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INSTITUTE OF DIAGNOSTICS,
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DEPARTMENT OF CHILD AND ADOLESCENT HEALTH

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Abstract

Chlamydia pneumoniae is an obligate intracellular gram-negative bacterium, which causes respiratory infections in humans and may participate in the development of chronic diseases like atherosclerosis, chronic obstructive lung disease, adult-onset asthma and late-onset Alzheimer's disease. It can infect various cell types, e.g. vascular endothelial cells, smooth muscle cells and monocyte-derived macrophages *in vitro*. It has been speculated that circulating macrophages disseminate the infection in the body, and that genetically susceptible individuals become chronically infected.

Quantification of *C. pneumoniae* growth inside cultured cells is needed when studying e.g. the effect of drugs or host cell factors on infectivity and replication. Conventionally this has been done by immunofluorescence staining and microscopic counting of chlamydial inclusions. However, this method is usable only if the cell numbers do not fluctuate in cell culture vials and the inclusions are uniform. In macrophages, inclusions are often aberrant, their sizes vary and multiple inclusions are also seen. Therefore we developed a new method based on the real-time PCR quantification of chlamydial genomes adjusted to the number of human genomes and used it to quantify the exact amounts of *C. pneumoniae* in infected cells.

The susceptibility of monocyte-macrophages from healthy individuals to *C. pneumoniae* infection *in vitro* was studied first. Intracellular growth of *C. pneumoniae* was used as an indicator of susceptibility to infection, and it was compared to serum levels of CRP, soluble CD14, human HSP-IgG, human HSP-IgA, *C. pneumoniae* IgG and IgA antibodies. The growth of *C. pneumoniae* in infected macrophages was highly variable, ranging from 0 to 638 chlamydial genomes per human genome. *C. pneumoniae* growth associated positively with serum *C. pneumoniae* IgA (titer ≥10) and hHSP-IgG and negatively with soluble CD14 concentration. The association between chlamydial IgA antibodies, hHSP-IgG and *C. pneumoniae* growth was statistically significant only among men. Age did not correlate with the growth. Therefore we hypothesize that persons whose macrophages cannot restrict the growth of *C. pneumoniae* are more prone to chronic infection by this agent.

In the next study, we evaluated the effects of innate immunity genes CD14 -260 C>T, TLR2 Arg753Gln, TLR4 Asp299Gly, LBP Phe436Leu and IL-6 -174 G>C polymorphisms on *C. pneumoniae* growth in human macrophages *in vitro*. The growth of *C. pneumoniae* was highest in CD14 -260 C>T TT genotype cells and the difference to CC or CT genotype was statistically significant. The G-allele of the IL6 -174 G>C polymorphism had a positive influence on chlamydial growth; the difference was statistically significant only between CC and GC genotypes. TLR2 Arg753Gln, TLR4 Asp299Gly, LBP Phe436Leu polymorphisms showed no effect on chlamydial growth.

Keywords: Chlamydia pneumoniae, gene polymorphisms, human macrophages, infection in vitro, innate immunity

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Abbreviations

ATP	adenosine triphosphate
CAD	coronary artery disease
CD14	cluster of differentiation 14
CHD	coronary heart disease
CPAF	chlamydial protease-like activity factor
CRP	C-reactive protein
DNA	deoxyribonucleic acid
DC	dendritic cell
EB	elementary body
EIA	enzyme immunoassay
HSP	heat shock protein
IB	inclusion body
IFU	inclusion forming unit
IL-6	interleukin-6
IFNγ	interferon-γ
LBP	lipopolysaccharide binding protein
LPS	lipopolysaccharide
NF-κB	nuclear factor-κB
MyD88	myeloid differentiation primary-response protein-88
NOD	nucleotide-binding oligomerization domain
PAMPs	pathogen-associated molecular patterns
PBMC	peripheral blood mononuclear cell
PDI	protein disulfide isomerase
PCR	polymerase chain reaction
PMN	polymorphonuclear neutrophils
PRR	pattern recognition receptor
RB	reticulate body
RNA	ribonucleic acid
SNP	single nucleotide polymorphism
TIR	toll-interleukin-1-receptor homology domain
TLR	toll-like receptor
TNF-α	tumor necrosis factor-α
TTSS	type three secretion system

List of original articles

This thesis is based on the following articles, which are referred by their Roman numerals.

I Poikonen K, Lajunen T, Silvennoinen-Kassinen S, Leinonen M & Saikku P (2010) Quantification of *Chlamydia pneumoniae* in cultured human macrophages and HL cells. Comparison of real-time PCR, immunofluorescence and ELISA methods. APMIS 118(1): 45–48.

II Poikonen K, Lajunen T, Silvennoinen-Kassinen S, Paldanius M, Leinonen M & Saikku P (2008) Susceptibility of human monocyte-macrophages to *Chlamydia pneumoniae* infection *in vitro* is highly variable and associated with levels of soluble CD14 and *C. pneumoniae* IgA and human HSP-IgG antibodies in serum. Scand J Immunol 67(3): 279–284.

III Poikonen K, Lajunen T, Silvennoinen-Kassinen S, Leinonen M & Saikku P (2009) Effects of CD14, TLR2, TLR4, LPB, and IL-6 gene polymorphisms on *Chlamydia pneumoniae* growth in human macrophages *in vitro*. Scand J Immunol 70(1): 34–39.

Contents

1 Introduction

Chlamydia pneumoniae, an obligatory intracellular gram-negative bacterium, is a human pathogen, which causes acute upper and lower respiratory tract infections (Grayston *et al.* 1990); it is responsible for up to 10% of community-acquired pneumonia and 5% of bronchitis and sinusitis cases in adults (Kuo *et al.* 1995). *C. pneumoniae* infections are very common, and almost everyone gets infected at some point during their lifetime. Most infected persons recover without complications, and the host's genetic background may predetermine the outcome of the infection. The prevalence of *C. pneumoniae*-specific Cpn-IgG and Cpn-IgA antibodies increases with age, with males more often having antibodies in their sera than females (Saikku 1992). Since serum antibodies usually disappear within a few years following the infection, the continuous presence of elevated IgG and especially short-living IgA antibodies can be considered a marker of recurrent or chronic infection (Saikku *et al.* 1992). All chlamydial species have a tendency to cause chronic infections, and it has been suggested that chronic *C. pneumoniae* infection contributes to the development of cardiovascular diseases (Leinonen & Saikku 2002), asthma (Hahn *et al.* 1991), chronic obstructive pulmonary disease (von Hertzen *et al.* 1997), lung cancer (Laurila *et al.* 1997, Littman *et al.* 2005) and late-onset Alzheimer's disease (Balin *et al.* 2008).

C. pneumoniae infection is transmitted by a metabolically dormant elementary body (EB). Inside the cell, EBs develop into metabolically active reticulate bodies (RBs). RBs replicate in a vacuole, an inclusion body (IB), which isolates RBs from the endocytic pathway of the host cell. After replication, RBs differentiate back to EBs, which are then liberated from the cell (Kuo *et al.* 1995). *C. pneumoniae* primarily infects respiratory tract epithelial cells. During acute bacterial pneumonia the amount of polymorphonuclear neutrophils (PMN) increases in the lung, accounting for more than 80% of the cells. PMNs phagocytise *C. pneumoniae* and they multiply in the cells (van Zandbergen *et al.* 2004). Bronchoalveolar macrophages phagocytose apoptotic PMNs growing bacteria; this leads to productive infection (Rupp *et al.* 2009). Inside macrophages, replicating *C. pneumoniae* may disseminate to other tissues actively from the lung and infect susceptible cells like vascular endothelial cells (Kaukoranta-Tolvanen *et al.* 1994, Godzik *et al.* 1995) and smooth muscle cells (Gaydos *et al.* 1996).

Innate immune cells recognize invading pathogens through pattern recognition receptors. The receptors involved are cell surface molecules, like CD14 (Medzhitov & Janeway 1997) and Toll-like receptors TLR2 and TLR4,

which play an important role in the recognition of pathogen-derived pattern ligands such as lipopolysaccharide (LPS), peptidoglycan, lipoarabinomannan and lipoproteins (Takeuchi *et al.* 1999). Macrophages become activated when LPS is bound to CD14 and further interact with the TLR4-MD2 complex. This initiates signal transduction cascades inside cells finally leading to the translocation of NF-κB into the nucleus, where it regulates the expression of pro-inflammatory cytokines (Arancibia *et al.* 2007). Activated macrophages secrete pro-inflammatory cytokines like TNF-α, IL-1 and IL-6 (Dobrovolskaia & Vogel, 2002). IL-6 is the predominant mediator of the acute phase response, and it activates the synthesis of acute phase reactants such as lipopolysaccharide binding protein (LBP) (Graeve *et al.* 1993). Chlamydial LPS and heat shock protein 60 (HSP60) are able to activate macrophages through TLR4 (Bea *et al.* 2003, Kol *et al.* 2000, Bulut *et al.* 2002) but, primarily, recognition of *C. pneumoniae* depends on TLR2, because the production of pro-inflammatory proteins is mainly mediated by TLR2 and not TLR4 (Netea *et al.* 2002, Prebeck *et al.* 2001).

The innate immune system is the first line of defence against infection but it also influences the development of the adaptive immune response (Iwasaki & Medzhitov 2004, Sabroe *et al.* 2008). Hence the genes encoding proteins of the innate immune system may have an effect on an organism's susceptibility to infections and recovery. Polymorphism -260C>T in the CD14 promoter gene regulates the density of CD14 receptors on monocytes. The T variant causes a higher expression of CD14 and leads to an enhanced inflammatory response. The T-allele has also been associated with increased risk of myocardial infarction and to the presence of *C. pneumoniae* antibodies in healthy subjects (Hubacek *et al.* 1999, Eng *et al.* 2003). Asp299Gly polymorphism in the TLR4 gene causes hyporesponsiveness to LPS in human alveolar macrophages (Arbour *et al.* 2000); however, this was not observed with blood-derived monocytes (Erridge *et al.* 2003). Regardless of being more susceptible to infections, individuals carrying the TLR4 299Gly allele exhibit a slower progression of atherosclerosis (Kiechl *et al.* 2002) and have a reduced risk of acute coronary syndromes (Ameziane *et al.* 2003). The Arg753Gln mutation in the TLR2 gene affects the ability to respond to bacterial peptides *in vitro* (Lorenz *et al.* 2000) and increases the risk of the development of tuberculosis (Ogus *et al.* 2004). The IL-6 gene -174G>C polymorphism affects the promoter function and causes differences in IL-6 plasma levels (Fisman *et al.* 1998). This polymorphism has been associated with a risk of coronary heart disease and myocardial infarction (Humphries *et al.* 2001, Chiappelli *et al.* 2005). The LBP Phe436Leu polymorphism seems to be

associated with an increased risk of the development of sepsis but does not influence the risk of myocardial infarction (Hubacek *et al.* 2001, Hubacek *et al.* 2002).

2 Review of literature

2.1 *Chlamydiales*

The order *Chlamydiales* are among the most successful bacterial pathogens of humans, and *Chlamydia pneumoniae* is undoubtedly the most successful among the family *Chlamydiace* as it infects almost all human beings at least once during their lifetime. The phylum *Chlamydiae* have earlier been considered a human pathogen group of few unique bacteria. Based on ribosomal 16S RNA (rRNA) phylogenetic trees *Chlamydiae* diverged about 2,000 million years ago from other *Eubacteria* (Weisburg *et al.* 1986). The order *Chlamydiales* evolved from their free living ancestors about 500–1,000 million years ago (Stephens 2002). After recovering the genus *Chlamydia*-related endosymbionts in free-living amoeba (Amann *et al.* 1997), the known diversity of the order *Chlamydiales* has increased from one, *Chlamydia*, to currently eight families during the last decade. A large number of phygenetically diverse 16S rRNA sequences similar to those recognized in *Chlamydia* have been detected in a variety of habitats like soils, sediments, aquatic environments, hydrothermal vent fluid, and engineered environments such as activated sludge and anaerobic bioreactors (Horn 2008). Environmental *Chlamydiales* share the unique biphasic chlamydial developmental cycle and growth only occurs in a vacuole inside eukaryotic host cells. It is approximated that pathogenic and environmental lines diverged from a common ancestor about 350–800 million years ago and the human pathogens *Chlamydia trachomatis* and *Chlamydia pneumoniae* diverged about 50–200 million years ago (Stephens 2002).

All pathogenic *Chlamydia* have reduced their genomic size to about 1 Mb while adapting to an intracellular niche. In contrast, environmental *Chlamydiales* have a genome the size of which is about 2-fold compared to pathogens. It is likely that pathogens lost genes because they live in a more homeostatic niche than environmental species (Ochman & Moran 2001). It is remarkable that horizontal gene transfer (HGT) has not played any role in the evolution of the *Chlamydiales* (Ortutay *et al.* 2003).

As with the other obligate intracellular parasites, all *Chlamydiales* exhibit environment species to a lesser extent, as well as reduced central metabolic and biosynthetic pathways, and are also auxotrophic for amino acids and nucleotides.

Despite some minor differences the metabolic pathways indicate that their common ancestor was already adapted to intracellular life (Horn 2004).

Pathogenic and environmental *Chlamydiales* share many virulence factors, like type III secretion system (TTSS), which delivers effector molecules into eukaryotic host cell cytoplasm (Hueck 1998, Horn 2004). Another example is chlamydial protease-like activity factor (CPAF) that is able to degrade the human transcription factor required for major histocompatibility complex (MHC) expression (Zhong *et al.* 2001). It is also expressed in environmental *Chlamydiales*, whose hosts do not have MCH system, indicating that CPAF has evolved from protease with different function in lower eukaryote host. Thus, the virulence mechanisms of pathogenic *Chlamydia* have evolved over millions of years from the interactions of ancestral *Chlamydia* with early eukaryotes.

2.2 Pathogenic *Chlamydiales*

The family *Chlamydiacea* comprises a single genus *Chlamydia*, which contains 8 pathogenic species. A decade ago it was proposed that the family should be divided into two genera, the *Chlamydia* and the *Chlamydophila.* The proposal was based on16 S rRNA sequence analysis (Everett *et al.* 1999) but was rejected by numerous researchers (Schachter *et al.* 2001). Indeed, with obligatory intracellular bacteria the 16S rRNA is not a proper indicator to determine evolutive differences between species (Stephens 2002, 2009). However, the classification of members within the genus *Chlamydia* that cause infections in livestock and other veterinary based groups was entangled and needed reclassification. Thus, a compromise classification was presented (Table 1) (Kalayoglu &Byrne 2006).

2.2.1 Chlamydia pneumoniae

In addition to humans, *Chlamydia pneumoniae* has been detected in horses, koalas, frogs (3 species), green sea turtles, snakes (3 species), chameleons, and iguanas (Horn 2008).The genetic diversity of *C. pneumoniae* has been evaluated by synonymous single nucleotide polymorphisms (sSNP) in 38 isolates, 36 strain isolates derived from humans, one from a frog and one from a koala (Rattei *et al.* 2007). The isolates differentiate into 15 genotypes in four major clusters, one cluster of which consisted of animal strains and human strains branched into three clusters. It was suggested that animal lineages are basal on the *C. pneumoniae*

phylogeny and the transmission to humans happened through a successive bottleneck some 150,000 years ago. A lack of detectable diversification in 17 isolates emphasizes the extraordinary conservation of *C. pneumoniae* and the high clonality of the population. The largest cluster, which contained 80% of the analyzed strains, is a young clade that went through a population expansion some 3,300 years ago. In a recent study (Myers *et al.* 2009) the genome of koala strain of *C. pneumoniae* (LPcoLN) was sequenced. The sequence combined with phylogenetic analysis of all *C. pneumoniae* SNPs and conserved chlamydial coding sequences in human derived isolates, and so provided evidence that human isolates of *C. pneumoniae* have zoonotic origins. The authors proposed that *C. pneumoniae* was originally an animal pathogen which crossed the species barrier to humans and has adapted to the point where it no longer requires an animal reservoir for transmission.

Table 1. The compromise approach to Chlamydial taxonomy.

Old taxonomy	Compromise taxonomy
Human strains	Human strains
Chlamydia trachomatis L1-L3	*Chlamydia trachomatis* – similar serologic
Chlamydia trachomatis A, B, Ba, C	and genotypic groupings as before
Chlamydia trachomatis D-K	
Chlamydia pneumoniae	*Chlamydia pneumoniae*
Non-human strains	Non-human strains
Chlamydia trachomatis – mouse pneumonitis	*Chlamydia muridarum* – same as before
Chlamydia trachomatis – pig strains	*Chlamydia suis*
Chlamydia psittaci	*Chlamydia psittaci* – mainly bird strains, can
Birds and mammals, associated with arthritis or	cause respiratory and systematic zoonotic
abortion in live stocks, especially sheep and cattle	diseases in humans
Chlamydia pecorum found in various species	*Chlamydia abortus* (former *C. psittaci*)
including koalas and ruminants, causing arthritis	associated with cattle and sheep abortion
and systemic infection	*Chlamydia caviae* (former *C. psittaci* guinea
Chlamydia pneumoniae strains found in horses and	pig inclusion conjunctivitis strains)
koalas	*Chlamydia felis* (former *C. psittaci* feline
	pneumonitis strains)
	Chlamydia pecorum –same as before
	Chlamydia pneumoniae

C. pneumoniae *as human pathogen*

C. pneumoniae causes acute upper and lower respiratory tract infections (Grayston *et al.* 1990), and is responsible for up to 10% of community–acquired pneumonia and 5% bronchitis and sinusitis cases in adults (Kuo *et al.* 1995). Most patients recover from infections without complications, and the host's genetic background may predetermine the outcome of the infection. The prevalence of *C. pneumoniae*- specific Cpn-IgG and Cpn-IgA antibodies increases with age, with males having antibodies more often in their sera than females (Saikku 1992). Antibodies in serum usually disappear after a few years of infection, thus the continuous presence of elevated Cpn-IgG and specially Cpn-IgA antibodies can be considered a marker of recurrent or chronic infection (Saikku *et al.* 1992).

C. pneumoniae has a tendency to cause chronic infections, like all other chlamydial species, and chronic infections have been associated with cardiovascular diseases (Leinonen & Saikku 2002), asthma (Hahn *et al.* 1991), chronic obstructive pulmonary disease (von Herzen *et al.* 1997) and lung cancer (Laurila *et al.* 1997, Littman *et al.* 2005).

C. pneumoniae is transmitted by respiratory droplets into the upper respiratory tract where it infects epithelial cells. The first symptoms are sinusitis, pharyngitis and bronchitis. Infection of respiratory epithelium induces ciliostasis (Shemer-Avni & Lieberman 1995), which may result in transmission of the pathogen to the lower respiratory tract causing atypical pneumonia characterized by cough, mild fever and pleuritic pain. Most pneumonias resolve without treatment; severe cases do occur, especially in patients with chronic obstructive lung disease and immunocompromised patients (Kalayoglu & Byrne 2006).

Alveolar macrophages are the predominant immune system's cells in the human lung and represent the first line of defence in respiratory infections. However, during acute bacterial infection the amount of polymorphonuclear neutrophils (PMN) increases in the lung, accounting for more than 80% of the cells. PMNs phagocytise *C. pneumoniae* and they multiply in the cells and delay their spontaneous apoptosis but induce apoptosis in surrounding neutrophils (van Zandbergen *et al.* 2004). Infected PMNs secrete MIP-1β that attracts macrophages. The macrophages phagocytose apoptotic PMNs hiding *C. pneumoniae*. This leads to multiple, round and large inclusions seen in productive infection; in contrast, direct infection of macrophages results in persistent-like infection characterized by aberrant small inclusions. Efficient uptake and intracellular growth of *C. pneumoniae* is dependent on phosphatidylserine

expression (the “eat-me” signal) on infected PMNs (Rupp *et al.* 2009). Inside macrophages, actively replicating bacteria may disseminate to other tissues from the lung and infect susceptible cells like vascular endothelial cells (Kaukoranta-Tolvanen *et al.* 1994, Godzik *et al.* 1995) and smooth muscle cells (Gaydos *et al.* 1996).

2.3 *Chlamydia*l developmental cycle

Chlamydia exists in two different morphological forms, as extracellular elementary body (EB) and as intracellular, vegetative reticulate body (RB) (Moulder 1991). *Chlamydia* infection disseminates to susceptible cells by EBs. The chlamydial development cycle takes place within vacuole, termed inclusion, which does not fuse with lysosomes. After internalization to cells, EBs differentiate inside inclusions to metabolically active, non-infectious reticulate bodies (RB). During the growth cycle RBs undergo repeated binary fissions and then differentiate back to EBs, which are released as host cell lyses.

2.3.1 Elementary and reticulate bodies

Elementary body (EB) is a small (about 0.3 µm) metabolically inactive, but infectious form of the organism. Its chromatin is highly condensated by the histone-like proteins Hc1 and Hc2 (Brickman *et al.* 1993). With using scanning electron microscopic techniques, in EB membrane hexagonally, regularly organized projections with a centre to centre about 50 nm can be seen (Matsumoto 1973 and 1982). It has been suggested that these structures are type III secretion system (TTSS) “needles”. This theory was in line with a study which demonstrated that secreted inclusion proteins (Inc) appeared in the inclusion membrane before TTSS proteins were synthesized (Field *et al.* 2003).

The attachment of EB to a host cell has two separate phases: initial attachment is reversible and occurs through electrostatic interactions, and the second binding step is irreversible (Carabeo & Hackstadt 2001, Fudyk *et al.* 2002). It is suggested that cell surface heparin sulphate glycosaminoglycans are involved in the first stage of attachment. *Chlamydia* cysteine-rich outer membrane OmcB complex is able to bind native sulphate-like invasin, mammalian proteoglycans and exogenous heparin (Stephens *et al.* 2001). However, exogenous heparin inhibited *Chlamydia* infection of CHO-18.4 cells that lack cell surface heparin sulphate. This finding is consistent with a hypothesis

that heparin inhibits competitively a functional component on the surface of EB (Zhan & Stephens 1992). Several host proteins have been shown to influence *Chlamydia* attachment, such as estrogen receptor complex (Davis *et al.* 2002), mannose-6-phosohate receptor (Puolakkainen *et al.* 2005), platelet-derived growth factor receptor (Elwell *et al.* 2008) and protein disulfide isomerase (PDI) (Davis *et al.* 2002, Conant & Stephens 2007). PDI is a multifunctional protein that resides in various subcellular locations and catalyzes the reduction, oxidation, and isomerisation of disulfide bonds; it has also chaperone and anti-chaperone activities (Primm *et al.* 1996). PDI is the only mammalian protein that is involved in attachment of multiple species and serovars of *Chlamydia*. Recently, it was demonstrated that PDI is necessary for *Chlamydia* attachment to cells but it does not serve as a receptor. Instead, EB binds to some cell surface protein that requires structural association with PDI and subsequently the oxido-reductive function of PDI is required for entry (Abromaitis & Stephens 2009). Immediately afterwards irreversible binding chlamydial effector protein Tarp (Translocated actin-requiting phosphoprotein) is delivered to the host by TTSS. Tarp is rapidly phosphorylated at tyrosine residues followed with reorganisation of filamentous actin and internalization of EB (Clifton *et al.* 2002).

After the entry, differentiation of EB to RB begins. Hc1-DNA interaction is disrupted by a small metabolite in the methylerythritol phosphate (MEP) pathway of isoprenoid biosynthesis. It is not known how Hc2 is released from the chromatin (Grieshaber *et al.* 2004). Gene transcription begins in the EB almost immediately and protein expression can be detected within 15 min post-infection (Plaunt *et al.* 1998). The EB contains significant quantities of carryover mRNA and ribosomes; however, late RB-phase proteins encoded by carryover mRNA is not expressed, which means that in the differentiating EB there must be a mechanism to differentiate new mRNA from old. Carryover RNA is rapidly degraded and is not detectable after 6 hpi (Belland *et al.* 2003). Temporal gene transcription during chlamydial growth has been studied with microarray technique in C. trachomatis serovar D and L2 infected cells (Belland *et al.* 2003, Nicholson *et al.* 2003). On the basis of these analyses gene expression can be classified to early, mid-cycle and late categories with subgrouping genes expressed at 1h post-infection (hpi) as immediate-early.

During first hour of infection, numerous inclusion-associated protein genes (*inc*) and *euo* gene (early upstream open reading frame) (Shaw *et al.* 2000, Wichlan & Hatch 1993) are expressed. e*uo* codes a minor protein that is expressed within 1 h of infection of host cells with *C. psittaci* 6BC. The

expression lasts through logarithmic growth phase of RBs and declines late in the infection when RBs reorganize to infectious EBs. EUO protein binds to promotor region of *crp* and may repress the crp operons during growth phases. *crp* codes cysteine-rich proteins which are important constituents of EB outer membrane (Hatch 1996).

Activation of early phase (1–8 h) genes is involved with biogenesis of the developing inclusion and transition from EB to RB (Valdivia 2008). After primary differentiation to RB, bacterial cell division begins. Chlamydia lack an identifiable gene *ftsZ* ortholog that encodes protein needed in bacterial division (AbdelRahman & Belland 2005). Instead RB may synthesize small amounts of peptidoglycan to substitute the lack of the protein FtsZ in formatting cell division septa (Chopra *et al.* 1998). Then, afterwards mid-cycle (16–24 h) begins with rapid multiplication of RBs and inclusion expanding. Characteristic for this phase is numerous activated genes involved in cell envelope biogenesis, energy metabolism, TTSS, protein folding, and DNA synthesis and repair. Late phase (>24 h) consists of two temporal clusters, late and very late. First begins the activation of genes involved in EB differentiation, like *crp, omcA* and *omcB,* which encode cysteine-rich membrane proteins found only in EB. DNA topoisomerase, DNA gyrase and histone-like proteins are also induced at this stage and are implicated in the mechanism of the terminal stages of condensed nucleoid formation. The largest functional category expressed at this phase is the hypothetical protein group consisting of genes of unknown function unique to *Chlamydiae*; however, the coordinated regulation of these genes suggests that they have essential functions in EB formation. At 36 hpi inclusion body contains mixed population of RBs and EBs, with the majority of transcriptionally inactive EBs. At this stage some genes are still active and they code proteins of undermined functions (Stephens *et al.* 1998, Nicholson *et al.* 2003).

2.3.2 Inclusion body

EB is endocytoced by the host cell, where it is tightly bound within a membranous vesicle. Within this vesicle, unknown signals trigger an initial round of chlamydial transcription which actively modifies the properties of the nascent vacuole. The inclusion associates intimately with recycling endosomes and is moved to the peri-Golgi region where the inclusion remains for the duration of intracellular growth (Hackstadt 1995). The inclusion body uses host microtubules for moving to the minus end anchored at the microtubules-organizing center

(MTOC). This movement requires chlamydial protein synthesis because treatment of infected cells with chloramphicol completely inhibits the migration (Grieshaber *et al.* 2003). During the migration Rab GTPases, key regulators of membrane trafficking, associates with inclusions. This association with different Rab GTPases is species-specific, which suggests that different host interactions with the inclusion may play significant roles (Rzomp *et al.* 2003).

From Golgi apparatus *Chlamydia* acquires host-derived lipids: sterols, sphingolipids, glyserophospholipids and neutral lipids (Wylie *et al.* 1997, Carabeo *et al.* 2003). Recently it was demonstrated that C. trachomatis infection in human epithelial cells induces fragmentation of Golgi apparatus to generate Golgi ministacks surrounding the inclusion. Fragmentation is triggered by the proteolytic cleavage of the Golgi matrix protein golgin-84 (Heuer *et al.* 2009). Inclusion is fusogenic with these lipid-containing exocytic vesicles. Chlamydial proteins mediate the fusogenecity and actively transport lipids in inclusion. Inhibition of chlamydial protein synthesis results gradually in a loss of ability to acquire lipids from the host (Scidmore *et al.* 2003).

Genomic sequence analysis has demonstrated that *Chlamydiales* are auxotrophic for virtually all amino acids and most nucleotides (Stephens *et al.* 1998). The inclusion membrane excludes soluble molecules larger than 520 Da (Heinzen & Hackstadt 1997). It is suggested that simple diffusion may be a possible mechanism for acquisition of amino acids and nucleotides which have molecular masses of 100–300Da (Grieshaber *et al.* 2002). The genome analysis revealed numerous orthologs involved in eubacterial membrane transport system, like transporters primarily associated with amino acids and oligopeptide transport and Na^+ and H^+ amino acid symporters, permeases for magnesium phosphate, nitrate and sulphate were also identified. The relative preponderance of amino acid and peptide transporters is consistent with the limited amino acid biosynthetic capacity of *Chlamydia* (Stephens *et al.* 1998). Genomic and experimental data indicates that *Chlamydia* is able to synthesize ATP (Stephens *et al.* 1998, Iliffe-Lee & McCarthy 1999). However, many of the intermediary genes are not expressed until mid-cycle but two ATP transporters are expressed at the early phase of the development cycle (Shaw *et al.* 2000).

First, the inclusion membrane protein IncA was detected by screening with *C. psittaci* EB-antiserum bacteriophage expression libraries for genes encoding the infection-specific antigens (Rockey *et al.* 1995). Later, IncB and IncC were localized to the inclusion membrane (Bannantine *et al.* 1998). Different Inc proteins share minimal primary sequence identity with each other or any other

proteins in the sequence database. However, each Inc protein has a unique bi-lobed hydrophobic domain of about 50–80 amino acids. In screening of all predicted protein sequences within genomes of *C. pneumoniae* and *C. trachomatis* 40 sequences were identified that possessed the characteristics of the hydrophobicity domain (Bannantine *et al.* 2000). This study led to the identification of four novel Inc proteins, termed IncD-G, which are produced early in the infection and thus may be involved in initial pathogen-host interactions that lead to the formation of the inclusion (Scidmore at al. 1999). After this, at least ten independent proteins that share the common structure motif are localized to the inclusion membrane (Rockey *et al.* 2002).

Two proteins in inclusion membrane have been detected that lack the hydrophobic domain. CopN is member of a family of proteins common to pathogens *C. psittaci, C. trachomatis*, *Pseudomonas aeruginosa* and *Yersinia* species. Following contact of the *Yersinia* with a eukaryotic cell, YopN is translocated by TTSS into the cytoplasm of the host cell (Day *et al.* 1998). Chlamydial CopN is secreted by the *Yersinia* TTSS machinery, demonstrating that this protein is a TTSS substrate in heterologous system (Fields & Hackstadt 2000). Heterologous expression of CopN in yeast or mammalian cells affect the formation of microtubule structures and causes a cell cycle phase-specific cell division block. Function of CopN during infection can be prevent with small molecule inhibitors 043YC1 (ChemDiv 5947-0064) and 0433YC2 (ChemDiv C303-0665). These molecules inhibit the growth of *C. pneumoniae* in a dose-depended manner without any toxic effects to BGMK-host cell (Huang *et al.* 2008). Class 1 accessible protein (Cap 1), localized on C. trachomatis inclusion, is another protein that lacks the hydrophobicity motif. Cap 1 has been identified as a target protein for $CD8^+$ T cells, however nothing is known about its function. It is likely that Cap 1 is exposed at the cytosolic face of the inclusion, as peptides of this protein enter the class I processing pathway (Fling *et al.* 2001).

As the inclusion expands, chlamydial replication becomes asynchronous and RBs begin to differentiate into EBs upon an unidentified signal. It is suggested that the RBs grow and replicate predominantly when in physiological contact with the inclusion membrane surface. However, the RBs attached to the surface continue to replicate at the same rate and thus are physically squeezed out of contact with the surface, and this triggers the differentiation from RB to EB (Hackstadt *et al.* 1997, Wilson *et al.* 2006). At the end of the developmental cycle EBs are released from host cells either by cellular lysis or by packaged release, termed extrusion (Hybiske & Stephens 2007). Lysis consists of an ordered

sequence of permeabilizations within the host cell. The inclusion membrane ruptures first; thereafter intracellular compartments and finally plasma membrane permeabilize resulting in dispersion of EBs. Lysis of inclusion is abolished with protease inhibitors, and intracellular calcium signalling is needed for plasma membrane breakdown. Packaged release, extrusion, is a slow process resulting in a pinching of the inclusion, protrusion out of the cell with a cell membrane compartment and ultimately detachment from the cell. Extrusion requires polymerization of actin, myosin and at the late phase Rho GTPase. It is suggested that initiating signals for *Chlamydia* release may come from the bacteria, and that the release is a consequence of a biological interaction established between the inclusion and the host cell at the late phase of infection (Hybiske & Stephens 2007). Very late (30 hpi) gene expression supports the view that a late-expressed chlamydial protein could induce the release (Nicholson *et al.* 2003).

2.3.3 Chlamydial effector mechanisms

Throughout the infection cycle *Chlamydia* modulates the functions of the host cell by secreting effector proteins. All Chlamydia code the core components of TTSS (Peters *et al.* 2007). Therefore it is suggested that many chlamydial effector proteins are translocated to the host by TTSS, but TTSS-independent secretion also exists. Chlamydial protease-like activity factor (CPAF) has type II secretion signal and it is first secreted in the inclusion lumen and then translocated into the host cytoplasm (Heuer *et al.* 2003).

The first chlamydial effector molecules identified were Inc proteins (see above). It is remarkable that about 5% of chlamydial genome codes for Inc like proteins (Rockey *et al.* 2002), so they are probably central regulators of bacterial-host interactions. Inc proteins seem to participate in the recruitment of Rab proteins to inclusion. Inc CT229 interacts with Rab4-GTP protein both *in vitro* and *in vivo* (Rzomp *et al.* 2006). Similarly, the *C. pneumoniae* inclusion membrane protein Inc Cpn0585 interacts with Rab1, Rab10 and Rab11 (Cortes *et al.* 2007). Rab GTPases regulate host vesicle fusion and species-specific recruitment of Rabs to inclusion membrane probably represents essential events in creating a productive intracellular niche (Moorhead *et al.* 2007).

Inhibition of host cell apoptosis is fundamental for *Chlamydia* growth. *In vitro Chlamydia*-infected cells are resistant to apoptosis induction and host cells can continue to undergo DNA synthesis and mitosis (Greene *et al.* 2004). *C. pneumoniae* infected epithelial HL-cells and macrophages (U937-line) are able to

inhibit stauroporine or TNFα induced apoptosis and the inhibition depends on the protein synthesis (Airenne *et al.* 2002). *C. pneumoniae* induces the activation of nuclear factor kappa B (NF-κB) which is required for apoptosis resistance in persistently infected cells, where it upregulates inhibitor of apoptosis protein 2 (cIAP-2) synthesis (Paland *et al.* 2006). Early in infection with *C. trachomatis*, Inc G prevents pro-apoptotic phosphorylated BAD from binding to mitochondria by sequestering its binding partner 14-3-3β protein (Verbecke *et al.* 2006). Later in the infection, the secreted CPAF degrades BH3 proapoptotic proteins (Pirbhai *et al.* 2006).

Downregulating antigen presentation may constitute a molecular mechanism of chlamydial evasion of immune surveillance. In mid-cycle, secreted chlamydial protease CPAF disables adaptive immune responses by degradation of RFX-5, USF1 and CD1d, factors required for MCH expression and lipid antigen presentation (Zhang *et al.* 2001, Kawana *et al.* 2007).

C. trachomatis infection has been associated with cervical cancers (Koskela *et al.* 2000). *C. trachomatis* affects the cell cycle of the host cell by cleaving mitotic cyclin B (Balsara *et al.* 2006), by delaying cytokinesis (Greene & Zhong 2003) and by inducing centromere supernumeracy (Grieshaber *et al.* 2006). Combination of these to genomic instability leading factors and the anti-apoptotic effect of infection could predispose to cancer.

2.3.4 Persistent C. pneumoniae*–infection* in vitro

Many chlamydial diseases are associated with a long-term or chronic infectious state. However, it is often difficult to realise whether chronic or recurrent infections arise through the host's incapability to resolve the infection or repeated infections with similar species or genotypes. Therefore persistence models of chlamydial infection have been studied to provide an insight into the nature of chronic diseases (AbdelRahman *et al.* 2005). Persistence is defined as a long-term association between *Chlamydia* and its host cell. With persistent infection *Chlamydia* remains viable but is non-cultivable. Typical to persistence are pleomorphic EBs and multinucleated, numerous inclusions in one cell. RBs do not undergo binary fission and differentiate to EBs but nevertheless continue to replicate DNA. These changes are mostly reversible and the developmental cycle continues normally if inhibitory factors are removed (reviewed in Hogan *et al.* 2004).

Chlamydia persistence *in vitro* has been induced by penicillin (Matsumoto & Manire 1970), iron depletion (Roulston *et al.* 1997), amino acid deficiency (Coles *et al.* 1993) and IFNγ-induction (Beatty *et al.* 1993). IFNγ-induction is the most studied persistence model and it relies on the inducible enzyme indoleamine-2,3-dioxygenase (IDO) which depletes host tryptophan pool (Hogan *et al.* 2004). In addition *Chlamydia* causes persistent infection in monocytic cells without any additive mechanism (Koehler *et al.* 1997).

Induced persistent infection

Chlamydial gene expression in different persistent models has been evaluated initially with reverse transcriptase PCR (RT-PCR) and subsequently with proteomic analysis and DNA microarray methods. The first study demonstrated that DNA synthesis and replication involved the genes *dnaA, polA, mutS*, *minD* in *C. pneumonia* infected HEp-2 cells. These genes were expressed in both IFNγ treated and untreated cells. However, bacterial cytokinesis involved the genes *ftsK* and *ftsW* that were expressed only by IFNγ untreated cells (Byrne *et al.* 2001). Mathews *et al.* (2001) studied RT-PCR with different levels of gene transcription of 14 genes between untreated and IFNγ treated *C. pneumoniae* infected HEp-2 cells. They detected five upregulated genes: *ompA, ompB, pyk, nlpD* and *Cpn0585*. Two of these genes, *ompA* and *ompB*, were code structural proteins thought to be important in cell wall rigidity. *Cpn0585* was the most upregulated gene. *Cpn0585* is involved in the recruitment of Rab GTPases to the inclusion membrane (Cortes *et al.* 2007) and upregulating this function may impact the inclusion for surviving under stress. The *C. pneumoniae nlpD* gene product has significant homology with major extracellular protein family, p60, found in many pathogens. In *Listeria* the homologous gene product, invasion associated protein, has been shown to be required for adherence to and invasion of nonphagocytic cells (Bubert *et al.* 1990). Its function in *C. pneumoniae* infection is unknown.

Proteomic analysis of HEp-2 cells persistently infected with *C. pneumoniae* showed a marked upregulation of major outer membrane protein (MOMP), heat shock protein 60 (HSP60/GroEL), and proteins with functions in DNA replication (GyrA), transcription (RpoA, PnP), translation (Rrf), glycolysis (PgK, GlgP), and type III secretion (SctN) at 24 hpi. In contrast, no significant decreases in bacterial protein expression were found in *C. pneumoniae*-infected cells due to IFN-gamma treatment (Molestina *et al.* 2002).

In a recent study (Timms *et al.* 2009) quantitative real time-PCR was used to compare the IFN-γ induction and iron-limitation models of *C. pneumoniae* persistence at the transcriptional level by analyzing selected genes in each of 5 distinct, functionally relevant subcategories. The models showed minimal evidence of a general transcriptional stress response in persistence, with only 1 of the 7 genes analyzed in the IFN-γ induction model (*htrA*) and 4 of the genes in the iron-limitation model (*htrA*, *clpB*, *clpP1*, *ahpC*) showing increased mRNA levels. Both models showed similar expression of genes which associated with reticulate body to elementary body conversion (*ctcB*, *lcrH1)*, and *hctB* levels were all unchanged or downregulated. The models also showed similar responses to the key cell wall and envelope genes, *ompA*, *omcB*, and *crpA*, exhibiting lower mRNA levels in both models. The authors suggested that *Chlamydia* spp. have evolved more than one mechanism to respond to different persistence-inducing conditions, but ultimately the pathways probably converge through a common persistence regulon.

In another recent study transcript patterns were examined using real-time reverse transcriptase-PCR in four *in vitro* models of persistence for *C. pneumoniae* strain CWL 029, using HeLa cells and normal human monocytes as host (Klos *et al.* 2009). Differential expression of genes encoding cell division proteins was variable when persistence was induced by IFNγ, penicillin G, or deferoxamine mesylate (DAM) treatment, and in the monocyte model of persistence. Their conclusion was that these *in vitro* observations indicate that chlamydial persistence is not solely and simply a single fixed default transcriptional response to stress. Rather, it is an evolutionarily determined complex and flexible metabolic strategy designed to enhance long-term survival in the host via evasion of immune surveillance.

Persistence infection in mononuclear cells

Freshly isolated human peripheral blood mononuclear cells (PBMC) were infected with *C. pneumoniae in vitro* secrete TNFα, IL-1β, IL-6 and IFNα. Viable pathogen could not be recovered from freshly infected monocytes but if the cells were cultured 3 to 9 days *in vitro* before infection they were able to maintain growth of *C. pneumoniae* (Kaukoranta-Tolvanen *et al.* 1996).

Airenne *et al.* (1999) also infected freshly isolated human blood mononuclear cells with *C. pneumoniae* and were able to show with confocal and electron microscopy that the morphology of the chlamydial inclusions was abnormal. The

Chlamydia was non-cultivable and addition of tryptophan or anti- IFNα-antibodies did not diminish the inhibition. Chlamydial mRNA was expressed at least 3 days post infection (dpi) and bacteria remained metabolically active at at least 7 dpi.

Viable *C. pneumoniae* has been isolated and cultured from CD14-positive cells (macrophages) of coronary artery disease patients (Gieffers *et al.* 2001). It was remarkable in that patients had undergone azitromycin treatment. In addition, azitromycin or rifampin did not protect cultured human monocytes from *in vitro* infection with *C. pneumoniae*. The chlamydial inclusions and their contents were morphologically different, something normally found in acute infection of epithelial cells, and the pathogen remained viable without initiating the host cell lysis. This study demonstrated that monocytes can disseminate viable *C. pneumoniae* within the systemic circulation and that the pathogen cannot be eliminated from monocytes by standard antichlamydial treatment.

Dentritic cell (DC) is an antigen presenting cell (APC) and initiator and modulator of immune response (Banchereau & Steinman 1998). Adherent peripheral blood mononuclear cells (PBMCs) can differentiate into DCs *in vitro*. These monocyte-derived DCs are susceptible to *C. pneumoniae in vitro*, and the infection remains persistent. Chlamydial inclusions in DCs differ morphologically from those observed in infected HEp-2 or HeLa cells and over 10 inclusions are detected in one cell at 14 to 25 dpi. During infection cells secrete TNFα in cell culture medium from 2 to 25 dpi with a maximum amount on day 2 (Wittkop *et al.* 2006). Chlamydial transcripts of *groEL-1* and *omcB* genes are expressed in infected cells but the expression of *ftsK* is limited. Cells also secrete IFNγ in the culture medium, but its presence is not the major factor that restricts the growth of *C. pneumoniae*, because neutralization of IFNγ does not restore the normal developmental cycle (Kis *et al.* 2008). In a recent study Njau *et al.* (2009) demonstrated that in cell culture medium TNFα induced IDO activity only occurs in *C. pneumoniae* infected DCs. The TNFα-induced anti-chlamydial effect can be blocked with TNFα neutralizing antibody (adalimumab). *C. pneumoniae* infection significantly increased the expression of 1469 host genes and most up-regulated was the IDO coding gene *INDO* (over 140-fold). Respectively, chlamydial *euo* expression was significantly down-regulated in the presence of tryptophan, whereas *groEL1* was up-regulated. In the absence of TNFα a similar trend was observed for both genes. However, supplementation with tryptophan resulted in a slight (not significant) increase in expression of *omcB* and *ompA*. The expression of *dnaA* gene was unchanged, whereas the addition of tryptophan to the cultured

cells abrogated the effect of TNFα on the expression of *ftsK* and *tal* which was strongly attenuated by TNF-α treatment in a concentration-dependent manner.

2.4 Innate immunity

Innate immunity is the first line of defence against infection. It is an ancient form of host defence that evolved under selective pressure imposed by infectious microorganisms, and it is shared by almost all multicellular organisms. For instance mammals and *Drosophila* (common fruit fly) show many similarities between pathogen recognition, signalling pathways inside cells, and effector mechanisms of innate immunity (Hoffmann *et al.* 1999). Similar defence mechanisms to those in insect and mammalian cells are also found in plant cells (Baker *et al.* 1997). So, it is intriguing to presume that innate immunity in both plants and animals has evolved from common modules descending beyond one billion years.

2.4.1 Basic aspects of innate immunity

All metazoans live in different microbial communities which colonize them. The distributions of microbes in and on the human body descend from adaptation of life on land, about 400 million years ago. Terrestrial vertebrates developed relatively impermeable skin, and mucous membranes were confined to the protection of respiratory, intestine and urogenital tracts. These constructs provided to be niches that could be colonized by microbes. The skin, mouth and upper respiratory tract and the intestinal lumen are colonized constantly by endogenous microbiota. In an immunocompetent host the lower respiratory tract and the inner organs are kept sterile (Medzhitov 2007). It is estimated that the human intestine contains 10^{13}–10^{14} microbes whose collective genome has at least 100 times as many genes as our own genome (Gill *et al.* 2006). The effect of microbial colonization can provide benefits to the host. The microbes in the distal alimentary tract, most notably at the colon, synthesize essential amino acids and vitamins and process indigestible components like lipopolysaccharides (LPS) (Backhed *et al.* 2005). The gut microbiota may influence the maturation of the immune system (Mazmanian *et al.* 2005) and modulate response to epithelial cell injury (Rakoff-Nahoum *et al.* 2004). In many cases microbes can be detrimental to the host. Pathogenic bacteria use different virulence factors to spread and adapt in the host. They express adhesive factors, adhesins, or secrete toxins, which

facilitate invasion of tissues or inside cells (Finlay & Falkow 1997). For the host it is fundamental to distinguish pathogenic bacteria from commensal bacteria. It is not known at present how the host separates "good" bacteria from "bad" bacteria. However, when pathogens invade a normally sterile place in the host or pass through epithelial barriers they activate the host's innate immunity.

Three basic resistance mechanisms against microbial infections are already well developed in *Drosophila*: (1) phagocytosis of invading microorganisms, (2) proteolytic cascades leading to localized blood clotting and opsonisation, and (3) transient synthesis of potent antimicrobial peptides (Hoffmann *et al.* 1999). Mammals have developed additional mechanisms specialized against viruses and parasites. The innate immunity consists of distinct subsystems which appeared at different stages of evolution and carry out different functions in host defence (Table 2).

Table 2. Basic modules of mammalian innate immunity (Marshall 2004, Komiya *et al.* 2006, Medzhitov 2007, Driss *et al.* 2009).

Module	Primary sensors (PRRs)	Typical responses
Mucosal epithelia	TLRs and NOD proteins	Production of antimicrobial peptides Production of mucins
Phagocytes	TLRs, CD14, NODs, RLRs (RIG-I-like receptors), NLRs, CLRs, PYHIN family proteins	Production of antimicrobial proteins Production of cytokines:IL-1β, TNFα and IL-6
Acute-phase proteins and complement system	Collectins, pentraxins and ficolins, LBP	Lysis or opsonisation of pathogens Chemotactic attraction of leukocytes
Type-I-IFN-induced antiviral proteins	TLRs, RIG-I, MDA5, DAI	Induction of antiviral state Apoptosis of infected cells
NK cells	Not determined	Apoptosis of infected cells
Eosinophils and basophils	TLR2, TLR4	Contraction of smooth muscle Production of mucins, biogenic amines and cytokines: IL-4, IL-5, IL-9, IL13 and TNFα
Mast cells	TLRs	Contraction of smooth muscle Production of mucins, biogenic amines and cytokines: IL-1, 3, 4,5, 9,10, 12,13,15,16 and TNF

Pattern recognition receptors (PRR) are germ line coded and each of them has broad specifities for conserved features of microorganisms (Janeway 1998). Pathogen-associated molecular patterns (PAMPs) are well-suited targets of

recognition because they are invariant and unique among a given class of microorganisms, allowing the host to recognize between self and non-self molecules. Moreover PAMPs are essential in microbial physiology and the generation of escape mutants is unlikely (Medzhitov 2007). Thus, it is obvious that mutations in PRR encoding genes or their promotors may affect innate immunity and predispose the host to infections. An important aspect of pattern recognition is that PRRs do not distinguish between pathogenic and non-pathogenic (symbiotic) microorganisms.

Innate defence mechanisms consist of a cellular and a humoral arm. Cellular PRRs can be divided into several functionally distinct classes. Toll-like receptors (TLRs) are transmembrane proteins that recognize a variety of pathogen-associated molecules, including lipids, lipoarabinomannan, LPS, and nucleic acids (Akira *et al.* 2006). CD14 is the major receptor for bacterial LPS on the cell membranes of mononuclear cells and macrophages (Wright *et al.* 1990). Nod proteins are cytosolic receptor molecules which contain a nucleotide-binding oligomerization domain (NOD). Together with TLRs NOD1 and NOD2 are important sensors of bacterial components (Inohara 2005). Extracellular viral RNA is recognized by TLR3, TLR4, TLR8 and TLR9 (Alexopoulou *et al.* 2004, Lund *et al.* 2004). RIG-I (RNA helicase retinoic acid inducible gene I) interacts with viral dsRNA inside the cells and augments interferon production (Yoneyama *et al.* 2004). DAI (DNA-dependent activator of interferon-regulatory factor) is a cytosolic DNA sensor that activates innate immune response during viral infection (Takaoka *et al.* 2007). The humoral arm of innate immunity is also diverse; it includes collectins (mannose-binding lectin, surfactant protein A and D), ficolins (H-ficolin, M-ficolin and L-ficolin), and pentraxins. The human collectins and ficolins are oligomeric proteins that contain carbohydrate-recognition domains (Holmskov *et al.* 2003). Pentraxins are a superfamily of evolutionarily conserved proteins which are characterized by a structural motif, the pentraxin domain. C- reactive protein (CRP), the first innate immunity receptor identified, and serum amyloid P component are classic short pentraxins produced in the liver in response to inflammatory signals, most prominently interleukin-6 (IL-6). CRP was originally described and named for its ability to bind to C-polysaccharide of *Streptococcus pneumoniae*. Long pentraxins are produced by a variety of cells and tissues, most notably dendritic cells and macrophages, in response to TLR engagement and inflammatory cytokines. PTX3 acts as a functional ancestor of antibodies, recognizing microbes, activating complement, and facilitating pathogen recognition by phagocytes (Botazzi *et al.*

2006). Lipopolysaccharide binding protein (LBP) is a hepatic secretory protein that potentiates LPS recognition by transferring it from bacterial membranes to CD14 (Schumann *et al.* 1990).

During infection the modules of innate host defence recognize invading pathogens in most cases through PRRs. TLRs can activate multiple modules (mucosal epithelia, phagocytes, eosinophils, basophils, Mast cells and type I IFNs) (Medzhitov 2007). Recognition of pathogen initiates signal transduction cascades inside cells finally leading to the translocation of NF-κB into the nucleus, where it regulates the expression and secretion of a large repertoire of effector molecules (Arancibia *et al.* 2007). Macrophages for example secrete pro-inflammatory cytokines, like TNFα, IL-1 and IL-6 (Dobrovolskaia *et al.* 2002). IL-6 is the predominant mediator of the acute phase response and it activates the synthesis of other acute phase reactants such as CRP and LBP (Graeve *et al.* 1993).

Innate immunity also contributes to the induction of adaptive immune response. Dendritic cells (DCs) which reside in peripheral tissues use various PRRs and phagocytosize pathogens. In lymph nodes DCs present pathogen derived antigenic peptides at their cell surfaces with the MHC class I (major histocompatibility molecule) or MHC class II molecules, together with PRR induced effector molecules to T cells (Blander & Medzhitov 2006). This induces T-cell activation and in the case of helper-T-cells, the cells differentiate into several types of effector Th-cells (Banchereau 1998). T-independent antigens like LPS or flagellin are themselves recognized by the B-cell receptor and corresponding TLR expressed by a B-cell. In the case of T-dependent antigen PAMP and antigen are not physically linked and hence B-cell response is driven by Th-cells, which have previously been activated by DCs. On the other hand the adaptive immune response results in an antigen-specific activation of the same innate immunity module that started the sequence, because the Th-cells secrete cytokines that activate those modules (Medzhitov 2007). This positive feed-back from adaptive immunity to innate immunity may be crucial for controlling infections if innate immunity is insufficient for pathogen clearance, indicating that to clear intracellular *Listeria monocytogenes* infection T-cell help is required (Unanue 1997).

2.4.2 Lipopolysaccharide receptor CD14

CD14 was originally referred to as a differentiation marker of myeloid cells and used in leukemia phenotyping. Later, its LPS-binding capacity was discovered

and CD14 is still the major endotoxin receptor that also binds several products of different bacteria, fungi and parasites, and as such has a central role in innate immunity (Antal-Szalmas 2000).

The CD14 receptor exists as a cell membrane bound receptor (mCD14) and as a soluble molecule (sCD14) with two different molecular weights in serum (Labeta *et al.* 1993). mCD14 receptor is a 356 amino acids long glycoprotein (55-kDa) (Ferrero & Goyert 1988). The C-terminal leader sequence, 28–30 amino acids, is replaced after translation by a glycosyl phosphatidyl inositol (GPI) anchor that binds the receptor on the cell membrane (Haziot *et al.* 1988). The anchored receptors reside on the membrane in lipid rafts which are involved in cellular processes like transcytosis, protocytosis, and transmembrane signalling (Simons & Toomre 2000). Activation of cells with various stimuli, like IFNγ or LPS, induce shedding of mCD14 by a membrane associated serine protease and results in sCD14 with molecular mass of 48–49 kDa (Bazil *et al.* 1991, Bufler *et al.* 1995). Polymorphonuclear neutrophils can down-regulate mCD14 expression by human leukocyte elastase, a serine protease, that releases mCD14 from cell membranes as sCD14 (Le-Barillec *et al.* 1999). Some CD14 molecules keep the C-terminal leader sequence, resulting in a molecular weight of 55–56 kDa. These molecules are stored intracellulary and released upon cell activation (Labeta *et al.* 1993, Bufler *et al.* 1995).

Binding of PAMPs to CD14 relies on groups of charged residues, which are located on one side of the protein surface in close proximity to each other (Cunningham *et al.* 2000). The interaction is not restricted to any particular pathogen. Serum LBP, an acute phase protein, enhances binding of LPS to CD14 100–1,000 times (Hailman *et al.* 1994). CD14 is not able to activate intracellular signalling pathways, because it has no cytoplasmic domain and GPI anchor is not sufficient to signal transduction (Lee *et al.* 1993). Human TLR2 and TLR4 co-operate with CD14 receptor in recognition of Gram-negative and Gram-positive bacteria cell wall components, respectively (Takeuchi *et al.* 1999). The most important co-receptor is TLR4, which activates cells to the synthesis and release of inflammatory mediators (Guha & Mackman 2001).

Expression of CD14 is transcriptionally upregulated by LPS and other bacterial cell wall constituents. A 2-day incubation of human mononuclear cells and macrophages *in vitro* with LPS (≥1ng/ml) increased the levels of CD14 mRNA, mCD14 and sCD14 about 2-fold. Lipid A, heat-killed *Escherichia coli*, lipoteichoic acid and *Staphylococcus aureus* cell wall extract caused similar

increases of mCD14. The effect was not caused by TNFα, IL-6, IFNγ or LBP because antibodies were ineffective on them (Landmann *et al.* 1996).

Single nucleotide polymorphism (SNP) -260C>T (rs2569190) in the proximal promoter region of CD14 affects its expression. In this region a GC box contains binding sites for Sp1, Sp2 and Sp3 transcription factors (Zhang *et al.* 1994, Hata *et al.* 1999). Transcription activity of the T allele was increased in monocytic cells, which express low level of SP3 that has inhibitory potential relative to activating Sp1 and Sp2. In hepatocytic HepG2 cells the Sp3 expression is higher and differences between C and T alleles was not seen. Thus, the affinity of the promotor GC box and the ratio between activating and repressing transcription factors of the Sp family regulate the transcription of these allelic variants (LeVan *et al.* 2001). This polymorphism influences sSD14 and mCD14 levels *in vivo*. Among children with asthma or allergies the TT homozygotes had significantly higher serum levels of sCD14 than carriers of CC and CT genotypes (Baldini *et al.* 1999). The density of mCD14 receptor on monocytes from healthy persons was significantly higher in the TT homozygotes than in the CT heterozygotes or in CC homozygotes (Hubacek *et al.* 1999, Eng *et al.* 2004).

The association of the polymorphism to sepsis has been evaluated in case-control studies. In a multicenter study 90 sepsis patients and 122 age- and gender-matched controls were followed (Gibot *et al.* 2002). The frequency of TT genotype was higher in patients than in controls, and the mortality rate of TT genotype was significantly increased compared to patients with CC or CT genotypes. In critically ill patients with systemic inflammatory response syndrome TT genotype was associated with increased prevalence of positive bacterial cultures and with Gram-negative bacteria at admission; however, the polymorphism did not associate with increased prevalence of septic shock or altered 28-day survival (Sutherland *et al.* 2005). Thus, at the least the polymorphism may alter the recognition and clearance of bacteria or at the worst affects susceptibility to septic shock and could be a genetic risk for death.

The -260C>T polymorphism seems associated with a variety of chronic diseases that possess an immunopathogenic component. The T allele was associated with advanced alcoholic hepatitis and cirrhosis (Järveläinen *et al.* 2001) and the TT genotype was significantly more frequent in alcoholic cirrhosis patients compared to alcoholic controls without cirrhosis (Meiler *et al.* 2005). Biliary atresia and idiopathic neonatal cholestasis patients had significantly higher frequencies of the T allele and TT homozygosity compared to healthy controls (Shih *et al.* 2005). The extent of periodontal disease has been reported to be

higher in subjects with T allele containing genotype (Tervonen *et al.* 2007). Association of the T allele to cardiovascular diseases has been reported in many studies (Hubacek *et al.* 1999, Hohda *et al.* 2003, Kondo *et al.* 2003, Giacconi *et al.* 2007, Porch-Özcurumez *et al.* 2007). The -260C>T polymorphism may be the common factor between chronic *C. pneumoniae* infection and cardiovascular diseases. Eng *et al.* (2003) demonstrated in a study with healthy subjects that the T allele appeared to influence individual susceptibility to *C. pneumoniae* infection. The genotype dependent expression of CD14 has an effect on TNFα secretion and after *C. pneumoniae* stimulation TT genotype mononuclear cells secrete significantly more TNFα compared to CC type cells (Eng *et al.* 2004). It is remarkable that monocytes with high CD14 and CD16 expression are more prone to transform to foam cells when incubated with enzymatically modified low density lipoprotein (Kapinsky *et al.* 2001). Thus, increased proinflammatory activity and the capability to interact with lipids might contribute to the susceptibility towards atherogenesis.

2.4.3 Interleukin-6

Interleukin-6 is involved in numerous physiological and pathophysiological processes in the body, thus it has also been called B-cell stimulatory factor-2, B cell differentiation factor, T cell-replacing factor, interferon-ß2, 26-kDa protein, hybridoma growth factor, interleukin hybridoma plasmacytoma factor 1, plasmacytoma growth factor, hepatocyte-stimulating factor, macrophage granulocyte-inducing factor 2, cytotoxic T cell differentiation factor, and thrombopoietin (Akira *et al.* 1993). In innate immune response IL-6 has a crucial role as an activator of acute phase inflammatory response, i.e., secretion of hepatic acute phase proteins like CRP, LBP and complement components. It is also important for proliferation and differentiation of different T-cells and immunoglobulin secretion by B-cells (Keller *et al.* 1996).

Human IL-6 peptide consists of 184 amino acids and has a molecular weight between 21 and 28 kD depending on post-translational glycosylation and phosphorylation (Van Snick, 1990). It belongs to the family of four-helical cytokines that comprise IL-6, ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1), cardiotrophin like cytokine, leukemia inhibitory factor (LIF), neuropoietin (NPN), oncostatin M (OSM), IL-27 and IL-31 (Scheller *et al.* 2006). On cell membrane IL-6 it interacts with its receptor complex, which consists of IL-6R and two signal transducing proteins gp130. While gp130 is expressed on most

cells of the body the IL-6R is found only on hepatocytes and some leukocytes (Scheller & Rose-John 2005). Membrane bound IL-6R could be shed as soluble sIL-6R after a limited enzymatic proteolysis (Müllberg *et al.* 1993). Macrophages, granulocytes and T-cells can release sIL-6R which is generated by translation from alternatively spliced mRNA (Horiuchi *et al.* 1994). The sIL-6R-IL-6 complex can bind to gb130 and activate cells which do not express mIL-6R. This mechanism is called trans-signalling (Mackiewicz *et al.* 1992).

The human IL-6 gene locates in chromosome 7p21 and it has a very complex control region where the proximal 300 nucleotides are highly conserved (Tanabe *et al.* 1988). IL-6 is produced during infection and it has been demonstrated to be a marker of severity in sepsis (Calandra *et al.* 1991). IL-6 is rapidly cleared from blood and is not stored in cells, thus its presence in the circulation requires precisely regulated gene transcription. The expression is regulated by activating the promotor with transcriptional factors NF-κB and NF-IL6, which are produced in mononuclear cells after challenge with PAMPs (Keller *et al.* 1996). SNPs located in the promoter region, which affect the expression of the IL-6 gene, are -597G>A, -572G>C, -373AnTn, and -174G>C (Terry *et al.* 2000). The first SNP, which has an effect on gene expression, IL-6 plasma levels and association to particulate disease, was evaluated and was -174G>C (Fishman *et al.* 1998). After stimulation with LPS or IL-1 the -174G constructs in HeLa cells showed significantly higher expression than -174C constructs. C-allele associated to lower plasma levels of IL-6 in healthy subjects, and the frequency of, in this case potentially protective, CC genotype, was reduced among juvenile chronic arthritis patients. GG genotype seems to be associated with improved sepsis outcome and, respectively, CC genotype seems to be associated with septic shock. Thus, it is suggested that CC genotype is less protective in the Caucasian population, however, the mechanism is not known (Schluter *et al.* 2002, Michalek *et al.* 2007). GG genotype can predispose children to recurrent acute otitis media (Patel *et al.* 2006, Emonts *et al.* 2007). On the other hand a lower frequency of CC genotype has reported to be associated with the common cold (Nieters *et al.* 2001), which is known to predispose for recurrent otitis media.

Inflammation is involved in the development of atherogenesis and so IL-6 may play an important role in regulation of both inflammatory and atherosclerotic processes. Elevated levels of the IL-6, which correlated with CRP levels and was associated with disease prognosis, was first demonstrated in patients with unstable angina; in patients with stable angina the IL-6 levels were below detection limit (Biasucci *et al.* 1996). In a prospective study apparently healthy

men was followed up to 6 years (Ridker *et al.* 2000). Median concentrations of IL-6 were higher among men who subsequently had myocardial infarction (MI) than among control subjects matched for age and smoking status. The risk of future MI increased with increasing IL-6 concentration and in the highest quartile the relative risk was 2.3 times higher than in the lowest quartile. The relationship of IL-6 concentration with subsequent risk remained after adjustment for other cardiovascular risk factors. Because the IL-6 seemed to effect the development of cardiovascular diseases, the next step was to evaluate how the -174G>C polymorphism associated with diseases. Results from these studies were inconsistent. In some studies the polymorphism had no effect (Nauck *et al.* 2002, Lieb *et al.* 2003, Sie *et al.*, 2006). Other studies showed that the C allele was a risk factor for diseases (Jenny *et al.* 2002, Chiappelli *et al.* 2005), and in some cases the GG genotype associated with increased risk (Flex *et al.* 2002, Giacconi *et al.* 2004, Antonicelli *et al.* 2005). Recently, Danesh *et al.* (2008) reported results from a large prospective study where they evaluated associations of long-term circulating IL-6 levels with coronary heart disease (CHD). In addition they evaluated 15 relevant previous population based prospective studies of IL-6 and clinical coronary outcomes. Samples were obtained from 2,138 patients who had a first-ever nonfatal MI or died of CHD during follow-up and from 4,263 controls. Correction for within-person variability was made using data from repeat measurements during several years. Final results showed that increasing IL-6 levels were associated with progressively increasing CHD risk. The risk was about as strong as major established risk factors and meta-analysis of the other prospective studies gave the same result.

2.4.4 Lipopolysaccharide binding protein LBP

LBP is a 50 kDa secretory class I acute phase protein that is synthesized mainly in liver cells. After glycosylation it is secreted in the bloodstream as a 58 to 60 kDa glycoprotein (Schumann *et al.* 1990). Human type II pneumocytes and lung epithelial cell line A549 cells secrete LBP upon stimulation *in vitro*. Secretion of LBP is activated by IL-1 alone or synergistically by IL-1 and IL-6. Maximum LBP concentration is achieved within 24–48 h after stimulation and the response can be enhanced by TNFα and dexamethasone (Schumann *et al.* 1996, Dentener *et al.* 2000).

LBP has a central role in mediating responses to LPS. It binds to the A moiety of LPS and catalyzes the transfer of the complex to mCD14 (Wright *et al.* 1990).

This enzymatic reaction follows first order kinetics and it is proposed that a single LBP molecule is able to transfer hundreds of LPS molecules from aggregates to sCD14 (Yo and Wright 1996). In addition, LBP-LPS complexes react with sCD14 and form LPS-sCD14 complexes, which activate mCD14 negative endothelial and epithelial cells (Pugin *et al.* 1993). High concentration of LBP inhibits LPS induced activation of human monocytes *in vitro*. The balance between stimulatory and inhibitory effect depends primarily upon the ratio of LPS and LBP concentrations: if LPS concentration increases then the level of inhibitory LBP concentration needs to be higher respectively (Lamping *et al.* 1998). *In vivo* LBP removes LPS from the bloodstream. LBP is associated physically with apoA containing lipoproteins and thus transfers LPS into low- and high-density lipoproteins and clearances LPS from plasma (Wurfel *et al.* 1994).

Acute phase proteins, CRP and LBP, are serum markers that indicate the activation of innate immune response. CRP has proposed a reliable marker for atherosclerosis (Koenig *et al.* 2004). Recently, Lepper *et al.* (2007) showed that LBP can also be used as marker for coronary artery disease (CAD) in man. In their study serum LBP concentration was significantly increased in patients with angiographically confirmed CAD compared to controls without coronary atherosclerosis and the LBP levels increased with the severity of disease. In adjusted multivariance logistic regression analyses LBP was a significant predictor of prevalent CAD and independent of IL-6 and CRP levels.

The efficiency of LBP gene transcription is regulated by its promotor region that is activated by cytokine inducible nuclear proteins. SNP in the promoter region results in variable blood levels of LBP and may affect the ability to resolve bacteremia. SNP 1683 (rs2232571) confers a C/T substitution in the CAAT box at position -778 (Schumann *et al.* 1996). This polymorphism associated with a risk for Gram-negative bacteremia and mortality among patients after allogeneic hematopoietic cell transplantation (Chien *et al.* 2007). In CC genotype basal serum LBP level was significantly 2-fold higher compared to TT genotype. Patients with C allele who developed bacteremia had a significant 5-fold increase in mortality risk.

LBP gene Cys98Gly polymorphism has reported to associate with increased risk for the development of sepsis and lethality, the association was significant only among men (Hubacek *et al.* 2001). However, in another study this polymorphism was not found. Instead a synonymous (Pro->Pro) SNP at the adjacent nucleotide was identified (Barber *et al.* 2003). The LBP Leu436Phe (rs2232618) polymorphism seems to be associated with an increased risk of the

development of sepsis but does not influence the risk of myocardial infarction (Hubacek *et al.* 2001, 2002).

2.4.5 Toll-like receptors TLR2 and TLR4

Toll molecule was first found in *Drosophila* as a transmembrane receptor required for the regulation of dorso-ventral polarity during embryogenesis (Hashimoto *et al.* 1988). Subsequent studies revealed that Toll was also involved in the insect innate immune response against fungal infection (Lemaitre *et al.* 1996). A year later a mammalian homologue of the *Drosophila* Toll was identified (Medzhitov *et al.* 1997). Thereafter, numerous homologous molecules to Toll, referred to as the Toll-like receptors, were identified through data base searches, and to date at least 10 human TLRs have been identified and characterized (Ishii *et al.* 2008).

TLRs are type I integral membrane glycoproteins and are characterized by the extracellular leucine rich repeat (LRR) domain. TLRs contain 9–25 tandem copies of the LRR motif; each of them contains 24–29 amino acids – a leucine rich sequence and another conserved sequence. The LRRs form concave surfaces which are involved in the recognition of PAMs (Bell *et al.* 2003). Intracellular domains of TLRs are homologous with each other, and on the basis of homology to interleukin-1-receptor domain TLRs belong to the IL-1R-superfamily (Akira & Takeda 2004). The homologous domain is called Toll/IL-1R domain (TIR) and it has a conserved region of about 200 amino acids. The homologous regions are located in three conserved boxes, which are crucial for signalling (Slakat *et al.* 2000).

TLR2 and TLR4 are the main receptors involved in the recognition of bacterial products, as shown in Table 3. TLR4 has been established as a receptor for LPS. Evidence for this came from a C3H/HeJ mouse that is hyposensitive to LPS because of a missense mutation in the *Tlr4* gene, which abrogates LPS signalling (Poltorak *et al.* 1998). LPS is generally bound to LBP and this complex is first recognized by CD14. Once bound to CD14, LPS comes in close proximity with TLR4 (da Silva Correia *et al.* 2001). TLR4 is coexpressed with polymeric protein MD-2 and without it signalling will not occur (Shimadzu *et al.* 1999). Human MD-2 and TLR4 associate already in the endoplasmic reticulum where MD-2 is required for glycosylation of TLR. Without glycosylation TLR4 cannot get to the cell surface (Ohnisni *et al.* 2003).

Table 3. Ligands of TLR2 and TLR4 (adapted from Takeda *et al.* 2003).

TLR	Ligands	Origin
TLR2	Lipoprotein/lipopeptides	a variety of pathogens
	Peptidoglycan	Gram-positive bacteria
	Lipoteichoic acid	Gram-positive bacteria
	Lipoarabinomannan	mycobacteria
	A phenol- soluble modulin	*Staphylococcus epidermidis*
	Glycoinositolphospholipids	*Trypanosoma Cruzi*
	Glycolipids	*Treonema maltophilum*
	Porins	*Neisseria*
	Zymosan	fungi
	Atypical LPS	*Leptospira interrogans*
	Atypical LPS	*Porphyromonas gingivalis*
	HSP70	host
TLR4	LPS	Gram-negative bacteria
	Taxol	plant
	Fusion protein	RSV
	Envelope proteins	MMTV
	HSP60	*Chlamydia pneumoniae*
	HSP60, HSP70	host
	Oligosaccharides of hyaluronic acid	host
	Polysaccharides fragments of heparin sulfate	host
	Fibronectin	host

TLR4 can induce two independent signalling pathways, which are regulated by pairs of TIRAP–MyD88 (TIR-domain-containing adaptor protein – Myeloid differentiation primary response protein) and TRIF–TRAM (TIR-domain containing adaptor-inducing IFNβ – TRIF-related adaptor molecule). These pathways lead to the production of proinflammatory cytokines and IFNβ, respectively. TIRAP – MyD88 signalling is induced first at the plasma membrane and the TRIF–TRAM dependent signalling begins in early endosomes after endocytosis of the TLR4 complex (Kagan *et al.* 2008). After binding ligand TIR domain of TLR4 dimerize and conformational change recruits downstream signalling molecules. These adaptor molecules include MyD88 (myeloid differentiation primary response protein), TIRAP (TIR-domain-containing adaptor protein), IRAKs (IL1-1R-associated kinases), TAKI1 (transforming factor-β (TGF-β) activated kinase), TAB1 (TAK1-binding protein), TAB2 and TRAF6 (TNF-receptor-associated factor 6). TLR4 mediated response to LPS can be divided into MyD88-dependent and a delayed MyD88-independent response

(Akira & Takida, 2004, Pålsson-McDermott *et al.* 2004). MyD88 has at C-terminus TIR containing region that associates with TLR-TIR domain (Hultmark 1994). At N-terminus there is a death domain (DD) that interacts with other DD-containing proteins. MyD88 has also an intermediate domain (ID) that interacts with IRAKs (Boldin *et al.* 1995). Another necessary adaptor at this phase is TIRAP, which probably acts upstream of MyD88 (Akira & Takeda 2004). Downstream from MyD88 the signal is transduced by IRAKs. IRAKs1, 2, 3 and M belong to a family of serine/threonine kinases and each of them contains DD at N-terminus. IRAK1 and IRAK4 have auto- and cross-phosphorylation kinase activity; IRAK2 and IRAK-M lack an aspartatic residue in the kinase domain and have therefore no enzymatic activity (Janssens & Beyaert 2003). IRAK4 interacts with MyD88-ID domain and promotes phosphorylation of IRAK1, after that TRAF6 is recruited to the TLR complex. Finally, they react with membrane associated TAK1, TAB1 and TAB2, which are part of the multicomplex IRAK-TRAF6-TAK1-TAB1-TAB2 (Takaesu *et al.* 2001). After TAK1 and TAB2 phosphorylation IRAK1 and TRAF6 dissociate from the complex and further activate the IKK complex (inhibitor of nuclear factor (IκB)-kinase complex) (Wang *et al.* 2001). The IKK complex contains two catalytic subunits IKKα and IKKβ and a regulatory subunit IKKγ (also known as NF-κB essential modulator NEMO). Activated IKK phosphorylates IκBs lead to their polyubiquitylation and proteasome mediated degration. NF-κB transcription factor family contains five members: p65 (RelA), RelB, cytoplasmic cRel, p50 and p52, which function as homo- and heterodimers. NF-κB dimers are usually in the cytoplasm in inactive forms controlled by inhibitor molecules of the IκB-family. They are released after degradation of the IκB proteins and translocate as active molecules in the nucleus (Karin & Ben-Neriah 2000).

TLR induced pro-inflammatory cytokine expression and secretion in excess can induce serious systemic disorders. Thus, the balance between activation and inactivation is crucial in avoiding disastrous inflammatory response in autoimmune, chronic inflammatory and infectious diseases. Soluble forms of TLR2 and TLR4 have been described as regulators. Soluble forms of TLR2 that can modulate TLR2 signalling are present in human plasma and milk (Lebouder *et al.* 2003). The soluble TLR4 has been shown to inhibit LPS mediated TNFα production (Iwami *et al.* 2000). Intracellularly several adaptors and kinases regulate the expression of pro-inflammatory molecules. IRAK-M inhibits in monocytes and macrophages the dissociation of IRAK1-IRAK4 complex from the MyD88 and formation of the IRAK1-TRAF6 complex (Koboyashi *et al.*

2002). In macrophages SOCS1 (suppressor of cytokine signalling 1) associates, probably, with IRAK1 and inhibits its activity (Gingras *et al.* 2004). TLR-mediated activation can also be regulated with another PRR. NOD2 is an intracellular PRR that recognizes bacterial peptidoglycan and muramyl dipeptide. NOD2 signalling normally inhibits TLR2-driven TH-1 response by downregulating NF-κB activation. In the absence of NOD2 signalling, as in the case of a mutation, NOD2 mediated inhibition is abrogated, resulting in increased TLR2 mediated NF-κB activation and more IL-12 production (Watanabe *et al.* 2004).

It is remarkable that TLR4 is able to recognize HSP60 that has been implicated in inflammation accompanying atherosclerosis, which is associated with chronic infection by *C. pneumoniae*. *Chlamydia* derived cHPS60 can also activate vascular smooth muscle cells, macrophages and endothelial cells (Sasu *et al.* 2001, Bulut *et al.* 2002). *Chlamydia*l LPS stimulates cells through TLR4, but its activity is 100–1,000-fold weaker compared to endotoxin derived from *Salmonella minnesota* (Ingalls *et al.* 1995). Primarily the recognition of *C. pneumoniae* depends on TLR2. Human freshly isolated peripheral mononuclear cells incubated with sonicated *C. pneumoniae* secreted significant amounts of pro-inflammatory cytokines TNFα and IL-1β and anti-inflammatory cytokine IL-10. Secretion was not blocked with the addition either of anti-TLR4- or anti-CD14-antibodies. In contrast anti-TLR2-antibodies decreased TNFα- and IL-1β – secretion significantly (Netea *et al.* 2002). Upon contact with *C. pneumoniae* murine bone marrow derived dendritic cells were activated and secreted cytokines TNFα and IL-12p40, and expression of MHC class I was upregulated. Secretion of cytokines was dependent on the presence of TLR2 and independent from TLR4, with the exception of IL-12p40, which attenuated if either of TLR2 or 4 were deficient (Prebeck *et al.* 2001). These results have been confirmed with experiments in which TLR2- and TLR4-deficient mice were intranasally infected with *C. pneumoniae* (Rodriguez *et al.* 2006). The study showed that TLRs, in particular TLR2 and to some extent TLR4, are of crucial importance in the control of *C. pneumoniae* infection *in vivo*.

The TLR2 polymorphism Arg753Gln, within conserved part of the C-terminal region, has been reported to associate with an increased incidence of septic shock caused by *Staphylococcus aureus* (Lorenz *et al.* 2000). However, a larger study came to the conclusion that this SNP is not associated with severe diseases caused by *S. aureus* (Moore *et al.* 2004). Later, Ogus *et al.* (2004) reported that Arg753Gln polymorphism influenced the risk of developing

tuberculosis and that SNP was strongly associated with acute rheumatic fever in children (Berdeli *et al.* 2005).

TLR4 gene has two co-segregating SNPs: Asp299Gly and Thr399Ile in the extracellular recognition domain. They were found at a substantially higher proportion among people hyporesponsive to inhaled LPS compared to normal controls. The transfection of THP-1 cells demonstrated that Asp299Gly mutation interrupted TLR4 mediated LPS response (Arbour *et al.* 2000). After this report several studies have demonstrated the prevalence of this mutation with contrasting results. Some studies suggested an association of mutation to increased susceptibility to infections but others were unable to confirm these results (reviewed in Schröder & Schuman 2005). In two *in vitro* studies human blood mononuclear cells were stimulated with LPS derived from different bacteria and HSP60. No differences in either production of the TNFα or the anti-inflammatory cytokine IL-10 were observed between cells with different genotypes (Erridge *et al.* 2003, van der Graaf *et al.* 2005). However, in a five-year follow-up study the subjects with Asp299Gly allele had in their sera lower levels of proinflammatory cytokines, acute-phase reactants, and adhesion molecules compared to subjects with wild-type TLR4. The subjects with Asp299Gly allele were more susceptible to severe infections but they had lower risk of carotid atherosclerosis (Kiechl *et al.* 2002). Ameziane *et al.* (2003) demonstrated in a case-control study that the Asp299Gly allele associated with a decreased risk of acute coronary events independently of standard coronary risk factors. These findings suggest that innate immunity may play a part in atherogenesis.

3 Aims of the study

The aims of this study were:

1. To develop a reliable method of quantifying exactly the degree of *C. pneumoniae* infection in cultured cells.
2. To evaluate individual susceptibility of human macrophages to *C. pneumoniae* infection *in vitro* and compare it to serological markers of chronic infection.
3. To determine the effects of innate immunity gene polymorphisms on the *C. pneumoniae* growth in cultured human macrophages.

4 Materials and methods

4.1 Subjects

Buffy coat cells of 393 blood donors were obtained from the local Finnish Red Cross Blood Service, Oulu, Finland (permission no. 48). The cells of 258 donors (192 males and 66 females, aged 45.3 ± 11.2 and 43.7 ± 14.5 years, respectively) were used for the *in vitro* infectivity study.

4.1.1 Preparation of cells

Fifty ml of buffy coat was diluted in 150 ml of phosphate-buffered saline (PBS), layered over Ficoll-Isopaque (Amersham Biosciences, Uppsala, Sweden) in polypropylene tubes, and centrifuged at 400 G at room temperature for 20 minutes, after which mononuclear cells were recovered and washed three times with PBS. The cells were suspended in RPMI-1640 medium (RPMI) supplemented with 10% fetal bovine serum (FBS) and 20 µg /ml streptomycin at a concentration of 2×10^6 cells/ml. One ml of cells was distributed into each well of 24-well plates with a glass cover. The plates were incubated for one hour in 5% CO_2 at 37 °C. After incubation, the wells were emptied of medium, and the attached cells on the cover slips were washed twice with PBS. The wells were then filled with 2 ml of RPMI supplemented with 20% FBS + 10% human AB serum (Finnish Red Cross Blood Service, Helsinki, Finland) and incubated for two weeks at 37 °C with 5% CO_2.

4.1.2 Infection of macrophages with C. pneumoniae

The matured macrophages were washed twice with PBS and infected with *C. pneumoniae* (strain K7, National Public Health Institute, Oulu, Finland). The infective dose was 87,500 inclusion-forming units (IFU) in 300 µl PBS/well. The plates were centrifuged at 400 G for one hour at 4 °C. The cells were washed twice with PBS, covered with 1 ml of RPMI supplemented with 7% human AB serum and incubated at 35 °C with 5% CO_2 for 72 hours.

4.1.3 *Quantitative PCR for* C. pneumoniae *16S rDNA and human LBP gene*

The degree of infection was determined by measuring the number of chlamydial genomes per human genome. The cells infected with *C. pneumoniae* were collected and frozen together with the culture supernatant. The samples were stored at -70 °C and, prior to DNA extraction, allowed to melt at +4 °C overnight. The cells were centrifuged at 13,500 rpm for 5 min, and the supernatant was removed. The cells were suspended with 400 µl of homogenization buffer (50 mM Tris-HCl, 10 mM NaCl, 50 mM EDTA, 1% SDS, pH 8.0) and homogenized using BIO101® Systems lysing matrix B tubes (Qbiogene, Illkirch, France) and FastPrep homogenizer FP120 (Thermo Savant, Basingstoke, UK). Homogenization was done twice at a speed of 4 m/s for 20 s. The samples were then centrifuged at 13,500 rpm for 10 min, and 300 µl of the lysate was used for DNA extraction. DNA was extracted using the MagNa Pure LC Instrument (Roche Diagnostics GmbH, Mannheim, Germany) and the Large Volume MagNa Pure LC DNA Isolation Kit (Roche Applied Science, Penzberg, Germany) protocol for cultured cells, according to the manufacturer's instructions. The elution volume was 100 µl. The elutes were stored at -70 °C until analysis and, after melting, kept at +4 °C between the two PCR analyses. Negative controls (sample replaced by water) were included in DNA extraction at least as every 16th sample. Negative controls were tested in PCR to detect possible contamination during extraction.

Quantitative LightCycler® (Roche Diagnostics GmbH) real-time PCR assay was used to detect *C. pneumoniae* 16S rDNA (Reischl *et al.* 2003). A dilution series of 80,000 to 8 genome equivalents of *C. pneumoniae* DNA (diluted with MS RNA solution, Roche Diagnostics GmbH) extracted from *C. pneumoniae* elementary bodies (strain Kajaani 7) was used to generate a standard curve. 8 µl of undiluted DNA was used for PCR, and a negative control was included as every 7th sample. In addition, the possible presence of PCR inhibitors was tested by analysing 1:100 dilutions of 105 samples in PCR and comparing the results to those obtained from undiluted samples. PCR was carried out using the LightCycler® real-time PCR machine 1.2 (Roche Diagnostics GmbH) and the software versions 3.5 and 4.0. For quantification analysis, the second derivative maximum method with proportional baseline adjustment (software version 3.5) or absolute quantification with the automated F'' max method (software version 4.0.) was used.

A quantitative LightCycler® (Roche Diagnostics GmbH) real-time PCR assay was developed for quantifying the amount of human genomes in the samples. Primers and probes for this method were designed at and obtained from Tib MolBiol GmbH, Berlin, Germany. The primers LBP S and LBP A (5' CTT TCA TCG TAA CCA CCG3' and 5' GGC CGT GTT GAA GAC ATA A 3') amplified a 155 bp fragment of the lipopolysaccharide binding protein (LBP) gene, exon 8. The hybridization probes, LBP FL and LBP LC (5' CAG TCA TGA GCC TTC CTG AGG A– FL 3' and 5' Red 640- CAC AAC AAA ATG GTC TAC TTT GCC AT –P 3'), bound to the amplified fragment specifically and thus enabled accurate quantification of the PCR product. The PCR mixture contained 0.5 µM of both primers, 0.2 µM of both probes, 4 mM $MgCl_2$ and 2 µl of the fast-start master hybridization probes mix (Roche Diagnostics GmbH). The sample volume was 8 µl and the whole reaction volume 20 µl. The PCR program was as follows: initial denaturation at 95 °C for 10 min, 50 cycles of denaturation at 95 °C for 10 s, annealing at 46 °C for 10 s and elongation at 72 °C for 20 s, finally followed by cooling to 40 °C for 30 s. Fluorescence was measured at the end of each annealing step at the channel 640/530. For the standard curve, a dilution series of 36508 to 3.6508 genome equivalents (diploid genome, mtDNA was not taken into account) of pathogen-free human genomic DNA (Roche Diagnostics GmbH) was run along in each PCR. The quantification analysis was done as described in the *C. pneumoniae* 16S rDNA PCR procedure.

4.1.4 Measurement of chlamydial LPS concentration

Chlamydial LPS concentration in culture supernatants was measured with IDEIA™ PCE Chlamydia kit (DakoCytomation Ltd, Cambridgeshire, UK) according to manufacturer's instructions.

4.1.5 Measurement of C. pneumoniae *antibodies by microimmunofluorescence (MIF) test*

Elementary bodies of *C. pneumoniae*, strain K6 (National Public Health Institute, Oulu, Finland), stored in 0.02% formalin were diluted with egg emulsion according to the instructions for the antigen lot (generally 1:2). The egg emulsion was made by diluting 250 µl of egg yolk (Biotrading Benelux B.V., 3640 AG Mijdreht, Netherlands) in 100 ml of PBS. The diluted antigen solution was dotted with a pen nib (Hunt 104, Hunt Manufacturing Co., Statsville, W.C., USA) on 30-

well microscopic slides. The slides were dried for one hour and fixed with acetone for 10–15 minutes. The serum samples were diluted with sample diluent, 0.25% egg yolk in PBS, starting from 1:16 for IgG and from 1:5 for IgA antibodies. The sera were tested in serial two-fold dilution to the end point. The diluted specimens were pipetted on to slides, 5µl/well. A known positive serum was used as a control. The slides were placed in a moist chamber, incubated at 37 °C for one hour and washed four times in PBS and twice in distilled water. The diluted (1:20 in PBS) conjugates, FITC-conjugated goat anti-human IgG (Kallestad, Austin, USA) and FITC-conjugated goat anti-human IgA (Kallestadt, Austin, USA), were pipetted. The slides were incubated in a moist chamber at 37 °C for 30 min, washed and dried. Finally, the samples were covered with mounting medium (0.1 M veronal buffer with 0.15 M NaCl, pH 8.5) and slips and read under a fluorescence microscope using a 50 × oil immersion objective and 10 × oculars.

4.1.6 Measurement of LBP, sCD14, IL6 and CRP concentrations

Serum levels of LBP, sCD14, IL6 and CRP were measured using commercial ELISA kits: Hycult HK315, Hycult HK320 (Hycult biotechnology, Uden, The Netherlands), PeliKine Compact TM human IL-6 ELISA kit (Sanquin Reagents, Amsterdam, The Netherlands) and highly sensitive hs-CRP (Oy Medix Biochemica AB, Kauniainen, Finland), respectively, according to the manufacturers' instructions. The sensitivity of the hs-CRP test was 0.08 mg/l and the assay range from 0.3 to 30 mg/l. For LBP measurements, the sample dilution ratio was 1:1000 (first dilution 1:10 and second 1:100), while for sCD14 measurements it was 1:80 (first 1:8 and second 1:10) and for CRP measurements 1:51. The sera for LBP and sCD14 measurements were diluted in two phases since the amount of dilution buffer was restricted. Absorbances were measured with a photometer, and concentrations were calculated using the Genesis program (Labsystems, Helsinki, Finland) based on a standard curve drawn from the control samples.

4.1.7 Measurement of hHSP-IgG and hHSP-IgA concentrations

IgG and IgA antibodies to human specific heat shock protein 60 (hHsp60) were measured by an enzyme immunoassay (EIA) as described earlier (Huittinen *et al.* 2002. Briefly, the microtiter plates were coated with diluted (2 µg/ml dilution)

recombinant hHsp60 (Stressgen, Victoria, Canada). The sera were diluted 1:50 for IgA and 1:200 for IgG antibodies in PBS-10% fetal bovine serum (FBS). Alkaline phosphatase-conjugated goat anti-human IgG and IgA conjugates (Sigma, St. Louis, USA) were used with 1:1,000 dilutions in PBS-10% FBS. Absorbances were measured against blank at 405 nm, and the results were expressed as EIA units (EIU) by multiplying the optical densities by 100.

4.1.8 Genotyping

Genotyping of the polymorphisms was done with a real-time PCR (Roche Diagnostics GmbH) according to previously published methods: LBP Phe436Leu (1341 T>C) (Korhonen *et al.* 2006), CD14 -260 C>T (Heesen *et al.* 2000, Heesen *et al.* 2003) TLR-4 Asp299Gly (896 A>G) (Heesen *et al.* 2003), TLR2 Arg 753Gln (2408 G>A) (Hamann *et al.* 2004), and IL-6 -174 G>C (Bertch *et al.* 2001). For CD14 genotyping, the protocol was modified as follows: the detection probe was LC Red 640- 5'TTC CTG TTA CGG CCC CCC T 3'-phosphate and the anchor probe was 5'GGA GAC ACA GAA CCC TAG ATG CCC TGC A 3'-fluorescein (Proligo, Boulder, CO, USA), the final $MgCl_2$ concentration was 2.5 mM in a reaction volume of 20 µl. A FastStart DNA Master Hybridization probe buffer (Roche Diagnostics GmbH) was used, and initial denaturation at 95 °C was prolonged to 10 min and the denaturation phase during cycling to 5 sec. For TLR-4 genotyping, the protocol was also slightly modified: the detection probe was 5'ACT ACC TCG ATG ATA TTA TTG ACT TAT T 3'-fluorescein and the anchor probe was LC Red640- 5' AAT TGT TTG ACA AAT GTT TCT TCA TTT TCC 3'-phosphate (Tib MolBiol GmbH). The concentration of both primers was 0.5 µM, in a 20 µl reaction volume. The FastStart DNA Master Hybridization probe buffer (Roche Diagnostics GmbH) was used. Initial denaturation at 95 °C was prolonged to 10 min and cycling was performed at 95 °C for 2 sec, at 49 °C for 15 sec, and at 72 °C for 20 sec. For the TLR-2 genotyping probe a phosphate was added to the 3' end of the anchor to prevent priming by the probes, the primer concentration was 0.5 µM, and the final $MgCl_2$ concentration was 3.0 mM.

4.1.9 Statistical analysis

Statistical analysis was performed using SPSS (release 14.0 or 15.0 SPSS Inc., USA). Spearman's correlation coefficients were used to compare correlation between inclusion numbers and chlamydial genomes or Cpn/Hum. Mann-

Whitney U-test was used to compare the means of serological variables, age and Chlamydia growth between males and females. Categorical variables were compared with linear-by-linear association *chi*-square test. Fisher's exact test was used to determine the significance of the differences of negative or positive *C. pneumoniae* IgA antibodies in the production quartiles. Student's t-test was used to compare the mean values of *C. pneumoniae* production in different gene polymorphism categories. A p-value ≤ 0.05 was considered statistically significant.

5 Results

5.1 Quantification of infection

The correlation was first examined between the number of chlamydial inclusions and genome counts quantified by PCR in cultures of the human epithelial HL cell line. Numbers of *C. pneumoniae* inclusions and genomes in HL cell cultures correlated significantly ($r = 0.990$, $p < 0.001$). The correlation was also significant between the *C. pneumoniae* inclusion numbers and the ratio of *C. pneumonia* genomes/human genome (Cpn/Hum) ($r = 0.978$, $p < 0.001$) as shown in Figure 1.

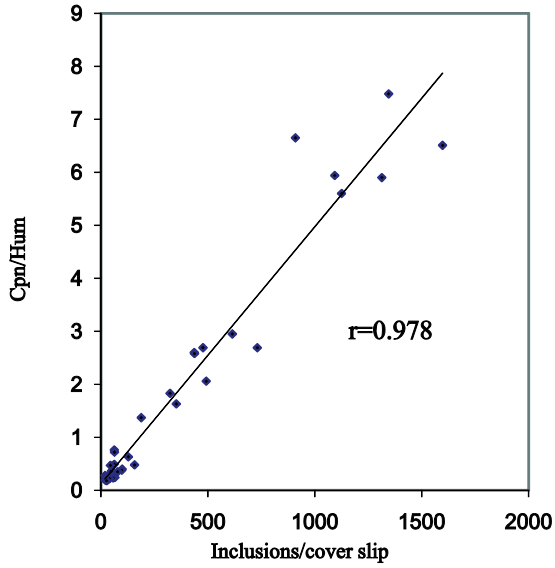

Fig. 1. Correlation between *Chlamydia pneumoniae* inclusions and the ratio *Chlamydia pneumoniae* genomes/human genome (Cpn/Hum) in infected HL cells. Data obtained from three independent assays (n = 42).

The correlation between inclusion numbers and chlamydial genomes or Cpn/Hum was not as high in macrophage cultures as in HL cell cultures (Table 4). Chlamydial LPS concentrations in the supernatants correlated with chlamydial genomes and Cpn/Hum, but not at all with the number of inclusions.

Table 4. Correlation coefficients between *C. pneumoniae* inclusions in cultured human macrophages, chlamydial LPS-concentration in culture supernatants (cLPS), chlamydial genomes (Cpn gen) and the ratio of chlamydial genomes/human genome (Cpn/Hum).

	cLPS	Cpn gen	Cpn / Hum
Inclusions	-0.003	0.237	0.133
n	209	249	249
p	0.968	< 0.001	0.036
cLPS		0.430	0.332
n		212	212
p		< 0.001	< 0.001
Cpn gen			0.428
n			257
p			< 0.001

5.2 Serological markers

We compared serological markers between females and males and found two statistically significant differences (Table 5). Males had more sCD14 in their sera than females (1.67 ± 0.16 and 1.43 ± 0.58, respectively, $p = 0.013$). CRP concentration was over two-fold among females compared to males (2.06 ± 3.90 and 0.97 ± 1.43, respectively, $p = 0.011$). The concentrations of hHSP-IgG, hHSP-IgA, LBP and IL6 did not differ significantly between females and males.

Table 5. Comparison of serological markers, age and *in vitro Chlamydia pneumoniae* growth between males and females. Results are expressed as mean ± S.D.

	Males (n=192)	Females (n=66)	p
Age ± S.D. (y)	45.3 ± 11.0	43.7 ± 14.5	0.600
sCD14 ± S.D. (µg/ml)	1.67 ± 0.60	1.43 ± 0.58	0.013
CRP ± S.D. (mg/l)	0.94 ± 1.43	2.06 ± 3.90	0.011
hHSP-IgG ± S.D. (EIU)	87.6 ± 67.6	91.4 ± 75.1	0.791
hHSP-IgA ± S.D. (EIU)	31.6 ± 38.2	27.0 ± 23.3	0.137
LBP ± S.D. (µg/ml)	11.4 ± 7.1	12.4 ± 7.3	0.115
IL6 ± S.D. (pg/ml)	0.79 ± 1.49	1.08 ± 1.78	0.336
Cpn/Hum ± S.D. (genes/genome)	30.9 ± 78.6	48.2 ± 126.3	0.833

5.2.1 *Growth of* C. pneumoniae *vs. serological markers*

Growth of *C. pneumoniae* in infected macrophages was highly variable, ranging individually from 0 to 638 *C. pneumoniae* genomes per human genome (Cpn/Hum). For further analysis, the study group was subdivided into quartiles according to *C. pneumoniae* production, the ranges for each quartile (Q) being: Q1: 0–1.9, Q2: 2.0–5.6, Q3: 5.7–18.8 and Q4: 18.9–638 Cpn/Hum, respectively, and the distribution of subjects with low or high (≤ or > median) values of sCD14, CRP, hHSP-IgG, hHSP-IgA, LBP and IL6 in the quartiles was compared (Table 6). hHSP-IgG level associated positively with *Chlamydia* production (p for trend = 0.024). CRP concentration had a negative association with *Chlamydia* production (p for trend = 0.035). However, when males and females were analysed separately, only the hHSP-IgG association with *Chlamydia* production among males was statistically significant (p for trend = 0.042). In addition, among males, the sCD14 concentration associated negatively with *Chlamydia* production (p for trend = 0.034). Among females, none of the variables associated with *Chlamydia* production (data not shown).

Table 6. Distribution of subjects with low or high (≤ or > median) values of sCD14, CRP, hHSP-IgG, hHSP-IgA, LBP and IL6 in quartiles of *C. pneumoniae* growth expressed as the number of chlamydial genomes per human genome (Cpn/Hum).

	Quartile	Q1	Q2	Q3	Q4	
	Cpn/Hum	0–1.9	2.0–5.6	5.7–18.8	18.9–638	p for trend
All						
sCD14	< median	29	31	31	38	0.148
	≥ median	35	34	33	27	
CRP	< median	31	24	33	41	0.035
	≥ median	33	41	31	24	
hHSP-IgG	< median	39	33	30	27	0.024
	≥ median	25	31	34	38	
hHSP-IgG	< median	35	34	26	33	0.389
	≥ median	29	30	38	32	
LBP	< median	33	30	26	39	0.470
	≥ median	31	35	38	26	
IL6	< median	26	35	31	36	0.156
	≥ median	38	30	32	29	
Males						
sCD14	< median	22	20	23	32	0.034
	≥ median	26	28	25	16	
CRP	< median	24	16	27	29	0.094
	≥ median	24	32	21	19	
hHSP-IgG	< median	30	22	24	19	0.042
	≥ median	18	25	24	29	
hHSP-IgA	< median	25	23	25	23	0.772
	≥ median	23	24	25	25	
LBP	< median	25	21	18	32	0.223
	≥ median	23	27	29	16	
IL6	< median	19	26	23	26	0.234
	≥ median	29	22	24	22	

The presence of *C. pneumoniae* IgA antibodies (titre ≥10) associated positively with *C. pneumoniae* production, and a statistically significant association was detected only in male subjects (Table 7). There was no association between anti-chlamydial IgG titres and *C. pneumoniae* production (data not shown).

Table 7. Distributions of subjects with negative or positive (≥10) *C. pneumoniae* IgA antibodies (Cpn-IgA) in quartiles of *C. pneumoniae* growth expressed as the number of chlamydial genomes per human genome (Cpn/Hum) in macrophages.

	Quartile	Q1	Q2	Q3	Q4
	Cpn/Hum	0–1.9	2.0–5.6	5.7–18.8	18.9–638
Males					
Cpn-IgA	Neg, n (%)	45 (28)	39 (25)	40 (25)	35 (22)
	Pos, n (%)	5 (15)	7 (21)	9 (27)	12 (37)
	p	-	0.321	0.118	0.040
Females					
Cpn-IgA	Neg, n (%)	13 (25)	15 (28)	11 (21)	14 (26)
	Pos, n (%)	2 (14)	3 (21)	5 (36)	4 (29)
	p	-	0.521	0.224	0.562
All					
Cpn-IgA	Neg, n (%)	58 (27)	54 (26)	51 (24)	49 (23)
	Pos, n (%)	7 (15)	10 (21)	14 (30)	16 (34)
	p	-	0.290	0.076	0.046

Q1 vs. Q2, Q3, or Q4 by Fisher's exact test

5.3 Effects of polymorphisms on growth of *C. pneumoniae*

The genotype and allele frequencies of polymorphisms studied are shown in Table 8. In the study group (n = 258) used for *in vitro* infection tests, CD14 polymorphisms deviated from Hardy-Weinberg equilibrium ($p < 0.001$); in the whole blood donor group (n = 393) no deviation was seen (data not shown). No deviation from Hardy-Weinberg equilibrium was observed in any other genotype frequencies.

Table 8. Genotype and allele frequencies of the studied polymorphisms.

	CD14	TLR-2	TLR-4	LBP	IL-6
	-260C→T	Arg_{753}→Gln	Asp_{299}→Gly	Phe_{436}→Leu	-174G→C
Genotype 1, n (%)	CC, 122 (47.3)	GG, 246 (95.7)	AA, 216 (83.7)	TT, 211 (82.1)	GG, 48 (18.7)
Genotype 2, n (%)	CT, 89 (34.5)	AG, 11 (4.3)	AG, 39 (15.1)	CT, 45 (17.5)	GC, 130 (50.6)
Genotype 3, n (%)	TT, 47 (18.2)	AA, 0 (0)	GG, 3 (1.2)	CC, 1 (0.4)	CC, 79 (30.7)
Total n	258	257	258	257	257
Allele 1, (%)	T, 64.5	G, 97.9	A, 91.3	T, 90.9	G, 44.0
Allele 2, (%)	C, 35.5	A, 2.1	G, 8.7	C, 9.1	C, 56.0

The effects of CD14 -260C>T, TLR2 Arg753Gln (2408 G>A), TLR4 Asp299Gly (896 A>G), LBP Phe436Leu (1341 T>C) and IL-6 -174G>C polymorphisms on

the production of *C. pneumoniae* in human macrophages infected *in vitro* are shown in Table 9.

Table 9. Growth of *Chlamydia pneumoniae* in cultured human macrophages (Cpn/hum) in CD14, TLR2, TLR4, LBP and IL6 gene polymorphism genotypes.

Gene	Genotype	n	Cpn/Hum ± SEM	p
CD14	TT	47	80.4 ± 23.2	-
	CT	89	26.3 ± 8.5	0.032
	CC	122	24.5 ± 5.2	0.022
TLR2	AG	11	28.8 ± 18.5	-
	GG	246	35.7 ± 6.0	0.811
TLR4	AA	216	30.5 ± 5.3	-
	AG	39	52.2 ± 22.6	0.356
	GG	3	160.2 ± 141.3	0.456
LBP	TT	211	35.2 ± 6.8	-
	TC	45	37.1 ± 22.7	0.456
	CC	1	1.0	
IL6	CC	79	18.3 ± 5.9	-
	CG	130	43.4 ± 8.7	0.018
	GG	48	42.0 ± 17.9	0.214

The CD14 gene T-allele increased *C. pneumoniae* production in cells and production was higher in the TT genotype than in TC or CC genotypes ($p = 0.032$ and $p = 0.022$, respectively).

The polymorphisms of TLR2 or TLR4 genes had no significant effect on the amount of *C. pneumoniae* in cells. In the TLR2 GG genotype the production was slightly higher than in the AG genotype, though the difference was not statistically significant ($p = 0.811$). The TLR4 gene G allele seemed to potentiate the production of *C. pneumoniae*, however the difference between AA and GA or GG genotypes was not significant ($p = 0.356$ and $p = 0.456$, respectively).

LBP gene polymorphism had no influence on *C. pneumoniae* production and the outcome was the same for TT and TC genotypes ($p = 0.465$).

The production of *C. pneumoniae* was lowest in IL-6 genotype CC; in CG and GG genotypes the values were over two-fold compared to the CC genotype ($p = 0.018$ and $p = 0.214$, respectively). For a more detailed analysis of the effects of IL-6 polymorphism, the study group was subdivided by CD14 genotypes (Table 10). In the CD14 genotype CC, the *C. pneumoniae* yield was highest in the IL-6 GC heterozygote, the difference being statistically significant compared to GG and CC genotypes ($p = 0.006$ and $p = 0.013$, respectively). There was no

difference between CC and GG genotypes. In the CD14 CT genotype, the production was also higher in IL-6 GC than in GG and CC genotypes ($p = 0.032$ and $p = 0.060$, respectively) and there was no difference between CC and GG genotypes. In the CD14 TT genotype, no significant differences between various genotypes were detectable.

Table 10. Growth of *Chlamydia pneumoniae* in cultured macrophages (Cpn/hum) in CD14 and IL6 genotypes.

Genes and genotypes		n	Cpn/hum ± SEM	p*
CD14	IL-6			
CC	CC	40	11.6 ± 2.7	0.013
	GC	61	38.2 ± 10.0	-
	GG	21	9.0 ± 2.0	0.006
CT	CC	30	10.8 ± 4.1	0.060
	GC	45	42.7 ± 16.0	-
	GG	13	7.1 ± 1.7	0.032
TT	CC	9	72.7 ± 47.4	0.777
	GC	24	58.0 ± 26.0	-
	GG	14	123.8 ± 56.8	0.305

* IL-6 GC vs. CC or GG

6 Discussion

6.1 Methodological aspects (I)

The chlamydial growth *in vitro* has traditionally been quantified by calculating microscopically the number of stained inclusions in cells. This method is reliable when epithelial cells, like HL or HeLa cells, are used as hosts because they usually contain only one, large and uniform inclusion. This is in line with our results, as the correlation was high between inclusion numbers and the amount of chlamydial genes or the ratio Cpn/Hum in infected HL cells. So, it is likely that the developed PCR-method reliably indicates exact quantity of *C. pneumoniae* in cells.

The correlation between inclusion numbers and chlamydial genomes or the ratio Cpn/Hum was not as high in macrophage cultures as in HL cell cultures. One explanation for this was the inconsistent size of inclusions in macrophages compared to HL cells and many of the macrophages also contained multiple inclusions, which is characteristic for persistent infections (Gieffers *et al.* 2001). Chlamydial LPS concentrations in the supernatants correlated with chlamydial genomes and Cpn/Hum but not at all with the number of inclusions. The same ELISA-kit as used in our study has been used to measure *C. pneumoniae* numbers in murine alveolar macrophages (Haranaga *et al.* 2001). However, in our study the ELISA-method was semi-quantitative, because we used only one positive standard that was included in the kit. Thus, the method gave arbitrary EIU-units and the scale was not linear, which may partly lead to the low correlations.

In conclusion, with this method both chlamydial and human genomes are quantified with real time quantitative PCR, providing more exact measurements of chlamydial infectivity and growth *in vitro*. The advantage of this method is its independence from microscopic counting of inclusions, which is an inherently subjective and laborious procedure. The variable size of inclusions which reflect different numbers of chlamydial particles inside inclusions, as seen in monocyte-macrophage cultures in this study, leads to a poor correlation between chlamydial genome counts and number of inclusions. This means that it is not possible to quantify *C. pneumoniae* growth exactly inside infected cells by calculating microscopically the numbers of stained inclusions. Therefore, the developed PCR-method may be used for reliable quantification of *C. pneumoniae* growth in macrophages with characteristic persistent chlamydial infection.

6.2 Chlamydial growth *in vitro* vs. serological markers (II)

In this study we showed that the individual susceptibility of cultured human macrophages to *C. pneumoniae* infection is highly variable. Godzik *et al.* (1995) have shown earlier that human monocytes vary in their susceptibility to *C. pneumoniae*, but they used cells from three donors only. Thus, our study confirms this preliminary finding in a much larger population of healthy persons.

Kaukoranta-Tolvanen *et al.* (1996) reported that it is not possible to infect human peripheral blood monocytes with *C. pneumoniae* (strain K6) on the day of isolation. Freshly isolated monocytes could be infected with *C. pneumoniae* when cultured for three to nine days *in vitro*. Human monocytes cultured for less than seven days have also been shown to be microbicidal against the human lymphogranuloma venereum biovar (LGV) and the trachoma biovar of *C. trachomatis* (Yong *et al.* 1987). In cultures older than 7 days, chlamydiocidal activity against LGV vanishes, but cells cultured for up to 21 days are still able to kill the trachoma biovar with the same effectiveness as those cultured for seven days. No respective studies on the survival of *C. pneumoniae* in human monocytes have been done. In this study we used *C. pneumoniae*, isolate K7, and further studies are needed to find out the possible differences in the survival and growth of different *C. pneumoniae* strains in human monocyte-macrophages.

Elevated IgG and especially IgA antibody titres to *C. pneumoniae* measured by MIF have been considered markers of chronic *C. pneumoniae* infection (Saikku *et al.* 1992, Falck *et al.* 2002). The prevalence of *C. pneumoniae*-specific antibodies is higher in males compared to females (Saikku 1992). Interestingly, in the present study, elevated *C. pneumoniae* IgA levels among males were associated with abundant *in vitro* growth of *C. pneumoniae*. There was also a positive association between hHSP60-IgG antibodies and *C. pneumoniae* production among males. This finding suggests that males are more susceptible to chronic *C. pneumoniae* infections than females. The direct effect of the male hormonal milieu or men's elevated iron levels could be excluded (Schwartz *et al.* 2002, Freidank *et al.* 2001), since the macrophages were cultured *in vitro.*

The serum concentrations of acute phase reactants (CRP, LBP) and mediators of innate immunity (IL-6, sCD14) were measured to find if any of them predict the degree of chlamydial growth *in vitro*. The mean concentration of CRP was higher among females compared to males. The likely explanation is the use of oral contraception or hormone replacement therapy, both of which have been shown to significantly increase CRP concentrations (Frölich *et al.* 1999, Ridker *et*

al. 1999). The mean sCD14 concentration was significantly higher and also associated negatively with *C. pneumoniae* production among males. CRP concentration associated negatively with *C. pneumoniae* production, but the association was not significant when the study group was subdivided into females and males. The explanation for these findings remains open.

Taken together, the association between elevated *C. pneumoniae* IgA and hHSP60 IgG antibody levels and abundant growth of *C. pneumoniae* in monocyte-macrophage cultures suggests that persons with abundant growth are prone to chronic infection. The genetic background may predispose the host to susceptibility to chronic infection and predetermine the outcome of the infection in the host. Thus, a further study was done to elucidate some genetic factors predetermining individual susceptibility to *C. pneumoniae* infection.

6.3 Chlamydial growth *in vitro* and innate immunity gene polymorphisms (III)

The studied genotypes were in Hardy-Weinberg equilibrium, except for CD14 genotypes. However, because among the entire blood donor group the CD14 genotypes were in equilibrium, the deviation among the *in vitro* study group was likely accidental.

Seropositivity to *C. pneumoniae* has been reported by Eng *et al.* (2003) to be associated with CD14 promoter gene polymorphism. They studied the distribution of the -260C>T polymorphism among healthy subjects and found the TT genotype to be a risk factor for the presence of *C. pneumoniae* antibodies, suggesting that persons with the TT genotype are more susceptible to *C. pneumoniae*. Our results show that the growth of *C. pneumoniae* was highest in TT genotype cells and is thus in line with the earlier *in vivo* finding. This association has also been demonstrated among chronically *C. pneumoniae* infected coronary artery disease patients: the patients, whose peripheral blood monocytes are repeatedly positive for *C. pneumoniae* DNA, were also significantly more likely to carry the TT genotype than *C. pneumoniae*-negative patients (Rupp *et al.* 2004).

Activated macrophages secrete proinflammatory cytokines TNFα, IL-1, and IL-6 (Dobrovolskaia & Vogel 2002). The results on the influence of CD14 polymorphism on cytokine secretion *in vitro* are contradictory. LPS has been found to induce significantly higher levels of TNFα and IL-6 production in the TT than in CC genotype (Kondo *et al.* 2003, Lin *et al.* 2007, Härtel *et al.* 2008), but

other reports have not shown any effect (Heesen *et al.* 2001, von Aulock *et al.* 2005). In whole blood cell cultures, *C. pneumoniae* (strain TW183, purified elementary bodies) stimulated higher TNFα secretion in the TT genotype than in TC or CC genotypes (Eng *et al.* 2004). However, this finding was not supported in another study (Härtel *et al.* 2008). Although the TT genotype would improve the effectiveness of the immune response, it does not necessarily aid in the eradication of an intracellular pathogen. *C. pneumoniae* infection of Mono Mac 6 cells has been shown to induce rapid activation of NF-κB and subsequent production of TNFα, IL-1β and IL-6 (Wahl *et al.* 2001). The activation was essential for survival and replication of *C. pneumoniae* (Heinemann *et al.* 1996). In human endothelial EVLC2 cells *C. pneumoniae* growth was inhibited in a dose-dependent manner with aspirin, and the growth inhibition was specific to NF-κB inhibition (Tiran *et al.* 2002). It is apparent that *C. pneumoniae* has developed a strategy to benefit from the activation of host cells to promote its own replication, thus the TT genotype cell would be the most optimal environment for its growth as seen in our study.

The association between CAD and pathogen burden has shown to be modulated by IL-6 -174G>C polymorphism, the odds ratio being higher in heterozygotes (GC) than in both homozygotes (GG or CC) (Georges *et al.* 2003). In a prospective study of dialysis patients the polymorphism predicted the incidence of CVD and mortality, and the cumulative incidence of CVD was highest among heterozygotes (Liu *et al.* 2006). In our study the IL-6 gene -174 G allele enhanced the growth of *C. pneumoniae* in GC and GG genotypes compared with the CC genotype. However, the difference was statistically significant only between CC and GC genotypes. In persons with CD14 CC or CT genotypes, the IL-6 GC genotype potentiated *C. pneumoniae* growth was about four-fold compared to CC or GG genotypes, though in the CD14 TT genotype this difference was not seen. This could be explained by the pronounced effect of the CD14 TT genotype on *C. pneumoniae* growth. IL-6 has been shown to up-regulate CD14 expression in PMA-differentiated monocytic leukemia cells (THR1) over ten-fold (Zarember & Godovski 2002). It has been suggested that IL-6 may alter the phosphorylation state of the nuclear proteins binding to the Sp1 binding site (Gierens *et al.* 2000). Because CD14 -260C>T polymorphism is located at the Sp1 binding site that regulates transcription of CD14, IL-6 could influence CD14 transcription in an allele-specific manner.

Polymorphism, -174G> C, affecting transcription of the IL-6 gene is located in its promoter region. In HeLa cells, the -174G construct showed higher

expression than the -174C construct. The C allele was associated with significantly lower IL-6 plasma levels (Fishman *et al.* 1998). Other known polymorphisms, which regulate transcription of the IL-6 gene, are -597G>A, -572G>C and -373A_nT_n. The different polymorphisms had an influence on transcription, but they did not function individually, since the final expression resulted from the combination of alleles, i.e. the haplotype (Terry *et al.* 2000). In our study only the effect of -174G>C polymorphism to *C. pneumoniae* growth was evaluated, and thus the effects of other polymorphisms and alleles remain to be solved.

In conclusion, in this study we demonstrated a positive effect of the CD14 -260C>T polymorphism on *C. pneumoniae* growth in human macrophages. The G allele of the IL-6 -174G> C polymorphism was also associated with enhanced *C. pneumoniae* proliferation. The intracellular growth of *C. pneumoniae* requires activation of the host cell. The CD14 -260C>T polymorphism results in a higher activation level of macrophages and thus may favour the growth of *C. pneumoniae.*

7 Concluding remarks

In this study we developed a new, reliable PCR-based method to quantify *Chlamydia pneumoniae* growth in cultured cells. The new method was used to evaluate individual susceptibility of human monocyte-derived macrophages to *C. pneumoniae* infection *in vitro*. The mononuclear cells were obtained from healthy blood donors. We found that the intracellular growth of *C. pneumoniae* was highly variable and it correlated positively with serum *C. pneumoniae* IgA titer and hHSP60-IgG antibodies. These antibodies may indicate current or persistent chlamydial infection. The blood donors were apparently healthy, thus we suggest that these antibodies associated with persistent infection.

Because of the high number of studied subjects, we were also able to run genetic analyses to determine the effects of innate immunity gene polymorphisms on *C. pneumoniae* infection *in vitro*, which has not been done previously. Indeed, innate immunity gene polymorphisms affected the growth of *C. pneumoniae* in macrophages. CD14 -260C>T polymorphism showed the strongest effect and the growth was higher in TT-genotype cells than in CT or CC cells, and also the G-allele of the IL-6 -174G> C polymorphism associated with enhanced *C. pneumoniae* proliferation. In this *in vitro* study we used cultured macrophages only, thus all other immunity factors were excluded and only the intrinsic properties of the cells interacted on the chlamydial growth. Although our studies were conducted on the properties of cells *in vitro*, the finding of enhanced growth in the CD14 -260C>T promoter gene polymorphism TT-genotype cells is intriguingly in line with earlier *in vivo* study which reported *C. pneumoniae* seropositivity to associate with the TT-genotype (Eng *et al.* 2003). Furthermore, our finding that the IL-6 -174G> C polymorphism G-allele cells had enhanced *C. pneumoniae* proliferation is in line with the *in vivo* conclusion that the association between CAD and pathogen burden is modulated by the same polymorphism (Georges *et al.* 2003).

Intracellulary PAMPs are recognized by highly conserved proteins of NLR (Nod like receptor) family, which initiate innate and adaptive immune responses upon pattern-specific sensing of pathogens (Inohara & Nunez 2003). After sensing, various substructures of bacterial peptidoglycan NOD1 and NOD2 trigger NF-κB dependent activation of host cell (Fritz *et al.* 2006). NLRP3 containing inflammasome recognize microbial motifs and danger-associated host components and thereafter activates caspase-1, which mediates cleavage and release of biologically active cytokine IL-1β (Latz 2010). A recent study done

with mice genetically deficient in NOD1 and NOD2 demonstrates that those proteins are essential in defence against *C. pneumoniae* infection (Shimada *et al.* 2009). Mutations in NOD1 and NOD2 are linked to inflammatory mucosal barrier diseases in humans (Fritz *et al.* 2006). Also the polymorphism NOD1 -796G>A in association with *C. pneumoniae* infection has proved to be a risk factor for stroke (Tiszlavicz *et al.* 2009). Approximately 40 mutations have been found in gene encoding NLRP3 and in most of these lead to increased release of IL-1β from peripheral blood monocytes and leukocytes (Fritz *et al.* 2006). Thus, further studies are required to elucidate how the polymorphisms of NOD1, NOD2 and NLRP3 affect the growth of *C. pneumoniae* and infection induced IL-1β secretion in human macrophages.

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I Poikonen K, Lajunen T, Silvennoinen-Kassinen S, Leinonen M & Saikku P (2010) Quantification of *Chlamydia pneumoniae* in cultured human macrophages and HL cells. Comparison of real-time PCR, immunofluorescence and ELISA methods. APMIS 118(1): 45–48.

II Poikonen K, Lajunen T, Silvennoinen-Kassinen S, Paldanius M, Leinonen M & Saikku P (2008) Susceptibility of human monocyte-macrophages to *Chlamydia pneumoniae* infection *in vitro* is highly variable and associated with levels of soluble CD14 and *C. pneumoniae* IgA and human HSP-IgG antibodies in serum. Scand J Immunol 67(3): 279–284.

III Poikonen K, Lajunen T, Silvennoinen-Kassinen S, Leinonen M & Saikku P (2009) Effects of CD14, TLR2, TLR4, LPB, and IL-6 gene polymorphisms on *Chlamydia pneumoniae* growth in human macrophages *in vitro*. Scand J Immunol 70(1): 34–39.

Original publications are not included in the electronic dissertation.

1034. Sipola, Annina (2009) Effects of vascular endothelial growth factor (VEGF-A) and endostatin on bone

1035. Kokkonen, Hanna (2009) Aspects of bone sugar biology. Pectin nanocoatings of hard tissue implants

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1037. Arkkola, Tuula (2009) Diet during pregnancy. Dietary patterns and weight gain rate among Finnish pregnant women

1038. Seppinen, Lotta (2009) The roles of collagen XVIII and its endostatin domain in wound healing, hair follicle cycling and bone development

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