≥16 and the use of a single elevated IgG titer as diagnostic is discouraged. Past exposure is indicated by an IgG titer of ≥16 (Dowell *et al.*, 2001). In a primary infection,

the IgG antibody response may not appear until 6 to 8 weeks after the onset of illness, whereas the IgM antibody has been estimated to appear in three weeks after the onset (Kuo *et al.*, 1995). The IgM response is low or absent in reinfection, and the IgG antibody rise can be measured earlier, within one to two weeks (Boman and Hammerschlag, 2002).

Enzyme immununoassays (EIA) have potential for standardized performance, automation, and objective end points. EIA is also able to distinguish between IgA, IgG, and IgM antibody classes. In EIA, recombinant LPS antigen, LPS-extracted EB or synthetic peptides have been used as antigens. However, there have been problems with the sensitivity and specificity of the current EIA tests and cross-reactions between the chlamydial species have been observed (Boman and Hammerschlag, 2002, Hermann *et al.*, 2002, Hoymans *et al.*, 2003, Paldanius *et al.*, 2006).

Also a complement fixation test (CF) is used to study *C. pneumoniae* antibody levels. It measures antibody response against chlamydial LPS and therefore is unable to distinguish between *Chlamydia* species (Kuo *et al.*, 1995). The advantage of the CF test is that chlamydial CF antibodies are produced rapidly and may be detected earlier than MIF IgM antibodies. On the other hand, the CF test is not suitable for detecting reinfections, where CF antibodies are often at low levels or absent (Kuo *et al.*, 1995).

Isolation of *C. pneumoniae* in cell culture is considered the only reliable method to confirm the viability of the organism in specimen (Dowell *et al.*, 2001). However, it is not able to detect a viable RB form of *Chlamydia*. Detection of chlamydial RNA also suggests viable *Chlamydia* in a specimen, but RNA detection methods are not routinely used in *C. pneumoniae* diagnostics (Beagley *et al.*, 2009). The propagation and isolation of *C. pneumoniae* in cell cultures is an important tool in research laboratories, but it is technically difficult and laborious, and therefore rarely used in routine diagnostics.

PCR has established itself as a sensitive and specific technique that has become important to all areas of microbiology. However, it is essential that such parameters as sensitivity and specificity, reproducibility, and optimal sample types are determined before PCR becomes part of the routine diagnostic workflow. Also, the PCR protocols used in the research laboratory need to be thoroughly validated in order to produce reliable research results. In general, *C. pneumoniae* PCR can be considered at least as sensitive as culture (Murdoch, 2003). *C. pneumoniae* DNA can be detected in both upper and lower respiratory tract samples, but it is unclear which respiratory sample is optimal for PCR testing (Murdoch, 2003). Some investigators have found more PCR positive results for upper than for lower respiratory tract samples, but *C. pneumoniae* DNA positive upper respiratory samples may represent an asymptomatic infection (Verkooyen *et al.*, 1998,

Murdoch, 2003). Vascular tissue specimens and PBMC have been subjected to PCR to investigate the association between *C. pneumoniae* and cardiovascular disease. However, the rate of positive findings varies considerably between different studies (Boman and Hammerschlag, 2002) and agreement is poor between different laboratories even when the same samples are analyzed (Apfalter *et al.*, 2001) highlighting the need for careful validation and standardization of the used techniques. Currently, real time PCR techniques are commonly used both for diagnostic and research purposes. The method has several advantages: the specificity (if a probe is included in the PCR reaction) and sensitivity are good and comparable to conventional PCR combined to hybridization. The method is rapid and no post PCR manipulations are needed, thus preventing contamination problems (Espy *et al.*, 2006). Multiplex PCR-methods in combination with microarray technology are becoming routine in microbial diagnostics (Tissari *et al.*, 2010) allowing simultaneous identification of several microbial targets in one specimen. This technology can also be used to genotype microbes and recently a genotyping assay for *C. trachomatis* has been published (Ruettger *et al.*, 2011). In addition to PCR, the presence of *C. pneumoniae* organism, nucleic acid or antigen can been detected from various samples by, electron microscopy, immunohistochemistry or *in situ* hybridization (Kuo *et al.*, 1995).

At present there is no widely accepted serologic marker for persistent or chronic *C. pneumoniae* infection. High IgA antibody titers have been proposed as a marker of chronic *C. pneumoniae* infection, since the half-life of serum IgA is less than 7 days, compared to several weeks for IgG (Boman and Hammerschlag, 2002). Elevated serum *C. pneumoniae* IgA and IgG antibodies and C-reactive protein has been associated with acute coronary syndromes and asthma (Huittinen *et al.*, 2003, Miya *et al.*, 2004, Sävykoski *et al.*, 2004). Recently Tiirola *et al.* developed an assay for the measurement of chlamydial lipopolysaccharide in serum. The chlamydial LPS was observed more frequently and in higher amounts in patients with acute coronary syndrome and they concluded that the method has potential, at least on an epidemiological level, for detecting chronic *C. pneumoniae* infection (Tiirola *et al.*, 2006).

2.1.10 Treatment

Macrolides (erythromycin, roxithromycin, clarithromycin, and azithromycin), fluoroquinolones (levofloxasin, gatifloxacin, moxifloxasin, gemifloxasin and garenoxacin), tetracyclines, and the new ketolide antibiotic telithromycin exhibit good *in vitro* activity against *C. pneumoniae* (Hammerschlag *et al.*, 2001, Blasi, 2004). Current treatment recommendations for *C. pneumoniae* pneumonia include macrolide, telithromycin or tetracycline as first-line therapy and fluoroquinolone for alternative antimicrobial (Mandell *et al.*, 2007, Honkanen *et al.*, 2008) and prolonged therapy of 10-14 days has been recommended (Kuo *et al.*, 1995, Hammerschlag *et al.*, 2001, Blasi, 2004). However, the treatment of chronic *C. pneumoniae* infections may be problematic.

The normal dosage and duration of antibiotic monotherapy for *C. pneumoniae* infection may not be sufficient to eradicate a persistent infection, whereas suboptimal antibiotic treatment may trigger persistent *C. pneumoniae* infection (Matsumoto and Manire, 1970, Gieffers *et al.*, 2001, Kutlin *et al.*, 2002). In the tissue the delivery of the drugs to the inclusion may be inefficient resulting in suboptimal concentrations inside the inclusion. Thus, new strategies for managing *C. pneumoniae* infection are needed and several new promising natural and synthetic compounds with antichlamydial activity have been identified (Donati *et al.*, 2005, Erkkilä *et al.*, 2005, Törmäkangas *et al.*, 2005b, Alvesalo *et al.*, 2006a, 2006b, Wolf *et al.*, 2006, Muschiol *et al.*, 2009). Polyphenolic compounds have anti-inflammatory and anti-microbial potential and plant polyphenolic compounds may play a role in protection against cardiovascular diseases through their antioxidative properties (Reed, 2002, Aviram *et al.*, 2002, Vuorela *et al.*, 2004). Several flavonoids possess antichlamydial activity and luteolin is among the compounds with perhaps the greatest potential in restricting chlamydial gowth and reducing inflammatory responses (Törmäkangas *et al.*, 2005b, Alvesalo *et al.*, 2006b). Cathelicidins are a family of antimicrobial peptides acting as effector molecules of innate immunity and exerting potent antimicrobial activities. In a study by Donati *et al.* cathelicidin peptide SMAP-29 proved to be most active against chlamydial infection (Donati *et al.*, 2005). Statins are efficient drugs that are used to reduce serum LDL cholesterol levels. In addition, statins have been shown to reduce *C. pneumoniae* growth both in cell cultures and experimental infections in mice (Kothe *et al.* 2000, Erkkilä *et al.*, 2005). The availability of synthetic small molecule inhibitors of the Yersinia type III secretion system (INPs) is providing new tools to modulate T3SS activity, and INPs have been reported to disrupt progression of the chlamydial developmental cycle and to have a strong inhibitory effect on chlamydial growth (Wolf *et al.*, 2006, Muschiol *et al.*, 2009). Also, vitamin D has recently been reported to have antichlamydial activity both in cell cultures and in experimental infections through induction of antimicrobial peptides (He *et al.*, 2010).

3. PERSISTENT CHLAMYDIA PNEUMONIAE INFECTION

Clamydial persistence has been defined as a viable but noncultivable growth stage resulting in a long-term relationship with the infected host cell (Beatty et el., 1994a). The persistence is best characterized *in vitro* (Figure 3.) but evidence for its existence *in vivo* has also been obtained from experimental animal models and from natural infections in humans. As already discussed earlier in chapter 2.1.7. (Cell tropism), morphologically aberrant chlamydial forms have been observed in diseased tissues in association with human chronic disease. Persistent pulmonary *C. pneumoniae* infections with recurrences despite of antibiotic treatment have been described. (Falck *et al.*, 1994, Falck *et al.*, 1996, Miyashita *et al.*, 2002). Additional evidence supporting the occurrence of persistent *C. pneumoniae* infection *in vivo* comes from experimental infection models where culture-negative *C. pneumoniae* infection have been reactivated with corticosteroid treatment (Malinverni *et al.*, 1995, Laitinen *et al.*, 1996).

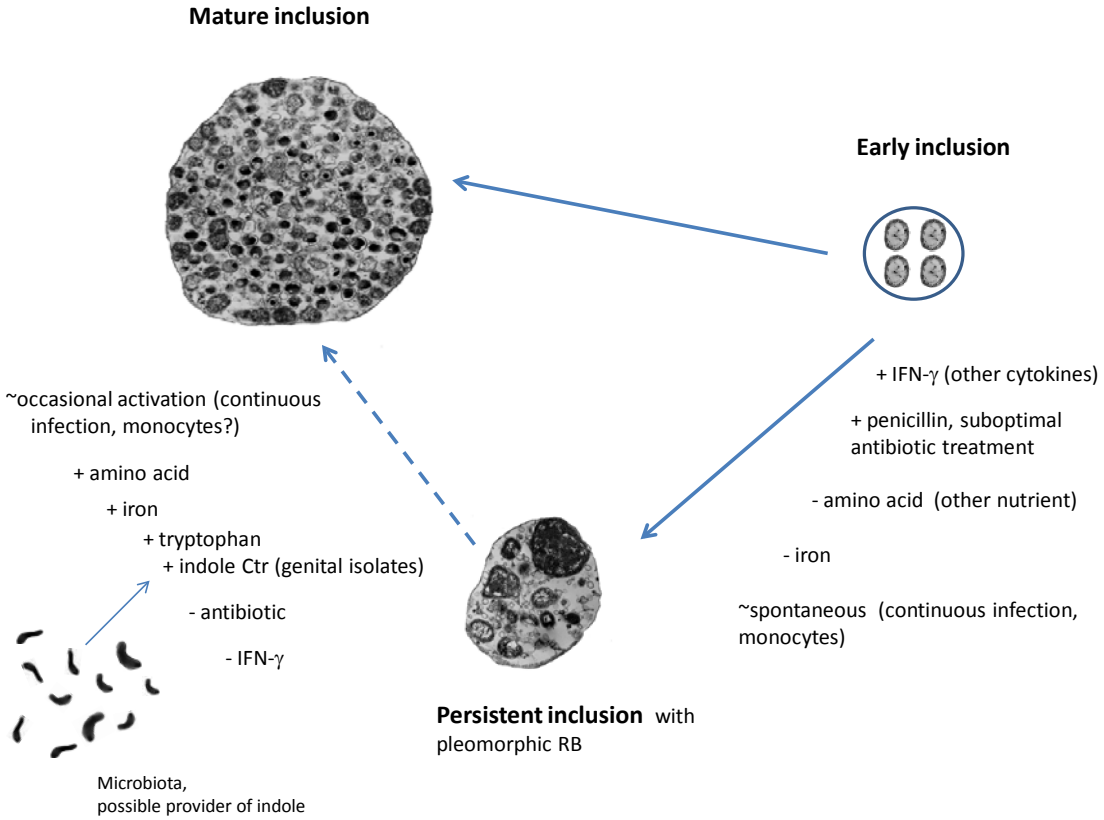


Figure 3. Schematic presentation of establishment and activation of a persistent *C. pneumoniae* infection in an eukaryotic cell.

3.1 In vitro models of persistent Chlamydia infection

It would be ideal if the mechanisms of persistent chlamydia infection could be studied *in vivo* in humans. However, there are several limitations in practice. To study the basic mechanisms of persistence e.g. by microarray analysis, a large number of *C. pneumoniae* infected cells are needed, which is difficult and in some cases even impossible to obtain from one individual. Furthermore, the biological variance between individuals would interfere with interpretation of the results. Animal models e.g. the mouse model is a good option, but the found mechanisms might not reflect the mechanisms in humans, therefore also studies in cell culture systems are needed. *In vitro* infection models provide a

practical approach, although a simplified model, to study mechanisms of persistent *C. pneumoniae* infection with an unlimited source of human cells.

Ultrastructurally characterized penicillin-induced *in vitro* persistence of *C. psittaci* infection has been described already in 1970 by Matsumoto and Manire. Upon exposure to penicillin, the chlamydial RB exhibited aberrant enlarged morphology typical for persistent *Chlamydia* infection and were unable to divide. Removal of penicillin from the culture media recovered the aberrant RB back to normal ones (Matsumoto and Manire, 1970). The effect of penicillin exposure is similar for *C. trachomatis* and *C. pneumoniae* (Skilton *et al.*, 2009, Peters *et al.*, 2005). Also, exposure to suboptimal levels of antibiotics used in treatment such as tetracycline, erythromycin, rifampin, and ciprofloxacin have been shown to induce *in vitro* persistent infections of *C. trachomatis* and *C. pneumoniae* (Kramer and Gordon, 1971, Clark *et al.*, 1982, Hammerschlag and Vuletin, 1985, Gieffers *et al.*, 2004a). Thus, suboptimal antibiotic treatment may cause chlamydial persistence also *in vivo*.

The importance of nutrients such as amino acids, glucose and vitamins to active growth of *C. psittaci* was shown already 50 years ago (Morgan, 1956, Bader and Morgan, 1958, 1961). In these initial *in vitro* studies, *Chlamydia* was shown to be able to invade the host cell in a nutrient deficient medium and remain noninfectious but viable in the cells (Bader and Morgan, 1958, Bader and Morgan, 1961). More recently, Coles *et al.* showed decreased infectivity and production of aberrant *C. psittaci* and *C. trachomatis* morphology when all 13 amino acids were depleted progressively. Upon restoration of amino acids in the growth media the productive infection was resumed (Coles *et al.*, 1993). Amino acid levels present in plasma have been shown to produce aberrant chlamydial forms with reduced infectivity, suggesting that nutrient levels *in vivo* could promote abnormal chlamydial development (Harper *et al.*, 2000).

Iron depletion by addition of the chelating compound deferoxamine mesylate (DAM) has also been shown to restrict the growth of *C. pneumoniae*, *C. trachomatis* and *C. psittaci*. Upon iron depletion, the RB are enlarged and pleomorphic, exhibiting a wavy appearance of the outer membrane (Raulston, 1997, Al-Younes *et al.*, 2001, Goellner *et al.*, 2006). A number of chlamydial enzymes require iron for their activities and therefore the growth restriction may be directely due to the lack of iron (Al-Younes *et al.*, 2001). The growth restriction can be reversed by adding iron loaded transferrin to the growth media (Al-Younes *et al.*, 2001, Kutlin *et al*, 2001, Goellner *et al.*, 2006). One of the host cell's defense mechanisms against bacterial infections is the induction of iron-chelating agents, which may lead to iron deficiency *in vivo*.

Exposure of host cells to moderate levels of IFN-γ restricts chlamydial growth and leads to the development of atypical aberrant RB. The RB are typically enlarged due to the inhibition of cell division and the maturation of RB to infectious EB is minimal. Like in other described models, the alterations in the developmental cycle are reversible and upon removal of IFN-γ aberrant RB restart division and maturate into infectious EB (Beatty *et al.*, 1994a). Well characterized models of IFN-γ induced persistent infection have been described to *C. psittaci*, *C. trachomatis* and *C. pneumoniae* (Byrne *et al.*, 1986, Beatty *et al.*, 1993, Pantoja *et al.*, 2001). The major mechanism of IFN-γ action on chlamydia is based on the induction of indoleamine 2,3-dioxygenase (IDO), a host cell enzyme involved in tryptophan catabolism. In other words, the IFN-γ model of persistent *Chlamydia* infection is also based on amino acid depletion and addition of exogenous tryptophan, and in the case of genital *C. trachomatis* serovars also indole reverses IFN-γ induced growth restriction (Beatty *et al.*, 1994c, Caldwell *et al.*, 2003). Genital serovars of *C. trachomatis* possess functional tryptophan synthase genes and are able to convert indole into tryptophan. Other microbes in vaginal flora may provide the indole into the environment allowing the escape of *C. trachomatis* from the growth inhibitory mechanisms of IFN-γ. *C. pneumoniae* lacks the genes for tryptophan synthase and ocular serovars *C. trachomatis* have frame-shift mutation in the *trpA* gene resulting in a nonfunctional enzyme (Caldwell *et al.*, 2003). In addition to induction of IDO, IFN-γ is known to induce tryptophanyl-tRNA synthetase in fibroblasts and epithelial cells (Rubin *et al.*, 1991, Flohr *et al.*, 1992), which possibly further depletes the tryptophan pool available for *Chlamydia*.

The IFN-γ model for persistent *Chlamydia* infection is perhaps the most widely used and has particular relevance *in vivo* since *Chlamydia* infection is known to elicit the IFN-γ response (Numazaki and Chiba, 1996, Penttilä *et al.*, 1998). High levels of IFN-γ successfully eradicate *Chlamydia*, however low levels of IFN-γ may be a result of a too weak Th1 type immune response or a dominant Th2 type immune response favoring the establishment of a persistent infection. The situation *in vivo* is more complex than that in the *in vitro* model. The level of IDO expression is affected by numerous factors including the host cell type and the anatomical location (Sakash *et al.*, 2002, Sedlmayr *et al.*, 2002). A recent report implicates that hypoxia abrogates anti-chlamydial effects of IFN-γ, reduces IDO expression, and rescues genital *C. trachomatis* from IFN-γ induced persistence (Roth *et al.*, 2010), while another recent study reports that IFN-γ secretion by CD4+ T-cells is significantly enhanced under hypoxic conditions (Roman *et al.*, 2010). Also, synergy in restricting chlamydial growth with other cytokines, especially with tumor necrosis factor alpha (TNF-α) has been described *in vitro* (Summersgill *et al.*, 1995, Cheshire and Baldwin, 1997, Igietseme *et al.*, 1998, Matsushima *et al.*, 1999) and is likely relevant also *in vivo*. Recently Tietzel *et al.* reported that human guanylate binding proteins (hGBP) 1 and 2 potentiate the anti-chlamydial effect of IFN-γ (Tietzel *et al.*, 2009) and in addition to IFN-γ, hGBP1 and 2 are induced by TNF-α and IL1 (Tripal *et al.*, 2007). Alone or together with other cytokines IFN-γ may also contribute to other

inhibitory systems restricting chlamydial growth such as nitric oxide induction and iron deprivation (Byrd and Horwitz, 1993, Igietseme *et al.*, 1998).

Continuous chlamydial cultures were initially described for *C. psittaci* (Moulder *et al.*, 1980) and later also for *C. trachomatis* and *C. pneumoniae* (Lee, 1981, Kutlin *et al.*, 2001). A continuous *Chlamydia* infection can be established without external induction and resembles the other persistent *in vitro* models with the appearance of spontaneous abnormal chlamydial inclusions. These cultures alter between periods of chlamydial multiplication, induced host cell destruction, and periods of cell proliferation, and these cells can be cultured indefinitely (Moulder *et al.*, 1980, Lee, 1981, Kutlin *et al.*, 2001).

C. pneumoniae and *C. trachomatis* infection of monocytes resembles a persistent infection with minimal bacterial cell division, and with a low level of infectious progeny. Aberrant RB morphology is detected and viability of *Chlamydia* can be shown by detection of bacterial mRNA (Koehler *et al.*, 1997, Airenne *et al.*, 1999). The mechanism of chlamydial growth restriction in mononuclear cells remains unknown, but IDO is not likely to play a role, since *Chlamydia* remains nonproductive in monocytic cultures after the addition of tryptophan (Koehler *et al.*, 1997, Airenne *et al.*, 1999).

Tobacco smoke has been reported to induce *C. pneumoniae* infections with characteristics of persistent infections in epithelial and endothelial cell lines (Wiedeman *et al.*, 2004, 2005). Infection of epithelial cells with *C. psittaci* and bacteriophage ϕCPG1 have been shown to switch the bacteria into an altered developmental cycle resembling persistent infection (Hsia *et al.*, 2000) and persistent chlamydia forms have been observed in coinfections of *C. trachomatis* with HSV-2 and *Chlamydia abortus* or *Chlamydia pecorum* with porcine epidemic diarrhea virus (Deka *et al.*, 2006, Vanover *et al.*, 2008, Borel *et al.*, 2010). Also, *C. pneumoniae* is susceptible to phage infection, *C. pneumoniae* AR-39 harbors phage sequenses in its genome (Read *et al.*, 2000, Karunakaran *et al.*, 2002, Rupp *et al.*, 2007) and coinfections of *C. pneumoniae* with an other respiratory pathogens are common (Kauppinen *et al.*, 1995, 1996, Heiskanen-Kosma *et al.*, 1999, Monno *et al.*, 2002). Whether superinfection with a bacteriophage or other microbial pathogen can induce persistent appearance of *C. pneumoniae*, is still unknown.

4. MOLECULAR STUDIES OF CHLAMYDIA PNEUMONIAE – HOST INTERACTIONS

4.1. Transcriptomics

There are several options to study *C. pneumoniae* – host cell interactions on the level of gene transcription. Conventionally Northern blot analysis has been used to study differential transcription of host cells and pathogens, and later reverse transcriptase PCR (RT-PCR) became available. Presently, "real-time" formats of this technique are widely used to study the expression of focused gene sets. More recently, microarray techniques have become available allowing for the simultaneous determination of the RNA levels of thousands of specific mRNA species.

There are varieties of technical solutions for performing microarray analysis, but in essence they are all miniaturized hybridization assays, where the array contains immobilized nucleic acid sequences and detection is based on the hybridization of labeled complementary sequences. In gene expression profiling, mRNA is isolated from the samples of interest and usually reverse transcribed into cDNA or amplified into cRNA and labeled with a fluorochrome. The labeled cDNA is hybridized to the array containing nucleic acid sequences, representing a specific gene, spotted to the array in an orderly pattern. After the hybridization step, the microarrays are scanned and fluorescence intensities are measured. In transcription studies, the two-color hybridization is often used where cDNA molecules from two different conditions are compared to each other and are labeled with different colors. By measuring the different fluorescent signals associated to each spot on the array, the relative abundance of a specific transcript of each studied condition can be determined (Leroy and Raoult, 2010).

Microarray techniques have been used to study transcriptional host cell responses to *C. pneumoniae* infection (Coombes and Mahony 2001, Hess *et al.*, 2003, Virok *et al.*, 2003, Shi and Tokunaga, 2004, Eickhoff *et al.*, 2007, Wang *et al.*, 2010) and to target putative drug molecules (Alvesalo *et al.*, 2008). In some studies, the importance of up-regulated host cell molecules in *C. pneumoniae* infection has been further characterized by knocking down the gene transcript with small inhibitory RNA molecule (Alvesalo *et al.*, 2008, Wang *et al.*, 2010). Most information of host cell responses to *C. pneumoniae* infection comes from *in vitro* models of active infection. Also, the genomic transcriptional activity of *C. pneumoniae* during the developmental cycle has been characterized by microarrays (Ouellette *et al.*, 2006, Mäurer *et al.*, 2007).

Very little is known about host cell response to persistent *C. pneumoniae* infection. *Chlamydia* infection of monocytes is considered to represent persistent *Chlamydia* infection and Virok *et al.* have studied host cell gene expression by microarray in monocytic cell line infected with *C. pneumoniae*. They found that several molecules linked to the development of atherosclerosis and other chronic inflammatory diseases were up-regulated in monocytes in response to *C. pneumoniae* infection (Virok *et al.*, 2003). Peters *et al.* studied seven selected key host molecules of cellular signaling pathways known to be up-regulated in *C. pneumoniae* infection. The expression of the selected host molecules was studied in monocytes and in three different *in vitro* models of persistent *C. pneumoniae* infection in epithelial cells by RT-PCR (CTGF, IL-8, LIF, EGR-1 and ETV4) or ELISA (IL-6 and IL-11). It was demonstrated that depending on the cell type, different signaling cascades are turned on in persistence and depending on the mode that persistent infection is induced in epithelial cells, either permanent activation or silencing of the studied host gene is observed (Peters *et al.*, 2005). A later study by the same group identified 17 human host cell genes related to apoptosis, the cell cycle, or metabolism as both up- or down-regulated in IFN-γ induced persistent infection of epithelial cells by microarray analysis and real-time PCR (Eickhoff *et al.*, 2007).

Transcription of chlamydial genes in different models of persistent *C. pneumoniae* infection in epithelial cells has been conducted both by microarray and RT-PCR studies (Byrne *et al.*, 2001, Mathews *et al.*, 2001, Hogan *et al.*, 2003, Mäurer *et al.*, 2007, Ouellette *et al.*, 2006, Polkinghorne *et al.*, 2006, Slepenkin *et al.*, 2003, Timms *et al.*, 2009). By microarray analysis Ouellette *et al.* noticed global transcriptional up-regulation of *C. pneumoniae* gene transcription in IFN-γ induced persistent infection of epithelial cells without increased translation (Ouellette *et al.*, 2006). In contrast, Mäurer *et al.*, observed global down-regulation or arrest of the transcription during mid phase in the iron limited persistent *C. pneumoniae* infection model. They found σ^{28}, which regulates some of the late genes, to be specifically down regulated (Mäurer *et al.*, 2007). The low level of infection attained in mononuclear cells has hindered the use of genome-wide approaches to analyze bacterial transcription in mononuclear cells and thus there is only limited knowledge of the transcription of *C. pneumoniae* genes in monocyte/macrophages (Airenne *et al.*, 1999, Gieffers *et al.*, 2001, Haranaga *et al.*, 2003, Klos *et al.*, 2009). Before the entire chlamydial genome was sequenced, molecular studies focused on small group of genes thought to be key antigens in persistence. Early studies with *C. trachomatis* showed decreased expression of MOMP and Omp2 and increased expression of Hsp60 (Beatty *et al.*, 1993, Beatty *et al.*, 1994b). Since then, these molecules have been extensively studied in models for persistent *C. pneumoniae* infection without uniform outcome (Airenne *et al.*, 1999, Gieffers *et al.*, 2001, Mathews *et al.*, 2001, Mäurer *et al.*, 2007, Ouellette *et al.*, 2006, Polkinghorne *et al.*, 2006, Haranaga *et al.*, 2003, Slepenkin *et al.*, 2003) and therefore cannot be considered as universal markers of persistent chlamydia infection. In 2001 Byrne *et al.* showed in the IFN-γ induced persistent infection model attenuated levels of expression of genes involved in cell division while genes involved in DNA replication were unaffected agreeing with the observation that in persistent *C.*

pneumoniae infection enlarged RB are formed (Byrne *et al.*, 2001). Other molecules recently reported to be differentially expressed during persistent *Chlamydia* infection include the stress response related gene *htrA*, the early transcribed gene *euo*, genes encoding phospholipase D-like enzymes, ribosomal proteins, histone-like proteins, signal transduction genes, proteases, peptide transporters, and T3S associated genes among others (Belland *et al.*, 2003b, Hogan *et al.*, 2004, Polkinghorne *et al.*, 2006, Ouellette *et al.*, 2006, Mäurer *et al.*, 2007, Timms *et al.*, 2009).

4.2. Proteomics

Proteomics refers to a procedure that characterizes large sets of proteins. The ideal proteome study would provide quantitative information and identification of all proteins in a biological sample analyzed. Such a technique does not exist yet but there has been promising development in the methodology (Malmström *et al.*, 2007, Sleno and Emili, 2008). Proteomic approaches can be used e.g. for proteome profiling, comparative expression analysis, identification of posttranslational modifications, and to study protein–protein interactions (interactomics). The proteome approaches lack sensitivity in comparison to transcriptome methodologies, but they have some advantages. The stability of an RNA transcript affects its translation and protein production from a transcript may be post-transcriptionally regulated, and hence information obtained at the transcriptional level does not always reflect the situation at the protein level. An advantage of a proteomic approach is also its ability to detect proteins in their post-translationally modified and processed form. In the most used proteome approach proteins are separated in two-dimensional polyacrylamide gel electrophoresis and separated spots are identified by mass spectrometry methodologies. Protein arrays are an emerging technology in proteomics and allow the detection and the comparison of large number of different proteins simultaneously (Sleno and Emili, 2008). The protein array technology is still in its infancy, but it has great potential.

Proteome analyses have been used to construct a proteome map of *C. pneumoniae* (http://www.gram.au.dk, http://www.mpiib-berlin.mpg.de/2D-PAGE), to identify secreted *C. pneumoniae* proteins, to characterize constituents of chlamydial OMC, and to identify proteins differentially expressed in persistent infection models (Vandahl *et al.*, 2004). The first proteomic analysis of persistent *C. pneumoniae* infection was published by Molestina *et al.* in 2002. They observed up-regulation of MOMP, Hsp60, and seven other proteins with functions in DNA replication, transcription, translation, glycolysis, and type III secretion at 24 hours after infection in IFN-γ induced persistent infection of HEp-2 epithelial cell line (Molestina *et al.*, 2002). In a study by Wehrl *et al.* 11 proteins were identified as up-regulated and eight as down-regulated in an iron limitation model of persistent *C. pneumoniae* infection in HEp-2 cells (Wehrl *et al.*, 2004b). In an extensive study by Mukhopadhyay *et al.*, protein expression profiles of IFN-γ and iron limitation

models of persistent *C. pneumoniae* infection in HEp-2 cells were compared to those of heat shock stress response. They showed that although persistent *C. pneumoniae* infection contains a stress component it is clearly more than just a stress response. All three studied conditions induced up-regulation of stress response proteins DnaK, HtrA, ClpP_1, and ClpP_2, and down-regulation of Gcp_1 involved in folding, assembly, and modification of proteins and down-regulation of Ndk involved in amino acid biosynthesis. In IFN-γ induced persistent infection many proteins involved in the biosynthesis of amino acids and nucleotides were specifically up-regulated and in the iron limitation model proteins functioning in biosynthesis of cofactors, cellular processes, energy metabolism, transcription, and translation were specifically up-regulated (Mukhopadhyay *et al.*, 2006).

5. AIMS OF THE STUDY

The overall aim of this work was to study *Chlamydia*-host cell interactions in persistent infection models and to identify possible host cell or bacterial genes important for pathogenesis of persistent infection or as putative markers of persistent *C. pneumoniae* infection.

The specific aims of this study were:

i. to set up active and persistent *C. pneumoniae* infection models in epithelial and monocyte/macrophage cell lines

ii. to establish detection methods for *C. pneumoniae* genomes and transcripts in the infection models

iii. to study host cell responses to persistent *C. pneumoniae* infection and compare the obtained results to those from an active infection

iv. to study transcription of selected *C. pneumoniae* genes during the infection of monocytes

6. MATERIALS AND METHODS

Bacterial strains, cell lines and plasmids used in this study are listed in Table 4. The methods used in this study are described in detail in the indicated articles and are summarized in Table 5.

Table 4. Bacterial strains, cell lines and plasmids used in this study

Strain or cell line	Description	Article	Source/Reference
Bacterial strains:			
K6	*C. pneumoniae* isolate Kajaani 6	I-III	Prof.Saikku/Ekman *et al.*, 1993
JM109	*E. coli*	I, III	
Cell lines:			
HL	Human line, an epithelial cell line	I-III	Kuo and Grayston, 1990
Mono Mac 6	Human monocytic cell line	I,III	DSMZ 124/ Ziegler-Heitbrock *et al.*, 1988
Plasmids:			
pGEM-T	Procaryotic cloning vector	I	Promega
pSTBlue-1	Procaryotic cloning vector	III	Novagen

Table 5. Methods used in this study

Method	Described and used in
***C. pneumoniae* culture conditions:**	
Active infection in HL cells	I-III
Persistent infection in HL-cells	I-III
Infection in monocytes	I,III
Molecular biology methods:	
Molecular cloning	I,III
In vitro transcription	III
PCR	I-III
RT-PCR	I-III
Microarray	II
Microscopy:	
Electron microscopy	I
Immunofluorescence microscopy	I-III

7. RESULTS AND DISCUSSION

7.1. The quantitative PCR method (qPCR) for C. pneumoniae DNA (I) and transcripts (III)

To measure *C. pneumoniae* genomes in cell cultures, a quantitative real-time PCR (qPCR) method, using dual-labeled hydrolysis probes targeting *ompA* gene, was set up and optimized. After optimization and validation, it was used in all studies (**I-III**) to measure *C. pneumoniae* genomes during active and persistent infection. The qPCR assay could reproducibly detect 10 *C. pneumoniae* genome equivalents (GE) per reaction showing good analytical sensitivity comparable to that of previously reported quantitative PCR assays for *C. pneumoniae* (Berger *et al.*, 2000, Huang *et al.*, 2001, Mygind *et al.*, 2001). The efficiency of amplification was 95-100%. The intratest coefficient of variation (CV) was 5.0% - 12.2% depending on the used *C. pneumoniae* amount and the intertest CV was at the highest 21%, when 100 genomes/reaction were used, indicating good reproducibility for the method. When the results of the qPCR were compared to those of quantitative culture of *C. pneumoniae* with purified EB preparations and infected murine tissues as starting material, there was in general a 100-fold difference in the amount of *C. pneumoniae* detected by these methods. This suggests that the amplification method is more sensitive than the culture method in detection of *C. pneumoniae*, and that the purified chlamydial preparations as well as the tissue specimens from infected animals contain a mixture of infectious and non-infectious chlamydial particles (**I**).

To analyze transcription of 11 *C. pneumoniae* genes in Mono Mac 6 cells during infection real time RT-PCR methods with dual-labeled hydrolysis probes were set up for each gene. Our primary interest was to study the genes for secreted proteins (incA, cpaf, cpn0572, cpn0809, and lcrE) and the genes (ompA, omp2, ftsK, euo, htrA and hsp60) that were previously found differentially expressed in persistent infection models in epithelial cell lines. The analytical sensitivity of the set up assays were 50-100 RNA copies/reaction as determined by dilution series of *in vitro* transcripts of the corresponding gene. The sensitivities of the assays are comparable to those reported for other RT-qPCR assays (Escaffre *et al.*, 2010, Kitajima *et al.*, 2010). Both the amount of *C. pneumoniae* DNA and the number of 16S rRNA transcripts have been used to normalize *C. pneumoniae* gene transcription data (Polkinghorne *et al.*, 2006, Ouellette *et al.*, 2006, Timms *et al.*, 2009). We normalized our data for both the *C. pneumoniae* DNA measured as genome equivalents (GE) and the 16S rRNA transcripts. There were no significant differences in the transcription data between the two normalization methods **(III)**. However, deviations between the replicate samples were slightly lower (CV% 41 vs. CV% 49) when the 16S rRNA was used for normalization (**III**) and the DNA from nonviable chlamydial particles in Mono Mac 6 cells is known to degrade slowly (**I**). Hence, the *C. pneumoniae* gene transcription data normalized to 16S rRNA transcripts is presented here, although

normalization based on the DNA content was preferred for normalization of chlamydial gene expression in epithelial cells under different growth conditions (Ouellette *et al.*, 2006). As controls, IFN-γ exposed (representing persistent infection) and unexposed (representing active infection) HL cells were infected with *C. pneumoniae*. Our aim was to ascertain that the results based on our infection model in epithelial cells, including the use of Kajaani-6 isolate of *C. pneumoniae*, and the developed methodology for measuring *C. pneumoniae* transcripts were in agreement with those obtained in earlier studies on *C. pneumoniae* grown in epithelial cells. The transcription of the panel of 11 genes was studied with RT-PCR analysis. The panel included three stage-specific genes: 1. Early-cycle gene *euo*, 2. Mid-cycle gene *ompA*, and 3. Late-cycle gene *omp2* (*omcB*) (Ouellette *et al.* 2006, Mäurer *et al.* 2007). Transcription profiles of the three stage-specific genes in HL cells were as reported earlier and the expression profiles of *incA*, *hsp60*, *cpn0809*, *ftsK*, and *htrA* genes during infection of HL cells were similar to those described earlier for epithelial cell cultures (**III**, Mathews *et al.*, 2001, Polkinghorne *et al.*, 2006, Mäurer *et al.*, 2007, Timms *et al.*, 2009).

7.2. Model for active C. pneumoniae infection in epithelial cell line (I)

C. pneumoniae culture is most successful in HL (Cles and Stamm, 1990, Kuo and Grayston 1990) and Hep-2 (Roblin *et al.*, 1992) epithelial cells. Here growth of the *C.pneumoniae* K6 isolate was characterized by qPCR, an infectivity assay, and microscopy in HL cells. The cell cultures were monitored for 8 days (192 hours) after inoculation. In the cells collected early at the developmental cycle, immediately after the inoculation or 24 h later, very few infective EB could be detected (Figure 1./**I**) suggesting that nearly all EB inside the cells had transformed into RB. The RB differentiation to EB occurred most rapidly between 36 and 48 hours after inoculation, but the highest amount of EB was cultured from the cells at 72 hours after inoculation (hai) (Figure 1, **I**). The number of *C. pneumoniae* genomes decreased rapidly between 0 and 12 hai and a similar decrease was observed when cells were inoculated with heat and UV inactivated *C. pneumoniae*. At 12 hours after inoculation only ~10% of the GE detected at 0 h post inoculation still existed and at 72 hours after inoculation only a minor fraction (3-6%) of the initial GE remained in the cells inoculated with heat or UV inactivated *C. pneumoniae*. The observed decrease in the GE number early in the cycle may reflect the presence of nonviable EB in the used *C. pneumoniae* stock or EB being unable to initiate productive infection for other reasons. Accumulation of the *C. pneumoniae* genomes started at 12-24 hours after infection (Figure 1./**I**) and the replication of the genomes still continued after 48 hours after infection although most RB had, based on the growth curve (Figure 1./**I**), already matured into EB, reflecting the asynchronous nature of the later stages of *C. pneumoniae* developmental cycle (Wolf *et al.*, 2000). Why relatively synchronous developmental cycle becomes asynchronous at later stages is not known, but it has been speculated that the dissociation of dividing RB from the inclusion membrane in consequence of the lack of space could trigger RB to EB differentiation, and thus

contribute to the asynchronous nature of the late developmental cycle (Bavoil *et al.*, 2000, Hoare *et al.*, 2008).

By quantitating *C. pneumoniae* genomes by qPCR, we predicted the doubling time of *C. pneumoniae* genome in HL cells using the method of Mathews *et al.* with modifications (Mathews *et al.*, 1999). Between 12 and 24 h after inoculation, there was already an increase in the number of *C. pneumoniae* genomes. Thus, 12 h after infection was assigned as the replication starting point for this model, and then theoretical DNA replication profiles assuming a doubling time of 6, 7 and 9 hours were calculated using 12 hai as a starting point. These replication profiles were then compared to the observed genome replication rates. Based on the accumulation of genome sequences during the exponential phase of genome replication between 12 and 48 h after inoculation, the curve of active *C. pneumoniae* K6 culture lies between 6 and 7 hours doubling time (Figure 4./ **I**). The observed doubling time of 6-7 hours is longer than the doubling time observed for faster growing *C. trachomatis* L2 (Mathews *et al.*, 1999). In a recent study Mitchell *et al.* found similar doubling time (5.9-8.7 h) for the *C. pneumoniae* human isolate AR39 grown in Hep-2 cells. However, a much faster doubling time (3.4-4.9 h) was observed for the *C. pneumoniae* koala isolate LPCoLN in Hep-2 cells (Mitchell *et al.*, 2009). Whether this reflects differences between human and koala isolates or different pathotypes, is unknown.

The growth curve of *C. pneumoniae* K6 in HL cells was found similar to that reported earlier for *C. pneumoniae* AR-39 in HL cells and in HeLa 229 cells (Kuo and Grayston 1990, Wolf *et al.*, 2000). In agreement with previous study (Kuo and Grayston, 1990), a second cycle of *C. pneumoniae* growth in HL cells was detected at 72-96 h after inoculation, as the number of EB recovered and genomes detected decreased, but increased again at 92 to 168 h after infection (Figure 1./**I**). This ability to spread to new cells and initiate a new cycle of growth is critical for *Chlamydia* in order to survive and disseminate.

7.3. Model for persistent C. pneumoniae infection in epithelial cells (I)

Chlamydia is able to modulate the gene expression and apoptosis of host cells, which may assist *Chlamydia* to evade the hosts' immune responses. This, in turn, may lead to extended survival of the organism inside epithelial cells and promote the development of persistent infection. To simulate persistent *C. pneumoniae* infection *in vivo*, we set up a persistent infection model, exposing the HL cell cultures to IFN-γ. At that time little information was available on the kinetics of chlamydial DNA replication and genome load during persistence in host cells. We studied the effect of different concentrations of the cytokine to *C. pneumoniae* growth and chose the appropriate IFN-γ concentration to establish the persistent infection model. This model was characterized by measuring the

number of *C. pneumoniae* genomes by qPCR, maturation by infectivity assay and assessing the morphology of inclusions and the bacteria by immunoflurescence and electron microscope. Growth of *C. pneumoniae* was monitored in the cell cultures for 8 days (192 hours) after inoculation. In agreement with previous studies, exposure of HL cells to IFN-γ dose-dependently inhibited differentiation (Mehta *et al.*, 1998, Summersgill *et al.*, 1995) and DNA-replication of *C. pneumoniae*, but the amount of cytokine required to reduce DNA-replication was higher than that needed to stop the production of *C. pneumoniae* able to infect new cells (Byrne *et al.*, 2001) (Table 1./**I**). Altered inclusion morphology was observed at IFNγ concentrations of 15-25 U/ml: The inclusions were smaller in size and stained less intensely than the inclusions observed in untreated cells. At an IFN-γ concentration of 25 U/ml, the number of inclusion forming units produced per well decreased 20-fold ($P<0,01$) when compared to that of untreated cultures, whereas the number of *C. pneumoniae* genomes remained unaltered ($P>0,05$). At concentrations of >25 U/ml, IFN-γ abolished the production of infectious *C. pneumoniae* EB and reduced the number of GE in HL cells (Table 1./**I**). An IFN-γ concentration of 25 U/ml, which significantly reduces the production of infectious EB, but does not affect DNA replication, was chosen to induce persistent *C. pneumoniae* infection in HL cells in the following experiments.

During IFN-γ induced persistent infection, the growth kinetics of *C. pneumoniae* in HL cells was similar to that of active growth during the first 72 h (Figure 1./**I**). There was also no significant difference in the doubling time of the *C. pneumoniae* genome during active infection and IFN-γ induced persistent infection (Figure 4./**I**). However, no second cycle of infection could be observed, and the number of infectious *C. pneumoniae* particles and GE remained rather stable after 72 h after inoculation (Figure 1./**I**). Also, with fluorescence microscopy small inclusions were seen to remain in the IFN-γ treated cells through the observation period of 8 days, whereas in untreated cells a remarkable reduction in the number of inclusions was observed at 110 h after inoculation.

We estimated the level of *C. pneumoniae* persistence in cultured cells by calculating the ratio of infectious particles/ GE at different time points (Table 2./**I**). During active infection, the highest ratio (0.77) was achieved at 72 h after inoculation, when *C. pneumoniae* is considered to complete its development cycle in the epithelial cell culture (Kuo and Grayston, 1990). This value was used as a reference value for the highest level of maturation obtained in this model. By comparing the ratios of IFUs/GEs observed in IFN-γ treated cells to the reference value, we estimated the percentage of chlamydial particles during IFN-γ induced persistent infection that matured into infectious EB [(ratio in IFN-γ treated cells/ratio in untreated cells at 72 hai)x100]. During the IFN-γ induced persistent infection, 8% of *C. pneumoniae* genomes were in infectious particles at 72 h after inoculation, while approximately 90% of the particles did not maturate and/or they became inactivated. The level of successful maturation remained rather stable (up to 20 %) at 2 to 6 days after inoculation.

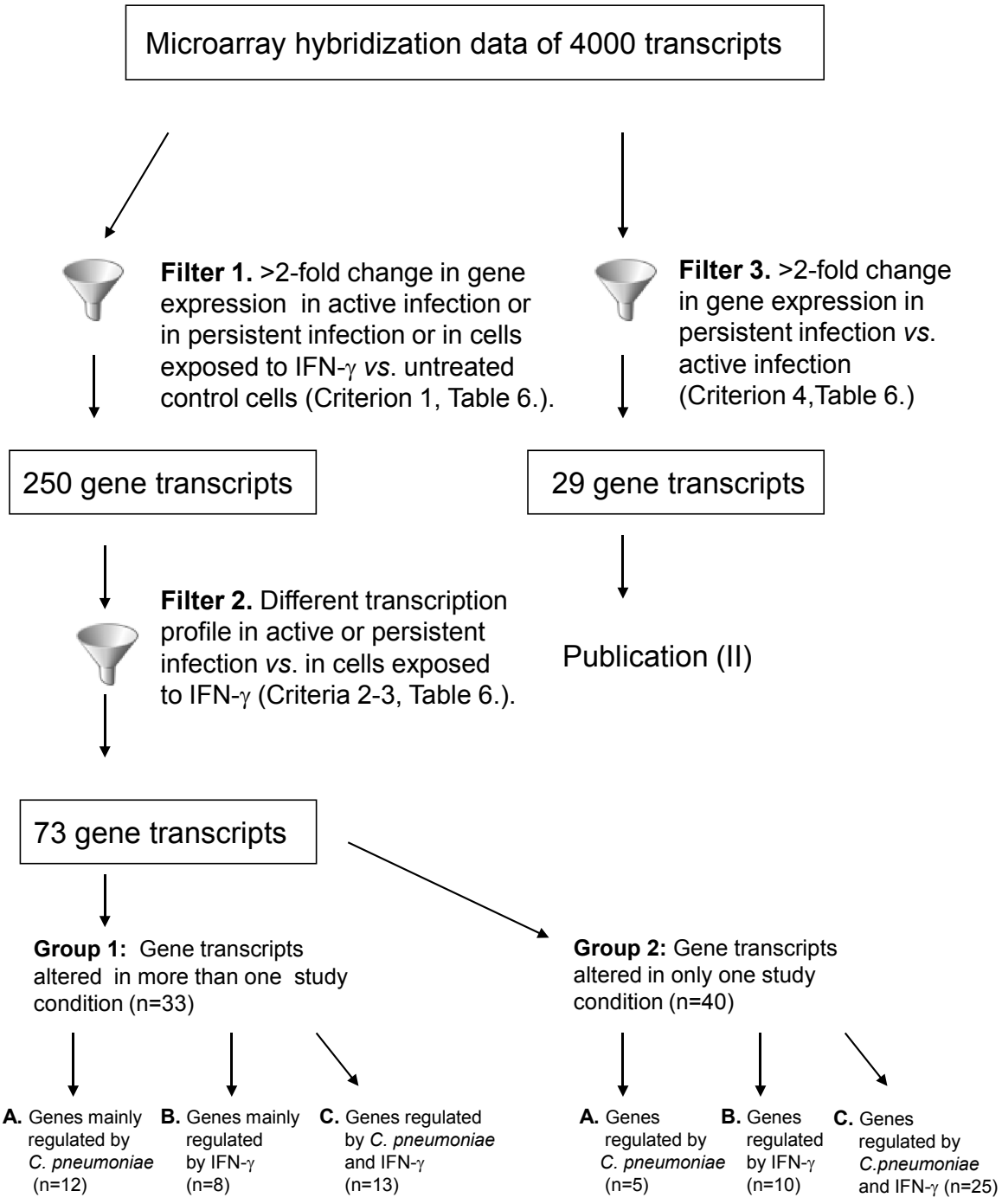


Figure 4. Overview of the microarray analysis.

Transmission electron microscopy identified a mixture of different types of inclusions in IFN-γ treated HL cells in agreement with earlier observations in IFN-γ induced persistent *C. pneumoniae* infection (Mathews *et al.*, 2001, Pantoja *et al.*, 2001). Small inclusions containing aberrant and large RB, small inclusions containing aberrant, pleomorphic RB normal in size, and small inclusions containing a mixture of mature-looking EB, intermediate forms and pleomorphic altered RB were observed (Figure 2/**I**). The major mechanism IFN-γ action on chlamydia is based on the induction of the host cell enzyme indoleamine 2,3-dioxygenase (IDO) involved in tryptophan catabolism (Mehta *et al.*, 1998). Pantoja *et al.* has suggested that tryptophan depletion affects more RB in their early stages and arrests them in a persistent state, and that the RB and EB at the later stages are less affected (Pantoja *et al.*, 2001). This could explain why a mixture of different inclusions was observed in this study and by others when a low IFN-γ concentration was used (Mathews *et al.*, 2001), whereas mainly inclusions typical for persistent infection are seen under higher IFN-γ concentrations (Pantoja *et al.*, 2001). However, our data suggests that inclusions containing more mature-looking chlamydial developmental forms might also represent persistence, since no second cycle of infection could be detected by qPCR, passage, or staining of inclusions with fluorescein-labeled antibodies in these cultures.

7.4. Host cell response to persistent C. pneumoniae infection in epithelial cells (II)

Although *Chlamydia* multiplies in its own vacuole surrounded by the inclusion membrane, it is capable of interacting with its host cell and modulating host cell gene expression. This modulation requires chlamydial protein synthesis and it may assist the bacteria to establish persistence. In this study, we compared changes in host cell transcription of 4000 genes (available at that time) in response to productive and IFN-γ induced persistent *C. pneumoniae* infection in HL cells with cDNA array. To better understand the kinetics of the responses in active versus persistent *C. pneumoniae* infection models, changes in transcription profiles were studied at four time points between 6 hours and 6 days after infection. We established criteria to select host cell genes whose transcription was different between the studied conditions and we further analyzed the data to identify the genes specifically regulated in persistent *C. pneumoniae* infection and confirmed the results with RT-PCR analysis. Only the results focused on specific changes in persistent infection model at 6-48 hai and confirmed by RT-PCR analysis have been previously published. Although the microarray experiments were not repeated and therefore the results are only preliminary or suggestive, the screening results of the microarray data are presented here since the data on host cell responses to persistent *Chlamydia* infection is exiguous. Nevertheless, individual genes are only discussed if the observed response in transcription has been confirmed by another method.

When the same RNA preparations were studied by both microarray and RT-PCR (N = 21), approximately 70 % of the responses detected by microarray analysis could be confirmed by RT-PCR. In most unconfirmed cases the microarray analysis had obviously failed to detect a response to IFN-γ or to chlamydial infection at one time point. When the biological replicate samples were analyzed by RT-PCR, results of 43% of the genes correlated moderately well with the microarray results. In the context that others have reported RT-PCR validation percentages of 60-100% for microarray findings, our confirmation percentage by RT-PCR in biological replicate samples is somewhat low. One potential reason affecting the observed lower validation percentage is that in most reports up- or down-regulation of only one condition was studied, whereas in our study each of the studied conditions needed to correlate (also in magnitude) to microarray results in order to one gene transcript to be confirmed. Also, the inclusion of genes with low responses to RT-PCR confirmation based on their biological importance potentially affected the lower confirmation percentage. In addition, there was a considerable time lag between our microarray sample and biological replicate sample. Even though the different IFN-γ preparations used to induce persistent infection were carefully titrated and evaluated to ensure equal biological effect, it can not be excluded that the different preparations used might have had slightly different potencies.

7.4.1. Initial analysis of the microarray screening

The transcriptional responses of the HL cells in three different conditions were studied by a cDNA array: 1. Productive *C. pneumoniae* infection, 2. persistent *C. pneumoniae* infection induced by IFN-γ, and 3. IFN-γ treatment (to control the effect of IFN-γ alone on the gene transcription in HL cells). To confirm the nature of infection (active vs. persistent) in the used models, the number of infectious bacteria and GE present in cultures were quantified by their passage into permissive cell culture and qPCR, respectively. In the persistently infected cell cultures, < 3 % of the chlamydial particles were in infectious form at 3 days after infection when compared to the level reached during active infection.

Initially three different selection criteria were used to extract significant results from the microarray data. First, genes with a significant change in relation to the untreated control cells were selected for each studied condition (criterion 1, Table 6.). Second, two-fold changes in the comparative analysis of the three samples representing different conditions were used to filter genes that had met the first criterion (criterion 2, Table 6.). Third, 1.5-fold changes were selected among those genes that had met the first criterion in only one of the studied conditions but had not met the second criterion (criterion 3, Table 6.). Overview of the microarray analysis is presented in Figure 4. To confirm that the first criterion for a significant change in expression was sufficiently strict, and to control that vancomycin, an antibiotic used in the cell cultures, was not affecting HL cell transcription,

Table 6. Criteria used to filter the microarray data

Criterion number	Criterion equation
Criterion 1	Mean of $\log_2$(sample/control) of the 3 replicate hybridizations ≥ 1 or ≤ -1, Coefficient of variation (CV) < 0.25 (25%).
Criterion 2	[("acute infection" fold change /IFN-γ fold change) ≥ 2 OR ≤ 0.5] OR [("persistent infection" fold change / IFN-g fold change) ≥ 2 OR ≤ 0.5], in addition, at least the other fold change in the division equation has to fulfill criterion 1.
Criterion 3	If only one of the samples was fulfilling the criterion 1 (= sample x_1), then [(sample x_1 fold change/sample x_2 fold change) OR (sample x_1 fold change/sample x_3 fold change)] ≥ 1.5 OR ≤ 0.67.
Criterion 4 (Criterion 2 in **II**)	[("persistent infection" fold change / "acute infection" fold change) ≥ 2 OR ≤ 0.5] AND [("persistent infection" fold change / IFN-γ fold change) ≥ 2 OR ≤ 0.5]

RNA from HL cells grown in the presence of vancomycin was hybridized against RNA from HL cells grown without vancomycin on the microarray. When these data were analyzed using the above-mentioned criterion 1, a significant change was detected in the transcription level of 3 genes only. Then, the RNA extracted from a pool of HL cell cultures grown with vancomycin was used as a hybridization control against which the transcription levels in studied samples were normalized. Of the approximately 4000 genes, 250 genes initially fulfilled the criterion for a significant change in expression (criterion 1, Table 6.). Generally, the HL cell gene transcription was more often up-regulated than down-regulated upon infection with *C. pneumoniae* and treatment with IFN-γ. However, the IFN-γ treated culture at the latest time point studied (at 150 hpi) was an exception. At that time point the host cell gene expression, if altered, was mostly down-regulated. Interestingly, at the same time point the number of genes whose transcription was down-regulated was lower in cells infected persistently with *C. pneumoniae* than in cells only treated with IFN-γ (Figure 5.). When the different conditions were compared, it was also noteworthy that at 48 hpi there were more up-regulated genes in the cells of the persistent infection model than together in the cells only infected with chlamydia or only treated with IFN-γ. At this time point, most of the *C. pneumoniae* RB forms have been transformed into infectious EB forms during the productive infection, whereas in the persistent nonproductive infection RB forms persist (Figure 5.). Similarly, in a study by Burian *et al.*, *C. trachomatis* L2 infection of murine epithelial cells alone up-regulated 185 genes, exposure to IFN-γ alone up-regulated 82 genes, and when combined, up-regulation of 503 genes was observed. A similar enhancing effect was observed for IFN-γ induced persistent infection of *C. muridarum* in murine epithelial cells, but it was less prominent (Burian *et al.*, 2010).

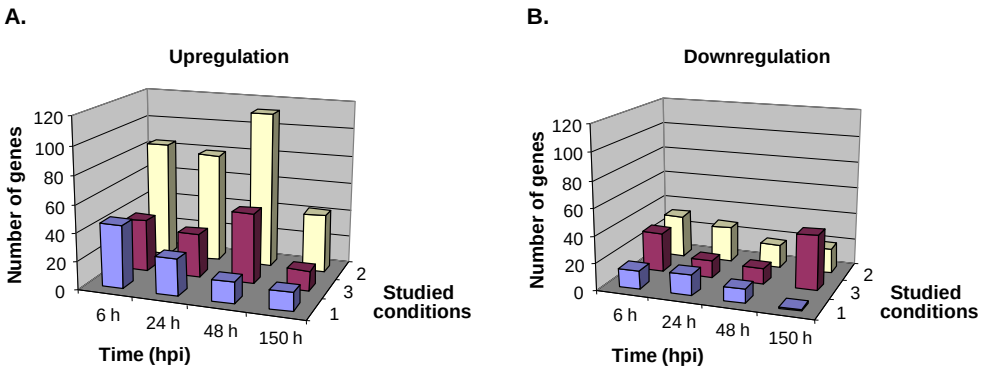


Figure 5. General pattern of gene regulation in HL cells in response to 1. productive *C. pneumoniae* infection, 2. IFN-γ induced nonproductive *C. pneumoniae* infection and 3. IFN-γ. Cells were collected at 6, 24, 48 and 150 h post infection (hpi), total RNA was isolated and hybridized against untreated HL cell pool (control) on a cDNA array containing 4000 genes. A two-fold change in transcription level of a gene in relation to control was considered up-regulation (A.) or down-regulation (B.).

Comparative analysis of the C. pneumoniae and IFN-γ effects on HL cell gene regulation

The transcriptional profiles of the 250 genes that had fulfilled criterion 1 were then compared between the persistent and active infection models and the IFN-γ control. The 73 genes that, in addition to meeting the criterion 1, fulfilled either criterion 2 or 3 were considered to have a different transcription profile in the active or persistent infection in comparison to the HL cells treated with IFN-γ. To further facilitate the management of the data, the genes were assigned to two groups. The 33 genes whose transcription was altered in more than one condition studied (criterion 1 was fulfilled in at least two conditions) were assigned to group 1. The 40 genes whose transcription was altered only in one of the studied conditions (criterion 1 was fulfilled only in one condition) were assigned to group 2. Three subgroups were then formed inside each of these two main groups to facilitate comparisons of the different study conditions (Figure 6.).

The genes whose transcription was mainly regulated by *C. pneumoniae* were assigned to subgroup 1 A. In this group the host cell transcriptional responses were usually stronger or lasted longer under the conditions representing persistent infection than in active infection. However, some genes, were similarly regulated both during active and persistent infection (Figure 6., 1A). The genes whose transcription was regulated by IFN-γ were assigned to

subgroup 1B. In this subgroup, consisting of IFN-γ regulated genes, the transcription was not directly regulated by *C. pneumoniae* alone, but still the responses of genes differed between the persistent infection model and the IFN-γ treatment. The up-regulation of transcription was in many cases seen earlier in the persistent infection model, and sometimes also stronger, than in the IFN-γ treated cells (Figure 6., 1B). The genes whose transcription was regulated by both *C. pneumoniae* and IFN-γ were assigned to subgroup 1C. Among the genes assigned to this subgroup, regulation of the genes was seen in both *C. pneumoniae* infected and IFN-γ treated cells and when combined, in condition 2, the transcriptional changes were stronger, and in many cases, also the kinetics of the response was different. Toll-like receptor 4 (TLR4) induced core cluster of genes in human macrophages has recently been described (Nau *et al.*, 2003). From the defined cluster of 43 genes, 11 were present on the cDNA array used in this study. Seven of the 11 genes fulfilled criterion 1, and 4 out of the 7 genes were found to be differentially expressed between the studied conditions. All the 4 differentially transcribed genes, IFIT1 (interferon-induced protein with tetratricopeptide repeats 1), IFITM1 (interferon induced transmembrane protein 1), ISG15 (15 kD interferon-stimulated protein) and PMAIP1 (phorbol-12-myristate-13-acetate-induced protein 1.), fell into the subgroup 1C (Figure 6., 1C). In agreement with our study, more potent up-regulation of many genes was observed upon IFN-γ induced persistent infection of *C. trachomatis* L2, and although less prominent also of *C. muridarium*, in murine epithelial cells. It was suggested that at least partially this enhancement effect may be due to the simultaneous switch-on of the TLR signaling pathway and IFN-γ receptor signaling pathway (Burian *et al.*, 2010).

The genes whose transcription was altered only by *C. pneumoniae* alone (condition 1) were assigned to subgroup 2A. The transcription of the genes assigned to this subgroup was significantly changed only upon productive *C. pneumoniae* infection. Four of the five changes specific for active infection were seen at 150 hpi. At this time point, *C. pneumoniae* is completing the second cycle of infection in these cells (Kuo and Grayston, 1990, **I**). BCL2/adenovirus E1B 19kD-interacting protein 3 (BNIP3), which is involved in the induction of apoptosis (Chen *et al.*, 1997), was up-regulated. The other up-regulated genes are involved in the metabolism. Also, down-regulation of the transcription of one gene was seen at 48 hpi (Figure 6., 2A). The genes whose transcription was altered only by IFN-γ were assigned to subgroup 2B. In the subgroup 2B, transcription of the genes was only changed upon IFN-γ treatment and again, the changes often appeared at the late time points. Both up-regulation and down-regulation of transcription was seen and these changes were absent in the persistent infection. This suggests that *C. pneumoniae* is able to counteract some of the responses induced by IFN-γ. For example, transcription of cell adhesion molecule ALCAM was specifically down-regulated and transcription of tumor protein p53 (TP53) was specifically up-regulated by IFN-γ (Figure 6., 2B). The specific down-regulation of ALCAM and up-regulation of TP53 at 150 hai in the IFN-γ control, but not in the persistent infection induced by IFN-γ was later also observed in an Affymetrix experiment (data not shown).

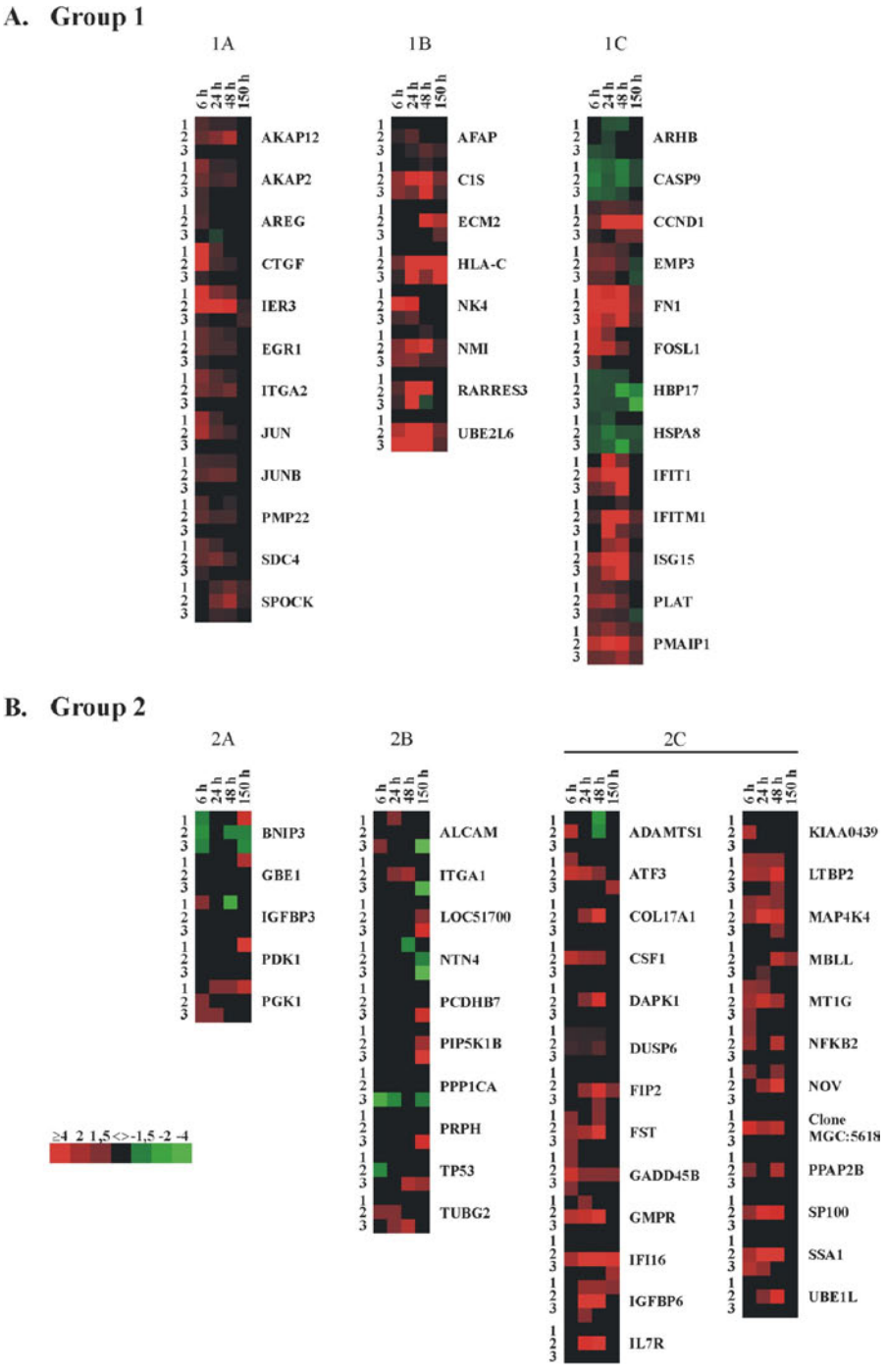


Figure 6. Tree views of genes differentially expressed in the three conditions studied (1. productive *C. pneumoniae* infection, 2. IFN-γ induced nonproductive *C. pneumoniae* infection and 3. IFN-γ). Group 1 includes the genes, whose transcription was affected in more than one condition studied and group 2 includes the genes, whose transcription was affected only in one condition studied. Each row represents normalized transcription level of a gene (the gene in question is indicated at the right side of a row) under one condition studied in relation to untreated HL cells: Red squares indicate up-regulation, green squares down-regulation and black squares unchanged transcription. The scale bar indicates fold difference in gene expression. The columns represent the time points 6, 24, 48 and 150 h post inoculation.

Furthermore, the ability of *C. pneumoniae* to overcome TP53 up-regulation was also shown by RT-PCR in the same samples as used in the cDNA microarray (Figure 7.) and also in biological replicate sample, although lesser amount of TP53 was produced in the replicate samples.Recently IFN-γ has been shown to induce TP53 expression also in human endothelial cells (Kim *et al.*, 2009). The absence of p53 has been implicated to have a role in atherosclerosis acceleration by increasing cell proliferation (Guevara *et al.*, 1999). In view that *C. pneumoniae* is associated to lung cancer (Laurila *et al.*, 1997, Littman *et al.*, 2005) and TP53 is widely known as a suppressing factor for cancer, our finding that *C. pneumoniae* can antagonize IFN-γ induced up-regulation of TP53 in epithelial cells is intriguing. The genes whose transcription was altered only when cells were both inoculated with *Chlamydia* and exposed to IFN-γ were assigned to subgroup 2C, forming the largest subgroup (Figure 6, 2C). In this subgroup, a significant change in transcription was detected only in the persistent infection. Up-regulation of several genes was seen at time points 6-48 hai. The genes assigned to this subgroup were heterogeneous in function, but more than half of the gene products have a function in apoptosis, in growth and proliferation, in immunity and inflammation, in signal transduction, or in transcription and translation.

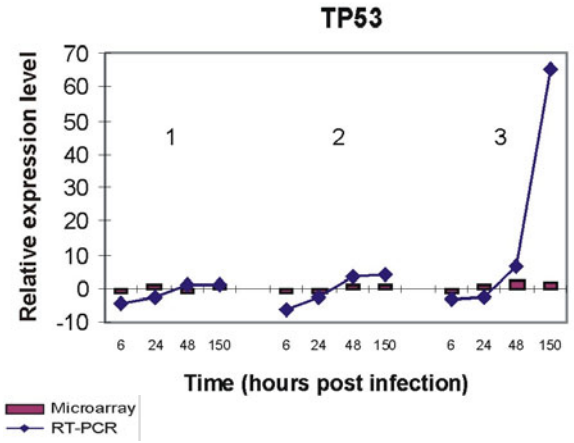


Figure 7. Real-time RT-PCR analysis of TP53 gene in sample preparations that were studied by microarray. In the model for active infection HL cells were inoculated with *C. pneumoniae* (1.), in the model for persistent infection HL cells were inoculated with *C. pneumoniae* and treated with IFN-γ (2.) and in a control for persistent infection HL cells were treated solely with IFN-γ (3.). Cells were harvested at 6, 24, 48 and 150 h post inoculation (hpi). The relative expression levels are expressed as fold changes of a sample in relation to untreated HL cells. For expression levels obtained by the microarray (bars), a > two-fold change was considered significant, whereas for expression levels obtained by the RT-PCR (line), a > three-fold change was considered significant (see text).

7.4.2. Transcription analysis focused on genes differentially regulated in persistent infection and verification by RT-PCR

After initial analysis the data analysis was focused on genes having ≥two-fold change in gene expression in persistent infection vs. active infection and IFN-γ treatment alone (criterion 4, Table 6.). After refiltering the data 29 genes (from subgroups 1A-C and 2C) were identified as having ≥two-fold change in gene expression in persistent infection vs. active infection and IFN-γ treatment alone. To verify this data, we employed Applied Biosystems TaqMan® Gene Expression Assays to study expression of 28 genes using RNA extracted from the biological replicate samples. At each time point, cells collected from two different wells were analyzed by RT-PCR. After normalization to a housekeeping gene ACTB (β-actin), the mean difference in expression levels between two replicate cell preparations (28 genes, 3 time points, n=84) was 21% (median 15 %). In addition, the RNA preparations used in the microarray hybridization were used to study expression of 21 genes by real time RT-PCR. When the amount of ACTB mRNA was normalized to the amount of another housekeeping gene HSPCB (heat shock 90kD protein 1, beta) mRNA in 48 biological replicate samples (including samples not analyzed in this study), a mean value of 2,48 (SEM=0,36) was obtained, but with 95% confidence interval the true mean lies between 1,76-3,2. This result and the normalization of the mRNA levels of studied samples to the levels of both housekeeping genes suggested that a 3-fold difference should be used as a cutoff for a significant difference detected by RT-PCR, although 2-fold differences could be distinguished using the standard curve.

Of the 28 genes, verified by RT-PCR in biological replicate samples, the gene expression results of nine genes obtained by microarray and RT-PCR correlated well at all time points, and the changes observed in the expression of these nine genes were therefore considered to be specific for persistent *C. pneumoniae* infection. Of the nine genes, four have a function in immunity and inflammation related processes (IL7R, IFIT1, NMI and ISG15), two (FN1 and COL17A1) are adhesion-related and two play a role in growth and proliferation (IGFBP6 and CCND1). Interestingly, three of the genes involved in immune responses, IL7R, ISG15 and IFIT1, were also up-regulated in HeLa 229 cells infected with a *C. trachomatis* variant with nonfusing inclusions as compared to cells infected with a prototype strain of *C. trachomatis* D (Xia *et al.*, 2005). The variant carries a mutated IncA gene and lacks IncA protein in the inclusion membrane (Suchland *et al.*, 2000, Rockey *et al.*, 2002). The IncA variant strain grows more slowly than the prototype strain and by inducing transcription of immune-related genes could lead to increased inflammation and pathogenicity (Xia *et al.*, 2005). *C. pneumoniae* resembles the variant *C. trachomatis* in slow growth rate and low production of progeny, and the inclusions are not fusogenic. Yet *C. pneumoniae* encodes proteins localized to the inclusion membrane, including IncA, but the *C. pneumoniae* IncA shares only limited primary sequence homology with *C. trachomatis* IncA.

The up-regulation in persistent infection in comparison to active infection and IFN-γ control was most prominent for IGFBP6 (>500-fold up-regulation by RT-PCR), NMI and interleukin 7 receptor (IL7R) transcripts (Table 1./**II**). The IGFBP6 gene is involved in growth and proliferation. IGFBP6 belongs to a family of six insulin-like growth factor binding proteins that regulate IGF (insulin-like growth factor) activity, but can also have IGF independent actions (Firth and Baxter, 2002). Other members of the family, including IGFBP3, IGFBP5 as well as IGFBP6 were up-regulated upon L2 infection in monocytic cells (Ren *et al.*, 2003) and HeLa 229 cells (Xia *et al.*, 2003), but their potential role during infection has remained uninvestigated. IGFBP6 is considered anti-tumorigenic *per se*, however, in some cases, IGFBP-6 is associated with increased cancer cell tumorigenicity (Bach, 2005, Fu *et al.*, 2010). IGFBP-6 is a rather specific inhibitor of IGF2 (Bach, 2005) and IGF2 has been reported to specifically enhance infectivity of *C. pneumoniae* in endothelial cells *in vitro* (Lin *et al.*, 2001). The mechanism of enhancement by IGF2 has remained unknown, but it has been hypothesized that the simultaneous binding of chlamydial organisms and IGF-2 to the IGF2/M6P (mannose-6 phosphate) receptor may inhibit the uptake of lysosomal enzymes into endocytic vesicles, promoting the survival of *C. pneumoniae* in phagosomes (Lin *et al.*, 2001). Two other ligands capable of binding to the IGF2/M6P receptor at separate binding sites, M6P and retinoic acid, have been reported to decrease infectivity of *C. pneumoniae* in endothelial cells (Puolakkainen *et al.*, 2005). Increased expression of IGFBP6 could be a host's attempt to limit infection of endothelial cells by inhibiting IGF2, although transmission of the infection from respiratory epithelium to endothelial cells of the vessel wall, via infected mononuclear cells may remain effective (Gieffers *et al.*, 2004b).

The persistent infection associated up-regulation of IL-7 receptor, a protein associated with both immune responses and human malignancies, was observed at 24 hai and according to RT-PCR analysis at 6 days after inoculation the transcription was still enhanced (data not shown). IL7R is a type 1 membrane glycoprotein and the functional receptor consists of two components, an alpha chain and a common gamma chain that is shared by the receptors for other cytokines (IL-2, 4, 7, 9, 15 and 21). IL-7 is needed for the development and proliferation of lymphocytes. A role for IL-7 mediated inflammation in atherogenesis has been suggested (Damås *et al.*, 2003) and an increased serum level of IL-7 has been found in patients with coronary heart diseases (Carratelli *et al.*, 2006). In a recent report by Konstantinidou *et al.* olive oil polyphenols were indicated to play a significant role in the down-regulation of inflammatory genes including IL7R (Konstantinidou *et al.*, 2010). The function of the IL-7R complex in epithelial tissues has remained unknown. However, up-regulation of IL7R in the respiratory epithelial cells might result in increased IL-7 signaling during *C. pneumoniae* infection. Bacterial invasion into intestinal epithelial cells has been shown to induce expression of IL7R that may, in turn, participate in the modulation of mucosal inflammation during infection (Yamada *et al.*, 1997). In addition, IL-7 receptor signaling has been suggested to participate in the development of gastritis induced by Helicobacter (Ohana *et al.*, 2001), a common cause of chronic gastritis and even gastric cancer. Whether IL-7 is actually

produced during persistent *C. pneumoniae* infection and could hence participate in the pathogenesis through interaction with its receptor, remains to be studied.

Also, for a 15 kD interferon-stimulated protein (ISG15), IFIT1 and Cyclin D1 (CCND1), the observed up-regulation of transcripts in persistent infection was substantially stronger (more than 5-fold) than in the other conditions studied (Table 1./**II**). ISG15 is an interferon-stimulated, ubiquitin-like protein that becomes conjugated to cellular proteins (Ritchie and Zhang, 2004). ISG15 expression and conjugation to target proteins (ISGylation) is highly up-regulated by bacterial and viral infection as well as by LPS treatment (Kim and Zhang, 2003). ISG15 has antiviral effects in mice (Ritchie *et al.*, 2004), but unlike ubiquitination, ISGylation might not target proteins for degradation. Several ISG15 targets including both viral and host cell proteins have tentatively been identified (Giannakopoulos *et al.*, 2005, Zhao *et al.*, 2005, Skaug and Chen, 2010) including a number of cellular proteins associated with the cytoskeleton, e.g. cytokeratin 8 (Loeb and Haas, 1994). Association of ISG15 with cytoskeleton filaments may exert antiviral activity by inhibiting binding of viral proteins to the cytoskeleton and therefore interfering with the assembly of viral particles (Loeb and Haas, 1994). The host cell cytoskeleton has also been suggested to play a role in chlamydial vacuole expansion, as a chlamydial secreted protease (CPAF) cleaves cytokeratin 8, potentially resulting in increased solubility of the host cell cytoskeleton (Dong *et al.*, 2004). As CPAF is produced also during IFN-γ induced persistent infection (Heuer *et al.*, 2003), it could be speculated that during persistent infection, ISGylation of host cell cytoskeleton together with at least partial inhibition of the CPAF translocation into cytoplasm could interfere with cytokeratin 8 cleavage by the chlamydial protease. This could affect the inclusion expansion and chlamydia release from host cells resulting in small inclusions and fewer infectious progeny during persistence.

The CCND1 gene product plays a role in growth and cell proliferation. Up-regulation of CCND1 was sustained (still seen as late as 6 days after infection, Figure 5., also verified by RT-PCR, data not shown). To exert their biochemical functions, cyclins, including CCND1, form heterodimers with cyclin-dependent kinases (CDKs), but CCND1 also has CDK-independent properties (Fu *et al.*, 2004). CCND1 promotes progression of the cell cycle and overexpression is associated with tumorgenesis, possibly due to the shortened time available for DNA repair during accelerated progression of the cell cycle (Stacey, 2003). Whether cyclin D1 is a player in malignant transformation associated with chlamydial infections (Littman *et al.*, 2005), remains to be elucidated.

For COL17A1, a tissue type plasminogen activator (PLAT) and FN1, the observed up-regulation of transcripts during persistent infection was less than 5-fold but more than 3–fold higher than that observed in acute infection or in IFN-γ treated cells (Table 1./**II**). Fibronectin could contribute to the development of a sustained inflammatory response often accompanying chlamydial infection, as fibronectin 1 is a potential ligand of TLR4

(Okamura *et al.*, 2001). A characteristic of diseased tissue both in atherosclerosis and asthma is an increased deposition of fibronectin and other structural molecules e.g. collagens in the extracellular matrix (ECM). It has been proposed that excess fibronectin deposition into the subepithelial space of the lung airways may contribute to the abnormal tissue remodelling and inhibit reepithelization in asthma (Hocking, 2002, Dolhnikoff *et al.*, 2009) and fibronectin may be also an important regulator of vascular remodeling (Chiang *et al.*, 2009). In addition, fibronectin can bind IL-7 in ECM and therefore may regulate the availability of IL-7 within the tissue microenvironment (Ariel *et al.*, 1997). Increased expression of fibronectin could also result in modification of the bacterial surface and affect its pathogenic properties: host cell derived fibronectin has indeed been found in association with the surface *of C. trachomatis* EB (serovars D and L2) (Kleba *et al.*, 2002), and could participate in the infectious process, although exogenous fibronectin did not alter the infectivity of *C. trachomatis* grown in fibronectin deficient cells (Kleba and Stephens, 2005). However, FN1 has been found to be slightly down-regulated in HeLa 299 cells upon *C. trachomatis* L2 infection (Xia *et al.*, 2003).

The genes identified here as specifically regulated upon persistent *C. pneumoniae* infection (IGFBP6, NMI, FN1, CCND1, COL 17A1, ISG15, IFIT 1, PLAT, and IL7R) had not previously been reported to change upon *C. pneumoniae* infection. Although shortly after the up-regulation of ISG15 and IFIT1 were also observed in IFN-γ induced persistent *C. pneumoniae* infection (Eickhoff *et al.*, 2007). Quite recently, in a study by Burian *et al.* more potent up regulation of IFIT1, NMI, and PLAT was observed in IFN-γ induced persistent infection of *C. trachomatis* L2 in murine epithelial cells (Burian *et al.*, 2010). Infection of HeLa 229 cells with *C. trachomatis* has been reported to up-regulate transcription of IGFBP6, ISG15 (also known as G1P2), and IFIT1, and to down-regulate that of FN1 (Xia *et al.*, 2003, Lad *et al.*, 2005), although also up-regulation have been observed (Burian *et al.*, 2010). Microarray analysis of *C. trachomatis* L2 infected THP-1 cells has shown up-regulated transcription of ISG15 and IFIT1 (Ren *et al.*, 2003). Since the altered mRNA profiles may not reflect altered production of corresponding proteins (Gygi *et al.*, 1999), additional techniques, such as Western blot analysis, are needed to support the findings in this study.

7.4.3. Other microarray studies on host cell transcription in persistent C. pneumoniae infection

Presently, there is only one other published study that has used microarray technology to screen host cell changes upon persistent *C. pneumoniae* infection of epithelial cells (Eickhoff *et al.*, 2007). They used Affymetrix human genome U133A chips to study IFN-γ induced persistent infection of *C. pneumoniae* CWL-029 in an epithelial cell line (HeLa). They produced samples for two independent microarray experiments at 24 hours after infection for both active and persistent *C. pneumoniae* infection and an additional sample at 96 h after infection for persistent infection. Hybridization intensities of host cell transcripts in infected cultures were compared to those of mock infected cultures to create

fold changes. The mock infected culture of persistent infection contained IFN-γ to subtract responses created solely by IFN-γ. Fiftyseven genes were identified as differentially regulated by *C. pneumoniae*, 19 genes were selected for RT-PCR analysis, and responses of 17 genes could be verified (Eickhoff *et al.*, 2007). Of the 57 genes identified by Eickhoff *et al.* 27 gene sequences were present on the cDNA array used in our study and only seven of the genes were here identified as differently regulated upon *C. pneumoniae* and/or IFN-γ induced persistent *C. pneumoniae* infection (Table 7.). Six of the genes had similar responses in both studies and one gene (BNIP3) was identified as down-regulated in all studied conditions by Eickhoff *et al.*, while we found up-regulation of this transcript at 150 h after infection by active infection but not by persistent infection (unverified observation). However, slight down-regulation of BNIP3 was also seen in our experiment in *C. pneumoniae* infected samples at 6-48 hai, although it did not fulfill our criteria for a significant change. Of the nine genes identified as specifically up-regulated by microarray and RT-PCR in independent samples of persistent *C. pneumoniae* infection in our study, only two genes were identified by Affymetrix, which contains probe sets for all nine genes, in the study by Eickhoff *et al.* indicating moderately low agreement between the studies. This may be due to different *C. pneumoniae* isolates used, different MOI used for infection (30 vs. 0.5), different amount of IFN-γ used to induce persistence (100 U vs. 25 U) and different cell lines used in the studies. The transcription kinetics may also be different as already seen in the similar gene responses between the studies (Table 7.). The differences may also be due to technical reasons relating to microarray technology. Thus, more studies on host cell transcription during persistent *C. pneumoniae* infection are needed in order to identify the genes involved in the interactomics of persistent *C. pneumoniae* infection. However, there were also similarities between the studies. In agreement with our study, Eickhoff *et al.* observed longer lasting up- or down-regulation of gene transcripts in persistent *C. pneumoniae* infection in comparison to active infection. They also showed that some of the responses were due to bacterial metabolism, since several responses were absent when rifampin was introduced to cultures directly after infection (Eickhoff *et al.*, 2007). Supporting this observation, in our study most differences in transcription between persistent and active infection were observed at 48 h after infection, when most of the metabolically active RB-forms are transformed into EB-forms in active infection. The longer lasting up or down regulations of genes observed in the studies may also be due to continuing bacterial metabolism in the cultures of persistent infection.

Table 7. Comparison of the two published microarray studies on IFN-γ induced persistent infection of epithelial cells (Eickhof et al., 2007 and **II**). Results from **II** are presented with gray background.

			Change in transcription									
Gene	Affymetrix probe set[a]	Accession Number	6h Cpn	6h Cpn + IFN-γ	24h Cpn	24 h Cpn	24h Cpn+IFN-γ	24 h Cpn + IFN-γ	48 h Cpn	48 h Cpn + IFN-γ	96h Cpn+IFN-γ	150h Cpn	150h Cpn + IFN-γ
dual specificity phosphatase 6 (DUSP6)	208892_s_at	BC003143.1	NC	NC	I	NC	NC	NC	NC	I	NC	NC	NC
follistatin (FST)	204948_s_at	NM_013409.1	NC	NC	I	NC	I	NC	NC	I	NC	NC	NC
FOS-like antigen 1 (FOSL1)	204420_at	NM_005438	I	I	I	I	I	I	NC	I	NC	NC	NC
Homo sapiens BCL2 adenovirus E1B 19kD-interacting protein 3 (BNIP3)	201848_s_at	NM_004052	NC	NC	D	NC	NC	NC	NC	NC	D	I	NC
	221478_at	NM_004052			D		D				D		
Human connective tissue growth factor (CTGF)	209101_at	M92934.1	I	I	I	I	NC	I	NC	NC	NC	NC	NC
interferon-induced protein with tetratricopeptide repeats 1 (IFIT1)	203153_at	NM_001548.1	NC	NC	NC	I	I	I	I	NC	I	NC	NC
interferon-stimulated protein, 15 kDa (ISG15)	205483_s_at	NM_005101.1	NC	NC	I	I	I	NC	I	I	NC	NC	NC

[a] from experiment by Eickhof et al. 2007; h = time point after infection; Cpn = active *C. pneumoniae* infection; Cpn + IFN-g = persistent *C. pneumoniae* infection; D= decrease in transcripts; I= increase in transcripts; NC= no change

7.5. C. pneumoniae infection model in a monocytic cell line (I)

In addition to providing possible means of transport out of the lungs to other anatomical sites, infection of monocytes/macrophages with *C. pneumoniae* affects the adherence and migration properties of the macrophages, potentially leading to enhanced accumulation of macrophages in atherosclerotic plaques. To mimic infection of macrophages *in vivo*, we set up a *C. pneumoniae* infection model in the Mono Mac 6 cell line, which is a macrophage-like cell line that expresses phenotypic and functional properties of mature monocytes. We characterized the growth of the *C. pneumoniae* K6 isolate in the Mono Mac 6 cell line in the presence and absence of IFN-γ by qPCR, infectivity assay, and microscopy. A growth curve of *C. pneumoniae* in Mono Mac 6 cell line showed a decrease in the number of infectious EB immediately after inoculation and at 12 h post infection. After that the number of EB increased clearly up to 48 h after infection suggesting that most differentiation of EB into RB took place between 36-48 h after infection. Unlike in HL cells, the amount of infectious *C. pneumoniae* cultivable from Mono Mac 6 cells remained rather stable up to 192 h post infection and no evidence of a second infectious cycle of *C. pneumoniae* in the Mono Mac 6 cells was observed (Figure 3./**I**).

The number of GE decreased during the first 12 h after inoculation and the *C. pneumoniae* genomes accumulated between 12-48 h after inoculation in the Mono Mac 6 cells. At later time points, the number of *C. pneumoniae* GE remained at a rather stable level during the observation period of 8 days (Figure 3./**I**). The doubling time of *C. pneumoniae* genomes in Mono Mac 6 cells (10-14 h) was found to be much longer than that of in HL cells (6-7 h). However, in Mono Mac 6 cultures infected with heat and UV-inactivated *C. pneumoniae* a significant portion of DNA detected in cells immediately after inoculation still persisted at 72 hours post infection, which may interfere with the results. By TEM inclusions containing a mixture of RB, intermediate, and EB forms could be detected 72 hours post infection in the untreated cultures. As observed earlier by others (Koehler *et al.*, 1997, Airenne *et al.*, 1999), we found many RB forms in monocytic cell line that exhibited pleomorphic morphology (Figure 2./**I**).

The effect of IFN-γ on *C. pneumoniae* growth in Mono Mac 6 cells was followed after treating the cells with increasing concentrations of IFN-γ. A concentration of 1000 U/ml was chosen for further experiments, since it was the lowest concentration that had an effect on inclusion morphology and infectivity of *C. pneumoniae*. In the presence of 1000 U/ml of IFN-γ, the number of infectious particles recovered from the infected cells decreased initially, and increased a little at 36 – 48 h post infection. However, after 72 hours post infection the number of EB decreased and no *C. pneumoniae* could be cultured at 168 hours post infection. The *C. pneumoniae* genomes decreased during the first 12 h after inoculation, increased slightly at 12-36 h post infection suggesting that *C.*

pneumoniae DNA was replicated during this period, after which the number of genomes decreased and stayed at a rather stable level to the end of the observation period (Figure 3./**I**).

Human macrophages are able to support the growth of *C. pneumoniae* (Godzik *et al.*, 1995), although the burst size of infection is low, suggesting that macrophages can to some extent naturally restrict the growth of *C. pneumoniae*. *Chlamydia* infection of monocytes resembles persistent infection with minimal bacterial cell division and with a low level of infectious progeny. In our experment, a relatively high concentration of IFN-γ (1000 U/ml) was needed to further restrict the growth of *C. pneumoniae* in human monocytic cell line. With this IFN-γ concentration, *C. pneumoniae* genomes were still detectable and remained at a rather stable level, although the cultures did not produce infectious EB. Also, Airenne *et al.* (Airenne *et al.*, 1999) have suggested that human monocyte-derived macrophages require a higher concentration of IFN-γ than epithelial cells to suppress production of infectious progeny. However, even under these conditions, *C. pneumoniae* still stays viable, since *C. pneumoniae* transcripts can be detected in IFN-γ treated cultures still at 8 days after infection.

7.6. Transcription of selected genes in C. pneumoniae infection of monocytes (III)

Immune cells offer an important link between the initial site of *C. pneumoniae* infection and the chronic inflammatory disease. The infection of the Mono Mac 6 cell line without the IFN-γ exposure may represent a persistent infection *per se*, since the level of infectious progeny in relation to the detected number of chlamydial genomes is low. However, the IFN-γ exposure further restricts chlamydial growth and *C. pneumoniae* is known to elicit an IFN-γ response *in vivo*. In addition, as IFN-γ exposure is known to affect the transcription of *C. pneumoniae* genes in epithelial cells, we decided to study the transcription of *C. pneumoniae* genes both in the presence and the absence of IFN-γ in the monocytic cell culture

The transcription of 11 *C. pneumoniae* genes in Mono Mac 6 cells were analyzed during infection by real time RT-PCR. Our primary interest was to study the secreted genes (incA, cpaf, cpn0572, cpn0809 and lcrE) and the genes (ompA, omp2, ftsK, euo, htrA and hsp60) that were previously found differentially expressed in persistent infection models in epithelial cell lines. To control the *C. pneumoniae* infection in the monocyte - macrophage cell culture model, *C. pneumoniae* genomes, 16S rRNA -transcripts and Mono Mac 6 cell concentration were measured quantitatively from all three individual experiments. In addition, the viability of *C. pneumoniae* in Mono Mac 6 cells was analyzed by passaging the samples into permissive cells (HL) to measure recovery of live

bacteria (inclusion forming units, IFUs). The growth of *C. pneumoniae* in IFN-γ exposed and unexposed cultures was found to be similar to our previous study (**I**).

Samples from infected and infected and IFN-γ treated Mono Mac 6 cells were collected at 6, 36, and 72 hours after infection in three independent experiments and analyzed by real time RT-PCR. According to the observed transcription kinetics, the 11 analyzed genes were divided into 4 groups: I. early-cycle genes: the transcripts were abundant already at 6 hours after infection and decreased between 6 and 36 hours after infection, II. mid-cycle genes: the accumulation of transcripts was most abundant between 6 and 36 hours after infection, representing the metabolically most active stage when chlamydial genomes replicate and cells divide (**I**), III. late-cycle genes: the number of transcripts was increasing between 36 and 72 hours after infection during the late developmental stage of *C. pneumoniae*, when the RB forms are transforming into the EB forms in Mono Mac 6 cells (**I**), and IV. Constitutively expressed genes (Table 2./**III**).

The 16SrRNA normalized transcription levels of the studied *C. pneumoniae* genes tended to be slightly lower in the IFN-γ exposed monocyte cultures for most of the studied genes at 72 hours after infection. However, statistically significant differences in the transcription levels between IFN-γ exposed and unexposed cultures were observed only for *omp2* and *cpaf* transcripts (Table 2./**III**). The kinetics of transcription of most of the 11 studied genes was similar in both conditions, with only the transcription of *incA* and *euo* being exceptions. This finding is in contrast to the earlier reports on *C. pneumoniae* transcription in persistent infection of epithelial cells, whereas also Airenne *et al.* reported that IFN-γ had no significant effect on *C. pneumoniae* mRNA (omp2 and hsp60) levels in infected PBMC (Airenne *et al.*, 1999, Hogan *et al.*, 2004).

7.6.1. Early cycle genes

In consistence with the previous studies in fibroblasts and epithelial cells (Wichlan and Hatch, 1993, Shaw *et al.*, 2000, Belland *et al.*, 2003a) the *C. pneumoniae euo* gene was identified to have an early-cycle transcription profile (group I). When the cultures were exposed to IFN-γ, the transcription profile of the *euo* gene resembled constitutive expression (Table 2./**III**). The Euo protein, which is capable to bind DNA *in vitro*, has been suggested to have a role in chlamydial chromosome decondensation early in the infection by degrading the histone like protein Hc1 (Kaul *et al.*, 1997, Zhang *et al.*, 1998). Hence, it is likely that Euo has a role in transcription regulation. In some studies, *euo* transcription has been up-regulated in the persistent infection model (Ouellette *et al.*, 2006, Timms *et al.*, 2009). However, also no change in the transcription or even down-regulation of the *euo* transcript during the persistent *C. pneumoniae* infection have been reported (Mäurer *et al.*, 2007, Timms *et al.*, 2009).

7.6.2. Mid-cycle genes

Transcription profiles of *C. pneumoniae* genes *ompA* and *htrA* were identified as mid-cycle genes in mononuclear cells and thus were assigned to group II (Table 2./**III**). The observed transcription profile is in consistence with earlier reports on *ompA* transcription in epithelial cells (Hogan *et al.*, 2003, Nicholson *et al.*, 2003). There was no difference in the transcription of the *ompA* gene either between the IFN-γ exposed and unexposed monocyte cultures, or between the infected monocytes and epithelial cells, suggesting a similar transcription profile in both cell types. Both up-regulation and down-regulation of *C. pneumoniae* MOMP expression have been reported in persistent infection models (Timms *et al.*, 2009, Hogan *et al.*, 2003, Mathews *et al.*, 2001). Up-regulation of *htrA* transcription and translation have been reported in both IFN-γ and iron depletion induced persistent models of *C. pneumoniae* infection in epithelial cells (Mukhopadhyay *et al.*, 2006, Polkinghorne *et al.*, 2006, Mäurer *et al.*, 2007, Timms *et al.*, 2009). Also, here *htrA* was up-regulated in the mid-cycle (group II), whereas the kinetics of transcription during the infection of the HL control cultures showed up-regulation only after exposure to IFN-γ. The expression of *htrA* seems to be consistently up-regulated during persistence, which makes it a potential biomarker of persistent *Chlamydia* infection.

In addition, the transcription of *incA* and *cpaf* genes followed the profile of the mid-cycle genes when the cells were grown in IFN-γ exposed cultures. The transcription of the *C. pneumoniae* cpaf gene was similar also in bacteria grown without IFN-γ, but no statistical difference was found for the transcript accumulation between 6 and 36 hours after infection (Table 2./ **III**). IncA protein seems to play role in inclusion fusion during *C. trachomatis* growth and it may also prevent fusion of chlamydial endosome/inclusion and lysosomes. In addition, IncA may play an important role in obtaining nutrients into the host cell (Paumet *et al.*, 2009, Wyrick, 2000, Hackstadt *et al.*, 1997, 1999) and in the study by Slepenkin *et al*, incA was not expressed in cultures exposed to a T3S inhibitor (Slepenkin *et al.*, 2007). In monocytes exposed to IFN-γ, the transcription of *incA* was up-regulated during the developmental cycle. In contrast to the unexposed cultures, the highest level of *incA* transcript was observed in the middle of the developmental cycle (at 36 hpi) when the RB are converting into the EB. Interestingly, Matthews and co-workers found another inclusion membrane protein, *cpn0585*, which resembles *C. psittaci* incA, to be up-regulated in a model of persistent infection and speculated that the up-regulation is a mechanism for *C. pneumoniae* to modulate its survival in stressful conditions such as in the IFN-γ induced persistence or during infection of macrophages (Mathews *et al.*, 2001). By immunofluorescence microscopy we showed that the IncA protein is also produced and localizes to the chlamydial inclusion membrane during infection as reported earlier for *C. pneumoniae* infection in epithelial cells (Bannantine *et al.*, 2000), suggesting that the T3S apparatus is functional also during *C. pneumoniae* infection in monocytes. Upon IFN-γ exposure, IncA protein could also be stained, but the staining pattern appeared more often granular (Figure 2., A and B/ **III**).

Also, transcription of the chlamydial proteasome-like activity factor CPAF gene was found to be up-regulated in the mid cycle. In addition, a statistically significant difference was noted at 72 hai in the normalized transcription levels between IFN-γ treated and untreated cultures, the transcription level being lower in the IFN-γ exposed cultures. Heuer *et al.* have previously shown by immunofluorescence microscopy that CPAF is expressed in *C. pneumoniae* infected epithelial cells at 1-2 day post infection (dpi), but remains in the inclusion until 3 dpi after which it is translocated into the host cell cytosol (Heuer *et al.*, 2003). However, in persistent *C. pneumoniae* infection models CPAF has been shown to be produced, but the transport of the protein into host cell cytosol is at least partially inhibited (Heuer *et al.*, 2003). In our model, the immunofluorescence staining of CPAF protein was predominantly in inclusions at 72 hour post infection, and both inclusions and the host cell cytosol were stained at 96 hour post infection suggesting partial secretion into the host cell cytosol (Figure 2 C and D/**III**). The host cell cytoskeleton, a network of microtubules, actin filaments, and intermediate filaments (IF), are implicated to be involved in chlamydial entry, inclusion expansion, and exit. In addition to cleaving host cell transcription factors, CPAF has been shown to be capable of also cleaving host cell IF-proteins cytokeratin 8, cytokeratin 18, and vimentin (Heurer *et al.*, 2003, Dong *et al.*, 2004, Kumar and Valdivia, 2008). A possible outcome of CPAF cleaving activity of IF is to make the cytoskeletal structures surrounding the chlamydial inclusion more flexible and allow expansion of the inclusion during the developmental cycle (Kumar and Valdivia, 2008). The observed down-regulation of CPAF transcription in monocytic cell line when exposed to IFN-γ may affect the ability of *C. pneumoniae* inclusion to expand, explaining in part why normal inclusions are not observed in these cells.

7.6.3. Late-cycle genes

The late transcription profile was observed for *omp2*, *lcrE*, *cpn0572*, and *cpn0809*. Although no statistically significant changes could be detected for *lcrE* transcription, the gene was assigned to the group III, since the transcripts were absent (at 6 hai) or at very low level (at 36 hai) before 72 hours after infection. Accumulation of the *cpn0572* transcripts was statistically significantly increased between 36-72 hours after infection for cultures without IFN-γ, and the transcription profile in the IFN-γ exposed cultures was rather similar, but did not reach statistical significance (Table 2./**III**). Transcription of the *cpn0572*, which encodes a *C. pneumoniae* TARP homolog is a putative T3S-effector protein and it has been suggested to play a role in chlamydial entry into the host cells by recruiting actin at the entry site (Clifton *et al.*, 2004). The observed late transcription profile is consistent with the finding by Mäurer and co-workers, that the TARP protein is present in the EB (Mäurer *et al.*, 2007). Also the transcription of another putative T3S substrate, the *cpn0809*, was induced late in the developmental cycle both in the IFN-γ exposed and unexposed cultures, as has been described earlier in epithelial cells (Mäurer *et al.*, 2007, Lugert *et al.*, 2004). The late transcription profile combined with the localization of the protein to the host cell cytosol suggests that it may have a role in the

exit of *Chlamydia* from the host cell. The transcription of lcrE, a component of the T3S, was rather stable in our study, although it has been shown to be down-regulated in persistent *C. pneumoniae* infection models of epithelial cells (Mäurer *et al.*, 2007, Slepenkin *et al.*, 2003). Like its homolog YopN in Yersinia, LcrE of *C. pneumoniae* may regulate the release of T3S effector proteins by controlling their access to the secretory channel. In a recent report by Huang *et al.*, LcrE was described to modify the host cell microtubule network and in "functional LcrE knock outs" *C. pneumoniae* replication was inhibited in a dose dependent manner, suggesting an essential role for the LcrE protein in *C. pneumoniae* development (Huang *et al.*, 2008).

The transcription of the chlamydial membrane protein gene *omp2*, was assessed in this study. In agreement with previous studies performed in epithelial cells (Watson *et al.*, 1995, Hogan *et al.*, 2003, Nicholson *et al.*, 2003), the transcription of *omp2* was induced in the late cycle. On the contrary, the transcription of the *omp2* gene was found to be significantly down regulated in the IFN-γ exposed monocyte cultures. Our finding is in agreement with an earlier report, in which, using murine alveolar macrophages, Haranaga *et al.* found that the *omp2* transcripts were attenuated late in the developmental cycle (at 36-48 hpi) (Haranaga *et al.*, 2003). The Omp2 protein has been suggested to have a role in the RB to EB differentiation (Watson *et al.*, 1995). In our infection model, more infectious EB were formed in the absence of IFN-γ, whereas the reduced production of *omp2* mRNA could well be related to the absence of infectious progeny (**I**) in the IFN-γ exposed monocyte cultures. Omp2 has been suggested to be repressed by the Euo protein during the active growth phase of chlamydiae (Zhang *et al.*, 1998) and Belland *et al.* suggested that elevated expression of Euo protein during IFN-γ induced persistent *C. trachomatis* infection in epithelial cells contributes towards silencing of the late gene expression in persistence (Belland *et al.*, 2003b). Despite the rather stable transcription of the *euo* gene observed in this study, *omp2* transcription was down-regulated in the IFN-γ exposed cultures. This suggests that additional regulatory elements exist for *omp2* transcription.

7.6.4. Constitutively expressed genes

For two genes, *ftsK* and *hsp60*, no significant differences in the transcript levels were noted during the observation period. Therefore, the genes were assigned to group IV of constitutive expression (Table 2./**III**). The observed low level of constitutive expression of ftsK, whose product is required for chlamydial cell division, supports the hypothesis that infection in monocytes resembles persistent infection with minimal bacterial cell division. The *ftsK* is often down-regulated in epithelial cells exposed to IFN-γ or upon iron depletion (Byrne *et al.*, 2001, Slepenkin *et al.*, 2003, Polkinghorne *et al.*, 2006, Timms *et al.*, 2009).

The expression of Hsp60 has been extensively studied in various models of persistent infection without a uniform outcome (Matthews *et al.*, 2001, Slepenkin *et al.*, 2003, Polkinghorne *et al.*, 2006, Ouellette *et al.*, 2006, Mäurer *et al.*, 2007). Also, in monocyte cultures, both up-regulation and down-regulation of *hsp60* mRNA have been reported (Airenne *et al.*, 1999, Haranaga *et al.*, 2003, Gieffers *et al.*, 2001). In our study, a rather stable (constitutive) expression of hsp60 was observed both in the presence and absence of IFN-γ. Taken together, this suggests that up-regulation of *hsp60* during the persistent stage of *C. pneumoniae* infection is not as marked as in *C. trachomatis* infection (Hogan *et al.*, 2004). However, Beatty *et al.* speculated that direct control at the translational level may explain increased chlamydial hsp60 to MOMP ratio in IFN-γ induced *C. trachomatis* infection, since MOMP, but not Hsp60, contain significant number of tryptophan residues (Beatty *et al.*, 1994c). This kind of direct regulation would yield increased hsp60 to MOMP ratio also in IFN-γ induced persistent *C. pneumoniae* infection in epithelial cells, since *C. pneumoniae* MOMP contains 10 tryptophan residues whereas Hsp60 contains none (as determined from *C. pneumoniae* Tw183, Swiss-Prot IDprotien sequence: MOMP, P27455; Hsp60, P31681).

Our results suggest that the transcriptional profile of the studied genes in monocytes is different from that seen in epithelial cells and that IFN-γ exposure affects less *C. pneumoniae* transcription in monocytes in genes studied. Furthermore, our study shows that genes related to bacterial secretion are transcribed and IncA and CPAF proteins are produced. The detection of IncA protein in the inclusion membrane suggests that T3SS is fully functional during *C. pneumoniae* infection in monocytes.

8. CONCLUSIONS AND FUTURE PROSPECTS

Interactions between *Chlamydia* and its host may have several outcomes. These interactions may lead to a mild or asymptomatic infection which is followed by successful eradication of the pathogen or to longer relationship between the pathogen and its host potentially leading to sustained inflammation and tissue damage. This longer relationship with the host, termed persistence, is characterized in chlamydial infection by presence of abnormal RB unable to divide. The molecular details of host–pathogen interaction that support persistence are still largely unknown, but such interactions have been mostly studied in the cell culture models for persistent *Chlamydia* infection. Even though the aberrant RB morphology of persistent *C. pneumoniae* appears similar in all *in vitro* models the transcription profile is dependent on the means by which persistence is induced (Klos *et al.*, 2009, Timms *et al.*, 2009). However, there are a few genes showing consistent regulation across the used models, including the *htrA* gene. It has been shown to be up-regulated in the IFN-γ and iron depletion induced persistent infection in epithelial cells, and now also during *C. pneumoniae* infection of monocytes (**III**). Hence, HtrA has potential as a biomarker of a persistent *C. pneumoniae* infection and its usefulness in diagnostic applications should be further studied, since currently there is no reliable method to identify chronic *C. pneumoniae* infection *in vivo*.

Common antibiotic agents have proved inefficient in eradication of persistent chlamydial infection and circulating monocytes carrying *C. pneumoniae* may also have reduced antibiotic susceptibility. Hence, there is a need for new methods and compounds effective against persistent *Chlamydia* infection and against the spreading of *Chlamydia* from lungs to other tissues. In this study, we observed that the IncA protein is translated and localized into the inclusion membrane suggesting the presence of a functional T3S during infection of monocytes. Since small molecule inhibitors of T3S are able block *C. pneumoniae* growth in epithelial cells (Slepenkin *et al.*, 2007, Bailey *et al.*, 2007), testing whether the use of small inhibitory molecules targeting the T3S during infection of monocytes could lead to the development of novel treatment to chronic chlamydial infection is warranted (**III**). Successful treatment of *C. pneumoniae* residing in monocytes could also potentially prevent spreading of the organisim from the initial site of the infection.

In addition to elucidating molecular mechanisms of persistent *Chlamydia* infection, analysis of host cell transcriptome during persistence may also reveal new targets for therapeutic interventions or diagnostic applications. This study involved investigation of host cell responses in the persistent *C. pneumoniae* infection by microarray. The results revealed several previously unknown alterations of host gene expression induced by *C. pneumoniae* which provide new targets for further analysis of their role in this particular host-pathogen interaction. Some responses in the host cell gene expression were specific for persistent infection and some responses were potentiated in the persistent infection

perhaps through overlapping pathways of TLR4 and IFN-γ. These responses, if also occurring at the protein level, are likely to have an impact in chlamydial infection or in chlamydial disease. Another important finding of the present study was that some responses induced by IFN-γ were absent in persistent infection. Especially the ability of *C. pneumoniae* to overcome up-regulation of the TP53 gene might be important. Further studies are required to evaluate the significance of the genes identified in the present study. However, the results obtained from this microarray study will provide a basis for the elucidation of selected genes and pathways for the host response in productive and persistent *C. pneumoniae* infection (**II**). Although the host cell transcriptome during persistence is at present only studied in the IFN-γ model, it is likely that also the host cell responses are dependent on the stimulus of which persistent state is achieved. On the other hand, they may overlap and provide universal markers of persistent *C. pneumoniae* infection. However, if such markers are not found among bacteria or the host genes, identification of gene and protein expression patterns representing persistent infection would also allow development of diagnostic applications.

Host cell responses and host-pathogen interactions in persistent *C. pneumoniae* infection are still to a large degree unraveled. Studies focusing on the molecular interactions of *Chlamydia* and its host during persistent infection should be the primary interest of future studies in both cell culture and experimental models as well as *in vivo*. In future investigations on parallel kinetic analysis of host and *Chlamydia* transcriptome and proteome from the same samples would allow for correlating the expression of chlamydial genes with effects on the host. Relevance of the identified host cell genes or pathways can be further studied by RNAi, whereas relevance of both the identified chlamydial and host cell genes can be studied by overexpressing the protein during active *C. pneumoniae* infection. Interactions of the identified proteins can also be studied by yeast two-hybrid system or protein arrays. Since these methods complement each other combined use is beneficial. A major challenge of the future is to convert these model systems to clinical reality in form of new diagnostic methods and therapy of chronic *C. pneumoniae* infection.

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