

Leena Erkkilä

PULMONARY INFECTION AND
ATHEROSCLEROSIS IN AN EXPERIMENTAL
CHLAMYDIA PNEUMONIAE MODEL

ACADEMIC DISSERTATION

*To be presented with the permission of the Faculty of Biosciences, Department of
Biological and Environmental Sciences,
University of Helsinki, for public examination in the auditorium no 1041,
Viikinkaari 5, on the 21st of October, 2005, at 12 noon.*

National Public Health Institute, Department of Viral Diseases and Immunology, Laboratory for Chlamydia and Respiratory Tract Bacteria, Oulu, Finland

and

University of Helsinki, Faculty of Biosciences, Department of Biological and Environmental Sciences, Division of General Microbiology, Helsinki, Finland

and

University of Oulu, Faculty of Medicine, Department of Medical Microbiology, Oulu, Finland

Helsinki 2005

**Publications of the National Public Health Institute
KTL A17 / 2005**

Copyright National Public Health Institute

Julkaisija-Utgivare-Publisher

Kansanterveyslaitos (KTL)

Mannerheimintie 166
00300 Helsinki
Puh. vaihde (09) 474 41, telefax (09) 4744 8408

Folkhälsoinstitutet

Mannerheimvägen 166
00300 Helsingfors
Tel. växel (09) 474 41, telefax (09) 4744 8408

National Public Health Institute

Mannerheimintie 166
FIN-00300 Helsinki, Finland
Telephone +358 9 474 41, telefax +358 9 4744 8408

ISBN 951-740-564-2

ISSN 0359-3584

ISBN 951-740-565-0 (pdf)

ISSN 1458-6290 (pdf)

Kannen kuva - cover graphic: Leena Erkkilä

Painopaikka:

Edita Prima Oy
Helsinki 2005

S u p e r v i s e d b y

Research Professor Maija Leinonen, PhD
Department of Viral Diseases and Immunology
National Public Health Institute
Oulu, Finland

Docent Kirsi Laitinen, PhD
Department of Public Health
University of Helsinki
Helsinki, Finland

Emeritus Professor Pekka Saikku, MD, PhD
Department of Medical Microbiology
University of Oulu
Oulu, Finland

R e v i e w e d b y

Professor Petri Kovanen, MD, PhD
Wihuri Research Institute
Helsinki, Finland

and

Professor Matthias Maass, MD
Institute of Medical Microbiology
University of Lübeck
Lübeck, Germany

O p p o n e n t

Professor Gerald I. Byrne, PhD
Department of Molecular Sciences
University of Tennessee Health Sciences Center
Memphis, USA

Leena Erkkilä, Pulmonary infection and atherosclerosis in an experimental *Chlamydia pneumoniae* model

Publications of the National Public Health Institute, A17/2005, 78 Pages

ISBN 951-740-564-2; 951-740-565-0 (pdf-version)

ISSN 0359-3584; 1458-6290 (pdf-version)

http://www.ktl.fi/portal/suomi/julkaisut/julkaisusarjat/kansanterveyslaitoksen_julkaisu_a/

ABSTRACT

Chlamydia pneumoniae is a Gram-negative bacterium, which has a unique intracellular developmental cycle for replication inside host cells. *C. pneumoniae* infects the respiratory tract and is a common cause of upper respiratory tract infections as well as bronchitis and atypical pneumonia, which may be mild or life-threatening. Similarly to all chlamydial species, *C. pneumoniae* has a tendency to cause chronic infections, which may lead to severe disease sequelae, such as chronic pulmonary diseases and atherosclerosis. In an experimental *C. pneumoniae* infection model, *C. pneumoniae* and chronic inflammation persist in mouse lung. Among the individual chlamydial antigens, chlamydial Heat shock protein 60 (cHsp60) is associated with chronic diseases. The immune response against cHsp60 and, further, the development of autoimmunity against host Hsp60 are associated with a chronic inflammatory disease, namely atherosclerosis. In the prevention of cardiovascular diseases, lipid-lowering statin therapies are widely used. They inhibit the rate-limiting step in the mevalonate pathway and cholesterol biosynthesis and have been shown to have antimicrobial effects.

In the present study, a mouse model for *C. pneumoniae* infection was used. Specifically, the technical aspects, the pulmonary infection, the development of atherosclerotic lesions and Hsp60 autoimmunity, and the possible effect of simvastatin treatment on *C. pneumoniae* infection were studied. Mice were infected with *C. pneumoniae* and, in some experiments, fed a diet with some cholesterol supplementation or a high-fat diet.

One of the most important findings of the present study was that the host cell components in the *C. pneumoniae* inoculum may induce a weak inflammatory response. This may affect the course of infection by, for instance, reactivating a culture-negative infection. It was also shown that chronic *C. pneumoniae* infection led to the development of a humoral Hsp60 autoimmune response along with the development of initial aortic atherosclerotic lesions. The Hsp60 autoimmune response developed after repeated *C. pneumoniae* exposures and was exaggerated if the mice were also fed a cholesterol-supplemented diet. In addition, treatment with simvastatin was shown to exhibit an antichlamydial effect *in vivo*. The effect was seen as diminished chlamydial growth in the lungs.

Keywords: *Chlamydia pneumoniae*; *Chlamydomydia pneumoniae*; Mice; Models, animal; Disease models, animal; Pneumonia; Pulmonary infection; Hsp60 autoimmune response; Chronic infection; Arteriosclerosis; Atherosclerosis; Simvastatin

Leena Erkkilä, Keuhkoinfektio ja ateroskleroosi kokeellisessa *Chlamydia pneumoniae* mallissa

Kansanterveyslaitoksen julkaisuja, A17/2005, 78 sivua

ISBN 951-740-564-2; 951-740-565-0 (pdf-versio)

ISSN 0359-3584; 1458-6290 (pdf-versio)

http://www.ktl.fi/portal/suomi/julkaisut/julkaisusarjat/kansanterveyslaitoksen_julkaisuja_a/

TIIVISTELMÄ

Chlamydia pneumoniae on Gram-negatiivinen bakteeri, jolla on solunsisäinen lisääntymiskierto. *C. pneumoniae* infektoi hengitysteitä ja on yleinen ylähengitystieinfetioiden, ja myöskin bronkiitin ja atyyppisen pneumonian aiheuttaja. Infektio voi olla lievä tai hengenvaarallinen. *C. pneumoniae*:lla kuten muillakin klamydialajeilla on taipumus aiheuttaa kroonisia infektiota, jotka voivat johtaa vakaviimpiin jälkiseurauksiin, kuten kroonisiin keuhkotauteihin ja ateroskleroosiin. Kokeellisessa infektiomallissa, *C. pneumoniae* ja krooninen tulehdus persistoivat hiiren keuhkoissa. Yksi tärkeimmistä klamydian antigeneistä, joka on yhdistetty kroonisiin tautimuotoihin, on klamydian 60kD:n stressiproteiini Hsp60. Sitä vastaan syntyvä immuunivaste ja mahdollinen autoimmuunivaste isännän omaa Hsp60:a vastaan voivat osallistua kroonisen tulehdustaudin, ateroskleroosin kehittymiseen. Sydän- ja verisuonitautien ehkäisyssä ja hoidossa käytetään laajasti kolesterolia alentavia statiineja. Ne inhiboivat kynnyksreaktiota mevalonaatti-synteesipolulla ja kolesterolin biosynteesissä ja niillä on havaittu olevan antimikrobisia vaikutuksia.

Tässä tutkimuksessa käytettiin hiirimallia *C. pneumoniae* infektiolle. Tutkimuksen kohteena olivat hiirimallin tekninen suorittaminen, keuhkoinfektio ja siihen vaikuttavat tekijät, ateroskleroosin ja autoimmuunivasteen kehittyminen Hsp60:lle, sekä kolesterolilääke simvastatiinin mahdollinen vaikutus *C. pneumoniae* infektiioon. Hiiret infektoitiin nenän kautta *C. pneumoniae*:lla, ja joissakin kokeissa käytettiin rehua, jossa oli ylimääräinen annos kolesterolia tai rasvaa.

Tutkimuksen yksi tärkeimmistä tuloksista oli, että infektoinnissa käytettävän *C. pneumoniae* -preparaatin sisältämät isäntäsolujen rippeet saattavat aiheuttaa lievää tulehdusreaktiota. Tämä voi vaikuttaa infektion kulkuun mm. reaktivoimalla piilevän infektion. Lisäksi osoitettiin, että krooninen *C. pneumoniae* infektiota johtaa Hsp60-autovasta-aineiden lisääntymiseen, sekä alkuvaiheessa olevien ateroskleroottisten plakkien kasvamiseen. Hsp60 autoimmuunivaste kehittyi toistuvien *C. pneumoniae* infektioiden myötä, ja voimistui mikäli hiiriä syötettiin rehulla, jossa oli kolesterolilisiä. Simvastatiini-hoidolla havaittiin lisäksi olevan klamydian kasvua hillitsevä vaikutus, joka näkyi vähentyneenä klamydian lisääntymisenä hiiren keuhkoissa.

Avainsanat: *Chlamydia pneumoniae*; Keuhkoklamydia; Hiirimalli; Keuhkokuume; Keuhkoinfektio; Hsp60 autoimmuunivaste; Krooninen infektiota; Ateroskleroosi; Simvastatiini

Contents

Abstract	8
Tiivistelmä	8
Contents.....	8
Abbreviations	8
List of original publications.....	10
1 Review of the literature	11
1.1 Bacterial pathogens and the respiratory tract.....	11
1.2 <i>C. pneumoniae</i> diseases of the respiratory tract.....	12
1.2.1 Acute respiratory diseases.....	12
1.2.2 Chronic pulmonary diseases	13
1.3 <i>C. pneumoniae</i>	14
1.3.1 History and taxonomy	14
1.3.2 Cell biology	15
1.3.3 Experimental in vivo models for <i>C. pneumoniae</i> infection	16
1.4 Factors determining the persistence of <i>C. pneumoniae</i> infection in lung.....	20
1.4.1 Pulmonary inflammation	20
1.4.2 Persistence of <i>C. pneumoniae</i>	21
1.4.3 Hsp60 in chlamydial persistence	22
1.5 Association of atherosclerosis and <i>C. pneumoniae</i> infection.....	23
1.5.1 Inflammation in atherogenesis.....	24
1.5.2 Mouse models for <i>C. pneumoniae</i> and atherosclerosis.....	25
1.5.3 Role of <i>C. pneumoniae</i> in lesion development	27
1.5.4 Hsp60 autoimmunity	28
1.5.4.1 Hsp60 autoimmunity in atherogenesis.....	28
1.5.4.2 Chlamydial infection and Hsp60 autoimmunity in atherogenesis	29
1.5.5 Statins and their antimicrobial effects.....	29
2 Aims of the study.....	31
3 Materials and methods.....	32
3.1 Mouse model	32
3.1.1 Mice and diets.....	32

3.1.2	<i>C. pneumoniae</i> challenge.....	32
3.1.3	Other treatments (IV).....	33
3.2	Detection of <i>C. pneumoniae</i>	33
3.2.1	Chlamydial culture (I, II, III, IV).....	33
3.2.2	Detection of <i>C. pneumoniae</i> DNA by PCR (II, III, IV).....	34
3.3	Inflammation	34
3.3.1	Pulmonary inflammation (II, III, IV).....	34
3.3.2	Circulating inflammatory markers (IV).....	34
3.4	<i>C. pneumoniae</i> -specific and autoimmune responses (II, III, IV).....	35
3.5	Quantification of atherosclerotic lesions and serum lipids (III, IV)	35
3.6	Statistics.....	36
4	Results	37
4.1	Technical considerations of <i>C. pneumoniae</i> mouse model	37
4.1.1	Effect of anesthetic agent (I).....	37
4.1.2	Effect of host cell debris in challenge inoculum (II)	37
4.2	Pulmonary infection and inflammation	38
4.2.1	Comparison of the rates of growth of two different <i>C. pneumoniae</i> Kajaani isolates (I).....	38
4.2.2	Development of pulmonary infection and inflammatory cell infiltrations after different challenging routes.....	38
4.2.3	Effect of cholesterol feeding on chronic <i>C. pneumoniae</i> pulmonary infection (III)	39
4.3	Development of Hsp60 responses and atherosclerotic lesions	40
4.3.1	Chlamydial and mHsp60 responses (II, III).....	40
4.3.2	Aortic lesions and serum lipids (III)	41
4.4	Effect of simvastatin	41
4.4.1	Effect of simvastatin treatment on the growth of <i>C. pneumoniae</i> in mouse lungs (IV).....	41
4.4.2	Immunomodulatory effect of simvastatin treatment (IV)	42
5	Discussion	43
5.1	Technical considerations of the <i>C. pneumoniae</i> mouse model	43
5.2	Aspects of <i>C. pneumoniae</i> pulmonary infection and inflammation.....	44
5.3	Chlamydial infection and induction of immune response against Hsp60s	46
5.4	Atherosclerosis in the chronic <i>C. pneumoniae</i> infection model of C57BL/6J mice ..	47
5.5	Antichlamydial effect of simvastatin	48
6	Conclusions and future prospects	50
7	Acknowledgments	52
8	References	54

Abbreviations

AAA	abdominal aortic aneurysm
AM	alveolar macrophage
ApoE-KO	Apolipoprotein E knockout
bp	base pair
CAD	coronary artery disease
CAP	community-acquired pneumonia
cHsp60	chlamydial heat shock protein 60
CO ₂	carbon dioxide
COPD	chronic obstructive pulmonary disease
CRP	C-reactive protein
DMSO	dimethylsulfoxide
EB	elementary body
EIA	enzyme immunoassay
HDL	high-density lipoprotein
hHsp60	human heat shock protein 60
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
Hsp	heat shock protein
i.c.	intracerebral
IFN- γ	interferon - γ
IFU	inclusion-forming unit
Ig	immunoglobulin
i.g.	intra-gastric
IL	interleukin
i.n.	intranasal
iNOS	inducible nitric oxide synthase
i.p.	intraperitoneal
i.v.	intravenous
K6	Kajaani 6, <i>Chlamydia pneumoniae</i> isolate
K7	Kajaani 7, <i>Chlamydia pneumoniae</i> isolate
LBP	lipopolysaccharide-binding protein
LDL	low-density lipoprotein

LDLR-KO	low-density lipoprotein receptor knockout
LPS	lipopolysaccharide
mbHsp65	mycobacterial heat shock protein 65
MBL	mannose-binding lectin
MCP-1	monocyte chemoattractant protein-1
mHsp60	mouse Hsp60
MOMP	major outer membrane protein
Nod	nucleotide-binding oligomerization domain
OmpA	outer membrane protein A
OVA	ovalbumin
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
p.i.	postinfection
PMN	polymorphonuclear
Pmp	polymorphic membrane protein
RB	reticulate body
rRNA	ribosomal RNA
SAA	serum amyloid A
SPG	sucrose-phosphate-glutamate
Th	T-helper
TLR	toll-like receptor
TNF- α	tumour necrosis factor- α
TW-183	<i>Chlamydia pneumoniae</i> isolate
TWAR	synonym for <i>Chlamydia pneumoniae</i>

List of original publications

This thesis is based on the following articles, which are referred to in the text by their Roman numerals. These articles are reproduced with the kind permission of their copyright holders.

I Erkkilä L, Rottenberg ME, Laitinen K (2000) Comparison of anesthetics for inoculation of mice with *Chlamydia pneumoniae*. *Comp Med* 50:46-48

II Erkkilä L, Laitinen K, Laurila A, Saikku P, Leinonen M (2002) Experimental *Chlamydia pneumoniae* infection in NIH/S mice: effect of reinoculation with chlamydial or cell preparation on culture, PCR and histological findings of the lung tissue. *Vaccine* 20:2318-2324

III Erkkilä L, Laitinen K, Haasio K, Tiirola T, Jauhiainen M, Lehr HA, Aalto-Setälä K, Saikku P, Leinonen M (2004) Heat shock protein 60 autoimmunity and early lipid lesions in cholesterol-fed C57BL/6JBom mice during *Chlamydia pneumoniae* infection. *Atherosclerosis* 177:321-328

IV Erkkilä L, Jauhiainen M, Laitinen K, Haasio K, Tiirola T, Saikku P, Leinonen M (2005) Effect of simvastatin, an established lipid-lowering drug, on pulmonary *Chlamydia pneumoniae* infection in mice. *Antimicrob Agents Chemother* 49:3959-3962.

In addition, some unpublished data are presented.

1 Review of the literature

1.1 Bacterial pathogens and the respiratory tract

The human respiratory tract is divided into the upper and lower airways. The ascending bronchi narrow through dichotomous branching, and only particles smaller than 10 μm in diameter are able to reach the lung along with the airflow, and particles smaller than 3-5 μm have direct access into the bronchioles.

The bacteria causing respiratory infections may be part of the normal flora in the upper respiratory tract or may be acquired from the environment within aerosols or droplets. In the respiratory tract, bacteria are in contact with the ciliated epithelium, which attempts to push the trapped particles upward within mucus. The alveoli are lined by a thin layer of alveolar lining fluid, which contains soluble immune molecules, complement, collectins (e.g. surfactants A and D), and immunoglobulins. Soluble factors and cells of the innate immune system (lymphocyte-independent immunity) serve as the first-line defence against invading microbes. Dendritic cells are present in the submucosa of the nasopharynx, trachea and bronchial tree, and alveolar macrophages (AM) within the alveoli, with approximately one AM per alveolus. In addition to the immune cells in tissues, those residing in blood, i.e. monocytes and phagocytic neutrophils, are rapidly extravasated from blood when needed in defence against pulmonary infection. Blood-derived monocytes differentiate into macrophages in tissues. Monocyte and neutrophil cytokine signalling activates other immune cells. Cytokine signalling also induces the secretion of acute phase proteins as C-reactive protein (CRP). For innate immune recognition and subsequent phagocytosis and/or pro- or anti-inflammatory activation, pre-exposure is not needed, but molecular detection is based on structural divergence between procaryotes and eucaryotes. Procaryotes have conserved motifs, called pathogen-associated molecular pattern, e.g. lipopolysaccharide (LPS) and lipoteichoic acid. Immune cells recognize these through pattern recognition receptors, which are surface or cytosolic molecules, e.g. CD14, toll-like receptors (TLR), cytosolic nucleotide-binding oligomerization domain (Nod) receptors, LPS-binding protein (LBP), and mannose-

binding lectin (MBL) (Aderem and Ulevitch, 2000; Gordon and Read, 2002). Innate immune responses activate lymphocyte-dependent immunity, i.e. T- and B-cell responses.

If a pathogen is able to reach the alveoli and remains alive, it is widely dispersed onto the epithelium and may disseminate from one alveolus to another through, for instance, alveolar pores (pores of Kohn). Further, though there are effective anti-infective mechanisms to protect the organism against airborne pathogens entering the circulating blood in gas-exchanging areas, common bacteria causing pneumonia can also be detected in circulation.

1.2 *C. pneumoniae* diseases of the respiratory tract

1.2.1 Acute respiratory diseases

The outcome in host-microbe interaction, in general, is influenced by microbial and host-related factors. The outcome and possible damage vary between different microbes and hosts and between a certain microbe and its host over time, and the process keeps changing continuously (Fig. 1). (Casadevall and Pirofski, 1999). In commensalism and symbiosis, no damage to the host occurs, and in symbiosis, both benefit from the interaction. Colonization is a continuum of damage, which may progress to disease or persistence. In persistence, the host suffers continuous damage, and microbial persistence may be reactivated into overt disease. (Casadevall and Pirofski, 2000).

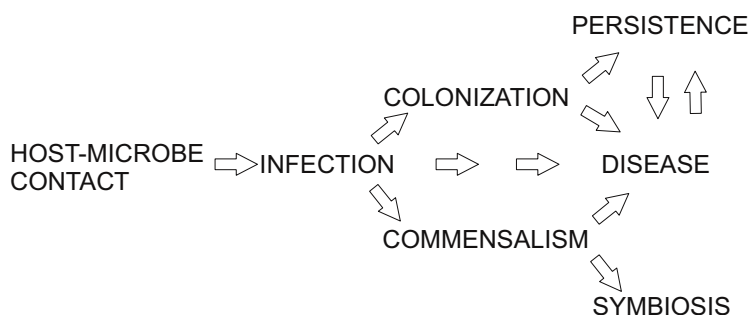


Fig. 1. Outcome of host-microbe interaction in infection. If the microbe is not eradicated, infection may result in colonization, disease, or commensalism. Colonization may further develop into persistence (chronicity/latency) and/or disease, and commensalism into symbiosis or disease if sufficient damage ensues from the interaction. Modified from (Casadevall and Pirofski, 2000).

C. pneumoniae, which spreads through the respiratory tract, is transmitted from person to person relatively inefficiently (Kleemola et al., 1988; Grayston et al., 1990; Aldous et al., 1992; Grayston et al., 1993), but it is still assumed that almost all people get infected with it at least once during their lifetime. Adults have 60-70 % seroprevalence, and men have higher seroprevalence than women (Grayston, 1992). The outcome of *C. pneumoniae*

infection in the respiratory tract may vary from asymptomatic nasopharyngeal carriage (Hammerschlag, 2004) through mild upper respiratory tract infections, sinusitis, pharyngitis, and otitis media to lower respiratory tract infections such as bronchitis (Grayston, 1992) and life-threatening pneumonia (Troy et al., 1997), and even severe systemic diseases have been reported (Grayston, 1994). As the high prevalence rates suggest, most (70 %) *C. pneumoniae* infections remain asymptomatic or mild, while a smaller proportion (30 %) result in more severe diseases (Hahn et al., 2002). Although some typical features exist, e.g. prolonged cough, (Miyashita et al., 2003) and some patient groups may be more likely to have *C. pneumoniae* disease than others, there are no specific clinical findings associated with *C. pneumoniae* respiratory infections (Grayston, 1992). One study showed that the highest predictive values for *C. pneumoniae* etiology was found in patients from nursing homes, with low CRP levels, non-productive cough, and normal urine analysis (Socan et al., 2004). In industrialized countries, community-acquired pneumonia (CAP) is the most common serious infectious disease. In Finland, the most common etiological agent of bacterial pneumonia, *Streptococcus pneumoniae*, causes 40-50 % of all CAP cases, while atypical bacteria, *Mycoplasma pneumoniae* and *C. pneumoniae*, cause 10-20 % of pneumonia cases during interepidemic periods (Jokinen et al., 2001). *C. pneumoniae* is evidently not an important cause of severe infections in small children (Chirgwin et al., 1991; Grayston, 1994), though severe pulmonary involvement has been reported to occur in otherwise healthy children and in adults (Hammerschlag, 2004), but most commonly, it causes mild pneumonias in school-aged children and healthy adolescents, e.g. college students and military conscripts (Kleemola et al., 1988; Grayston et al., 1993).

In *C. pneumoniae* disease, the damage is both pathogen- and host-mediated, and immune defence evidently plays a significant role in directing the respiratory outcome and cardiovascular dissemination. *C. pneumoniae* reinfections or recurrent infections may occur at short intervals. In fact, at least part of recurrent infections may actually be reactivations of chronic infections (Kleemola et al., 1988; Ekman et al., 1993). *C. pneumoniae* may also persist in the lungs of subjects with normal lung function (Wu et al., 2000; Kotsimbos et al., 2005). Chronic quiescent infection is possibly reactivated when appropriate, e.g. immunosuppressive, conditions emerge, as has been reported during hospitalization or in experimental animals on immunosuppressive cortisone treatment (Grayston et al., 1989a; Malinverni et al., 1995a; Laitinen et al., 1996).

1.2.2 Chronic pulmonary diseases

Evidently, persistent *C. pneumoniae* infection in the lungs contributes to several chronic pulmonary diseases (Laurila et al., 1997b), asthma and chronic obstructive pulmonary disease (COPD) being the most intensively studied. Adult-onset asthma, wheezing, asthmatic bronchitis, more severe asthma, and in asthmatic children, acute wheezing episodes and frequent exacerbations may be *C. pneumoniae*-associated (Hahn et al., 1991; Emre et al., 1994; Hahn et al., 1996; Cunningham et al., 1998; Black et al., 2000). Polymorphism of the host MBL gene seems to affect the association between *C. pneumoniae* and asthma (Nagy et al., 2003). COPD and its acute exacerbations may also be *C. pneumo-*

niae-associated (Blasi et al., 1993; von Hertzen et al., 1995; von Hertzen et al., 1996; von Hertzen et al., 1997; Wu et al., 2000). Other chronic lung processes whose serological associations with *C. pneumoniae* infection have been studied include sarcoidosis (Puolakkainen et al., 1996) and lung cancer (Laurila et al., 1997a; Jackson et al., 2000; Littman et al., 2005).

1.3 *C. pneumoniae*

1.3.1 History and taxonomy

C. pneumoniae was first found to be a human pathogen during mild pneumonia epidemics in Finland, as demonstrated by the antibody response against the first *C. pneumoniae* isolate TW-183 (Saikku et al., 1985). Later, respiratory tract-derived isolates were obtained from patients during acute respiratory tract infection (Grayston et al., 1986). The two first isolates (TW-183 and AR-39) were originally given the strain name TWAR (Grayston et al., 1986). They were serologically unique, and less pathogenic in mice than the other *C. psittaci* and *C. trachomatis* strains. Based on their morphology and staining with iodine (*C. psittaci* inclusions do not stain with iodine contrary to *C. trachomatis* inclusions), however, they were considered as *C. psittaci* (Kuo et al., 1986). Eventually, in 1989, the strain TWAR was classified as a new species based on elementary body (EB) ultrastructure (Chi et al., 1987), DNA analysis (Campbell et al., 1987; Cox et al., 1988), and serology (Kuo et al., 1986) and given the name *C. pneumoniae* (Grayston et al., 1989b).

In the order *Chlamydiales*, only the family *Chlamydiaceae* and its pathogenic *Chlamydia* species - *C. pneumoniae*, *C. psittaci* and *C. trachomatis* - were identified. Recently, environmental species with similar characteristics - obligate intracellular growth and unique developmental cycle - belonging to three more families, *Parachlamydiaceae*, *Simkaniaceae* and *Waddliaceae*, have been recognized, and the possibility of emerging pathogens within these families has been recognized (Amann et al., 1997; Kahane et al., 1998; Everett et al., 1999; Rurangirwa et al., 1999; Friedman et al., 2003). Environmental chlamydiae may have twice as large genomes as pathogenic chlamydiae, and although there is little evidence of recent lateral gene acquisition, they contain the same virulence factors as pathogenic chlamydiae (Horn et al., 2004). In 1999, based on 16S and 23S ribosomal RNA (rRNA) sequences, the family *Chlamydiaceae*, which contains the pathogenic chlamydiae, was taxonomically reclassified (Everett et al., 1999), and one genus in the former, namely *Chlamydia*, was divided into two genera: *Chlamydia* and *Chlamydophila* (Fig. 2). *C. pneumoniae* was then renamed as belonging to the genus *Chlamydophila*. However, chlamydiae are still considered to constitute a unique group of bacteria that should not yet be separated (Schachter et al., 2001), and the original name *Chlamydia pneumoniae* is used alongside the proposed new one, and actually even more frequently (PubMed, year 2004, *Chlamydia pneumoniae* or *Chlamydophila pneumoniae* as a title/abstract word: 238 vs. 42, respectively). In addition, the new classification also iden-

tified the animal pathogen species in the *Chlamydiaceae* family (Fig. 2). Recently, the previous belief that *C. pneumoniae* is only a human pathogen, has changed. As a possible disease reservoir, *C. pneumoniae* infections do occur in wild and domestic animals. Natural hosts include horse, koala and amphibians (reviewed in (Longbottom and Coulter, 2003)) as well as reptiles (Bodetti et al., 2002) and dogs (Sako et al., 2002). Varying clinical signs, including atherosclerosis, have been reported in these animals, too.

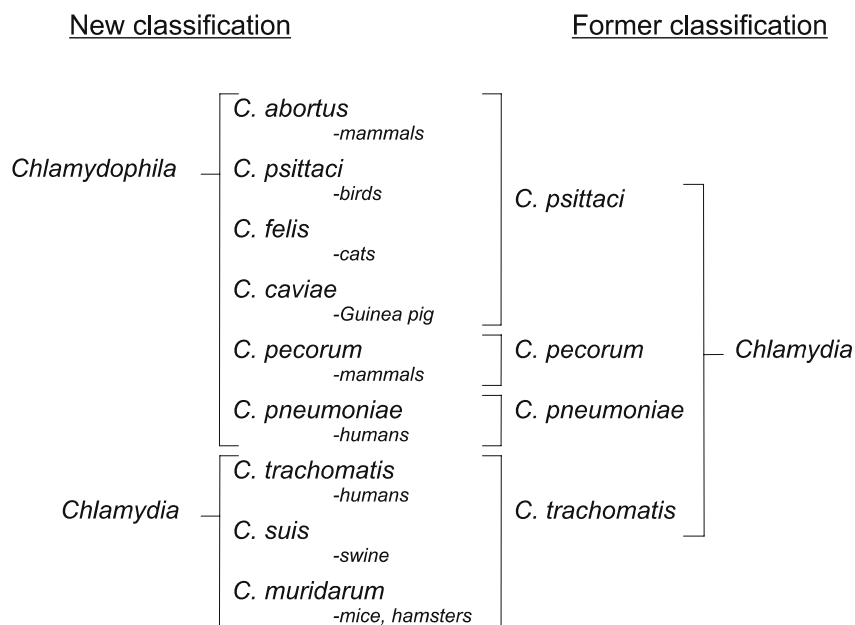


Fig. 2. New and former classification of *Chlamydiaceae*. The most common hosts for each species are listed. For details concerning the genus name of *C. pneumoniae*, see the text. Modified from (Bush and Everett, 2001).

1.3.2 Cell biology

The complete *C. pneumoniae* genome size is 1.23×10^6 base pairs (bp) (Kalman et al., 1999), and the interstrain identity between the thus far sequenced strains (AR-39, J138, CWL029 and TW-183) is $>99.9\%$ (Read et al., 2000a; Shirai et al., 2000). The chlamydial nucleoid consists of the chromosome and of two eucaryotic histone-like proteins (Hackstadt et al., 1991). Some biovars may contain a plasmid (Everett et al., 1999), and a 4524-nucleotide single-stranded DNA bacteriophage (ϕ Cpn1) has been found in the strain AR39 but not in the other strains studied (Read et al., 2000a; Read et al., 2000b). Chlamydiae are strictly intracellular bacteria that multiply within inclusions in host cells and avoid lysosomal fusion (Moulder, 1991; Al-Younes et al., 1999). Chlamydial particles are non-motile and have a large periplasmic space (Fig. 3.a.). The pointed end of the pear-shaped EB particle may be the first part to contact the host cell (Kuo et al., 1988). The cell wall of *C. pneumoniae* resembles that of gram-negative bacteria, and the outer

membrane contains LPS. Chlamydiae have genes for peptidoglycan synthesis, but the issue of whether peptidoglycan exists is controversial (Ghuysen and Goffin, 1999). Systematic genome studies of *C. pneumoniae* surface proteins (outer membrane, periplasmic and inner membrane proteins) have identified 53 proteins, which include a major outer membrane protein (MOMP)/outer membrane protein A (OmpA), PorB, a 76-kDa protein homolog, 11 proteins of the polymorphic membrane protein (Pmp) family, a cysteine-rich 60-kDa protein encoded by *omcB* (Omp3), a 9-kDa cysteine-rich outer membrane complex lipoprotein OmcA (Omp2), DnaK and Mip-like proteins, hypothetical protein, and type-three secretion system proteins (Montigiani et al., 2002), which may provide a direct connection between chlamydial particles and the host cell cytoplasm (Miyashita et al., 1993). Several molecules are involved in the attachment and subsequent endocytosis of chlamydiae, e.g. the host cell's heparan sulfate-like glycosaminoglycan (Wuppermann et al., 2001), and the cholesterol-containing lipid rafts on the host cell plasma membrane (Stuart et al., 2003). Once internalized within an inclusion, *C. pneumoniae* EBs reorganize into vegetative reticulate bodies (RBs), which multiply by binary fission during their logarithmic growth and occasionally possibly by budding (Wolf et al., 2000) (Fig. 3.b.). At the end of the cycle, RBs reorganize back into more condensed and rigid EBs, and oxidation of the outer membrane cysteine-rich proteins takes place. Both nonlytic and lytic release of infectious progeny occurs (Gieffers et al., 2002). The ATP required for *C. pneumoniae* growth is derived both from the host cell (ATP/ADP translocase) and from chlamydial *de novo* synthesis (Read et al., 2000a).

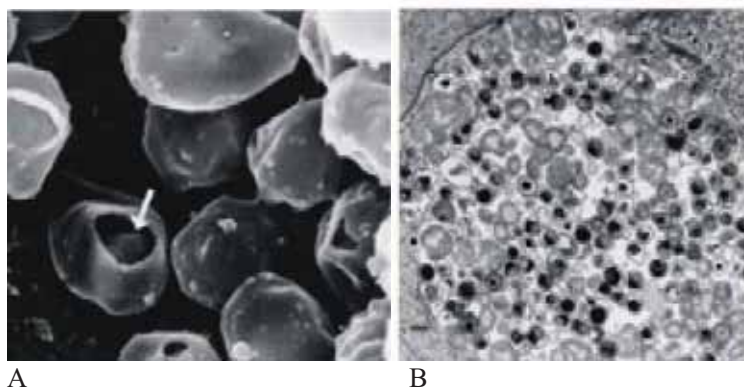


Fig. 3. A) Scanning electron micrograph of *C. pneumoniae* AR-39 EBs inside an inclusion in a HeLa cell. The arrow points to the large periplasmic space between the outer membrane and the cytoplasmic membrane of EB at the breaking point. B) Transmission electron micrograph of *C. pneumoniae* AR-39 inclusion in a HeLa cell 60 hours after infection. Both RBs and EBs are present. (Wolf et al., 2000).

1.3.3 Experimental in vivo models for C. pneumoniae infection

Mouse is most commonly used in experimental *C. pneumoniae* *in vivo* studies (Tables 1 and 2). In the first reported study, Swiss Webster mice were infected intranasally (i.n.), intrave-

nously (i.v.), or intracerebrally (i.c.), and the organism was recovered from different tissues: from the brain after an i.c. challenge, from the lungs after an i.n. challenge, and from the spleen, liver, and lungs after an i.v. challenge. In the same tissues, inflammatory reactions were detected by histology (Kuo et al., 1986). The course of mouse lung infection after i.n. inoculation during primary and reinfection was later described in more detail (Kaukoranta-Tolvanen et al., 1993; Yang et al., 1993), and i.n. infection became the most commonly used route of challenge in lung infections as well as later in atherosclerosis models. In primary infection, culture positivity in the lungs lasts for a few weeks. Inflammation, as detected histologically, persists in the lungs beyond the culture-positive period. After reinfection, the culture-positive period is shorter, and the inflammatory reaction is exaggerated. Variations occur between different mouse strains, *C. pneumoniae* isolates, and challenging doses. The use of more sensitive methods, such as polymerase chain reaction (PCR), in detecting *C. pneumoniae* in animal tissues has extended the *C. pneumoniae*-positive period to several months after the last challenge. Clinical signs in mice and other species are usually scarce, unless a high inoculation dose is given.

Table 1. *C. pneumoniae* studies in mouse models.

Study topic	Reference	Infection route	Cpn strain	Mouse strain				
Infection course	(Kuo et al., 1986)	i.n., i.c., i.v.	AR-39 TW-183	Swiss Webster				
	(Kishimoto, 1990)	i.n.	TW-183	ICR				
	(Kaukoranta-Tolvanen et al., 1993)	i.n.	TW-183	K6	Swiss Webster NIH/S			
				Helsinki 12	BALB/c			
	(Yang et al., 1993)	i.n.	AR-39		Swiss Webster ICR BALB/cAnN C57BL/6N B6C3F1 C3H/HeN			
					(Kimura, 1994)	i.n.	TW-183	IHC
					(Yang et al., 1994)	i.n.	AR-39	Swiss Webster
					(Kaukoranta-Tolvanen et al., 1995)	i.n.	K6	NIH/S
					(Yang et al., 1995)	i.n., i.v., s.c.	AR-39	Swiss Webster
					(Laitinen et al., 1996)	i.n.	K6	NIH/S
					(Moazed et al., 1998)	i.p.	AR-39	C57BL/6J
					(Erkkilä et al., 2002)	i.n.	K6	NIH/S
					(May et al., 2003)	i.v.	CM1	C57BL/6J
	(Shi et al., 2003)	i.n., i.v.	CWL-029	Icr				
	(Little et al., 2005)	i.n.	AR-39	BALB/c				

Table 1. Continued

Technique	(Erkkilä et al., 2000)	i.n.	K6	NIH/S
			K7	C57BL/6J
Atherosclerosis	(Moazed et al., 1997)	i.n.	AR-39	ApoE-KO
	(Hu et al., 1999)	i.n.	AR-39	C57BL/6J
	(Moazed et al., 1999)	i.n.	AR-39	LDLR-KO
	(Blessing et al., 2000)	i.n.	AR-39	ApoE-KO
	(Liu et al., 2000)	i.n.	AR-39	C57BL/6J
	(Liuba et al., 2000)	i.n.	AR-39	LDLR-KO
	(Aalto-Setälä et al., 2001)	i.n.	IOL 207	ApoE-KO
	(Blessing et al., 2001)	i.n.	K7	ApoE-KO
	(Burian et al., 2001a)	i.n.	AR-39	C57BL/6J
	(Burnett et al., 2001)	i.n.	TWAR	BALB/c
	(Caligiuri et al., 2001)	i.n.	na	ApoE-KO
			na	C57BL/6J
				ApoE-KO
	(Rothstein et al., 2001)	i.n.	A-03	ApoE-KO
	(Blessing et al., 2002a)	i.n.	AR-39	C57BL/6J
	(Ezzahiri et al., 2002)	i.p.	TWAR2043	ApoE3-Leiden
	(Chesebro et al., 2003)	i.n.	AR-39	eNOS-/-
				iNOS-/-
	(Ezzahiri et al., 2003)	i.p.	TWAR2043	LDLr/ApoE-/-
	(Erkkilä et al., 2004)	i.n.	K7	C57BL/6JBom
	(Sharma et al., 2004)	i.n.	AR-39	LDLR-KO
Lipids	(Tirola et al., 2002)	i.n.	K7	NIH/S
Alzheimer	(Little et al., 2004)	i.n.	96-41	BALB/c
Immunology	(Peterson et al., 1996)	i.p.	TW-183	BALB/c
				C57BL/6
	(Penttilä et al., 1998b)	i.n.	K6	NIH/S
				BALB/c
				C57BL/6J
	(Penttilä et al., 1998a)	i.n.	K6	BALB/c
	(Peterson et al., 1998)	i.n.	TW-183	Swiss Webster
			2043	
			CM-1	
	(Penttilä et al., 1999)	i.n.	K6	BALB/c
				nu/nu
	(Rottenberg et al., 1999)	i.n.	K6	C57BL/6
				BALB/c
				multiple KOs
	(Geng et al., 2000a)	i.n.	TW-183	BALB/c
				129
				G129
	(Murdin et al., 2000)	i.n.	AR-39	BALB/c
	(Pal et al., 2000)	i.n.	TW-183	129sv
				Nramp1d
	(Penttilä et al., 2000)	i.n.	K6	BALB/c
	(Rottenberg et al., 2000)	i.n.	K6	C57BL/6
				multiple KOs
	(Svanholm et al., 2000)	i.n.	K	C57BL/6
				multiple KOs

Table 1. Continued

	(Vuola et al., 2000)	i.n.	K6	BALB/c C57BL/6JBom
	(Bandholtz et al., 2002)	i.n.	K6	C57BL/6 multiple KOs
	(Airaksinen et al., 2003)	i.n.	K6	BALB/c
	(Burian et al., 2003)	i.n.	TW-183	BALB/c +KO
	(Mueller et al., 2004)	i.n.	HK	Swiss Webster C3H/HeN C3H/HeJ TLR2+/+, -/-
	(Mygind et al., 2004)	i.n.	na	C57BL/6J
	(Penttilä et al., 2004)	i.n.	K6	BALB/c
	(Rothfuchs et al., 2004)	i.n.	K6	C57BL/6 multiple KOs
Antibiotics	(Nakata et al., 1994)	i.n.	K6 IOL 207	MHC-ICR
	(Malinverni et al., 1995b)	i.n.	AR-39	Swiss Webster
	(Masson et al., 1995)	i.n.	TW-183	NIH/S BALB/c MF1 C3H/He
	(Wolf and Malinverni, 1999)	i.n.	AR-39	NMRI
	(Bin et al., 2000)	i.n.	AR-39	NMRI
	(Törmäkangas et al., 2004b)	i.n.	K7	C57BL/6J
	(Törmäkangas et al., 2004a)	i.n.	K7	C57BL/6J
	(Blessing et al., 2005)	i.n.	AR-39	ApoE-KO

Abbreviations used in this table: i.n.=intranasal; i.c.=intracranial; i.v.=intravenous; s.c.=subcutaneous; i.p.=intraperitoneal; Cpn=C. pneumoniae; na=not assessed; ApoE-KO=ApoE knockout; LDLR-KO=LDL receptor knockout.

Table 2. Other *C. pneumoniae* animal model studies.

Animal species	Reference	Infection route	Cpn strain	Study topic
Monkey	(Kuo et al., 1986)	conjunctiva	TW-183	Infection course
	(Holland et al., 1990)	several mucosal sites	TW-183	Infection course
Baboon	(Bell et al., 1989)	i.t., several mucosal sites	AR-39	Infection course
Rabbit	(Moazed et al., 1996)	i.n., i.t.	AR-39	Atherosclerosis
	(Fong et al., 1997)	nasopharynx	VR1310	Atherosclerosis
	(Laitinen et al., 1997)	i.n.	K7	Atherosclerosis
	(Muhlestein et al., 1998)	i.n.	VR1310	Atherosclerosis
	(Fong et al., 1999a)	i.n.	AR-39	Atherosclerosis
	(Fong et al., 1999b)	i.n.	VR1310	Atherosclerosis
	(Tambiah et al., 2001)	laparotomy	na	AAA
	(Coombes et al., 2002)	i.n.	na	Atherosclerosis
	(Tambiah and Powell, 2002)	laparotomy	na	AAA
	(Gieffers et al., 2004b)	i.t.	CV-6	Infection course
Pig	(Liuba et al., 2003b)	i.t.	IOL-207	Atherosclerosis
	(Pislaru et al., 2003)	i.c., i.b.	AR-39	Atherosclerosis
Rat	(Herrera et al., 2003)	i.n.	AR-39	Atherosclerosis
Hamster	(Sambri et al., 2004)	i.p., i.n.	FB/96	Infection course
	(Finco et al., 2005)	i.p.	FB/96	Immunology

Abbreviations used in this table: i.t.=intratracheal; i.n.=intranasal; i.c.=intracoronary; i.b.=intra-bronchial; na=not assessed; i.p.=intraperitoneal; Cpn=*C. pneumoniae*; AAA=abdominal aortic aneurysm.

1.4 Factors determining the persistence of *C. pneumoniae* infection in lung

1.4.1 Pulmonary inflammation

C. pneumoniae readily infects *in vitro* and *in vivo* cells from both the upper and the lower respiratory tract (Wong et al., 1992; Yang et al., 1994; Shemer-Avni and Lieberman, 1995; Alakärppä et al., 1999; Jahn et al., 2000; Meijer et al., 2000; Haralambieva et al., 2004). Deciliation of ciliated cells also occurs (Yang et al., 1994; Shemer-Avni and Lieberman, 1995). Attachment and invasion into epithelial cells activate directly e.g. nuclear factor- κ B activation/translocation and secretion of pro-inflammatory cytokines (IL-8), interferon- γ (IFN- γ), tumor necrosis factor- α (TNF- α), expression of leukocyte adhesion molecules, and subsequent enhanced polymorphonuclear (PMN) leukocyte transepithelial migration (Jahn et al., 2000; Yang et al., 2003). In mice, the expression of histidine decarboxylase, which generates histamine from histidine, is increased, especially in bronchial epithelial cells, which may have a deleterious role in *C. pneumoniae* infection

(Burian et al., 2003). The surfactant proteins A and D enhance the phagocytosis of *C. pneumoniae* (Oberley et al., 2004). In the alveoli, release of surfactant protein A by Type II pneumocytes may be inhibited during *C. pneumoniae* infection (Wissel et al., 2003).

The presence of inflammatory cells and inflammatory mediators in lungs reflect the local defense against invading *C. pneumoniae*. In mice, histology implies interstitial pneumonitis of varying severity during *C. pneumoniae* infection. The cellular picture during infection is of chronic type, i.e. it mainly consists of mononuclear cells, in peribronchial and perivascular infiltrations, although in the first few days PMN leukocytes may also infiltrate in large amounts into the bronchioles, alveolar space, and interstitium. Lymphoid follicles are also seen. (Kaukoranta-Tolvanen et al., 1993; Yang et al., 1993; Yang et al., 1994). In the *C. pneumoniae* mouse model, primary infection gives partial protection against reinfection. The role of various cell types in the overall outcome of infection or in the protection against reinfection has been studied, and T cells, both CD8+ and CD4+, were found protective (Penttilä et al., 1999; Rottenberg et al., 1999). *C. pneumoniae*-specific CD8+ have a T-helper 1 (Th1) type cytokine pattern, which is considered favorable for clearance, and they are able to lyse *C. pneumoniae*-infected cells. Multiple CD8+ cytolytic antigens/epitopes have been screened from the genome, some of which are protective (Saren et al., 2002; Wizel et al., 2002). The Th1 type also predominates against complete Pmps8, 20, and 21, and both CD8+ and CD4+ are needed for maximal responses (Mygind et al., 2004). Natural killer cell cytotoxicity does not seem to be necessary for controlling *C. pneumoniae* infection (Rottenberg et al., 2000).

The various protective effects of CD4+ cells, CD8+ cells, and macrophages seem to be mediated through IFN- γ secretion (Rothfuchs et al., 2004). IFN- γ also participates in the induction of e.g. tryptophan deprivation, inducible nitric oxide synthase (iNOS), iron deprivation, Th1/Th2 balance, and chemokine and adhesion molecule expression. In mouse pulmonary infection, IFN- γ restricts the growth of chlamydiae and histopathological changes and strengthens the Th1 type response (Penttilä et al., 1998a; Rottenberg et al., 1999; Vuola et al., 2000). Deleterious effects in *C. pneumoniae* infection and more severe disease are suggested to be mediated through delayed IFN- γ transcription and enhanced interleukin-4 (IL-4) and IL-10 transcription in lungs (shift from Th1 to Th2) (Rottenberg et al., 1999), high nitric oxide responders (Huang et al., 2002), and reduced iNOS transcription (Rottenberg et al., 1999).

1.4.2 Persistence of *C. pneumoniae*

Importantly, *C. pneumoniae* is able to resist immune defense and replicates and persists within immune cells. In addition to being able to replicate within phagocytes, macrophages, and neutrophils, *C. pneumoniae* replicates within nonphagocytic lymphocytes as well (Gaydos et al., 1996; Haranaga et al., 2001; van Zandbergen et al., 2004). Failure to clear the infection is due to several factors. The predominance of the Th2 type response in chlamydial diseases is usually associated with a failure in clearance (Holland et al., 1996). Chlamydiae may hamper the presentation of its antigens - chlamydial protease-like activity factor has been shown to degrade transcription factors and to suppress major histocompatibility complex class I and II expression (Zhong et al., 1999; Zhong et al.,

2000). Inhibition of apoptosis in several cell types, including immune cells, such as blood mononuclears and neutrophils (Geng et al., 2000b; Rajalingam et al., 2001; Airene et al., 2002; van Zandbergen et al., 2004), may also predispose to chronicity. A certain cell type, type II alveolar epithelial cells (surfactant protein-A positive cells), has been found to be the host for *C. pneumoniae* in chronic lung disease (COPD) (Rupp et al., 2004a).

In vitro, chlamydial persistence is characterized by an altered developmental cycle (reviewed in (Beatty et al., 1994b)): development of aberrant forms and interruption in the development of infective EB forms. Transcription and translation are also altered in persistent chlamydial infection (Mathews et al., 2001; Molestina et al., 2002). In persistent *C. pneumoniae* infection, the expression of genes needed for DNA replication, repair, and chromosome segregation continues, but those encoding proteins for cell division are not involved (Byrne et al., 2001). ATP is probably derived from the host cell during persistence (Gerard et al., 2002). Importantly, persistent *in vitro* chlamydial infection induced by concentrations of IFN- γ not high enough to kill can be reactivated by anti-IFN- γ treatment or by replacing IFN- γ -containing medium with medium that does not contain IFN- γ (Shemer and Sarov, 1985; Beatty et al., 1993b; Mehta et al., 1998) through reorganization of persistent forms to infective EBs (Beatty et al., 1995). In experimental animals, *C. pneumoniae* infection can be reactivated with immunosuppressive treatment (Malinverni et al., 1995a; Laitinen et al., 1996). Concerning *C. pneumoniae in vitro*, spontaneous persistence takes place in blood mononuclear cells and alveolar macrophages (Airene et al., 1999; Haranaga et al., 2003). In addition, persistence of *C. pneumoniae* can be induced by several agents *in vitro*, including IFN- γ alone or in combination with TNF- α (Summersgill et al., 1995), tobacco smoke (Wiedeman et al., 2004), and several antibiotics (Gieffers et al., 2004a).

1.4.3 Hsp60 in chlamydial persistence

In persisting chlamydial infections and associated chronic inflammation, the 60 kD Heat shock protein (Hsp), called chlamydial Hsp60 (cHsp60), is of special interest among the chlamydial antigens (Morrison et al., 1989; Toye et al., 1993). Hsps function as chaperones, and both microbial and host cells express large amounts of Hsps during infection. Both mammalian and microbial Hsps are able to induce activation of the immune system, and microbial Hsps are likely targets of the host immune response, because they may be processed along with other antigens and presented to macrophages, and they further contain conserved epitopes enabling interspecies cross-reaction (Young et al., 1988; Young and Elliott, 1989; Zügel and Kaufmann, 1999).

Chlamydiae have three Hsp60 genes (Stephens et al., 1998), and the proteins Hsp60 1-3 are encoded by the genes *groEL1* – 3 (Kalman et al., 1999). At the amino acid level, GroEL1 is the most conserved among the chlamydial species (91-98%), while GroEL2 and GroEL3 are conserved at a lower level (26-37%) (Karunakaran et al., 2003). There is a negative regulator protein HrcA, which binds to the CIRCE element found in *groEL1* containing *groE* operon (Wilson and Tan, 2002). Expression of Hsp60 (Hsp60 1) during actively replicating *C. trachomatis* infection *in vitro* occurs throughout the developmental cycle and may correlate with metabolic activity (Shaw et al., 2000). cHsp60 is detectable

inside a *C. pneumoniae* inclusion and in the culture supernatant, and it stimulates innate immune cells both *in vitro* and *in vivo* (da Costa et al., 2002; da Costa et al., 2004). During persistent IFN- γ -induced *C. trachomatis* infection, cHsp60 is found at almost normal levels compared to the low levels of, e.g., MOMP (Beatty et al., 1993a; Beatty et al., 1995). In *C. pneumoniae*, Hsp60/GroEL protein expression is also increased in persistent IFN- γ -induced infection (Molestina et al., 2002), and the Hsp60 expression of *C. pneumoniae* is similarly high in persistent alveolar macrophage infection (Haranaga et al., 2003) and in *ex vivo* COPD lung specimens (Rupp et al., 2004a). In persistent *C. trachomatis* serovar K infection, different transcription patterns were identified for the three different genes compared to active infection, and this pattern correlated with that of synovial tissue samples from *C. trachomatis* reactive arthritis patients (Gerard et al., 2004).

1.5 Association of atherosclerosis and *C. pneumoniae* infection

Although there are reports from as early as the mid-20th century focusing on the association of chlamydial (psittacosis and lymphogranuloma venereum) with arterial and cardiac diseases (Coutts and Davila, 1945; Sutton et al., 1967), the initial seroepidemiological study on the association of *C. pneumoniae* and atherosclerosis was published in 1988 (Saikku et al., 1988). Thereafter, other seroepidemiological studies as well as studies on the detection of *C. pneumoniae* in atherosclerotic lesions by electron microscopy, immunohistochemistry, PCR, and culture have been published (Shor et al., 1992; Kuo et al., 1993; Ramirez, 1996). Animal experiments revealed the development of atherosclerotic changes due to infection (Fong et al., 1997; Laitinen et al., 1997). Results from the first two small antibiotic prevention studies in humans were positive, but larger studies conducted thereafter have been controversial (Gupta et al., 1997; Gurfinkel et al., 1997; Wells et al., 2004; Anderson, 2005).

Chronic and relapsing *C. pneumoniae* infections are difficult to diagnose, which hampers association studies (Boman and Hammerschlag, 2002). In addition, studies on the effects of microbial and host gene polymorphism on the outcome of infection are just emerging. Yet, it is known that *C. pneumoniae* isolates vary in the copy number of the *tyrP* encoding an aromatic amino acid transporter - respiratory isolates have multiple copies, and cardiovascular isolates have only one copy of the gene (Gieffers et al., 2003). Diversity in *omp1* between the respiratory isolates and the coronary isolate has also been reported (Molestina et al., 1998). The presence of *C. pneumoniae* bacteriophage (ϕ Cpn1) has been associated with AAA, although ϕ Cpn1 does not contain any known virulence genes (Karunakaran et al., 2002). Certain host gene polymorphisms in the CD14 promoter with enhanced expression have been associated with *C. pneumoniae* infection (Heinemann et al., 1996; Eng et al., 2003) and, further, with chronic *C. pneumoniae* infection diagnosed by the presence of chlamydial DNA in peripheral blood monocytes of coronary artery disease (CAD) patients (Rupp et al., 2004b). In addition, IL-1 polymorphism has been linked with CAD in connection with *C. pneumoniae* infection (Momiya et al., 2001), and *C. pneumoniae* seropositivity seems to be associated with high Lipoprotein(a) levels and certain human leukocyte antigen class II genotypes (Dahlen et al., 1995).

1.5.1 Inflammation in atherogenesis

Atherosclerosis is considered a low-grade chronic inflammatory disease of the arterial wall. The hallmark of the disease is lipid accumulation into the blood vessel walls. Lipids and inflammatory markers are present in circulating blood (reviewed in (Libby et al., 2002)). Slightly elevated levels of CRP have been shown to predict subsequent cardiovascular events (Libby and Ridker, 2004). The levels of CRP and other acute phase proteins increase to various degrees in several stress conditions, e.g. ongoing infection, inflammation, and trauma (Gabay and Kushner, 1999). During *C. pneumoniae* pneumonia, CRP levels are elevated, but remain lower than in, for instance, *S. pneumoniae* pneumonia or in coinfection by these two microbes (Kauppinen et al., 1996). Overall, the aggregate number of pathogens (pathogen burden) has been associated with the degree of inflammation, the extent of atherosclerosis, and the risk for death (Zhu et al., 2000; Zhu et al., 2001; Espinola-Klein et al., 2002a; Espinola-Klein et al., 2002b).

In the development of atherosclerotic lesions, endothelial dysfunction is the initial event, which leads to increased adhesion of leukocytes to the vascular wall and to inflammation. The earliest atherosclerotic lesion type, the fatty streak, is an inflammatory lesion consisting of T lymphocytes (both CD4+ and CD8+) and monocyte-derived macrophages. Foam cells are formed from macrophages in the presence of lipids. (Fig. 4) If the inflammatory process continues, more complicated lesions evolve, i.e. intermediate lesions, with smooth muscle cell migration and proliferation in the inflammation area. In an advanced, complicated lesion, the fibrous cap overlies the lipid and necrotic tissue containing core. Such lesions expand in their shoulder region, where leukocytes adhere (Ross, 1999). For the histological identification of different lesion types, American Heart Association has published instructions for the differentiation of each lesion type (Stary et al., 1994; Stary et al., 1995; Stary, 2000). The initial fatty streak and the intermediate lesion types I, II, and III are able to regress to normality. Most clinical events occur when type VI lesions and also type IV and V lesions are present. These lesion types are non-regressive.

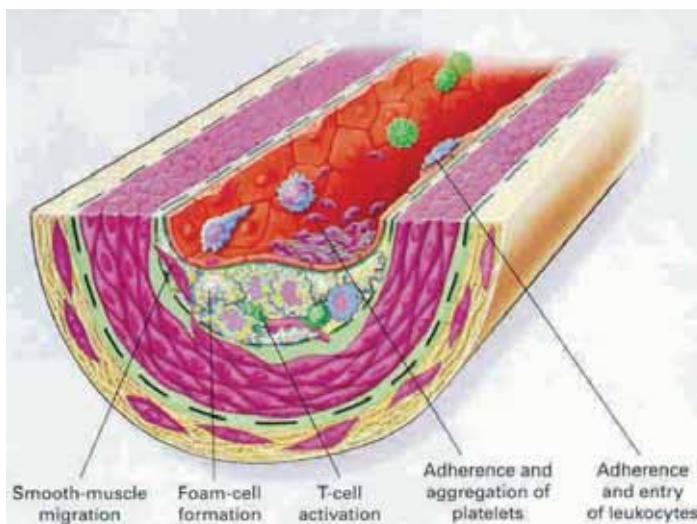


Fig. 4. Fatty streak formation in atherosclerosis development (Ross, 1999). (With permission, Copyright © 2005 Massachusetts Medical Society).

1.5.2 Mouse models for C. pneumoniae and atherosclerosis

Mice primarily have a non-atherogenic lipid profile, as most of their cholesterol is carried in high-density lipoprotein (HDL) particles. They are thus quite resistant to atherosclerosis, but disturbances of normal cholesterol metabolism lead to atherosclerotic changes. Atherosclerosis experiments have conventionally been done in wild-type mice by modifying the diet to obtain high fat and high cholesterol concentrations in the circulation. Another approach is genetical alterations of the mouse to have excess cholesterol in the circulation and altered lipid metabolism. ApoE and LDL receptor are important molecules in the clearance of harmful low-density lipoprotein (LDL) cholesterol (Breslow, 1993).

In *C. pneumoniae* mouse models, both genetic and diet-induced approaches have been used. Both extension and progression of lesions have been reported. To obtain chronic infection in mice, repeated exposures with *C. pneumoniae* are used (Grayston et al., 1985; Beatty et al., 1994a). The impact of *C. pneumoniae* infections on atherosclerotic lesions in ApoE-deficient mice has varied, but in other genetically modified mouse strains, studies have shown uniformly that repeated infections have increased either the lesion area or the severity of lesions (Table 3). Even advanced lesions develop in these genetically modified mouse strains.

Table 3. Mouse models for *C. pneumoniae* (as a sole pathogen) and atherosclerosis using genetically modified mouse strains.

Deficiency	Infection		Diet		Impact on lesions	Reference
	No	Age*	Age**	Type		
ApoE	3	8	-	chow	Increase in area	(Moazed et al., 1999)
ApoE	1-3	na	-	chow	Endoth. dysfunction	(Liuba et al., 2000)
ApoE	3, 4	8	-	chow	No effect	(Aalto-Setälä et al., 2001)
	3, 4	8	na	high fat	No effect	
ApoE	2	6	-	chow	Increase in area***	(Burnett et al., 2001)
ApoE	1, 2	6-8	-	chow	No effect	(Caligiuri et al., 2001)
ApoE	2	10	-	chow	No effect	(Rothstein et al., 2001)
	2	12	-	chow	Increase in area	
ApoE	3	7	-	chow	No effect	(Liuba et al., 2003a)
ApoE3****	2	9	5	high fat	Increase in severity	(Ezzahiri et al., 2002)
LDLR	9	4-5	-	chow	No effect	(Hu et al., 1999)
	9	4-5	na	2% chol.	Increase in area and severity	
LDLR	12	4-6	na	2% chol.	Increase in area	(Liu et al., 2000)
LDLR	4-6	4-6	2-4	2% chol	Increase in area	(Sharma et al., 2004)
ApoE+LDLR	2, 6	16	-	chow	Reduce fibrous cap	(Ezzahiri et al., 2003)
eNOS	3	8	8	high fat	No effect	(Chesebro et al., 2003)
iNOS	3	8	8	high fat	Increase in area	

* age of the mouse at the first *C. pneumoniae* challenge, in weeks

** if a modified diet was used, the age of the mouse when the diet was initiated, in weeks

*** in males only

**** transgenic mouse strain, which has ApoE3-Leiden insertion

Abbreviations used in this table: ApoE=Apolipoprotein E; LDLR=LDL receptor; eNOS=endothelial nitric oxide synthase; iNOS=inducible nitric oxide synthase; na=not assessed; chow=maintenance regular diet; chol=cholesterol.

Among several wild-type strains, the C57BL/6J strain has been shown to be most prone to lesion development when on a high-fat diet (Paigen et al., 1985), although only initial type I and fatty streak type II lesions are seen. In *C. pneumoniae* models, the high-fat diet must be initiated either before the first *C. pneumoniae* inoculation or simultaneously to it to obtain the atherogenic effect of *C. pneumoniae* (Table 4). If a wild-type C57BL/6J mouse is repeatedly infected with *C. pneumoniae* and fed a non-modified diet, only inflammatory changes in the aortic wall are induced.

Table 4. Mouse models for *C. pneumoniae* (as a sole pathogen) and atherosclerosis using wild-type mouse strains.

Mouse strain	Infection		Diet		Impact on lesions***	Reference
	No	Age*	Age**	Type		
C57BL/6J	1, 3	8	-	chow	Inflammation	(Blessing et al., 2000)
C57BL/6J	3	8	-	chow	No effect	(Blessing et al., 2001)
	3	8	8	high fat	Increase in area	
C57BL/6J	1	6-8	-	chow	No effect	(Caligiuri et al., 2001)
C57BL/6J	3	8	13, 15	high fat	No effect	(Blessing et al., 2002a)
C57BL/6J	3	8	8	high fat	Increase in area	(Chesebro et al., 2003)
C57BL/6J	3	8	6	0.2% chol.	Increase in area	(Erkkilä et al., 2004)
BALB/c***	1-3	6-8	-	chow	Inflammation	(Burian et al., 2001a)

* age of the mouse at the first *C. pneumoniae* challenge, in weeks

** if a modified diet was used, the age of the mouse when the diet was initiated, in weeks

*** the mice were γ -irradiated.

Abbreviations used in this table: chow=maintenance regular diet; chol=cholesterol.

1.5.3 Role of *C. pneumoniae* in lesion development

C. pneumoniae infection may contribute to lesion development in several stages (reviewed in (Mahony and Coombes, 2001)). Being a respiratory pathogen, *C. pneumoniae* may be directly shed into the bloodstream from the lungs or carried within infected monocytes/macrophages through bronchus-associated lymphatic tissue and lymphatics into the circulation (Yang et al., 1995; Moazed et al., 1998; Gieffers et al., 2004b). *C. pneumoniae*-infected monocytes adhere through activation of their integrin adhesion receptor system to noninfected arterial endothelial cells (Kalayoglu et al., 2001; May et al., 2003) and transfer *C. pneumoniae* to endothelial cells (Lin et al., 2000) and further to smooth muscle cells beneath the endothelial cell layer (Rupp et al., 2005), in which *C. pneumoniae* is able to multiply (Kaukoranta-Tolvanen et al., 1994; Godzik et al., 1995; Gaydos et al., 1996). In endothelial cells, *C. pneumoniae* infection induces inflammation (Kaukoranta-Tolvanen et al., 1996; Molestina et al., 1998; Krüll et al., 1999; Molestina et al., 1999; Coombes and Mahony, 2001). Cytosolic Nod proteins participate in the recognition and activation of inflammation due to intracellular *C. pneumoniae* (Opitz et al., 2005). TLR2-dependent activation in endothelial cells occurred only by extracellular *C. pneumoniae* (Opitz et al., 2005). Also, TLR4 has been reported to participate in the recognition of *C. pneumoniae* (Prebeck et al., 2001). cHsp60 and LPS may provide a source for a continuous inflammatory stimulus. Chlamydial LPS gives only a weak inflammatory stimulus, which probably serves to allow persistence. Chlamydial LPS-containing immune complexes are present in acute myocardial infarction patients (Leinonen et al., 1990). cHsp60 can be found in human atheroma, and it induces TNF- α expression (Kol et al., 1998) and activates vascular adhesion molecules as well as the production of proinflammatory IL-6 in vascular cells: endothelial cells, smooth muscle cells, and macro-

phages (Kol et al., 1999). cHsp60-responding T cells are found in the lesion (Curry et al., 2000; Mosorin et al., 2000).

Along with inflammatory activation, *C. pneumoniae* is able to induce the formation of foam cells, which is the initial step in lipid lesion development (Kalayoglu and Byrne, 1998). *Vice versa*, macrophage foam cells can be infected with *C. pneumoniae* (Blessing et al., 2002b). In addition, *C. pneumoniae* and cHsp60, but not LPS, alone were able to induce cellular oxidation of LDL in the supernatant (Kalayoglu et al., 1999).

Smooth muscle cell proliferation is an important step in lesion progression and fibrous cap formation, and *C. pneumoniae* and cHsp60 are able to trigger it (Sasu et al., 2001; Hirono et al., 2003). Although these steps lead to narrowing of the artery, they simultaneously add stability to the atherosclerotic lesion. However, *C. pneumoniae* infection as well as cHsp60 and human Hsp60 (hHsp60) may also induce events that lead to instability and fibrous cap degradation, by expression and secretion of matrix metalloproteinase, which contributes to tissue damage by proteolytic activity (Kol et al., 1998; Vehmaan-Kreula et al., 2001).

1.5.4 Hsp60 autoimmunity

1.5.4.1 Hsp60 autoimmunity in atherogenesis

The protective stress response may ultimately lead to the development of autoimmune response against the conserved epitopes of self-stress proteins. It may develop as a consequence of repeated microbial exposure and is suggested to be beneficial for the removal of defective host cells, the maintenance of a general level of protection, the first-line defence in infection, and the elimination of stressed cells infected by intracellular bacteria (reviewed by (Young and Elliott, 1989)). Autoimmunity to Hsps may, however, contribute to the pathogenesis of several chronic inflammatory diseases (Zügel and Kaufmann, 1999). Indeed, Hsp60 autoimmunity has been suggested to participate in atherogenesis (Wick et al., 1995). In humans, mycobacterial Hsp65 (mbHsp65) antibodies, which cross-react with hHsp60, were increased in patients with carotid atherosclerosis (Xu et al., 1993b; Xu et al., 1993c). Soluble Hsp60 levels in serum also strongly correlate with atherosclerosis as well as with markers of inflammation and infection (Xu et al., 2000). Parenteral mbHsp65 immunization of normocholesterolemic rabbits leads to atherosclerotic lesion development (Xu et al., 1992), and in combination with a cholesterol-rich diet, advanced lesions develop (Xu et al., 1996). MbHsp65-reactive antibodies and T cells are found in the lesions of mbHsp65-immunized animals (Xu et al., 1993a). Interestingly, mucosal administration of mbHsp65 in high-cholesterol/fat-fed LDL receptor-deficient mice decreases atherosclerotic plaques (Harats et al., 2002; Maron et al., 2002).

1.5.4.2 *Chlamydial infection and Hsp60 autoimmunity in atherogenesis*

In cHsp60, B-cell and T-cell epitopes conserved and cross-reactive with hHsp60 have been detected (Yi et al., 1993; Witkin et al., 1994). Self-Hsp60-reactive antibodies and T cells are induced in mice simultaneously immunized with cHsp60 and mouse Hsp60 (mHsp60), but not if either agent is given alone (Yi et al., 1997). In humans, cross-reactive Hsp60 antibodies exist (Domeika et al., 1998). In atherosclerosis, mbHsp65 immunity correlates with *C. pneumoniae* and *Helicobacter pylori* infections (Mayr et al., 2000), and high levels of immunoglobulin G (IgG) antibodies to both cHsp60 and hHsp60 are independent and combined risk factors for atherosclerosis (Burian et al., 2001b; Mahdi et al., 2002). In addition, cHsp60 IgG antibodies correlate with the detection of *C. pneumoniae* in human atheroma (Fong et al., 2002). For coronary risk, elevated levels of hHsp60 IgA, *C. pneumoniae* IgA and CRP are powerful predictors when present together, and especially when persistently elevated (Huittinen et al., 2002; Huittinen et al., 2003).

The mechanisms in atherogenic Hsp60 autoimmunity are proposed to act through reactivity with Hsp60 expressed on the surface of stressed endothelial cells and endothelial cytotoxicity (Xu et al., 1994; Schett et al., 1995). Hsp60 expression is increased in endothelial cells in atherosclerotic lesions in humans and in rabbits, but not in disease-free rabbit vessels (Kleindienst et al., 1993; Xu et al., 1993a). In experimental animals, the expression of Hsps in aortic endothelial cells is enhanced in stress condition, such as cholesterol-rich diet (Xu et al., 1993a), hypercholesterolemia (expression correlated with the development of lesions) (Kanwar et al., 2001), exposure to LPS (Seitz et al., 1996), fluctuation in blood flow (Hochleitner et al., 2000), or *in vitro* in monocytic cells by oxidized LDL (Frostedgard et al., 1996). *C. pneumoniae* infection in endothelial cells also upregulates Hsp60 expression (Shi and Tokunaga, 2004). Hsp65/60 antibodies that cross-react with hHsp60 and cHsp60, as well as *E. coli* GroEL, are able to mediate endothelial cytotoxicity (Mayr et al., 1999).

1.5.5 *Statins and their antimicrobial effects*

Statins are lipid-lowering drugs that are widely used to prevent cardiovascular diseases (reviewed in (Maron et al., 2000)). They competitively inhibit the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase in the mevalonate pathway of isoprenoid biosynthesis, which is also the rate-limiting enzyme in the cholesterol biosynthesis pathway. This inhibition ultimately leads to decreased LDL cholesterol levels in serum (Endo et al., 1977; Corsini et al., 1995). In addition to their lipid-lowering effect on circulating and lesional lipids, statins have other effects also considered protective against atherosclerosis and its complications, e.g. immunomodulation, reduction of cellular activation and proliferation, and improvement of endothelial function and vasomotion (reviewed in (Vaughan et al., 1996)). It has been shown that pravastatin also decreases coronary events in patients with average cholesterol levels (Sacks et al., 1996) and with slightly elevated CRP levels, and further, that they decrease elevated CRP levels (Ridker et al., 1999; Strandberg et al., 1999). Several immunomodulatory effects have been re-

ported (Kwak et al., 2000; Loike et al., 2004; Schonbeck and Libby, 2004), and both innate and acquired types of immunity are affected. *In vivo*, the influx of different leukocytes, PMN leukocytes, eosinophils, macrophages, and lymphocytes due to inflammatory stimuli is inhibited, (Diomedea et al., 2001; Sparrow et al., 2001; McKay et al., 2004; Williams et al., 2004; Yeh and Huang, 2004). Different statins may, however, have different immunomodulatory effects (Kiener et al., 2001).

Through inhibition of HMG-CoA reductase in the mevalonate pathway, statins also inhibit isoprenoid biosynthesis in eucaryotes and also in some bacteria (Hedl et al., 2002). *C. pneumoniae* does not have the HMG-CoA reductase enzyme, but *in vitro* studies have shown that cerivastatin tends to reduce infection rates in macrophages, and the secretion of monocyte chemoattractant protein-1 (MCP-1) and IL-8 decreased both in macrophages and endothelial cells (Kothe et al., 2000). Dechend *et al.* have shown that cerivastatin reduces *C. pneumoniae* infection of smooth muscle cells via infected human macrophages and blocks cell membrane-associated RhoA and Rac1 through inhibition of prenylation, reactive oxygen production, and RANTES and MCP-1 (Dechend et al., 2003). Statins also reduce the intracellular growth of *Salmonella enterica* serovar Typhimurium *in vitro* and *in vivo* in mouse (Catron et al., 2004) and inhibit *in vitro* the growth of two viruses, cytomegalovirus (Potena et al., 2004) and HIV-1. Inhibition of HIV-1 also occurred *in vivo* in infected patients (del Real et al., 2004). In a sepsis mouse model (gram-negative rods, gram-positive cocci), simvastatin treatment improved survival and reversed increased monocyte adhesion to endothelium (Merx et al., 2004), and in a LPS-induced sepsis model, cerivastatin improved survival and reduced the levels of inflammatory mediators (Ando et al., 2000). In humans, too, statin treatment has been shown to reduce mortality in bacteremic patients (Liappis et al., 2001) and to reduce the rate of severe sepsis (Almog et al., 2004).

2 Aims of the study

The present project was carried out using mouse models for *C. pneumoniae* infection. The specific aims were to analyze:

1. Technical aspects of the mouse model, i.e. the impact of the anesthetic agent on inoculating the mice and the presence of host cell remnants in the inoculum
2. Pulmonary infection and inflammation caused by different *C. pneumoniae* isolates administered via different inoculation routes and during cholesterol feeding
3. Development of cHsp60 and mHsp60 antibody responses and development of atherosclerotic lesions
4. Effects of simvastatin treatment on immune and inflammatory responses and *C. pneumoniae* growth

3 Materials and methods

3.1 Mouse model

3.1.1 *Mice and diets*

The Animal Care and Use Committee of National Public Health Institute, Helsinki, approved all procedures involving experimental animals in this project. The following mouse strains were used in the experiments: female inbred C57BL/6JBom mice (M&B A/S, Ry, Denmark) (I, III) and female outbred NIH/S mice (National Public Health Institute, Kuopio, Finland) (I, II, IV). The mice were specifically pathogen-free, and they were maintained under pathogen-free conditions and handled using Biosafety level-II precautions. The temperature, relative humidity, number of air changes, and photoperiod were automatically controlled in the animal facilities.

The mice were allowed an autoclaved or irradiated regular chow diet (Altromin 1324, Chr. Petersen A/S, Ringsted, Denmark) and water *ad libitum*. In study III, a group of mice were fed an autoclaved regular chow diet with 0.2% cholesterol supplement. In study IV, a group of mice were fed a high-fat diet consisting of 21% total fat, 0.2% cholesterol, and 19.5% casein (Harlan Teklad, Horst, The Netherlands).

3.1.2 *C. pneumoniae challenge*

The mice were given an infectious challenge i.n. under light carbon dioxide (CO₂) or methoxyflurane (Metofane; Pitman-Moore, Mundelein, IL, USA) anesthesia. Control mice were given similarly sucrose-phosphate-glutamate (SPG) solution. Two different *C. pneumoniae* isolates, which are both throat swab isolates, Kajaani 6 (K6) (I, II) or Kajaani 7 (K7) (I, III, IV), from an epidemic in Finnish military conscripts (Ekman et al., 1993) were used to

challenge the mice. Both isolates have been propagated in HL (a human epithelial cell line) cells and stored in SPG solution at -70°C (National Public Health Institute, strain collection). The infectious challenge was a crude chlamydial preparation consisting of *C. pneumoniae* particles, mainly EBs, and HL host cell remnants. The infectious dose per mouse, which was quantified directly after the challenge, varied between 1.8×10^5 and 2.0×10^6 inclusion-forming units (IFU) in different experiments.

In one experiment, a cellular preparation consisting of HL host cell remnants was given to a control group of NIH/S mice and also before or after the *C. pneumoniae* challenge (II). In study III, C57BL/6J mice were fed either a regular chow diet or a regular chow diet with 0.2% cholesterol supplementation and repeatedly infected. The 1st reinfection was given 7 weeks after the primary infection, and the 2nd reinfection was given 11 weeks after the primary infection, i.e. 4 weeks after the 1st reinfection (III). A separate experiment was also performed where a group of C57BL/6J mice were infected i.n. and another group intragastrically (i.g.). The i.g. inoculation was given under Hypnorm sedation.

3.1.3 Other treatments (IV)

In study IV, a group of NIH/S mice on a regular chow diet and a group on a high-fat diet were treated with simvastatin. Simvastatin (L-644, a gift from Merck) was dissolved in 1 % dimethylsulfoxide (1 % DMSO), and the selected daily dose (0.5 mg/kg) was given as i.p. injections (100 μl) for 24 consecutive days. Control groups of NIH/S mice were treated with the same volume of vehicle.

3.2 Detection of *C. pneumoniae*

3.2.1 Chlamydial culture (I, II, III, IV)

For the detection of infective chlamydia in different tissues of the mice, the tissue samples were mechanically homogenized (Stomacher blender) in SPG, and the supernatant of the tissue homogenate was inoculated in several volumes into either HL or Vero cells (Green monkey kidney cell line) grown in 24-well plates. After pipetting the samples into the wells, the plates were centrifuged at $550 \times g$ for 1 h, and the media were replaced with media containing 0.5 $\mu\text{g/ml}$ cycloheximide and incubated for 72 hours. Cells were fixed with methanol and stained with fluorescein isothiocyanate-conjugated chlamydia LPS-specific antibodies (Pathfinder Chlamydia Confirmation System, Kallestad Diagnostics, Chaska, MN).

3.2.2 Detection of *C. pneumoniae* DNA by PCR (II, III, IV)

For the detection of *C. pneumoniae* DNA from samples of mouse tissue, DNA was extracted using the QIAamp Tissue Kit (Qiagen GmbH) by following the manufacturer's instructions. One to 10 µl of DNA extract was used for the PCR reaction. Three different PCR protocols were used in this project. In the first PCR protocol (II), the PCR reaction was performed using the *C. pneumoniae* 16S rRNA gene-specific primers CpnA and CpnB, which result in a 463 bp product (Gaydos et al., 1992). The reaction was done in a 25 µl mixture with AmpliTaq GOLD polymerase (PE Biosystems), and the cycle number was 40. The PCR products were visualized in a 2% agarose gel electrophoresis. The second PCR protocol (III), hot-start PCR, was performed using *C. pneumoniae omp1* -specific biotinylated primers (Institute of Biotechnology, Helsinki, Finland) with AmpliTaq polymerase (Promega) and AmpliWax Gem 50 bed (PE Biosystems) in a total reaction volume of 50 µl. The cycle number was 50. The presence of a specific PCR product was confirmed by measuring time-resolved fluorescence in liquid hybridization with Eu-labeled SJ1 probes (Institute of Biotechnology) in streptavidin-coated microplates (Wallac). The third (IV) and fourth (i.g. infection experiment) PCR protocols were real-time quantitative protocols for Light Cycler machine (Roche), using *pmp4* (Mygind et al., 2001) and 16S rRNA (Gaydos et al., 1992) -specific primers and a hybridization probe in a total reaction volume of 25 µl (Reischl et al., 2003). The number of cycles was 50.

3.3 Inflammation

3.3.1 Pulmonary inflammation (II, III, IV)

Histopathological analysis was performed on lung samples. The tissue samples were fixed using phosphate-buffered formaldehyde and embedded in paraffin. Sections of 4 µm in thickness were cut from the paraffin blocks using a microtome, and the sections were stained with hematoxylin and eosin. The degree of pulmonary inflammation was assessed from the mononuclear and polymorphonuclear leukocytes present in the stained sections. Grading depended on the number of cells and the area affected. In the mild forms, the inflammatory cell infiltrates were limited to the focal areas or occurred in small scattered foci, while in the severe cases, large tissue areas were affected.

3.3.2 Circulating inflammatory markers (IV)

Serum IL-10 levels were measured using the Quantikine® M mouse IL-10 immunoassay kit according to the manufacturer's instructions (i.g. experiment, R&D Systems). Serum amyloid A (SAA) concentrations were measured using a commercial EIA method (IV, BioSource International, Camarillo, CA, USA), and serum LBP concentrations were

measured from pooled (n=6) mouse sera (HyCult Biotechnology b.v., Uden, The Netherlands).

3.4 *C. pneumoniae*-specific and autoimmune responses (II, III, IV)

The sera of mice were tested for *C. pneumoniae*-specific IgG antibodies using enzyme immunoassay (EIA). Commercial plates coated with whole *C. pneumoniae* EBs were used (AniLabsystems, Helsinki, Finland) in accordance with the manufacturer's instructions. Mouse sera were tested in 1:100 dilution, and for the detection of bound IgG antibodies, alkaline phosphatase-conjugated rabbit anti-mouse IgG (Sigma Chemical Co., St. Louis, MO, USA) diluted 1:3000 with PBS containing 10% fetal bovine serum was used.

From the lungs, using a supernatant of lung tissue homogenate, *C. pneumoniae*-specific IgA antibodies were measured using EIA and commercial plates coated with whole *C. pneumoniae* EBs. The supernatant was diluted 1:5, and for the detection of IgA antibodies, alkaline phosphatase-conjugated rabbit anti-mouse IgA (Sigma Chemical Co., St. Louis, MO, USA) diluted 1:1000 with PBS containing 10% fetal bovine serum was used.

C. pneumoniae Hsp60-specific IgG antibodies were measured. In EIA, recombinant *C. pneumoniae* Hsp60 protein produced in *Bacillus subtilis* was used as an antigen ((Airaksinen et al., 2003), kindly provided by professor Matti Sarvas, National Public Health Institute, Helsinki, Finland). The serum samples were diluted 1:1000 and bound antibodies were detected using alkaline phosphatase-conjugated anti-mouse immunoglobulin diluted in PBS-Tween (Sigma).

Serum IgG autoantibodies against mHsp60 were measured by EIA using recombinant mHsp60 protein as an antigen (StressGen Biotechnologies Corp, Canada). The serum samples were diluted 1:10, and bound antibodies were detected using alkaline phosphatase-conjugated anti-mouse immunoglobulin (Sigma).

3.5 Quantification of atherosclerotic lesions and serum lipids (III, IV)

A method of lesion quantification described by Paigen et al and computer-assisted morphometric measurement were used to quantitate lipid lesions (Paigen et al., 1987). The upper part of the heart was fixed in 10% phosphate-buffered formalin and embedded sequentially in 5%, 10%, and 25% gelatin. The sections were cut in a plane parallel to the atria. Every second of the consecutive 12-14 μm frozen sections was stained with Oil-Red-O lipid stain, and lipids were counterstained with hematoxylin and Light green. True-color digital images were captured with a ColorView 12 video camera (resolution 1300 x 1300) attached to an Olympus BX-50-F3 light microscope with 20x or 40x objectives. Every third stained section was analyzed for Oil-Red-O stainings using the analysis software (Soft Imaging System, GmbH). The number of sections (n=4) taken for statistical analyses was chosen according to the lesions that extended most widely along the ascending aorta. The data are expressed as a geometric mean of the Oil-Red-O-stained lesion areas with a 95 % confidence interval.

Serum total cholesterol and triglycerides were measured with fully enzymatic methods (Roche Diagnostics, Basel, Switzerland and Wako Chemicals GmbH, Nuess, Germany).

3.6 Statistics

To determine the statistical differences, non-parametric Mann-Whitney U-test, χ^2 test, or 2-sided Fisher's Exact Test was used. *P*-values of <0.05 were considered statistically significant.

4 Results

4.1 Technical considerations of *C. pneumoniae* mouse model

4.1.1 *Effect of anesthetic agent (I)*

Originally, in this mouse model, the mice have been immobilized with CO₂ for effective delivery of intranasal inoculation. In the present study, methoxyflurane, which is used in short- and long-term surgical anesthesia (Danneman and Mandrell, 1997), was compared to CO₂. C57BL/6J mice were inoculated i.n. with K6 isolate. In the C57BL/6J mice inoculated i.n. under methoxyflurane anesthesia, chlamydial culture findings in the lungs showed more consistent infection at the days 6 and 12 postinfection (p.i.) than in the mice that had been infected under CO₂ (I, Table 1).

4.1.2 *Effect of host cell debris in challenge inoculum (II)*

For inoculation of mice with infective *C. pneumoniae*, a crude preparation, which also contained host cell components, was used instead of highly purified EB preparations. Possible inflammatory or immune responses against host cell components, such as Hsps, were recorded. The results show that only some mild lymphocyte infiltrations were seen in the lungs, and no serum IgG response against *C. pneumoniae* or mHsp60s developed (II, Table 1).

When the mock-inoculated mice were infected 30 days later with K6, the course of infection resembled primary infection, and the chlamydial titres in the lungs were lower (day 3 p.i., median 0.7 IFU/sample log₁₀) compared to the lungs of mice which had received two K6 infections (day 3 p.i., median 1.5 IFU/sample log₁₀) (II, Table 1). PCR positivity correlated with the culture findings in each group. The inflammatory reaction in

the lungs, however, was milder compared to the primary infection without mock pre-treatment and milder than in normal *C. pneumoniae* reinfection, where the cellular inflammatory response in the lungs is fast and strong. (II, Table 1, Fig. 1). In each study group, the inflammation began to subside at day 28-30 at the latest. *C. pneumoniae* Hsp60 IgG levels rose, as did IgG antibodies against the whole EBs. No mHsp60 IgG antibodies were detected.

A group of mice were primarily infected with *C. pneumoniae*, and the culture-negative mice were inoculated 30 days later with a host cell preparation. After the host cell inoculum, reactivation of infection was detected by culture (II, Table 1). *C. pneumoniae* PCR from lung tissue was also positive, and the lung inflammation was enhanced compared to the primary mock inoculation or the mock/mock rechallenge, but was milder than after a *C. pneumoniae* reinfection (II, Table 1). In addition to showing IgG antibodies against whole *C. pneumoniae* EBs and *C. pneumoniae* Hsp60, some of these mice also developed elevated mHsp60 IgG levels (II, Fig. 2).

4.2 Pulmonary infection and inflammation

4.2.1 *Comparison of the rates of growth of two different C. pneumoniae Kajaani isolates (I)*

Both of the Kajaani isolates used in the present studies, K6 and K7, are consecutive throat swab isolates that have been propagated in HL cells. The K6 isolate has been used in mouse and K7 in rabbit studies. The two isolates were now compared in parallel, and slightly different infection courses were demonstrated by chlamydial cultures from the lungs of NIH/S mice after i.n. infection: inoculation with the K7 isolate led to higher chlamydial titers at the early stage than inoculation with the K6 isolate, while opposite results were obtained at later time points (I, Table 2).

4.2.2 *Development of pulmonary infection and inflammatory cell infiltrations after different challenging routes*

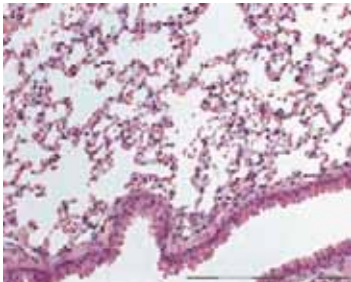
The C57BL/6J mice were infected either i.n. or i.g. After i.n. infection, chlamydia could be cultured in large amounts from the lungs, and PCR positivity was also high. The inflammation in the lungs, which was seen as patchy mononuclear cell infiltrations around the bronchi and veins, was severe and continued till the end of the follow-up. On the contrary, after i.g. infection, infective chlamydiae were not cultured from any of the tissues, including lungs, liver, and rectal swab, but PCR was positive in a few lung and liver samples (Table 5). In addition, mild lymphocyte infiltrations were detected in the lungs (bronchointerstitial pneumonia) at day 9 p.i., and they increased steadily up to day 34 p.i.

(Fig. 5). In addition, in both groups of mice, IL-10 levels in serum were slightly elevated at the early time points, and in i.g.-infected mice, a second peak occurred after the lymphocyte infiltrations at day 14 p.i. *C. pneumoniae* IgG antibodies in serum developed (OD>0.10) after i.n. infection at day 9 p.i., and in i.g.-infected mice at day 24 p.i. In the lungs, *C. pneumoniae* IgA antibodies were present after i.n. infection, but not after i.g. infection.

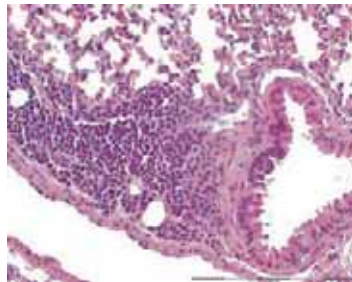
Table 5. Incidence rates (pos./studied) of C. pneumoniae PCR-positive lung and liver samples of primarily i.n. and i.g. infected C57BL/6JBom mice at different time points.

Days postinfection	Lung		Liver	
	i.n.	i.g.	i.n.	i.g.
3	6/7	0/7	7/7	2/7
6	5/7	1/7	5/7	0/7
9	5/7	0/7	4/7	0/7
14	3/7	1/7	3/7	1/7
24	6/7	3/7	2/7	0/7
34	5/7	1/3	0/7	1/7

Abbreviations used in this table: i.n.=intranasal; i.g.=intra-gastric.



A



B

Fig. 5. Hematoxylin and eosin stained lung section from A) a control mouse showing normal histopathology of C57BL/6J mouse lung, and B) from an i.g. infected mouse showing mono-nuclear cell/lymphocyte infiltrates.

4.2.3 Effect of cholesterol feeding on chronic C. pneumoniae pulmonary infection (III)

C57BL/6J mice, which were fed either a regular chow diet or a regular chow diet with 0.2% cholesterol supplementation, were repeatedly infected with K7. *C. pneumoniae* DNA could be detected by PCR at each time point, and the rate varied between the different diet groups (III, Fig. 6). After the first reinfection, the rate tended to be higher in the mice on a cholesterol-enriched diet than in those fed a regular diet, but after the second reinfection, DNA positivity was slightly lower in the mice on a cholesterol-enriched diet compared to those fed a regular diet. In the other tissues studied, i.e. hearts, aortic arches,

and abdominal aortas, *C. pneumoniae* DNA was detected intermittently, and in all, the DNA positivity was slightly higher in the group of mice fed a cholesterol-enriched diet than in the regular diet group.

In the lungs of repeatedly infected C57BL/6J mice, *C. pneumoniae* induced typical inflammatory changes, i.e. infiltrates of mononuclear cells mainly around the bronchi and veins (III, Fig. 4). These inflammatory cell infiltrates persisted up to the last time point studied, i.e. 12 weeks after the second reinfection. The cholesterol-enriched diet slightly exaggerated the inflammation at the time points after the first reinfection and up to 8 weeks after the second reinfection (III, Fig. 5).

4.3 Development of Hsp60 responses and atherosclerotic lesions

4.3.1 Chlamydial and mHsp60 responses (II, III)

When NIH/S mice were primary infected with K6 and reinfected 30 days later, the serum IgG antibody levels against *C. pneumoniae* Hsp60 started to rise at day 10 p.i. The levels further rose after the reinfection. The *C. pneumoniae* Hsp60 IgG levels correlated with the respective IgG antibody levels against whole *C. pneumoniae* EBs ($r=0.9$)(Fig. 6).

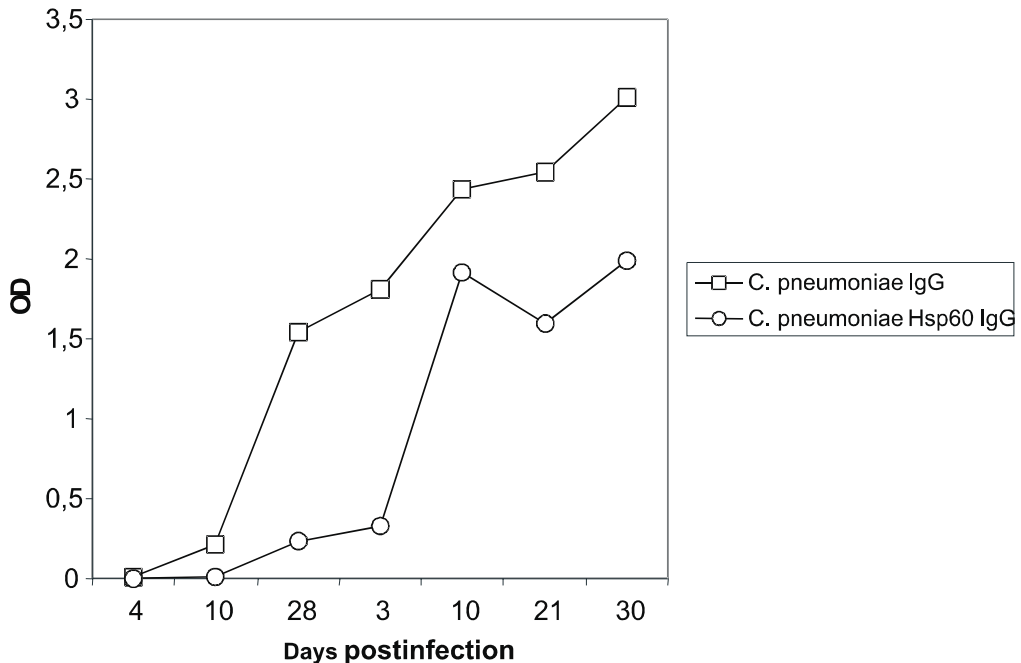


Fig. 6. *C. pneumoniae* IgG and *C. pneumoniae* Hsp60 IgG levels. NIH/S mice were primary infected and reinfected 30 days later. Different serum dilutions were used (for details, see chapter 3.4).

In the reinfected NIH/S mice, highly elevated levels (OD 405nm > 0.5) of mHsp60 IgG antibodies were also present in some individuals (II, Fig. 2). Most of the mice with elevated levels also had high *C. pneumoniae* Hsp60 antibodies present in serum. However, not all mice with high *C. pneumoniae* Hsp60 IgG levels had mHsp60 IgG antibodies in their serum. mHsp60 IgG was also found in the repeatedly infected C57BL/6J mice that were fed a regular chow diet or a regular chow diet with 0.2% cholesterol supplementation (III, Fig. 2). These IgG autoantibody levels were significantly increased in reinfected mice, and a faster increase was detected in the mice fed a cholesterol-enriched diet compared to those fed a regular diet. The cholesterol-enriched diet even alone increased the levels transiently.

4.3.2 Aortic lesions and serum lipids (III)

Foam cell lesions of two different types were detected in the aortic valve cross-sections from repeatedly infected (K7) C57BL/6J mice. One type was present exclusively in the media layers in the arterial walls between valve cusps and was not enhanced by *C. pneumoniae*. Another lesion type predominated in the subendothelium at valve cusps and resembled individual and aggregated foam cells in the intima layer (III, Fig. 3). These foam cell lesions were significantly increased by the repeated *C. pneumoniae* infections compared to SPG inoculations of the mice on a cholesterol-enriched diet; 116 μm^2 (95% CI 32-416 μm^2) versus 7 μm^2 (95% CI 1-62 μm^2), $P=0.022$, respectively. Only occasional foam cells were detected in the mice on a regular diet independently of the *C. pneumoniae* infection, 2 μm^2 (95% CI 1-5 μm^2) in both groups.

The cholesterol-enriched diet with 0.2% cholesterol supplementation did not affect the serum lipid levels, triglycerides, or total cholesterol. Nor were there differences in these circulatory lipid levels due to *C. pneumoniae* infections.

4.4 Effect of simvastatin

4.4.1 Effect of simvastatin treatment on the growth of C. pneumoniae in mouse lungs (IV)

In the simvastatin-treated NIH/S mice, infective chlamydiae were detected in decreased amounts compared to the vehicle-treated mice. These reductions (55-82%) were seen at the early stages of chlamydial infection (days 3 and 6 p.i., respectively) both in the mice fed a regular diet and in those fed with a high-fat diet (Fig. 7 and IV, Fig 2). At later time points, no marked differences in the counts of viable chlamydiae due to simvastatin treatment could be detected. By quantitative PCR, the amount of *C. pneumoniae* DNA

also decreased at same time points and to the same degree as the amount of infective chlamydiae (Fig. 7).

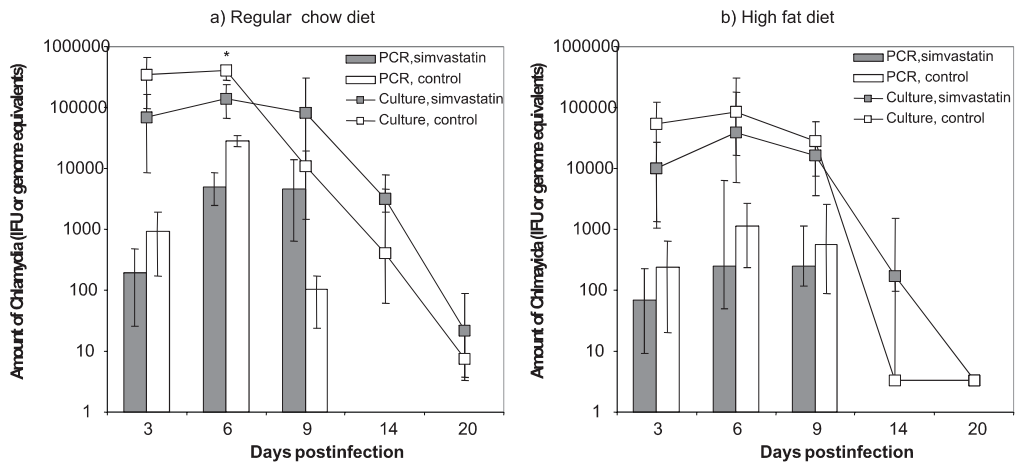


Fig. 7. Chlamydia culture (IFU) and *C. pneumoniae* PCR (genome equivalent) results from the lung tissue of mice fed a) a regular chow diet, or b) a high-fat diet. The results are shown as median±interquartile range (IQR, 25th and 75th percentile). Culture results were calculated for the whole right lung and PCR results for 8 μ l of DNA extract. * P=0.026.

Simvastatin treatment of mice did not lower the serum lipid levels of either triglycerides or total cholesterol. The high-fat diet caused a rise of the serum lipid levels. (IV, Table 1).

4.4.2 Immunomodulatory effect of simvastatin treatment (IV)

In the sera of infected mice, a moderate increase in the concentrations of acute phase proteins was seen. Both SAA and LBP levels started to rise at day 3 p.i. and were highest at day 6 p.i.. The increase of SAA was over 200-fold and that of LBP about 10-fold. Toward the days 14-20 p.i., the levels returned to the baseline. Simvastatin treatment did not affect the levels significantly.

Simvastatin treatment did not affect the *C. pneumoniae*-specific IgG antibody response, either. There was, however, a slight increase in the levels in the high-fat mouse group, but the background levels in the sera of the mice on a high-fat diet were already significantly higher at the beginning of the experiment compared to those in the sera of the mice on a regular diet, and the finding was thus considered an artefact (increased turbidity of lipemic sera). In the lungs, the effect of simvastatin treatment on the course of infection was seen as an increase of mononuclear cell infiltrates (IV, Fig. 2). In the mice fed a regular diet, at each time point, there was a slight increase in these infiltrates due to the simvastatin treatment compared to the vehicle-treated mice, and an increase in the infiltrates due to the simvastatin treatment was also seen in the mice on a high-fat diet.

5 Discussion

5.1 Technical considerations of the *C. pneumoniae* mouse model

In *C. pneumoniae* mouse models, CO₂, ketamine+xylozine, and methoxyflurane have mostly been used as anesthetizing agents. Two different anesthetics, CO₂ and methoxyflurane, were compared here in inoculating mice i.n. with *C. pneumoniae*. The use of methoxyflurane led to a more consistent distribution of bacteria in the respiratory tract in individual mice and to higher chlamydial titers. Interestingly, in a pneumococcal pneumonia mouse model, too, the use of inhaled anesthetics, methoxyflurane or halothane, in i.n. infection led to higher bacterial counts in the lungs compared to anesthesia with an i.p. injection of pentobarbital (Rubins and Charboneau, 2000). That study further showed differences in the levels of pro-inflammatory cytokines, which were not assessed in the present study. The depth of anesthesia was easily controlled with methoxyflurane, and the mice breathed normally without gasping, which is frequently seen during CO₂ anesthesia. CO₂-anesthetized mice showed more variation in culture positivity. However, the high antibody levels also seen in culture-negative mice, both CO₂- and methoxyflurane-anesthetized, indicate that they have all become infected with chlamydia (data not shown). Thus, it is possible that, due to incomplete anesthesia with CO₂ in some mice, most of the chlamydial inoculum may have remained in the upper respiratory tract, and too few organisms may have entered the lung to be detected with lung tissue culture.

When chlamydiae are grown in different hosts and tissues, the composition and infectivity of the chlamydial preparations is affected (Kuo et al., 1975; Kordova et al., 1977; Allan and Pearce, 1979; Neuman et al., 1980). As demonstrated in early human vaccination trials with *C. trachomatis*, and later in monkeys (MacDonald et al., 1984; Grayston et al., 1985), vaccination with whole organisms led to a more exaggerated disease during a natural *C. trachomatis* infection compared to nonvaccinated subjects. Although the vaccine preparations used in the above studies may have contained host components, the exaggerated disease after repeated chlamydial infections has been well documented later. The crude chlamydial preparations used in most mouse studies certainly contain more cellular contaminants than vaccine preparations, but based on the results of the present

study, the main factors associated with exaggerated pathology in the present *C. pneumoniae* mouse model are not those included in the host cell components. When mice were i.n. mock-inoculated with cellular components alone, mild inflammation was detected in the mouse lungs. In BALB/c mice, i.n. inoculation with cell components has also been reported to induce only mild histological changes in the lungs (Stack et al., 2000), but no IL-12, IFN- γ , TNF- α , IL-10, or IL-4 production (Geng et al., 2000a). In the present study, IgG antibodies against mHsp60 were not produced after mock inoculation. However, the cellular preparation consisted of HL cell components, and HL is a cell line of human origin. Thus, no mHsp60 antibodies were expected to be produced, although there is high sequence identity between the conserved epitopes of mHsp60 and hHsp60s to allow cross-reaction between these and mammalian Hsp60s. Instead, the mock inoculation may have induced local antibody production against the cellular components, and the reinoculation with the crude chlamydial preparation may have activated the immunological memory mechanisms against cell components, because a partial, although low, protective effect against consecutive *C. pneumoniae* infection was detected. The protection was also seen in histology.

An important finding was the reactivation of culture-negative *C. pneumoniae* infection with a cellular mock preparation. In this mouse model, after i.n. inoculation of the mice with a *C. pneumoniae* challenge dose containing approximately 10^6 IFUs, the lungs of NIH/S mice usually only remain culture-positive for 30 days p.i. However, it has been shown earlier that a culture-negative persistent infection in the lungs can be reactivated as culture-positive by immunosuppressive cortisone treatment (Malinverni et al., 1995a; Laitinen et al., 1996). A boosting effect of *C. trachomatis* infection on *C. pneumoniae* IgG levels, but not cultures, after two *C. pneumoniae* infections in primates has been reported (Holland et al., 1990). Mock reactivation importantly indicates that a latent infection can be reactivated by environmental stimuli in addition to immunosuppression with cortisone. Intermittent detection of *C. pneumoniae* is frequently seen in animal models with prolonged follow-up times (Moazed et al., 1997). In humans, too, periods of PCR positivity and PCR negativity have been reported (Falck et al., 1996). In addition, *C. pneumoniae* IgA reactivation has been associated with inflammation in the airways in acute asthma (Wark et al., 2002). On the other hand, it is also possible that no reactivation actually took place here, but that the chlamydia had remained viable for 34 days after the primary infection, and the culture positivity merely reflected the prolonged primary infection.

5.2 Aspects of *C. pneumoniae* pulmonary infection and inflammation

Though *C. pneumoniae* strains possess only one serotype, antigenic variation and differential expression of surface proteins occur (Black et al., 1991; Christiansen et al., 1999). Possible microbial polymorphism concerning the virulence of *C. pneumoniae* strains is still not well known. In mouse models, parallel comparison of isolates has shown different infection courses between isolates (Kaukoranta-Tolvanen et al., 1993). In the present study, a difference between two Finnish *C. pneumoniae* isolates, K6 and K7, in the recovery of viable organisms from lung tissue of NIH/S mice was demonstrated. This could be due to the small structural differences between the isolates. On the other hand, K6 and

K7 were isolated from consecutive patients during a *C. pneumoniae* epidemic in Finland, and they have been propagated in HL cells, suggesting that the isolates are closely related, and that some other factors such as the number of passages may be involved. The two isolates have not been sequenced. Thus, the conclusions concerning the possible structural or other genetic differences between the two isolates and the consequences on the course of infection are not proven but inconclusive. In addition, we do not know yet if these two Finnish isolates harbor the already identified atherogenic properties or have as yet unidentified ones. And several hypothetical proteins without known function and homologs in other organisms exist in *C. pneumoniae*, but have not yet been characterized.

C. pneumoniae disseminates into different organs after i.n., i.v., and s.c. inoculation of experimental animals. In primates, *C. pneumoniae* is detectable in the rectum of animals infected into the conjunctiva or nasopharynx (Holland et al., 1990). In the intestinal mucosal biopsies in humans, too, *C. pneumoniae* can be detected with PCR (Chen et al., 2002). The i.g. challenging route for *C. pneumoniae* in animal models has not been reported earlier, but *C. trachomatis* MoPn (*C. muridarum*), a mouse pathogen, is able to cause a persistent gastrointestinal infection/colonization in mouse (Igietseme et al., 2001). When the infectious challenge was given to the mice i.g. here, *C. pneumoniae* DNA could be detected in their liver and lungs. In addition, inflammation (lung histology, IL-10 levels) and a humoral immune response against *C. pneumoniae* (serum IgG) could be demonstrated. Contrary to *C. trachomatis* MoPn, which is able to cause a persistent gastrointestinal infection/colonization in mouse (Igietseme et al., 2001), viable chlamydiae could not be cultured from rectal swabs. It is, indeed, possible that there were too few viable chlamydiae in the gastrointestinal tract to be detected by culture from the rectum. This culture negativity of rectal swabs in the present study indicates that no autoinoculation took place. Development of peribronchial and perivascular mononuclear cellular infiltrates could indicate either the development of pulmonary infection with actively replicating *C. pneumoniae* in the lungs or the recruitment of homing lymphocytes into distinct mucosal sites, e.g. the lungs, as a function of the common mucosal system and without active pulmonary infection (Quiding-Jarbrink et al., 1997).

In C57BL/6J mice, repeated challenges with *C. pneumoniae* K7 led to persistent infection, as demonstrated by PCR and histology. DNA was present in lung tissue with variable PCR positivity rates, suggesting the insidious nature of infection or the difficulty of recovering *C. pneumoniae* DNA uniformly from tissue samples (Chernesky et al., 2002). The mice on a cholesterol-enriched diet had slightly increased PCR positivity in their heart and lung at early time points, but this effect was lost and even reversed at later time points. Previously, atherogenic high-cholesterol diet has been shown to increase the presence of *C. pneumoniae* antigen in the aorta of mice (Hu et al., 1999). The effect of cholesterol feeding on *C. pneumoniae*-induced pulmonary inflammation has not been reported earlier, though other mouse models have shown that hypercholesterolemia impairs innate immunity, suppresses T cell responses, hence increasing infectious mortality (Roselaar and Daugherty, 1998; Ludewig et al., 2001), as well as changes the Th1/Th2 balance during the development of atherosclerotic lesions in ApoE-deficient mice (Zhou et al., 1998). In the present study, the pulmonary inflammation in repeatedly infected mice was slightly intensified by a cholesterol-enriched diet during the first reinfection and in the acute phase (4-8 weeks) of the second reinfection. Thus, even though the mice in the present study were not overtly hypercholesterolemic, the findings suggest that even

a small cholesterol increment in the diet has deleterious effects on the course and immunopathology of *C. pneumoniae* infection.

5.3 Chlamydial infection and induction of immune response against Hsp60s

A clear IgG antibody response against *C. pneumoniae* Hsp60 developed in infected NIH/S mice, and the levels correlated with those against whole EBs. No comparisons with the severity of infection (culture or pulmonary inflammation) could be performed due to the small group sizes. In a *C. trachomatis* monkey model, the persistence of cHsp60 antibodies correlated with chlamydial presence, and further, high levels correlated with more severe pathology (Peeling et al., 1999).

Importantly, autoantibodies against self-Hsp60 also developed in the mice of the present study. Subcutaneous immunization of mice with heat-inactivated *Mycobacterium tuberculosis* or mbHsp65 has been found to induce formation of antibodies reacting with mbHsp65 and to accelerate atherosclerosis (George et al., 1999; Afek et al., 2000; George et al., 2000). Furthermore, the immunization of LDL receptor-deficient mice with ovalbumin (OVA) fed a cholesterol-enriched diet has also been shown to increase the levels of antibodies to mbHsp65 (Maron et al., 2002). Recently, microbial Hsp60/65 (*E. coli* GroEL, cHsp60 and mbHsp65) antibodies have been shown to bind to certain hHsp60 epitopes, which are detected in apparently normal arterial walls but even more abundantly in atherosclerotic arterial walls (Perschinka et al., 2003). Interestingly, elevated mHsp60 antibody levels were seen in mice primarily infected with *C. pneumoniae* and then challenged with either *C. pneumoniae* or mock (cell remnants), which suggests that the primary *C. pneumoniae* infection had primed the mice for the booster effect of the infection or the mock inoculation. High mHsp60 IgG levels were demonstrated in mice with high *C. pneumoniae* Hsp60 IgG levels, but not all of the mice with high cHsp60 IgG levels exhibited high mHsp60 levels. In C57BL/6J mice, too, the mHsp60-reactive IgG antibody levels were elevated after the second exposure to *C. pneumoniae*, regardless of diet, suggesting that the first infection had primed the mice to elicit a response to the host Hsp60 protein. Compared to the transient increase in mHsp60 antibodies induced by the cholesterol-enriched diet alone, the autoantibody response was prolonged when induced by *C. pneumoniae* infection. In the present study, C57BL/6J mice did not have equally high levels of mHsp60 IgG antibodies as NIH/S mice. Indeed, C57BL/6J has been shown to be a low responder to chlamydial Hsp60 and 70 proteins (Zhong and Brunham, 1992). Repeated microbial exposures starting early in life are suggested to be the reason for the generation of antibodies capable of reacting with self-epitopes in Hsps. In pathogen-free animals, self-reactive Hsp antibodies do not naturally occur (Grandia et al., 1991).

5.4 Atherosclerosis in the chronic *C. pneumoniae* infection model of C57BL/6J mice

The C57BL/6J mice repeatedly challenged with *C. pneumoniae* were chronically infected with *C. pneumoniae*, as shown by their persistent lung inflammation and the PCR positivity of different tissues for *C. pneumoniae*. The early lipid lesions, which were scattered focally in the subendothelium at the base of valve cusps in C57BL/6J mice, were significantly increased by *C. pneumoniae* infections. Indeed, lipid lesions of this kind bear some similarity to the earliest stages of atherosclerotic lesion development in human subjects (Ross, 1999). Previous mouse studies have shown that *C. pneumoniae* infection leads to accelerated atherosclerosis in C57BL/6J mice only if the mice are hypercholesterolemic, and further, only if the hypercholesterolemia has been initiated prior or simultaneously to the first *C. pneumoniae* challenge (Moazed et al., 1997; Blessing et al., 2000; Blessing et al., 2001; Blessing et al., 2002a). In the above studies, however, the low-fat diet with only cholesterol supplementation was not studied. In the present study, the feeding of mice with a 0.2 % cholesterol-supplemented diet was initiated two weeks prior to the first *C. pneumoniae* challenge, but the cholesterol homeostasis in circulation was not markedly affected, nor was hypercholesterolemia induced with this diet. The distribution of cholesterol between the lipoprotein subclasses (i.e. levels of VLDL, LDL, and HDL) was not studied. If there had been even minor changes, the data might have produced relevant information. In the cholesterol-supplemented diet, the total fat content was identical to that of the regular chow diet (4 %) used. Further, the diet did not contain a cholate supplement, which contributes to the elevation of cholesterol levels in the circulation of C57BL/6J mice, and which was included in the diets of the previous studies with hypercholesterolemic mice (Vergnes et al., 2003). It is essential in C57BL/6 mice to suppress hepatic sterol-7-hydroxylase (CYP7A1) by cholate, the major regulator of the synthesis of bile acid from cholesterol (Dueland et al., 1993), whose overexpression diminishes the progression of atherosclerosis induced by an atherogenic diet (Miyake et al., 2002). Thus, the present study suggests that a cholesterol increment in the diet, although not associated with changes in plasma total cholesterol, is needed for the accelerated development induced by the infectious burden (i.e. persistent *C. pneumoniae* infection here). The cholesterol and cholate components of an atherogenic diet have been shown in C57BL/6J mice to induce the expression of genes involved in the proatherogenic inflammatory responses, such as acute inflammatory response and fibrinogenesis (Vergnes et al., 2003). Interestingly, dietary cholesterol, without cholate, is critical in the activation of acute inflammatory genes in C57BL/6J mice (Vergnes et al., 2003). As was shown in the present study, the dietary cholesterol-supplement exaggerated the *C. pneumoniae* pulmonary infection in C57BL/6J mice. The mechanisms leading to this remain to be studied.

The effects of repeated *C. pneumoniae* infections on serum lipid levels were, in agreement with the previous studies, virtually absent in C57BL/6J mice on both diets at the time points studied several weeks after the *C. pneumoniae* challenges (Blessing et al., 2001). In the acute phase of *C. pneumoniae* infection, changes in lipid levels are present in NIH/S mice, and may thus also be present in C57BL/6J mice (Tirola et al., 2002). Also in agreement with the previous studies is the finding that *C. pneumoniae* infections do not induce atherosclerosis in C57BL/6J mice without a modification of diet (Moazed

et al., 1999; Blessing et al., 2000). The development of autoimmunity to mHsp60, as previously proposed by Wick and coworkers, may have participated in the development of early atherosclerotic lesions.

5.5 Antichlamydial effect of simvastatin

In a wider sense, statins, i.e. the lipid-lowering inhibitors of cholesterol biosynthesis, may act as inhibitors of intracellular signaling in eucaryotic cells and in the procaryotes that have HMG-CoA reductase of their own. The present study showed that the treatment of mice with lipophilic simvastatin at a dose corresponding to the therapeutic dose for treating hypercholesterolemia in humans (0.5 mg/kg) affected the course of acute *C. pneumoniae* infection by decreasing chlamydial counts and *C. pneumoniae* DNA in the lungs during the early phases of the infection. The present study does not allow interpretations of the long-term effects of statin treatment on the persistence of *C. pneumoniae* infection, which is considered important for the development of atherosclerosis.

The observed anti-chlamydial effect of simvastatin could have been mediated through a disturbance in the intracellular trafficking of cholesterol (Kempen et al., 1991; Keidar et al., 1994). Chlamydiae, and another gram-negative bacterium, *Salmonella*, which are inhibited by statin treatment (Kothe et al., 2000; Catron et al., 2004), do not have the capacity to synthesize cholesterol, but cholesterol is required for their successful multiplication in the intracellular compartment (Lange et al., 2000; Carabeo et al., 2003; Catron et al., 2004). Chlamydiae may use cholesterol of the infected cells, derived from either the extracellular space, like that derived from LDL uptake, or the intracellular cholesterol stores from *de novo* synthesis (Carabeo et al., 2003). An important step where statins may interfere with chlamydial infection is the chlamydial entry into cells, as in the case of HIV infection (del Real et al., 2004). Detergent-resistant lipid microdomains in the plasma membrane, caveolae, or lipid rafts are rich in cholesterol, and these domains have been shown to be important for the entry of several *Chlamydia* species and biovars, including *C. pneumoniae* (Stuart et al., 2003). The statin may have taken over numerous biochemical functions of host cell, which are important for chlamydial infection, through inhibition of isoprenoid biosynthesis (Kothe et al., 2000).

This inhibition may also be partly immune-mediated. In the present study, the pulmonary inflammatory response to *C. pneumoniae* infection was – opposite to most pre-existing literature reports – increased by the statin treatment of mice on both diets. The anti-inflammatory action that statins exhibit has been reported in several studies conducted in different experimental conditions. In one mouse model, oral administration of simvastatin to mice inhibited innate immunity by reducing the oedema (influx of polymorphonuclear leukocytes) of a carrageenan-induced inflammatory reaction in the mouse foot pad (Sparrow et al., 2001). In another study, atorvastatin diminished histological changes in Th1-mediated central nervous system autoimmune disease by promoting Th2 type immunity and resting Th1 type immunity, which resulted in decreased IFN- γ secretion among other things (Youssef et al., 2002). Airway inflammation in an OVA-induced asthma model has also been reported to be decreased by simvastatin treatment (McKay et al., 2004). Another group has further demonstrated that pravastatin treatment

of OVA-challenged mice reduces total cell counts and eosinophils, but not neutrophils, lymphocytes, or macrophages in the lungs (Yeh and Huang, 2004). A common feature of all these studies is the use of noninfectious inflammatory stimuli. The present study is the first to focus on the effect of statin treatment on immunological parameters after an active infection caused by a live, replicating pathogen *in vivo* under controlled experimental conditions. Kiener *et al* previously pointed out the proinflammatory effects of statins. Pretreatment of mice with simvastatin before an inflammatory challenge with thioglycollate transiently increased the influx of inflammatory cells into the peritoneal cavity (Kiener et al., 2001). In the present study, the increased inflammatory response may reflect the intensified reaction, which would be beneficial in defense against the invading pathogen. Statins also induce apoptosis, and thus, in simvastatin treated mice, *C. pneumoniae* liberated from infected cells after apoptosis could have been more extensively exposed to immune defense. In general, SAA levels parallel those of CRP. SAA binds to HDL and may affect cholesterol metabolism and also contribute to the oxidation of LDL (reviewed in (Gabay and Kushner, 1999)). In the present study, both SAA and LBP levels increased moderately and returned to baseline, but simvastatin therapy did not modulate this acute phase response during primary infection.

In mice, a cholesterol-lowering effect of statins has been achieved with high treatment doses even in individuals lacking functional low-density lipoprotein receptors (Bisgaier et al., 1997). However, with the simvastatin dose used in the present study, no decrease in the total cholesterol or triglyceride levels either in the mice on a regular diet or in those on a high-fat diet was seen. The response to a high-fat diet was as expected, and elevated levels of serum total cholesterol and triglycerides were observed.

6 Conclusions and future prospects

The present study highlighted the importance of the appropriate performance of the infectious challenge by comparing the different anesthetic agents used to inoculate animals. It also showed that the host cell debris in *C. pneumoniae* inoculum may possibly have some contribution to the infection model: inoculation with host cell debris alone induced a weak inflammatory response and reactivated culture-negative *C. pneumoniae* infection in the lungs.

When investigating the lung histopathology of mice infected via different routes, it turned out that either *C. pneumoniae* was able to disseminate from the gastrointestinal tract into the lungs through circulation and/or lymphatics or the lymphocytes that had encountered gastrointestinal tract-derived *C. pneumoniae* were homing into the lungs.

When two different Finnish *C. pneumoniae* isolates, K6 and K7, were used in parallel in the present study, the courses of pulmonary infection differed slightly.

The present results further indicated that repeated inoculations led to the development of a Hsp60 autoimmune response, and further, without overt hypercholesterolemia, repeated *C. pneumoniae* infections enhanced the development of early atherosclerosis lesions, offering an experimental model to study chronic *C. pneumoniae* infection and early atherosclerotic events. It was also shown that cholesterol feeding alone transiently increased Hsp60 autoimmune antibodies, but when it was combined with repeated *C. pneumoniae* infections, a stronger response developed than with either *C. pneumoniae* or cholesterol feeding alone. Thus, even a small increment of dietary cholesterol alone may induce the formation of antibodies to mHsp60, but for persistent Hsp60 autoimmunity, the presence of cHsp60 in the stressed cells may possibly also be needed. Further, the chronic pulmonary inflammation due to *C. pneumoniae* was shown to be exaggerated in mice on a cholesterol-supplemented diet.

In the present study, treatment of mice with simvastatin decreased chlamydial growth, as has been seen in previous *in vitro* studies using cerivastatin. The statin used in the present study is lipophilic, like cerivastatin, and thus able to access cells in which *C. pneumoniae* is able to multiply. Simvastatin treatment also exerted an immunomodulatory effect, but an unexpected one, by increasing the amount of inflammatory cells in lungs.

Further questions arise from the present findings. A cellular preparation consisting only of host cells was shown to reactivate quiescent *C. pneumoniae* infection in the lungs.

We can speculate that other microbial infections or any immunological stimulus might also have a similar reactivating effect on chronic infection, thus accelerating the development of chronic sequelae such as atherosclerosis. Further animal studies using other microbial challenges after established chronic *C. pneumoniae* infection are needed to find answers to these questions.

The experimental *C. pneumoniae* mouse models developed to study infection-induced atherosclerosis are still poorly characterized. The application of genomics and proteomics to study microbe-host interaction during the development of chronic infection in mouse models will provide valuable information and allow comparisons with *C. pneumoniae* infection in human.

The interesting finding on the antichlamydial effect of simvastatin in acute infection should be expanded to a chronic infection model as well as to studies on the markers of systemic inflammation during chronic *C. pneumoniae* infection. These studies will hopefully provide us with the information of whether the beneficial effect of statins on cardiovascular diseases may, at least partly, be explained by their antichlamydial (and more generally, antimicrobial) effects.

7 Acknowledgments

This work was carried out at the Department of Viral Diseases and Immunology at National Public Health Institute, Helsinki and Oulu, between 1997 and 2005. I would like to thank Professor Jussi Huttunen, the former head of the Institute, and Professor Pekka Puska, the present Director General, for providing excellent working facilities. I also thank Research Professor Tapani Hovi, the head of the Department of Viral Disease and Immunology, for friendly and constructive comments concerning the studies.

I am grateful to Professor Timo Korhonen, head of the Division of General Microbiology in the University of Helsinki for graduate education, and Emeritus Professor Pekka Saikku and Professor Olli Vainio, the former and the present head of the Department of Medical Microbiology in the University of Oulu, for postgraduate education.

I am most grateful to my three supervisors, Research Professor Maija Leinonen, Emeritus Professor Pekka Saikku, and Docent Kirsi Laitinen. I thank them for continuous and encouraging support and guidance and enthusiasm. And I appreciate their extensive knowledge concerning chlamydiae and animal experiments.

I thank all collaborators and co-authors for sharing their expertise during these studies, Docent Katriina Aalto-Setälä, Kristiina Haasio, Docent Matti Jauhiainen, Docent Aino Laurila, Professor Hans-Anton Lehr, Martin Rottenberg, Elise Saario and Terttu Tiirola. I especially want to thank Matti for his contribution in studies III and IV, and enthusiastic discussions concerning lipid metabolism. I also want to thank Terttu for friendship and good sense of humour. There haven't been many discussions without laughing. In addition, I want to express my appreciation to Hans-Anton Lehr for giving me the opportunity to visit his laboratory and introducing to me the histopathology of atherosclerosis.

I acknowledge Aini Bloigu for her assistance with the statistical work during the studies. In addition, I wish to thank Academician Pirjo Mäkelä, Docent Mirja Puolakkainen, Emeritus Professor Matti Sarvas and Jenni Vuola for giving valuable comments for paper I, and Docent Sylvi Silvennoinen-Kassinen for paper IV.

I also want to thank Liisa Pyhälä for providing excellent facilities for performing animal infection experiments at National Public Health Institute, and the staff in the facilities for helping during the experiments.

I am grateful to the official reviewers of this study, Professor Petri Kovanen, the head of Wihuri Research Institute, and Professor Matthias Maass from the University of

Lübeck, for their quick and constructive reviewing of this thesis. I am also grateful to Sirkka-Liisa Leinonen for efficiently revising the English language of this thesis and all the studies included.

I also wish to thank Docent Markku Koskela for his contribution concerning my education in the field of clinical microbiology.

I wish to thank all the staff at KTL in Oulu for their support. I am especially indebted to my friends and colleagues Tiina Sävykoski, Liisa Törmäkangas, Annika Saukkoriipi, Mika Paldanius, and Taina Korhonen. I wish also thank Carola Andersson-Parkkonen, Terttu Korpela, Maija Holtinkoski, Eeva-Liisa Korhonen, Leena Kuisma, and Anu Ojala for their expert assistance in these studies.

I warmly thank all my friends and family for their important support and interest towards my work, and all the happy events. My warmest thanks belong to Pekka, for his continuous support, unreserved faith and the joy he brings to my life.

Oulu, September 2005

Leena Erkkilä

8 References

- Aalto-Setälä, K., Laitinen, K., Erkkilä, L., Leinonen, M., Jauhiainen, M., Ehnholm, C., Tamminen, M., Puolakkainen, M., Penttilä, I., and Saikku, P. (2001). *Chlamydia pneumoniae* does not increase atherosclerosis in the aortic root of apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol* 21, 578-584.
- Aderem, A., and Ulevitch, R. J. (2000). Toll-like receptors in the induction of the innate immune response. *Nature* 406, 782-787.
- Afek, A., George, J., Gilburd, B., Rauova, L., Goldberg, I., Kopolovic, J., Harats, D., and Shoenfeld, Y. (2000). Immunization of low-density lipoprotein receptor deficient (LDL-RD) mice with heat shock protein 65 (HSP-65) promotes early atherosclerosis. *J Autoimmun* 14, 115-121.
- Airaksinen, U., Penttilä, T., Wahlström, E., Vuola, J. M., Puolakkainen, M., and Sarvas, M. (2003). Production of *Chlamydia pneumoniae* proteins in *Bacillus subtilis* and their use in characterizing immune responses in the experimental infection model. *Clin Diagn Lab Immunol* 10, 367-375.
- Airenne, S., Surcel, H. M., Alakärppä, H., Laitinen, K., Paavonen, J., Saikku, P., and Laurila, A. (1999). *Chlamydia pneumoniae* infection in human monocytes. *Infect Immun* 67, 1445-1449.
- Airenne, S., Surcel, H. M., Tuukkanen, J., Leinonen, M., and Saikku, P. (2002). *Chlamydia pneumoniae* inhibits apoptosis in human epithelial and monocyte cell lines. *Scand J Immunol* 55, 390-398.
- Alakärppä, H., Surcel, H. M., Laitinen, K., Juvonen, T., Saikku, P., and Laurila, A. (1999). Detection of *Chlamydia pneumoniae* by colorimetric in situ hybridization. *Apmis* 107, 451-454.
- Aldous, M. B., Grayston, J. T., Wang, S. P., and Foy, H. M. (1992). Seroepidemiology of *Chlamydia pneumoniae* TWAR infection in Seattle families, 1966-1979. *J Infect Dis* 166, 646-649.
- Allan, I., and Pearce, J. H. (1979). Host modification of chlamydiae: differential infectivity for cell monolayers of chlamydiae grown in eggs and monolayers. *J Gen Microbiol* 112, 53-59.
- Almog, Y., Shefer, A., Novack, V., Maimon, N., Barski, L., Eizinger, M., Friger, M., Zeller, L., and Danon, A. (2004). Prior statin therapy is associated with a decreased rate of severe sepsis. *Circulation* 110, 880-885.

- Al-Younes, H. M., Rudel, T., and Meyer, T. F. (1999). Characterization and intracellular trafficking pattern of vacuoles containing *Chlamydia pneumoniae* in human epithelial cells. *Cell Microbiol* 1, 237-247.
- Amann, R., Springer, N., Schönhuber, W., Ludwig, W., Schmid, E. N., Müller, K. D., and Michel, R. (1997). Obligate intracellular bacterial parasites of acanthamoebae related to *Chlamydia* spp. *Appl Environ Microbiol* 63, 115-121.
- Anderson, J. L. (2005). Infection, antibiotics, and atherothrombosis--end of the road or new beginnings? *N Engl J Med* 352, 1706-1709.
- Ando, H., Takamura, T., Ota, T., Nagai, Y., and Kobayashi, K. (2000). Cerivastatin improves survival of mice with lipopolysaccharide-induced sepsis. *J Pharmacol Exp Ther* 294, 1043-1046.
- Bandholtz, L., Kreuger, M. R., Svanholm, C., Wigzell, H., and Rottenberg, M. E. (2002). Adjuvant modulation of the immune responses and the outcome of infection with *Chlamydia pneumoniae*. *Clin Exp Immunol* 130, 393-403.
- Beatty, W. L., Byrne, G. I., and Morrison, R. P. (1993a). Morphologic and antigenic characterization of interferon gamma-mediated persistent *Chlamydia trachomatis* infection in vitro. *Proc Natl Acad Sci U S A* 90, 3998-4002.
- Beatty, W. L., Byrne, G. I., and Morrison, R. P. (1993b). Morphologic and antigenic characterization of interferon gamma-mediated persistent *Chlamydia trachomatis* infection in vitro. *Proc Natl Acad Sci U S A* 90, 3998-4002.
- Beatty, W. L., Byrne, G. I., and Morrison, R. P. (1994a). Repeated and persistent infection with *Chlamydia* and the development of chronic inflammation and disease. *Trends Microbiol* 2, 94-98.
- Beatty, W. L., Morrison, R. P., and Byrne, G. I. (1994b). Persistent chlamydiae: from cell culture to a paradigm for chlamydial pathogenesis. *Microbiol Rev* 58, 686-699.
- Beatty, W. L., Morrison, R. P., and Byrne, G. I. (1995). Reactivation of persistent *Chlamydia trachomatis* infection in cell culture. *Infect Immun* 63, 199-205.
- Bell, T. A., Kuo, C. C., Wang, S. P., and Grayston, J. T. (1989). Experimental infection of baboons (*Papio cynocephalus anubis*) with *Chlamydia pneumoniae* strain 'TWAR'. *J Infect* 19, 47-49.
- Bin, X. X., Wolf, K., Schaffner, T., and Malinverni, R. (2000). Effect of azithromycin plus rifampin versus amoxicillin alone on eradication and inflammation in the chronic course of *Chlamydia pneumoniae* pneumonitis in mice. *Antimicrob Agents Chemother* 44, 1761-1764.
- Bisgaier, C. L., Essenburg, A. D., Auerbach, B. J., Pape, M. E., Sekerke, C. S., Gee, A., Wollé, S., and Newton, R. S. (1997). Attenuation of plasma low density lipoprotein cholesterol by select 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors in mice devoid of low density lipoprotein receptors. *J Lipid Res* 38, 2502-2515.
- Black, C. M., Johnson, J. E., Farshy, C. E., Brown, T. M., and Berdal, B. P. (1991). Antigenic variation among strains of *Chlamydia pneumoniae*. *J Clin Microbiol* 29, 1312-1316.
- Black, P. N., Scicchitano, R., Jenkins, C. R., Blasi, F., Allegra, L., Włodarczyk, J., and Cooper, B. C. (2000). Serological evidence of infection with *Chlamydia pneumoniae* is related to the severity of asthma. *Eur Respir J* 15, 254-259.

- Blasi, F., Legnani, D., Lombardo, V. M., Negretto, G. G., Magliano, E., Pozzoli, R., Chiodo, F., Fasoli, A., and Allegra, L. (1993). *Chlamydia pneumoniae* infection in acute exacerbations of COPD. *Eur Respir J* 6, 19-22.
- Blessing, E., Campbell, L. A., Rosenfeld, M. E., Chesebro, B., and Kuo, C. C. (2005). A 6 week course of azithromycin treatment has no beneficial effect on atherosclerotic lesion development in apolipoprotein E-deficient mice chronically infected with *Chlamydia pneumoniae*. *J Antimicrob Chemother.* 55, 1037-1040
- Blessing, E., Campbell, L. A., Rosenfeld, M. E., Chough, N., and Kuo, C. C. (2001). *Chlamydia pneumoniae* infection accelerates hyperlipidemia induced atherosclerotic lesion development in C57BL/6J mice. *Atherosclerosis* 158, 13-17.
- Blessing, E., Campbell, L. A., Rosenfeld, M. E., and Kuo, C. C. (2002a). *Chlamydia pneumoniae* and Hyperlipidemia Are Co-Risk Factors for Atherosclerosis: Infection Prior to Induction of Hyperlipidemia Does Not Accelerate Development of Atherosclerotic Lesions in C57BL/6J Mice. *Infect Immun* 70, 5332-5334.
- Blessing, E., Kuo, C. C., Lin, T. M., Campbell, L. A., Bea, F., Chesebro, B., and Rosenfeld, M. E. (2002b). Foam cell formation inhibits growth of *Chlamydia pneumoniae* but does not attenuate *Chlamydia pneumoniae*-induced secretion of proinflammatory cytokines. *Circulation* 105, 1976-1982.
- Blessing, E., Lin, T. M., Campbell, L. A., Rosenfeld, M. E., Lloyd, D., and Kuo, C. (2000). *Chlamydia pneumoniae* induces inflammatory changes in the heart and aorta of normocholesterolemic C57BL/6J mice. *Infect Immun* 68, 4765-4768.
- Bodetti, T. J., Jacobson, E., Wan, C., Hafner, L., Pospischil, A., Rose, K., and Timms, P. (2002). Molecular evidence to support the expansion of the hostrange of *Chlamydo-philum pneumoniae* to include reptiles as well as humans, horses, koalas and amphibians. *Syst Appl Microbiol* 25, 146-152.
- Boman, J., and Hammerschlag, M. R. (2002). *Chlamydia pneumoniae* and atherosclerosis: critical assessment of diagnostic methods and relevance to treatment studies. *Clin Microbiol Rev* 15, 1-20.
- Breslow, J. L. (1993). Transgenic mouse models of lipoprotein metabolism and atherosclerosis. *Proc Natl Acad Sci U S A* 90, 8314-8318.
- Burian, K., Berencsi, K., Endresz, V., Gyulai, Z., Valyi-Nagy, T., Valyi-Nagy, I., Bakay, M., Geng, Y., Virok, D., Kari, L., *et al.* (2001a). *Chlamydia pneumoniae* exacerbates aortic inflammatory foci caused by murine cytomegalovirus infection in normocholesterolemic mice. *Clin Diagn Lab Immunol* 8, 1263-1266.
- Burian, K., Hegyesi, H., Buzas, E., Endresz, V., Kis, Z., Falus, A., and Gonczol, E. (2003). *Chlamydo-philum (Chlamydia) pneumoniae* induces histidine decarboxylase production in the mouse lung. *Immunol Lett* 89, 229-236.
- Burian, K., Kis, Z., Virok, D., Endresz, V., Prohaszka, Z., Duba, J., Berencsi, K., Boda, K., Horvath, L., Romics, L., *et al.* (2001b). Independent and joint effects of antibodies to human heat-shock protein 60 and *Chlamydia pneumoniae* infection in the development of coronary atherosclerosis. *Circulation* 103, 1503-1508.
- Burnett, M. S., Gaydos, C. A., Madico, G. E., Glad, S. M., Paigen, B., Quinn, T. C., and Epstein, S. E. (2001). Atherosclerosis in apoE knockout mice infected with multiple pathogens. *J Infect Dis* 183, 226-231.
- Bush, R. M., and Everett, K. D. (2001). Molecular evolution of the *Chlamydiaceae*. *Int J Syst Evol Microbiol* 51, 203-220.

- Byrne, G. I., Ouellette, S. P., Wang, Z., Rao, J. P., Lu, L., Beatty, W. L., and Hudson, A. P. (2001). *Chlamydia pneumoniae* expresses genes required for DNA replication but not cytokinesis during persistent infection of HEp-2 cells. *Infect Immun* 69, 5423-5429.
- Caligiuri, G., Rottenberg, M., Nicoletti, A., Wigzell, H., and Hansson, G. K. (2001). *Chlamydia pneumoniae* infection does not induce or modify atherosclerosis in mice. *Circulation* 103, 2834-2838.
- Campbell, L. A., Kuo, C. C., and Grayston, J. T. (1987). Characterization of the new *Chlamydia* agent, TWAR, as a unique organism by restriction endonuclease analysis and DNA-DNA hybridization. *J Clin Microbiol* 25, 1911-1916.
- Carabeo, R. A., Mead, D. J., and Hackstadt, T. (2003). Golgi-dependent transport of cholesterol to the *Chlamydia trachomatis* inclusion. *Proc Natl Acad Sci U S A* 100, 6771-6776.
- Casadevall, A., and Pirofski, L. A. (1999). Host-pathogen interactions: redefining the basic concepts of virulence and pathogenicity. *Infect Immun* 67, 3703-3713.
- Casadevall, A., and Pirofski, L. A. (2000). Host-pathogen interactions: basic concepts of microbial commensalism, colonization, infection, and disease. *Infect Immun* 68, 6511-6518.
- Catron, D. M., Lange, Y., Borensztajn, J., Sylvester, M. D., Jones, B. D., and Haldar, K. (2004). *Salmonella enterica* serovar Typhimurium requires nonsterol precursors of the cholesterol biosynthetic pathway for intracellular proliferation. *Infect Immun* 72, 1036-1042.
- Chen, W., Li, D., Wilson, I., and Chadwick, V. S. (2002). Detection of *Chlamydia pneumoniae* by polymerase chain reaction-enzyme immunoassay in intestinal mucosal biopsies from patients with inflammatory bowel disease and controls. *J Gastroenterol Hepatol* 17, 987-993.
- Chernesky, M., Smieja, M., Schachter, J., Summersgill, J., Schindler, L., Solomon, N., Campbell, K., Campbell, L., Cappuccio, A., Gaydos, C., *et al.* (2002). Comparison of an industry-derived LCx *Chlamydia pneumoniae* PCR research kit to in-house assays performed in five laboratories. *J Clin Microbiol* 40, 2357-2362.
- Chesebro, B. B., Blessing, E., Kuo, C. C., Rosenfeld, M. E., Puolakkainen, M., and Campbell, L. A. (2003). Nitric oxide synthase plays a role in *Chlamydia pneumoniae*-induced atherosclerosis. *Cardiovasc Res* 60, 170-174.
- Chi, E. Y., Kuo, C. C., and Grayston, J. T. (1987). Unique ultrastructure in the elementary body of *Chlamydia* sp. strain TWAR. *J Bacteriol* 169, 3757-3763.
- Chirgwin, K., Roblin, P. M., Gelling, M., Hammerschlag, M. R., and Schachter, J. (1991). Infection with *Chlamydia pneumoniae* in Brooklyn. *J Infect Dis* 163, 757-761.
- Christiansen, G., Boesen, T., Hjerno, K., Daugaard, L., Mygind, P., Madsen, A. S., Knudsen, K., Falk, E., and Birkelund, S. (1999). Molecular biology of *Chlamydia pneumoniae* surface proteins and their role in immunopathogenicity. *Am Heart J* 138, S491-495.
- Coombes, B. K., Chiu, B., Fong, I. W., and Mahony, J. B. (2002). *Chlamydia pneumoniae* Infection of Endothelial Cells Induces Transcriptional Activation of Platelet-Derived Growth Factor-B: A Potential Link to Intimal Thickening in a Rabbit Model of Atherosclerosis. *J Infect Dis* 185, 1621-1630.
- Coombes, B. K., and Mahony, J. B. (2001). cDNA array analysis of altered gene expression in human endothelial cells in response to *Chlamydia pneumoniae* infection. *Infect Immun* 69, 1420-1427.

- Corsini, A., Maggi, F. M., and Catapano, A. L. (1995). Pharmacology of competitive inhibitors of HMG-CoA reductase. *Pharmacol Res* 31, 9-27.
- Coutts, W. E., and Davila, M. (1945). Lymphogranuloma Venereum as a possible cause of arterio-sclerosis and other arterial conditions. *J Trop Med Hyg* 48, 46-51.
- Cox, R. L., Kuo, C. C., Grayston, J. T., and Campbell, L. A. (1988). Deoxyribonucleic acid relatedness of *Chlamydia* sp. strain TWAR to *Chlamydia trachomatis* and *Chlamydia psittaci*. *Int J Syst Bacteriol* 38, 265-268.
- Cunningham, A. F., Johnston, S. L., Julious, S. A., Lampe, F. C., and Ward, M. E. (1998). Chronic *Chlamydia pneumoniae* infection and asthma exacerbations in children. *Eur Respir J* 11, 345-349.
- Curry, A. J., Portig, I., Goodall, J. C., Kirkpatrick, P. J., and Gaston, J. S. (2000). T lymphocyte lines isolated from atheromatous plaque contain cells capable of responding to *Chlamydia* antigens. *Clin Exp Immunol* 121, 261-269.
- da Costa, C. P., Kirschning, C. J., Busch, D., Dürr, S., Jennen, L., Heinzmann, U., Prebeck, S., Wagner, H., and Miethke, T. (2002). Role of chlamydial heat shock protein 60 in the stimulation of innate immune cells by *Chlamydia pneumoniae*. *Eur J Immunol* 32, 2460-2470.
- da Costa, C. U. P., Wantia, N., Kirschning, C. J., Busch, D. H., Rodriguez, N., Wagner, H., and Miethke, T. (2004). Heat shock protein 60 from *Chlamydia pneumoniae* elicits an unusual set of inflammatory responses via Toll-like receptor 2 and 4 in vivo. *Eur J Immunol* 34, 2874-2884.
- Dahlen, G. H., Boman, J., Birgander, L. S., and Lindblom, B. (1995). Lp(a) lipoprotein, IgG, IgA and IgM antibodies to *Chlamydia pneumoniae* and HLA class II genotype in early coronary artery disease. *Atherosclerosis* 114, 165-174.
- Danneman, P. J., and Mandrell, T. D. (1997). Evaluation of five agents/methods for anesthesia of neonatal rats. *Lab Anim Sci* 47, 386-395.
- Dechend, R., Gieffers, J., Dietz, R., Joerres, A., Rupp, J., Luft, F. C., and Maass, M. (2003). Hydroxymethylglutaryl coenzyme A reductase inhibition reduces *Chlamydia pneumoniae*-induced cell interaction and activation. *Circulation* 108, 261-265.
- del Real, G., Jimenez-Baranda, S., Mira, E., Lacalle, R. A., Lucas, P., Gomez-Mouton, C., Alegret, M., Pena, J. M., Rodriguez-Zapata, M., Alvarez-Mon, M., *et al.* (2004). Statins inhibit HIV-1 infection by down-regulating Rho activity. *J Exp Med* 200, 541-547.
- Diomede, L., Albani, D., Sottocorno, M., Donati, M. B., Bianchi, M., Fruscella, P., and Salmona, M. (2001). In vivo anti-inflammatory effect of statins is mediated by non-sterol mevalonate products. *Arterioscler Thromb Vasc Biol* 21, 1327-1332.
- Domeika, M., Domeika, K., Paavonen, J., Mardh, P. A., and Witkin, S. S. (1998). Humoral immune response to conserved epitopes of *Chlamydia trachomatis* and human 60-kDa heat-shock protein in women with pelvic inflammatory disease. *J Infect Dis* 177, 714-719.
- Dueland, S., Drisko, J., Graf, L., Machleder, D., Lusi, A. J., and Davis, R. A. (1993). Effect of dietary cholesterol and taurocholate on cholesterol 7 alpha-hydroxylase and hepatic LDL receptors in inbred mice. *J Lipid Res* 34, 923-931.
- Ekman, M. R., Grayston, J. T., Visakorpi, R., Kleemola, M., Kuo, C. C., and Saikku, P. (1993). An epidemic of infections due to *Chlamydia pneumoniae* in military conscripts. *Clin Infect Dis* 17, 420-425.

- Emre, U., Roblin, P. M., Gelling, M., Dumornay, W., Rao, M., Hammerschlag, M. R., and Schachter, J. (1994). The association of *Chlamydia pneumoniae* infection and reactive airway disease in children. *Arch Pediatr Adolesc Med* 148, 727-732.
- Endo, A., Tsujita, Y., Kuroda, M., and Tanzawa, K. (1977). Inhibition of cholesterol synthesis in vitro and in vivo by ML-236A and ML-236B, competitive inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A reductase. *Eur J Biochem* 77, 31-36.
- Eng, H. L., Chen, C. H., Kuo, C. C., Wu, J. S., Wang, C. H., and Lin, T. M. (2003). Association of CD14 promoter gene polymorphism and *Chlamydia pneumoniae* infection. *J Infect Dis* 188, 90-97.
- Erkkilä, L., Laitinen, K., Haasio, K., Tiirola, T., Jauhiainen, M., Lehr, H. A., Aalto-Setälä, K., Saikku, P., and Leinonen, M. (2004). Heat shock protein 60 autoimmunity and early lipid lesions in cholesterol-fed C57BL/6JBom mice during *Chlamydia pneumoniae* infection. *Atherosclerosis* 177, 321-328.
- Erkkilä, L., Laitinen, K., Laurila, A., Saikku, P., and Leinonen, M. (2002). Experimental *Chlamydia pneumoniae* infection in NIH/S mice: effect of reinoculation with chlamydial or cell preparation on culture, PCR and histological findings of lung tissue. *Vaccine* 20, 2318-2324.
- Erkkilä, L., Rottenberg, M. E., and Laitinen, K. (2000). Comparison of anesthetics for inoculation of mice with *Chlamydia pneumoniae*. *Comp Med* 50, 46-48.
- Espinola-Klein, C., Rupprecht, H. J., Blankenberg, S., Bickel, C., Kopp, H., Rippin, G., Victor, A., Hafner, G., Schlumberger, W., and Meyer, J. (2002a). Impact of infectious burden on extent and long-term prognosis of atherosclerosis. *Circulation* 105, 15-21.
- Espinola-Klein, C., Rupprecht, H. J., Blankenberg, S., Bickel, C., Kopp, H., Victor, A., Hafner, G., Prellwitz, W., Schlumberger, W., and Meyer, J. (2002b). Impact of infectious burden on progression of carotid atherosclerosis. *Stroke* 33, 2581-2586.
- Everett, K. D., Bush, R. M., and Andersen, A. A. (1999). Emended description of the order *Chlamydiales*, proposal of *Parachlamydiaceae* fam. nov. and *Simkaniaceae* fam. nov., each containing one monotypic genus, revised taxonomy of the family *Chlamydiaceae*, including a new genus and five new species, and standards for the identification of organisms. *Int J Syst Bacteriol* 2, 415-440.
- Ezzahiri, R., Nelissen-Vrancken, H. J., Kurvers, H. A., Stassen, F. R., Vliegen, I., Grauls, G. E., van Pul, M. M., Kitslaar, P. J., and Bruggeman, C. A. (2002). *Chlamydophila pneumoniae* (*Chlamydia pneumoniae*) accelerates the formation of complex atherosclerotic lesions in Apo E3-Leiden mice. *Cardiovasc Res* 56, 269-276.
- Ezzahiri, R., Stassen, F. R., Kurvers, H. A., van Pul, M. M., Kitslaar, P. J., and Bruggeman, C. A. (2003). *Chlamydia pneumoniae* infection induces an unstable atherosclerotic plaque phenotype in LDL-receptor, ApoE double knockout mice. *Eur J Vasc Endovasc Surg* 26, 88-95.
- Falck, G., Gnarpe, J., and Gnarpe, H. (1996). Persistent *Chlamydia pneumoniae* infection in a Swedish family. *Scand J Infect Dis* 28, 271-273.
- Finco, O., Bonci, A., Agnusdei, M., Scarselli, M., Petracca, R., Norais, N., Ferrari, G., Garaguso, I., Donati, M., Sambri, V., et al. (2005). Identification of new potential vaccine candidates against *Chlamydia pneumoniae* by multiple screenings. *Vaccine* 23, 1178-1188.
- Fong, I. W., Chiu, B., Viira, E., Fong, M. W., Jang, D., and Mahony, J. (1997). Rabbit model for *Chlamydia pneumoniae* infection. *J Clin Microbiol* 35, 48-52.

- Fong, I. W., Chiu, B., Viira, E., Jang, D., Fong, M. W., Peeling, R., and Mahony, J. B. (1999a). Can an antibiotic (macrolide) prevent *Chlamydia pneumoniae*-induced atherosclerosis in a rabbit model? *Clin Diagn Lab Immunol* 6, 891-894.
- Fong, I. W., Chiu, B., Viira, E., Jang, D., and Mahony, J. B. (1999b). De Novo induction of atherosclerosis by *Chlamydia pneumoniae* in a rabbit model. *Infect Immun* 67, 6048-6055.
- Fong, I. W., Chiu, B., Viira, E., Tucker, W., Wood, H., and Peeling, R. W. (2002). Chlamydial heat-shock protein-60 antibody and correlation with *Chlamydia pneumoniae* in atherosclerotic plaques. *J Infect Dis* 186, 1469-1473.
- Friedman, M. G., Dvoskin, B., and Kahane, S. (2003). Infections with the chlamydia-like microorganism *Simkania negevensis*, a possible emerging pathogen. *Microbes Infect* 5, 1013-1021.
- Frostegard, J., Kjellman, B., Gidlund, M., Andersson, B., Jindal, S., and Kiessling, R. (1996). Induction of heat shock protein in monocytic cells by oxidized low density lipoprotein. *Atherosclerosis* 121, 93-103.
- Gabay, C., and Kushner, I. (1999). Acute-phase proteins and other systemic responses to inflammation. *N Engl J Med* 340, 448-454.
- Gaydos, C. A., Quinn, T. C., and Eiden, J. J. (1992). Identification of *Chlamydia pneumoniae* by DNA amplification of the 16S rRNA gene. *J Clin Microbiol* 30, 796-800.
- Gaydos, C. A., Summersgill, J. T., Sahney, N. N., Ramirez, J. A., and Quinn, T. C. (1996). Replication of *Chlamydia pneumoniae* in vitro in human macrophages, endothelial cells, and aortic artery smooth muscle cells. *Infect Immun* 64, 1614-1620.
- Geng, Y., Berencsi, K., Gyulai, Z., Valyi-Nagy, T., Gonczol, E., and Trinchieri, G. (2000a). Roles of interleukin-12 and gamma interferon in murine *Chlamydia pneumoniae* infection. *Infect Immun* 68, 2245-2253.
- Geng, Y., Shane, R. B., Berencsi, K., Gonczol, E., Zaki, M. H., Margolis, D. J., Trinchieri, G., and Rook, A. H. (2000b). *Chlamydia pneumoniae* inhibits apoptosis in human peripheral blood mononuclear cells through induction of IL-10. *J Immunol* 164, 5522-5529.
- George, J., Shoenfeld, Y., Afek, A., Gilburd, B., Keren, P., Shaish, A., Kopolovic, J., Wick, G., and Harats, D. (1999). Enhanced fatty streak formation in C57BL/6J mice by immunization with heat shock protein-65. *Arterioscler Thromb Vasc Biol* 19, 505-510.
- George, J., Shoenfeld, Y., Gilburd, B., Afek, A., Shaish, A., and Harats, D. (2000). Requisite role for interleukin-4 in the acceleration of fatty streaks induced by heat shock protein 65 or *Mycobacterium tuberculosis*. *Circ Res* 86, 1203-1210.
- Gerard, H. C., Freise, J., Wang, Z., Roberts, G., Rudy, D., Krauss-Opatz, B., Kohler, L., Zeidler, H., Schumacher, H. R., Whittum-Hudson, J. A., and Hudson, A. P. (2002). *Chlamydia trachomatis* genes whose products are related to energy metabolism are expressed differentially in active vs. persistent infection. *Microbes Infect* 4, 13-22.
- Gerard, H. C., Whittum-Hudson, J. A., Schumacher, H. R., and Hudson, A. P. (2004). Differential expression of three *Chlamydia trachomatis* hsp60-encoding genes in active vs. persistent infections. *Microb Pathog* 36, 35-39.
- Ghuysen, J. M., and Goffin, C. (1999). Lack of cell wall peptidoglycan versus penicillin sensitivity: new insights into the chlamydial anomaly. *Antimicrob Agents Chemother* 43, 2339-2344.

- Gieffers, J., Belland, R. J., Whitmire, W., Ouellette, S., Crane, D., Maass, M., Byrne, G. I., and Caldwell, H. D. (2002). Isolation of *Chlamydia pneumoniae* clonal variants by a focus-forming assay. *Infect Immun* 70, 5827-5834.
- Gieffers, J., Durling, L., Ouellette, S. P., Rupp, J., Maass, M., Byrne, G. I., Caldwell, H. D., and Belland, R. J. (2003). Genotypic differences in the *Chlamydia pneumoniae* tyrP locus related to vascular tropism and pathogenicity. *J Infect Dis* 188, 1085-1093.
- Gieffers, J., Rupp, J., Gebert, A., Solbach, W., and Klinger, M. (2004a). First-choice antibiotics at subinhibitory concentrations induce persistence of *Chlamydia pneumoniae*. *Antimicrob Agents Chemother* 48, 1402-1405.
- Gieffers, J., van Zandbergen, G., Rupp, J., Sayk, F., Kruger, S., Ehlers, S., Solbach, W., and Maass, M. (2004b). Phagocytes transmit *Chlamydia pneumoniae* from the lungs to the vasculature. *Eur Respir J* 23, 506-510.
- Godzik, K. L., O'Brien, E. R., Wang, S. K., and Kuo, C. C. (1995). In vitro susceptibility of human vascular wall cells to infection with *Chlamydia pneumoniae*. *J Clin Microbiol* 33, 2411-2414.
- Gordon, S. B., and Read, R. C. (2002). Macrophage defences against respiratory tract infections. *Br Med Bull* 61, 45-61.
- Grandia, A. A., de Visser, H., van Embden, J. D., van der Zee, R., van den Berg, W. B., and Hazenberg, M. P. (1991). Natural antibodies to 65 kD mycobacterial heat shock protein in rats do not correlate with susceptibility for *Mycobacterium tuberculosis* induced adjuvant arthritis. *Immunobiology* 182, 127-134.
- Grayston, J. T. (1992). Infections caused by *Chlamydia pneumoniae* strain TWAR. *Clin Infect Dis* 15, 757-761.
- Grayston, J. T. (1994). *Chlamydia pneumoniae* (TWAR) infections in children. *Pediatr Infect Dis J* 13, 675-684.
- Grayston, J. T., Aldous, M. B., Easton, A., Wang, S. P., Kuo, C. C., Campbell, L. A., and Altman, J. (1993). Evidence that *Chlamydia pneumoniae* causes pneumonia and bronchitis. *J Infect Dis* 168, 1231-1235.
- Grayston, J. T., Campbell, L. A., Kuo, C. C., Mordhorst, C. H., Saikku, P., Thom, D. H., and Wang, S. P. (1990). A new respiratory tract pathogen: *Chlamydia pneumoniae* strain TWAR. *J Infect Dis* 161, 618-625.
- Grayston, J. T., Diwan, V. K., Cooney, M., and Wang, S. P. (1989a). Community- and hospital-acquired pneumonia associated with *Chlamydia* TWAR infection demonstrated serologically. *Arch Intern Med* 149, 169-173.
- Grayston, J. T., Kuo, C. C., Campbell, L. A., and Wang, S. P. (1989b). *Chlamydia pneumoniae* sp. nov. for *Chlamydia* sp. strain TWAR. *Int J Syst Bacteriol* 39, 88-90.
- Grayston, J. T., Kuo, C. C., Wang, S. P., and Altman, J. (1986). A new *Chlamydia psittaci* strain, TWAR, isolated in acute respiratory tract infections. *N Engl J Med* 315, 161-168.
- Grayston, J. T., Wang, S. P., Yeh, L. J., and Kuo, C. C. (1985). Importance of reinfection in the pathogenesis of trachoma. *Rev Infect Dis* 7, 717-725.
- Gupta, S., Leatham, E. W., Carrington, D., Mendall, M. A., Kaski, J. C., and Camm, A. J. (1997). Elevated *Chlamydia pneumoniae* antibodies, cardiovascular events, and azithromycin in male survivors of myocardial infarction. *Circulation* 96, 404-407.

- Gurfinkel, E., Bozovich, G., Daroca, A., Beck, E., and Mautner, B. (1997). Randomised trial of roxithromycin in non-Q-wave coronary syndromes: ROXIS Pilot Study. ROXIS Study Group. *Lancet* *350*, 404-407.
- Hackstadt, T., Baehr, W., and Ying, Y. (1991). *Chlamydia trachomatis* developmentally regulated protein is homologous to eukaryotic histone H1. *Proc Natl Acad Sci U S A* *88*, 3937-3941.
- Hahn, D. L., Anttila, T., and Saikku, P. (1996). Association of *Chlamydia pneumoniae* IgA antibodies with recently symptomatic asthma. *Epidemiol Infect* *117*, 513-517.
- Hahn, D. L., Azenabor, A. A., Beatty, W. L., and Byrne, G. I. (2002). *Chlamydia pneumoniae* as a respiratory pathogen. *Front Biosci* *7*, e66-76.
- Hahn, D. L., Dodge, R. W., and Golubjatnikov, R. (1991). Association of *Chlamydia pneumoniae* (strain TWAR) infection with wheezing, asthmatic bronchitis, and adult-onset asthma. *Jama* *266*, 225-230.
- Hammerschlag, M. R. (2004). *Chlamydia trachomatis* and *Chlamydia pneumoniae* infections in children and adolescents. *Pediatr Rev* *25*, 43-51.
- Haralambieva, I. H., Iankov, I. D., Ivanova, P. V., Mitev, V., and Mitov, I. G. (2004). *Chlamydia pneumoniae* induces p44/p42 mitogen-activated protein kinase activation in human fibroblasts through Toll-like receptor 4. *J Med Microbiol* *53*, 1187-1193.
- Haranaga, S., Yamaguchi, H., Friedman, H., Izumi, S., and Yamamoto, Y. (2001). *Chlamydia pneumoniae* infects and multiplies in lymphocytes in vitro. *Infect Immun* *69*, 7753-7759.
- Haranaga, S., Yamaguchi, H., Ikejima, H., Friedman, H., and Yamamoto, Y. (2003). *Chlamydia pneumoniae* infection of alveolar macrophages: a model. *J Infect Dis* *187*, 1107-1115.
- Harats, D., Yacov, N., Gilburd, B., Shoenfeld, Y., and George, J. (2002). Oral tolerance with heat shock protein 65 attenuates Mycobacterium tuberculosis-induced and high-fat-diet-driven atherosclerotic lesions. *J Am Coll Cardiol* *40*, 1333-1338.
- Hedl, M., Sutherlin, A., Wilding, E. I., Mazzulla, M., McDevitt, D., Lane, P., Burgner, J. W., 2nd, Lehnbeuter, K. R., Stauffacher, C. V., Gwynn, M. N., and Rodwell, V. W. (2002). Enterococcus faecalis acetoacetyl-coenzyme A thiolase/3-hydroxy-3-methylglutaryl-coenzyme A reductase, a dual-function protein of isopentenyl diphosphate biosynthesis. *J Bacteriol* *184*, 2116-2122.
- Heinemann, M., Susa, M., Simnacher, U., Marre, R., and Essig, A. (1996). Growth of *Chlamydia pneumoniae* induces cytokine production and expression of CD14 in a human monocytic cell line. *Infect Immun* *64*, 4872-4875.
- Herrera, V. L., Shen, L., Lopez, L. V., Didishvili, T., Zhang, Y. X., and Ruiz-Opazo, N. (2003). *Chlamydia pneumoniae* accelerates coronary artery disease progression in transgenic hyperlipidemia-genetic hypertension rat model. *Mol Med* *9*, 135-142.
- Hirono, S., Dibrov, E., Hurtado, C., Kostenuk, A., Ducas, R., and Pierce, G. N. (2003). *Chlamydia pneumoniae* stimulates proliferation of vascular smooth muscle cells through induction of endogenous heat shock protein 60. *Circ Res* *93*, 710-716.
- Hochleitner, B. W., Hochleitner, E. O., Obrist, P., Eberl, T., Amberger, A., Xu, Q., Margreiter, R., and Wick, G. (2000). Fluid shear stress induces heat shock protein 60 expression in endothelial cells in vitro and in vivo. *Arterioscler Thromb Vasc Biol* *20*, 617-623.

- Holland, M. J., Bailey, R. L., Conway, D. J., Culley, F., Miranpuri, G., Byrne, G. I., Whittle, H. C., and Mabey, D. C. (1996). T helper type-1 (Th1)/Th2 profiles of peripheral blood mononuclear cells (PBMC); responses to antigens of *Chlamydia trachomatis* in subjects with severe trachomatous scarring. *Clin Exp Immunol* 105, 429-435.
- Holland, S. M., Taylor, H. R., Gaydos, C. A., Kappus, E. W., and Quinn, T. C. (1990). Experimental infection with *Chlamydia pneumoniae* in nonhuman primates. *Infect Immun* 58, 593-597.
- Horn, M., Collingro, A., Schmitz-Esser, S., Beier, C. L., Purkhold, U., Fartmann, B., Brandt, P., Nyakatura, G. J., Droege, M., Frishman, D., *et al.* (2004). Illuminating the evolutionary history of chlamydiae. *Science* 304, 728-730.
- Hu, H., Pierce, G. N., and Zhong, G. (1999). The atherogenic effects of chlamydia are dependent on serum cholesterol and specific to *Chlamydia pneumoniae*. *J Clin Invest* 103, 747-753.
- Huang, J., DeGraves, F. J., Lenz, S. D., Gao, D., Feng, P., Li, D., Schlapp, T., and Kaltenboeck, B. (2002). The quantity of nitric oxide released by macrophages regulates *Chlamydia*-induced disease. *Proc Natl Acad Sci U S A* 99, 3914-3919.
- Huittinen, T., Leinonen, M., Tenkanen, L., Mänttari, M., Virkkunen, H., Pitkänen, T., Wahlström, E., Palosuo, T., Manninen, V., and Saikku, P. (2002). Autoimmunity to human heat shock protein 60, *Chlamydia pneumoniae* infection, and inflammation in predicting coronary risk. *Arterioscler Thromb Vasc Biol* 22, 431-437.
- Huittinen, T., Leinonen, M., Tenkanen, L., Virkkunen, H., Mänttari, M., Palosuo, T., Manninen, V., and Saikku, P. (2003). Synergistic effect of persistent *Chlamydia pneumoniae* infection, autoimmunity, and inflammation on coronary risk. *Circulation* 107, 2566-2570.
- Igietseme, J. U., Portis, J. L., and Perry, L. L. (2001). Inflammation and clearance of *Chlamydia trachomatis* in enteric and nonenteric mucosae. *Infect Immun* 69, 1832-1840.
- Jackson, L. A., Wang, S. P., Nazar-Stewart, V., Grayston, J. T., and Vaughan, T. L. (2000). Association of *Chlamydia pneumoniae* immunoglobulin A seropositivity and risk of lung cancer. *Cancer Epidemiol Biomarkers Prev* 9, 1263-1266.
- Jahn, H. U., Krull, M., Wuppermann, F. N., Klucken, A. C., Rosseau, S., Seybold, J., Hegemann, J. H., Jantos, C. A., and Suttorp, N. (2000). Infection and activation of airway epithelial cells by *Chlamydia pneumoniae*. *J Infect Dis* 182, 1678-1687.
- Jokinen, C., Heiskanen, L., Juvonen, H., Kallinen, S., Kleemola, M., Koskela, M., Leinonen, M., Rönneberg, P. R., Saikku, P., Stén, M., *et al.* (2001). Microbial etiology of community-acquired pneumonia in the adult population of 4 municipalities in eastern Finland. *Clin Infect Dis* 32, 1141-1154.
- Kahane, S., Greenberg, D., Friedman, M. G., Haikin, H., and Dagan, R. (1998). High prevalence of "Simkania Z," a novel *Chlamydia*-like bacterium, in infants with acute bronchiolitis. *J Infect Dis* 177, 1425-1429.
- Kalayoglu, M. V., and Byrne, G. I. (1998). Induction of macrophage foam cell formation by *Chlamydia pneumoniae*. *J Infect Dis* 177, 725-729.
- Kalayoglu, M. V., Hoerneman, B., LaVerda, D., Morrison, S. G., Morrison, R. P., and Byrne, G. I. (1999). Cellular oxidation of low-density lipoprotein by *Chlamydia pneumoniae*. *J Infect Dis* 180, 780-790.

- Kalayoglu, M. V., Perkins, B. N., and Byrne, G. I. (2001). *Chlamydia pneumoniae*-infected monocytes exhibit increased adherence to human aortic endothelial cells. *Microbes Infect* 3, 963-969.
- Kalman, S., Mitchell, W., Marathe, R., Lammel, C., Fan, J., Hyman, R. W., Olinger, L., Grimwood, J., Davis, R. W., and Stephens, R. S. (1999). Comparative genomes of *Chlamydia pneumoniae* and *C. trachomatis*. *Nat Genet* 21, 385-389.
- Kanwar, R. K., Kanwar, J. R., Wang, D., Ormrod, D. J., and Krissansen, G. W. (2001). Temporal expression of heat shock proteins 60 and 70 at lesion-prone sites during atherogenesis in ApoE-deficient mice. *Arterioscler Thromb Vasc Biol* 21, 1991-1997.
- Karunakaran, K. P., Blanchard, J. F., Raudonikiene, A., Shen, C., Murdin, A. D., and Brunham, R. C. (2002). Molecular detection and seroepidemiology of the *Chlamydia pneumoniae* bacteriophage (ϕ Cpn1). *J Clin Microbiol* 40, 4010-4014.
- Karunakaran, K. P., Noguchi, Y., Read, T. D., Cherkasov, A., Kwee, J., Shen, C., Nelson, C. C., and Brunham, R. C. (2003). Molecular analysis of the multiple GroEL proteins of *Chlamydiae*. *J Bacteriol* 185, 1958-1966.
- Kaukoranta-Tolvanen, S. E., Laurila, A. L., Saikku, P., Leinonen, M., and Laitinen, K. (1995). Experimental *Chlamydia pneumoniae* infection in mice: effect of reinfection and passive immunization. *Microb Pathog* 18, 279-288.
- Kaukoranta-Tolvanen, S. S., Laitinen, K., Saikku, P., and Leinonen, M. (1994). *Chlamydia pneumoniae* multiplies in human endothelial cells in vitro. *Microb Pathog* 16, 313-319.
- Kaukoranta-Tolvanen, S. S., Laurila, A. L., Saikku, P., Leinonen, M., Liesirova, L., and Laitinen, K. (1993). Experimental infection of *Chlamydia pneumoniae* in mice. *Microb Pathog* 15, 293-302.
- Kaukoranta-Tolvanen, S. S., Ronni, T., Leinonen, M., Saikku, P., and Laitinen, K. (1996). Expression of adhesion molecules on endothelial cells stimulated by *Chlamydia pneumoniae*. *Microb Pathog* 21, 407-411.
- Kauppinen, M. T., Saikku, P., Kujala, P., Herva, E., and Syrjälä, H. (1996). Clinical picture of community-acquired *Chlamydia pneumoniae* pneumonia requiring hospital treatment: a comparison between chlamydial and pneumococcal pneumonia. *Thorax* 51, 185-189.
- Keidar, S., Aviram, M., Maor, I., Oiknine, J., and Brook, J. G. (1994). Pravastatin inhibits cellular cholesterol synthesis and increases low density lipoprotein receptor activity in macrophages: in vitro and in vivo studies. *Br J Clin Pharmacol* 38, 513-519.
- Kempen, H. J., Vermeer, M., de Wit, E., and Havekes, L. M. (1991). Vastatins inhibit cholesterol ester accumulation in human monocyte-derived macrophages. *Arterioscler Thromb* 11, 146-153.
- Kiener, P. A., Davis, P. M., Murray, J. L., Youssef, S., Rankin, B. M., and Kowala, M. (2001). Stimulation of inflammatory responses in vitro and in vivo by lipophilic HMG-CoA reductase inhibitors. *International Immunopharmacology* 1, 105-118.
- Kimura, M. (1994). [Experimental study on the mechanisms of *Chlamydia pneumoniae* respiratory infection in mice with former chlamydial exposure]. *Kansenshogaku Zasshi* 68, 50-58.
- Kishimoto, T. (1990). [Studies on *Chlamydia pneumoniae*, strain TWAR, infection. I. Experimental infection of *C. pneumoniae* in mice and serum antibodies against TWAR by MFA.]. *Kansenshogaku Zasshi* 64, 124-131.

- Kleemola, M., Saikku, P., Visakorpi, R., Wang, S. P., and Grayston, J. T. (1988). Epidemics of pneumonia caused by TWAR, a new *Chlamydia* organism, in military trainees in Finland. *J Infect Dis* 157, 230-236.
- Kleindienst, R., Xu, Q., Willeit, J., Waldenberger, F. R., Weimann, S., and Wick, G. (1993). Immunology of atherosclerosis. Demonstration of heat shock protein 60 expression and T lymphocytes bearing alpha/beta or gamma/delta receptor in human atherosclerotic lesions. *Am J Pathol* 142, 1927-1937.
- Kol, A., Bourcier, T., Lichtman, A. H., and Libby, P. (1999). Chlamydial and human heat shock protein 60s activate human vascular endothelium, smooth muscle cells, and macrophages. *J Clin Invest* 103, 571-577.
- Kol, A., Sukhova, G. K., Lichtman, A. H., and Libby, P. (1998). Chlamydial heat shock protein 60 localizes in human atheroma and regulates macrophage tumor necrosis factor-alpha and matrix metalloproteinase expression. *Circulation* 98, 300-307.
- Kordova, N., Martin, C., Wilt, J. C., and Neuman, M. (1977). Sucrose density differences of *Chlamydia psittaci* 6BC in relation to its host. *Can J Microbiol* 23, 649-652.
- Kothe, H., Dalhoff, K., Rupp, J., Muller, A., Kreuzer, J., Maass, M., and Katus, H. A. (2000). Hydroxymethylglutaryl coenzyme A reductase inhibitors modify the inflammatory response of human macrophages and endothelial cells infected with *Chlamydia pneumoniae*. *Circulation* 101, 1760-1763.
- Kotsimbos, T. C., Snell, G. I., Levvey, B., Spelman, D. W., Fuller, A. J., Wesselingh, S. L., Williams, T. J., and Ostergaard, L. (2005). *Chlamydia pneumoniae* serology in donors and recipients and the risk of bronchiolitis obliterans syndrome after lung transplantation. *Transplantation* 79, 269-275.
- Krüll, M., Klucken, A. C., Wuppermann, F. N., Fuhrmann, O., Magerl, C., Seybold, J., Hippenstiel, S., Hegemann, J. H., Jantos, C. A., and Suttorp, N. (1999). Signal transduction pathways activated in endothelial cells following infection with *Chlamydia pneumoniae*. *J Immunol* 162, 4834-4841.
- Kuo, C. C., Chen, H. H., Wang, S. P., and Grayston, J. T. (1986). Identification of a new group of *Chlamydia psittaci* strains called TWAR. *J Clin Microbiol* 24, 1034-1037.
- Kuo, C. C., Chi, E. Y., and Grayston, J. T. (1988). Ultrastructural study of entry of *Chlamydia* strain TWAR into HeLa cells. *Infect Immun* 56, 1668-1672.
- Kuo, C. C., Shor, A., Campbell, L. A., Fukushi, H., Patton, D. L., and Grayston, J. T. (1993). Demonstration of *Chlamydia pneumoniae* in atherosclerotic lesions of coronary arteries. *J Infect Dis* 167, 841-849.
- Kuo, C. C., Wang, S. P., and Grayston, J. T. (1975). Comparative infectivity of trachoma organisms in HeLa 229 cells and egg cultures. *Infect Immun* 12, 1078-1082.
- Kwak, B., Mulhaupt, F., Myit, S., and Mach, F. (2000). Statins as a newly recognized type of immunomodulator. *Nat Med* 6, 1399-1402.
- Laitinen, K., Laurila, A., Pyhälä, L., Leinonen, M., and Saikku, P. (1997). *Chlamydia pneumoniae* infection induces inflammatory changes in the aortas of rabbits. *Infect Immun* 65, 4832-4835.
- Laitinen, K., Laurila, A. L., Leinonen, M., and Saikku, P. (1996). Reactivation of *Chlamydia pneumoniae* infection in mice by cortisone treatment. *Infect Immun* 64, 1488-1490.

- Lange, B. M., Rujan, T., Martin, W., and Croteau, R. (2000). Isoprenoid biosynthesis: the evolution of two ancient and distinct pathways across genomes. *Proc Natl Acad Sci U S A* *97*, 13172-13177.
- Laurila, A. L., Anttila, T., Läärä, E., Bloigu, A., Virtamo, J., Albanes, D., Leinonen, M., and Saikku, P. (1997a). Serological evidence of an association between *Chlamydia pneumoniae* infection and lung cancer. *Int J Cancer* *74*, 31-34.
- Laurila, A. L., Von Hertzen, L., and Saikku, P. (1997b). *Chlamydia pneumoniae* and chronic lung diseases. *Scand J Infect Dis Suppl* *104*, 34-36.
- Leinonen, M., Linnanmäki, E., Mattila, K., Nieminen, M. S., Valtonen, V., Leirisalo-Repo, M., and Saikku, P. (1990). Circulating immune complexes containing chlamydial lipopolysaccharide in acute myocardial infarction. *Microb Pathog* *9*, 67-73.
- Liappis, A. P., Kan, V. L., Rochester, C. G., and Simon, G. L. (2001). The effect of statins on mortality in patients with bacteremia. *Clin Infect Dis* *33*, 1352-1357.
- Libby, P., and Ridker, P. M. (2004). Inflammation and atherosclerosis: role of C-reactive protein in risk assessment. *Am J Med* *116*, 9S-16S.
- Libby, P., Ridker, P. M., and Maseri, A. (2002). Inflammation and atherosclerosis. *Circulation* *105*, 1135-1143.
- Lin, T. M., Campbell, L. A., Rosenfeld, M. E., and Kuo, C. C. (2000). Monocyte-endothelial cell coculture enhances infection of endothelial cells with *Chlamydia pneumoniae*. *J Infect Dis* *181*, 1096-1100.
- Little, C. S., Bowe, A., Lin, R., Litsky, J., Fogel, R. M., Balin, B. J., and Fresa-Dillon, K. L. (2005). Age alterations in extent and severity of experimental intranasal infection with *Chlamydoxiphila pneumoniae* in BALB/c mice. *Infect Immun* *73*, 1723-1734.
- Little, C. S., Hammond, C. J., MacIntyre, A., Balin, B. J., and Appelt, D. M. (2004). *Chlamydia pneumoniae* induces Alzheimer-like amyloid plaques in brains of BALB/c mice. *Neurobiol Aging* *25*, 419-429.
- Littman, A. J., Jackson, L. A., and Vaughan, T. L. (2005). *Chlamydia pneumoniae* and lung cancer: epidemiologic evidence. *Cancer Epidemiol Biomarkers Prev* *14*, 773-778.
- Liu, L., Hu, H., Ji, H., Murdin, A. D., Pierce, G. N., and Zhong, G. (2000). *Chlamydia pneumoniae* infection significantly exacerbates aortic atherosclerosis in an LDLR-/- mouse model within six months. *Mol Cell Biochem* *215*, 123-128.
- Liuba, P., Karnani, P., Pesonen, E., Paakkari, I., Forslid, A., Johansson, L., Persson, K., Wadstrom, T., and Laurini, R. (2000). Endothelial dysfunction after repeated *Chlamydia pneumoniae* infection in apolipoprotein E-knockout mice. *Circulation* *102*, 1039-1044.
- Liuba, P., Pesonen, E., Paakkari, I., Batra, S., Andersen, L., Forslid, A., Yla-Herttuala, S., Persson, K., Wadstrom, T., Wang, X., and Laurini, R. (2003a). Co-infection with *Chlamydia pneumoniae* and *Helicobacter pylori* results in vascular endothelial dysfunction and enhanced VCAM-1 expression in apoE-knockout mice. *J Vasc Res* *40*, 115-122.
- Liuba, P., Pesonen, E., Paakkari, I., Batra, S., Forslid, A., Kovanen, P., Pentikainen, M., Persson, K., and Sandstrom, S. (2003b). Acute *Chlamydia pneumoniae* infection causes coronary endothelial dysfunction in pigs. *Atherosclerosis* *167*, 215-222.
- Loike, J. D., Shabtai, D. Y., Neuhut, R., Malitzky, S., Lu, E., Husemann, J., Goldberg, I. J., and Silverstein, S. C. (2004). Statin inhibition of Fc receptor-mediated phagocytosis

- sis by macrophages is modulated by cell activation and cholesterol. *Arterioscler Thromb Vasc Biol* 24, 2051-2056.
- Longbottom, D., and Coulter, L. J. (2003). Animal chlamydioses and zoonotic implications. *J Comp Pathol* 128, 217-244.
- Ludewig, B., Jaggi, M., Dumrese, T., Brduscha-Riem, K., Odermatt, B., Hengartner, H., and Zinkernagel, R. M. (2001). Hypercholesterolemia exacerbates virus-induced immunopathologic liver disease via suppression of antiviral cytotoxic T cell responses. *J Immunol* 166, 3369-3376.
- MacDonald, A. B., McComb, D., and Howard, L. (1984). Immune response of owl monkeys to topical vaccination with irradiated *Chlamydia trachomatis*. *J Infect Dis* 149, 439-442.
- Mahdi, O. S., Horne, B. D., Mullen, K., Muhlestein, J. B., and Byrne, G. I. (2002). Serum immunoglobulin G antibodies to chlamydial heat shock protein 60 but not to human and bacterial homologs are associated with coronary artery disease. *Circulation* 106, 1659-1663.
- Mahony, J. B., and Coombes, B. K. (2001). *Chlamydia pneumoniae* and atherosclerosis: does the evidence support a causal or contributory role? *FEMS Microbiol Lett* 197, 1-9.
- Malinverni, R., Kuo, C. C., Campbell, L. A., and Grayston, J. T. (1995a). Reactivation of *Chlamydia pneumoniae* lung infection in mice by cortisone. *J Infect Dis* 172, 593-594.
- Malinverni, R., Kuo, C. C., Campbell, L. A., Lee, A., and Grayston, J. T. (1995b). Effects of two antibiotic regimens on course and persistence of experimental *Chlamydia pneumoniae* TWAR pneumonitis. *Antimicrob Agents Chemother* 39, 45-49.
- Maron, D. J., Fazio, S., and Linton, M. F. (2000). Current perspectives on statins. *Circulation* 101, 207-213.
- Maron, R., Sukhova, G., Faria, A. M., Hoffmann, E., Mach, F., Libby, P., and Weiner, H. L. (2002). Mucosal administration of heat shock protein-65 decreases atherosclerosis and inflammation in aortic arch of low-density lipoprotein receptor-deficient mice. *Circulation* 106, 1708-1715.
- Masson, N. D., Toseland, C. D., and Beale, A. S. (1995). Relevance of *Chlamydia pneumoniae* murine pneumonitis model to evaluation of antimicrobial agents. *Antimicrob Agents Chemother* 39, 1959-1964.
- Mathews, S., George, C., Flegg, C., Stenzel, D., and Timms, P. (2001). Differential expression of ompA, ompB, pyk, nlpD and Cpn0585 genes between normal and interferon-gamma treated cultures of *Chlamydia pneumoniae*. *Microb Pathog* 30, 337-345.
- May, A. E., Redecke, V., Grüner, S., Schmidt, R., Massberg, S., Miethke, T., Ryba, B., Prazeres da Costa, C., Schömig, A., and Neumann, F. J. (2003). Recruitment of *Chlamydia pneumoniae*-infected macrophages to the carotid artery wall in noninfected, nonatherosclerotic mice. *Arterioscler Thromb Vasc Biol* 23, 789-794.
- Mayr, M., Kiechl, S., Willeit, J., Wick, G., and Xu, Q. (2000). Infections, immunity, and atherosclerosis: associations of antibodies to *Chlamydia pneumoniae*, *Helicobacter pylori*, and cytomegalovirus with immune reactions to heat-shock protein 60 and carotid or femoral atherosclerosis. *Circulation* 102, 833-839.
- Mayr, M., Metzler, B., Kiechl, S., Willeit, J., Schett, G., Xu, Q., and Wick, G. (1999). Endothelial cytotoxicity mediated by serum antibodies to heat shock proteins of *Escherichia coli* and *Chlamydia pneumoniae*: immune reactions to heat shock proteins as a possible link between infection and atherosclerosis. *Circulation* 99, 1560-1566.

- McKay, A., Leung, B. P., McInnes, I. B., Thomson, N. C., and Liew, F. Y. (2004). A novel anti-inflammatory role of simvastatin in a murine model of allergic asthma. *J Immunol* *172*, 2903-2908.
- Mehta, S. J., Miller, R. D., Ramirez, J. A., and Summersgill, J. T. (1998). Inhibition of *Chlamydia pneumoniae* replication in HEp-2 cells by interferon-gamma: role of tryptophan catabolism. *J Infect Dis* *177*, 1326-1331.
- Meijer, A., Roholl, P. J., Gielis-Proper, S. K., Meulenber, Y. F., and Ossewaarde, J. M. (2000). *Chlamydia pneumoniae* in vitro and in vivo: a critical evaluation of in situ detection methods. *J Clin Pathol* *53*, 904-910.
- Merx, M. W., Liehn, E. A., Janssens, U., Luttkien, R., Schrader, J., Hanrath, P., and Weber, C. (2004). HMG-CoA reductase inhibitor simvastatin profoundly improves survival in a murine model of sepsis. *Circulation* *109*, 2560-2565.
- Miyake, J. H., Duong-Polk, X. T., Taylor, J. M., Du, E. Z., Castellani, L. W., Lusic, A. J., and Davis, R. A. (2002). Transgenic expression of cholesterol-7-alpha-hydroxylase prevents atherosclerosis in C57BL/6J mice. *Arterioscler Thromb Vasc Biol* *22*, 121-126.
- Miyashita, N., Fukano, H., Yoshida, K., Niki, Y., and Matsushima, T. (2003). *Chlamydia pneumoniae* infection in adult patients with persistent cough. *J Med Microbiol* *52*, 265-269.
- Miyashita, N., Kanamoto, Y., and Matsumoto, A. (1993). The morphology of *Chlamydia pneumoniae*. *J Med Microbiol* *38*, 418-425.
- Moazed, T. C., Campbell, L. A., Rosenfeld, M. E., Grayston, J. T., and Kuo, C. C. (1999). *Chlamydia pneumoniae* infection accelerates the progression of atherosclerosis in apolipoprotein E-deficient mice. *J Infect Dis* *180*, 238-241.
- Moazed, T. C., Kuo, C., Grayston, J. T., and Campbell, L. A. (1997). Murine models of *Chlamydia pneumoniae* infection and atherosclerosis. *J Infect Dis* *175*, 883-890.
- Moazed, T. C., Kuo, C., Patton, D. L., Grayston, J. T., and Campbell, L. A. (1996). Experimental rabbit models of *Chlamydia pneumoniae* infection. *Am J Pathol* *148*, 667-676.
- Moazed, T. C., Kuo, C. C., Grayston, J. T., and Campbell, L. A. (1998). Evidence of systemic dissemination of *Chlamydia pneumoniae* via macrophages in the mouse. *J Infect Dis* *177*, 1322-1325.
- Molestina, R. E., Dean, D., Miller, R. D., Ramirez, J. A., and Summersgill, J. T. (1998). Characterization of a strain of *Chlamydia pneumoniae* isolated from a coronary atheroma by analysis of the omp1 gene and biological activity in human endothelial cells. *Infect Immun* *66*, 1370-1376.
- Molestina, R. E., Klein, J. B., Miller, R. D., Pierce, W. H., Ramirez, J. A., and Summersgill, J. T. (2002). Proteomic analysis of differentially expressed *Chlamydia pneumoniae* genes during persistent infection of HEp-2 cells. *Infect Immun* *70*, 2976-2981.
- Molestina, R. E., Miller, R. D., Ramirez, J. A., and Summersgill, J. T. (1999). Infection of human endothelial cells with *Chlamydia pneumoniae* stimulates transendothelial migration of neutrophils and monocytes. *Infect Immun* *67*, 1323-1330.
- Momiyama, Y., Hirano, R., Taniguchi, H., Nakamura, H., and Ohsuzu, F. (2001). Effects of interleukin-1 gene polymorphisms on the development of coronary artery disease associated with *Chlamydia pneumoniae* infection. *J Am Coll Cardiol* *38*, 712-717.

- Montigiani, S., Falugi, F., Scarselli, M., Finco, O., Petracca, R., Galli, G., Mariani, M., Manetti, R., Agnusdei, M., Cevenini, R., *et al.* (2002). Genomic approach for analysis of surface proteins in *Chlamydia pneumoniae*. *Infect Immun* 70, 368-379.
- Morrison, R. P., Belland, R. J., Lyng, K., and Caldwell, H. D. (1989). Chlamydial disease pathogenesis. The 57-kD chlamydial hypersensitivity antigen is a stress response protein. *J Exp Med* 170, 1271-1283.
- Mosorin, M., Surcel, H. M., Laurila, A., Lehtinen, M., Karttunen, R., Juvonen, J., Paa-vonen, J., Morrison, R. P., Saikku, P., and Juvonen, T. (2000). Detection of *Chlamydia pneumoniae*-reactive T lymphocytes in human atherosclerotic plaques of carotid artery. *Arterioscler Thromb Vasc Biol* 20, 1061-1067.
- Moulder, J. W. (1991). Interaction of chlamydiae and host cells in vitro. *Microbiol Rev* 55, 143-190.
- Mueller, M., Postius, S., Thimm, J. G., Gueinzus, K., Muehldorfer, I., and Hermann, C. (2004). Toll-like receptors 2 and 4 do not contribute to clearance of *Chlamydomytila pneumoniae* in mice, but are necessary for the release of monokines. *Immunobiology* 209, 599-608.
- Muhlestein, J. B., Anderson, J. L., Hammond, E. H., Zhao, L., Trehan, S., Schwobe, E. P., and Carlquist, J. F. (1998). Infection with *Chlamydia pneumoniae* accelerates the development of atherosclerosis and treatment with azithromycin prevents it in a rabbit model. *Circulation* 97, 633-636.
- Murdin, A. D., Dunn, P., Sodayer, R., Wang, J., Caterini, J., Brunham, R. C., Aujame, L., and Oomen, R. (2000). Use of a mouse lung challenge model to identify antigens protective against *Chlamydia pneumoniae* lung infection. *J Infect Dis* 181, S544-551.
- Mygind, T., Birkelund, S., Falk, E., and Christiansen, G. (2001). Evaluation of real-time quantitative PCR for identification and quantification of *Chlamydia pneumoniae* by comparison with immunohistochemistry. *J Microbiol Methods* 46, 241-251.
- Mygind, T., Vandahl, B., Pedersen, A. S., Christiansen, G., Hollsberg, P., and Birkelund, S. (2004). Identification of an in vivo CD4+ T cell-mediated response to polymorphic membrane proteins of *Chlamydia pneumoniae* during experimental infection. *FEMS Immunol Med Microbiol* 40, 129-137.
- Nagy, A., Kozma, G. T., Keszei, M., Treszl, A., Falus, A., and Szalai, C. (2003). The development of asthma in children infected with *Chlamydia pneumoniae* is dependent on the modifying effect of mannose-binding lectin. *J Allergy Clin Immunol* 112, 729-734.
- Nakata, K., Okazaki, Y., Hattori, H., and Nakamura, S. (1994). Protective effects of sparfloxacin in experimental pneumonia caused by *Chlamydia pneumoniae* in leukopenic mice. *Antimicrob Agents Chemother* 38, 1757-1762.
- Neuman, M. R., Kordova, N., and Wilt, J. C. (1980). Host influence on the density of chlamydiae in Renografin gradients. *Zentralbl Bakteriol A* 247, 526-529.
- Oberley, R. E., Ault, K. A., Neff, T. L., Khubchandani, K. R., Crouch, E. C., and Snyder, J. M. (2004). Surfactant proteins A and D enhance the phagocytosis of *Chlamydia* into THP-1 cells. *Am J Physiol Lung Cell Mol Physiol* 287, L296-306.
- Opitz, B., Forster, S., Hocke, A. C., Maass, M., Schmeck, B., Hippenstiel, S., Suttorp, N., and Krull, M. (2005). Nod1-mediated endothelial cell activation by *Chlamydomytila pneumoniae*. *Circ Res* 96, 319-326.
- Paigen, B., Morrow, A., Brandon, C., Mitchell, D., and Holmes, P. (1985). Variation in susceptibility to atherosclerosis among inbred strains of mice. *Atherosclerosis* 57, 65-73.

- Paigen, B., Morrow, A., Holmes, P. A., Mitchell, D., and Williams, R. A. (1987). Quantitative assessment of atherosclerotic lesions in mice. *Atherosclerosis* 68, 231-240.
- Pal, S., Peterson, E. M., and de La Maza, L. M. (2000). Role of Nramp1 deletion in *Chlamydia* infection in mice. *Infect Immun* 68, 4831-4833.
- Peeling, R. W., Patton, D. L., Cosgrove Sweeney, Y. T., Cheang, M. S., Lichtenwalner, A. B., Brunham, R. C., and Stamm, W. E. (1999). Antibody response to the chlamydial heat-shock protein 60 in an experimental model of chronic pelvic inflammatory disease in monkeys (*Macaca nemestrina*). *J Infect Dis* 180, 774-779.
- Penttilä, J. M., Anttila, M., Puolakkainen, M., Laurila, A., Varkila, K., Sarvas, M., Mäkelä, P. H., and Rautonen, N. (1998a). Local immune responses to *Chlamydia pneumoniae* in the lungs of BALB/c mice during primary infection and reinfection. *Infect Immun* 66, 5113-5118.
- Penttilä, J. M., Anttila, M., Varkila, K., Puolakkainen, M., Sarvas, M., Mäkelä, P. H., and Rautonen, N. (1999). Depletion of CD8+ cells abolishes memory in acquired immunity against *Chlamydia pneumoniae* in BALB/c mice. *Immunology* 97, 490-496.
- Penttilä, J. M., Pyhälä, R., Sarvas, M., and Rautonen, N. (1998b). Expansion of a novel pulmonary CD3(-) CD4(+) CD8(+) cell population in mice during *Chlamydia pneumoniae* infection. *Infect Immun* 66, 3290-3294.
- Penttilä, T., Tammiruusu, A., Liljeström, P., Sarvas, M., Mäkelä, P. H., Vuola, J. M., and Puolakkainen, M. (2004). DNA immunization followed by a viral vector booster in a *Chlamydia pneumoniae* mouse model. *Vaccine* 22, 3386-3394.
- Penttilä, T., Vuola, J. M., Puurula, V., Anttila, M., Sarvas, M., Rautonen, N., Mäkelä, P. H., and Puolakkainen, M. (2000). Immunity to *Chlamydia pneumoniae* induced by vaccination with DNA vectors expressing a cytoplasmic protein (Hsp60) or outer membrane proteins (MOMP and Omp2). *Vaccine* 19, 1256-1265.
- Perschinka, H., Mayr, M., Millonig, G., Mayerl, C., van der Zee, R., Morrison, S. G., Morrison, R. P., Xu, Q., and Wick, G. (2003). Cross-reactive B-cell epitopes of microbial and human heat shock protein 60/65 in atherosclerosis. *Arterioscler Thromb Vasc Biol* 23, 1060-1065.
- Peterson, E. M., Cheng, X., Qu, Z., and de La Maza, L. M. (1996). Characterization of the murine antibody response to peptides representing the variable domains of the major outer membrane protein of *Chlamydia pneumoniae*. *Infect Immun* 64, 3354-3359.
- Peterson, E. M., de la Maza, L. M., Brade, L., and Brade, H. (1998). Characterization of a neutralizing monoclonal antibody directed at the lipopolysaccharide of *Chlamydia pneumoniae*. *Infect Immun* 66, 3848-3855.
- Pislaru, S. V., Van Ranst, M., Pislaru, C., Szelid, Z., Theilmeier, G., Ossewaarde, J. M., Holvoet, P., Janssens, S., Verbeken, E., and Van de Werf, F. J. (2003). *Chlamydia pneumoniae* induces neointima formation in coronary arteries of normal pigs. *Cardiovasc Res* 57, 834-842.
- Potena, L., Frascaroli, G., Grigioni, F., Lazzarotto, T., Magnani, G., Tomasi, L., Coccolo, F., Gabrielli, L., Magelli, C., Landini, M. P., and Branzi, A. (2004). Hydroxymethyl-glutaryl coenzyme a reductase inhibition limits cytomegalovirus infection in human endothelial cells. *Circulation* 109, 532-536.
- Prebeck, S., Kirschning, C., Durr, S., da Costa, C., Donath, B., Brand, K., Redecke, V., Wagner, H., and Miethke, T. (2001). Predominant role of toll-like receptor 2 versus 4 in *Chlamydia pneumoniae*-induced activation of dendritic cells. *J Immunol* 167, 3316-3323.

- Puolakkainen, M., Campbell, L. A., Kuo, C. C., Leinonen, M., Grönhagen-Riska, C., and Saikku, P. (1996). Serological response to *Chlamydia pneumoniae* in patients with sarcoidosis. *J Infect* 33, 199-205.
- Quiding-Jarbrink, M., Nordstrom, I., Granstrom, G., Kilander, A., Jertborn, M., Butcher, E. C., Lazarovits, A. I., Holmgren, J., and Czerkinsky, C. (1997). Differential expression of tissue-specific adhesion molecules on human circulating antibody-forming cells after systemic, enteric, and nasal immunizations. A molecular basis for the compartmentalization of effector B cell responses. *J Clin Invest* 99, 1281-1286.
- Rajalingam, K., Al-Younes, H., Muller, A., Meyer, T. F., Szczepek, A. J., and Rudel, T. (2001). Epithelial cells infected with *Chlamydophila pneumoniae* (*Chlamydia pneumoniae*) are resistant to apoptosis. *Infect Immun* 69, 7880-7888.
- Ramirez, J. A. (1996). Isolation of *Chlamydia pneumoniae* from the coronary artery of a patient with coronary atherosclerosis. The *Chlamydia pneumoniae*/Atherosclerosis Study Group. *Ann Intern Med* 125, 979-982.
- Read, T. D., Brunham, R. C., Shen, C., Gill, S. R., Heidelberg, J. F., White, O., Hickey, E. K., Peterson, J., Utterback, T., Berry, K., *et al.* (2000a). Genome sequences of *Chlamydia trachomatis* MoPn and *Chlamydia pneumoniae* AR39. *Nucleic Acids Res* 28, 1397-1406.
- Read, T. D., Fraser, C. M., Hsia, R. C., and Bavoil, P. M. (2000b). Comparative analysis of *Chlamydia* bacteriophages reveals variation localized to a putative receptor binding domain. *Microb Comp Genomics* 5, 223-231.
- Reischl, U., Lehn, N., Simnacher, U., Marre, R., and Essig, A. (2003). Rapid and standardized detection of *Chlamydia pneumoniae* using LightCycler real-time fluorescence PCR. *Eur J Clin Microbiol Infect Dis* 22, 54-57.
- Ridker, P. M., Rifai, N., Pfeffer, M. A., Sacks, F., and Braunwald, E. (1999). Long-term effects of pravastatin on plasma concentration of C-reactive protein. The Cholesterol and Recurrent Events (CARE) Investigators. *Circulation* 100, 230-235.
- Roselaar, S. E., and Daugherty, A. (1998). Apolipoprotein E-deficient mice have impaired innate immune responses to *Listeria monocytogenes* in vivo. *J Lipid Res* 39, 1740-1743.
- Ross, R. (1999). Atherosclerosis--an inflammatory disease. *N Engl J Med* 340, 115-126.
- Rothfuchs, A. G., Kreuger, M. R., Wigzell, H., and Rottenberg, M. E. (2004). Macrophages, CD4(+) or CD8(+) cells are each sufficient for protection against *Chlamydia pneumoniae* infection through their ability to secrete IFN-gamma. *J Immunol* 172, 2407-2415.
- Rothstein, N. M., Quinn, T. C., Madico, G., Gaydos, C. A., and Lowenstein, C. J. (2001). Effect of azithromycin on murine arteriosclerosis exacerbated by *Chlamydia pneumoniae*. *J Infect Dis* 183, 232-238.
- Rottenberg, M. E., Gigliotti Rothfuchs, A., Gigliotti, D., Ceausu, M., Une, C., Levitsky, V., and Wigzell, H. (2000). Regulation and role of IFN-gamma in the innate resistance to infection with *Chlamydia pneumoniae*. *J Immunol* 164, 4812-4818.
- Rottenberg, M. E., Gigliotti Rothfuchs, A. C., Gigliotti, D., Svanholm, C., Bandholtz, L., and Wigzell, H. (1999). Role of innate and adaptive immunity in the outcome of primary infection with *Chlamydia pneumoniae*, as analyzed in genetically modified mice. *J Immunol* 162, 2829-2836.
- Rubins, J. B., and Charboneau, D. (2000). Effect of anesthetics on pathogenesis of experimentally induced murine pneumococcal pneumonia. *Comp Med* 50, 292-295.

- Rupp, J., Droemann, D., Goldmann, T., Zabel, P., Solbach, W., Vollmer, E., Branscheid, D., Dalhoff, K., and Maass, M. (2004a). Alveolar epithelial cells type II are major target cells for *C. pneumoniae* in chronic but not in acute respiratory infection. *FEMS Immunol Med Microbiol* 41, 197-203.
- Rupp, J., Goepel, W., Kramme, E., Jahn, J., Solbach, W., and Maass, M. (2004b). CD14 promoter polymorphism -159C>T is associated with susceptibility to chronic *Chlamydia pneumoniae* infection in peripheral blood monocytes. *Genes Immun* 5, 435-438.
- Rupp, J., Koch, M., vanZandbergen, G., Solbach, W., Brandt, E., and Maass, M. (2005). Transmission of *Chlamydia pneumoniae* infection from blood monocytes to vascular cells in a novel transendothelial migration model. *FEMS Microbiol Lett* 242, 203-208.
- Rurangirwa, F. R., Dilbeck, P. M., Crawford, T. B., McGuire, T. C., and McElwain, T. F. (1999). Analysis of the 16S rRNA gene of micro-organism WSU 86-1044 from an aborted bovine foetus reveals that it is a member of the order Chlamydiales: proposal of Waddliaceae fam. nov., *Waddlia chondrophila* gen. nov., sp. nov. *Int J Syst Bacteriol* 49, 577-581.
- Sacks, F. M., Pfeffer, M. A., Moye, L. A., Rouleau, J. L., Rutherford, J. D., Cole, T. G., Brown, L., Warnica, J. W., Arnold, J. M., Wun, C. C., *et al.* (1996). The effect of pravastatin on coronary events after myocardial infarction in patients with average cholesterol levels. Cholesterol and Recurrent Events Trial investigators. *N Engl J Med* 335, 1001-1009.
- Saikku, P., Leinonen, M., Mattila, K., Ekman, M. R., Nieminen, M. S., Mäkelä, P. H., Huttunen, J. K., and Valtonen, V. (1988). Serological evidence of an association of a novel *Chlamydia*, TWAR, with chronic coronary heart disease and acute myocardial infarction. *Lancet* 2, 983-986.
- Saikku, P., Wang, S. P., Kleemola, M., Brander, E., Rusanen, E., and Grayston, J. T. (1985). An epidemic of mild pneumonia due to an unusual strain of *Chlamydia psittaci*. *J Infect Dis* 151, 832-839.
- Sako, T., Takahashi, T., Takehana, K., Uchida, E., Nakade, T., Umemura, T., and Taniyama, H. (2002). Chlamydial infection in canine atherosclerotic lesions. *Atherosclerosis* 162, 253-259.
- Sambri, V., Donati, M., Storni, E., Leo, K. D., Agnusdei, M., Petracca, R., Finco, O., Grandi, G., Ratti, G., and Cevenini, R. (2004). Experimental infection by *Chlamydia pneumoniae* in the hamster. *Vaccine* 22, 1131-1137.
- Saren, A., Pascolo, S., Stevanovic, S., Dumrese, T., Puolakkainen, M., Sarvas, M., Rammensee, H. G., and Vuola, J. M. (2002). Identification of *Chlamydia pneumoniae*-derived mouse CD8 epitopes. *Infect Immun* 70, 3336-3343.
- Sasu, S., LaVerda, D., Qureshi, N., Golenbock, D. T., and Beasley, D. (2001). *Chlamydia pneumoniae* and chlamydial heat shock protein 60 stimulate proliferation of human vascular smooth muscle cells via toll-like receptor 4 and p44/p42 mitogen-activated protein kinase activation. *Circ Res* 89, 244-250.
- Schachter, J., Stephens, R. S., Timms, P., Kuo, C., Bavoi, P. M., Birkelund, S., Boman, J., Caldwell, H., Campbell, L. A., Chernesky, M., *et al.* (2001). Radical changes to chlamydial taxonomy are not necessary just yet. *Int J Syst Evol Microbiol* 51, 249.
- Schett, G., Xu, Q., Amberger, A., Van der Zee, R., Recheis, H., Willeit, J., and Wick, G. (1995). Autoantibodies against heat shock protein 60 mediate endothelial cytotoxicity. *J Clin Invest* 96, 2569-2577.

- Schonbeck, U., and Libby, P. (2004). Inflammation, immunity, and HMG-CoA reductase inhibitors: statins as antiinflammatory agents? *Circulation* 109, II18-26.
- Seitz, C. S., Kleindienst, R., Xu, Q., and Wick, G. (1996). Coexpression of heat-shock protein 60 and intercellular-adhesion molecule-1 is related to increased adhesion of monocytes and T cells to aortic endothelium of rats in response to endotoxin. *Lab Invest* 74, 241-252.
- Sharma, J., Niu, Y., Ge, J., Pierce, G. N., and Zhong, G. (2004). Heat-inactivated *C. pneumoniae* organisms are not atherogenic. *Mol Cell Biochem* 260, 147-152.
- Shaw, E. I., Dooley, C. A., Fischer, E. R., Scidmore, M. A., Fields, K. A., and Hackstadt, T. (2000). Three temporal classes of gene expression during the *Chlamydia trachomatis* developmental cycle. *Mol Microbiol* 37, 913-925.
- Shemer, Y., and Sarov, I. (1985). Inhibition of growth of *Chlamydia trachomatis* by human gamma interferon. *Infect Immun* 48, 592-596.
- Shemer-Avni, Y., and Lieberman, D. (1995). *Chlamydia pneumoniae*-induced ciliostasis in ciliated bronchial epithelial cells. *J Infect Dis* 171, 1274-1278.
- Shi, Y., and Tokunaga, O. (2004). *Chlamydia pneumoniae* (*C. pneumoniae*) infection upregulates atherosclerosis-related gene expression in human umbilical vein endothelial cells (HUVECs). *Atherosclerosis* 177, 245-253.
- Shi, Y., Yin, J., Zhan, H., Feng, G., Zhang, X., Su, X., Song, Y., Xia, X., Zhou, X., and Shen, P. (2003). The pathogenesis of *Chlamydia pneumoniae*-type pneumonitis in mice. *Chin Med J (Engl)* 116, 328-332.
- Shirai, M., Hirakawa, H., Kimoto, M., Tabuchi, M., Kishi, F., Ouchi, K., Shiba, T., Ishii, K., Hattori, M., Kuhara, S., and Nakazawa, T. (2000). Comparison of whole genome sequences of *Chlamydia pneumoniae* J138 from Japan and CWL029 from USA. *Nucleic Acids Res* 28, 2311-2314.
- Shor, A., Kuo, C. C., and Patton, D. L. (1992). Detection of *Chlamydia pneumoniae* in coronary arterial fatty streaks and atheromatous plaques. *S Afr Med J* 82, 158-161.
- Socan, M., Kosmelj, K., Marinic-Fiser, N., and Vidmar, L. (2004). A prediction model for community-acquired *Chlamydia pneumoniae* pneumonia in hospitalized patients. *Infection* 32, 204-209.
- Sparrow, C. P., Burton, C. A., Hernandez, M., Mundt, S., Hassing, H., Patel, S., Rosa, R., Hermanowski-Vosatka, A., Wang, P. R., Zhang, D., *et al.* (2001). Simvastatin has anti-inflammatory and antiatherosclerotic activities independent of plasma cholesterol lowering. *Arterioscler Thromb Vasc Biol* 21, 115-121.
- Stack, A. M., Malley, R., Saladino, R. A., Montana, J. B., MacDonald, K. L., and Molrine, D. C. (2000). Primary respiratory syncytial virus infection: pathology, immune response, and evaluation of vaccine challenge strains in a new mouse model. *Vaccine* 18, 1412-1418.
- Sтары, H. C. (2000). Natural history and histological classification of atherosclerotic lesions: an update. *Arterioscler Thromb Vasc Biol* 20, 1177-1178.
- Sтары, H. C., Chandler, A. B., Dinsmore, R. E., Fuster, V., Glagov, S., Insull, W., Jr., Rosenfeld, M. E., Schwartz, C. J., Wagner, W. D., and Wissler, R. W. (1995). A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Circulation* 92, 1355-1374.

- Stary, H. C., Chandler, A. B., Glagov, S., Guyton, J. R., Insull, W., Jr., Rosenfeld, M. E., Schaffer, S. A., Schwartz, C. J., Wagner, W. D., and Wissler, R. W. (1994). A definition of initial, fatty streak, and intermediate lesions of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Circulation* 89, 2462-2478.
- Stephens, R. S., Kalman, S., Lammel, C., Fan, J., Marathe, R., Aravind, L., Mitchell, W., Olinger, L., Tatusov, R. L., Zhao, Q., *et al.* (1998). Genome sequence of an obligate intracellular pathogen of humans: *Chlamydia trachomatis*. *Science* 282, 754-759.
- Strandberg, T. E., Vanhanen, H., and Tikkanen, M. J. (1999). Effect of statins on C-reactive protein in patients with coronary artery disease. *Lancet* 353, 118-119.
- Stuart, E. S., Webley, W. C., and Norkin, L. C. (2003). Lipid rafts, caveolae, caveolin-1, and entry by *Chlamydiae* into host cells. *Exp Cell Res* 287, 67-78.
- Summersgill, J. T., Sahney, N. N., Gaydos, C. A., Quinn, T. C., and Ramirez, J. A. (1995). Inhibition of *Chlamydia pneumoniae* growth in HEp-2 cells pretreated with gamma interferon and tumor necrosis factor alpha. *Infect Immun* 63, 2801-2803.
- Sutton, G. C., Morrissey, R. A., Tobin, J. R., and Anderson, T. O. (1967). Pericardial and myocardial disease associated with serological evidence of infection by agents of the Psittacosis-Lymphogranuloma Venereum group (*Chlamydiaceae*). *Circulation* 36, 830-838.
- Svanholm, C., Bandholtz, L., Castanos-Velez, E., Wigzell, H., and Rottenberg, M. E. (2000). Protective DNA immunization against *Chlamydia pneumoniae*. *Scand J Immunol* 51, 345-353.
- Tambiah, J., Franklin, I. J., Trendell-Smith, N., Peston, D., and Powell, J. T. (2001). Provocation of experimental aortic inflammation and dilatation by inflammatory mediators and *Chlamydia pneumoniae*. *Br J Surg* 88, 935-940.
- Tambiah, J., and Powell, J. T. (2002). *Chlamydia pneumoniae* antigens facilitate experimental aortic dilatation: prevention with azithromycin. *J Vasc Surg* 36, 1011-1017.
- Tirola, T., Erkkilä, L., Laitinen, K., Leinonen, M., Saikku, P., Bloigu, A., and Jauhainen, M. (2002). Effect of acute *Chlamydia pneumoniae* infection on lipoprotein metabolism in NIH/S mice. *Scand J Clin Lab Invest* 62, 477-484.
- Toye, B., Laferriere, C., Claman, P., Jessamine, P., and Peeling, R. (1993). Association between antibody to the chlamydial heat-shock protein and tubal infertility. *J Infect Dis* 168, 1236-1240.
- Troy, C. J., Peeling, R. W., Ellis, A. G., Hockin, J. C., Bennett, D. A., Murphy, M. R., and Spika, J. S. (1997). *Chlamydia pneumoniae* as a new source of infectious outbreaks in nursing homes. *Jama* 277, 1214-1218.
- Törmäkangas, L., Alakärppä, H., David, D. B., Leinonen, M., and Saikku, P. (2004a). Telithromycin treatment of chronic *Chlamydia pneumoniae* infection in C57BL/6J mice. *Antimicrob Agents Chemother* 48, 3655-3661.
- Törmäkangas, L., Saario, E., Bem David, D., Bryskier, A., Leinonen, M., and Saikku, P. (2004b). Treatment of acute *Chlamydia pneumoniae* infection with telithromycin in C57BL/6J mice. *J Antimicrob Chemother*. 53, 1101-1104
- van Zandbergen, G., Gieffers, J., Kothe, H., Rupp, J., Bollinger, A., Aga, E., Klinger, M., Brade, H., Dalhoff, K., Maass, M., *et al.* (2004). *Chlamydia pneumoniae* multiply in neutrophil granulocytes and delay their spontaneous apoptosis. *J Immunol* 172, 1768-1776.

- Wark, P. A., Johnston, S. L., Simpson, J. L., Hensley, M. J., and Gibson, P. G. (2002). *Chlamydia pneumoniae* immunoglobulin A reactivation and airway inflammation in acute asthma. *Eur Respir J* 20, 834-840.
- Vaughan, C. J., Murphy, M. B., and Buckley, B. M. (1996). Statins do more than just lower cholesterol. *Lancet* 348, 1079-1082.
- Vehmaan-Kreula, P., Puolakkainen, M., Sarvas, M., Welgus, H. G., and Kovanen, P. T. (2001). *Chlamydia pneumoniae* proteins induce secretion of the 92-kDa gelatinase by human monocyte-derived macrophages. *Arterioscler Thromb Vasc Biol* 21, E1-8.
- Wells, B. J., Mainous, A. G., 3rd, and Dickerson, L. M. (2004). Antibiotics for the secondary prevention of ischemic heart disease: a meta-analysis of randomized controlled trials. *Arch Intern Med* 164, 2156-2161.
- Vergnes, L., Phan, J., Strauss, M., Tafuri, S., and Reue, K. (2003). Cholesterol and cholate components of an atherogenic diet induce distinct stages of hepatic inflammatory gene expression. *J Biol Chem* 278, 42774-42784.
- Wick, G., Schett, G., Amberger, A., Kleindienst, R., and Xu, Q. (1995). Is atherosclerosis an immunologically mediated disease? *Immunol Today* 16, 27-33.
- Wiedeman, J. A., Kaul, R., Heuer, L. S., Thao, N. N., Pinkerton, K. E., and Wenman, W. M. (2004). Tobacco smoke induces persistent infection of *Chlamydomytila pneumoniae* in HEP-2 cells. *Microb Pathog* 37, 141-148.
- Williams, J. P., Hernady, E., Johnston, C. J., Reed, C. M., Fenton, B., Okunieff, P., and Finkelstein, J. N. (2004). Effect of administration of lovastatin on the development of late pulmonary effects after whole-lung irradiation in a murine model. *Radiat Res* 161, 560-567.
- Wilson, A. C., and Tan, M. (2002). Functional analysis of the heat shock regulator HrcA of *Chlamydia trachomatis*. *J Bacteriol* 184, 6566-6571.
- Wissel, H., Schulz, C., Rudiger, M., Krull, M., Stevens, P. A., and Wauer, R. R. (2003). *Chlamydia pneumoniae* affect surfactant trafficking and secretion due to changes of type II cell cytoskeleton. *Am J Respir Cell Mol Biol* 29, 303-313.
- Witkin, S. S., Jeremias, J., Toth, M., and Ledger, W. J. (1994). Proliferative response to conserved epitopes of the *Chlamydia trachomatis* and human 60-kilodalton heat-shock proteins by lymphocytes from women with salpingitis. *Am J Obstet Gynecol* 171, 455-460.
- Wizel, B., Starcher, B. C., Samten, B., Chroneos, Z., Barnes, P. F., Dzuris, J., Higashimoto, Y., Appella, E., and Sette, A. (2002). Multiple *Chlamydia pneumoniae* antigens prime CD8⁺ Tc1 responses that inhibit intracellular growth of this vacuolar pathogen. *J Immunol* 169, 2524-2535.
- Wolf, K., Fischer, E., and Hackstadt, T. (2000). Ultrastructural analysis of developmental events in *Chlamydia pneumoniae*-infected cells. *Infect Immun* 68, 2379-2385.
- Wolf, K., and Malinverni, R. (1999). Effect of azithromycin plus rifampin versus that of azithromycin alone on the eradication of *Chlamydia pneumoniae* from lung tissue in experimental pneumonitis. *Antimicrob Agents Chemother* 43, 1491-1493.
- von Hertzen, L., Alakärppä, H., Koskinen, R., Liippo, K., Surcel, H. M., Leinonen, M., and Saikku, P. (1997). *Chlamydia pneumoniae* infection in patients with chronic obstructive pulmonary disease. *Epidemiol Infect* 118, 155-164.

- von Hertzen, L., Isoaho, R., Leinonen, M., Koskinen, R., Laippala, P., Töyrylä, M., Kivelä, S. L., and Saikku, P. (1996). *Chlamydia pneumoniae* antibodies in chronic obstructive pulmonary disease. *Int J Epidemiol* 25, 658-664.
- von Hertzen, L., Leinonen, M., Surcel, H. M., Karjalainen, J., and Saikku, P. (1995). Measurement of sputum antibodies in the diagnosis of acute and chronic respiratory infections associated with *Chlamydia pneumoniae*. *Clin Diagn Lab Immunol* 2, 454-457.
- Wong, K. H., Skelton, S. K., and Chan, Y. K. (1992). Efficient culture of *Chlamydia pneumoniae* with cell lines derived from the human respiratory tract. *J Clin Microbiol* 30, 1625-1630.
- Wu, L., Skinner, S. J., Lambie, N., Vuletic, J. C., Blasi, F., and Black, P. N. (2000). Immunohistochemical staining for *Chlamydia pneumoniae* is increased in lung tissue from subjects with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 162, 1148-1151.
- Vuola, J. M., Puurula, V., Anttila, M., Mäkelä, P. H., and Rautonen, N. (2000). Acquired immunity to *Chlamydia pneumoniae* is dependent on gamma interferon in two mouse strains that initially differ in this respect after primary challenge. *Infect Immun* 68, 960-964.
- Wuppermann, F. N., Hegemann, J. H., and Jantos, C. A. (2001). Heparan Sulfate-like Glycosaminoglycan Is a Cellular Receptor for *Chlamydia pneumoniae*. *J Infect Dis* 184, 181-187.
- Xu, Q., Dietrich, H., Steiner, H. J., Gown, A. M., Schoel, B., Mikuz, G., Kaufmann, S. H., and Wick, G. (1992). Induction of arteriosclerosis in normocholesterolemic rabbits by immunization with heat shock protein 65. *Arterioscler Thromb* 12, 789-799.
- Xu, Q., Kleindienst, R., Schett, G., Waitz, W., Jindal, S., Gupta, R. S., Dietrich, H., and Wick, G. (1996). Regression of arteriosclerotic lesions induced by immunization with heat shock protein 65-containing material in normocholesterolemic, but not hypercholesterolemic, rabbits. *Atherosclerosis* 123, 145-155.
- Xu, Q., Kleindienst, R., Waitz, W., Dietrich, H., and Wick, G. (1993a). Increased expression of heat shock protein 65 coincides with a population of infiltrating T lymphocytes in atherosclerotic lesions of rabbits specifically responding to heat shock protein 65. *J Clin Invest* 91, 2693-2702.
- Xu, Q., Luef, G., Weimann, S., Gupta, R. S., Wolf, H., and Wick, G. (1993b). Staining of endothelial cells and macrophages in atherosclerotic lesions with human heat-shock protein-reactive antisera. *Arterioscler Thromb* 13, 1763-1769.
- Xu, Q., Schett, G., Perschinka, H., Mayr, M., Egger, G., Oberhollenzer, F., Willeit, J., Kiechl, S., and Wick, G. (2000). Serum soluble heat shock protein 60 is elevated in subjects with atherosclerosis in a general population. *Circulation* 102, 14-20.
- Xu, Q., Schett, G., Seitz, C. S., Hu, Y., Gupta, R. S., and Wick, G. (1994). Surface staining and cytotoxic activity of heat-shock protein 60 antibody in stressed aortic endothelial cells. *Circ Res* 75, 1078-1085.
- Xu, Q., Willeit, J., Marosi, M., Kleindienst, R., Oberhollenzer, F., Kiechl, S., Stulnig, T., Luef, G., and Wick, G. (1993c). Association of serum antibodies to heat-shock protein 65 with carotid atherosclerosis. *Lancet* 341, 255-259.
- Yang, J., Hooper, W. C., Phillips, D. J., Tondella, M. L., and Talkington, D. F. (2003). Induction of proinflammatory cytokines in human lung epithelial cells during *Chlamydia pneumoniae* infection. *Infect Immun* 71, 614-620.

- Yang, Z. P., Cummings, P. K., Patton, D. L., and Kuo, C. C. (1994). Ultrastructural lung pathology of experimental *Chlamydia pneumoniae* pneumonitis in mice. *J Infect Dis* 170, 464-467.
- Yang, Z. P., Kuo, C. C., and Grayston, J. T. (1993). A mouse model of *Chlamydia pneumoniae* strain TWAR pneumonitis. *Infect Immun* 61, 2037-2040.
- Yang, Z. P., Kuo, C. C., and Grayston, J. T. (1995). Systemic dissemination of *Chlamydia pneumoniae* following intranasal inoculation in mice. *J Infect Dis* 171, 736-738.
- Yeh, Y. F., and Huang, S. L. (2004). Enhancing effect of dietary cholesterol and inhibitory effect of pravastatin on allergic pulmonary inflammation. *J Biomed Sci* 11, 599-606.
- Yi, Y., Yang, X., and Brunham, R. C. (1997). Autoimmunity to heat shock protein 60 and antigen-specific production of interleukin-10. *Infect Immun* 65, 1669-1674.
- Yi, Y., Zhong, G., and Brunham, R. C. (1993). Continuous B-cell epitopes in *Chlamydia trachomatis* heat shock protein 60. *Infect Immun* 61, 1117-1120.
- Young, D., Lathigra, R., Hendrix, R., Sweetser, D., and Young, R. A. (1988). Stress proteins are immune targets in leprosy and tuberculosis. *Proc Natl Acad Sci U S A* 85, 4267-4270.
- Young, R. A., and Elliott, T. J. (1989). Stress proteins, infection, and immune surveillance. *Cell* 59, 5-8.
- Youssef, S., Stuve, O., Patarroyo, J. C., Ruiz, P. J., Radosevich, J. L., Hur, E. M., Bravo, M., Mitchell, D. J., Sobel, R. A., Steinman, L., and Zamvil, S. S. (2002). The HMG-CoA reductase inhibitor, atorvastatin, promotes a Th2 bias and reverses paralysis in central nervous system autoimmune disease. *Nature* 420, 78-84.
- Zhong, G., and Brunham, R. C. (1992). Antibody responses to the chlamydial heat shock proteins hsp60 and hsp70 are H-2 linked. *Infect Immun* 60, 3143-3149.
- Zhong, G., Fan, T., and Liu, L. (1999). Chlamydia inhibits interferon gamma-inducible major histocompatibility complex class II expression by degradation of upstream stimulatory factor 1. *J Exp Med* 189, 1931-1938.
- Zhong, G., Liu, L., Fan, T., Fan, P., and Ji, H. (2000). Degradation of transcription factor RFX5 during the inhibition of both constitutive and interferon gamma-inducible major histocompatibility complex class I expression in chlamydia-infected cells. *J Exp Med* 191, 1525-1534.
- Zhou, X., Paulsson, G., Stemme, S., and Hansson, G. K. (1998). Hypercholesterolemia is associated with a T helper (Th) 1/Th2 switch of the autoimmune response in atherosclerotic apo E-knockout mice. *J Clin Invest* 101, 1717-1725.
- Zhu, J., Nieto, F. J., Horne, B. D., Anderson, J. L., Muhlestein, J. B., and Epstein, S. E. (2001). Prospective study of pathogen burden and risk of myocardial infarction or death. *Circulation* 103, 45-51.
- Zhu, J., Quyyumi, A. A., Norman, J. E., Csako, G., Waclawiw, M. A., Shearer, G. M., and Epstein, S. E. (2000). Effects of total pathogen burden on coronary artery disease risk and C-reactive protein levels. *Am J Cardiol* 85, 140-146.
- Zügel, U., and Kaufmann, S. H. (1999). Role of heat shock proteins in protection from and pathogenesis of infectious diseases. *Clin Microbiol Rev* 12, 19-39.