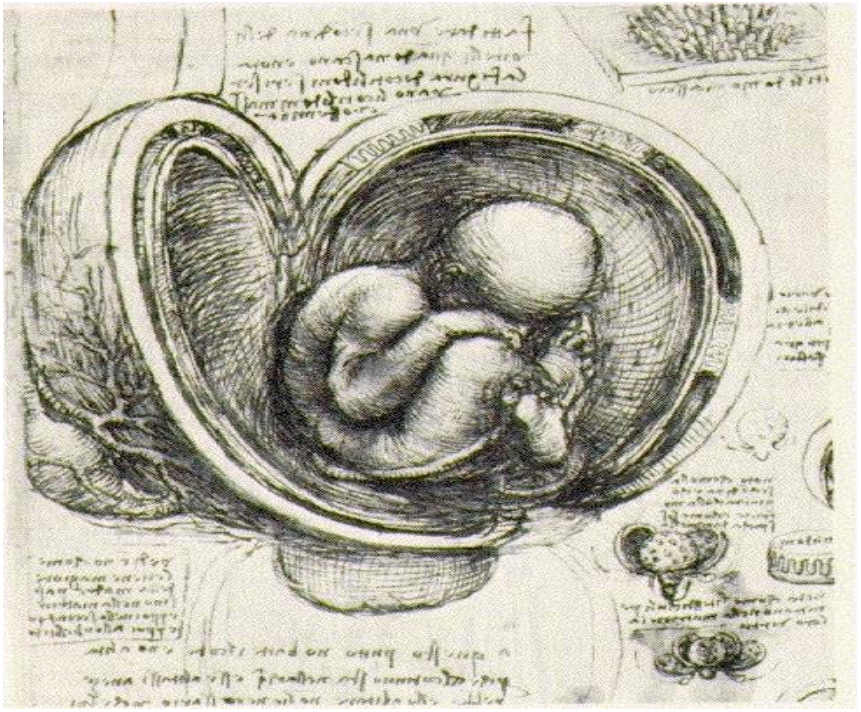
Anne Kinnunen

CHLAMYDIAL HEAT SHOCK PROTEIN 60 AND CELL-MEDIATED IMMUNITY IN TUBAL FACTOR INFERTILITY



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By

Anne Kinnunen

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A scetch of the womb by Leonardo da Vinci (1452 -1519).

To My Family –Anerrifto kybos

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Abstract

The purpose of this study was to elucidate the role of chlamydial 60 kDa heat shock protein (CHSP60) induced cell-mediated immune (CMI) response in women with tubal factor infertility (TFI). *Chlamydia trachomatis* is the major cause of sexually transmitted bacterial infections (STIs) worlwide. Most chlamydial infections are asymptomatic or show mild symptoms and are thus often left untreated. Reinfections with same or different serovars are common. The long-term consequences of recurrent or persistent chlamydial infections are severe leading to structural damage of the inflamed tissue. Chronic *C. trachomatis* infection in women has been linked with ectopic pregnancy and TFI. Animal models for chlamydial infections have shown that CD4+ T-cells are the key players both in resolution of and protection from chlamydial infection, but have been implicated also in the pathogenesis of the disease sequelae. Generation of pathologic changes in the fallopian tube tissue is mostly an unknown process but is thought to be due to repeated or persistent infections. In addition, a balance of pro- and anti-inflammatory cytokines, especially that of interferon-gamma (IFN-γ) and interleukin-10 (IL-10) may have an important role during both primary and recurrent or persistent infection.

We established *C. trachomatis*-specific T-lymphocyte lines (TLLs) and T-lymphocyte clones (TLCs) from the obstructed fallopian tube tissue specimens of infertile women (TFI) and from endometrial biopsies of patients with acute pelvic inflammatory disease. CMI responses of isolated T-cells in response to chlamydial elementary body (EB) antigens and CHSP60 were measured using lymphocyte proliferation (LP) assay. Frequency of CHSP60-responding T-cells was determined. Production of pro-inflammatory (IFN-γ) and anti-inflammatory (IL-10) cytokines by the *Chlamydia*-specific TLLs and TLCs in response to recall antigens was determined.

According to our results *C. trachomatis*-specific T-cells were frequently found in peripheral blood and in the inflamed fallopian tube tissue of TFI patients. One third of the T-cells derived from fallopian tube tissue specimens of TFI patients responded to CHSP60. We also found that CHSP60-specific T-cells produce IL-10 which may down-regulate appropriate IFN-γ-mediated immune response needed for elimination of chlamydial infection. In addition, the CHSP60-specific CMI response was associated with HLA-DQA1*0102 and HLA-DQB1*0602 alleles and IL-10 – 1082 AA genotype in the TFI patients.

Taken together, these data suggest that the etiopathogenesis of *C. trachomatis*-related TFI is associated with impaired CMI response caused by CHSP60-reactive T-cells. Larger clinical and population-based follow-up studies are warranted to further evaluate the role of specific HLA class II and IL-10 geno(haplo)types in association with CHSP60-reactive T-cells in the pathogenesis of TFI.

Keywords: Chlamydia trachomatis, heat shock protein, tubal factor infertility (TFI), cell-mediated immunity (CMI)

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Oulu, September 2002 Anne Kinnunen

Abbreviations

APC	Antigen presenting cell
ATP	Adenosine triphosphate
bp	base pair
CMI	Cell-mediated immunity/ immune response
(C)HSP60	60 kDa (chlamydial) heat shock protein
CTL	Cytotoxic T lymphocyte/ CD8+ T cell
DFA	Direct fluorescence assay
DNA	Deoxyribonucleic acid
DTH	Delayed type hypersensitivity
EB	Elementary body
ELISA	Enzyme-linked immunosorbent assay
HLA	Human leukocyte antigen
HSP	Heat-shock protein
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
LCR	Ligase chain reaction
LGV	Lymphogranuloma venereum
LP	Lymphocyte proliferation
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
MIF	Micro-immunofluorescence
MOMP/OmpA	Major outer membrane protein
NK cell	Natural killer cell
OMP2/OmcB	60 kDa cysteine-rich outer membrane protein
OMP3/OmcA	12 kDa outer membrane protein
PBL	Peripheral blood lymphocyte
PCR	Polymerase chain reaction
PHA	Phytohemagglutinine
PID	Pelvic inflammatory disease
Pomp/Pmp	Putative/ polymorphic outer membrane protein
PWM	Pokeweed mitogen

RA	Rheumatoid arthritis
ReA	Reactive arthritis
RB	Reticulate body
(r)RNA	(ribosomal) ribonucleic acid
SI	Stimulation index
TFI	Tubal factor infertility
TLC	T lymphocyte (cell) clone
TLL	T lymphocyte (cell) line
TNF	Tumour necrosis factor

List of original publications

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

I Kinnunen A, Surcel HM, Halttunen M, Tiitinen A, Morrison RP, Morrison SG, Koskela P, Lehtinen M and Paavonen J. *Chlamydia trachomatis* heat shock protein-60 induced interferon-γ and interleukin-10 production by peripheral blood lymphocytes in infertile women. Submitted.

II Kinnunen A, Molander P, Laurila A, Rantala I, Morrison R, Lehtinen M, Karttunen R, Tiitinen A, Paavonen J and Surcel HM. *Chlamydia trachomatis* reactive T lymphocytes from upper genital tract tissue specimens. Hum Reprod 2000; 15:1484-1489.

III Kinnunen A, Molander P, Morrison RP, Lehtinen M, Karttunen R, Tiitinen A, Paavonen J, Surcel HM. Chlamydial heat shock protein 60 specific T cells in inflamed salpingeal tissue. Fertil Steril 2002; 77:162-166.

IV Kinnunen A, Surcel HM, Lehtinen M, Karhukorpi J, Tiitinen A, Halttunen M, Bloigu A, Morrison RP, Karttunen R, Paavonen J. HLA DQ alleles and interleukin-10 polymorphism associated with *Chlamydia trachomatis*-related tubal factor infertility: A case–control study. Hum Reprod 2002; 17:2073-2078.

Contents

1 Introduction

Chlamydia trachomatis infection is the most common bacterial sexually transmitted disease worldwide. It has been estimated that about 90 million new infections occur annually. Most of the acute infections are asymptomatic and are thus left untreated. Reinfections with the same or different serovar are common because *C. trachomatis* immunity is short-lived and usually serovar-specific. In some women repeated or persistent *C. trachomatis* infection leads to scarring of the fallopian tube tissue and subsequent infertility because of occlusion of the tubes. In fact, *C. trachomatis*-associated tubal factor infertility (TFI) is one of the top three reasons for couples to seek help for involuntary infertility.

Chlamydiae are typical intracellular bacteria that are able to elicit a variety of humoral and cell-mediated immune (CMI) responses. Interplay between humoral and CMI responses in terms of protection or adverse sequelae of chlamydial infection, is not thoroughly studied. For a long period of time antibodies were considered to play the main role to confer protection. Trachoma vaccine trials in the 1960s and 1970s showed that some protective immunity develops following primary chlamydial infection, but it is short-lived and a different serovar can infect the individual shortly after resolution of the primary infection (Grayston and Wang 1978). It is probable that “protection” detected in these studies was mediated by antibodies. The duration of chlamydial ocular infection is shorter and the resolution of disease is more rapid in older individuals compared to children indicating that some kind of immunity develops over time (Bailey *et al.* 1999).

Immunity during chronic chlamydial infections is still at large a mystery although persistent or repeated infections are needed to pave the way to immunopathological changes. Until 1990s the only role of CMI was thought to be “the bad guy” eliciting pathological changes seen in repeatedly (persistently) infected individuals. Since then convincing amount of evidence has gathered suggesting that CMI is needed to eliminate chlamydial infection and also for protection. Recovery from chlamydial infections results from type 1 immune response (see 2.6.1) that is largely regulated by interferon-gamma (IFN–γ). What may be even more important is the balance of pro- and anti-inflammatory cytokines present at the site of infection. Chronic chlamydial infection may induce continuous inflammatory reactions through the action of secreted cytokines that provoke tissue injury. Serological studies with humans have suggested that one protein in particular, i.e. the 60 kDa chlamydial heat shock protein (CHSP60) may have a central

role in the pathogenesis of chlamydial infection. Experimental murine and primate models of chronic chlamydial disease have further supported a role for CHSP60 since repeated exposure to CHSP60 elicited delayed-type hypersensitivity (DTH) reactions and scarring in animals previously immunized with *C. trachomatis*. In human chlamydial infections especially in the TFI the role of CHSP60 in eliciting and maintaining inflammatory response in the tissue is not known.

2 Review of the literature

2.1 Chlamydia

Chlamydiae are obligate intracellular gram-negative-like bacteria that replicate in membrane-bound vacuoles (inclusions) in the cytoplasm of eukaryotic cells (reviewed by Ward 1983, Matsumoto 1988, and Ward 1988). They belong to the order *Chlamydiales*, which contains one family, *Chlamydiaceae* with one genus *Chlamydia* (Moulder *et al.* 1984, Fukushi and Hirai 1992, Pudjiatmoko *et al.* 1997). The genus is divided into four species, *C. trachomatis, C. pneumoniae, C. psittaci* and *C. pecorum*, which differ in their host cell tropism, but have similar cell structure and share certain biological properties in the course of their intracellular existence (reviewed by Hackstadt 1999). Recent discovery of new obligate intracellular organisms that have *Chlamydia*-like developmental cycle and analysis of the ribosomal operon lead Everett et al. (1999) to propose a new revised taxonomy to *Chlamydiaceae.* The new taxonomy is based on phylogenetic analyses of 16S and 23S rRNA genes corroborated with genetic and phenotypic information obtained during the past few years (Kaltenboeck *et al.* 1993, Everett and Andersen 1997, Pudjiatmoko *et al.* 1997, Takahashi *et al.* 1997). According to the proposed taxonomy the family *Chlamydiaceae* is divided into two genera, *Chlamydia* and *Chlamydophila* (Figure 1) with nine species (Everett *et al.* 1999). The new taxonomy, however, has not yet gained general acceptance (Schachter *et al.* 2001).

Chlamydiae cause infections both in humans and in animals. They spread by aerosol or by contact (reviewed by Bavoil *et al.* 1996) and have been demonstrated *in vitro* to infect and multiply in wide variety of cells including epithelial and endothelial cells. In addition, chlamydiae are able to infect and/or get into and remain viable in phagocytes, macrophages and monocytes (reviewed by Bavoil *et al.* 1996). This gives the organism an opportunity to disseminate from the original site of infection. Past studies focused mainly on infections caused by *C. trachomatis* but during the last decade *C. pneumoniae* has also been under keen investigation because of its association with the pathogenesis of cardiovascular disease (reviewed by Saikku 2000b, Saikku 2000a).

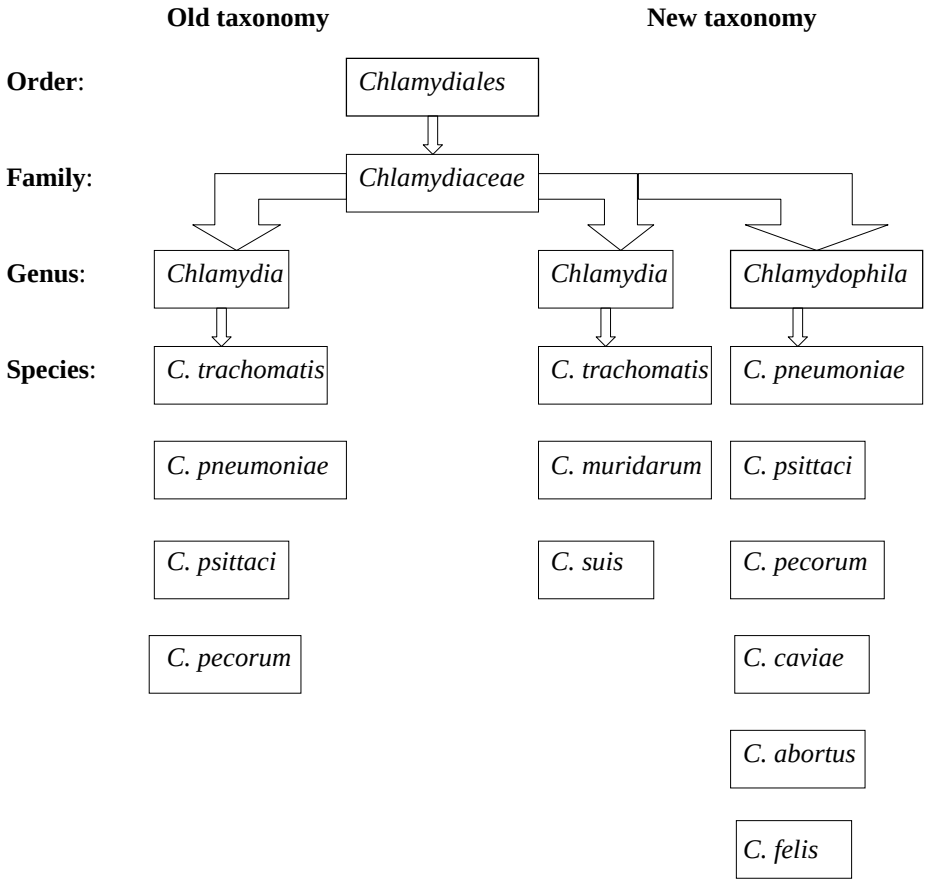

Fig. 1. Old and new taxonomy of chlamydiae (modified from Everett *et al.* 1999).

2.2 Developmental cycle of chlamydiae

Chlamydiae have a unique developmental cycle comprising two functionally and morphologically different forms to survive both outside and inside the host cell. The elementary body (EB) is the infectious form of the bacterium. It attaches to and enters into the host cell. EB is a small particle (Ø 300 –400 nm) with a rigid cell wall, which contains the major outer membrane protein (MOMP) and two cysteine-rich outer membrane proteins, OMP2 and OMP3 as well as other proteins (reviewed by Beatty *et al.* 1994c). EB cell wall does not have the kind of peptidoglycan that other gram-negative bacteria have (Barbour *et al.* 1982) although chlamydiae encode proteins forming a nearly complete peptidoglycan synthesis pathway (reviewed by Rockey *et al.* 2000). It has been proposed that chlamydiae synthesize a truncated form of peptidoglycan that

cannot be detected by conventional methods (Ghuysen and Goffin 1999). This could explain why *in vitro* chlamydiae are sensitive to drugs that inhibit peptidoglycan synthesis (Moulder 1993), such as penicillin.

The structure of EB makes it resistant to environmental factors permitting survival outside the host cell during transmission of the organism from cell to cell or from host to host. After its entry into the host cell EB undergoes morphological changes reorganizing to the reticulate body (RB). RB is a larger (Ø 800 –1000 nm), metabolically active form of chlamydiae capable of synthesizing DNA, RNA and proteins. Chlamydiae have lost their ability to produce their own energy i.e. adenosine triphosphate (ATP) and high-energy metabolites, hence the term energy-parasite (reviewed by Moulder 1991 and Beatty *et al.* 1994c). In addition, chlamydiae acquire many precursors such as nucleotides and amino acids from the host. Compared to EB, RB has a less rigid cell wall and is not able to survive outside the host cell (Beatty *et al.* 1994a). RBs divide by binary fission within chlamydial inclusion. After a few rounds of growth and division, RBs reorganize condensing to infectious EBs (Figure 2A). Redifferentiation of RBs to EBs starts with DNA condensation, which involves histone-like proteins that help pack the chlamydial chromosome (Wagar and Stephens 1988, Hackstadt *et al.* 1991, Tao *et al.* 1991). Redifferentiation is accompanied by the incorporation of MOMP and other outer membrane proteins into an outer membrane complex through extensive cross-linking with disulfide bonds. The developmental cycle is completed with host cell lysis or exocytosis of chlamydial particles allowing EBs to start new infectious cycles. *In vitro* studies have shown that the length of the cycle is approximately 48 –72 hours but it varies as a function of the infecting strain, host cell and environmental conditions (Figure 2A; reviewed by Beatty *et al.* 1994a).

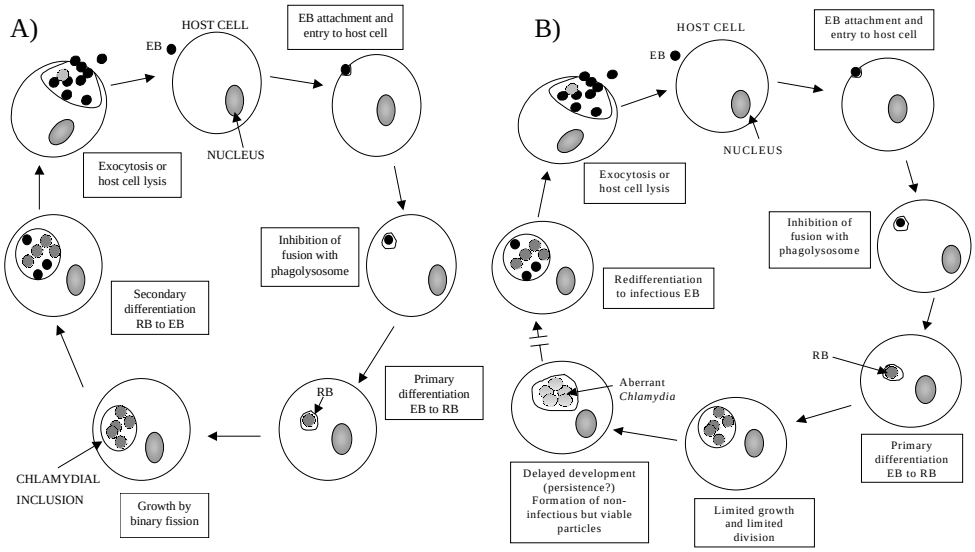

Fig 2. A) Chlamydial developmental cycle, and B) altered developmental cycle (Beatty *et al.* 1994a**).**

2.2.1 Molecular composition of chlamydiae

Both *C. trachomatis* and *C. pneumoniae* genomes consist of a more than one million base pair (bp) chromosome that has been completely sequenced (Stephens *et al.* 1998, Kalman *et al.* 1999, http:// chlamydia-www.berkeley.edu:4231). Most *C. trachomatis* strains have a ~7500 bp plasmid. Genes for some essential enzymes encompassing DNA replication, repair, transcription, and translation have been identified and a diverse set of genes have been found that provide considerable metabolic capabilities for chlamydial growth. Systems for nutrient (amino acids, ions etc.) transport from the host have been identified (Stephens *et al.* 1998, Kalman *et al.* 1999). Also, both genomes sequenced contain genes for ADP/ ATP translocases indicating that chlamydiae can acquire ATP from the host cell (Hatch 1988). Complete pathways to synthesize and degrade glycogen have also been found. Evaluation of putative amino acid sequences of the *C. trachomatis* and *C. pneumoniae* proteins revealed elements of glycolysis, respiration, and pentose biosynthesis as well as open reading frames for previously unknown proteins (Stephens *et al.* 1998, Kalman *et al.* 1999). Thus, chlamydiae are, in principle, able to produce and store high-energy compounds. This was unexpected given the obligate intracellular nature of chlamydiae. To what extent these genes and proteins are active in the infected cell remains to be settled.

Comparison of *C. trachomatis* and *C. pneumoniae* sequences has revealed certain differences in the number of genes. These include genes without homologs between the species or in other organisms. *C. trachomatis* had ~70 genes that were not present in *C. pneumoniae* genome while *C. pneumoniae* had ~200 genes that were not present in *C. trachomatis* genome. These genes occurred mainly in clusters indicating that they are probably responsible for species-specific systems that define their unique biological capacities (Stephens 1999).

Interestingly, chlamydial counterparts for genes of the type III secretion system, that is known to be essential basic virulence determinant in microbes (reviewed by Hueck 1998) have been discovered and appear to be active in chlamydiae (Hsia *et al.* 1997, Subtil *et al.* 2000, Kim 2001). It is presumed that surface projections on chlamydial EBs described twenty years ago by Matsumoto (1981) and Nichols et al. (1985) represent type III secretion system (Bavoil and Hsia 1998). Genes for type III secretion system may turn out to be important in *Chlamydia*-host cell interaction if the genes encoding these proteins form a system that enables chlamydiae to secrete effector molecules into the host cytoplasm that in turn may modulate host's immune responses. Proteins that are secreted by chlamydiae are yet to be identified although there is evidence that they may include inclusion membrane-associated (Inc) proteins (Subtil *et al.* 2001) and *Chlamydia* outer protein N (CopN) (Fields and Hackstadt 2000). In other pathogenic gram-negative bacteria type III secretion is usually activated by contact with host cell and specific proteins are injected directly into the host cell. The injected proteins disrupt host cell signal transduction mechanisms and cytoskeletal arrangements leading to endocytosis of the bacterium by epithelial cells or death of professional phagocytes or other events that help the bacterium establish a parasite-like relationship (reviewed by Hueck 1998 and Plano *et al.* 2001). In general type III secretion system plays a role in disease process, providing bacteria a powerful tool to invade the host. *Salmonella* and *Shigella* species use the type III secretion system to invade and replicate within the host cell (reviewed by

Hueck 1998). In this respect, they represent a similar model of type III secretion system that is detected in chlamydiae.

2.2.1.1 Lipopolysaccharide and cytotoxin

Cell wall of gram-negative bacteria contains lipopolysaccharide (LPS), also called endotoxin that is characteristic and essential for the viability of the bacteria. LPS is composed of lipid A and hydrophobic and hydrophilic polysaccharide portions. In chlamydiae, the polysaccharide part consists of 3-deoxy –D-*manno*-2 –octulosonic acid (KDO) (Dhir *et al.* 1971, Brade *et al.* 1986) but lacks the O-chain that is typically found in enterobacterial LPS (Brade *et al.* 1985). KDO contains two antigen determinants, one of which is chlamydial genus-specific group antigen. The other is similar to enterobacterial Re-type LPS (Nurminen *et al.* 1983, Brade *et al.* 1986). Chlamydial LPS is inactive in inducing lethal toxicity or pyrogenicity (Brade *et al.* 1986) and a weak inducer of the inflammatory cytokine response (Ingalls *et al.* 1995). Besides LPS, chlamydial cytotoxin with a significant homology to large clostridial cytotoxins has been described which may contribute to pathological changes found in chlamydial infections (Belland *et al.* 2001).

2.2.1.2 Outer membrane proteins

Outer membrane proteins (OMPs) are typically engaged in specific nutrient and metabolite acquisition, adhesion to host cells or tissues (Stephens and Lammel 2001). A 40 kDa MOMP, also known as OmpA, is the most abundant protein in both EBs and RBs composing about 60% of total protein mass (Caldwell *et al.* 1981, reviewed by Brunham and Peeling 1994). Its structure varies in the two developmental forms of chlamydiae being highly disulfide-linked in EBs and completely reduced in RBs. In all *C. trachomatis* strains the MOMP consists of four variable segments that specify individual *C. trachomatis* serovars flanked by relatively conserved sequences (Zhang *et al.* 1987b). Surface exposure of variable regions makes chlamydial MOMP an obvious target of the host's immune response. Consequently, chlamydial MOMP has been shown to induce antibody formation that is directed to both linear and conformational epitopes (Zhang *et al.* 1987a, Zhang *et al.* 1989, Wolf *et al.* 2001). Early *in vitro* studies showed that MOMP functions as a tribarrelled porin (Bavoil *et al.* 1984) allowing ATP to pass through the lipid bilayer (Wyllie *et al.* 1999, Jones *et al.* 2000). To do this MOMP has to be reduced and maintained in a non-cross-linked form, which suggests that only reduced MOMP can act as a porin (Bavoil *et al.* 1984). Thus, it has been speculated that MOMP functions as a porin only in the RB form i.e. when chlamydiae are intracellular.

First OMPs identified in EBs but not in RBs were two cystein-rich proteins OMP3 of 12 kDa and OMP2 of 60 kDa, also named OmcA and OmcB, respectively. OmcA is a lipoprotein, the structure of which resembles murein lipoproteins found in other gram-negative bacteria. OmcB is processed after transcription to two 60 kDa proteins. Studies

suggest that the doublet proteins may function as a pair, but in *C. trachomatis* only one mature OmcB has been detected. The cellular location of the OmcB proteins is controversial but it appears that they are located in the periplasm below the inner leaflet of the outer membrane in the EB. Furthermore, OmcA is suggested to be anchored in the inner leaflet as well through its lipid part (reviewed by Hatch 1999).

In addition, chlamydial cell wall contains a number of highly polymorphic membrane proteins (Pmps), also called putative outer membrane proteins (Pomps) with the approximate size of 90 –100 kDa (Campbell *et al.* 1990, Cevenini *et al.* 1991, Melgosa *et al.* 1993, Souriau *et al.* 1994, Longbottom *et al.* 1998a, Stephens *et al.* 1998, Knudsen *et al.* 1999, Kalman *et al.* 1999). Nine Pomp genes (*pmpA –pmpI)* have been identified in *C. trachomatis* by Stephens et al. (1998). In addition, *C. pneumoniae* contains as many as 21 Pomp genes. The function of the Pomps is mostly unknown but of obvious interest considering their surface exposition that has been demonstrated in *C. psittaci* (Longbottom *et al.* 1998b) and in *C. trachomatis* EBs (Tanzer and Hatch 2001). One of the Pomps has been identified as PorB, which functions as a substrate-specific porin facilitating efficient diffusion of dicarboxylates across the outer membrane and is thus responsible for the transport of essential metabolites for chlamydial growth (Stephens and Lammel 2001).

2.2.1.3 Heat shock proteins

Heat shock proteins (HSPs) are highly conserved proteins present in all organisms from bacteria to human. HSPs play central role in cell's normal processes including their function as molecular chaperones during folding, unfolding, assembly and translocation of newly synthetized or damaged proteins (Lindquist 1986, Lindquist and Craig 1988). They are constitutively expressed in most cells and their production is enhanced in response to a wide variety of stimuli such as elevated temperature, anoxia, infection and inflammation (Welch 1993, Zugel and Kaufmann 1999b, Zugel and Kaufmann 1999a). HSPs are divided into four major families according to their approximate molecular weight: HSP90, HSP70 (DnaK in *E. coli)*, HSP60 (GroEL) and small HSPs (Mw approximately 10 kDa GroES). HSPs are highly immunogenic (reviewed by Kaufmann 1990 and Young 1990). Because of their conserved structure (~50% to 60% identical residues among HSP60 protein family) the immune response initiated by the microbial HSP may also be evoked against self-HSP through cross-reactive epitopes as can be concluded from serological studies performed in patients with chronic chlamydial infection (Domeika *et al.* 1998, Witkin *et al.* 1998).

CHSP60 is expressed throughout the chlamydial developmental cycle (Engel *et al.* 1990, Shaw *et al.* 2000) and the amount of CHSP60 is increased after the heat shock (Engel *et al.* 1990) and during the course of other unfavourable growth conditions (Beatty *et al.* 1993, Beatty *et al.* 1994b). Sequencing of the chlamydial genome revealed that it contains three different genes coding for proteins with similarity to the HSP60 protein family (Stephens 1999). GroEL-1 is linked with the gene encoding GroES (i.e. CHSP10). GroEL-2 is not linked with other HSPs. The third gene, GroEL-3, was more distantly related but showed tremendous similarity to the 60 kDa HSP family. Comparable

multigene families of *groEL* genes are found also in *Mycobacteria* (Rinke de Wit *et al.* 1992, Kong *et al.* 1993) and in *Bradyrhizobium* (Fischer *et al.* 1993). These three forms of CHSP60 may have different though unknown roles during chlamydial developmental cycle and possibly in disease pathogenesis.

CHSP70. Chlamydial HSP70 is constitutively expressed (Birkelund *et al.* 1990, Danilition *et al.* 1990) and its expression raises after heat stress (Engel *et al.* 1990). CHSP70 is located in the outer membrane complexes of chlamydial EBs (Raulston *et al.* 1993) and it is thought to facilitate the entry of the EBs into the host cell (Raulston *et al.* 1998), especially in the events following the initial stage of attachment (Raulston *et al.* 2002).

CHSP10. Chlamydial GroES homolog CHSP10 is co-expressed with CHSP60 (LaVerda and Byrne 1997). Immune response to CHSP10 is associated with the pathogenetic sequelae of chronic chlamydial infections (Betsou *et al.* 1999) and tubal occlusion (Spandorfer *et al.* 1999, LaVerda *et al.* 2000).

2.3 *Chlamydia* and disease

2.3.1 Diseases caused by C. trachomatis

C. trachomatis infects superficial columnar epithelial cells in various anatomical sites such as endocervix, urethra, epididymis, endometrium, fallopian tube, conjunctiva and lower respiratory tract (reviewed by Schachter 1999). *C. trachomatis* has three different biotypes: mouse pneumonitis, lymphogranuloma venereum (LGV) and trachoma serovars that cause either ocular (serovars A, B, Ba and C) or genital tract infections (serovars D - K) (Grayston and Wang 1975 and reviewed by Schachter 1999, Mabey and Fraser-Hurt 2001). Mouse pneumonitis agent is used in animal models to mimic both human genital tract and respiratory tract infections. It has proven to be very useful in studying chlamydial immunity.

2.3.1.1 Trachoma

Trachoma is the world's leading cause of preventable blindness. The ocular infection manifests from mild conjunctival lesions (follicular conjunctivitis) to severe forms that eventually lead to scarring and blindness. Severe forms develop through repeated or persistent infections by *C. trachomatis* serovars A, B, Ba and C (Taylor *et al.* 1982, Abu el-Asrar *et al.* 2001). It has been estimated that about 500 million people have had the disease. In the developing countries about 7 to 9 million people are estimated to be blind because of *C. trachomatis* infection (WHO, http://www.who.int/). Trachoma is endemic mainly in tropical and subtropical countries. Main reservoir of the organism is eye of the

infected person, usually a child, and transmission may be potentiated by flies that carry infected secretions from person to person (Jones 1974, Emerson *et al.* 1999).

2.3.1.2 Sexually transmitted infections

C. trachomatis -infection causes important genital tract diseases in women and men; in infants it is the major contributor of inclusion conjunctivitis and pneumonitis. *C. trachomatis* infection is one of the most common sexually transmitted infections (STI), with estimated 90 million new cases occurring each year worldwide (WHO, http://www.who.int/). It has been estimated that in many populations about 10% of the sexually active people are infected with *C. trachomatis*. Highest incidence of positive isolation of chlamydiae is in the age group of less than 25 years (Barnes *et al.* 1990). The incidence gradually decreases with increasing age (Arno *et al.* 1994).

Acute genital tract infections manifest in women as urethritis, cervicitis, salpingitis (Stamm *et al.* 1980, Brunham *et al.* 1984, Dieterle *et al.* 1998), endometritis (Mårdh *et al.* 1981) and pelvic inflammatory disease (PID) (Mårdh *et al.* 1977, De Punzio *et al.* 1995, Paavonen and Lehtinen 1996). It appears that between 10% -40% of women with *C. trachomatis* infection develop PID (reviewed by Simms and Stephenson 2000 and Rogstad 2001). *C. trachomatis* infection may show mild symptoms but in about half of the cases it is asymptomatic (Lan *et al.* 1995, Rogstad 2001). Repeated infections with different or same serovar occur and have been shown to increase the risk for subsequent infertility (Patton *et al.* 1990, Rank *et al.* 1995, Pavletic *et al.* 1999). Long-term consequences of *C. trachomatis* infection are associated with ectopic pregnancy (Cates and Wasserheit 1991, Gerard *et al.* 1998a, Barlow *et al.* 2001), TFI (Punnonen *et al.* 1979, Rhoton-Vlasak 2000, Barlow *et al.* 2001), spontaneous (recurrent) abortions (Quinn *et al.* 1987, Witkin and Ledger 1992), and arthritis (Gerard *et al.* 1998b). It appears that 43% of ectopic pregnancy cases and 25% of TFI cases are due to *C. trachomatis* infection (reviewed by Simms and Stephenson 2000, Rogstad 2001). Recently, association of *C. trachomatis* infection and invasive cervical squamous cell carcinoma was found (Koskela *et al.* 2000, Anttila *et al.* 2001, Smith *et al.* 2002).

In men, *C. trachomatis* infection causes male urethritis and epididymitis (Berger *et al.* 1978, Stamm *et al.* 1984). Newborn babies can become infected with *C. trachomatis* while passing through the infected birth canal. Inclusion conjunctivitis and chlamydial pneumonitis are clinical manifestations of *C. trachomatis* infection in the newborn and have been shown to develop in 18 –50% and 11-20% of cases, respectively (Schachter *et al.* 1982, Alexander and Harrison 1983, Schachter *et al.* 1986, Numazaki *et al.* 1989, Rogstad 2001).

LGV differs considerably from other syndromes caused by *C. trachomatis*. LGV is a systemic disease caused by an invasive biovar (serovars L1, L2 and L3) of *C. trachomatis*. LGV infects lymphatic and subepithelial tissues. Although it is an important pathogen in some developing countries, it is relatively uncommon in industrialized countries. LGV spreads exclusively through sexual contact (reviewed by Schachter 1999).

2.3.2 Diseases caused by other chlamydiae

In the 1980's an atypical chlamydial strain caused outbreaks of acute lower respiratory infections in humans. This strain was originally named TWAR, i.e. Taiwan acute respiratory strain and was later renamed as *C. pneumoniae* by Grayston and colleagues (1989). Acute *C. pneumoniae* causes respiratory tract infections such as sinusitis, pharyngitis, bronchitis, but also pneumonia, which can be a serious disease in the immunocompromized and in the elderly. *C. pneumoniae* is one of the most common human pathogens (Saikku 2000a). Seroprevalence of *C. pneumoniae* increases with age and 60 –70% of the adult population has antibodies to *C. pneumoniae* worldwide (reviewed by Grayston 1992). Chronic *C. pneumoniae* infection is associated with coronary heart disease, atherosclerosis (reviewed by Saikku 2000a) and adult onset asthma (Hahn *et al.* 1991, Hahn *et al.* 1996). Evidence is accumulating to suggest that *C. pneumoniae* may predispose to lung cancer (Laurila *et al.* 1997, Anttila et al. unpublished data).

Both *C. psittaci* and *C. pecorum* typically infect animals. *C. psittaci* causes infections in sheep, cattle and cats but mainly in the avian species (ornithosis). Occasionally *C. psittaci* also infects humans causing an atypical pneumonia known as psittacosis. It mainly occurs in persons that process or handle birds. Human infection is usually acquired through respiratory route and manifests as flu-like (respiratory tract) symptoms such as cough, high fever, rigors and headache. Although some fatal cases have been reported psittacosis is treatable by antibiotics (tetracycline, erythromycine). *C. pecorum* infects swine, cattle and sheep causing spontaneous abortions. No human infections have been reported (reviewed by Schachter 1999).

2.4 **Diagnostic techniques to detect clinical chlamydial infection**

In assessing diagnostic tests for *C. trachomatis* infection it is important to realize that different methods measure distinct targets which have different properties and are present in different amounts in any given sample. Diagnostics of chlamydial infections include direct detection methods, enzyme-immuno assays (EIA) and direct fluorescence assays (DFA), nucleic acid hybridization and amplification tests (NAHTs and NAATS, respectively), and indirect serological techniques [microimmunofluorescence (MIF) and enzyme-linked immuno assays (ELISA)] (reviewed by Black 1997, Battle *et al.* 2001). Major problem in diagnosing acute chlamydial infections is their asymptomatic nature and obtaining appropriate specimen for direct detection. Serologic tests are mainly used to detect acute respiratory tract infections but they are not useful in the diagnosis of chronic chlamydial infections because antibodies are long-lived and a positive result does not distinguish between current and past infection (Clad *et al.* 2000, Tuuminen *et al.* 2000). MIF test was developed in the 1970s by Wang and Grayston (1970). It is considered the most specific of the serologic tests for chlamydial infections and the only serologic test that is able to detect species and serovar-specific responses. The technique itself is very laborious and antigens are expensive and available only on a limited basis (Dowell *et al.* 2001). MIF is used also by many laboratories for epidemiological studies.

In general, one of the biggest problems lies in diagnosing chronic chlamydial infections and markers for chronic infection are still under debate (Black 1997, Boman and Hammerschlag 2002). Nevertheless, at present NAATs must be considered the tests of choice for diagnosing *C. trachomatis* infection (Black 1997, Puolakkainen *et al.* 1998, Paavonen and Eggert-Kruse 1999). Table 1 shows an overview of methods used to diagnose *C. trachomatis* infection.

Table 1. Overview of diagnostic methods for detection of C. trachomatis EB or antigens in clinical specimens (modified from Caul and Herring 2001*).*

Diagnostic test	Based on	Advantages	Disadvantages
Culture	Detection of viable EBs in the specimen	-Only test for medico legal work -Specificity -Preserves viability of chlamydiae for additional testing	-Needs skilled laboratory staff -Sensitivity compared to NAATs -Needs viable EBs -Long time required to obtain results
Conventional EIAs	Detection of chlamydial LPS present outside of EBs	-Simple, high throughput, -LPS can be found in abundance in specimens with low number of mature EBs. -Standardization possible.	-Sensitivities vary greatly -Needs confirmation
Amplified EIAs	Detection of chlamydial LPS present outside of EBs	-Simple, high throughput -Good sensitivity and specificity -Standardization possible.	-Needs confirmation
Direct immuno-fluorescence	Detection of genus-specific (LPS) epitopes or species-specific epitopes on MOMP	-Good for confirmation of other tests and research. -Allows assessment of the quality of the specimen.	-Quality of results depends on experience of technician. -Throughput very limited.
NAATs	Detection of DNA and RNA	-Highest sensitivity and specificity -Easy sampling -Standardization possible -Rapid	-Needs a lot of laboratory space. -Contamination is constant threat.

2.5 Altered chlamydial development

The productive chlamydial developmental cycle that occurs in normal or optimal growth conditions can alter in *in vitro* culture into slowly growing i.e. persistent development with sporadic productions of infectious chlamydial EBs (Figure 2B; Moulder *et al.* 1980, Lee and Moulder 1981). The persistently infected cell cultures has been maintained for months (Moulder *et al.* 1980, Lee and Moulder 1981) or for years (Kutlin *et al.* 2001). Deviations from the typical developmental cycle of chlamydiae has been demonstrated *in vitro* to associate with a development of incomplete chlamydial infection and formation of morphologically aberrant RBs (Clark *et al.* 1982, Shemer and Sarov 1985, Beatty *et al.* 1993, Kutlin *et al.* 2001). In cell cultures chlamydial persistence has been accomplished by using penicillin or other antibiotics (Clark *et al.* 1982, Byrne *et al.* 1989, Dreses-Werringloer *et al.* 2000), nutrient-deficient conditions (depletions of essential amino acids such as tryptophan or cysteine) and immune system-regulated factors (cytokines like IFN-γ and tumour necrosis factor (TNF)-α) (Coles and Pearce 1987, Coles *et al.* 1993, Beatty *et al.* 1993, Beatty *et al.* 1995).

The incomplete chlamydial growth involves non-replicative (or slowly replicating), non-infectious but metabolically active stage of development (reviewed by Beatty *et al*., 1994 and Ward, 1999). During persistent infection the expression of MOMP is significantly reduced *in vitro*, while the expression of CHSP60 remains at nearly constant levels or is enhanced (Beatty *et al.* 1993, Kinnunen 1997, Gerard *et al.* 1998c, Dreses-Werringloer *et al.* 2000).

2.5.1 The evidence for persistence in animal models and in humans

Differing from naturally occurring chlamydial infection in humans, animal models are useful for evaluating chlamydial persistence *in vivo* because the time and onset of infection can be controlled. Specimens from different tissues are easily available and the role of the immune system can be investigated by using knock-out and transgenic animals (reviewed by Ward 1999). At present there are few experimental models focused specifically on the persistent chlamydial infection or at least on a prolonged infection beyond the time at which presence of chlamydiae can no longer be detected by culture. Chlamydiae can obviously remain in infected tissue after apparent resolution of infection and is detectable with polymerase chain reaction (PCR) techniques (Holland *et al.* 1992, reviewed by Ward 1999). Immunosuppression reactivates chlamydial shedding indicating the viability of the organism (Yang *et al.* 1983, Malinverni *et al.* 1995, Laitinen *et al.* 1996, Cotter *et al.* 1997). However, a true persistence of chlamydial infections remains unverified as the frequency of reactivation was found to fade with time in a mouse model of salpingitis (Cotter *et al.* 1997).

Animal models have enabled researchers to confirm chlamydial dissemination from a local infection into a systematic one involving detection of chlamydial antigen or DNA in

deep-seated tissues (reviewed by Ward 1999). The frequency of dissemination and the clearance of the infection depends, however, on genotype of immune response genes, previous immune sensitization and hormonal status of the host, as well as on the virulence of the chlamydial strain, the challenge dose and the route of immunization (reviewed by Ward 1999).

In humans chlamydial persistence has not conclusively been shown partly due to the difficulty in discriminating relapse from reinfection. However, there is indirect evidence to suggest that this phenomenon may occur during human ocular and genital infections. The evidence is based on collected data from studies where chlamydial organisms were cultivated from individuals with a disease that occurred years or decades after any evidence of active infection, or the chlamydial antigen or nucleic acids were detected in the absence of cultivable chlamydiae (Ward *et al.* 1990, Holland *et al.* 1993, Toth *et al.* 2000, reviewed by Beatty *et al.* 1994c and Ward 1999). One of the most convincing evidence for *in vivo* persistent chlamydial infection comes from a study conducted by Bell et al. (1992) who showed that approximately 35% of infants infected at birth remained infected at one year of age.

In genital tract infections, chlamydiae have been detected three months after appropriate treatment in 10% of the patients (Katz *et al.* 1998) and after postinfectious tubal infertility (Patton *et al.* 1994a). In addition, persistence of *C. trachomatis* infection was shown in cervixes of recurrently infected women by *omp1* (MOMP) genotyping (Dean *et al.* 2000). In this study, Dean et al. (2000) studied 45 chlamydial isolates from 7 women who had had 3 –10 repeated infections with the same serovar over the years. Analyses revealed that 5 of the women had identical genotypes at each new episode of infection and 2 women had minor amino acid changes after treatment. Interestingly, many intervening samples were culture-negative, although chlamydial DNA was detected in these samples by ligase chain reaction (LCR). In women with ectopic pregnancy *C. trachomatis*-specific DNA and RNA was detected in the tissue specimens indicating that metabolically active bacteria is present (Gerard *et al.* 1998a).

2.5.2 Suggested mechanisms of chlamydial persistence

Mechanisms how chlamydiae remain persistent are not known in detail but are probably partly explained by the ability of *Chlamydia* to infect and remain viable in professional phagocytes. A plausible molecular mechanism that chlamydiae may use to escape host immune responses and remain persistent involves the production of protease-like activity factor (CPAFct/cp) that has been demonstrated in both *C. trachomatis* and *C. pneumoniae* species. CPAFct/cp is secreted into the host cytoplasm where it degrades host transcription factor (RFX5) needed for major histocompatibility complex (MHC) gene activation (Zhong *et al.* 2000, Zhong *et al.* 2001, Fan *et al.* 2002). This is in accordance with the report where *C. trachomatis* infection decreased the IFN-γ-induced expression of MHC molecule on the infected cell (Rodel *et al.* 1998, Zhong *et al.* 1999).

Furthermore, chlamydiae are able to inhibit apoptosis of the host cell by interrupting different apoptotic pathways (Fan *et al.* 1998, Dean and Powers 2001, Coutinho-Silva *et al.* 2001). Apoptosis, also called programmed cell death is genetically programmed

process in which macromolecules are broken down and released from the cell without eliciting inflammation. Apoptosis is triggered by a variety of physiological and stress stimuli. It is involved in normal development and tissue homeostasis but it is also a way for the host to battle against intracellular micro-organisms. Apoptotic suicide of infected host cell can limit the spread of the invading bacteria by delivering it to professional phagocytes as has been shown with *Mycobacteria* (Fratazzi *et al.* 1997). Thus, intracellular bacteria including chlamydiae can benefit by employing anti-apoptotic mechanisms in order to evade host's immune responses and to survive in the host cell for prolonged periods of time.

Also, *Chlamydia*-infected macrophages in *in vitro* cultures induce the apoptosis of non-infected immune cells such as T-cells (Jendro *et al.* 2000) which may further augment the development of persistent infection. Although the apoptosis of immune cells detected in the vicinity of the *Chlamydia*-infected cells needs confirmation, it is an interesting notion in the light of recently discovered capacity of *Chlamydia* to produce cytotoxin (Belland *et al.* 2001). In addition, studies in our laboratory show that the lymphocyte proliferative (LP) responses typically observed after lymphocyte stimulation (Surcel *et al.* 1993) are dramatically reduced in the presence of infected monocytes (Airenne et al., unpublished observations). In a mouse model of chlamydial infection Perfettini et al. (2002) suggested that IFN-γ may contribute to pathogenesis of persistent *Chlamydia* infections *in vivo* by preventing apoptosis of infected cells (Perfettini *et al.* 2002). They showed that in addition to producing a persistent chlamydial infection IFN-γ treatment also resulted in inhibition of apoptosis of the infected cells. Instead of directly inducing inhibition of apoptosis IFN-γ may have produced a persistent chlamydial infection that induced the anti-apoptotic effect. Dean and Powers (2001) suggested that inhibition of host cell apoptosis may enable chlamydiae to form persistent infection while locally produced immunoregulatory molecules such as IFN-γ and interleukin (IL)-10 could favor the development of aberrant chlamydial form with elevated expression of CHSP60 which could sustain inflammation (Dean and Powers 2001). The differential expression of MOMP and CHSP60 during normal and altered chlamydial development have been known for a long time but a true meaning of this balance in terms of chlamydial persistence is not known.

2.6 *Chlamydia* and immune response

The humoral and CMI responses serve different functions and involve different effector mechanisms for generating immunity. Humoral immunity is mediated by secreted antibodies, which can neutralize soluble antigens while CMI exerts its function through T-cells. Infections caused by intracellular bacteria such as *Chlamydia* tend to induce CMI response. Both antigen-specific T-cells and non-specific antigen presenting cells (APCs) contribute to CMI response. Specific cells include CD4+ T-cells secreting different panels of cytokines and CD8+ cytotoxic T-lymphocytes (CTLs) (Kuby 1997).

In vitro studies have shown that antibodies generated during chlamydial infection are able to neutralize infectious chlamydiae. Neutralizing antibodies prevent attachment to host cell (Byrne and Moulder 1978, Su and Caldwell 1991) or inhibit replication

(Caldwell and Perry 1982). In humans a strong antibody response has been detected both in serum, genital secretions and tears but conclusive evidence for its role in chlamydial elimination or protection against chlamydial infection is lacking (Holland *et al.* 1993, Ghaem-Maghami *et al.* 1996, Ghaem-Maghami *et al.* 1997).

Due to silent nature of chlamydial diseases it is difficult to study development of CMI during primary chlamydial infections, although it is plausible that CMI responses take place concurrently with antibody response (Hanna *et al.* 1979, Surcel *et al.* 1993, Kelly and Rank 1997). Bailey et al. (1995) showed that lymphocyte proliferation to chlamydial antigens is enhanced in individuals who spontaneously resolve ocular chlamydial infection compared to those with persistent ocular disease. In association with human genital tract infection, CMI responses against chlamydiae measured as LP responses of peripheral blood lymphocytes (PBLs) has been detected (Hanna *et al.* 1979, Brunham *et al.* 1981, Hanna *et al.* 1982, Witkin *et al.* 1993a), but the situation at the site of infection i.e. in the genital tract tissue of infected individuals is not known. Role of CD8+ T-cells (i.e. CTL) during chlamydial infections has not been as extensively studied as that of CD4+ T-cells. CTL response in patients with trachoma was present only in children resolving current infection and in adults without scarring indicating that CTL responses may be important in the resolution of naturally acquired ocular infection (Holland *et al.* 1997). Nevertheless, despite the presence of *Chlamydia*-specific immune response, animals and humans do become reinfected.

The MHC molecules are central in the development of antigen-specific immune responses. In order to a T-cell to recognize an antigen it must be processed and presented in the context of a MHC or human leukocyte antigen (HLA) molecule, as it is called in humans. MHC class I molecules bind and present peptides derived from endogenous antigens that have been processed within the cytoplasm of the cell (e.g. normal cellular proteins or viral and bacterial proteins produced within infected cell). They present these peptides to CD8+ T-cells. HLA class II molecules bind and present peptides derived from exogenous antigens that have been internalized and processed within the endocytic pathway. They present peptides to CD4+ T-cells. Because MHC molecules function as antigen presenting structures, the particular set of MHC molecules expressed by a certain individual influences the T-cell repertoire responding to different antigens. For this reason MHC partly determines whether an individual is able to respond to antigens of an infectious organism and therefore MHC molecules have been associated with disease susceptibility (reviewed by Kuby 1997, Maksymowych and Kane 2000, Witkin *et al.* 2000).

2.6.1 Cytokines and T-cell response

After recognition of MHC-peptide complex by T-cell receptor the T-cell is activated. Activation is seen by the production of cytokines, a group of low-molecular weight proteins. Cytokines assist in regulating immune effector cells i.e. they function as messengers of the immune system (reviewed by Kuby 1997). In the mid 80's Mosmann et al. (1986) suggested that murine T-cells could be divided into two functionally different subsets according to the cytokines they produce. Type 1 (Th1) T-cells produce mainly

IFN–γ (and IL-2, TNF-α, TNF-β) while type 2 (Th2) T-cells produce IL-4 (and IL-5, IL-6, IL-10, IL-13 and possibly IL-9) (reviewed by Curfs *et al.* 1997). Thus, type 1 response is defined as a strong cellular immune response with normal or increased levels of IL-2, IFN–γ, TNF-β and/or IL-12 while type 2 response is defined as an impaired cellular response with an increase in one or more B-cell activities and an increase in the level of IL-4, IL-5, IL-6, IL-10 and/or IL-13. Importantly, in this definition the emphasis is on the relative predominance of type 1 or type 2 cytokines and not on the absolute dichotomy of either type of cytokine (Lucey *et al.* 1996). Main leukocyte sources of type 1 and type 2 cytokines are shown in Table 2 (Lucey *et al.* 1996). A simple division of T-cells to Th1 – Th2 type cells has not been demonstrated in humans. Evaluation of different cell types according to cytokines they produce have shown cells capable of producing both Th1 and Th2-type cytokines. These cells have been determined as intermediate or Th0 type cells.

*Table 2. Main leukocyte sources of type 1 and type 2 cytokines (*Lucey *et al.* 1996*)*

Cell source	Cytokine(s) Type 1	Type 2
CD4+ T-cell	IL-2, IFN-γ, IL-12, TNF-β	IL-4, IL-5, IL-6, IL-10, IL-13
CD8+ T-cell	IL-2, IFN-γ	IL-4, IL-5, IL-10
NK cell	IFN.γ, TNF-β	
Monocyte/ macrophage	IL-12	IL-6, IL-10
B cell	IL-12, TNF-β	IL-6, IL-10
Dendritic cell	IL-12	
Neutrophil	IL-12	
Mast cell		IL-4, IL-5, IL-6
Eosinophil		IL-4, IL-5, IL-6

Suppressor T cells (or regulatory T (Tr) cells) are able to suppress immune response *in vivo* and they are strong producers of IL-10. They are also able to inhibit harmful immunopathological responses directed against self or foreign antigens through suppression of other T-cells. Tr-cells occur naturally as a subset of CD4+ T-cells. Animal studies provide convincing evidence that specialized Tr-cells capable of controlling autoimmune responses exist as part of the T-cell repertoire in normal animals. Although the activation of Tr-cells depends on antigenic stimulation once activated these cells are able to suppress both CD4 and CD8 positive cells in an antigen-nonspecific manner. The mechanism of Tr cell action probably involves inhibition of IL-2 production by normally responsive T-cells **(**reviewed by Maloy and Powrie 2001).

2.6.1.1 Cytokines and Chlamydia

Outcome of chlamydial infection is influenced by the interaction and balance of pro- and anti-inflammatory cytokines present at the site of infection. Two cytokines, IFN–γ and IL-10, are especially important for immunity to chlamydial infections. In the murine system CD4+ T-cell mediated immune response alone is enough to resolve primary chlamydial infection and to provide immunity against reinfection. Protection is mainly mediated through the action of IFN–γ (Ramsey and Rank 1991, Rank *et al.* 1992, Igietseme *et al.* 1993, Su and Caldwell 1995, Van Voorhis *et al.* 1996). In a recent study with mice Morrison et al. (2000) showed that the co-operation of CD4+ T-cells and B-cells are important in recall immune responses in mice while CD8+ T-cells were not needed for protective immunity. On the other hand, resolution of secondary chlamydial infection in immune mice does not necessarily need CD4 and CD8+ cells but indicate that additional effector immune responses have a role in resistance to chlamydial genital tract reinfection (Morrison and Morrison 2001). These mechanisms may include TNF-α (Williams *et al.* 1997). Protective role for CD8+ T-cells may be underestimated since mouse is the only animal in which it has been studied.

In *in vitro* experiments IFN-γ has been closely linked with the development of persistent chlamydial infection through chlamydial growth inhibition. IFN-γ induces the production of the enzyme indoleamine 2,3-dioxygenase (IDO), which degrades tryptophan, an essential amino acid for chlamydial development (Summersgill *et al.* 1995). However, sensitivity to IFN–γ varies significantly among different serovars of *C. trachomatis* (Perry *et al.* 1999, Morrison 2000). Downregulation of the Th1 (IFN-γ) response at the site of inflammation during infection may lead to prolonged infection and inflammation (Yang *et al.* 1996). In addition, IFN-γ induces the expression of HLA-DR molecules in epithelial cells (Tabibzadeh *et al.* 1986), which can function as non-professional APC and become a target of immune response.

Emerging evidence in rodents suggests that prolonged (persistent?) infection is mediated by IL-10 (Yang *et al.* 1999, Igietseme *et al.* 2000). This is due to the ability of IL-10 to down-regulate the expression of Th1-type cytokines. The situation with chlamydial genital tract infections resembles that of reactive arthritis (ReA) which is triggered by an infection by intracellular bacteria such as *Yersinia*, *Chlamydia* or *Salmonella*. Effective elimination of these bacteria is mediated through the action of Th1-type cytokines (reviewed by van Roon *et al.* 2001) while the symptoms and progression of the disease is associated with a predominance of Th2-type cytokines in the synovial tissue of ReA patients (Yin *et al.* 1997).

2.6.1.2 Cytokine gene polymorphisms and disease

In vivo production of the cytokine is influenced by polymorphisms in the promoter region of the specific cytokine gene (Turner *et al.* 1997, Conway *et al.* 1997, Westendorp *et al.* 1997, Crawley *et al.* 1999, Reynard *et al.* 2000, Gibson *et al.* 2001, Hahn *et al.* 2001, Hulkkonen *et al.* 2001, Poli *et al.* 2001, Cox *et al.* 2001). Westendorp et al. (1997) found that approximately 60% of the variation in TNF-α and 75% of the variation in IL-10

production was due to genetic differences. Thus, it is not surprising that different cytokine gene polymorphisms have been associated with the outcome of a disease. In chlamydial diseases Conway et al. (1997) showed an association of scarring trachoma with polymorphism in the TNF-α gene promoter and elevated TNF-α levels in tear fluid (Conway *et al.* 1997). In another trachoma study, Mozzato–Chamay et al. (2000) found an association between the disease and the IL-10 -1082GG genotype, although the association was detected only in one out of five ethnic groups.

2.6.1.3 Innate immunity

Innate host response in chlamydial diseases has not been studied thoroughly. According to literature, innate host response plays a role in reducing the number of infectious EBs during initial stages of the infection, but it may also have an important role in the actual disease pathogenesis (reviewed by Bavoil *et al.* 1996 and Rank and Bavoil 1996). The primary responses seen in animal models and in humans comprise infiltration of polymorphonuclear leukocytes (PMNs), which clearly reduce or even eliminate infectivity of chlamydiae (Yong *et al.* 1986, Register *et al.* 1986, Register *et al.* 1987). Despite the chlamydiatoxic effect of PMNs many chlamydial organisms probably survive because of their ability to attach to susceptible epithelial cells and gain rapidly access to the intracellular environment (reviewed by Bavoil *et al.* 1996).

Animal models with mouse pneumonitis have shown an increase of IFN–γ producing cells in the genital tract (Tseng and Rank 1998) and in the respiratory tract (Williams *et al.* 1993) of infected animals early in the disease course. Increase in IFN–γ production was not affected by the elimination of CD4+ T-cells suggesting that IFN–γ was produced by the natural killer (NK) cells (Tseng and Rank 1998, Williams *et al.* 1993).

TNF-α has also been detected during chlamydial infection (Shemer-Avni *et al.* 1988, Kaukoranta-Tolvanen *et al.* 1996, Darville *et al.* 2001b). TNF-α eliminates chlamydiae in the early stage of infection in a murine model of lung (Williams *et al.* 1990) and tubal infection (Darville *et al.* 1997). During chronic or persistent chlamydial infection TNF-α seems to play a major role in the tissue damage and scarring at least in trachoma (Conway *et al.* 1997). Whether TNF-α exerts its effect directly (chlamydiatoxic) or whether it potentiates homing of various inflammatory cells to the site of infection remains to be elucidated. In addition to IFN–γ and TNF–α, nitric oxide (NO) is produced and may be one of the non-specific mechanisms by which chlamydiae are cleared, at least in murine model of chlamydial infection (Rottenberg *et al.* 1999, Rottenberg *et al.* 2000, Ramsey *et al.* 2001).

2.6.2 60 kDa Heat shock protein as an antigen

As discussed earlier (2.2.1.3) HSPs are highly conserved proteins that function as molecular chaperones under normal physiological conditions. They are important antigens inducing both humoral and CMI responses. In *Mycobacterium*, HSP60 has been

described as an immunodominant antigen because a considerable part of the *in vitro* activated T-cells seem to be targeted against HSP60 (Kaufmann *et al.* 1987). The HSP60-specific immune response is associated with several infectious diseases including tuberculosis, arthritis and chlamydial infections as well as in certain autoimmune diseases (reviewed by Kaufmann and Schoel 1994, Gaston 1998, Zugel and Kaufmann 1999b, Neuer *et al.* 2000 and Kinnunen *et al.* 2001). In addition to the infectious and autoimmune diseases, HSP60-specific antibodies have been detected in healthy individuals as well (Pockley *et al.* 1999). This has led to a suggestion that the HSP60-directed immune response is induced by frequent contact with low virulence or non-pathogenic organisms, which drives and maintains the immune response especially against the conserved regions of HSP-molecule and confers at least partial protection against pathogenic organisms invading the host (reviewed by Kaufmann and Schoel 1994).

Because of high amino acid and structural homology between HSP60s in different species, the immune response initially generated against the microbial HSP60 can eventually be targeted against cross-reactive epitopes in self-HSP60 molecule. The activation of self-HSP60-specific immune responses is generally considered to be detrimental for the well-being of the host and associated with a shift from protective immune response into pathological one (reviewed by Kaufmann and Schoel 1994). In *C. trachomatis*-induced diseases enhanced antibody and CMI response to CHSP60 are typically detected in the patients (Tables 3 and 4). CHSP60 is considered as the antigen mediating the immunopathogenesis of persistent chlamydial infections such as TFI and scarring trachoma (Morrison *et al.* 1989a, Morrison *et al.* 1989b, Patton *et al.* 1994a, Patton *et al.* 1994b). Antigenic structure of HSP60 has been studied in detail. Nevertheless, data on human responses against specific B-cell or T-cell epitopes of CHSP60 is limited. However, antibodies to specific B-cell epitopes of CHSP60 have been characterized in infants with chlamydial pneumonitis (Paavonen *et al.* 1994) and in women with PID (Domeika *et al.* 1998). Detection of self-HSP60-specific antibodies (Domeika *et al.* 1998, Witkin *et al.* 1998) or activation of self-HSP60-specific T-cells in peripheral blood (Witkin *et al.* 1994) in the patients suggests that the immunopathogenesis of chronic chlamydial diseases may involve self-HSP60-targeted immunity.

Table 3. Examples of humoral response to CHSP60 in association with different chlamydial diseases in humans.

Disease association	**Reference**
PID	Peeling *et al.* 1997
PID	Domeika *et al.* 1998
PID, ectopic pregnancy	Wagar *et al.* 1990
PID, occluded tubes	Eckert *et al.* 1997
Ectopic pregnancy	Sziller *et al.* 1998
Chronic salpingitis, TFI	Dieterle and Wollenhaupt 1996
TFI	Toye *et al.* 1993
TFI	Persson *et al.* 1999
TFI	Ault *et al.* 1998
Infertility	Arno *et al.* 1995
Infertile couples	Witkin *et al.* 1998
Infertile women	Freidank *et al.* 1995
Trachoma	Peeling *et al.* 1998,
Trachoma	Hessel *et al.* 2001

Table 4. Examples of cellular responses to CHSP60 and human HSP60 in association with chronic chlamydial diseases in human.

CMI response to	**Disease association**	**Reference**
CHSP60	Salpingitis	Witkin *et al.* 1993b
CHSP60 and human HSP60	Salpingitis	Witkin *et al.* 1994
Human HSP60	Spontaneous abortion, endometriosis	Kligman *et al.* 1998
CHSP60	Trachoma	Bailey *et al.* 1995
CHSP60	Trachoma	Holland *et al.* 1993
CHSP60	Trachoma	Holland *et al.* 1996

The functional role of HSP60-reactive T-cells in terms of cytokine secretion has been studied in association with *Yersinia*- or *Chlamydia*-induced ReA in which T-cell activation has been detected against both bacterial and self-HSP60 molecule (Hermann *et al.* 1991, Mertz *et al.* 1998). Studying function of synovial fluid derived T-cells in patients with *Yersinia*-induced ReA, Mertz et al. (1998) demonstrated that self-HSP60 directed T-cell responses involve a production of Th2-type cytokines. They suggested that the Th2-type cytokine milieu could be responsible for the impaired host defence against the microbe and lead to bacterial persistence within the synovium and eventually lead to pathological changes seen in the joints. Similar to ReA, activation of CHSP60-specific Th2-type cells was demonstrated in individuals with scarring trachoma but not in age- and sex-matched controls (Holland *et al.* 1996) further supporting the hypothesis that chlamydial pathogenesis involves activation of Th2 cells.

On the other hand, early rheumatoid arthritis (RA) is linked with lower and less frequently detected CMI responses to mycobacterial HSP60 compared to healthy controls (Ramage and Gaston 1999) suggesting that inflammatory conditions such as RA might be associated with defective regulatory responses to bacterial HSP60. In juvenile RA T-cell proliferative responses to self-HSP60 were associated with the disease remission (Prakken *et al.* 1996). van Roon et al. (1997) suggested that expression of self-HSP60 molecules might activate regulatory Th2-type cells, which can cause suppression of T-cells that induce arthritis (i.e. Th1-type cells). Further support for this has been provided by Macht et al. (2000) who showed that RA was less severe in those patients whose HSP60-stimulated PBLs produced Th2-type cytokine response. Taken together data from studies with experimental models of arthritis and chlamydial infections as well as from humans have shown that HSP60 have a potential to trigger the production of Th2-type cytokines.

2.6.3 Immunopathogenic responses in chlamydial infections

In addition to elimination of and protection from chlamydial infections CMI responses to *Chlamydia* are involved in the pathogenesis of chlamydial diseases (reviewed by Loomis and Starnbach 2002). This notion is originally based on animal studies, which showed that sensitization of guinea-pigs with a detergent soluble extract of chlamydial EBs accelerated accumulation of mononuclear lymphocytes in the regions of ocular inflammation (Watkins *et al.* 1986). In a comparable study with monkeys, Taylor et al. (1987) showed that the follicular response was observed only in *C. trachomatis*-immunized but not in naive animals. Later Morrison and coauthors (1989a, 1989b) demonstrated that a 57 kDa protein, identified as CHSP60, is the active component and responsible for the DTH response in trachoma. Experimental studies in the monkey “pocket” model of salpingitis have further supported the role of CHSP60-specific T-cells in the immunopathogenesis of salpingitis (Patton *et al.* 1994b). Pigtailed monkeys were sensitized by inoculation of live *C. trachomatis* organisms into subcutaneous pockets containing salpingeal autotransplants. When recombinant CHSP60 was injected into such pockets of previously sensitized monkeys, a typical DTH reaction was detected.

Serological studies have implicated that *C. trachomatis* has immunodominant proteins such as MOMP and CHSP60 that most of the host's immune response is directed at. During persistent infection the expression of CHSP60 is enhanced while the expression of MOMP is maintained at a low level (Beatty *et al.* 1993, Gerard *et al.* 1998c, Dreses-Werringloer *et al.* 2000). This is indirectly seen as appearance of CHSP60-specific antibodies in the patient's serum (Table 3). Appearance of CHSP60 antibodies in the patients' serum are associated with repeated or persistent *C. trachomatis* infections (Table 3). However, the role of the CHSP60 antibodies remains unclear since antibody to CHSP60 could also be mere indicator of chronic infection. Studying patients with ectopic pregnancy Yi et al. (1993) have found CHSP60-specific antibodies that are cross-reactive with self-HSP60 suggesting that these antibodies are possibly playing role in disease pathogenesis. Prolonged or repeated exposure to CHSP60 or to both human and chlamydial HSP60 could result in braking down of immunological tolerance and lead to self-HSP60-directed immunity through cross-reactive T- and B- cell epitopes.

The immunopathogenesis studies in humans have mainly focused on the role of CMI in disease pathogenesis. Holland et al. (1993) showed that scarring trachoma is linked to markedly depressed LP response to chlamydial antigens and involves downregulation of Th1 activity and IFN-γ response (Holland *et al.* 1996). They further showed that Th2-type of response was detected after stimulation of the patients' PBLs with CHSP60 while MOMP induced Th1-type (i.e. IFN–γ) response. Similar to the humoral responses, LP reactivity to CHSP60 is linked with repeated or chronic *C. trachomatis* infection (Beatty *et al.* 1993, Witkin *et al.* 1993a). Studying LP responses of circulating lymphocytes *in vitro*, Witkin et al. (1993a) demonstrated positive LP responses to CHSP60 more often in patients with salpingitis than in healthy controls. The responses were directed to chlamydial peptides that share substantial homology with human i.e. self-HSP60 (Witkin *et al.* 1994). More recently, Witkin et al. (1998) reported that cell-mediated immunity to human HSP60 is associated with a history of spontaneous abortion.

3 Aims of the present study

Purpose of this study was to investigate the immunopathological mechanisms of *C. trachomatis*–induced infertility in women. To better understand the relationship between CHSP60 and T-cells in the inflamed tissue, we established *Chlamydia*-specific T-lymphocyte lines (TLLs) from the obstructed fallopian tubes of infertile women (TFI) and from endometrial biopsies of patients with acute PID. The following questions were asked:

1) What is the relative role of chlamydial antigens in modulating immune responses in TFI (I)?

2) Are *in vivo* activated T-cells responding to *C. trachomatis* or CHSP60 present in PID and TFI patients' tissue (II)?

3) What is the role of CHSP60 in modulating the immune response in TFI (III)?

4) What is the role of HLA alleles and cytokines in the regulation of the host T-cell response to *C. trachomatis* (IV)

4 Materials and Methods

Detailed description of materials and methods has been given in the original articles (I-IV).

4.1 Study subjects and specimens

The study population consisted of 22 consecutive patients with a clinical diagnosis of PID, who attended the Department of Obstetrics and Gynecology at the University Hospital of Helsinki, between January 1997 –June 1998. The PID patients underwent an operative laparoscopy that confirmed the diagnosis of acute PID in 14 of the 22 (64%) patients. The laparoscopic diagnosis of PID was based on commonly accepted visual findings (Tukeva *et al.* 1999). The TFI group consisted of 57 women attending the infertility clinic of the same hospital because of tubal occlusion with or without severe tubo-ovarian adhesion during January 1997 –April 2000 (Papers II and III, Table 5). An additional study population consisted of 28 women with other infertility factor, such as endometriosis or ovulation disorder, 23 women with unexplained infertility, and women partners of 25 couples with male factor infertility (control group for immunological analyses in Papers I and IV, Table 5). The control group for HLA genotyping and IL-10 promoter gene polymorphism analysis (Paper IV) consisted of 61 Finnish female adults (age range 23 to 69 yrs) who were collected in the context of another study on HLA-association and *Helicobacter* carriage (Karhukorpi *et al.* 1999). HLA allele frequencies of the control group were found to be similar to those in general Finnish population (Ikäheimo *et al.* 1996).

Endometrial biopsy specimens were obtained from the PID (n=14) and TFI patients (n=22) for histology, immunohistochemistry, and lymphocyte cultures (II). In addition, fallopian tube tissue specimens were obtained from 6 infertility clinic patients who underwent elective salpingectomy because of hydrosalpinx formation (II, III). Tissue specimens for morphological studies were fixed in 10% neutral buffered formalin and embedded in paraffin. Specimens for lymphocyte cultures were immersed in tissue culture medium as described in Paper II. Heparinized blood samples were drawn from all subjects for immunological analyses and kept at room temperature for no longer than 24 hours prior to the separation of PBLs (I-IV).

4.2 Chlamydial strains and antigens

C. trachomatis serovars E (ATCC VR-348B), F (ATCC VR-346) and L2 (ATCC) were propagated in McCoy-cells and *C. pneumoniae* Kajaani 7 was propagated in HL-cells. EBs were purified using conventional Urografin (Schering AG, Berlin, Germany) density gradient centrifugation. Purified EBs were either formalin-inactivated (Surcel *et al.* 1993) or suspended in sucrose-phosphate-glutamic acid (SPG; 0.2 M sucrose, 3.8 mM KH_2PO_4, 6.7 mM Na_2HPO_4 x $2H_2O$, 5 mM glutamic acid; pH 7.4) buffer and stored in small stock aliquots at -70°C until used. The number of chlamydial inclusion forming units (IFU) was determined by infecting McCoy- or HL-cells with serially diluted EB stock suspension. Chlamydial inclusions were stained with chlamydial genus-specific fluorescein isothiocyanate (FITC)-conjugated antibody (Sanofi Pasteur Redmond, WA, USA) at 72 hours of incubation and counted using fluorescent microscope. Recombinant CHSP60 (LaVerda *et al.* 2000) was a generous gift from Dr. RP Morrison (Montana, USA) and contained <0.03 ng/ml of endotoxin as determined by *Limulus* assay.

4.3 Measurement of serum antibodies

Serum samples for the detection of *C. trachomatis*-specific immunoglobulin (Ig)G (I, IV) and IgA (IV) antibodies were diluted 1:10 and studied using commercially available synthetic *C. trachomatis*-specific peptide-based EIA kits (Labsystems, Helsinki, Finland) according to manufacturer's instructions. Results were obtained by calculating the mean absorbance of duplicate samples at 450 nm. OD ≥ 1 was used as positive cut-off level as recommended by the manufacturer.

4.4 Immunohistochemistry

For histological examination, paraffin sections of endometrial and fallopian tube tissue specimens were routinely stained with hematoxylin and eosin. T-cell populations were stained either on paraffin or cryostat sections with primary antibodies as follows: CD45RO (T memory cells, clone UCHL1, Dako a/s, Glostrup, Denmark), CD4 (T helper cells, clone SK3, Becton Dickinson Immunocytometry Systems, San Jose, CA, USA), CD8 (T suppressor cells, clone SK1, Becton Dickinson), CD15 (monocytes, clone MMA, Becton Dickinson), CD20 (B cells, clone L26, Dako) and CD25 (IL-2 receptor, clone 2A3, Becton Dickinson). Detailed description of immunohistochemical stainings is presented in Paper II. The presence of *C. trachomatis* in genital tract tissues was visualized by direct immunofluorescence microscopy of paraffin sections as earlier described by Rantala and Kivinen (1998).

4.5 Peripheral blood lymphocyte and T-lymphocyte cultures

PBLs were isolated from heparinized blood by Ficoll-Paque® (Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation. Cells were suspended in RPMI-1640 medium (Sigma, St.Louis, MO, USA) containing 10% heat-inactivated human AB serum (Finnish Red Cross, Helsinki, Finland) for the LP assay (I, II, IV), or 5% fetal calf serum (FCS) for cytokine analyses (I, II, IV).

C. trachomatis–specific TLLs were cultured from the endometrial biopsy and fallopian tube tissue specimens as described in Paper II. Briefly, IL-2 receptor expressing T-cells representing *in vivo* activation of these cells were propagated by adding IL-2 (10% vol/vol; Lymphocult, Biotest, Germany) into the culture medium and incubated for 10 days in 5% CO_2 atmosphere at 37°C. Half of the IL-2 containing medium was changed every third day. Growing T-cells were further augmented by stimulation with chlamydial EB antigen three times before antigen specificity tests were performed. Proliferating T-cells were suspended in RPMI-1640 medium containing 10% (vol/vol) IL-2 (Lymphocult) and cloned by the limiting dilution method as described previously (Halme *et al.* 1997 and Paper III). Briefly, 0.5 blast cells per well were cultured for 10 days with phytohemagglutinine (PHA; 2 μg/ml) and fresh irradiated PBL (10 000 cells/well) as the APC in a total volume of 20 μl. The expanding T-lymphocyte clones (TLCs) were restimulated with mitogen (PHA) three to four times for periods of ten days before they were used in the antigen specificity tests. Half of the IL-2 containing culture medium was changed every third day, but no later than four days before the antigen specificity test.

4.6 Antigen specificity of the lymphocytes

The PBL (5×10^4 cells/well) proliferative reactivity to chlamydial EB antigens (*C. trachomatis* strains E, F or L2, and *C. pneumoniae* K7) and CHSP60 was studied *in vitro* according to Surcel et al. (1993) and as described in Papers I and IV. Pokeweed mitogen (PWM, Gibco, Paisley, UK; 12.5 μg/ml) was used as a positive control mitogen in each experiment. The antigen specificity of the TLL and TLC was tested by culturing 20 000 cells in the presence of 20 000 irradiated autologous APC and chlamydial antigens according to Papers II and III. Results are expressed as stimulation indices (SI = mean cpm in the presence of the antigen divided by mean cpm in its absence) for triplicate cultures. Tetanus toxoid was used as control antigen. TLLs or TLCs giving a positive response to tetanus toxoid were omitted from the analysis. For PBL cultures SI > 2.5 was used as a positive value, while for TLL and TLC cultures SI > 3 was used as a positive cut-off value because the frequency of antigen-specific cells is clearly higher among TLLs and TLCs than that among the PBLs.

4.7 Surface antigens of the lymphocytes

Surface antigens of the lymphocytes were studied by double immunofluorescence flow cytometric analysis. Cultured lymphocytes were stained using phycoerythrin (PE)-conjugated anti -CD4 and FITC-conjugated anti -CD8 monoclonal antibodies (Caltag Lab., San Francisco, USA) and analyzed by FACScan (Becton Dickinson and Co., Mountain View, CA, USA).

4.8 Antigen or mitogen induced cytokine secretion

Cytokine production was induced by incubating PBL cells (I, II, IV), TLL (II) or TLC (III) with or without chlamydial antigens or mitogen (PHA). Cytokine analyses were performed on supernatants from lymphocyte cultures using commercially available enzyme-linked immunosorbent assay kits (ELISA; DuoSet® human IFN-γ, IL-10, IL-12p40 (IV) and IL-4 (II); R & D Systems, Minneapolis, USA; PeliKine Compact™ human IL-10 (III) ELISA, CLB, Amsterdam, Netherlands, DuoSet™ Human IFN-γ, Genzyme Diagnostics, Cambridge, MA, USA; II, III and Pharmingen Quantigen™ Human IL-5 Set, San Diego, California, USA; II) according to the manufacturer's instructions. PBLs were considered positive when the concentration of cytokine (pg/ml) in the presence of antigen was at least twice that in the absence of antigen. PBL responses were considered Th1-dominant when the ratio of IFN-γ to IL-10 was ≥ 2, Th2-dominant when the ratio was ≤ 0.5.

4.9 Analyses of HLA class II genotypes

HLA-DQA1 and HLA-DQB1 genotyping was performed using PCR-SSP method (Olerup *et al.* 1993). Genomic DNA for analyses was extracted from the peripheral leukocytes using proteinase K digestion in 10% SDS and 7.5 M guanidine-HCl and precipitated with ethanol. Primers and PCR conditions are described in Materials and Methods in Paper IV.

4.10 Detection of interleukin-10 promoter gene polymorphism

IL-10 promoter gene polymorphism of a single nucleotide at position -1082 (A/G) was determined using sequence-specific oligonucleotide primers in a bidirectional PCR amplification method according to Karhukorpi and Karttunen (2001). The primers and PCR conditions are as described in Paper IV (Materials and Methods and Table I in Paper IV).

4.11 Statistical Analyses

SPSS for Windows 9.1 statistical software (SPSS Inc. Chicago, Illinois, USA) was used to perform Mann-Whitney U-test (I, II), Wilcoxon Signed-Rank test (I), and to calculate Spearman's correlation (I) and χ^2 test (I, II). Fisher's exact test was used to compare proportions between groups. Confidence intervals were calculated by CIA program (IV). The significance of the HLA association results was corrected using the Bonferroni correction multiplying the p value by the factor 22 (8 tests for HLA-DQA1 and 14 tests for DQB1). Spearman correlation coefficient was used to test the association between lymphocyte responses to different antigens (IV).

5 Results

5.1 *C. trachomatis*-specific antibody and lymphocyte proliferative responses

C. trachomatis-specific IgG antibodies indicative of past *C. trachomatis* infection were detected more often in TFI patients (31 of 56; 55%) than in other groups (18 of 74; 24%, p<0.001; Paper I and Table 5).

Table 5. Selected characteristics of the study population of 133 females of infertile couples.

Infertility group	N	Mean age yrs (range)	Positive [a] anti-chlamydial IgG titer No. (%)	Positive LP [b] response to CTR EB No. (%)	CHSP60 No. (%)
TFI	57	34 (22-41)	31/56 (55%)**	49/57 (86%)	20/57 (35%)
Other female factor	28	33 (24-37)	8/27 (30%)	23/28 (82%)	9/28 (32%)
Unexplained	23	34 (25-40)	2/23 (9%)	15/23 (65%)	4/23 (17%)
Male factor	25	32 (23-39)	8/24 (33%)	17/25 (68%)	4/24 (17%)

Statistical comparison between TFI and all other groups (combined): **p<0.001

a) OD value at 450 nm ≥ 1

b) Stimulation index >2.5

TFI: tubal factor infertility; CTR EB: *Chlamydia trachomatis* elementary bodies

LP responses of the PBLs were assessed with chlamydial EB antigens and CHSP60. Positive LP responses (SI > 2.5) to *C. trachomatis* EB antigen were more common in the TFI group (49 of 57; 86%) than in the control group (17 of 25; 68%; Paper I and Table 5) but the difference did not reach statistical significance. Median SI to *C. trachomatis* EB was significantly higher in the TFI group (SI 11.5, range 0.4 to 247.6) than in the control

group (SI 3.9, range 0.2 to 74.6; $p<0.005$). There were no statistically significant differences in median SI between TFI group and other female factor group (SI 6.9, range 1.1 to 64.7) or between TFI group and unexplained infertility group (SI 7.7, range 0.3 to 59.8). Presence of *C. trachomatis*-specific IgG antibodies and the LP responses did not correlate.

LP responses to CHSP60 were highly variable (median SI 1.4, range 0.2 to 141) with no statistically significant differences between the study groups. The largest variance was seen in the TFI group. Positive responders (SI > 2.5) were found in 20 of 57 (35%) TFI patients compared to 4 of 24 (17%) controls (Paper I and Table 5) but the difference did not reach statistical significance. TFI patients who had a negative LP response to the EB antigen did not respond to the CHSP60 either. *C. trachomatis*-specific IgG antibodies were detected in 10 of 20 (50%) of TFI patients who had a positive LP response to CHSP60 compared to none of four controls with positive LP response to CHSP60.

5.2 Histopathology and immunohistochemistry

Endometrial biopsies from 13 PID patients and from 22 TFI patients were available for histopathological examination. Fallopian tube tissue samples were available from 6 TFI patients. Routine histology showed endometritis with plasma cell infiltrations in 7 of 13 (54%) PID patients and in 4 of 22 (18%) TFI patients (Paper II). The total number of lymphocytes varied remarkably, the median number of lymphocytes/ 10 high-power field (HPF) was clearly higher in patients with plasma cell endometritis than in the non-endometritis patients (613; range 137 to 2220 vs. 91; 27 to 890; $p<0.001$). Chronic salpingitis with mucosal atrophy was found in 5 of 6 TFI patients.

Overall, the number of inflammatory cells was greater in the PID patients' endometrial specimens than in the TFI patients' specimens. The majority of the endometrial lymphocytes in the PID group were T memory cells (Paper II and Table 6). T-cells were found only occasionally in the endometrial specimens of the TFI patients and no T-memory cell predominance was noted. Most of the T-memory cells were CD4+ T-cells in all tissues (Paper II and Table 6). On the other hand, fallopian tube tissue specimens of the TFI patients contained equal amounts of inflammatory cells compared to endometrial specimens of the PID patients. This suggests that in the TFI patients the inflammation was restricted in the fallopian tubes, while in the PID patients the inflammatory response took place in the endometrium.

5.3 Characterization of T lymphocyte lines

IL-2 induced growth of T-cells as TLLs more frequently in the PID patients' endometrial specimens (8 of 14; 57%) than in the TFI patients' endometrial specimens (4 of 22; 18%, $p<0.05$). In addition, IL-2 supported T-cell growth in 4 of 6 (67%) fallopian tube tissue specimens obtained from TFI patients indicating that *in vivo* activated T-cells are present in the inflamed tissue of both patient groups.

Table 6. Mononuclear lymphocyte subsets (number of positive staining cells in 10 high power fields) in the female upper genital tract tissue specimen from acute PID patients and TFI patients.

Tissue	T-memory cells	Monocytes	B-cells	CD4+ T-cells	CD8+ T-cells	CD4/CD8
PID patients' Endometrium	n=13	n=13	n=13	n=4	n=4	n=4
Median (Range)	330 (39-1500)	66 (16-1100)	22 (9-220)	445 (110-1100)	115 (5-310)	7 (1.6-22)
TFI patients' Endometrium	n=20	n=20	n=19	n=2	n=2	n=2
Median (Range)	72 (22-400)	17 (10-30)	11 (0-54)	185 (102-268)	91.5 (43-140)	2.1 (1.9-2.4)
TFI patients' Fallopian tube	n=6	n=6	n=6	n=4	n=4	n=4
Median (Range)	370 (121-700)	28 (9-700)	9 (9-45)	280 (9-500)	108 (5-175)	2.2 (1.1-5.7)

Three of 4 TLLs established from the endometrial specimens of the TFI patients and all 4 TLLs established from the fallopian tube tissue specimens of TFI patients responded to *C. trachomatis* EB antigen. Only 2 of 8 TLLs established from the endometrial specimens of PID patients responded to the EB antigen. Furthermore, the median LP responses of the TLLs established from fallopian tube specimens of the TFI patients were significantly higher than median responses of the endometrial TLLs (median 16 822 cpm, range 11 668 to 24 187 vs. 1502; 194 to 2035; $p<0.001$, Paper II). Background proliferation in the absence of antigen ranged from 31 to 608 cpm. CHSP60 was recognized by all 4 TLLs derived from the fallopian tube tissue specimen of the TFI patients. Thus, in patients with chronic chlamydial infection and its complications the CMI response is directed to both chlamydial EB antigen and CHSP60.

5.4 Characterization of T lymphocyte clones

To further evaluate the role of CHSP60-specific T-cells among the TLLs established from the inflamed fallopian tube tissue, 229 TLCs were successfully grown from three of five women with TFI. Antigen specificity of the TLCs was tested with *C. trachomatis* EB antigen. Seventy-seven (34 %) of the 229 TLCs were positive (SI > 3) to the recalled *C. trachomatis* EB antigen (median SI 20.7, range 3 to 580).

Antigen reactivity of the 77 *C. trachomatis* EB responsive TLCs was further characterized by studying LP responses to *C. pneumoniae* EB antigen and to CHSP60 (Paper III and Figure 3). Positive LP responses (SI > 3) to *C. pneumoniae* EB were found in 52 of 77 (67%) *C. trachomatis*-specific TLCs (median SI 11.6; range 3.3 to 235) suggesting that their target epitopes were *Chlamydia* genus-specific. CHSP60 induced positive proliferative responses in 26 of 77 (34%) TLCs (median SI 6.8; range 3 to 926). Twenty-three of the 26 (88%) CHSP60-specific TLCs responded also to both *C. trachomatis* and *C. pneumoniae* EB (Paper III and Figure 3).

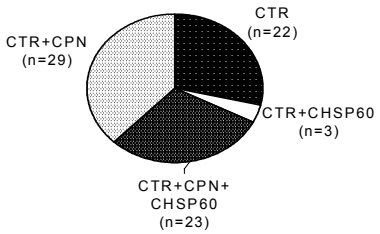

Fig. 3. Distribution of TLCs recognizing a target epitope on *C. trachomatis* EB only (CTR), on both *C. trachomatis* and *C. pneumoniae* EB (CTR + CPN), on *C. trachomatis* EB and chlamydial heat shock protein 60 (CTR+CHSP60) and on *C. trachomatis* EB, *C. pneumoniae* EB and CHSP60 (CTR+CPN+CHSP60) (Paper III).

5.5 Cytokine analyses of the peripheral blood lymphocytes

Secretion of pro-inflammatory cytokines (IFN-γ and IL-12) and anti-inflammatory IL-10 was studied from culture supernatants of chlamydial antigen stimulated PBLs. Median IFN-γ secretion induced by *C. trachomatis* EB antigen was greater in the TFI group (280 pg/ml,; range <10 to 8914 pg/ml) than in the control group (median 18 pg/ml, range <10 to 193 pg/ml; p<0.01; Paper I and Table 7). In addition, median IL-10 production in response to EB stimulation was significantly lower in the TFI group (median 138 pg/ml, range <3 to 6498 pg/ml) compared to controls (1150 pg/ml, <3 to 5529 pg/ml, p<0.001; Paper I and Table 7). No differences were observed in the IL-12 secretion after stimulation with EB antigen (data not shown).

Table 7. Median production of IFN-γ and IL-10 (pg/ml) by PBLs stimulated with C. trachomatis EBs (CTR EB) or CHSP60 antigen.

Group	PBL stimulated with	IFN-γ Median (range)	IL-10 Median (range)
TFI (N=57)	CTR EB	280 (<10 -8914)*	138 (<3 –6498)**
	CHSP60	32 (<10 –7483)**	1764 (<3 –7245)**
Other female Factor (N=28)	CTR EB	154 (<10 -1026)	871 (<3 –4270)
	CHSP60	<10 (<10 –31)	68 (<3 –2959)
Unexplained (N=23)	CTR EB	120 (<10 -638)	673 (<3 –6870)
	CHSP60	<10 (<10)	177 (<3 –3247)
Male factor (N=25)	CTR EB	18 (<10 -193)	1150 (<3 –5529)
	CHSP60	<10 (<10)	345 (<3 –2388)

Statistical comparison between TFI and Male factor group:
* p<0.01; ** p<0.001

CHSP60-stimulated PBL cultures produced significantly higher median amounts of IFN-γ (median 32 pg/ml, range <10 to 7483 pg/ml) and IL-10 (median 1764 pg/ml, range <3 to 7245 pg/ml) in the TFI group, compared to the IFN-γ (median <10 pg/ml, range <10 pg/ml) and IL-10 (median 345 pg/ml, range <3 to 2388 pg/ml) production found in the control group (p<0.001; Paper I and Table 7).

PBL cytokine responses were classified into Th1, Th2 and Th0 type responses according to the ratio of secreted IFN-γ and IL-10 (Paper I and Table 8). IFN-γ secretion i.e. Th1 type response dominated after stimulation with *C. trachomatis* EB antigen in 33 of 46 (72%) women with TFI compared to only 3 of 15 (20%) controls. On the contrary, after stimulation with CHSP60 secretion of IL-10, i.e. Th2-type response dominated in 20 of 49 (41%) women with TFI. The difference of the cytokine distribution following EB

and CHSP60 stimulation within the TFI group was statistically significant ($p<0.001$; Paper I and Table 8).

Table 8. Th1 vs. Th2 distribution (number of positive specimens/ responders) of peripheral blood lymphocyte response to C. trachomatis elementary bodies (CTR EB) or CHSP60 by infertility group.

	Cytokine response to CTR EB		CHSP60	
Group	Th1 [a]	Th2 [b]	Th1	Th2
TFI	33/46 (72%)*	3/46 (7%)*	7/49 (14%)*	20/49 (41%)*
Other female factor	8/13 (62%)	0/13 (0%)	3/15 (20%)	4/15 (27%)
Unexplained	6/9 (67%)	0/9 (0%)	0/11 (0%)	1/11 (9%)
Male factor	3/15 (20%)	3/15 (20%)	1/16 (6%)	4/16 (25%)
ALL	50/83	6/83	11/91	29/91

Statistical comparison between CTR EB and CHSP60 induced responses: *$p<0.001$

a) ratio of IFN-γ to IL-10 ≥ 2

b) ratio of IFN-γ to IL-10 $\geq$ 0.5

5.6 Cytokine analyses of the *C. trachomatis*-specific T-lymphocyte lines and clones

Cytokine production profile was analyzed from the culture supernatants of 5 representative *C. trachomatis*-specific TLL using ELISA. After stimulation with PHA all TLL secreted high quantities of IFN-γ (median 1007 pg/ml, range 765 to 1080 pg/ml), whereas IL-5 production varied considerably (median 779 pg/ml, range 91 to 1034 pg/ml). Two TLL produced high amounts of IFN-γ, suggesting a Th1-dominant response. Two TLLs produced comparable amounts of both IFN-γ and IL-5 indicative of both type 1 and type 0 T-cell reactivity. No IL-4 secretion was detected.

Forty-five randomly selected *C. trachomatis*-specific TLCs were characterized by studying their ability to produce IFN-γ and IL-10 in response to the chlamydial antigens. After stimulation with *C. trachomatis* EB antigen secretion of both IFN-γ and IL-10 was observed in 37 of 45 (82%) TLCs. Comparison of these TLCs showed an IL-10-dominant (Th2) response in 22 (59%) and an IFN-γ-dominant (Th1) response in 8 (22%) of 37 TLCs ($p<0.01$). Production of both IL-10 and IFN-γ, (i.e. a Th0-type response) was found in 7 of 37 (19 %) TLCs.

The distribution of 14 CHSP60-reactive TLCs according to Th1- and Th2-dominant cytokine response was evaluated as well. Seven (50%) TLCs showed a Th2-dominant response, two (14%) showed a Th1-dominant response, and five (36%) showed a Th0-response (both IL-10 and IFN-γ were found).

5.7 HLA-DQA1 and HLA-DQB1 alleles

Eight HLA-DQA1 alleles and 14 HLA-DQB1 alleles were found among 52 TFI patients and 61 control women. The allele frequencies were comparable between the groups (Paper IV and Table 9). The frequencies of HLA-DQA1*0102 and HLA-DQB*0602 alleles were slightly, although not significantly, higher among the TFI patients (0.31 and 0.23) than among the controls (0.16 and 0.10, respectively; Paper IV and Table 9). Twenty-two of 52 (42%) TFI patients had HLA-DQB1*0602 compared to 10 of 61 (16%) control women (p=0.04, Paper IV and Table 9).

5.8 IL-10 promoter gene polymorphism and HLA-DQ alleles

The allele frequency of IL-10 -1082A was 0.58 in the TFI patients and 0.51 in the controls, and corresponding frequencies of the IL-10 -1082G allele were 0.42 and 0.49, respectively. IL-10 -1082AG heterozygotes were found in 21 of 51 (41%) TFI patients and in 32 of 61 (52%) controls. IL-10 -1082AA homozygotes were found slightly more often among the TFI patients (19 of 51; 37%) than among the controls (15 of 61; 25%, Paper IV and Figure 4A), but the difference was not statistically significant.

A clear association of IL-10 –1082 AA homozygocity and both the HLA-DQA1*0102 and HLA-DQB1*0602 alleles were found among the TFI patients. The combination of DQA1*0102, DQB1*0602 alleles and IL-10 –1082AA was found significantly more often among the TFI patients than among the controls (0.18 and 0.02, respectively; p=0.005).

Table 9. Distribution of HLA-DQA1 and HLA-DQB1 alleles in 52 women with tubal factor infertility (TFI) and in 61 control subjects.

	TFI			Controls			
HLA-DQA1 allele	*Number of positive subjects*	*Number of alleles (2n=104)*	*Allele frequency (n=52)* ,	*Number of positive subjects*	*Number of alleles (2n=122)*	*Allele frequency* (n=61)	*Uncorrected p Allele frequency*
0101	15	16	0.15	21	21	0.17	0.711
0102	28	32	0.31	18	20	0.16	0.010
0103	12	14	0.13	12	12	0.10	0.395
0104	2	2	0.02	8	8	0.07	0.113
0201	4	4	0.04	4	4	0.03	1.00
0301	8	10	0.10	19	20	0.16	0.134
0401	5	5	0.05	14	14	0.11	0.072
0501	19	21	0.20	22	23	0.19	0.800
HLA-DQB1 allele							
0201	15	16	0.15	18	19	0.16	0.969
0301	6	8	0.08	9	9	0.07	0.929
0302	7	9	0.09	15	15	0.12	0.376
0303	4	4	0.04	4	4	0.03	1.00
0401	0	0	0.00	1	1	0.01	1.00
0402	5	5	0.05	12	12	0.10	0.153
0501	18	19	0.18	22	24	0.20	0.789
0502	1	1	0.01	0	0	0.00	0.278
0503	0	0	0.00	7	7	0.06	0.016
0601	0	0	0.00	1	1	0.01	1.00
0602	22	24	0.23	10	12	0.10	0.007
0603	12	14	0.13	11	11	0.09	0.288
0604	3	3	0.03	7	7	0.06	0.349
0608	1	1	0.01	0	0	0.00	0.460

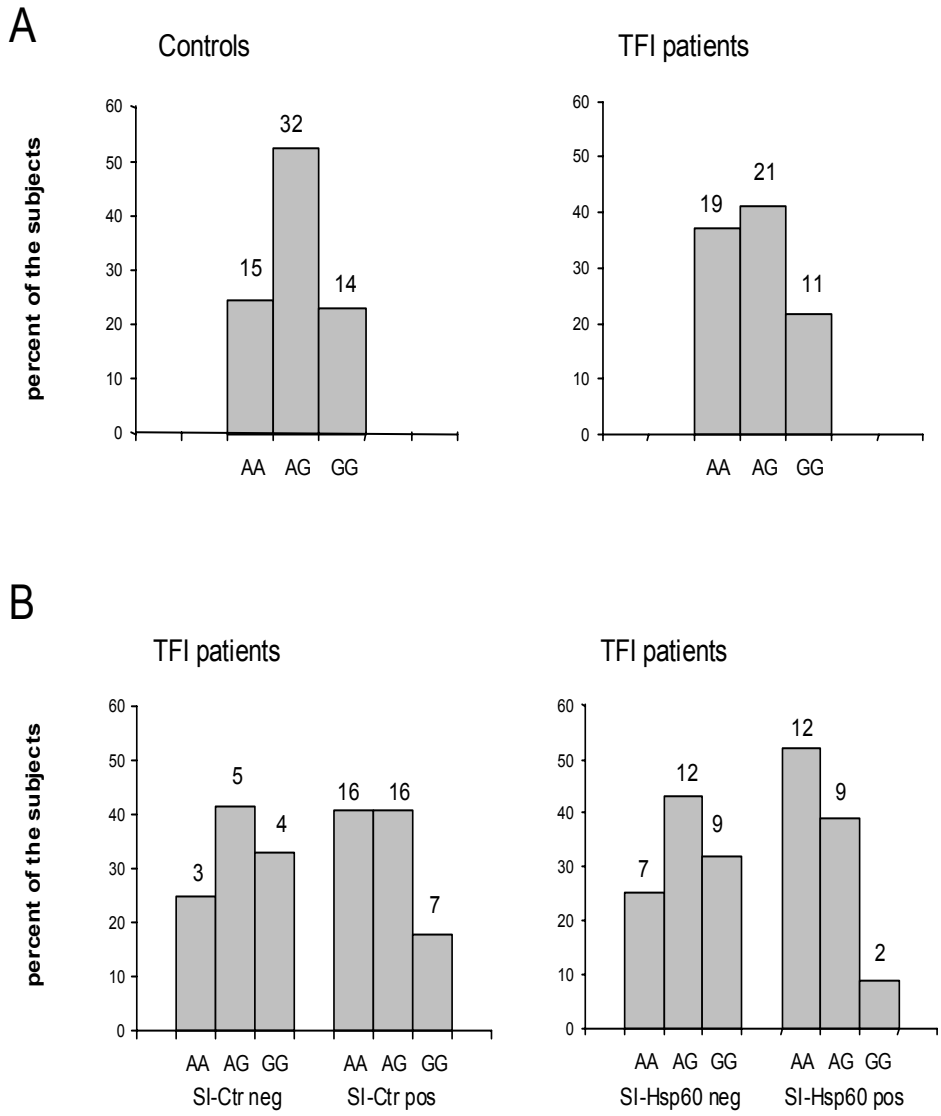

Fig. 4: Distribution of IL-10 –1082 AA, AG and GG genotypes (A) in 51 TFI patients and in 61 control women, and in TFI subgroups with patients who have negative or positive (B) cell-mediated response to *C. trachomatis* EB antigen (SI-Ctr neg and SI-Ctr pos) or chlamydial heat shock protein 60 (SI-HSP60 neg and SI-CHSP60 pos). p=0.046 when comparing the number of IL-10 AA genotypes between TFI patients with positive or negative SI-CHSP60.

5.9 *C. trachomatis*-specific immune response, HLA-DQ alleles and IL-10 promoter gene polymorphism

The possible association of humoral and/ or CMI responses to *C. trachomatis* with HLA-DQA1 and HLA-DQB1 alleles was analyzed for the ten most frequently found HLA alleles (DQA1* 0101, 0102, 0103, 0301, 0501 and DQB1* 0201, 0302, 0501, 0602, 0603; Paper IV and Table 9). Distribution of the HLA alleles did not differ between the *C. trachomatis* -IgG positive and negative or between *C. trachomatis* EB or CHSP60 LP positive and negative TFI patients (Figure 4B and data not shown).

On the other hand, TFI patients with a positive LP response to CHSP60 had IL-10 –1082A allele significantly more often (0.72) than TFI patients with a negative CHSP60 response (0.46, p=0.01, Paper IV and Figure 4B). Furthermore, IL-10 -1082 AA homozygotes were detected more frequently in the CHSP60-responsive than in the non-responsive TFI patients (52% vs. 25%, p=0.046; Paper IV and Figure 4B). Five of 23 (22%) TFI patients with positive LP response to CHSP60 were positive also for IL-10 -1082AA and for the HLA-DQA1*0102 and HLA-DQB1*0602 alleles, whereas only 1 of 61 (2%) controls had this combination (p=0.005). No significant associations between the IL-10 polymorphism and *C. trachomatis* antibody response or LP response against chlamydial EBs were found.

6 Discussion

6.1 Lymphocyte proliferative responses to chlamydial antigens

Chronic *C. trachomatis* infection is an important etiological factor in TFI (Paavonen and Lehtinen 1996, Paavonen 1998). *C. trachomatis*-specific antibody response is generally investigated when evaluating a cause for infertility and a positive antibody response is often accepted as a marker of *Chlamydia*-related TFI (Mol *et al.* 1997, Chernesky *et al.* 1998). However, little is known about the role of *C. trachomatis*-specific immune responses in laparoscopically verified tubal occlusion. Whether and to what extent grouping of patients on the basis of detectable *Chlamydia* humoral immunity results in misclassification of TFI patients is not known. In our study, *C. trachomatis*-induced immune responses, either humoral or CMI were detected more often in the women with TFI than in the controls (women in the male factor infertility group). The proportion of TFI patients with *C. trachomatis*-reactive antibodies was about 50 %, which is comparable to corresponding analyses reported by others (Veenemans and van Der Linden 2002). It is also in accordance with the low specificity of *C. trachomatis* antibody testing in screening TFI (Mol *et al.* 1997). According to our results positive LP response to *C. trachomatis* was frequently found in TFI group. One may argue that the LP assay is less specific than the antibody test as a marker of past *C. trachomatis* infection and may reflect immunization with any chlamydial species. However, we have previously observed that LP responses are clearly higher to *C. trachomatis* than to *C. pneumoniae* in women with PID related diseases (unpublished observations) while in “normal” population *C. pneumoniae*-induced LP responses dominate (Halme *et al.* 1998, von Hertzen *et al.* 1998, Halme *et al.* 2000). This suggests that in a selected population of subjects i.e. women with TFI, a positive LP response can be considered as a sign of past *C. trachomatis* infection.

When evaluating CMI responses against chlamydial EB antigen and CHSP60 we found that the PBLs of TFI patients responded more often to CHSP60 than those of the controls which is in accordance with a previous study on women with salpingitis (Witkin *et al.* 1993a). Witkin et al. (1994) has also shown that the CHSP60-induced PBL response is directed at conserved epitopes of the HSP60-molecule (Witkin *et al.* 1994).

Development of CHSP60-directed LP responses requires multiple episodes of salpingitis (Witkin *et al.* 1994). Alternatively, the CHSP60-specific LP responses may appear during persistent infection but there is no experimental evidence for this assumption. In our study, seropositivity did not overlap with the LP reactivity against the CHSP60 antigen since half of the CHSP60 responders had no *C. trachomatis*-specific antibodies. It is tempting to think that the CHSP60-induced LP responses are even more specific than antibody analyses to identify subjects with previous *C. trachomatis* infection although the specificity may be limited to chronic chlamydial infection. Also, it is possible that CHSP60-induced responses cannot differentiate chronic disease sequelae caused by *C. trachomatis* or *C. pneumoniae* and crossreactivity with HSP60 molecules from other micro-organisms can not be excluded.

While an enhanced CMI response to CHSP60 is linked with adverse disease sequelae in women with PID related diseases (Witkin *et al.* 1993a, Witkin *et al.* 1994, Kligman *et al.* 1998), the situation seems opposite in patients with scarring trachoma. Scarring trachoma is linked with markedly depressed LP response to chlamydial antigens (Holland *et al.* 1993) and subjects recovering from ocular chlamydial infection had enhanced CMI responses to chlamydial antigens including MOMP and CHSP60 (Bailey *et al.* 1995). It is difficult to know whether the discrepancy between the *C. trachomatis*-induced CMI in PID related diseases and trachoma is real. It could reflect differences in the *Chlamydia* burden and induced immunity in the control groups of different studies. In trachoma endemic areas nearly all subjects have acquired ocular chlamydial infection at some stage of their life. The risk for acquiring chlamydial genital infection is much smaller especially in the Western world and the controls are more likely to have no immunity to *C. trachomatis*.

6.2 Immune responses of tissue derived T-cells to chlamydial antigens

To get evidence for the attendance of *C. trachomatis* activated T-cells in the inflamed tissue we established *Chlamydia*-specific TLLs (Paper II) and TLCs (Paper III) from the obstructed fallopian tubes of TFI patients and from endometrial biopsies of acute PID patients. *C. trachomatis* was recognized as a specific target antigen by 25% of the TLL obtained from the PID patients and by 75% of the TLL obtained from the TFI patients. Inflammation detected as infiltration of mononuclear cells was found more frequently in the fallopian tube tissue. Thus, *C. trachomatis* when present in the fallopian tube tissue (Thejls *et al.* 1991, Campbell *et al.* 1993, Patton *et al.* 1994a) participates in maintaining the tissue inflammation (Paavonen and Lehtinen 1996, Stamm 1999, Cohen and Brunham 1999) and may thereby provoke the tissue injury and eventual tubal occlusion seen in TFI patients.

We found that the CHSP60 was recognized only by the TLL originating from the TFI patients but not by TLL originating from the PID patients. One third of the *C. trachomatis*-specific TLCs derived from TFI patients' inflamed fallopian tube tissue recognized CHSP60. Since chlamydial EB contains a wide variety of proteins capable of eliciting CMI responses (Halme *et al.* 1997), the frequency of the CHSP60-reactive TLCs in the inflamed fallopian tube tissue is high. This is the first direct evidence for a

dominant role of CHSP60 as the T-cell stimulating antigen in TFI and correlates with experimental (Morrison *et al.* 1989a) and human studies (Witkin *et al.* 1993b, Toye *et al.* 1993, Arno *et al.* 1995, Eckert *et al.* 1997, Peeling *et al.* 1997, Domeika *et al.* 1998), which have linked the pathogenetic reactions to chlamydial infection with CHSP60.

Recently, Goodall et al. (2001) showed that CHSP60-induced LP responses of PBLs or proportions of CHSP60-specific TLCs derived from synovial fluid were equal or higher than those detected with OMP2. This is noteworthy since OMP2 is the second most abundant protein in chlamydial membrane. Further support for CHSP60 capability of eliciting strong CMI responses comes from *C. trachomatis*-induced ReA where CHSP60 responding T-cells were found in synovia-derived cells (Gaston *et al.* 1996). In addition, in patients with *Yersinia*-triggered ReA *Yersinia*-reactive T-cells respond both to *Yersinia* antigens and to mycobacterial and the human HSP60 (Hermann *et al.* 1991, Mertz *et al.* 1998).

6.3 Cytokine responses against *Chlamydia*

The immuno regulative role of CHSP60-induced CMI in TFI was evaluated by analyzing the secretion and balance of IFN-γ and IL-10, the two cytokines suggested to influence the outcome of chronic chlamydial infection (Wang *et al.* 1999, Yang *et al.* 1999). Positive LP response of PBLs to *C. trachomatis* EB was associated with a dominant secretion of pro-inflammatory IFN-γ, which is needed for clearance of chlamydial infection (Rank *et al.* 1992, Igietseme *et al.* 1993, Van Voorhis *et al.* 1996).

Although PBL from all patients secreted IFN-γ when stimulated with the EBs, the greatest variation in IFN-γ production occurred in the TFI group. The variation in the *in vitro* IFN-γ secretion is comparable to the results of Arno *et al.* (1990) who studied IFN-γ in endocervical secretion of *C. trachomatis* infected women. The levels of IFN-γ, however, varied considerably among individuals (from high to undetectable levels). Unfortunately, we can not draw conclusions regarding the IFN-γ secreting capacity of the chlamydial EB-activated lymphocytes *in vivo*. It is also noteworthy that the link between IFN-γ concentration and elimination of *C. trachomatis* cannot be judged simply by measuring cytokine concentrations in clinical specimens. This is to a great extend due to fact that *C. trachomatis* strains differ in sensitivity to inhibition by IFN-γ (Perry *et al.* 1999, Morrison 2000).

We found that CHSP60 induced higher secretion of IL-10 in TFI patients than in the controls. Debattista et al. (2002) has recently reported that women with PID and those with a history of multiple *C. trachomatis* infections show reduced IFN-γ responses to CHSP60. They also found that the CHSP60-induced IL-10 secretion was somewhat higher in the patients compared to uninfected controls. In an analogous study in individuals with severe scarring trachoma, CHSP60 has also been reported to induce Th2-type immune response (Holland *et al.* 1996).

Using tissue derived TLCs we found that a majority of the CHSP60-specific TLCs produced IL-10, Th2-type cytokine. We were not able to investigate the physiological cytokine concentrations in the fallopian tubes. However, it is tempting to speculate that the high frequency of IL-10 producing TLCs derived from the TFI patients' fallopian tube

tissue reflects their capacity to reduce the effect of IFN-γ (Mosmann and Moore 1991) induced by *C. trachomatis* infection.

The immune response to CHSP60 is generally associated with chronic chlamydial infections (Witkin *et al.* 1993a, Witkin *et al.* 1994) and CHSP60 has been shown to induce marked inflammatory reactions in animals (Morrison *et al.* 1989a, Patton *et al.* 1994b, Van Voorhis *et al.* 1997) and in humans (Kol *et al.* 1999). However, the potential of HSP60s to trigger release of immunosuppressive cytokines has also been demonstrated (Holland *et al.* 1996, Mertz *et al.* 1998). Holland et al. (1996) showed that CHSP60 induces activation of IL-4 and IL-10 mRNA expression in patients with scarring trachoma but not in *C. trachomatis* immunized healthy controls. We found that more than half of the tissue derived TLCs produced IL-10 when stimulated with CHSP60. We did not study reactivity of the TLCs to human HSP60, but some of the TLCs might be cross-reactive and recognize an epitope also on human HSP60. *C. pneumoniae*-specific TLCs derived from atherosclerotic plaques do recognize both CHSP60 and human-HSP60 (Mosorin *et al.* 2000).

Enhanced IL-10 secretion, i.e. a shift towards the Th2-type immune response is associated with lymphocyte activation against self-HSP60 in mice (Yi *et al.* 1997) and leads to delayed resolution of chlamydial infection (Yang *et al.* 1996). In addition, Mertz et al. (1998) showed in *Yersinia*-triggered ReA that TLCs responding to both human- and bacterial HSP60 produced Th2-type cytokine response while TLCs responding only to bacterial HSP60s produced Th1-type cytokine response. Thus, responses to self-HSP60 might lead to partial or temporary down-regulation of an effective bacterial clearance through Th1 response and facilitate microbial persistence.

6.4 *Chlamydia*-induced immune response and HLA

Immune response to CHSP60 plays an important role in chlamydial TFI (reviewed by Paavonen and Lehtinen 1996, Neuer *et al.* 2000, Kinnunen *et al.* 2001). Experimental studies with mice implicate that host's genetic make-up partly determines whether a pathological response develops against *Chlamydia* (Zhong and Brunham 1992, Yang *et al.* 1996, Darville *et al.* 1997, Stagg *et al.* 1998, Darville *et al.* 2001b, Darville *et al.* 2001a). In a macaque model of PID Lichtenwalner et al. (1997) showed that susceptibility or relative resistance to rapid formation of tubal adhesions is correlated with MHC class I alleles (Lichtenwalner *et al.* 1997). However, only a few studies have elucidated the impact of HLA antigens and disease susceptibility in human chlamydial infections (Mabey *et al.* 1991, Conway *et al.* 1996, Kimani *et al.* 1996, White *et al.* 1997, Gaur *et al.* 1999, Cohen *et al.* 2000, Abu el-Asrar *et al.* 2001). Although most of these studies suggest potential role for HLA class I and II antigens in chlamydial disease pathogenesis, no clear or unambiguous results have been obtained. Thus, *C. trachomatis*-associated PID has been linked with HLA-A31 (Kimani *et al.* 1996) and on the other hand with HLA-DQA1*0401 and DQB1*0402 alleles (Gaur *et al.* 1999).

According to our results considerable proportions of *C. trachomatis*-specific T-cells established from the inflamed fallopian tube tissue are targeted to CHSP60 and produce IL-10. TFI patients had slightly more often HLA-DQA1*0102 and DQB1*0602 alleles

compared to controls. We also found that women with TFI had LP responses to CHSP60 in association with HLA-DQA1*0102 and DQB1*0602 alleles suggesting that these HLA alleles may present CHSP60-derived epitopes. The possible association of these alleles with TFI may reflect an association with other HLA genes such as HLA-DRB1*1501 because of linkage disequilibrium between these genes. These three alleles are strongly associated with DR2 antigen. Interestingly, White et al. (1997) have reported an association between HLA-DR16, one subtype of DR2 antigen, and scarring trachoma. We found a possible association of HLA-DQA1*0102 and DQB1*0602 alleles and TFI in Finland while in Nairobi an association of *C. trachomatis* seropositivity and HLA-DQA1*0101 and DQB1*0501 alleles in TFI was reported (Cohen *et al.* 2000). Geographic differences in *C. trachomatis* strains, the permissive nature of HLA molecules in binding available epitopes after processing or the criteria for patient definition are possible explanations for the discrepancy.

We did not compare IL-10 production and IL-10 promoter gene polymorphisms, but IL-10 –1082 AA genotype is associated with lower production of IL-10 compared to –1082 GG genotype (Turner *et al.* 1997). This contradicts our earlier results suggesting that CHSP60 induced increased production of IL-10 in TFI patients (I-III). On the other hand, Nieters et al. (2001) did not find an association of a given genotype and *in vitro* production of IL-10 (Nieters *et al.* 2001). Thus, the relationship between *in vivo* production of a cytokine and single nucleotide polymorphisms is not clear and may involve interactions of several single nucleotide polymorphisms (Eskdale *et al.* 1998, Eskdale *et al.* 1999, Helminen *et al.* 1999, Gibson *et al.* 2001) and differencies between ethnic groups are likely (Mozzato-Chamay *et al.* 2000).

7 Conclusions

C. trachomatis infection is the major cause of sexually transmitted diseases especially in the industrialized countries. Acute infections are often asymptomatic or subclinical and are thus left untreated. Reinfections with the same or different serovars are common indicating that individual immunity is short-lived and serovar-specific. Long-term consequences of repeated or chronic infections include scar formation of the inflamed fallopian tube tissue and subsequent infertility due to occlusion of the fallopian tubes. Only a proportion of the infected women develop scarring and TFI, however, suggesting that genetic factors modulate the immune responses and thereby the pathogenesis of chronic chlamydial disease. Major chlamydial component associated with disease sequelae is CHSP60. It has been shown to modulate immune responses both in animals and in humans during chronic chlamydial infection.

Our aim was to elucidate the role of CHSP60-induced immune response in women with TFI by studying *C. trachomatis*-specific TLLs and TLCs derived from the inflamed endometrial and salpingeal tissue specimens. Our results show that CHSP60 antigen is recognized by T-cells present in the peripheral blood and in the inflamed fallopian tube tissue of TFI patients. In addition, CHSP60 seems to induce Th2-type cytokine responses in *C. trachomatis*-specific T-cells and may thereby down-regulate IFN-γ mediated immune response that is needed to eliminate chlamydiae. Furthermore, the CHSP60-specific CMI response was associated with HLA-DQA1*0102 and HLA-DQB1*0602 alleles and IL-10 –1082 AA genotype in the TFI patients. In genetically predisposed persons the production of an improper cytokine response i.e. Th2-type cytokines in response to CHSP60 or self-HSP60 could lead to a cycle of persistent and apparent infections as well as continued antigenic stimulation and inflammation. End result would then be structural damage of the fallopian tube tissue and infertility. In conclusion, we showed that the characteristics of the immune response to CHSP60 involve genetic regulation and possibly collaboration between HLA and IL-10 genes. Whether HLA and IL-10 genes can be used as markers of the risk for developing TFI should be determined with larger study populations.

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