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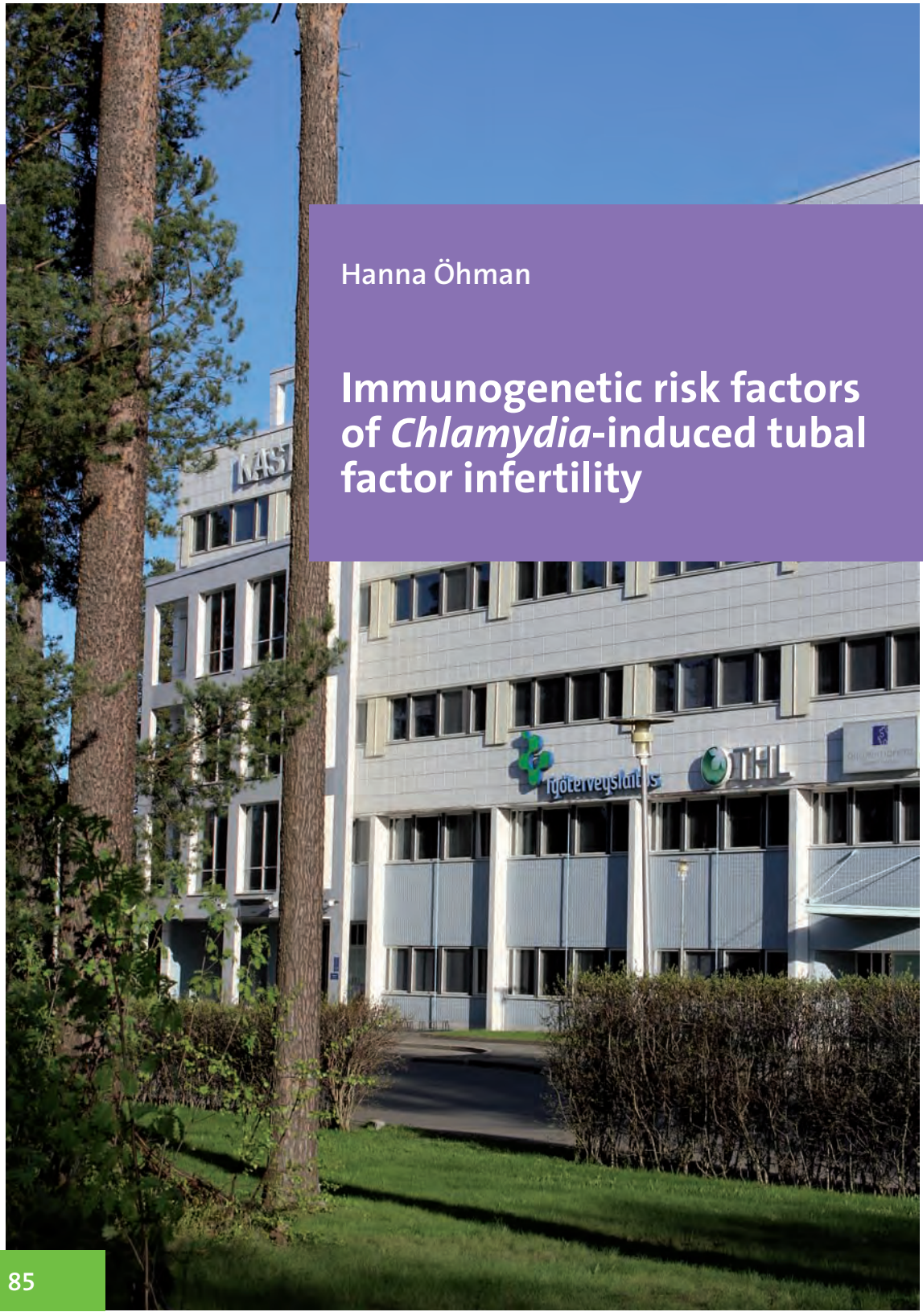

RESEARCH

Hanna Öhman

Immunogenetic risk factors of *Chlamydia*-induced tubal factor infertility

RESEARCH 85/2012

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ACADEMIC DISSERTATION

To be presented with the assent of the Doctoral Training Committee for Health and Biosciences, University of Oulu, for public discussion in the Auditorium of Kastelli Research Centre (Aapistie 1), on September 7th, 2012 at 12 noon.

National Institute for Health and Welfare, Oulu, Finland
University of Helsinki, Faculty of Medicine, Department of Obstetrics and Gynecology, Helsinki, Finland
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To my family

Abstract

Hanna Öhman. Immunogenetic risk factors of *Chlamydia*-induced tubal factor infertility. National Institute for Health and Welfare (THL). Research 85/2012. 135 pages. Helsinki, Finland 2012.
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Chlamydia trachomatis is the most common bacterial sexually transmitted disease worldwide. In women, persistent or repeated infection can cause tissue damage in the Fallopian tubes and impair their normal function, which increases the risk of infertility. The T lymphocyte immune response is crucial in the resolution of acute infection but T cells also probably play an important role in the pathogenesis of *C. trachomatis*-associated tubal damage.

The aim of this work was to study how interindividual differences in immunoregulatory genes affect the susceptibility to and severity of tubal damage and how related genes are associated with *C. trachomatis*-specific immune responses. The study population consisted of 164 infertile women with *C. trachomatis*-induced tubal factor infertility (TFI) and a control group consisting of 176 female blood donors and 179 pregnant women. The study population also included 17 women who were infertile for reasons other than TFI.

The immunosuppressive interleukin IL-10 and its genetic polymorphism *IL10* -1082 A/G had a strong influence on *C. trachomatis*-induced cytokine production. IL-10 levels also reflected the production of proinflammatory interferon-γ (IFN-γ) and tumor necrosis factor (TNF) as well as the intensity of the lymphocyte proliferative (LP) response. The *IL10* -1082 AA genotype, which was associated with low IL-10 production, increased the risk of severe tubal damage in women with *C. trachomatis*-induced TFI.

A single nucleotide polymorphism (SNP) at +874 A/T in the IFN-γ-coding *IFNG* gene was not associated with disease severity. However, this SNP was associated with the intensity of the LP response and the quantitative secretion of IFN-γ was in line with the LP responses. The *IFNG* +874 A/T and *IL10* -1082 A/G SNPs were found to have a cumulative effect on the intensity of *C. trachomatis*-induced LP responses. In addition, SNPs in TNF- and IL-12-coding genes, *TNF* -308 A/G and *IL12B* 1188 A/C, were associated with TFI and the severity of tubal damage. However, their roles in the cytokine profile need to be studied further.

The results of the present work support the hypothesis that genetic factors contribute to the risk and severity of TFI. Our results suggest that genetic predisposition to a strong inflammatory response to *C. trachomatis* infection may result in severe disease manifestation.

Keywords: cell-mediated immune response, *Chlamydia trachomatis*, cytokines, genetic polymorphism, interferon-γ, interleukin-10

Tiivistelmä

Hanna Öhman. Immunogenetic risk factors of *Chlamydia*-induced tubal factor infertility [Perimän vaikutus immuunivasteen säätelyyn klamydiaperäisen hedelmättömyyden tautimekanismeissa]. Terveyden ja hyvinvoinnin laitos (THL). Tutkimus 85/2012. 135 sivua. Helsinki, Finland 2012.
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Chlamydia trachomatis -bakteerin aiheuttama klamydiainfektio on maailmanlaajuisesti yleisin sukupuoliteitse tarttuva bakteeritauti. Naisilla pitkäkestoinen tai toistuva infektio voi vaurioittaa munanjohtimia, mikä saattaa johtaa hedelmättömyyteen. Soluvälitteistä immuunivastetta tarvitaan tartunnan parantumiseen, mutta se on osallisena myös kudosvaurioiden kehittymisessä.

Tämän väitöskirjatyön tavoitteena oli selvittää, miten yksilöiden väliset geneettiset erot sytokiinien tuotannossa vaikuttavat klamydiaperäisen hedelmättömyyden riskiin, kudosvaurioiden vakavuuteen ja soluvälitteisen immuunivasteen voimakkuuteen. Tutkimusaineistossa tautitapauksia edustivat 164 lapsettomuushoitoihin hakeutunutta naista, joilla hedelmättömyyden syyksi oli paljastunut munanjohdinvaurio. Kontrolleina oli 176 verenluovuttajaa ja 179 raskaana olevaa naista. Lisäksi tutkimusaineistoon kuului 17 hedelmöityshoidoissa käynyttä naista, joilla hedelmättömyyden syy oli jokin muu kuin munanjohdinvaurio.

Immuunivastetta vaimentavalla interleukiini-10-sytokiinilla (IL-10) ja sitä koodaavan *IL10*-geenin polymorfismilla -1082 A/G oli huomattava vaikutus klamydian indusoimaan sytokiinituotantoon. IL-10:n tuotanto heijastui myös tulehdusvastetta edistävien sytokiinien interferoni-γ:n (IFN-γ) ja tuumorinekroositekijän (TNF) pitoisuuksiin, sekä lymfosyyttien jakautumisvasteeseen. *IL10* -1082 AA -genotyyppi liittyi matalaan IL-10-tuotantoon, ja se yhdistettiin myös korkeaan vakavan munanjohdinvaurion riskiin.

IFN-γ-sytokiinia koodaavan *IFNG*-geenin polymorfismin +874 A/T ei havaittu liittyvän taudinkuvaan. Tämä polymorfismi oli kuitenkin yhteydessä lymfosyyttien jakautumisvasteeseen ja IFN-γ-tasoihin. Polymorfismit *IFNG* +874 A/T ja *IL10* -1082 A/G vaikuttivat yhdessä klamydian aiheuttaman lymfosyyttien jakautumisvasteen voimakkuuteen. Polymorfismit IL-12- ja TNF-sytokiineja koodaavissa geeneissä liittyivät myös taudinkuvaan, mutta niiden vaikutusmekanismien selvittäminen edellyttää vielä lisätutkimusta.

Tutkimustulokset osoittavat, että klamydian herättämän puolustusreaktion voimakkuus on geneettisesti säädelty, ja geneettinen taipumus voimakkaaseen tulehdusvasteeseen klamydiainfektion aikana lisää alttiutta kudosvaurioiden kehittymiselle.

Avainsanat: *Chlamydia trachomatis*, geenipolymorfismi, interferoni-γ, interleukiini-10, soluvälitteinen immuunivaste, sytokiinit

Contents

List of original publications

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:

I Öhman H, Tiitinen A, Halttunen M, Birkelund S, Christiansen G, Koskela P, Lehtinen M, Paavonen J, Surcel H-M. IL-10 polymorphism and cell-mediated immune response to *Chlamydia trachomatis*. Genes Immun 2006;7:243-9.

II Öhman H, Tiitinen A, Halttunen M, Lehtinen M, Paavonen J and Surcel H-M. Cytokine polymorphisms and severity of tubal damage in women with *Chlamydia*-associated infertility. J Infect Dis 2009;199:1353-9.

III Öhman H, Tiitinen A, Halttunen M, Paavonen J and Surcel H-M. Cytokine gene polymorphism and *Chlamydia trachomatis*-specific immune responses. Hum Immunol 2011;72:278–82.

IV Öhman H, Bailey R, Natividad A, Ragoussis J, Johnson L-L, Tiitinen A, Halttunen M, Paavonen J and Surcel H-M. Effect of *IL12A* and *IL12B* polymorphisms on the risk of *Chlamydia trachomatis*-induced tubal factor infertility and disease severity. Hum Reprod 2012; In press, doi:10.1093/humrep/des136.

Abbreviations

APC	Antigen-presenting cell
CMI	Cell-mediated immunity
EB	Elementary body
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal calf serum
FMC	Finnish Maternity Cohort
GWA	Genome-wide association
HLA	Human leukocyte antigen
HPV	Human papillomavirus
HSP	Heat shock protein
HSP60	Heat shock protein 60 kDa
HSV	Herpes simplex virus
IFN-γ	Interferon-gamma
IFU	Inclusion-forming unit
IL	Interleukin
LGV	Lymphogranuloma venereum
LP	Lymphocyte proliferation
LPS	Lipopolysaccharide
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight
MBL	Mannose-binding lectin

MHC	Major histocompatibility complex
MIF	Micro-immunofluorescence typing test
MOMP	Major outer membrane protein
NAAT	Nucleic acid amplification test
PBMC	Peripheral blood mononuclear cell
PID	Pelvic inflammatory disease
PRR	Pathogen recognition receptor
RB	Reticulate body
SI	Stimulation index
SNP	Single nucleotide polymorphism
STI	Sexually transmitted infection
TFI	Tubal factor infertility
TNF	Tumor necrosis factor

1 Introduction

Chlamydia trachomatis is the most common bacterial sexually transmitted disease worldwide, being diagnosed in over 90 million people each year (Starnbach & Roan 2008). In Finland *C. trachomatis* infection incidence is following the global trend, and the prevalence has risen by 60% since the mid-1990s, with over 13 000 cases being reported annually (The Statistical Database of the Infectious Diseases Register, 2011). The infection is typically asymptomatic but it can still cause long-term inflammation (Black 1997). Because of its symptomless nature, *C. trachomatis* infection is particularly difficult to confine (Stamm 1988, Zimmerman et al. 1990, Thejls et al. 1991). Asymptomatically infected persons do not seek testing or treatment and represent a reservoir for future transmission. In addition, repeat *C. trachomatis* infections are common and protective immunity seems to be short-lived (Batteiger et al. 2010).

The highest prevalence of *C. trachomatis* infection is seen in young sexually active men and women of 20–25 years of age (The Statistical Database of the Infectious Diseases Register, 2011) but complications may be discovered several years later when affected woman try to become pregnant. *C. trachomatis* infection can reduce fertility both in men (Joki-Korpela et al. 2009) and women but the damage to reproductive organs is seen more clearly in women (Paavonen and Eggert-Kruse, 1999). Persistent or repeated infection can cause changes in the Fallopian tube mucosa, adhesion development inside the tubes and eventually tubal blockage with accumulation of fluid (Paavonen & Eggert-Kruse 1999, Lyons et al. 2006). Tissue damage is permanent and impairs normal function of the Fallopian tubes, leading to subfertility and an increased risk of ectopic pregnancy (Lyons et al. 2006).

C. trachomatis lower genital tract infection may apparently become resolved without long-term complications and only some infected women develop infertility (Ramsey 2006). Tubal damage in infertile women also varies greatly in extent, anatomical location and nature (Akande 2007). The T lymphocyte response is crucial in the resolution of acute infection but T cells also probably play an important role in the pathogenesis of *C. trachomatis*-associated infertility (Loomis & Starnbach 2002). Interindividual variation in disease manifestation and the intensity and nature of the immune response is probably affected by the genetic background of the host (Morré et al. 2009).

The T cell response is regulated by a complex network of cytokines (Curfs et al. 1997). The resolution of genital chlamydial infection has been shown to be dependent on interferon-gamma (IFN-γ)-producing CD4+ T cells (Rottenberg et al. 2002), which activate macrophages and the cell-mediated immune response (Boehm et al. 1997). Interleukin-10 (IL-10) plays an opposing role by downregulating the expression of IFN-γ as well as major histocompatibility complex (MHC) class II antigens

and co-stimulatory molecules on macrophages (Moore et al. 2001). IL-10 may limit pathology in the upper genital tract (Hvid et al. 2007) but excessive or mistimed production may also inhibit pathogen clearance and result in chronic infection (Yang 2003, Couper et al. 2008). On the other hand, lack of IL-10 has been linked with inflammation-driven pathogenesis in some infectious diseases (Mege et al. 2006). Because T cell- and IFN-γ-mediated inflammatory responses are also considered to cause tissue scarring (Loomis & Starnbach 2002), careful regulation of the balance between IL-10 and IFN-γ and possibly also other cytokines is probably important as regards disease outcome.

The aim of this study was to improve understanding of disease mechanisms in *C. trachomatis*-induced tubal damage. A candidate gene approach was used to identify cytokine genes related to susceptibility to and severity of *C. trachomatis*-induced tubal damage. The study was also extended to the mechanism behind genetic association. We analyzed how related polymorphisms affect *C. trachomatis*-specific immune responses. Better knowledge of disease mechanisms is needed to develop new preventive strategies. At present the challenge in controlling *Chlamydia* associated pathogenesis is to identify and treat infected individuals before tissue damage occurs. This may be difficult because infections are often asymptomatic. The genetic markers that are associated with disease could be used as diagnostic tools to determine an individual's risk to develop later complications. The understanding of disease mechanisms is also needed for the development of *Chlamydia* vaccine which would be the most effective approach to control infections.

2 Review of the literature

2.1 *Chlamydia trachomatis*

2.1.1 History

Chlamydia trachomatis infections have been known to afflict mankind throughout history. Ancient medical texts from Egypt, China, Rome, Greece and Arabia all make reference to trachoma (Wright et al. 2008). The etiologic agent was described at the beginning of the 20th century. Chlamydial cytoplasmic inclusions were first found in 1907 in conjuctival scrapings from orangutans that were infected with ocular scrapings from a patient who suffered from trachoma (Halberstaedter & von Prowazek 1907). The first suggestion of *C. trachomatis* genital tract infection was made a hundred years ago in 1911 when Lindner described inclusions in urethral epithelia in men with non-gonococcal urethritis (Lindner 1911). At about the same time, cytoplasmic inclusions were reported in cervical cells from mothers of infants with non-gonococcal inflammation of the eye (Heymann 1910). This discovery was confirmed in 1911 by Lindner, who detected similar cytoplasmic inclusions in inflamed eyes of newborns as well as in cervical and urethral cells from their parents (Lindner 1911).

Since then, knowledge of *C. trachomatis* has increased enormously as detection and study of intracellular pathogens, including *Chlamydia*, have passed through a period of substantial evolution. *C. trachomatis* was first isolated from the genital tract in 1959 from the mother of an infant with conjunctivitis (Jones et al. 1959). The development of *in vitro* cell culture methods enabled the use of new research tools such as electron microscopy. Because of the small size and intracellular nature of *Chlamydia*, the microbes were first considered as parasites and then as viruses (Budai 2007). In the mid-1960s, after cell culture, cell wall, and antibiotic susceptibility tests had been applied in chlamydial research, it was proven that *Chlamydia* organisms are obligate intracellular bacterial pathogens of higher cells (Moulder 1964, Budai 2007).

C. trachomatis was associated with acute salpingitis and pelvic inflammatory disease in women in the 1970s (Mårdh et al. 1977, Paavonen et al. 1979, Treharne et al. 1979, Paavonen 1980). The development of the micro-immunofluorescence typing test (MIF) (Wang & Grayston 1970) and enzyme immunoassay (EIA) (Saikku et al. 1983) methods in the 70s and 80s gave the first prevalence and serotype data. Development of the PCR technique in the 1980s (Mullis & Faloona 1987) opened a new diagnostic and research field as regards chlamydial infections. The first successful *C. trachomatis* nucleic acid amplification by PCR was performed in 1989

(Griffais & Thibon 1989) and the first commercial nucleic acid amplification test was introduced in 1993 (Jaschek et al. 1993).

Two other species of the *Chlamydia* genus, *C. pneumoniae* and *C. psittaci*, have also been recognized to cause disease in humans. *C. pneumoniae* is a very common respiratory pathogen (Kuo et al. 1995) that was classified as a new species of the genus *Chlamydia* in 1989 (Grayston et al. 1989). It has been associated with coronary artery diseases (Saikku et al. 1988). *C. psittaci* typically infects avian species. In humans it can cause a zoonotic respiratory infection called psittacosis (Beeckman & Vanrompay 2009). *C. psittaci* caused a large outbreak of severe psittacosis with a 20% mortality rate in Europe and North America in 1929–1930. At that time, the pathogenic agent was found microscopically to resemble the "trachoma virus" (Bedson et al. 1930, Bedson & Bland 1932).

2.1.2 Developmental cycle

Chlamydia is an obligate intracellular bacterium that has a unique two-phase developmental cycle. The bacterium alternates between an infectious extracellular form, the elementary body (EB) and a noninfectious intracellular replicating form, the reticulate body (RB) (Moulder 1991). EBs are extracellular, metabolically inert forms that are able to attach to and invade susceptible host cells. They are internalized in membrane-bound vacuoles, named inclusions. Inside the cell the EBs develop into metabolically active RBs, which divide by binary fission. After multiple divisions, the RBs begin conversion back to EBs. The host cell then lyses, releasing a new generation of EBs that infect neighboring cells (AbdelRahman & Belland 2005). The bacteria stay inside the inclusion throughout the intracellular stage, which according to the results of *in vitro* studies lasts for approximately 48–72 h for *C. trachomatis*. The length can vary depending on the infecting strain, host cell and environmental conditions (Beatty et al. 1994b).

In vitro, the developmental cycle can be stalled into a state of persistence with morphologically enlarged, aberrant and nondividing RBs. Inducers of this state include IFN-γ (Beatty et al. 1993), penicillin (Nicholson & Stephens 2002), iron deprivation (Raulston 1997), nutrient starvation (Coles et al. 1993), concomitant herpes simplex virus infection (Deka et al. 2006, Deka et al. 2007) and maturation of the host cell into a physiologically different state (reviewed by Wyrick 2010). This persistent condition is reversible, yielding infectious EBs on removal of the inducers. However, it is still unknown whether aberrant chlamydial RBs occur *in vivo* and whether they contribute to prolonged inflammation and disease complications (Wyrick 2010).

2.1.3 Genome

C. trachomatis has a relatively small genome. The following *C. trachomatis* genomes have been sequenced: trachoma strain A (Carlson et al. 2005), sexually

transmitted strain D (Stephens et al. 1998), Swedish variant strain (Seth-Smith et al. 2009, Unemo et al. 2010) and lymphogranuloma venereum (LGV) strain L2 (Thomson et al. 2008) and they all contain approximately 1040 kbps. They vary in size only by a maximum of 5000 bps and they also share a high level of genome sequence conservation (Thomson & Clarke 2010). *C. trachomatis* strains normally have a plasmid, an extrachromosomal element, which is 7.5 kbps in size and present at a rate of around 4–8 copies per bacterium. The plasmid seems to be a virulence factor and its presence is linked to the ability of the host bacterium to synthesize glycogen (Carlson et al. 2008). Only a few viable isolates of plasmid-free *C. trachomatis* strains have been isolated (An et al. 1992, Farencena et al. 1997, Magbanua et al. 2007). Because the plasmid is present in multiple copies and the sequence is conserved, it is used as a target in many commercial nucleic acid amplification tests (NAATs) (Thomson & Clarke 2010).

2.1.4 *C. trachomatis* infections

C. trachomatis can be divided into two biological types or biovars on the basis of their target cells. The trachoma biovar is restricted to the epithelial cells of the eye and genital tract whereas the LGV biovar is invasive and proliferates in monocytes within lymphatic tissues (Mabey & Peeling 2002, Manavi 2006). The trachoma biovar is classified into 15 distinct serovars based on antigenic variation in *ompA* genes that encode the organism's major outer protein (MOMP). Serovars A, B, Ba and C are causative agents of trachoma, a major cause of blindness in sub-Saharan Africa, the Middle East and Asia (Burton & Mabey 2009). Serovars D–K are transmitted sexually and are a cause of urogenital tract infection worldwide (Bébéar & de Barbeyrac 2009). There are three main LGV serovars, L1, L2 and L3 (Mabey & Peeling 2002).

Genital infections

Globally, *C. trachomatis* is a major cause of sexually transmitted bacterial infections affecting over 90 million people annually (Starnbach & Roan 2008) and the number of reported cases has been rising during the past 10 years in Europe and the USA (Bébéar and de Barbeyrac, 2009). In both men and women, silent, asymptomatic infection is common (Stamm 1988, Zimmerman et al. 1990). Genital chlamydial infections are typically diagnosed in young people between 16 and 24 years of age. Risk factors of infection include a high frequency of partner change and number of partners (LaMontagne et al. 2004).

In men, *C. trachomatis* is the commonest cause of non-gonococcal (non-specific) urethritis. The symptoms can include dysuria and a moderate clear or whitish urethral discharge (Peipert 2003). Reactive arthritis has also been associated with genital *C. trachomatis* infection (Keat et al. 1978). In women, the infection can cause cervicitis, which can be asymptomatic, or it may cause vaginal discharge or postcoi-

tal bleeding. Urethral infection can also be associated with cervicitis. Infection can ascend to the upper genital tract and cause endometritis, salpingitis and pelvic inflammatory disease (PID) (Paavonen & Eggert-Kruse 1999). In the long term, *C. trachomatis* reduces fertility both in men (Joki-Korpela et al. 2009) and women (Paavonen & Eggert-Kruse 1999).

C. trachomatis infection has also been linked to adverse pregnancy outcomes, including chorioamnionitis, placentitis, premature rupture of membranes, and preterm birth. However, the existing evidence is relatively weak and mostly based on case-control studies (Paavonen 2012). Although *C. trachomatis* is a common cause of endometritis, no association between *C. trachomatis* infection and abnormal placentation has been demonstrated (Tikkanen et al. 2008).

C. trachomatis infection may be transmitted from mother to the infant at delivery. It can cause conjunctivitis, nasopharyngeal infection and chlamydial pneumonia in infants, with a transmission rate of 50–75% (Peipert 2003).

Trachoma

Trachoma is the leading infectious cause of blindness worldwide (Resnikoff et al. 2008) and it is estimated that approximately 1.3 million people are blind and a further 1.8 million have low vision because of this disease (Burton & Mabey 2009). Trachoma is transmitted by flies, fingers or shared clothes or towels. The disease affects the poor living in rural areas where people have limited access to water and personal hygiene is difficult (WHO 2006).

Repeated or prolonged infections produce scarring of the inner part of the upper eyelid, which turns the eyelashes inwards so that they scratch the eyeball. This condition is called trichiasis. Poor tear secretion due to scarring leads to drying of the eye. These things may lead to scarring of the cornea, which impairs vision (WHO 2006). Visual loss from trachoma often starts in adulthood and is more common in women than men. Trichiasis is a disabling disease causing visual impairment, pain and photophobia. Trachoma is therefore a significant health problem in affected communities, with a high disability burden (Burton & Mabey 2009).

Lymphogranuloma venereum

The LGV serovars may cause disseminated infection. They predominantly infect monocytes and macrophages and pass through the epithelial surface to regional lymph nodes. In contrast, the A–K serovars are largely confined to mucosal epithelial surfaces of the genital tract and eye (Mabey & Peeling 2002).

LGV infections are rare in industrialized countries, although the number of reported cases has increased in recent years. Until 2003, LGV was seen only sporadically in Europe and the small regional outbreaks usually involved migrants or tourists from tropical regions. LGV is endemic in East and West Africa, India, Southeast Asia and the Caribbean (Stary & Stary 2008). In 2003 there was an outbreak of LGV in Rotterdam, followed by additional reports from other European countries and

North America. Most patients have been HIV-positive men (Stary & Stary 2008, White 2009).

2.1.5 Natural history of genital *C. trachomatis* infection

Interindividual differences in the clinical course of *C. trachomatis* infection occur, including transmission of the pathogen (Lin et al. 1998, van Valkengoed et al. 2002), duration of untreated infection (McCormack et al. 1979, Morré et al. 2002, Molano et al. 2005) and development of tissue damage (Akande 2007) and late complications (Weström et al. 1992). However, knowledge of the natural history of untreated genital chlamydial infection in humans is limited and more information on factors affecting infection outcome, including spontaneous resolution, persistence and development of complications are needed to optimize *C. trachomatis* prevention strategies and to estimate the cost-effectiveness of screening (Geisler 2010).

Studies indicate that the transmission rate of *C. trachomatis* infection is relatively high. When the infection prevalence was studied in partners of *C. trachomatis*-positive patients the transmission rate from patient to partner was between 48% and 65%. A lower rate was found among asymptomatic populations (screening population) (van Valkengoed et al. 2002) and a higher one among subjects attending an STD clinic because of symptoms (Lin et al. 1998).

Three studies have concerned evaluation of the duration of untreated, uncomplicated chlamydial infection in humans with follow-up at intervals of at least one year (Geisler 2010). In these studies approximately half of the participants cleared the infection spontaneously within one year after initial testing (McCormack et al. 1979, Morré et al. 2002, Molano et al. 2005). The follow-up time was longest in the study by Molano et al., where *C. trachomatis* clearance was evaluated retrospectively using stored cervical specimens from 1995 Colombian women who were enrolled in a human papillomavirus (HPV) natural history study. The study was conducted among 82 women who tested positive at baseline. The infection had cleared in 54% by 1 year of follow-up, 82% by two years and 94% by four years (Molano et al. 2005). The accuracy of estimates of *C. trachomatis* infection duration is limited because the studies lack exact timing of initial infection, and bias due to the fact that repeated infections cannot be considered. Animal models of persistent infections differ fundamentally from human infection, limiting their use as regards mimicking human natural infection. In mice the infection is cleared within 3–4 weeks (Miyairi et al. 2010). Compared with mice, in pigtailed macaques the infection is more persistent (Wolner-Hanssen et al. 1991).

Chlamydial IgG antibodies can persist at stable levels for years after infection (Puolakkainen et al. 1986). Many studies have shown that serological markers of past *C. trachomatis* infection are associated with infertility (Robertson et al. 1987, Toye et al. 1993) and an increased risk of ectopic pregnancy (Chow et al. 1990, Brunham et al. 1992). However, only some women develop tubal damage as a con-

sequence of infection, while most women clear the infection without long-term complications. Studies among symptomatic patients visiting STD clinics or emergency departments have shown that 2–4.5% of women with untreated *C. trachomatis* infection develop PID within a two-week interval between testing and returning for treatment (Bachmann et al. 1999, Geisler et al. 2008). Symptomatic PID increases the risk of infertility (Weström et al. 1992, Ness et al. 2002). In a study by Weström et al. a patient group of 1844 women with laparoscopically verified, symptomatic PID were followed up for several years in regard to adverse outcomes. In this study population there were various reasons for PID and not all were *Chlamydia* related. The study showed that severe inflammation predicted later fertility problems. In addition, each repeated episode of PID roughly doubled the rate of TFI. After 1, 2, and 3 or more episodes the rates were 8.0%, 19.5% and 40.0% (Weström et al. 1992). Epidemiologic studies have shown that the cumulative risk of PID (Kimani et al. 1996, Hillis et al. 1997) and long-term reproductive consequences (Hillis et al. 1997, Bakken et al. 2007) increase according to the number of repeat *C. trachomatis* infections. However, it remains unclear whether the increased risk is due to longer exposure time (additive risk connected with each infection) or does the repeated infection induce more immunopathology (more than the additive risk connected with each infection).

Antibodies to chlamydial heat shock protein 60 kDa (HSP60) (Paavonen et al. 2003) and *C. trachomatis* EBs (Koskela et al. 2000, Anttila et al. 2001) as well as diagnosed *C. trachomatis* infection (Lehtinen et al. 2011) have been associated with cervical carcinoma. Although human papillomavirus (HPV) is necessary as regards the development of cervical carcinoma (Bosch et al. 1995, Walboomers et al. 1999, Wallin et al. 1999) *C. trachomatis* lower genital tract infection may act as a cofactor in carcinogenesis. *Chlamydia* induces inflammation and metaplasia and metaplastic cells are potential target cells for HPV. Also, *C. trachomatis* modulates host immune response and inhibits apoptosis (Paavonen 2012).

2.1.6 Diagnostics and treatment

Nucleic acid amplification tests have become the dominant tests for diagnosing genital infections with *C. trachomatis* during the last five years. Such tests became commercially available less than 20 years ago and their evolution has continued ever since, as has the understanding of their use (Schachter 2010). A number of the tests utilize the cryptic plasmid that exists in about 4–8 copies per EB. Currently the most sensitive *C. trachomatis* test, GenProbe Aptima Combo2, targets the rRNA sequence that exists in several hundred copies per EB and thus has an inherent advantage over assays that target DNA (Ikeda-Dantsuji et al. 2005).

Specimen collection has also become easier as the tests have evolved in efficacy. Before the era of NAATs the specimen types required for culture were endocervical swabs from women and urethral swabs from men. With NAATs, clinical examina-

tion is not required and self-collected first-catch urine specimens perform well in NAATs both in men and women (Schachter 2010). In women, self-collected vaginal swab specimens are also used and they have been shown to be better than urine samples (Chernesky et al. 2005, Schachter et al. 2005).

Single-dose therapy with azithromycin (1 g) is an efficient treatment for uncomplicated lower genital tract *C. trachomatis* infection. A 7-day course of doxycycline also gives comparable results. Both patients and their partners should be treated (Workowski & Berman 2010).

Lessons learned from a new variant of *C. trachomatis*

A reminder of the importance of diagnostic test design arose in Sweden in 2006 when a sudden fall in numbers of laboratory-confirmed *C. trachomatis* infections in some Swedish counties led to recognition of a highly prevalent new variant of *C. trachomatis* (Söderblom et al. 2006). Because of a 377-bp deletion in a cryptic plasmid at the target sequence for amplification, the variant had escaped detection by two widely used commercial NAATs, those manufactured by Abbott Laboratories and by Roche Diagnostics. The new variant belongs to serovar E and does not have any obvious biological differences compared with the wild-type strain (Unemo et al. 2010).

The rapid transmission of the new variant in Sweden was due to a strong diagnostic selective advantage – the variant became clearly more prevalent in counties where the Roche and Abbott tests were used (Herrmann et al. 2008). The emergence and spread of the new variant highlighted the fact that selection of the target gene for amplification is important. Although the plasmid region is highly conserved, its biological function and necessity to pathogen is not well known and it is therefore a risky target for diagnostics. Multitarget assays detecting essential conserved non-cryptic multicopy species-specific DNA or RNA sequences should be used for detection (Unemo & Clarke 2011).

Serology

C. trachomatis serology is no longer used as diagnostic tool for acute infections, but it is still used in the fertility work-up as a screening test to estimate the risk for tubal pathology (Akande et al. 2003). Nowadays serology has a new role in population-based epidemiological studies and has proven useful in evaluating on-going trends of infection prevalence and incidence rates at a population level (Johnson & Horner 2008, Lyytikäinen et al. 2008a, Lyytikäinen et al. 2008b). Serology is also used to evaluate the impact of *C. trachomatis* screening programs at a population level. Various serological tests have been developed for the detection of *C. trachomatis*-specific antibodies, including a complement fixation test, MIF, EIA and immunoblotting (Paavonen 2012).

2.2 Immune response to *C. trachomatis* infection

Because of difficulties in specifying the time of exposure to infection and following the development of a local immune response at the site of infection in humans, researchers have used several animal models of chlamydial infection to examine the immune response that occurs in the female genital tract after infection (Darville & Hiltke 2010). Mouse models show that the response to primary chlamydial infection occurs within a few days after inoculation of infection and is characterized by mucosal and submucosal infiltration of polymorphonuclear neutrophils and monocytes, and T cells accumulate at the site later (Morrison 2000, Morrison & Caldwell 2002).

Unlike other mucosal surfaces, genital mucosa lacks underlying organized lymphoid elements (Neutra et al. 1996, Kelly 2006). In a mouse model, antigen-specific naive T cells are activated and differentiate into effector T cells in the iliac lymph nodes that drain antigen from the genital tract (Cain & Rank 1995, Roan et al. 2006). Thereafter, the T cells migrate to the genital tract and accumulate at the site of infection (Kelly & Rank 1997), where they play a critical role in controlling it (Rank & Barron 1983, Ramsey & Rank 1991, Morrison et al. 1995).

2.2.1 Innate immunity

The mucosal barrier of the genital tract provides the first line of host defense against invading microbes (Quayle, 2002). Proliferation and maturation of vaginal epithelial cells is under hormonal regulation (Darville 2006) and in a mouse model the ability of *C. trachomatis* to go through this physical barrier is influenced by the stage of the estrus cycle (Roan & Starnbach 2008). At peak estrogen levels, the vaginal epithelium reaches its maximum thickness (Quayle 2002).

C. trachomatis primarily infects epithelial cells, which recognize microbial invariant structures via pathogen recognition receptors (PRRs) (Joyee & Yang 2008). The infected epithelial cells initiate immunity effector mechanisms by inducing the production of proinflammatory cytokines that also have chemoattractant functions, such as IL-1α, IL-6, IL-8, TNF, GROα and GM-CSF (Rasmussen et al. 1997). Chemokines recruit neutrophils and monocytes to the site of infection (Molestina et al. 1999, Dessus-Babus et al. 2000). Neutrophils play an important role in the first stage of infection. They are the only myeloid cells whose lysosomes readily fuse with phagosomes containing EBs (Darville 2006). They are capable of killing EBs and both oxygen-dependent and oxygen-independent mechanisms are active in the process (Yong et al. 1982, Söderlund et al. 1984, Zvillich & Sarov 1985, Yong et al. 1986). In addition, NK cells are important at the early stage of infection as they are able to produce IFN-γ, which is an essential cytokine in defense against *C. trachomatis* infection (Tseng & Rank 1998).

Macrophages and monocytes play central roles in both innate and adaptive immunity. Macrophages are present as Langerhans-like cells in the vagina and cervix (Booker et al. 1994, Darville 2006). Compared with neutrophils, macrophages per-

sist much longer at the site of inflammation. They are longer-lived than neutrophils, and can undergo cell division at the inflammatory site (reviewed by Darville 2006). Macrophages aid in eradication of *Chlamydia* in infected cells but *Chlamydia* can potentially survive and replicate within infected macrophages (Airenne et al. 1999). The ability of *Chlamydia* to inhibit phagosome-lysosome fusion varies between chlamydial species and serovars (La Verda & Byrne 1994). Macrophages activate and regulate the development of adaptive immunity by producing the cytokines IL-12 and IL-10 (O'Garra & Murphy 2009). They also process and present antigens to T cells (Trombetta & Mellman 2005) and therefore provide a link between innate and adaptive immunity.

2.2.2 Adaptive immunity

An adaptive immune response is necessary to limit *C. trachomatis* infection and generate antigen-specific immunity. Both T cells and B cells are activated during chlamydial infection (Morrison & Morrison 2000) but T cells have a more important role in host defense. The necessity of T cells in control of *C. trachomatis* infection was documented in the mid-1980s, when it was observed that nude mice, which lack T cells, suffer from chronic infection, whereas wild-type mice are able to clear the infection within 20 days (Rank et al. 1985). The role of B cells is to produce antibodies and mediate immunity to *C. trachomatis* by neutralizing infectivity and enhancing phagocytosis of EBs (Peeling et al. 1984, Peterson et al. 1991). However, antibodies are unable to access the *Chlamydia* organisms established in intracellular infection and in mouse models antibodies have not been found to be essential in controlling primary *C. trachomatis* infection (Su et al. 1997).

Both CD4+ and CD8+ T cells contribute to defense (Loomis & Starnbach 2002), but CD4+ T cells appear to be more important during natural infection (Magee et al. 1995, Morrison et al. 1995, Williams et al. 1997). CD4+ and CD8+ T cells recognize antigens that are processed through different pathways. CD4+ cells recognize antigens that are engulfed by antigen-presenting cells (APCs). Antigens are processed by proteases within the lysosomal compartments of APCs, and the resulting peptides are presented by MHC class II molecules to CD4+ T cells (Trombetta & Mellman 2005). APCs can process *Chlamydia* EBs phagocytosed from extracellular spaces within tissues and they also present RB antigens by engulfing infected cells that harbor RB organisms. Therefore, CD4+ T cells may recognize antigens from multiple stages of *C. trachomatis* infection (Roan & Starnbach 2008).

Infected epithelial cells present chlamydial antigens in complexes with MHC class I to CD8+ T cells. A number of chlamydial proteins are secreted during the intracellular stage of the developmental cycle from inclusion into the host cell cytoplasm (Zhong et al. 2001, Clifton et al. 2004) and these proteins serve as CD8+ T cell antigens (Fling et al. 2001, Starnbach et al. 2003). The cytosolic antigens are processed by the proteasome into peptide fragments (Cresswell et al. 2005).

Because reinfection is common (Niccolai et al. 2007), it seems that individuals infected with *Chlamydia* do not develop long-term protective immunity (Batteiger et al. 2010). Some degree of immunity develops, but it is not necessarily able to prevent infection (Katz et al. 1987). According to epidemiological data, previous *C. trachomatis* infection does not seem to protect against reinfection, but, in contrast, some studies have shown that a documented history of chlamydial infection is associated with an increased risk of current infection (Hiltunen-Back et al. 2001, Rietmeijer et al. 2002). In studies carried out in the US, the median time to reoccurrence of *C. trachomatis* infection has typically been found to be 5–7 months, with the risk being higher among young women than among older women (Burstein et al. 1998, Burstein et al. 2001). However, repeat infections are associated with number of partners (Hiltunen-Back et al. 2001), suggesting that risk-taking sexual behavior is associated with *C. trachomatis* infection. Therefore, these observations do not exclude the concept of protective immunity, but suggest that it is partial at best. The short term protective immunity is probably due to the inability of CD4+ cells to be retained in the genital tract. CD4+ cells are required for immunity, but when the chlamydial organisms and antigens are eliminated from the genital tract, homing receptors for CD4+ cells are downregulated and T cells cannot remain or home to the site (Kelly & Rank 1997, Rank 2006).

2.2.3 Cytokine profile and T helper subpopulations

The cytokine environment during the early stages of infection has a significant role in development of the T cell-mediated immune response, and it is also demonstrated to affect the outcome in many diseases (Belardelli 1995). Classification of CD4+ T cells into Th1 and Th2 cells (also known as Type 1 and Type 2 T helper cells) is based on the different functions and cytokine profiles of the cell types (Mosmann et al. 1986). Th1 cells activate macrophages and phagocytic functions via production of IFN-γ and the promote secretion of IgG2a opsonizing antibodies by the B cells. In contrast, Th2 cells mediate phagocyte-independent responses. Th2 cells produce IL-4, which activates B cell-mediated antibody responses at a broad level, and it also inhibits macrophage function (Romagnani 1996). Th1 and Th2 subsets cross-regulate each other's functions. The cytokines released from Th1 and Th2 cells stimulate their own subsets and, at the same time, they inhibit cytokine production and function of the other subset (Belardelli 1995).

Extensive evidence supports the concept that the Th1 and Th2 subsets and their main cytokines, IFN-γ and IL-4, mediate opposite effects on a variety of immune parameters, including several functional activities of macrophages and the development of T cell cytokine profiles (Kelso 1995). After introduction of the Th1/Th2 paradigm in the 1980s (Mosmann et al. 1986), the theory became widely accepted in the field of immunology. However, many murine and human T cell clones do not fit the Th1/Th2 classification and the model was soon recognized to be oversimplified

(Kelso 1995). In addition, experimental evidence from studies on autoimmune diseases has contradicted the Th1/Th2 paradigm, suggesting that some piece of the puzzle is missing. Although Th1 cells were thought to play a major role in tissue damage, the administration IFN-γ did not worsen autoimmune disease, and neither did blocking of IFN-γ ameliorate it – just the opposite (Basso et al. 2009). The discovery of a third member of the T helper set, IL-17-producing Th17 cells (Harrington et al. 2005) started to explain the mechanisms of autoimmune disease and immunopathology (Bettelli et al. 2007). Th17 cells are a distinct lineage that does not share developmental pathways with either Th1 or Th2 cells (Harrington et al. 2005, Park et al. 2005). The Th17 subset is highly proinflammatory and has been associated with autoimmune disorders (Bettelli et al. 2007). The Th17 response has also been implicated in the development of immunopathology in other models of infection (Torrado & Cooper 2010, Yang et al. 2011).

At present, CD4+ T helper cells are divided into four main subsets: Th1, Th2, Th17 and regulatory T (Treg) cells (Kaufmann 2007), presented in Figure 1. In addition, Th9, Th22 (Annunziato & Romagnani 2009) and follicular helper T (T_{FH}) cell (Crotty 2011) subpopulations have been introduced, suggesting that CD4+ cells do not fit into a simple classification. Of Treg cells, two main groups have been identified: naturally occurring CD4+CD25+ Tregs, which may develop in the thymus and regulate self-reactive T cells in the periphery, and inducible Tregs, which develop in the periphery from conventional CD4+ T cells after exposure to signals such as regulatory cytokines (O'Garra & Vieira 2004). Treg cells can limit Th1 and Th2 cell responses indirectly by modulating APC function or directly by way of cell–cell contact (Belkaid 2007).

In murine models of *C. trachomatis* infection, a Th1 type of immune response has been shown to mediate resolution of infection and a Th2 type of immune response is associated with an inadequate cell-mediated response and persistence of *C. trachomatis* infection (Perry et al. 1997, Yang 2003). However, in human *C. trachomatis* sexually transmitted infection a Th2 type of response has not been reported (Stephens 2003), suggesting that activation of the Th2 subset is not likely to be the reason for the pathological picture. Tregs and Th17 cells are probably also involved in pathogenesis but their roles have not yet been studied widely.

The cytokine profile also influences host defense in *C. trachomatis* infection. In particular, the balance between IFN-γ and IL-10 is a potential determinant of infection outcome, because these two cytokines play opposing roles in regulating the immune response. IFN-γ activates macrophages to control intracellular infections (Boehm et al. 1997), while IL-10 has a strong immunosuppressive effect on cell-mediated immunity (CMI) (Couper et al. 2008), which can inhibit pathogen clearance (Mege et al. 2006). On the other hand, IL-10 has also been shown to prevent inflammation-driven tissue pathology (Couper et al. 2008). Production of IFN-γ from NK cells and T cells is induced by IL-12, which is secreted in the early phase of infection (Trinchieri 2003) and might therefore be an important modulator of a

favorable cytokine environment. TNF is also an interesting cytokine because of its multifunctional effects on host immune responses (Locksley et al. 2001) and its possible involvement in fibrosis and tissue remodeling in disease pathogenesis (Banno et al. 2004).

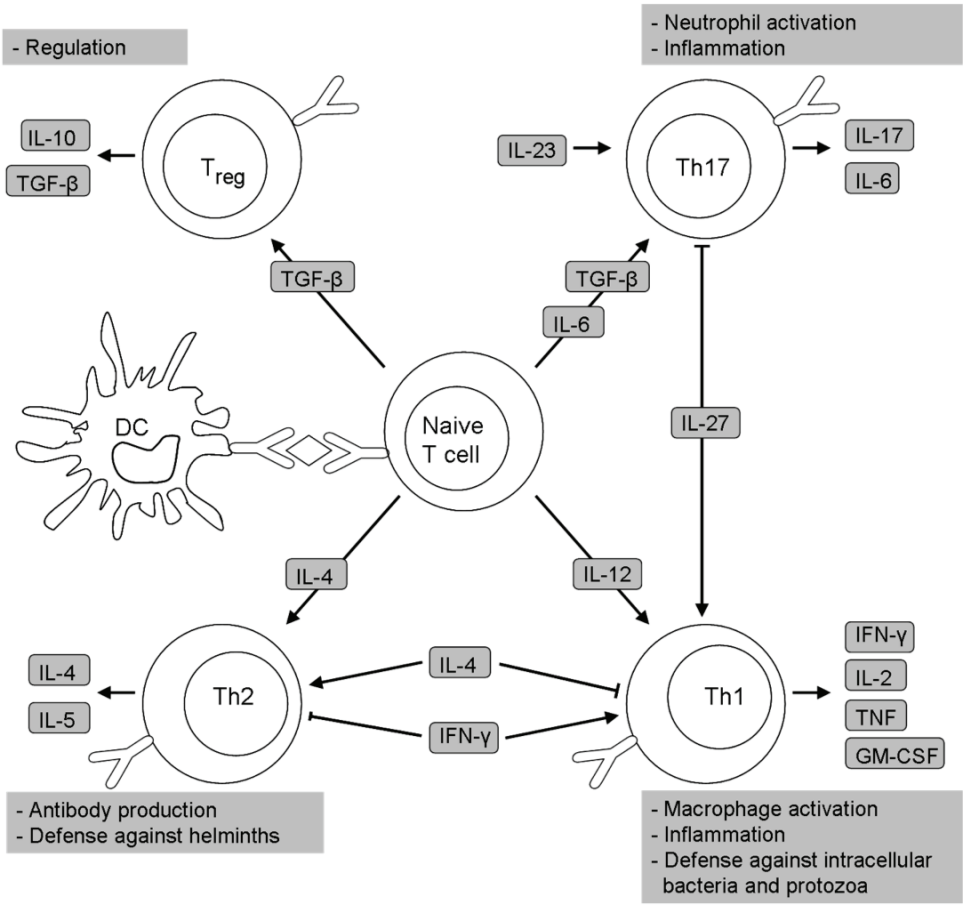

Figure 1. Four main populations of CD4+ T cells. Regulatory T cells (Treg cells) control and prevent excessive immune responses. T helper 1 (Th1) cells activate macrophages and promote cell-mediated immunity by producing interferon gamma (IFN-γ) and interleukin 2 (IL-2) cytokines. T helper 2 (Th2) cells promote humoral immunity and defense against helminths by producing IL-4 and IL-5 cytokines. Th17 cells produce IL-17. (Modified after Kaufmann 2007) DC, dendritic cell; GM-CSF, granulocyte-macrophage colony-stimulating factor; TGF, transforming growth factor.

2.2.3.1 IFN-γ

The functions of IFN-γ are important in CMI against intracellular microbes. IFN-γ is produced by CD4+ and CD8+ T cells and NK cells and it activates macrophages to kill phagocytosed microbes (Boehm et al. 1997). IFN-γ is necessary for protection against *C. trachomatis* infection (Rottenberg et al. 2002, Gondek et al. 2009) and deficiency in its production *in vitro* has been shown to promote persistent chlamydial infection (Beatty et al. 1993).

IFN-γ has been shown to limit *C. trachomatis* infection directly through a variety of mechanisms (Loomis & Starnbach 2002). It induces the expression of indoleamine 2,3-dioxygenase (IDO), which degrades intracellular tryptophan, limiting *Chlamydia* replication (Taylor & Feng 1991). IFN-γ also increases host nitric oxide (NO) production, which restricts *Chlamydia* growth *in vitro* (Ramsey et al. 2001) and downregulates transferrin receptors (Byrd & Horwitz 1993), resulting in intracellular iron deficiency, which may also limit chlamydial growth (Igietseme et al. 1998). It also indirectly controls intracellular infections by augmenting host immune responses. IFN-γ upregulates macrophage phagocytic potential and the expression of MHC I and MHC II antigens, leading to enhanced presentation of microbial antigens to both CD4+ and CD8+ T cells (Boehm et al. 1997), which enhances the CMI response.

The inhibitory effect of IFN-γ on chlamydial growth differs markedly among *C. trachomatis* strains. Strains associated with sexually transmitted infections (STIs) are less sensitive to the direct action of IFN-γ than ocular serotypes in epithelial cell cultures (Morrison 2000). This is probably because the STI serotypes are able to synthesize tryptophan of indole, while the ocular serotypes are not (McClarty et al. 2007). There is also variation in sensitivity to IFN-γ between different *C. trachomatis* STI strains, which can influence the virulence of the bacteria and clinical outcome of the infection (Morrison 2000).

An unsuccessful immune response that leads to persistent infection may be due to an insufficient amount of IFN-γ. The level of IFN-γ is affected by the expression of other cytokines that regulate its production, such as IL-10, IL-4 or IL-12, and probably also by genetic predisposition to IFN-γ expression. The IFN-γ gene *IFNG* is located in chromosome 12 and contains 4 exons that span 5.4 kb. Intronic polymorphisms, +874 A/T and a CA repeat microsatellite, have been associated with IFN-γ production levels (Pravica et al. 2000). The promoter SNP -179 T/G has also been associated with IFN-γ production levels (Bream et al. 2002, Gonsky et al. 2006). In the field of *C. trachomatis* research, certain *IFNG* polymorphisms have been associated with scarring trachoma (Natividad et al. 2005).

2.2.3.2 IL-12

IL-12 is an important mediator of the early innate immune response to intracellular microbes. IL-12 induces the production of IFN-γ and promotes the differentiation of

Th1 cells (Trinchieri 2003). It is mainly produced by monocytes, macrophages and dendritic cells, which are the first leukocytes to interact with pathogens (O'Garra & Murphy 2009). IL-12 is a heterodimeric cytokine, encoded by two separate genes, i.e. *IL12A*, which encodes subunit p35 and *IL12B*, which encodes subunit p40. The bioactive form of IL-12 is a 75-kDa heterodimer (IL-12p70) comprised of disulfide-linked p40 and p35 subunits (Gately et al. 1998).

Subunit p40 is a common building block of IL-12 and IL-23 cytokines (Trinchieri 2003). IL-12 and IL-23 cytokines are closely related and they are both involved in the regulation of T cell responses, yet they have distinct roles. IL-12 is important in induction of IFN-γ production and activation of Th1-type immunity, whereas IL-23 has been shown to play a key role in induction of the newly described Th17 cells that currently are considered to be responsible for the inflammation-driven pathogenesis, rather than the Th1 subset (Goriely et al. 2009).

The host genetic background can potentially affect the production level of IL-12. Because IL-12 induces IFN-γ production, its expression may also be reflected in IFN-γ secretion. Several SNPs of both the *IL12A* and *IL12B* genes have been identified and some of them have been associated with infectious diseases. In particular, the SNP marker 1188 (rs3212227) on the 3'-flanking region of *IL12B* (Figure 2) has been linked to susceptibility to psoriasis (Cargill et al. 2007, Nair et al. 2008) and also to recurrent chlamydial infections (Geisler et al. 2010).

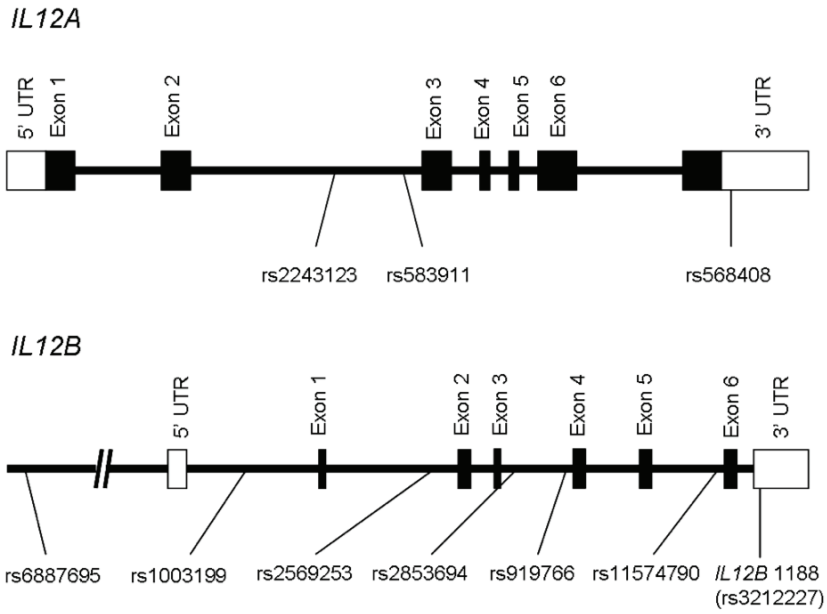

Figure 2. The figure shows the SNP locations in *IL12A* and *IL12B* genes.

2.2.3.3 IL-10

IL-10 was first described as a cytokine synthesis-inhibitory factor (Fiorentino et al. 1989) and it was soon recognized to have a wide range of immunosuppressive functions (Moore et al. 1993). IL-10 is a key immunosuppressive cytokine during infection with viruses, bacteria, fungi, protozoa and helminths. It inhibits the activity of Th1 cells, NK cells and macrophages by downregulating the expression of proinflammatory cytokines such as IFN-γ, IL-12 and TNF. It also inhibits the expression of MHC class II antigens and co-stimulatory molecules on macrophages (Couper et al. 2008). IL-10 can be produced by many different types of cells such as macrophages, dendritic cells, B cells and also various subsets of CD4+ and CD8+ T cells (Moore et al. 2001).

Because of its immunosuppressive effect on CMI, IL-10 has a double-edged nature in infectious diseases. On one hand, excessive or mistimed IL-10 production is connected with impaired immune responses and persistence of bacteria and viruses. On the other hand IL-10 has an important role in the prevention of immunopathology that may result from excessive inflammatory responses (Mege et al. 2006). In humans, IL-10 has been reported to be involved in the persistence of infections caused by bacteria such as *Coxiella burnetii* (Capo et al. 1996, Honstettre et al. 2003), *M. tuberculosis* (Tufariello et al. 2003) and *Bartonella* spp. (Capo et al. 2003, Mege et al. 2006). IL-10 has been shown to prevent the tissue pathology associated with excessive Th1 and CD8+ T cell responses in studies on infections involving *Toxoplasma gondii* (Wilson et al. 2005), *Trypanosoma* spp. (Hunter et al. 1997, Magez et al. 2002), *Plasmodium* spp. (Rudin et al. 1997), *Mycobacterium* spp. (Bekker et al. 1998) and herpes simplex virus (HSV) (Suvas et al. 2004). In addition, IL-10 has been reported to diminish the destructive effect of *C. trachomatis* induced by IL-1 on the epithelium in a human Fallopian tube culture model (Hvid et al. 2007).

Genetic predisposition to infection-induced IL-10 production may affect the clinical outcome of *C. trachomatis* infection. Interindividual variance is seen in the levels of IL-10 secretion from stimulated peripheral blood mononuclear cells (PBMCs) and the variability has been found to have a hereditary component (Westendorp et al. 1997). Several common polymorphisms have been identified in the promoter region of the gene, including *IL10* -1082 A/G, -819 C/T and -592 C/A SNPs. These SNPs are in linkage disequilibrium and form three haplotypes: GCC, ACC and ATA, where GCC is usually associated with high IL-10 production and ACC and ATA with low production (Turner et al. 1997). The functionality of the *IL10* -1082/-819/-592 haplotypes has been studied by using luciferase reporter assays and the ATA construct was found to have weaker transcriptional activity than the GCC construct (Crawley et al. 1999).

2.2.3.4 TNF

Tumor necrosis factor is a multifunctional cytokine that mediates inflammation, the immune response and apoptosis (Locksley et al. 2001). Lipopolysaccharide (LPS) is strong stimulus for TNF production and large amounts of this cytokine may be produced in infections by gram-negative bacteria. It is mainly produced by monocytes and macrophages but activated T cells, NK cells and mast cells can also produce TNF (Vassalli 1992).

TNF stimulates the recruitment of neutrophils and monocytes to sites of infection and activates these cells to eradicate microbes (Vassalli 1992). It has antichlamydial effects both *in vitro* (Shemer-Avni et al. 1988) and *in vivo* in a murine model (Williams et al. 1990). Besides its effects on host immune responses, TNF is also involved in fibrosis and tissue remodeling (Piguet et al. 1989, Banno et al. 2004), making it an interesting target in *Chlamydia* pathogenesis research. In trachoma studies increased levels of TNF transcripts in tear fluid have been associated with scarring trachoma (Conway et al. 1997).

TNF -308 A/G is the most commonly studied *TNF* polymorphism. The SNP is located in the promoter region of the gene and has been associated with many diseases. However, the literature covering the role of the *TNF* -308 A/G polymorphism in TNF production is inconsistent (Bayley et al. 2004) and the stimulus and studied cell type can affect TNF expression and its association with the -308 SNP (Kroeger et al. 2000). It is also possible that the association with disease manifestation is derived from a gene nearby that is linked to *TNF*, because the gene lies in an MHC III area which exhibits extensive linkage disequilibrium. Trachoma studies indicate that the *TNF* -308 A marker is part of a more complex picture. The *TNF* locus and also related neighboring genes such as that for lymphotoxin alpha affect the risk of severe outcome in ocular *C. trachomatis* infection (Natividad et al. 2007).

2.3 Pathogenesis of *C. trachomatis* infection

The hallmark of an adverse outcome of genital and ocular *C. trachomatis* infection is fibrosis (Ramsey 2006), which is the ultimate reason for the fertility problems in genital infections and impaired sight in trachoma disease. In the Fallopian tube, *C. trachomatis* has a direct cytotoxic effect on the mucosa, resulting in loss of microvilli (Cooper et al. 1990). In acute infection the inflammation and edema associated with chlamydial salpingitis also reduce ciliary activity. However, the irreversible and permanent tubal damage is consequence of a host immune response to persistent or repeated infections (Lyons et al. 2006).

The pathologic consequences of infections are well established but the mechanisms that result in tissue damage are not fully understood. Persistent and repeated infections are key concepts in chlamydial pathogenesis. In women, *C. trachomatis* genital infection can persist for years (Molano et al. 2005) and during long-term inflammation tissue remodeling and scarring have time to progress.

2.3.1 Role of epithelial cells and the adaptive immune response

At present it seems clear that chlamydial pathogenesis is a consequence of the host immune response to infection. Along with adaptive responses, innate immunity mechanisms and infected epithelial cells are important factors in host response and pathogenesis (Debattista et al. 2003).

Infected epithelial cells initiate defense mechanisms by secreting chemokines that recruit inflammatory leukocytes to the site of infection (Rasmussen et al. 1997), plus dedicated innate immune cells such as monocytes, macrophages and neutrophils, and adaptive immune cells such as lymphocytes contribute to inflammation and pathogenesis (reviewed by Darville & Hiltke 2010). Infected host epithelial cells also serve as reservoir of infection (Stephens 2003). Infected epithelial cells release the cytokine IL-1, which stimulates additional cytokine secretion and augments the inflammatory response. A study by Hvid et al. demonstrated that in a Fallopian tube organ culture model IL-1 is the initiator of Fallopian tube destruction. The pathology could be prevented by using IL-1 receptor antagonist (IL-1RA), indicating a direct role of this cytokine in pathogenesis. In addition, immunosuppressive IL-10 was found to diminish the effect of IL-1 (Hvid et al. 2007).

Because the pathogenesis occurs during repeated or persistent infection (Hillis et al. 1997), *Chlamydia*-specific adaptive T cell responses are considered to be important players in the development of tissue damage (Brunham & Peeling 1994, Loomis & Starnbach 2002). It is paradoxical that the primary protective action mediated by T cells and CMI is also associated with pathology. A failed or weak Th1 cell response can result in persistent infection and pathology (Beatty et al. 1994a, Mittal et al. 1996), but an exaggerated or over-stimulated Th1 response can also mediate inflammation-driven pathogenesis (Morrison et al. 1989, Beatty et al. 1994a, Ault et al. 1996). Therefore, the immune response needs to be balanced and carefully regulated (Debattista et al. 2003). Treg and Th17 cells might also have a role in the disease process, but as yet it is unclear.

2.3.2 Response to heat shock proteins

Chlamydial HSP60 is a major immune target of both humoral and cell-mediated immune mechanisms (Persson et al. 1999, Kinnunen et al. 2002a) and might play a role in pathogenesis. Heat shock proteins (HSPs) are conserved proteins present in all prokaryotic and eukaryotic organisms and they are divided into four structurally related groups based on their molecular weights. HSPs function as chaperones in folding, assembly and translocation of newly synthesized or damaged proteins. They have important roles in cell survival and recovery from metabolic disturbances, reflected in increased HSP synthesis during diverse forms of stress, such as infection, inflammation and exposure to harmful chemical or environmental factors (Kinnunen et al. 2001, Borges & Ramos 2005). Stressful growth conditions such as nutrient depletion, the presence of penicillin or low levels of IFN-γ can induce incomplete

Chlamydia growth and organism persistence *in vitro* (Beatty et al. 1994a). In persistent stage, *Chlamydia* is in a nonreplicative and noninfectious but metabolically active form, which explains why the production of chlamydial HSP60 in comparison with other chlamydial proteins increases during persistent infection (Beatty et al. 1993, Gérard et al. 1998, LaVerda et al. 1999).

Elevated levels of chlamydial HSP60-specific antibodies have been found in individuals with severe disease outcomes. In both genital tract disease (Toye et al. 1993, Peeling et al. 1997) and trachoma (Peeling et al. 1998, Hessel et al. 2001), chlamydial HSP60 has been associated with pathology. Chlamydial HSP60 and human mitochondrial HSP60 share 50% sequence identity and cross-reactive antibody-binding epitopes from chlamydial HSP60 and human HSP60 have been identified (Yi et al. 1993). The cross-reactive epitopes, recognition of self-HSP and immune tolerance breakdown via an autoimmune mechanism have been hypothesized as disease mechanisms. However, the evidence does not support the hypothesis of an autoimmune mechanism (Stephens 2003). In addition, in *C. pneumoniae* infection and coronary artery disease it has been reported that serological reactivity to chlamydial HSP60 is an independent risk factor and independent of reactivity to human HSP60 (Mahdi et al. 2002).

According to recent research results, HSPs are natural immune regulators. HSP60 has the capacity to act as a self-antigen and a foreign antigen and it can mediate both proinflammatory and anti-inflammatory signals (Quintana & Cohen 2011). Self-HSP recognition can lead to an anti-inflammatory phenotype in T cells, where upregulated HSP in inflamed tissue can be target for HSP-specific regulatory T cells (Wieten et al. 2007). Self-HSP cross-reactive T cells are found to produce regulatory cytokines such as IL-10 (Zanin-Zhorov et al. 2006). In the case of chlamydial HSP60 immune responses, the regulatory T cell response has not been studied, but it is likely that chlamydial HSP60 and human HSP60 cross-reactive epitopes are recognized by regulatory T cells. In a study by Kinnunen et al., 30% of T cell clones isolated from salpingeal tissue of TFI patients recognized chlamydial HSP60 and when chlamydial HSP60-induced IL-10 and IFN-γ cytokine secretion was compared, 50% of T cell clones showed an IL-10-dominant response (Kinnunen et al. 2002a). By producing IL-10, HSP-reactive regulatory T cells may dampen the inflammatory response to *Chlamydia* and postpone infection resolution. On the other hand, it may also prevent excessive inflammation and subsequent tissue damage.

2.4 Risk factors of disease pathogenesis

Only some infected women develop late complications after *C. trachomatis* infection (Bakken & Ghaderi 2009). Several factors (host, pathogen and environment) affect the probability of infection transmission, persistence, ascension to the Fallopian tubes and finally tissue remodeling and scarring, reflecting the clinical course of the disease and the risk of long-term complications. In general, *C. trachomatis*-

induced fertility disorders in women result from tissue damage in the Fallopian tubes (Darville & Hiltke 2010). Therefore, factors that prevent ascension of bacteria to the Fallopian tubes lower the risk of tubal damage.

2.4.1 Pathogens and environmental factors

Factors such as infecting strain, normal flora, co-infections and hormonal status can affect infection transmission and bacterial ascension to the upper genital tract. Differences in virulence have not been consistently associated with specific *C. trachomatis* strains (Byrne 2010). Some serotypes seem to be more resistant to IFN-γ than others (Morrison 2000), which might influence virulence.

Normal vaginal flora plays a role in defense and commonly includes *Lactobacillus* spp., *Gardnerella vaginalis*, coagulase-negative staphylococci, *Enterococcus* spp., *Ureaplasma urealyticum* and *E. coli*. The lactobacilli metabolize glycogen released by vaginal epithelial cells to lactic acid, thus producing a low vaginal pH (3.5–5) (Quayle 2002). *In vitro* studies have demonstrated that this pH prevents the growth of *C. trachomatis* and thus may reduce the load of this pathogen in upper genital tract epithelium (Mahmoud et al. 1995).

Bacterial vaginosis, which is characterized by a shift in vaginal bacterial flora and elevated pH, is, together with *C. trachomatis* or *N. gonorrhoea* infection, associated with the development of PID (Paavonen et al. 1987). The altered microbial flora in cases of bacterial vaginosis often contains microbes that produce indole, such as *Bacteroides* species (DeMoss & Moser 1969), *E. coli* (DeMoss & Moser 1969, Han et al. 2011) and *Trichomonas vaginalis* (Lloyd et al. 1991). Because genital strains of *C. trachomatis* are able to metabolize tryptophan of indole (Fehlner-Gardiner et al. 2002), bacterial vaginosis might increase chlamydial resistance to host defense mechanisms (Batteiger et al. 2010). Co-infections with other pathogens can impair defense against *C. trachomatis*. *In vitro*, *C. trachomatis* enters a persistent state in HSV-co-infected cells (Deka et al. 2006), suggesting that disease severity might be increased in individuals with concomitant infections.

Hormones affect the mucosal epithelium (Patton et al. 2000) and therefore the phase of the menstrual cycle and oral contraceptive use can influence the transmission and spread of a pathogen. Prior to ovulation the cervical barrier is compromised, as the mucus covering the cervix changes from viscous material to watery fluid to allow sperm penetration (Quayle 2002). This might also allow infection to ascend to the upper genital tract. However, upper genital tract infection is rare and it is possible that the temporary permeability is balanced by increased synthesis of antimicrobial molecules in the periovulatory period (Eggert-Kruse et al. 2000). Contraceptive use has been associated with an increased risk of *C. trachomatis* infection (Kimani et al. 1996, Morrison et al. 2009) but also with a reduced risk of acute salpingitis (Wolner-Hanssen et al. 1985) and clinical PID (Kimani et al. 1996).

2.4.2 Genetic factors of the host

Host genetic factors probably play a role at all stages of infection from transmission to tissue repair and scarring (Morré et al. 2009). Diversity in genes that regulate the immune response, such as activation of CMI and IFN-γ production by CD4+ T cells, is likely to explain the interindividual differences in the course of infection. Diversity in genes that are involved in tissue repair and scarring processes also probably contribute to clinical manifestation of pathogenesis.

Genetically inherited factors affect to interindividual differences in susceptibility to and severity of infectious diseases. In the field of *Chlamydia* immunogenetics, Bailey et al. published a twin-study where the relative contribution of host genetics to the total variation of lymphoproliferative responses to *C. trachomatis* antigen was estimated. The study population consisted of 64 Gambian twin-pairs from trachoma endemic areas. Proliferative responses to serovar A EB antigens were measured in 19 monozygotic twin pairs and 45 dizygotic twin pairs. They found a stronger correlation and lower within-pair variability in the response in monozygotic compared with dizygotic twin pairs. The heritability estimate of variation in immune response was 0.39, suggesting that host genetic factors contributed almost 40% of the variation (Bailey et al. 2009).

Several genetic association studies related to *C. trachomatis* genital and ocular diseases have been published (Tables 1 and 2). Candidate gene approaches have been used to study the genetic risk factors of susceptibility to and severity of *C. trachomatis* diseases. The studies have dealt with immunological regulation of infection, concentrating on genes that code cytokines, PRRs, MHCs, which in humans are also called human leukocyte antigens (HLAs), and genes related to scarring and tissue repair such as those for matrix metalloproteinases. Immunogenetic studies related to *C. trachomatis* genital infections and ocular infections are listed in Tables 1 and 2, respectively. However, comparison of the results of these studies is problematic because the study settings differ from each other in terms of case definition, classification of disease severity and selection of the control population.

Mannose-binding lectin (MBL) low-producing genotypes have been associated with an increased risk of tubal pathology in two studies (Sziller et al. 2007, Laisk et al. 2010). Several HLA allele associations have been found in connection with infection susceptibility and disease complications. The HLA-DQB1*0602 genotype has been associated with *C. trachomatis* in two independent studies. This genotype is associated with disease susceptibility (Geisler et al. 2004) and with TFI (Kinnunen et al. 2002b). HLA-DQB1*06 has also been associated with recurrent *C. trachomatis* infection (Wang et al. 2005b).

In both ocular and sexually transmitted *C. trachomatis* infections the IL-10 coding *IL10* gene has been associated with disease. As regards STIs, the *IL10* -1082/-819/-592 GCC haplotype is associated with a lower risk of recurrent infection (Wang et al. 2005b) and the *IL10* -1082 AA genotype has been found to be more

common among TFI cases (37%) than in controls (25%). Together with HLA-DQB1*0602 the *IL10* -1082 AA genotype has been associated with TFI ($p = 0.005$) (Kinnunen et al. 2002b). In contrast, the *IL10* -1082 GG genotype has been associated with an increased risk of trachoma in ocular infection. The opposite associations with *IL10* -1082 SNP in genital and ocular infections might be due to differences in defense at different anatomical sites and differences in virulence of the STI and ocular strains. STI strains are more resistant to IFN-γ-mediated defense than ocular strains (Morrison 2000) and capable to synthesize tryptophan from indole provided by vaginal microbial flora (Caldwell et al. 2003, McClarty et al. 2007).

Although the *IL10* SNPs are associated with *C. trachomatis* disease, their roles in *C. trachomatis*-induced IL-10 production and regulation of the *C. trachomatis*-specific immune response has not been studied in detail. In addition, information concerning other cytokine polymorphisms that regulate IFN-γ-mediated defense and interactions in the cytokine network is lacking.

Table 1. Immunogenetic association studies on *C. trachomatis* genital infection.

Gene	Polymorphism	Study population	Findings	Reference
CCR5 *TLR2* *TLR4* *MBL2*	Delta32 deletion (wt/del) 2477 A/G (Arg753Gln) 1187 A/G (Asp299Gly) 1487 C/T (Thr399Ile) -550 C/G (H/L) -221 C/G (X/Y) +4 A/G (P/Q) Codon 52 C/T (Arg52Cys) Codon 54 A/G (Gly54Asp)	163 women with tubal factor infertility and 400 controls.	*CCR5*, *TLR2* and *TLR4* genotypes were not associated with tubal factor infertility. *MBL2* low-producing genotypes were more common among TFI women with *C. trachomatis*-specific antibodies than in controls (p = 0.025). Low-producing *MBL* genotypes were more common among women with a history of infection by at least two different genital tract pathogens compared with patients with no such history (p = 0.001).	(Laisk et al. 2010)
microRNA-146a *NLRP3*	rs2910164 C/G rs4925663 C/T rs12065526 A/G	Cohort 1: 318 Dutch women attending an STD clinic. Cohort 2: 277 subfertile and 184 healthy Finnish women.	In the Dutch cohort, *NLPR* rs12065526 genotypes AG and AA were more likely to develop symptoms (OR = 2.9, p = 0.047) than women with the GG genotype. In the Finnish cohort, significant differences in genotype distribution of the studied SNPs were not found between cases and controls, or as regards the degree of tubal pathology.	(Wang et al. 2009)
TLR4	+896 A/G	STD cohort: 614 Dutch Caucasian women visiting an STD clinic. Subfertile cohort: 227 Dutch Caucasian women.	The studied SNP was not associated with susceptibility to infection or tubal pathology.	(den Hartog et al. 2009)

Gene	Polymorphism	Study population	Findings	Reference
TLR2	-16934 A/T 2477 A/G (Arg753Gln)	Dutch Caucasian women, STD cohort of 468 subjects, 56 women with tubal pathology and 321 controls.	Individually analyzed SNPs were not associated with susceptibility to disease. Haplotype -16934/2477 TG was associated with protection against tubal pathology following *C. trachomatis* infection (OR = 0.28, p = 0.015).	(Karimi et al. 2009)
MBL2	Codon 54 A/G (Gly54Asp)	Hungarian Caucasian women: 97 subjects with occluded and 104 subjects with patent Fallopian tubes.	Women with tubal occlusion who also had *C. trachomatis*-specific antibodies had the highest rate of variant *MBL2* Codon 54 allele A carriage (p < 0.001)	(Sziller et al. 2007)
TLR9 *TLR4* *CD14* *CARD15/NOD*	-1237 C/T +2948 A/G +896 A/G -260 C/T 3124:3125 -/C (Leu1007fsinsC)	Dutch Caucasian women: 227 subfertile women of whom 43 had tubal pathology, and 184 controls with patent Fallopian tubes.	No statistically significant associations. Carriage of two or more minor alleles of studied SNPs increased the risk of tubal pathology in subfertile women (p = 0.15).	(den Hartog et al. 2006)
CCR5	Delta32 deletion (wt/del)	256 subfertile Dutch Caucasian women, of whom 50 had severe tubal pathology, and 145 healthy Dutch Caucasian controls.	In *C. trachomatis* IgG-positive patients tubal pathology correlated with a low incidence of *CCR5* delta32 deletion (7%). Subfertile women without tubal pathology had a higher incidence of *CCR5* delta32 deletion (31%) compared with controls (19%).	(Barr et al. 2005)
CD14	-260 C/T	Dutch Caucasian women: 576 women attending an STD clinic, 253 subfertile women and 170 controls.	No significant differences were found in genotype distribution between the cohorts.	(Ouburg et al. 2005)

Gene	Polymorphism	Study population	Findings	Reference
IL2 *IL4* *IL6* *IL10* *IL12B* *TNF* HLA class I HLA class II	-330 G/T +160 G/T 1902 A/G -174 C/G +565 A/G -1082/-819-592 GCC/ACC/ATA -3575/-2763 TC/TA/AC/AA 1188 A/C -308 A/G -238 A/G HLA-A, -B and C DRB1 and DQB1	485 North American 13–18-year old adolescents at high risk of sexually transmitted infections (76% female, 71% African American, 61% HIV seropositive).	The *IL10* -1082/-819/-592 GCC haplotype was associated with a lower risk of recurrent infection (RO = 0.59, p = 0.046). Women who did not carry this haplotype were found to have elevated IL-10 concentrations in cervical secretions compared with women with the GCC haplotype (p = 0.056). No other cytokine gene alleles or haplotypes were associated with recurrent *Chlamydia* infection. HLA-A*36 and DQB1*06 were associated with increased risks of recurrent *C. trachomatis* infection (RO = 2.56, p = 0.040 and RO = 1.80, p = 0.018). HLA-Cw*16 was protective against recurrent infection (RO = 0.42, p = 0.031).	(Wang et al. 2005b)
HLA class I HLA class II	HLA-A, -B and –C DRB1 and DQB1	485 Adolescents at high risk of sexually transmitted infection (74% female, 70% African American, 68% HIV-positive).	HLA-A*23 (p = 0.02), DRB1*15 (p = 0.01), and DQB1*06 were more frequent in subjects with *C. trachomatis* infection than in control subjects. Of the DQB1*06 alleles, the most common, *0602 and the next most common, *0603 had similar associations (p = 0.006 and p = 0.08, respectively).	(Geisler et al. 2004)
TNF *TGFB1* *IL10* *IL6* *IFNG* HLA class II	-308 A/G Codon 10 C/T Codon 25 C/G -1082/-819/-592 GCC/ACC/ATA -174 C/G +874 A/T DRB1, DRB3, DRB4, DRB5 DQA and DQB	63 Kenyan women with tubal factor infertility (31 MIF+ as cases and 32 MIF- as controls).	DRB1*1503 (OR = 0.05, p = 0.01) and DRB5*0101 (OR = 0.2, p = 0.05) were found less commonly among *C. trachomatis*-associated infertility cases. No significant associations in genotype distribution of studied cytokine SNPs between cases and controls.	(Cohen et al. 2003)

Gene	Polymorphism	Study population	Findings	Reference
IL1B *IL1RN*	-511 C/T +3954 C/T Variable number of identical tandem repeats of 86 bp, six alleles	Infertile Dutch Caucasian women. Cases with severe tubal pathology (n = 40) and controls without tubal pathology (n = 95).	No significant associations.	(Murillo et al. 2003)
TLR4	1187 A/G (Asp299Gly)	240 Dutch Caucasian subfertile women: 35 with tubal pathology and 49 with patent tubes.	Not significant.	(Morré et al. 2003)
IL10 HLA class II	-1082 A/G DQA and DQB	Finnish women: 52 subjects with tubal factor infertility and 61 blood donors as controls.	HLA-DQB1*0602 was higher in the cases than in the controls (corrected p = 0.04). *IL10* -1082 AA genotype was more common among TFI cases (37%) than in the controls (25%, not significant). HLA-DQA1*0102 and HLA-DQB1*0602 together with *IL10* -1082 AA genotype were found more frequently in the TFI cases than in the controls (0.18 and 0.02 respectively, p = 0.005).	(Kinnunen et al. 2002b)
HLA class II	DQA and DQB	Kenyan women: 47 subjects with tubal infertility and 46 fertile controls.	DQA*0101 (OR = 4.9; 95% CI 1.3–18.6) and DQB*0501 (OR = 6.8; 95% CI 1.6–29.2) alleles were associated with *C. trachomatis* tubal infertility. DQA*0102 was protective against tubal pathology (OR = 0.2; 95% CI 0.005–0.6).	(Cohen et al. 2000)

Table 2. Immunogenetic association studies on *C. trachomatis* trachoma infection.

Gene	Polymorphism	Study population	Findings	Reference
IL8 loci		651 Gambians with scarring trachoma and pair-matched controls with normal eyelids	Homozygotes for *IL8* -251 T allele had reduced risk of scarring trachoma (OR = 0.29, p = 0.027). The intronic *CSF2* rs27348 A allele was associated with protection against trichiasis (p = 0.005). Also, there was evidence of epistasis, with effects at *IL8* and *CSF2* loci interacting with those previously reported at the *MMP9* locus.	(Natividad et al. 2009)
AFP	+8865 A/G			
AFM	+1666 A/G			
	+4530 A/G			
	+15790 C/T			
IL8	-251 A/T			
	+396 G/T			
	+37674 C/T			
	+39739 A/G			
	+40050 A/C			
CSF2 loci				
IL3	rs2069783 C/T			
	rs31480 A/G			
	rs40401 C/T			
	rs31481 A/G			
CSF2	rs27348 A/T			
	rs2069614 C/T			
	rs27438 A/G			
	rs2069632 C/T			
IL13	rs20541 C/T			
	rs1295686 A/G			
	rs1800925 A/G			
IL4	-589 C/T			
	+33 C/T			
	rs2243251 C/T			
	rs2227284 A/C			
	rs2243270 C/T			

Gene	Polymorphism	Study population	Findings	Reference
34 genes: Proinflammatory cytokine genes Adhesion molecule genes Th1/Th2/Th3 and related genes Chemokines and related genes Miscellaneous	49 biallelic SNPs: 8 SNPs (*TNF*, *LTA*, *IL1A*, *IL1B*, *IL6*) 6 SNPs (*ICAM1*, *VCAM1*, *SELE*, *SELP*) 8 SNPs (*L4*, *IL4R*, *IL5RA*, *IL9*, *IL10*, *IL13*) 6 SNPs (*SCYA11*, *CCR2*, *CCR3*, *CCR5*) 21 SNPs (*ADRB2*, *FCER1B*, *CD14*, *TGF1B*, *UGB*, *TCF7*, *C3*, *C5*, *CSF2*, *CTLA4*, *LTC4S*, *NOS2A*, *NOS3*, *SDF1*, *VDR*)	538 Nepalese subjects: 232 controls, 204 cases with trachomatous inflammation and 135 cases with trachomatous trichiasis	*TNF* -308 A allele, *LTA* 252 A allele, *VCAM1* -1594 C allele, *SCYA11* 228 A allele (Ala→Thr) and *IL9* 361 T allele (Thr→Met) were associated with decreased risk of trachomatous trichiasis. *IL4R* 465 G allele (Ile→Val) and *ICAM1* 486 T allele (Lys→Met) were associated with increased risk. Multiple comparison problem was not discussed. Frequency of *TNF* -308 allele A was higher than described in literature.	(Atik et al. 2008)

Gene	Polymorphism	Study population	Findings	Reference
IL10	713 C/T (rs3024496)	Sequencing: 13 Gambians Allele-specific PCR: 23 subjects heterozygous for the risk allele.	The minor allele *IL10* 713 C of SNP in 3'UTR of *IL10* transcript sequence was strongly associated with high risk (H-RISK) haplotype (*IL10* -3575/-1082/-591/+5009 ACGG). *IL10* 713 C allele was used as genetic marker of *IL10* transcripts that arise from H-RISK haplotype. Conjunctival *IL10* transcripts bearing the C allele were expressed more than those bearing the T allele.	(Natividad et al. 2008)
TNF *LTA* *lκBL*	-238 A/G -308 A/G +77 G/T +252 A/G -63 A/T	1315 Gambians 651 with scarring trachoma, of whom 371 additionally had trichiasis	*TNF* -308 A allele and its bearing haplotype correlated with increased TNF production and was associated with increased risk of pathological infection sequelae. *lκBL* -63T allele was associated with increased risk of trichiasis. *LTA* +77T was associated with lower risk of trichiasis.	(Natividad et al. 2007)
MMP9	855 A/G (Gln279Arg) 1740 C/G (Arg574Pro) 1840 A/C (Gly607Gly) 2101 A/G (Val694Val)	Gambian population: 651 cases with scarring trachoma (307 additionally had trichiasis) and 651 pair-matched controls with normal eyelids.	Presence of *MMP9* 855 G allele was associated with reduced risk of trachomatous scarring (OR = 0.74, p = 0.012) and with reduced risk of more severe trachomatous trichiasis phenotype (OR = 0.66, p = 0.021).	(Natividad et al. 2006)
IL10 *IFNG* 	-3575 A/T -1082 C/T -592 G/T +5009 A/G -1616 C/T +2200 C/T +3234 C/T +5612 C/T	1315 Gambians including 651 subjects with scarring trachoma of whom 307 additionally had trichiasis.	Two *IL10* high-risk haplotypes for scarring trachoma and trichiasis were identified: -3575/-1082/-592/+5009 ACGC (OR = 1.25, p = 0.045 and OR = 1.72, p = 0.001 respectively) and ATGG (OR = 1.99, p = 0.030 and OR = 4.11, p = 0.007 respectively). Both haplotypes contained alleles -3575 A and +5009 G, associated with increased risk of scarring trachoma and trichiasis. One *IFNG* haplotype, -1616/+2299/+3234/+5612 TTCC was associated with increased risk of scarring trachoma (OR = 1.27, p = 0.020)	(Natividad et al. 2005)

Gene	Polymorphism	Study population	Findings	Reference
lKBa	-881 A/G -826 C/T -297 C/T Haplotypes: ACC, GTC, ACT and GTT	393 Gambians: 199 patients with scarring trachoma and 194 matched controls	Differences in genotype distribution were not significant between cases and controls. *lKBa* -881G and -826T alleles, which were in complete linkage disequilibrium with each other, showed significantly lower frequency in trachoma patients than in controls.	(Mozzato-Chamay et al. 2001)
IL10 *IL4* *TNF*	-1082 A/G -819 C/T -592 A/C -590 C/T -376 A/G	Gambian population: 238 cases with scarring trachoma and 239 controls with normal eyelids	In the Mandinka ethnic group, the *IL10* -1082 GG genotype was more common among cases than among controls (OR = 5.10, p = 0.009). No other significant associations.	(Mozzato-Chamay et al. 2000)
TNF	-308 A/G -238 A/G	Gambian population: 153 cases with scarring trachoma and 153 controls with normal eyelids	*TNF* -308 A allele was more common among cases than in controls (p for trend = 0.032). For -238 SNP the association was not significant.	(Conway et al. 1997)
HLA class I HLA class II	A, B and Cw DRB1 and DQB1	Gambian population: 153 cases with trachomatous scarring and 153 matched healthy controls.	HLA-A28 was more common among cases than controls (OR = 1.88, p = 0.046). In subtyping of A28 specificity, the A*6802 allele was significantly overrepresented among the cases (OR = 3.14, p = 0.009).	(Conway et al. 1996)

3 Aims of the study

The aim of this work was to identify immunogenetic factors associated with susceptibility to and severity of *C. trachomatis*-induced tubal damage and to investigate how disease-related genetic markers affect phenotype, i.e. cytokine production and *C. trachomatis*-specific immune responses.

Specific aims were:

1) To define how immunosuppressive IL-10 and its genetic polymorphisms at the promoter site affect *C. trachomatis*-specific cytokine production and intensity of the cell-mediated immune response (I and III).
2) To investigate the role of the IFN-γ-coding gene *IFNG* and other immune regulatory genes in susceptibility to and severity of *Chlamydia*-induced tubal damage (II).
3) To study the interaction of IL-10 and IFN-γ cytokines and their genetic polymorphism in relation to *C. trachomatis*-specific immune responses (III).
4) To study whether the IFN-γ expression-inducing cytokine IL-12 and its coding genes are associated with susceptibility to *C. trachomatis*-induced TFI or severity of tubal damage (IV).

4 Materials and methods

4.1 Study subjects

The study population included 164 TFI women (median age 33, range 23–40 years) with various degrees of tubal damage who attended the Infertility Clinic of the Department of Obstetrics and Gynecology, Helsinki University Hospital during 1990–2005 and had accurate laparoscopic evaluation of tubal damage. Of the 164 TFI cases 137 women had at least one positive immunological marker out of four (cell mediated immune response or antibodies to *C. trachomatis* or chlamydial HSP60) as an evidence of past *C. trachomatis* infection. The controls consisted of 176 female blood donors (median age 41, range 18–59 years) whose buffy coat specimens were provided by the Finnish Red Cross Blood Transfusion Service (Oulu, Finland) in 2002 and 179 pregnant women (median age 36, range 27–44) from the Helsinki area, samples from whom were provided by the Finnish Maternity Cohort (FMC) serum bank collected in 2006. Blood donors were chosen as controls because they represent normal population and their blood cells are available for CMI studies. Pregnant women were chosen as controls because they represent fertile women and they were matched to the cases by age and residence. Unfortunately cells for CMI analyses were not available from these subjects. The study population also included 17 women (median age 33, range 25–38) who were infertile for reasons other than TFI, such as male factor infertility, endometriosis, anovulation and unexplained infertility. These subjects were used in CMI analyses. A description of the study population subcohorts and their use in the original papers is shown in Table 3.

The study protocol was approved by the Ethics Committee of the Department of Obstetrics and Gynecology, University Hospital, Helsinki, and the Finnish Red Cross Ethics Committee. Permission to link hospital register data to FMC data was obtained from the Ministry of Social Affairs and Health. The use of FMC samples was approved by the Institutional Ethics Committee and the FMC steering committee.

4.1.1 Specimens

The specimen types available in each subpopulation are shown in Table 3. With informed consent, peripheral blood samples were obtained from the study participants and transferred to the laboratory at room temperature. The specimens from pregnant women were delivered to the laboratory in regular vacuum tubes with no anticoagulant and therefore PBMCs were not available and blood clots were used as a source of DNA.

Plasma was separated and stored at -20 ºC for antibody analysis. Peripheral blood mononuclear cells were isolated from heparinized blood by Ficoll–Paques

(Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation. The cells were washed three times with Hanks' balanced salt solution (Sigma, St Louis, MO, USA) and suspended in RPMI 1640 medium (Sigma) containing 10% heat-inactivated human AB serum (Finnish Red Cross, Helsinki, Finland) for LP assay and intracellular cytokine staining, or 5% fetal calf serum (FCS) for cytokine production measurement. The rest of the cells were suspended in 60% AB serum/RPMI medium and stored at -150 ˚C. The leukocyte fraction was collected and used as a source of DNA for the genetic studies.

Table 3. Characteristics of the study populations.

Sample cohort	Study	Source	Geographic location of subjects	Age	Period of sampling	n	Specimens	
							Serum	Cells
Tubal factor infertile women	I, II, III and IV	Infertility Clinic, Helsinki University Hospital	Helsinki	23–40	1990–2005	164	163	72
Female blood donors	I, II and III	Finnish Red Cross Blood Transfusion Service	Oulu	18–59	2002	176	46	176 (viable n=142)
Pregnant women	IV	Finnish Maternity Cohort (FMC)	Helsinki	27–44	2006	179	179	-
Disease controls: Infertile due to reasons other than tubal factor infertility	III	Infertility Clinic, Helsinki University Hospital	Helsinki	25–38	1999–2000	17	17	17

4.1.2 Definition of *C. trachomatis*-induced tubal damage

Tubal damage was evaluated laparoscopically. In Studies II and IV the severity of damage was categorized according to the classification of Hull and Rutherford (Rutherford & Jenkins 2002) into three groups. The categories were:

- Minor damage: proximal or distal tubal occlusion but no tubal distension and at most, flimsy adhesions.
- Moderate damage: unilateral sactosalpinx or moderate tubal adhesions.
- Severe damage: bilateral sactosalpinx or extensive adhesions.

The immunity status to *C. trachomatis* infection was analyzed in TFI cases by measuring both CMI and antibody responses to *C. trachomatis* EB and to CHSP60. If at least one of four markers was found positive the subject was considered to have a history of *C. trachomatis* infection.

4.2 DNA extraction

Leukocyte fraction, blood clots and serum were used as DNA source material. From leukocytes the DNA was extracted by a guanidine hydrochloride method (Studies I, II, III and IV). DNA was extracted from blood clots by using a MagNA Pure LC instrument (Roche Diagnostics) and a DNA Isolation Kit - Large Volume (Roche Diagnostics), following the DNA LV Cells Protocol, and from serum by using a Total Nucleic Acid Isolation Kit – Large Volume (Roche Diagnostics), following the Total NA/LV Serum Plasma protocol (Studies II, III and IV). The quantity of DNA in serum samples was low and contributed to genotyping failure in some samples.

4.3 Genotyping

4.3.1 Conventional PCR (I)

The *IL10* promoter gene polymorphism of a single nucleotide at position -1082 (A/G) was investigated by using a polymerase chain reaction (PCR) technique. Alleles were amplified in separate tubes with sequence-specific oligonucleotide primers, as published by Karhukorpi and Karttunen (Karhukorpi & Karttunen 2001). Polymerase chain reactions were performed in a total volume of 10 µl using ~ 100 ng template DNA, 1 × PCR buffer (Promega, Madison, USA), 1.5 mmol/l $MgCl_2$ (Promega), 20 mmol/l of each nucleotide (Promega), 0.5 U Taq polymerase (Promega) and 0.25 mmol/l of each primer. After initial denaturation at 95 ºC for 5 min, 30 cycles, consisting of denaturation for 30 s at 95 ºC and annealing for 30 s at 64 ºC were run. Final elongation was run for 6 min at 72 ºC. The PCR products were separated on 2% agarose gel and visualized under UV light illumination with ethidium bromide stain. The results were documented photographically (Kodak DC 120 Zoom).

4.3.2 Cytokine genotyping tray (II and III)

Genotyping of cytokine polymorphisms (*TNF* -308 G/A [rs1800629], *TGFB1* codons 10 T/C [rs1982073] and 25 G/C [rs1800471], *IL10* -1082 A/G [rs1800896], -819 T/C [rs1800871] and -592 A/C [rs1800872], *IL6* -174 G/C [rs1800795], *IFNG* +874 T/A [rs2430561]) was performed by PCR using a Cytokine Genotyping Tray (One Lambda Inc., Canoga Park, CA). Briefly, preoptimized primers, 19 µl of purified DNA, 1 µl of Taq polymerase (5 U/µl, manufactured for Applied Biosystems by Roche Molecular Systems Inc., Branchburg, New Jersey, USA) and buffer mix containing deoxyribonucleoside triphosphate were used in PCR along with positive and negative controls, following the manufacturer's instructions. The PCR products were separated on 2.5% agarose gel and visualized under UV light illumination with ethidium bromide stain. The results were documented photographically (Kodak DC 120 Zoom).

4.3.3 MALDI-TOF (IV)

IL12A and *IL12B* SNPs were investigated by the Sequenom system using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Bradic et al. 2011). Three SNPs of *IL12A* (rs2243123, rs583911 and rs568408) and seven of *IL12B* (rs3212227 i.e. 1188, rs1003199, rs11574790, rs919766, rs2569253, rs2853694 and rs6887695) genes were studied.

4.4 Immune responses

4.4.1 Antibody analysis

Serum IgG antibodies specific to *C. trachomatis* and CHSP60 were analyzed using peptide-based ELISAs (Medac Diagnostika, Hamburg, Germany) according to the manufacturer's instructions. Results were measured as absorbance at 450 nm, and OD > 0.4 was considered positive.

4.4.2 Antigens used for cell-mediated immune response analysis

Lymphocyte-stimulating antigens included EB antigens of *C. trachomatis* E and F serovars, representing the most prevalent inducers of *C. trachomatis* infection among the Finnish population. Lymphocyte proliferation (LP) assays were performed using formalin-inactivated *C. trachomatis* strain E and F EB antigens at a total protein concentration of 0.6 µg/ml. Recombinant CHSP60 (kindly provided by professor Richard Morrison) was used at a protein concentration of 0.5 µg/ml. In Study I, formalin-inactivated *C. pneumoniae* serovar K7 EB was used as control antigen at a total protein concentration of 0.6 µg/ml. Pokeweed mitogen (12.5 µg/ml PWM, Gibco, Paisley, UK) served as a control mitogen for LP analysis.

Cytokine measurement was performed using cell culture supernatants of PBMCs infected *in vitro* with viable *C. trachomatis* EB. Optimum numbers of chlamydial

EBs were determined for cytokine analysis in preliminary experiments with diluted EB stocks ranging from 2.0 to 0.002 IFU/ml.

4.4.3 Lymphocyte proliferation

The intensity of cell-mediated responses was analyzed by using conventional LP assays. Peripheral blood mononuclear cells were isolated from heparinized blood by Ficoll–Paques (Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation. The cells were washed three times with Hanks' balanced salt solution (Sigma, St Louis, MO, USA) and suspended in RPMI 1640 medium (Sigma) containing 10% heat-inactivated human AB serum (Finnish Red Cross, Helsinki, Finland) for LP assays and intracellular cytokine staining, or 5% FCS for cytokine production measurement.

LP responses were studied by stimulation of 5×10^4 PBMCs/well in round-bottomed 96-well plates in a total volume of 200 µl with and without antigen. The cultures were incubated in a humidified atmosphere of 5% CO_2 at 37 °C for 6 days, and [methyl-^{3}H]thymidine (0.2 µCi/well; Amersham Life Science, Buckinghamshire, UK) was added to the cultures for the last 18 h of incubation. The LP responses were measured as counts per minute (cpm) of incorporated [methyl-^{3}H]thymidine by using a liquid scintillation counter (Wallac, Turku, Finland), and the results were expressed as median stimulation indices (SI = median cpm in the presence of antigen divided by median cpm in its absence) of triplicate cultures. The viability and reactivity of the cultured PBMCs were controlled in each experiment by requiring SI > 10 in response to the control Pokeweed mitogen.

4.4.4 Cytokine production (I and III)

The cytokines IL-2, IL-4, IL-5, IL-10, IFN-γ and TNF in Study I, and IL-10, IFN-γ and TNF in Study III were measured in infected and uninfected culture supernatants of 10^5 PBMCs/well. Human Th1/Th2 Cytokine Cytometric Bead Array kits (BD Biosciences, San Diego, CA, USA) were used in Study I and Cytokine Bead Array Flex Sets kits (BD Biosciences) were used in Study III according to the manufacturer's instructions. The PBMC supernatant was collected after 2 days and stored at -80 °C until analysis. The samples were analyzed by using a flow cytometer (FACSCalibur, BD Biosciences) and the data was analyzed by using BD CellQuest and BD CBA software packages (Study I) and BD FCAP Array software (Study III). Cytokine concentrations are presented after subtraction of the background level (cytokine production by unstimulated cells).

4.4.5 Effects of IL-10 and IFN-γ on the proliferative response (I)

The effects of cytokines on LP were studied by adding recombinant human IL-10 (0.25 pg/ml), IFN-γ (1 pg/ml) (R&D Systems, Minneapolis, MN, USA), or anti-IL-10 neutralizing antibody (2.5 µl/1*10^6 cells) (clone JES3-9D7, Caltag, Burlingame,

CA, USA) to the PBMC cultures. Optimum concentrations of cytokines and neutralizing anti-IL-10 antibody were determined in preliminary experiments. Stimulation indices of infected cells (0.2 IFU/cell) were calculated in the presence and absence of cytokines or neutralizing anti-IL-10 antibody. The effect of an exogenous agent was expressed as a relative change of LP response to *C. trachomatis* infection in its presence compared with its absence.

4.4.6 Intracellular cytokine analysis (I)

Intracellular cytokine analyses were carried out on *in vitro*-infected or uninfected PBMC cultures using an immunofluorescence technique and BD Cytofix/Cytoperm kits (BD Biosciences). Brefeldin A containing BD Golgi-Plug solution was added to the PBMC cultures after 30 h of incubation to prevent the secretion of cytokines. After 10 h, the cells were stained for surface and intracellular antigens. The cell-surface antigens were stained with PerCP-conjugated anti-CD3, PE-conjugated anti-CD4 and FITC-conjugated anti-CD8 in the case of IFN-γ analysis and with PerCP-conjugated anti-CD3 and APC-conjugated anti-CD14 in the case of IL-10 analysis. After fixation and permeabilization the intracellular cytokines were stained with FITC-conjugated anti-IFN-γ or PE-conjugated anti-IL-10 monoclonal antibodies. All antibodies and appropriate isotype control antibodies were purchased form BD Biosciences. Cells were measured in a FACS-Calibur cytometer. Cell populations were gated according to their surface antigens and cytokine production was determined in different subpopulations as percentage of cells staining positively for IFN-γ and IL-10.

4.5 Statistical analyses

In Study I, Pearson's Chi-Square test was used to compare genotype distributions in cases and controls. The Mann–Whitney *U*-test was used to compare cytokine production and LP responses in different *IL10* -1082 genotype groups and Wilcoxon's signed-rank test was used to assess the effect of infection dose on the number of cytokine-producing cells and the effect of exogenous cytokine or neutralizing-anti IL-10 antibody on the relative change in *C. trachomatis*-specific LP responses. The analyses were performed by using SPSS for Windows 11.5 software (SPSS Inc., Chicago, IL, USA).

In Studies II and IV, a $\chi 2$ test for trend was used to assess genotype distribution trends in cases and controls, and in cases with different degrees of severity of tubal damage (SPSS 15.0 and 18.0 software). The two-sample test of proportions was performed to compare genotype frequencies between cases and controls (Stata 5.0 statistical software). Genotypes conferring susceptibility were presented in terms of odds ratios (ORs) with 95% confidence intervals (95% CIs). In Study II, Pearson's Chi-Square test was used to test for differences in immunological markers between cases and controls (SPSS 15.0 software). In Study IV, Haploview 4.2 software (Bar-

rett et al. 2005) was used to analyse the linkage disequilibrium (LD) between SNPs in *IL12A* and *IL12B* genes.

In Study III, a test for trend was used to analyze the associations between *IL10* and *IFNG* polymorphisms and the intensities of immune responses (Stata 5.0 statistical software). The Mann–Whitney *U*-test (SPSS 15.0 software) was used to analyze associations between two *TNF* genotype groups and immune responses.

5 Results

5.1 *C. trachomatis*-specific immune responses in the study population

Cell-mediated and humoral responses in different subcohorts of study populations are shown in Table 4. Serum IgG antibodies to *C. trachomatis* were 3- to 4-fold and IgG antibodies to chlamydial HSP60 were 3- to 5-fold more common among TFI cases than in other groups. Cell-mediated immune responses were also found at a significantly higher frequency among TFI cases than in other groups. Altogether, 26 TFI cases were not reactive to the studied markers and were left out of genetic susceptibility analyses due to lack of evidence of *C. trachomatis* as an inductive factor in the development of tubal damage.

Table 4. Humoral and cell-mediated *C. trachomatis*-specific immunity variables in different sample cohorts of the study population.

Immunity variables	Study population			
	Tubal factor infertile women n (%)	Female blood donors n (%)	Pregnant women n (%)	Disease controls* n (%)
IgG *C. trachomatis*				
Negative	69 (42.3)	39 (84.8)	145 (86.8)	13 (76.5)
Positive	94 (57.7)	7 (15.2)	22 (13.2)	4 (23.5)
IgG chlamydial HSP60				
Negative	73 (44.8)	41 (89.1)	135 (80.8)	14 (82.4)
Positive	90 (55.2)	5 (10.9)	32 (19.2)	3 (17.6)
SI *C. trachomatis* E				
Negative	19 (26.4)	77 (54.2)	N/A	7 (41.2)
Positive	53 (73.6)	65 (45.8)		10 (58.8)
SI *C. trachomatis* F				
Negative	16 (30.8)	73 (51.4)	N/A	10 (58.8)
Positive	36 (69.2)	69 (48.6)		7 (41.2)
SI CHSP60				
Positive	39 (54.2)	104 (73.2)	N/A	12 (70.6)
Negative	33 (45.8)	38 (26.8)		5 (29.4)

* Women infertile for reasons other than tubal factor infertility

SI: stimulation index

The intensity of response differed between TFI cases and controls. LP responses were similar in the studied serovars, *C. trachomatis* E and F, and the responses were higher among TFI cases (median response to E 16.9; 25–75% percentiles 5.2–49.5 and F 14.0; 4.4–36.7) than in blood donors (median response to E 3.7; 25–75% percentiles 1.1–21.5 and F 4.5; 1.3–24.8). The median LP response to chlamydial HSP60 antigen was 2.1 (25–75% percentiles 0.8–7.5) among TFI cases and 0.9 (0.4–2.7) among blood donors. Differences in all studied variables were statistically significant ($p<0.005$). In addition, levels of antibodies against both *C. trachomatis* EB and chlamydial HSP60 antigens were higher among TFI cases than in other groups (between TFI and pregnant women or female blood donors, $p<0.001$; between TFI cases and disease controls, i.e. women infertile for reasons other than TFI, $p<0.01$).

5.2 Cytokine polymorphisms and susceptibility to *C. trachomatis*-induced TFI

The association between selected polymorphisms and susceptibility to and severity of *C. trachomatis*-induced tubal damage was studied. In Study II the roles of *IL10* -1082, -819, -592, *IFNG* +874, *TNF* -308, *TGFB1* codons 10 and 25 and *IL6* -174 cytokine SNPs were studied among 114 laparoscopically verified TFI cases and in 176 female blood donors as controls.

In Study IV the roles of *IL12A* and *IL12B* genes were studied in an enlarged study population. The population consisted of 163 TFI cases and 179 pregnant women who served as controls. Altogether, 26 TFI cases did not have an immune marker of past *C. trachomatis* infection and were excluded. Due to a low quantity of DNA in some samples, genotyping failures accumulated in some subjects. The subjects with three or more missing SNP genotypes (54 controls and 37 TFI cases) were excluded from genetic association analysis and the final study population contained 125 controls and 100 TFI cases. The overall genotyping success as regards *IL12A* and *IL12B* SNPs in the final study population was 83.6–99.6% depending on the SNP.

In Study II 96 of 137 (84.2%) TFI cases and in Study IV 137 of 163 (84.0%) TFI cases had at least one immune marker (cell-mediated or antibody response to *C. trachomatis* EB or CHSP60) as evidence of past *C. trachomatis* infection and were included in the genetic susceptibility analysis. The genotype distribution of selected polymorphisms is shown in Table 5. Among the controls, all studied polymorphisms were in Hardy–Weinberg equilibrium (HWE), except for *IL10* -1082 ($p = 0.042$). Among the TFI cases two of the studied *IL12B* SNPs, rs11574790 ($p = 0.001$) and rs919766 ($p = 0.004$), which exhibited strong linkage disequilibrium (Figure 3), were not in HWE in the TFI cases.

The *IL6* -174 CC genotype was more common in the TFI cases than in the controls (36.8% vs. 24.4%, $p = 0.03$). Although the controls differed from HWE, the

IL10 -1082/-819/-592 ATA/ATA genotype was found more commonly among TFI cases than in controls (OR = 5.04, 95% CI 1.17–21.63).

A divergent genotype distribution was found between cases and controls in connection with the *IL12B* 1188 (p for trend = 0.028). The minor allele C was rare; among TFI cases no CC homozygotes were found. The AC genotype was more common in the TFI women than in the controls (p = 0.009) and was associated with an increased risk of TFI (OR = 2.44, 95% CI = 1.23–4.87).

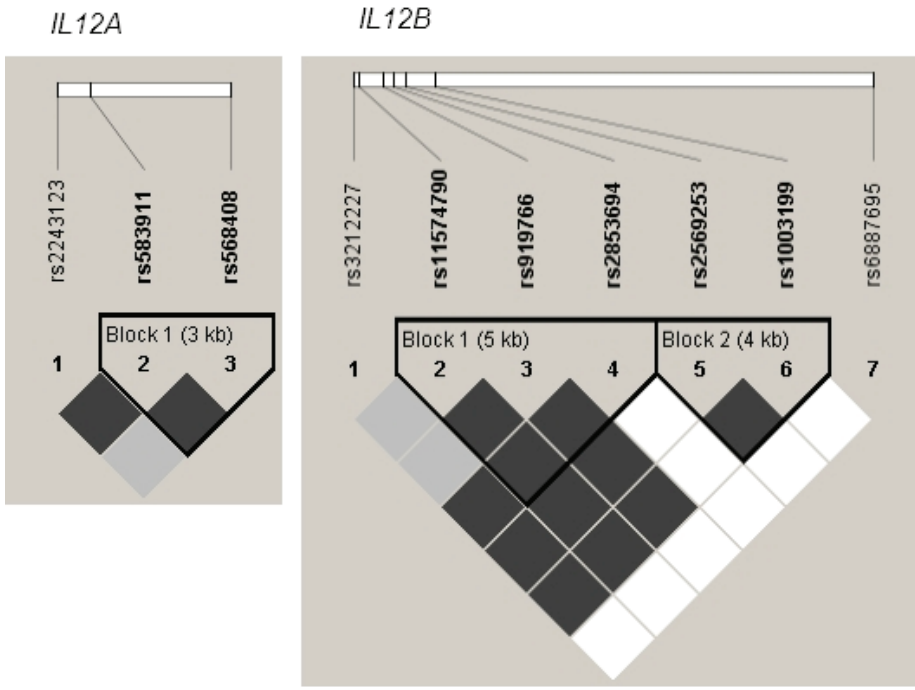

Figure 3. Linkage disequilibrium (LD) maps for the markers genotyped in the *IL12A* and *IL12B* genes created with Haploview program (Barrett et al. 2005). Black squares represent high LD; white squares represent low LD. Locations of single nucleotide polymorphisms are shown. Recall that rs3212227 is *IL12B* 1188.

Table 5. Distribution of cytokine genotypes in chlamydial TFI compared with controls. In Study II, the study population consisted of 96 cases with chlamydial TFI compared with 176 female blood donors as controls. In Study IV, the study population for genetic association analysis consisted of 100 TFI cases and 125 pregnant women as controls. The two-sample test of proportions was performed to compare the genotype frequencies between cases and controls. A $\chi 2$ test for trend was used to investigate a possible trend between disease and genotype. Genotypes conferring susceptibility are represented by odds ratios (ORs) with 95% confidence intervals (95% CIs).

Genotype	No. of genotypes (%)		Test of proportion, p value	$\chi 2$ for trend, p value	OR (95% CI)
	TFI women	Controls			
IL10 -1082/-819/-592 promoter (II)					
GCC/GCC (high)	19 (19.8)	41 (23.3)	0.505	0.053	Reference
GCC/ACC (intermediate)	32 (33.3)	60 (34.1)	0.900		1.15 (0.58-2.30)
GCC/ATA (intermediate)	17 (17.7)	42 (23.9)	0.239		0.87 (0.40-1.91)
ACC/ACC (low)	7 (7.3)	13 (7.4)	0.977		1.16 (0.40-3.38)
ACC/ATA (low)	14 (14.6)	17 (9.7)	0.222		1.78 (0.73-4.34)
ATA/ATA (low)	7 (7.3)	3 (1.7)	0.019		5.04 (1.17-21.63)
IFNG +874 intron 1 (II)					
TT (high)	12 (12.5)	21 (12.0)	0.904	0.773	Reference
TA (intermediate)	48 (50.0)	85 (48.6)	0.822		0.99 (0.45-2.18)
AA (low)	36 (37.5)	69 (39.4)	0.755		0.91 (0.40-2.06)
TNF -308 promoter (II)					
GG (low)	68 (70.8)	127 (72.2)	0.817	0.898	Reference
GA (high)	28 (29.2)	45 (25.6)	0.522		1.16 (0.67-2.03)
AA (high)	0	4 (2.3)	0.138		-
IL6 -174 promoter (II)					
GG (high)	22 (23.2)	42 (23.9)	0.896	0.154	Reference
GC (intermediate)	38 (40.0)	91 (51.7)	0.066		0.80 (0.42-1.51)
CC (low)	35 (36.8)	43 (24.4)	0.031		1.55 (0.79-3.07)
IL12B, 1188 3'UTR (IV)					
AA	66 (69.5)	89 (84.0)	0.015	0.028	Reference
AC	29 (30.5)	16 (15.1)	0.009		2.44 (1.23-4.87)
CC	0 (0)	1 (0.9)	0.343		-

5.3 Cytokine polymorphisms and severity of *C. trachomatis*-induced tubal damage

The TFI cases were categorized into three groups according to the severity of tubal damage, following the classification of Hull and Rutherford (Rutherford & Jenkins 2002). In Study II minor damage with proximal or distal tubal occlusion but no tubal distension and flimsy adhesions at most was found in 20 out of 96 (20.8%) cases with *C. trachomatis*-induced TFI, moderate damage with unilateral sactosalpinx or moderate tubal adhesions in 42 (43.8%) cases, and severe damage with bilateral sactosalpinx or extensive adhesions in 34 (35.4%) cases. In Study IV, minor damage was found in 24 of 100 (24.0%) cases, moderate damage in 41 (41.0%) cases, and severe damage in 35 (35.0%) cases.

The relationships between cytokine genotypes and the degree of tubal damage are shown in Table 6. The *IL10* -1082 A allele was associated with disease severity (p for trend = 0.03). The *IL10* -1082 AA and AG genotypes were associated with increased risks of moderate (OR = 5.33; 95% CI = 1.07–26.61 and OR = 2.67; 95% CI = 0.74–9.59 respectively) and severe (OR = 7.33; 95% CI = 1.27–42.29 and OR = 4.22; 95% CI = 1.00–17.80 respectively) tubal damage.

A trend towards increasing damage in the presence of the *TNF* -308 A allele was observed (p for trend = 0.036). The *TNF* -308 A allele was found in 15.0% of the cases with minor damage, 26.2% of the cases with moderate damage and in 41.2% of the cases with severe tubal damage. The *TNF* -308 GA genotype was associated with increased risks of moderate (OR = 2.01; 95% CI = 0.49–8.21) and severe (OR = 3.97; 95% CI = 0.97–16.16) tubal damage compared with women with milder disease. The *TNF* -308 A/G SNP was associated with both the severity of adhesions (p for trend = 0.025) and sactosalpinx (p for trend = 0.029).

The *IL12B* 1188 A/C SNP, which was associated with disease susceptibility was also linked to disease severity (p for trend=0.008) when compared with controls. The AC and CC genotypes were combined because of the small number of CC homozygotes. The subjects carrying allele C had increased risks of moderate (OR = 1.45, 95% CI = 1.06–5.95) and severe tubal damage (OR = 2.73, 95% CI =1.15–6.52) compared with pregnant women. When compared with minor damage a statistically significant risk was not found because of the small sample size (Table 6).

The distribution of the *TGFB* codon 25 genotype was not analyzed because the number of cases with a codon 25 C allele was small. The distribution of *IFNG* +874, *IL6* -174 and *TGFB1* codon 10 genotypes was similar among cases with different degrees of tubal damage. These polymorphisms were not associated with disease severity.

Table 6. Genotype distribution and risk estimates for TFI among women with different degrees of tubal damage. A $\chi 2$ test for trend was used to analyze the relationship between genotype and disease severity. Genotypes conferring susceptibility are represented by odds ratios (ORs) with 95% confidence intervals (95% CIs).

SNP and genotype (Study)		Severity of tubal damage (Hull and Rutherford)						$\chi 2$ for trend, p value	Minor vs. moderate OR (95% CI)		Minor vs. severe OR (95% CI)	
		Minor n (%)		Moderate n (%)		Severe n (%)						
IL10 -1082	GG	8	(40.0)	7	(16.7)	4	(11.8)	0.033	1		1	
(II)	AG	9	(45.0)	21	(50.0)	19	(55.9)		2.67	(0.74-9.59)	4.22	(1.00-17.80)
	AA	3	(15.0)	14	(33.3)	11	(32.4)		5.33	(1.07-26.61)	7.33	(1.27-42.29)
IFNG +874	TT	3	(15.0)	5	(11.9)	4	(11.8)	0.348	1		1	
(II)	TA	10	(50.0)	24	(57.1)	14	(41.2)		1.44	(0.29-7.21)	1.05	(0.19-5.76)
	AA	7	(35.0)	13	(31.0)	16	(47.1)		1.11	(0.20-6.11)	1.71	(0.30-9.77)
TNF -308	GG	17	(85.0)	31	(73.8)	20	(58.8)	0.036	1		1	
(II)	GA	3	(15.0)	11	(26.2)	14	(41.2)		2.01	(0.49-8.21)	3.97	(0.97-16.16)
TGFβ1 codon 10	TT	12	(60.0)	17	(40.5)	12	(35.3)	0.147	1		1	
(II)	TC	7	(35.0)	19	(45.2)	18	(52.9)		1.92	(0.61-5.98)	2.57	(0.79-8.40)
	CC	1	(5.0)	6	(14.3)	4	(11.8)		4.24	(0.45-39.87)	4.00	(0.39-41.23)
IL6 -174	GG	5	(25.0)	10	(24.4)	7	(20.6)	0.729	1		1	
(II)	GC	7	(35.0)	14	(34.1)	17	(50.0)		1.00	(0.25-4.08)	1.74	(0.41-7.37)
	CC	8	(40.0)	17	(41.5)	10	(29.4)		1.06	(0.27-4.15)	0.89	(0.20-3.91)
IL12B 1188	AA	18	(67.6)	25	(67.9)	23	(65.7)	0.338	1		1	
(IV)	AC	5	(21.7)	12	(32.4)	12	(34.3)		1.73	(0.52-5.77)	1.88	(0.56-6.31)

5.4 Genetic variability in *C. trachomatis*-specific immune responses

To better understand the genetic associations with susceptibility to and severity of tubal damage we studied genotypes in relation to *C. trachomatis*-induced immune responses (Studies I and III).

5.4.1 Cytokine polymorphisms and *in vitro* cytokine production

In Study I the role of the *IL10* -1082 polymorphism on IL-10 secretion, and indirectly on secretion and balance of type 1 and type 2 cytokines was studied by measuring type 1 (IL-2, IFN-γ and TNF) and type 2 (IL-4, IL-5 and IL-10) cytokines in culture supernatants of PBMCs infected*in vitro* . The experiments were performed in connection with 33 individuals who represented different *IL10* -1082 genotypes (11 AA, 17 AG and 5 GG) and who were selected on the basis of their positive LP responses to chlamydial EB. *C. trachomatis* (0.002 IFU)-induced IL-4 and IL-5 responses did not significantly differ from background levels (<13.2 pg/ml for IL-5 and <5.5 pg/ml for IL-4). *C. trachomatis*-induced IL-2 production differed clearly from the background level, although the concentrations were relatively low (3.1–29.0 pg/ml for *C. trachomatis* and 0.5–20.3 pg/ml for background, $p<0.001$).

In Study III the roles of cytokine SNPs in quantitative secretion of IL-10, IFN-γ and TNF were evaluated by analyzing the secreted cytokines in culture supernatants of *C. trachomatis*-infected (0.01 and 1 IFU/lymphocyte) and uninfected PBMCs. The study population consisted of 139 women with no selection based on LP responses. *C. trachomatis* infection induced significantly greater secretion of each cytokine compared with spontaneous secretion from uninfected lymphocytes ($p<0.001$), and the secretion was dose-dependent. Levels of IL-10 and IFN-γ presented in the following analysis are those induced by 1 IFU/lymphocyte, with the cytokine levels in uninfected lymphocytes subtracted.

5.4.1.1 IL10 polymorphisms

In Study I we found that *C. trachomatis*-induced IL-10 secretion was stronger among subjects with the *IL10* -1082 GG genotype (median IL-10 production 12702 pg/10^6 monocytes) than in those with the AG (3782 pg/10^6 monocytes) or AA genotype (4437 pg/10^6 monocytes; $p<0.001$) and variation was greatest in connection with the AG genotype. IL-10-induced Th1 downregulation was reflected in production of proinflammatory cytokines and *C. trachomatis*-induced IFN-γ and TNF secretion was clearly stronger in subjects with the AA (median IFN-γ production 356 pg/10^6 lymphocytes and TNF 1740 pg/10^6 monocytes) genotype compared with those with the AG (86 pg/10^6 lymphocytes and 1356 pg/10^6 monocytes) or GG genotype (0 pg/10^6 lymphocytes and 0 pg/10^6 monocytes, respectively; $p<0.05$). Concentrations of IL-2 also tended to be higher with the *IL10* -1082 AA genotype

than in those with the GG genotype, although the difference was not statistically significant (Figure 4).

In Study III the *IL10* polymorphism study was extended to cover the *IL10* -1082/-819/-592 haplotype in a larger study population. *C. trachomatis*-induced IL-10 production was found to vary slightly between the subjects with different genotypes. *IL10* -1082/-819/-592 form three haplotypes: GCC, ACC and ATA. A linear trend between secreted IL-10 levels and the studied *IL10* haplo-genotypes suggested that the GCC haplotype elicits the strongest IL-10 production, the ACC haplotype intermediate production and the ATA haplotype the lowest production (p for trend =0.06) (Figure 5A).

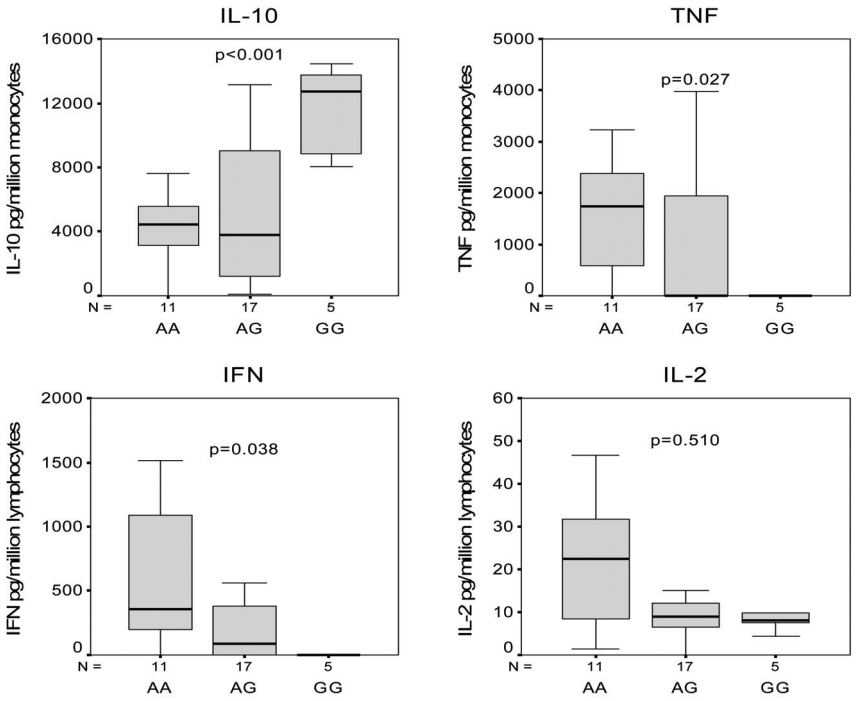

Figure 4. *C. trachomatis*-induced production of IL-10, TNF, IFN-γ and IL-2 cytokines (pg/1 × 10^6 cells) in subjects with *IL10* -1082 AA, AG and GG genotypes. Statistical differences between AA and GG genotype groups are shown.

5.4.1.2 IFNG and TNF polymorphisms

C. trachomatis-induced IFN-γ levels tended to be highest in the subjects with the *IFNG* +874 TT genotype (median 45.3 and 25–75% percentiles 22.9–308.0), intermediate in subjects with the TA genotype (53.3 and 14.8–199.5) and lowest in subjects with the AA genotype (30.8 and 6.7–122.2), although the trend was not statistically significant (p=0.06) (Figure 6A).

An association between the *TNF* -308 G/A genotype and spontaneous TNF secretion was detectable in cell culture supernatants of unstimulated cells (p=0.03). Secretion of TNF was higher in the women carrying the A allele (GA or AA genotype) than in the women with the *TNF* -308 GG genotype (median 68.6 pg/ml; 25–75% percentiles 26.7–125.6 vs. 29.2; 9.0–96.8 pg/ml; p = 0.031). TNF secretion increased after infection with 0.01 IFU/cell (GA or AA 132.9; 93.1–225.4 versus GG 97.8; 58.5–155.8 pg/ml; p=0.043) or 1 IFU/cell (GA or AA 634.0; 377.7–895.0 versus GG 679.2; 452.3–1134.6 pg/ml; p = 0.121), but linkage between TNF levels and *TNF* -308 genotypes was not detectable after subtracting the spontaneous secretion of TNF.

5.4.2 Cytokine polymorphisms and lymphocyte proliferative responses

The relationships between the cytokine SNPs and *C. trachomatis*-specific cell-mediated immune responses were analyzed in the subjects in the different genotype groups by using LP assays. Controls and cases were combined in analysis of associations between genetic factors and intensity of the immune response because the genetic effect was found to be parallel in all groups.

The *IL10* -1082/-819/-592 SNPs were associated with the intensity of LP responses to *C. trachomatis* serotypes E and F as well as to chlamydial HSP60 (Figure 5B). These SNPs form three haplotypes – GCC, ACC and ATA – and the *IL10* -819 and -592 SNPs are in perfect linkage disequilibrium, i.e. they form only two haplotypes, TA and CC. The *IL10* -1082 and *IL10* -819/-592 SNPs were analyzed separately to evaluate their contribution to regulation of LP responses. A significant association was found between LP responses to all chlamydial antigens and the *IL10* -1082 SNP (p<0.03), but not the *IL10* -819/-592 SNPs.

The relationship between the *IFNG* +874 (T/A) polymorphism and *C. trachomatis*-induced LP responses was less evident than that concerning the *IL10* polymorphisms. The LP responses to all antigens were greatest in the subjects with the *IFNG* +874 TT genotype and lowest among the subjects with the *IFNG* +874 AA genotype (Figure 6B). The trend was statistically significant as regards the *C. trachomatis* F antigen (p = 0.05). The *TNF* -308 polymorphism was not associated with the intensity of LP responses.

The combined effect of *IL10* and *IFNG* polymorphisms was analyzed to evaluate the interaction of these SNPs in *C. trachomatis*-specific cell-mediated responses. To enhance the power of statistical analysis, the subjects were sub-grouped into six

groups according to the *IL10* -1082 genotype and by the presence of a high- (TT or TA) or low-producing *IFNG* genotype (AA). As shown in Figure 7, subjects who are genetically predisposed to high IFN-γ and low IL-10 secretion (AA genotype) had the highest LP responses to the *C. trachomatis* antigens and, vice versa, the subjects who tend to produce high levels of IL-10 and low levels of IFN-γ had the lowest LP responses (p for trend: *C. trachomatis* E = 0.01, F = 0.01 and chlamydial HSP60 = 0.04).

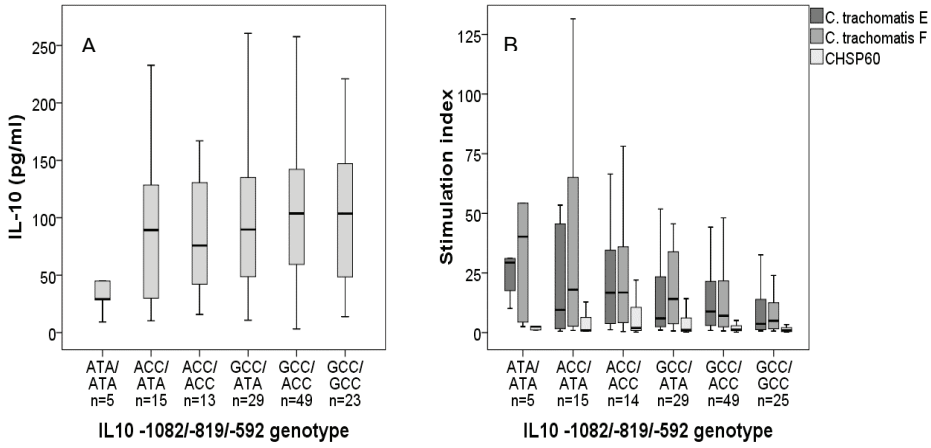

Figure 5. *IL10* -1082(A/G)/-819(C/T)/-592(C/A) polymorphism and *C. trachomatis*-specific immune responses. A) Production of IL-10 in different *IL10* genotype groups (p for trend = 0.06). B) LP responses to *C. trachomatis* E (p = 0.04), F (p = 0.01) and chlamydial HSP60 (p = 0.04) antigens in different *IL10* genotype groups.

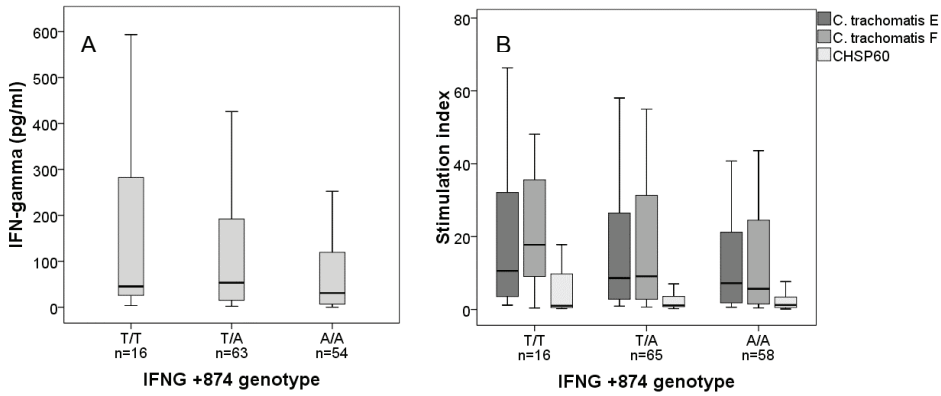

Figure 6. *IFNG* +874 (T/A) polymorphism and *C. trachomatis*-specific immune responses. A) Production of IFN-γ in different *IFNG* genotype groups. The cells were infected with 1 IFU/cell; p for trend = 0.06. B) LP responses to *C. trachomatis* E (p for trend = 0.13), F (p = 0.05) and chlamydial HSP60 (CHSP60) (p = 0.85) antigens in different *IFNG* genotype groups.

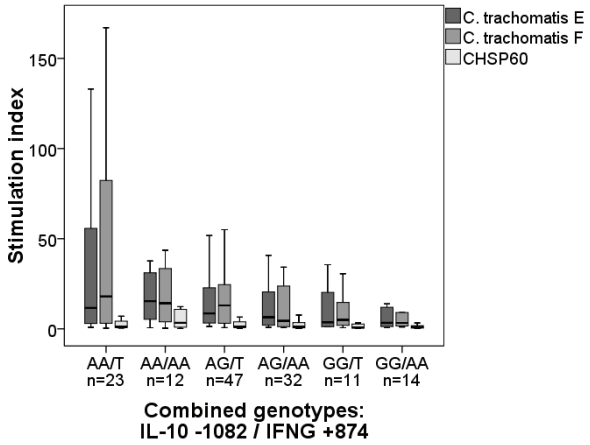

Figure 7. Combined effect of *IL10* and *IFNG* polymorphisms on proliferative responses to *C. trachomatis* E (p for trend <0.01), F (p<0.01) and chlamydial HSP60 (CHSP60) (p<0.04) antigens. The subjects were sub-grouped into 6 genotype groups according to the *IL10* -1082 genotype and by the presence of high- (1 or 2 copies of the *IFNG* +874 T allele) or low-producing *IFNG* genotypes (*IFNG* +874 AA).

5.5 *C. trachomatis* infection *in vitro* and biological characteristics of IL-10 and IFN-γ

5.5.1 IL-10- and IFN-γ-producing cells

In order to study the role of monocytes and CD4+ and CD8+ T cells as regulators of the immune response we analyzed intracellular IL-10 and IFN-γ production in *C. trachomatis*-infected cells by using an immunofluorescence technique and flow cytometry. Analysis of CD4+/CD3+ and CD8+/CD3+ T cells revealed that the median percentage of IFN-γ-secreting cells was higher among CD4+ (median 12.6%) than among CD8+ (median 4.8%) T cells (Study I). However, a statistically significant increase compared with uninfected cells was found in both T-cell subgroups.

A significant increase of IL-10-secreting CD14+ monocytes was detected in *C. trachomatis*-infected cells compared with uninfected cells (Study I), but there was only a marginal increase in IL-10-secreting CD3+ cells.

5.5.2 Influence of exogenous IL-10 and IFN-γ on *C. trachomatis*-specific cell responses

The influence of the cytokines IL-10 and IFN-γ on *C. trachomatis*-induced CMI responses was further studied by co-culturing PBMCs in the presence of recombinant IL-10 and IFN-γ. As expected, adding exogenous IFN-γ supported and adding exogenous IL-10 inhibited the LP responses (Figure 8). When IL-10 was blocked by adding anti-IL-10 antibody, LP responses increased in all genotype groups. The relative change in LP response increased most in the GG group, but because of the low number of subjects, it did not reach statistical significance. However, the responses remained lower than in the AA group.

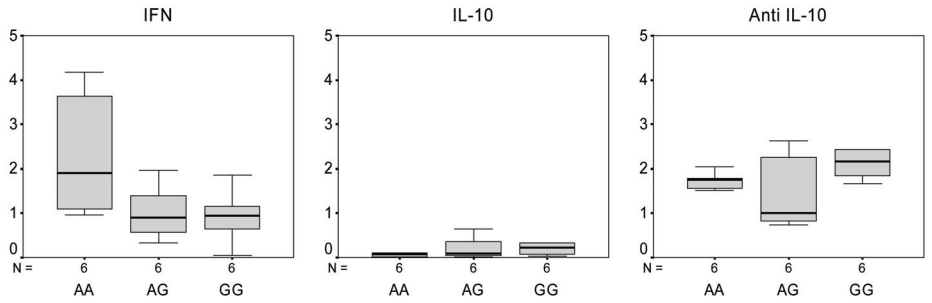

Figure 8. Relative change in PBMC proliferative responses to *C. trachomatis* infection in the presence of exogenous IFN-γ, IL-10 or IL-10 neutralizing anti-IL-10 antibody. The relative change is expressed as a ratio of the LP response to *C. trachomatis* antigen in the presence of exogenous agent to the LP response in its absence.

6 Discussion

The aim of this study was to identify immunogenetic risk factors of tubal damage and their functional effects on *C. trachomatis*-specific cell-mediated immune responses. The results show that variation in certain cytokine-coding genes is associated with disease susceptibility and also with interindividual differences in the intensity of cell-mediated immune responses.

6.1 Main findings

6.1.1 Effect of *IL10* polymorphisms (I and III)

Our studies showed that the *IL10* -1082 A allele increases the risk of severe tubal damage. This allele and the AA genotype have also been associated with susceptibility to TFI (Kinnunen et al. 2002b) and recurrent *C. trachomatis* infections (Geisler et al. 2004). IL-10 acts as a strong anti-inflammatory mediator (Moore et al. 1993) and its effect on proinflammatory cytokine production and the intensity of lymphocyte proliferation was also seen in our studies.

Of the *IL10* -1082, -819 and -592 polymorphisms studied in this work, the *IL10* -1082 SNP is most strongly associated with the intensity of LP responses against *C. trachomatis* antigens. The *IL10* -819/-592 SNPs play a minor role in the regulation of *C. trachomatis*-induced LP responses. Our results indicate that on the basis of *IL10* -1082 genotypes, *C. trachomatis*-induced immune responses could be divided into those with low IL-10 secretion and clearly positive LP responses, and those with high IL-10 secretion and diminished LP, IFN-γ and TNF responses. The central inhibiting role of IL-10 was confirmed by neutralizing IL-10 with specific anti-IL-10 antibody in cell culture. Enhanced lymphocyte proliferation responses were then observed in the cells from the study subjects and the increase was greatest among high IL-10 producers with the *IL10* -1082 GG genotype. Addition of recombinant IFN-γ was not able to prevent inhibition of LP responses, suggesting a dominant role of IL-10.

The influence of the *IL10* -1082 genotype on LP responses was similar with all studied antigens (*C. trachomatis* E and F, CHSP60 and *C. pneumoniae*) and among different study populations. Thus, the *IL10* -1082 genotype association with CMI response probably reflects the type of immune response in the presence of an intracellular microorganism. A similar association between *IL10* polymorphism and LP responses has been reported in connection with helminth antigen (Timmann et al. 2004).

6.1.2 Effect of *IFNG* +874 polymorphism (II and III)

Statistically significant association between *IFNG* +874 polymorphism and chlamydial TFI was not found. This might have been the result of small sample size. More cases with severe tubal damage had the genotype *IFNG* +874 AA than cases with milder damage. We also found that subjects with this *IFNG* +874 AA genotype tended to have lower LP responses compared with other *IFNG* +874 genotype groups and although not statistically significant, the quantitative secretion of IFN-γ was in line with the LP responses, the highest levels being observed in the subjects with the *IFNG* +874 TT genotype and the lowest levels in those with the AA genotype.

IFN-γ is necessary for protection against *C. trachomatis* infection (Rottenberg et al. 2002, Gondek et al. 2009) and deficiency in IFN-γ production is postulated to promote persistent *C. trachomatis* infection (Beatty et al. 1993). IFN-γ can limit *C. trachomatis* infection directly through a variety of mechanisms including induction of tryptophan catabolism and NO production (Loomis & Starnbach 2002), or indirectly by augmenting host immune responses (Boehm et al. 1997). Our results show that the indirect influence of IFN-γ is significant and higher IFN-γ levels among subjects with the *IFNG* +874 AA genotype are associated with enhanced intensity of the LP response compared with other genotype groups. IFN-γ upregulates the expression of MHC I and MHC II antigens, leading to enhanced presentation of microbial antigens to both CD4+ and CD8+ T cells (Boehm et al. 1997), and thereby an increased LP response.

Our results also fit in with the association between the *IFNG* +874 A allele and poor outcome in many intracellular infections, as described by others (Rossouw et al. 2003, Liu et al. 2006, Rasouli & Kiany 2007). The *IFNG* +874 A allele also lies within the disease-associated haplotype in trachoma, suggesting that less efficient IFN-γ production may be associated with trachomatous scarring (Natividad et al. 2005). We found a link between the *IFNG* +874 SNP and *C. trachomatis*-induced LP responses, but we did not find an association with disease or with IFN-γ production (p for trend = 0.06). This might be due to small sample size, but on the other hand, IFN-γ may also have a less important role in host defense during genital *C. trachomatis* infection than during ocular infections or many other intracellular infections, because genital *C. trachomatis* strains are less sensitive to IFN-γ-mediated defense (Morrison 2000). This is based on the capability of genital *C. trachomatis* strains to synthesize tryptophan from indole provided by vaginal microbial flora (Caldwell et al. 2003, McClarty et al. 2007).

6.1.3 Joint effect of *IL10* and *IFNG* polymorphisms (III)

According to our results, the combination of *IFNG* +874 and *IL10* -1082 genotypes has a cumulative effect on the intensity of *C. trachomatis*-induced LP responses. The subjects genetically predisposed to high IFN-γ (*IFNG* +874 TT or TA genotype)

and low IL-10 secretion (*IL10* -1082 AA genotype) had the highest LP responses to the *C. trachomatis* antigens and, vice versa, the subjects who tend to produce high levels of IL-10 and low levels of IFN-γ had the lowest LP responses. However, the *IL10* -1082 effect on LP responses was clearly stronger, indicating that genetic predisposition to abundant IL-10 secretion dominates over IFN-γ secretion in subjects with any *IFNG* +874 genotype.

The great variation in LP responses in subjects with a low IL-10- and a high IFN-γ-producing phenotype (Figure 7) suggest that these subjects have efficient IFN-γ-mediated protection when exposed to *C. trachomatis*. The results are consistent with those in our experiment in which addition of recombinant IFN-γ increased LP responses in low IL-10 producers but had little effect on the response in high producers (Figure 8).

6.1.4 Effect of *TNF* polymorphisms (II and III)

Chlamydia infection induces TNF secretion in human lymphocytes (Study I) and in Fallopian tube organ cultures (Ault et al. 1996). Because TNF is involved in fibrosis and tissue remodeling (Banno et al. 2004) and the presence of the *TNF* -308 A allele has been shown to increase the risk of scarring trachoma (Conway et al. 1997), this SNP was an interesting candidate as regards an association between *C. trachomatis* and TFI.

We found that carrying the *TNF* -308 A allele was associated with a risk of moderate and severe adhesions in TFI. However, when we studied the role of this SNP in *C. trachomatis*-induced cytokine production and LP response the results were ambiguous. In addition, the literature covering the role of the *TNF* -308 polymorphism in TNF production is inconsistent (Bayley et al. 2004) and the stimulus and studied cell type can affect TNF expression (Kroeger et al. 2000). Our results were somewhat discrepant, and infection dose-dependent. We found that cells from women carrying the A allele showed elevated levels of TNF in culture supernatants of unstimulated cells and in supernatants after infection with 0.01 IFU/cell, compared with women with the GG genotype. However, the association was not found after subtraction of spontaneous secretion of unstimulated cells, suggesting that *C. trachomatis*-induced TNF production is independent of the *TNF* -308 SNP, although this SNP is linked to the initial TNF level and could affect the development of the immune response at a very early stage of infection.

The involvement of TNF in pathology has been demonstrated in many inflammatory diseases (Clark 2007), in genital *Chlamydia* infection in mice (Perfettini et al. 2000) and in human trachoma (Conway et al. 1997). Although it is tempting to speculate that carrying the A allele could increase TNF secretion and thus increase the risk of severe tubal damage after *C. trachomatis* infection, the association between *TNF* -308 genotypes and TNF production needs to be studied further. In this investigation the sample size was relatively small for a quantitative study, consider-

ing that the A allele is uncommon – in our material there were only four AA homozygotes (Table 5). It is also possible that the association with disease manifestation is derived from a gene nearby that is linked to *TNF*, because the gene lies in an MHC III area which exhibits extensive linkage disequilibrium. Trachoma studies indicate that the *TNF* -308 A marker is part of a more complex picture. The *TNF* locus and also related neighboring genes such as that for lymphotoxin alpha affect the risk of severe outcome in ocular *C. trachomatis* infection (Natividad et al. 2007).

6.1.5 Effect of *IL12A* and *IL12B* polymorphisms (IV)

We found that one of the studied IL-12-related SNPs, *IL12B* 1188 A/C, was associated with TFI and the severity of tubal damage. This SNP is recognized as a susceptibility correlate in many inflammatory conditions from infections to autoimmune diseases (Cargill et al. 2007, Nair et al. 2008, McGovern et al. 2009, Phawong et al. 2010, Wang et al. 2010). The other SNPs studied were not associated with susceptibility to TFI or severity of tubal damage.

C. trachomatis infection increases endocervical IL-12 production *in vivo* (Wang et al. 2005a), indicating that IL-12 has an active role in the immune cascade provoked by *C. trachomatis*. The SNP *IL12B* 1188 A/C has also been associated with recurrent *C. trachomatis* infections (Geisler et al. 2010). In particular, the minor allele C, which in our data increased the risk and severity of TFI, was associated with recurrent *C. trachomatis* infections. As repeated infections and continuous inflammation increase the risk of tubal damage, our findings are in agreement with those reported by Geisler et al. In contrast, in inflammatory autoimmune illnesses such as psoriasis and Crohn's disease, the common genotype AA has been linked to disease susceptibility (Cargill et al. 2007, Nair et al. 2008, McGovern et al. 2009).

The results suggest that *IL12B* is involved in the disease process, but more data is needed to investigate the mechanism behind the genotype association. Differences in *IL12B* may be reflected in individual variation in levels of IL-12 and IL-23 cytokines, which are composed of a common IL-12p40 and distinct IL-12p35 or IL-23p19 subunits, respectively. The IL-12 effect is mediated through the IFN-γ pathway necessary in defense against *C. trachomatis*. The effects of IL-23 are less well known, but it seems to play a key role in the induction of Th17 cells. These cells induce production of proinflammatory cytokines and might be responsible for the inflammation-driven pathogenesis, rather than the Th1 subset (Goriely et al. 2009). Besides effects mediated through IL-12 and IL-23, it is also possible that IL-12p40 has biological activity on its own, as it has been reported that IL-12p40 can act as an antagonist of IL-12 *in vitro* (Zhang & Wang 2008) and thus mediate an anti-inflammatory effect. This hypothesis is also supported by Müller-Berghaus et al. (Müller-Berghaus et al. 2004), who showed that elevated levels of IL-12p40 were associated with lower levels of IL-12p70.

Although the mechanism behind the connection is still unsolved, the disease associations and the literature (Cargill et al. 2007, Nair et al. 2008, McGovern et al. 2009, Geisler et al. 2010) suggest that *IL12B* 1188 allele C is linked with a weaker and allele A with a stronger inflammatory response. Subjects with the AA genotype seem to cope with *C. trachomatis* infection better than subjects who carry allele C. This might be a result of more efficient *Chlamydia* clearance, and also protective immunity. The results may explain some individual variations in the manifestations of *C. trachomatis* disease. Further studies dealing with *IL12B* polymorphism and the immune response are needed to reveal the mechanism behind the disease association and to increase understanding of the pathogenesis of the disease, which is needed in the design of new therapeutic strategies.

6.1.6 IL-10- and IFN-γ-producing cells (I)

We found that both CD4+ and CD8+ cells seem to secrete IFN-γ, which is in accordance with animal data (Stagg et al. 1998, Igietseme et al. 2002). The number of IL-10-secreting CD3+ T cells increased marginally following *C. trachomatis* infection. This is in line with the results of a study by Kinnunen et al. in which IL-10-secreting *C. trachomatis*-specific T cells were occasionally detected in inflamed salpingeal tissue (Kinnunen et al. 2002a). However, IL-10 secretion predominantly originated from CD14+ monocytes, suggesting that the role of IL-10-secreting T cells in the course of TFI pathogenesis is less important than that of CD14+ monocytes. Their influence is especially strong among subjects with the *IL10* -1082 GG genotype.

6.2 Methodological considerations and limitations of the study

6.2.1 Study population

The study population consisted of TFI-cases, female blood donors, pregnant women and women infertile for reasons other than tubal factor infertility. TFI cases were used in all studies and the case population was enlarged in 2006 from 75 to 164 subjects. The number of study participants was relatively small for genetic analysis. In addition, a larger study population would be needed to test the interaction of different candidate genes in the development of TFI. Collection of cases is difficult, a factor that restricts sample size.

In Papers I–III the controls were healthy blood donors from Oulu, Northern Ostrobothnia, while the cases were collected from Helsinki, Southern Finland. This is problematic in candidate gene analysis because of regional heterogeneity and different population histories of eastern and western Finns (Lappalainen et al. 2006, Jakkula et al. 2008). However, Studies I and III were focused on association between genetic markers and immune responses. In Study II TFI cases with different levels of tubal pathology were compared and genetic marker association between cases and controls was not highlighted. The genotype distribution did not differ significantly

between TFI cases and blood donors, with the exception of the *IL10* -1082 genotype. In addition, the data on genetic associations with LP and cytokine responses were parallel, suggesting that these sample cohorts could be combined in analysis of genetic associations with *C. trachomatis*-specific immune responses.

In Study III the association between genetic markers and immune responses was investigated in subjects in whom a history of past clinically confirmed *C. trachomatis* infection was not known. Because we wanted to avoid selection bias, subjects with viable cell samples were included in the study regardless of their immune responses to *C. trachomatis* antigens. Exposure to *C. trachomatis* infection is similar in different genotype groups but genetic differences affect the intensity of immune responses. In addition, the studied cytokines IL-10, IFN-γ and TNF are produced by innate immunity cells (monocytes, dendritic- and NK-cells) and genetic differences may become manifest independently of previous exposure. This setting, which includes previously unexposed subjects, can, however, bias the results towards low lymphocyte reactivity. *In vitro* studies of *Chlamydia* immunity have shown that the intensities of LP and IFN-γ responses correlate with the amount of circulating memory T cells (Halme et al. 2000, Kinnunen et al. 2003).

For the fourth study the control population was enlarged by obtaining samples from the Finnish Maternity Cohort (FMC). Samples from pregnant women from the Helsinki area were collected to improve the validity of the results. Unfortunately, cell samples for LP analysis were not available from the FMC.

The strengths of our study include accurate TFI case definition by way of laparoscopic evaluation (Hubacher et al. 2004) and uniform classification of the degree of tubal damage (Rutherford & Jenkins 2002). This is necessary when analyzing genetic associations in connection with disease severity. Moreover, use of a combined test of *C. trachomatis*-specific humoral and CMI in predicting chlamydial TFI (Tiitinen et al. 2006) enhances the validity of our study.

6.2.2 Selected gene polymorphisms and genotyping methods

The cytokines for candidate gene analysis were selected on the basis of current knowledge of their central roles in *Chlamydia* immunology. The SNPs were selected on the basis of information in the literature, and available methodology.

Candidate gene approach has both benefits and drawbacks. Studies using this approach tend to have rather high statistical power but are incapable of discovering new genes or gene combinations (Amos et al. 2011). The candidate gene association studies have also been criticized to yield questionable genotype-phenotype associations that fail to replicate in independent studies (Chanock et al. 2007). The lack of replication may be a result of the complex disease pathogenesis which includes numerous genes and co-existent environmental risk factors. However, reproducibility is essential for establishing the credibility of a genotype-phenotype association (Zhang et al. 2009). The reliability of candidate gene approach can be increased by

selecting SNPs or haplotypes whose functional significance is shown from the basis of the effect on *in vivo* phenotype and on function *in vitro* (Daly & Day 2001).

The genetic markers associated with *C. trachomatis* induced tubal damage could be used as diagnostic tools to determine an individual's risk to develop later complications. However, the genes and SNPs studied in this dissertation explain only partly the genetic variation in disease susceptibility, and in addition to genetics, factors such as co-infections and hormonal status affect disease manifestation. Therefore, enabling clinical use of such genetic tests would require further studies.

Leukocyte fraction, blood clots and serum were used as source material for DNA isolation. Cells and blood clots were abundant sources of DNA, but, as expected, the amounts of DNA were low and its quality worse in serum samples, which contributed to genotyping failure in some of the samples. Three different methods were used for genotyping. Conventional PCR and the use of commercial genotyping trays were laborious methods. The results were read independently by two persons. MALDI-TOF genotyping used for IL-12-related genes was fast and the analysis was run in Oxford.

In the control population, all studied SNPs were in HWE except for *IL10* -1082 in blood donors. We obtained identical results regarding this genotype using two different methods (conventional PCR and a cytokine genotyping tray). This suggests that systematic genotyping errors are unlikely. In addition, allele frequencies in the *IL10* promoter region, and *IFNG* +874 A/T, *TNF* -308 A/G and *IL6* -174 C/G polymorphism data in our controls were similar to data reported by others in healthy Finns (Karhukorpi & Karttunen 2001, Wang et al. 2001, Nuolivirta et al. 2009, Heiskanen et al. 2010, Rantala et al. 2011).

6.3 Future prospects

We showed that *IL10* promoter polymorphisms are associated with the disease process and the intensity of the cell-mediated immune response in *C. trachomatis* infection. The immune responses are regulated by a complex network of cytokines and our results indicate that individual differences in *IFNG*, *TNF* and *IL12B* genes probably also contribute to variation in the clinical outcome of the infection.

We found an association between the *IFNG* +874 SNP and the intensity of the *C. trachomatis*-specific cell-mediated immune response, although the differences between genotype groups were small. However, associations between the studied SNP and disease susceptibility or severity were not found. In the future it would be interesting to study other *IFNG* SNPs and to carry out haplotype analysis as well, because IFN-γ has a central role in defense against *C. trachomatis*. Other factors that regulate IFN-γ production are also of interest. In this work we found an association between the *IL12B* 1188 SNP and disease susceptibility and severity of tissue damage. In the future, studies dealing with this SNP and *IL12B* gene expression as well

as production of functional IL-12, IL-23 and IL-12p40 cytokines are needed to increase understanding of the mechanisms of pathogenesis.

The *IL12B* gene might be also related to the Th17 cell population, which is highly proinflammatory and could be involved in pathogenesis. In addition, involvement of regulatory T cells can result in persistent infection and lead to complications. The involvement of these cell populations in *C. trachomatis* pathogenetic mechanisms would be interesting to study further.

The use of genome-wide association (GWA) tests could identify new genetic variants involved in *C. trachomatis* pathogenesis. Because the immune response is regulated by a complex network of genes, it would be interesting to study also epistatic gene-gene interactions in disease pathogenesis. These studies, however, would require a larger study population. Gathering a large cohort of women with proven *Chlamydia*-related tubal pathology could be accomplished by collaboration between study groups, establishment of research networks and by combining different cohorts.

7 Conclusions

In this work we showed that the immunosuppressive cytokine IL-10 and its genetic polymorphism *IL10* -1082 A/G strongly influence *C. trachomatis*-induced cytokine production. Levels of IL-10 also reflected the production of proinflammatory IFN-γ and TNF cytokines as well as the intensity of the LP response.

We found that the *IL10* -1082 AA genotype and *TNF* -308 A allele were associated with the severity of tubal damage in women with *C. trachomatis*-induced TFI. The *IL10* -1082 AA genotype was associated with low IL-10 production. The *TNF* -308 A/G SNP also potentially contributes to the cytokine profile during *C. trachomatis* infection, but the results were discrepant and the role of this SNP needs to be studied further. An association between the *IFNG* +874 A/T SNP and disease severity was not found, but this SNP was associated with the intensity of the LP response, and the quantitative secretion of IFN-γ was in line with the LP responses. The highest IFN-γ levels and LP responses were found in the subjects with the *IFNG* +874 TT genotype and the lowest levels in those with the AA genotype.

The *IFNG* +874 A/T SNP has a cumulative effect together with the *IL10* -1082 A/G SNP on the intensity of *C. trachomatis*-induced LP responses, although the *IL10* -1082 A/G SNP clearly had more influence on the LP response. However, the central role of IFN-γ in defense is also based on its direct *C. trachomatis* growth-limiting actions including induction of tryptophan catabolism and NO production (Loomis & Starnbach 2002), which were not evaluated here.

One of the studied IL-12-related SNPs, *IL12B* 1188 A/C, was associated with TFI and the severity of tubal damage. Carriage of the minor allele C, which has been associated with recurrent *C. trachomatis* infections (Geisler et al. 2010), was associated with increased risk and severity of *C. trachomatis*-induced tubal damage. The results suggest that *IL12B* is involved in the disease process, but more data is needed to investigate the mechanism behind the genotype association.

The results of this work increase understanding of disease mechanisms and support the hypothesis that polymorphisms in several cytokine-coding genes affect the risk and severity of TFI by contributing to the cytokine profile and host immune response during *C. trachomatis* infection. Our results suggest that the role of IL-10 is overwhelming relative to that of proinflammatory cytokines. IL-10 is needed to limit tissue pathology.

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