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Role of Inflammatory Mediators and their Genetics in Epstein-Barr Virus Infection, Febrile Seizures and Atopy

ACADEMIC DISSERTATION
To be presented, with the permission of
the Faculty of Medicine of the University of Tampere,
for public discussion in the Auditorium of Finn-Medi 1,
Biokatu 6, Tampere, on October 16th, 2009, at 12 o'clock.

UNIVERSITY OF TAMPERE

ACADEMIC DISSERTATION
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http://granum.uta.fi

Cover design by
Juha Siro

Acta Universitatis Tamperensis 1452
ISBN 978-951-44-7835-2 (print)
ISSN-L 1455-1616
ISSN 1455-1616

Acta Electronica Universitatis Tamperensis 885
ISBN 978-951-44-7836-9 (pdf)
ISSN 1456-954X
http://acta.uta.fi

Tampereen Yliopistopaino Oy – Juvenes Print
Tampere 2009

To my parents

Contents

List of original communications

This dissertation is based on the following original communications, which are referred to in the text by their Roman numerals (I-V).

I Virta M, Hurme M, Helminen M (2002): Increased frequency of interleukin-1β (-511) allele 2 in febrile seizures. Pediatr Neurol 26: 192-195.

II Virta M, Hurme M, Helminen M (2002): Increased plasma levels of pro- and anti-inflammatory cytokines in patients with febrile seizures. Epilepsia 43: 920-923.

III Helminen M, Kilpinen S, Virta M, Hurme M (2001): Susceptibility to primary Epstein-Barr virus infection is associated with interleukin-10 gene promoter polymorphism. J Infect Dis 184: 777-780.

IV Pessi T, Virta M, Ådjers K, Karjalainen J, Rautelin H, Kosunen T, Hurme M (2005): Genetic and environmental factors in the immunopathogenesis of atopy: Interaction of *Helicobacter pylori* infection and *IL4* genetics. Int Arch Allergy Immunol 137: 282-288.

V Virta M, Pessi T, Helminen M, Seiskari T, Kondrashova A, Knip M, Hyöty M, Hurme M (2008): Interaction between *CD14*-159C>T polymorphism and *Helicobacter pylori* is associated with serum total IgE. Clin exp allergy 38: 1929-1934.

In addition, this dissertation contains unpublished data.

Abbreviations

APC	antigen presenting cell
bp	base pair (only with numbers)
cDNA	complementary deoxyribonucleic acid
CI	confidence interval
CNS	central nervous system
CSF	cerebrospinal fluid
DNA	deoxyribonucleic acid
EBV	Epstein-Barr virus
ETS	environmental tobacco smoke
FEV1	forced expiratory volume in one second
FS	febrile seizure
HIV	human immunodefiency virus
hIL-10	human IL-10
IBD	inflammatory bowel disease
IFN	interferon
Ig	immunoglobulin
IL	interleukin e.g. interleukin-1
IL1RN	IL-1 receptor antagonist gene
IM	infectious mononucleosis
IU	international units
LPS	lipopolysaccharide
mCD14	membrane CD14
NK	natural killer
NS	non-significant
OR	odds ratio
PAGE	polyacrylamide gel
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PEF	peak expiratory flow rate
PGE	prostaglandin E
R	receptor
RA	rheumatoid arthritis
Ra	receptor antagonist (e.g. IL-1Ra)
RFLP	restriction fragment length polymorphism
rhIL-4	recombinant human IL-4
s	soluble (e.g. sCD14)
SLE	systemic lupus erythematosus
SNP	single nucleotide polymorphism

SPT	skin prick test
Th	T helper cell
TLE	temporal lobe epilepsy
TLE+HS	temporal lobe epilepsy with hippocampal sclerosis
TLR	Toll like receptor
TNF	tumor necrosis factor
Treg	T regulatory cell
UNCT	undifferentiated carcinoma of nasopharyngeal type
UV	ultraviolet
vIL-10	viral IL-10

Abbreviations are defined at first mention in the abstract and review of the literature and used only for concepts that occur more than twice.

Abstract

Inflammatory reactions are mediated by several molecules including cytokines, which can be divided into pro-inflammatory and anti-inflammatory depending on their main inflammatory functions. Cytokines interact with many other immunomodulators like innate immunity receptors, including CD14, in a very complex network. These inflammatory mediators are essential for normal host defense against pathogens, but they also participate in the pathogenesis of diseases. Genetic variations in inflammatory mediator genes can alter the function of the gene thereby possibly affecting susceptibility to or severity of several diseases.

This study was undertaken in order to investigate the role of *Interleukin (IL)1B, IL4, IL10* and *CD14* gene polymorphisms in three clinical conditions in which inflammatory mediators have an important role: Epstein-Barr virus (EBV) infection, febrile seizures (FSs) and atopy.

Associations between *IL1B*-511C>T polymorphism and FSs were studied among Finnish FS patients. The *IL1B*-511C>T allele T carriage was significantly increased in 35 FS patients compared to 400 adult blood donors (P=0.03). Relationships between plasma cytokines and FSs were also analyzed in Finnish children. Increased plasma IL-1Ra levels (P=0.0005), IL-6 levels (P=0.005) and IL-1Ra/IL-1β ratio (P<0.0001) and were found in 55 children with FSs compared to 20 control children with febrile illness without convulsions. However, there was no statistically significant association between *IL1B*-511C>T polymorphism and plasma cytokine levels in FS patients (n=35).

The relationship between *IL10* promoter -1082A>G/-819C>T/-592A>C haplotype and early EBV infection was investigated in 1-15 year-old Finnish children (n=116) and in adult blood donors (n=400). In children *IL10* promoter haplotype ATA was associated with EBV seronegativity (P=0.035). However, in adult blood donors this haplotype was not associated with EBV seronegativity (P=0.98).

Associations between *Helicobacter pylori* seropositivity, *IL4*-590C>T polymorphism and sensitization measured by skin prick test (SPT) were studied in Finnish asthmatic (n=245) and non-asthmatic (n=405) adults. *H.pylori* seronegativity was associated with increased risk of SPT positivity in both asthmatic and non-asthmatic groups (OR 2.28 95%CI 1.35-3.85 and OR 1.59 95%CI 1.06-2.39 respectively). When subjects were further divided into subgroups according to the number of positive SPT results, the number of subjects with more than one postitive SPT reaction was lower in *H.pylori* seropositive group compared to seronegative in both asthmatics and controls (P=0.0005 and P=0.004). This association between *H.pylori* and sensitization

was not seen among subjects with only one postitive reaction in SPT. There was no association between *IL4*-590C>T polymorphism and sensitization in these populations. The *IL4*-590 allele T was related to dimished risk of *H.pylori* seropositivity, but only in asthmatics (OR 0.485 95%CI 0.287-0.819). Gene-environment interactions between *IL4*-590 polymorphism and *H.pylori* infection having effect on sensitization or serum total IgE were also analyzed in both asthmatics and non-asthmatics, but no interactions were found.

In Russian Karelian schoolchildren (n=264) associations between *H.pylori* seropositivity, *Toxoplasma gondii* seropositivity, *CD14*-159C>T polymorphism, *TLR4*+896A>G polymorphism and serum total IgE were investigated. In this population serum total IgE median was 76.1 IU/L (interquartile range 30.9-236.0). Serum total IgE levels were increased in *T.gondii* seropositive children compared to seronegative (P=0.036). *H.pylori* seropositivity was not associated with serum total IgE. *CD14*-159 and *TLR4*+896 polymorphisms did not have any effect on serum total IgE. However, a significant interaction between *H.pylori* seropositivity and *CD14*-159 allele T carrier status on serum total IgE was found (P=0.004). In this population *H.pylori* seronegative children who were *CD14*-159 allele T non-carriers had higher serum total IgE levels than allele T carriers whereas in *H.pylori* seropositive children allele T non-carriers had lower IgE levels than allele T carriers. No other interactions were found.

As a conclusion, it seems that *IL10* promoter haplotype may be associated with delayed EBV infection and *IL1B*-511 polymorphism with FSs, whereas *IL4*-590, *CD14*-159 and *TLR4*+896 polymorphisms do not seem to be associated with sensitization or serum total IgE according to our results. However, candidate gene studies are known to have many limitations such as conflicting and unreplicable results especially in small populations. In addition, a single polymorphism rarely makes a remarkable contribution to the susceptibility or severity of multifactorial diseases like EBV infection, FSs and atopy. From this point of view approaches taking into account other factors in addition to a single polymorphism, like gene-environment interactions, could be more relevant. Therefore the scope of our investigations was widened from a candidate gene approach to gene-environment interactions. According to our results *CD14*-159 polymorphism and *H.pylori* seem to have interaction which is associated with serum total IgE in Russian Karelian children whereas *IL4*-590 polymorphism did not interact with *H.pylori* on sensitization or serum total IgE in Finnish adults. However, even though gene-environment interactions may explain some of the conficting results of candidate gene studies, caution should be exercised, because the interpretation of gene-environment interactions is very difficult due to the complex nature of these interactions.

Tiivistelmä

Tulehdusreaktioiden välittäjäaineina toimivat monenlaiset molekyylit kuten sytokiinit, jotka voidaan jakaa tulehdusta aiheuttaviin pro-inflammatorisiin ja tulehdusta estäviin eli anti-inflammatorisiin sytokiineihin. Sytokiinit toimivat monimutkaisessa vuorovaikutusverkostossa monien muiden puolustusjärjestelmän välittäjäaineiden, kuten synnynnäisen immuniteetin reseptoreiden (esim. CD14), kanssa. Nämä tulehdusvälittäjäaineet ovat välttämättömiä puolustusjärjestelmän normaalille toiminnalle, mutta ne vaikuttavat myös monien sairauksien kehittymiseen. Näiden välittäjäaineiden geeneissä on variaatiokohtia, jotka saattavat muuttaa geenien toimintaa, mikä puolestaan voi vaikuttaa sairauksien puhkeamiseen tai vaikeusasteeseen.

Tämän työn tarkoituksena oli tutkia *Interleukiini*(*IL)1B*, *IL4*, *IL10* ja *CD14* geenien variaatiokohtien yhteyttä Epstein-Barr virusinfektioihin, kuumekouristuksiin sekä atopiaan, joissa kaikissa tulehdusvälittäjäaineilla on tärkeä rooli.

IL1B-511C>T polymorfian ja kuumekouristusten välistä yhteyttä tutkittiin suomalaisilla lapsilla. *IL1B*-511C>T alleeli T:n kantajuus oli merkitsevästi lisääntynyt 35 kuumekouristajalla verrattuna 400 aikuiseen verenluovuttajaan (P=0.03). Suomalaisilla lapsilla tutkittiin myös plasman sytokiinitasojen yhteyttä kuumekouristuksiin. Kuumekouristajilla (n=55) plasman IL-1Ra ja IL-6 tasot sekä IL-1Ra/IL-1β suhde olivat kohonneet verrattuna 20 lapseen, joilla oli kuumesairaus ilman kouristuksia (P=0.0005, P=0.005 ja P<0.0001). Kuumekouristajien (n=35) *IL1B*-511 C>T polymorfian ja plasman sytokiinitasojen välillä ei kuitenkaan löytynyt yhteyttä.

IL10 geenin promootterialueen -1082A>G/-819C>T/-592A>C haplotyypin ja varhaisen EBV infektion välistä yhteyttä tutkittiin suomalaisilla 1-15 vuotiailla lapsilla (n=116) ja aikuisilla verenluovuttajilla (n=400). Lapsilla *IL10* promoottorin haplotyyppi ATA liittyi EBV seronegatiivisuuteen (P=0.035), mutta aikuisilla verenluovuttajilla tätä assosiaatiota ei löytynyt (P=0.98).

Helicobacter pylori seropositiivisuuden, *IL4*-590C>T polymorfian ja Prick-testeillä määritetyn allergeeneille herkistymisen välisiä yhteyksiä tutkittiin suomalaisilla astmaa sairastavilla (n=245) ja astmaa sairastamattomilla (n=405) aikuisilla. *H.pylori* seronegatiivisuus oli yhteydessä lisääntyneeseen Prick-testi positiivisuuteen niin astmaa sairastavilla kuin sairastamattomilla (OR 2.28 95%CI 1.35-3.85 ja OR 1.59 95%CI 1.06-2.39). *H.pylori* seropositiivisten joukossa useammalle kuin yhdelle allergeenille herkistyminen Prick-testillä mitattuna oli vähäisempää kuin *H.pylori* seronegatiivisilla niin astmaa sairastavien kuin sairastamattomien ryhmissä (P=0.0005 ja P=0.004). *H.pylori* ei kuitenkaan vaikuttanut vain yhdelle allergeenille herkistymisen riskiin. *IL4*-

590C>T polymorfian ja herkistymisen välillä ei löytynyt yhteyttä tässä tutkimuksessa. *IL4*-590 alleeli T liittyi kuitenkin pienentyneeseen *H.pylori* seropositiivisuuden riskiin astmaatikoilla (OR 0.485 95%CI 0.287-0.819). Tässä tutkimuksessa analysoitiin myös geenin ja ympäristötekijän vuorovaikutusta, mutta *IL4*-590 polymorfian ja *H.pylorin* välillä ei löytynyt allergeeneille herkistymiseen tai seerumin kokonais-IgE tasoon vaikuttavaa vuorovaikutusta.

Venäjänkarjalaisilla lapsilla (n=264) tutkittiin *H.pylori* seropositiivisuuden, *Toxoplasma gondii* seropositiivisuuden, *CD14*-159C>T polymorfian, *TLR4*+896A>G polymorfian ja seerumin kokonais-IgE:n välisiä yhteyksiä. Näillä lapsilla seerumin kokonais-IgE:n mediaani oli 76.1 IU/L (kvartiiliväli 30.9-236.0). Seerumin kokonais-IgE tasot olivat korkeammat *T.gondii* seropositiivisilla lapsilla verrattuna seronegatiivisiin (P=0.036). *H.pylori* seropositiivisuudella sekä *CD14*-159 ja *TLR4*+896 polymorfioilla ei ollut vaikutusta seerumin kokonais-IgE tasoihin. *H.pylori* seropositiivisuuden ja *CD14*-159 alleeli T:n kantajuuden välillä löytyi kuitenkin vuorovaikutus, joka vaikutti seerumin kokonais-IgE tasoon (P=0.004). *H.pylori* seronegatiivisilla lapsilla, jotka eivät olleet *CD14*-159 alleeli T:n kantajia, seerumin kokonais-IgE tasot olivat korkeampia kuin alleeli T:n kantajilla, kun taas *H.pylori* seropositiivisilla lapsilla, jotka eivät olleet alleeli T:n kantajia, seerumin kokonais-IgE tasot olivat matalampia kuin alleeli T:n kantajilla. Tutkittujen tekijöiden välillä ei löytynyt muita vuorovaikutuksia, joilla olisi ollut yhteyttä seerumin kokonais-IgE tasoihin.

Väitöskirjan tulosten mukaan *IL10* promoottorialueen haplotyypillä saattaa olla yhteyttä primaariin EBV infektioon ja *IL1B*-511 polymorfialla kuumekouristuksiin. *IL4*-590, *CD14*-159 ja *TLR4*+896 polymorfioilla ei sen sijaan näytä olevan yhteyttä allergeeneille herkistymiseen tai seerumin kokonais-IgE tasoihin. Kandidaattigeenitutkimuksiin tiedetään kuitenkin liittyvän monia ongelmia kuten ristiriitaiset tulokset erityisesti pienissä aineistoissa. Tämän lisäksi yksittäisellä polymorfialla on harvoin merkittävää vaikutusta monitekijäisten tautien, joihin EBV infektiot, kuumekouritukset ja atopia kuuluvat, alttiuteen ja vaikeusasteeseen. Nämä seikat huomioden muut tutkimusmenetelmät, jotka tarkastelevat geenien lisäksi sairauteen liittyviä ympäristötekijöitä, voisivat olla merkityksellisempiä kuin kandidaattigeenitutkimukset. Tämän vuoksi väitöskirjan tutkimuksia laajennettiin kandidaattigeenitutkimuksista geenien ja ympäristön vuorovaikutus-tutkimuksiin. Venäjänkarjalaisilla lapsilla löytyi *CD14*-159 polymorfian ja *H.pylori* välillä merkittävä vuorovaikutus, joka näyttää vaikuttavan seerumin kokonais-IgE tasoihin, mutta *IL4*-590 polymorfian ja *H.pylorin* välillä ei puolestaan löytynyt vuorovaikutusta, jolla olisi ollut vaikutusta allergeeneille herkistymiseen tai seerumin kokonais-IgE:hen. Geenien ja ympäristön välistä vuorovaikutusta analysoivien tutkimusten tulosten arvioinnissa pitää kuitenkin ottaa huomioon se, että näiden tulosten tulkinta on erittäin vaikeaa vuorovaikutusten monimutkaisuuden vuoksi.

Introduction

Inflammation is a complex biological process that occurs in response to tissue injury, microbial or allergen exposure. Inflammation is characterized by rapid activation of leukocytes including monocytes, macrophages and neutrophils. The contact of antigen or allergen with antigen presenting cells (APCs), like dendritic and monocyte/magrophage lineage cells, induces an inflammatory response mediated by pro-inflammatory cytokines such as interleukin (IL)-1, IL-6, IL-12, interferon (IFN)-γ and tumor necrosis factor (TNF)-α. The immune response is controlled, for example, by the negative feedback mechanism of anti-inflammatory cytokines like IL-1 receptor antagonist (IL-1Ra) and IL-10.

Innate immunity receptors, such as CD14 and Toll like receptors (TLRs) are essential to the host defense against microbes. Engagement of lipopolysaccharide (LPS) or other microbial components with CD14 results via TLRs in activation of APCs and subsequent release of pro-inflammatory cytokines and other inflammatory mediators (Koppelman et al. 2003). In allergy, allergens are taken up by dendritic cells and presented to T cells, which triggers specific IgE production by B cells to that allergen. This reaction is encouraged by T helper (Th) type 2 cells. Repeated exposure to allergen leads to binding of the allergen to the allergen-specific IgE on the surface of mast cells resulting in mast cell degranulation and release of numerous mediators such as histamines, prostaglandins and cytokines that triggers a secondary inflammatory reaction (Cookson 2004).

Cytokines are signaling proteins participating in cell-cell communication. Usually they act in a paracrine fashion affecting the adjacent cells, but they also have an effect on more distant cells (endocrine function) or the producing cell itself (autocrine function) (Callard et al. 1999). Cytokines are involved in every aspect of inflammatory reactions. Assessment of the function of an individual cytokine is complicated because the role of the cytokine may vary depending on the cellular source, target and phase of the immune reaction. Numerous cytokines have been shown to have both pro- and anti-inflammatory functions (Commins et al. 2008).

Variations in cytokine levels have been associated with disease susceptibility and progression, but in many cases the results have been contradictory. Many factors like local production, timing of the sample taking and measurement method affect cytokine levels and therefore the genetic background of the host could be more relevant in disease associations. Studies of cytokines and their genetics suggest that some of the inter-individual differences in cytokine profiles could be explained by allelic polymorphism within regulatory or coding regions of the cytokine genes (Bidwell et al. 1999).

There are several different types of polymorphisms in the genome including single nucleotide polymorphisms (SNPs), deletions, insertions and repeat polymorphism. SNPs are very common appearing in every 500-1000 base pairs in the human genome. Some of the SNPs alter the amino acids in the protein and some of them affect the protein indirectly, for example, by changing the function of regulatory sequences that control gene expression. Associations between SNPs and diseases have been widely analyzed (Hollegaard et al. 2006). However, the results have not been unambiguous.

Most common diseases like Epstein-Barr virus (EBV) infection, atopy and febrile seizures (FSs) studied in this dissertation have a multifactorial origin. Therefore a single polymorphism can explain only a fraction of the etiology of these diseases, which makes candidate gene studies challenging and quite often the results cannot be repeated (Zhang et al. 2008). It seems that environmental factors strongly influence the associations between single SNPs and diseases. For example, the same allele can be associated with either increased or decreased risk of disease depending on the environment the subject is exposed to (Vercelli 2006). Therefore the susceptibility or severity of disease may be better explained by interaction between genes and the environment.

In this dissertation inflammatory mediators and their genetics were studied in three different clinical conditions in which inflammatory mediators have an important role: EBV infection, FSs, and atopy. The first two studies focused on associations between polymorphisms and diseases. In the two last studies environmental factors and polymorphism were concomitantly investigated to ascertain the possibly gene-environment interactions having an effect on atopic phenotypes. Associations between FSs and pro- and anti-inflammatory cytokines were also analyzed.

Review of the literature

1. *Interleukin-1B* promoter polymorphism and febrile seizures

1.1. Interleukin-1

1.1.1. IL-1 family

The Interleukin-1 (IL-1) cytokine family was originally discovered independently in several institutes in the late 1970's, but the search for IL-1 was started as early as in the 1940's, when the factors causing fever were sought. The complementary deoxyribonucleic acid (cDNA) for human IL-1β and mouse IL-1α were cloned in 1984 (Dinarello 1996a). The so-called classic IL-1 family consists of two agonists IL-1α and IL-1β and of the specific IL-1 receptor antagonist (IL-1Ra). IL-18 has subsequently been accepted as the fourth member of the IL-1 superfamily, since its gene structure and tertiary protein structure are very similar to those of IL-1β and IL1-Ra (Bazan et al. 1996, Dinarello 2002). Recently seven other members of the IL-1 family (IL-1F5-10 and IL-33) have been identified by different research groups on the basis of sequence homology, three-dimensional structure, gene location and receptor binding. The exact functions of these novel members are still under investigation. The nomenclature of the IL-1 family has been revised and IL-1α, IL-1β, IL-1Ra, IL-18 and IL-33 are also known as IL-1F1, IL-1F2, IL-1F3, IL-1F4 and IL-1F11 respectively (Sims et al. 2001). An IL-1 receptor (IL-1R) family, which consists of at least nine receptors (IL-1R1-IL-1R9), has also been described. The function of some of these receptors is well known (e.g. IL-1R types I and II) whereas some are still under investigation (Sims et al. 2001, Dinarello 2002, Barksby et al. 2007).

1.1.2. Function of IL-1β

IL-1 is a pleiotropic inflammatory mediator. It affects nearly every cell type. There are two forms of IL-1 called IL-1α and IL-1β, which share a similar function profile. IL-1β is the mainly secreted form of IL-1 whereas IL-1α remains primary cell associated and acts as an intracellular transcriptional regulator (Dinarello 1996a). The principal cellular sources of IL-1β are

monocytes, specialized tissue macrophages like Langerhans cells, endothelial cells, mast cells, chondrocytes, alveolar and synovial macrophages, fibroblasts, astrocytes and glia cells (Rosenwasser 1998). Many proinflammatory mediators, like pathogen-associated molecule patterns (PAMPs) such as LPS, and proinflammatory cytokines, like TNF-α, IFN-α, IFN-β and IL-1β itself, stimulate production of IL-1 whereas cytokines with anti-inflammatory functions, like IL-4 and IL-10, and glucocorticoids have inhibitory effect on IL-1 production (Rothwell et al. 2000, Barksby et al. 2007).

IL-1β is primarily synthesized as an immature and inactive 31kDa protein called pro-IL-1β. Pro-IL-1β is cleaved to the 17 kDa active form intracellularly by IL-1β converting enzyme also known as caspase-1 (Dinarello 1996a). Caspase-1 is normally presented as an inactive precursor procaspase-1 in resting cells. It has been postulated that the initial stimulus, like LPS, causes large accumulation of pro-IL-1β in the cytosol. However, a second stimulus by extracellular adenosine triphosphate (ATP) via the P2X7R receptor causing procaspase-1 activation is needed for further IL-1β processing and secretion (Ferrari et al. 2006). IL-1β precursor can also be cleaved by some extracellular proteases like matrix metalloproteases 2 and elastase (Dinarello 2002).

The biological effect of IL-1β as well as IL-1α results from their ability to modulate gene expression in target cells. IL-1 has a variety of local and systemic effects. For example, IL-1 induces cyclooxygenase type 2, type 2 phospholipase A and inducible nitric oxide synthase. This accounts for the production of prostaglandin-E2 (PGE2), platelet activation factor and nitric oxide, which enhances inflammatory reactions. IL-1 also increases the expression of other cytokines, chemokines, adhesion molecules and vascular endothelial growth factor. It also acts as an adjuvant during antibody production and stimulates bone marrow stem cells for differentiation (Dinarello 2002). In central nervous system IL-1β participates, for example, in the production of fever, lethargy, slow-wave sleep and anorexia. IL-1β has also been found to promote oligodendrocyte cell death through glutamate excitotoxity (Rosenwasser 1998, Takahashi et al. 2003).

The effect of IL-1β and IL-1α is mediated by type I IL-1 receptor (IL-1RI). Binding of IL-1β (or IL-1α) to IL-1RI induces association of the receptor with IL-1 receptor-accessory protein (IL-1RacP), which initiates signal transduction events. The effect of IL-1 can be blocked by IL-1Ra binding to the IL-1RI. There is also type II receptor (IL-1RII), which binds IL-1α and IL-1β but does not induce signal transduction events (Colotta et al. 1993, Sims et al. 1993).

The systemic effects of IL-1β as well as IL-1α have been studied in animal models and also in humans. Intravenous injection of only a few hundred nanograms of IL-1β (or IL-1α) into humans causes chills, fever, hypotension, an increase in cortisol levels, a fall in serum glucose, an increase in adenocorticotropic hormone and thyroid stimulating hormone and a decrease in testosterone. IL-1 also stimulates production of other cytokines, like IL-6, which in turn induce the synthesis of hepatic acute phase proteins, like C-reactive protein (Dinarello 1998).

Due to its many biological effects, IL-1 has been shown to have a role in many diseases like rheumatoid arthritis (RA), inflammatory bowel disease

(IBD), cancers, atherosclerotic vascular disease, craft-versus-host disease, allergic diseases, psoriasis and central nervous system (CNS) degenerative diseases (Rosenwasser 1998, Rothwell et al. 2000).

1.1.3. IL-1Ra/ IL-1β ratio

Nearly all the cell types that produce IL-1α and IL-1β also produce IL-1Ra. This highly specific and naturally occurring receptor antagonism is quite unique in cytokine biology. After adequate stimulus, like LPS, plasma IL-1β levels have been seen to rise in a couple of hours followed by a peak of IL-1Ra levels a few hours later (Granowitz et al. 1991). 100-fold or greater levels of IL-1Ra over IL-1 are needed to inhibit the effects of IL-1 on target cells even though IL-1 and IL-1Ra have almost similar affinity to IL-1RI. It has been speculated that the need for excess IL-1Ra could result from high responsiveness to small amounts of IL-1 because maximal biological responses have been seen even when less than 5% of available receptors are occupied by IL-1 (Arend et al. 1990).

The delicate balance between IL-1 and IL-1Ra has an important role in the normal physiology of various organs and tissues including the CNS and the female reproductive system. This balance between IL-1 and IL-1Ra also has a profound effect on the pathogenesis of many inflammatory diseases like RA, IBD, kidney diseases, graft-versus-host disease, leukemia, osteoporosis, diabetes and arterial diseases (Arend 2002). Modification of impaired balance between IL-1/IL-Ra has provided a target for pharmacological intervention and for example recombinant IL-1Ra protein has been developed and proved to be efficient in treatment of RA (Arend 2002, Dinarello 2002).

1.1.4. IL1B gene polymorphisms

The *IL1* gene cluster including *IL1B* gene is located on the long arm of chromosome 2 (2q14) as seen in Figure 1. *IL1B* gene is about 7.0 kbp in length containing seven exons and six introns. The intron-exon organization of *IL1* gene complex genes suggests duplications of a common gene some 350 million years ago. *IL1B* regulatory regions are distributed over several thousand base pairs upstream and a few base pairs downstream from the transcriptional start site (Dinarello 2002, Barksby et al. 2007).

IL1B gene has several polymorphic sites. 83 SNPs have been listed in the *IL1B* gene region determined by NCBI (http://www.ncbi.nlm.nih.gov). However, many of these SNPs contain only one genotype, suggesting that these are artefacts of the database and thereby the number of SNPs in this gene area could be smaller. There are also many SNPs reported without allele frequencies and SNPs with very low minor allele frequency (<0.05). The structure of *IL1B* gene and the most studied polymorphisms, *IL1B*-511C>T (rs16944), *IL1B*-31T>C (rs1143627) and *IL1B*+3954C>T (rs1143633) are shown in Figure 1.

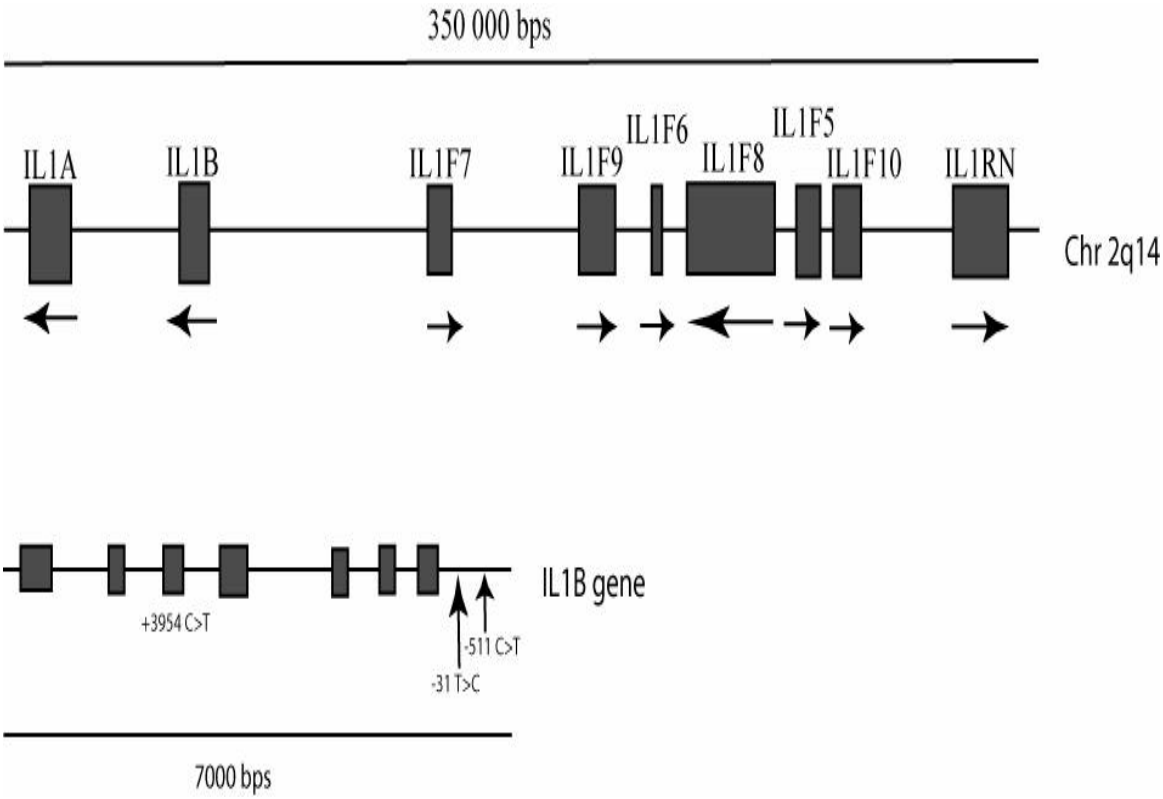
This dissertation focuses on *IL1B* promoter region C to T base-exchange polymorphism at position -511 from the transcription start site. This polymorphism was first described by di Giovine and colleagues in 1992 (di Giovine et al. 1992). The *ILIB*-511 polymorphism is in near-complete linkage disequilibrium with the TATA box polymorphism *IL1B*-31 in Caucasian population so that the *IL1B*-511 allele T is in linkage with the *IL1B*-31 allele C (El-Omar et al. 2000, El-Omar et al. 2001).

The exact biological role of *IL1B*-511 polymorphism is under investigation and the results have so far been somewhat confusing. The *IL1B*-511 allele T has been shown to increase the transcriptional activity more than allele C (Chen et al. 2006). The *IL1B*-511 TT genotype has been associated with higher gastric mucosa IL-1β levels compared to other genotypes in *H.pylori* positive Japanese adult population (Hwang et al. 2002). However, the *IL1B*-511 CC genotype has been associated with increased production of IL-1Ra and IL-1β in LPS stimulated PBMCs (Reich et al. 2002, Iacoviello et al. 2005). According to recent *IL1B* promoter haplotype studies, it seems that the functional role of *IL1B*-511 polymorphism may depend on the *IL1B* promoter region haplotype context. The *IL1B*-511 allele T has strongly enhanced transcription with the *IL1B*-31 allele C, whereas the enhancement was significantly lower in the context of *IL1B*-31 allele T (Chen et al. 2006). In addition, *IL1B* promoter haplotype -511T/-31C has been associated with 2-3 fold increase in LPS induced IL-1β secretion (Hall et al. 2004). However, another three locus haplotype -1470G/-511C/-31T have been shown to produce more IL-1β after LPS stimulation compared to -1470C/-511T/-31C haplotype. The former haplotype was also transcriptionally more active (Wen et al. 2006). *IL1B*-511 polymorphism may also have epistatic effects on protein production with other gene polymorphisms like *IL1RN* (Hurme et al. 1998, Hwang et al. 2002).

Associations between *IL1B*-511 polymorphism and diseases have been widely studied in recent years and over one hundred reports have been published. Associations have been found, for example, with Alzheimer's disease, asthma, chronic hepatitis C, gastric cancer, severe gastric inflammation, ischemic stroke, myocardial infarction, allergic rhinitis, EBV infection, Parkinson's disease, multiple sclerosis, bone marrow transplantation, schizophrenia, major depressive disorder, psoriasis, FSs, localization related epilepsy etc. (Hollegaard et al. 2006). The role of genotypes seems to differ in various diseases. For example, the *IL1B*-511 genotype TT has been associated with an increased risk for gastric inflammation whereas this genotype has been associated with decreased risk of ischemic stroke and myocardial infarction at young age (Hwang et al. 2002, Iacoviello et al. 2005). In several studies no associations between *IL1B*-511 polymorphism and diseases have been found (Hollegaard et al. 2006).

Figure 1. *IL1* gene cluster

The *IL1* gene cluster is located on the long arm of chromosome 2 (2q14). The arrows under the gene symbol indicate the direction of transcription. Exon - intron organization of *IL1B* gene is indicated in the picture and exons are shown as boxes. Information is based on Ensemble database (www.ensembl.org). Diagram is not to scale.



1.2. IL1B-511C>T polymorphism and febrile seizures

1.2.1. Febrile seizure

FS has been defined as a seizure occurring in childhood associated with febrile illness not caused by CNS infection, without previous neonatal or unprovoked seizures and not meeting the criteria of other acute symptomatic seizures. FSs are the most common type of convulsions in childhood affecting approximately 2-5% of children living in western countries. FSs usually occur between 6 months and 5 years of age with the peak incidence at 18 months (Waruiru et al. 2004, Fetveit 2008).

FSs have been classified as simple and complex. Simple FS has been defined as a self-limiting tonic-clonic seizure of short duration (usually less than 15 minutes), not recurring within 24 hours and without postictal pathology. Complex FSs have focal onset or focal features during seizure, are followed by neurological deficit, last over 15 minutes or recur during the same febrile illness within 24 hours. Most of the FSs are simple and the incidence of complex seizure is 9-35% of all FSs (Waruiru et al. 2004, Fetveit 2008).

Risk factors for the first FS are high fever and positive genetic background in first degree relatives. 25-40% of children having FSs have a positive family history of febrile convulsions (Berg AT et al. 1995). The rate of temperature rise has commonly been believed to be a risk factor for FS, but it has not been associated with FSs in every study (Berg AT et al. 1995, Waruiru et al. 2004). Other factors related to FSs are, for instance, daycare, exposure to passive smoking prenatally and immunization, but the results have been contradictory (Berg AT et al. 1995, Waruiru et al. 2004, Sillanpää et al. 2008). Both viral and bacterial infection causing fever can provoke FSs (Waruiru et al. 2004).

FSs are usually benign in nature. The risk of recurrence of febrile seizure is about 29-35%. Risk factors for recurrent FS are, for example, young age at the time of the first FS (<18 months), a positive family history of FS, relatively low fever during the first FS and short duration of fever before the first febrile seizure (Shinnar et al. 2002). The risk of developing epilepsy after simple FSs is 1-2.4% and 4.1-6% after complex FSs. Yet 10-15% of people with epilepsy or unprovoked seizures have a history of FSs. Risk factors for developing epilepsy after FSs are positive family history of epilepsy, complex features of FSs and the presence of early onset neurodevelopmental abnormalities (Waruiru et al. 2004, Fetveit 2008). It has been speculated that the associations between FSs and epilepsy may demonstrate a genetic link between these two diseases rather than a causal relationship, because the evidence of causality is not unambiguous (Waruiru et al. 2004, Fetveit 2008).

1.2.2. IL-1 and febrile seizures

Cytokines are important immunomodulators in CNS and they have been associated with FSs (Rothwell et al. 2000). However, the importance of fever-inducing cytokines, like IL-1, in FSs is disputed. Helminen and Vesikari first reported increased IL-1 production in LPS stimulated mononuclear cells isolated from FS patients (Helminen et al. 1990). This finding has been repeated in another study (Straussberg et al. 2001). Increased IL-1 production has also been seen in double-stranded ribonucleic acid stimulated leukocytes obtained from children with positive history of FSs (Matsuo et al. 2006). Elevated plasma IL-1β levels have been found in acute phase of FS, but cerebrospinal fluid (CSF) IL-1β levels were not associated with FSs in this study (Tütüncüoglu et al. 2001). Interestingly, in another study elevated CSF IL-1β levels were seen in FS children, but no association between plasma IL-1β levels and FSs was found (Haspolat et al. 2002). In some studies no associations between plasma or CSF IL-1β levels and FSs have been found (Lahat et al. 1997, Ichiyama et al. 1998, Tomoum et al. 2007).

The relationship between IL-1β and seizures has been studied in animal models. Expression of messenger RNA (mRNA) of many cytokines, like IL-1β, IL-6 and TNF-α, has been reported in the brain after kainic acid induced seizures (Minami et al. 1991). Intrahippocampally administrated kainic acid has been shown to induce IL-1β production in hippocampus whereas intrahippocampally administrated IL-1β increased the duration of kainic acid induced seizure activity and this effect was blocked by IL-1Ra (Vezzani et al. 1999). FSs have also been studied in experimental seizure model in mice with IL-1 receptor deficiency. The IL-1R deficient mice were more resistant to FS than wild type mice (Dube et al. 2005). In this study high IL-1β doses were able to induce seizures even without rise of temperature, but only in IL-1β receptor-expressing mice (Dube et al. 2005).

In addition to a putative role as a seizure inducing factor, IL-1β may also participate in the pathogenesis of FSs by regulating fever, which is the main trigger of FSs. IL-1β produced during infection triggers IL-1 receptors on the hypothalamic vascular network resulting in synthesis of cyclooxygenase type 2, which elevates brain PGE_2 levels leading to activation of the thermoregulatory center (Dinarello 1996b, Mackowiak et al. 1997, Davidson et al. 2001, Dinarello 2005). IL-1β can also cause fever by interacting with other pro-inflammatory cytokines, like IL-6 and TNF-α, which induce fever (Dinarello 1996b). Anti-inflammatory cytokines, such as IL-1Ra, may downregulate the effect of pro-inflammatory cytokines during the febrile response and therefore the balance between pro-and anti-inflammatory cytokines may contribute to the level of fever and could have a role in the pathogenesis of FSs (Opp et al. 1991, Miller et al. 1997, Fukuda et al. 2009). Hyperthermia itself may induce an excitatory effect in the brain especially in immature hippocampus (Schiff et al. 1985, Thompson et al. 1985, Moser et al. 1993, Liebregts et al. 2002, Baulac et al. 2004).

1.2.3. Associations between IL1B-511 and febrile seizures

Genetic predisposition to FSs has been shown in family and twin studies (Baulac et al. 2004, Nakayama et al. 2006). FSs most probably have multifactorial origin i.e. both genetic and environmental factors have a role in their pathogenesis. FSs have been reported to be linked to many genetic loci including 2q, 5q, 6q, 8q 18p and 19. However, it seems that simple sporadic FSs differ genetically from complex and familial FSs and most of the loci mentioned above do not have a role in simple FSs except for the chromosome 5 locus reported in Japanese population (Nakayama J et al. 2000, Waruiru et al. 2004).

Genes, like *IL1B*, encoding proteins involved in the regulation of inflammatory reaction and fever are also considered to be plausible candidate genes in the pathogenesis of FSs (Kauffman et al. 2008). Increased carriage of the *IL1B-511* allele T has been found in TLE patients with hippocampal sclerosis (TLE+HS) and in localization-related epilepsy patients (Peltola et al. 2001, Kanemoto et al. 2003). According to these results *IL1B*-511 polymorphism may have a role in the pathogenesis of convulsions. However, the results of recent association studies between *IL1B*-511 and FSs have been contradictory and in most studies no association has been found (Kauffman et al. 2008).

2. *Interleukin-10* promoter polymorphisms and Epstein-Barr virus infection

2.1. Interleukin-10

2.1.1. Function of IL-10

IL-10 is considered to be an anti-inflammatory multifunctional cytokine. It was first described as a cytokine synthesis inhibitory factor (CSIF) when the Th2 clones were shown to produce a factor that inhibited proliferation and cytokine production by activated mouse Th1 clones (Fiorentino et al. 1989). Human IL-10 cDNA was demonstrated in 1991 (Vieira et al. 1991). IL-10 is produced by many cells such as T cells, B cells (especially EBV infected or CD5+B cells), monocytes and keratinocytes (O'Garra et al. 2008).

IL-10 has an important role in the regulation of immune responses and affects many cell types. For example, IL-10 inhibits cytokine production and proliferation of T cells responding to antigens and IFN-γ production by natural killer (NK) cells. Most of the inhibitory effect of IL-10 on T cell cytokine production seems to be caused indirectly via suppressing crucial antigen-presenting cell (ACP) functions. IL-10 is able to down-regulate HLA II expression and antigen presentation of APC. IL-10 also inhibits, for example, the expression of CD80 and CD86 surface molecules on APCs. These molecules are

ligands for CD28 and CTLA4 on T cells and mediate co-stimulatory signals affecting T cell activation (Moore et al. 2001). IL-10 has also been shown to have a direct inhibitory effect on T cell activation by suppressing the expression of the T-cell co-stimulatory molecules CD28 and ICOS (Taylor et al. 2007). Additionally, IL-10 inhibits a number of inflammatory functions of monocytes and macrophages by inhibiting the synthesis of many cytokines (e.g. IL-1, TNF-α, IL-6, IL-10 itself), chemokines and PGE2. IL-10 has been shown to promote B cell activation and differentiation and induces immunoglobulin synthesis and autoantibody production (Llorente et al. 1995, Moore et al. 2001). IL-10 has also been found to be a potent suppressor of both total and specific IgE production, while it simultaneously increases IgG4 production (Blaser et al. 2004). In addition, IL-10 has shown direct inhibitory effects on mast cells and basophils (Pierkes et al. 1999, Royer et al. 2001).

Maintenance of peripheral tolerance has been associated with regulatory T cells (Tregs). Suppressive effects of inducible Tregs on Th1 and Th2 reactions are, at least partly, mediated by IL-10 and TGF-β (Vignali et al. 2008). Downregulation of T helper (Th)1 and Th2 responses by Tregs and IL-10 are presented in Figure 2. IL-10 has been shown to induce the differentiation of Tregs and also thereby mediate tolerence (Groux et al. 1997). In addition, IL-10 has been associated with inducing of T cell anergy (Groux et al. 1996). IL-10 also modulates the influence of TGF-β on T cells via regulation of the expression of TGF-β receptor (Cottrez et al. 2001).

The functions of IL-10 are mediated by IL-10 receptor (IL-10R), which is composed of two subunits, the ligand-binding IL-10R1 and the accessory subunit IL-10R2. These subunits are members of the IFN receptor family (Moore et al. 2001). IL-10R1 is mainly expressed by hemopoietic cells and IL-10R2 in most cells and tissues studied (Liu et al. 1994, Moore et al. 2001, Wolk et al. 2002).

IL-10 has closely related homologs in several virus genomes of which the homology to EBV *IL10* (ebv*IL10*) gene was found first (Moore et al. 1990, Moore et al. 2001). IL-10 superfamily has been described and includes IL-10, viral gene homologs of IL-10, IL-19, IL-20, IL-22, IL-24, IL-26, IL-28 and IL-29. These cytokines share genetic similarity through exon-intron gene structure, share receptors and have conserved signal cascades. However, the effects that cellular IL-10 family cytokines mediate differ significantly from immune suppression to enhancing antiviral activity (Commins et al. 2008).

2.1.2. Role of IL-10 in disease

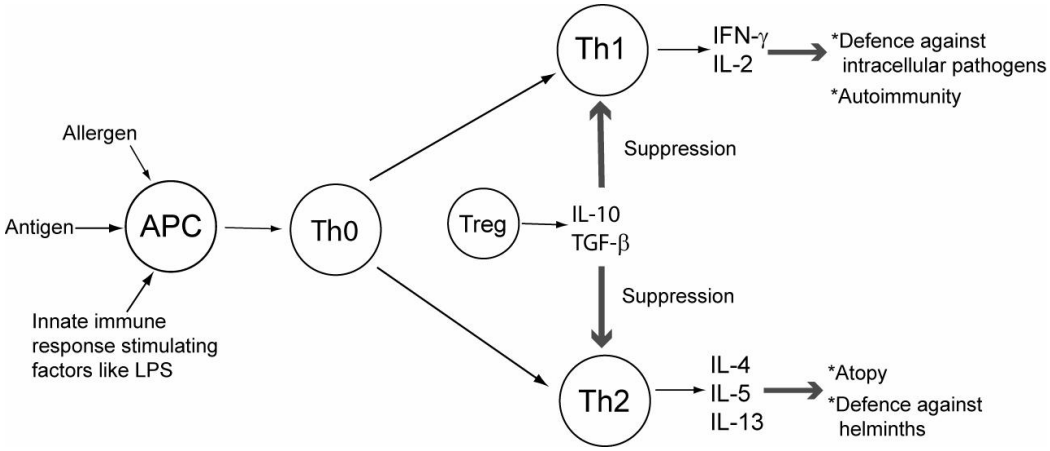
The major function of IL-10 seems to be limitation of host's harmful immune responses during infection and inflammation. IL-10 downregulates both Th1 and Th2 immune responses and thereby it seems to have important role in autoimmune and atopic diseases (von Hertzen et al. 2009). The role of IL-10 in infections and systemic inflammations has been studied in both humans and animals (Moore et al. 2001).

IL-10 deficient mice have been very susceptible to LPS-induced shock and administration of IL-10 has a protective effect against LPS-induced shock (Berg DJ et al. 1995). However, in human studies high IL-10 levels have been associated with fatal outcome in meningococcal disease (Lehmann et al. 1995, Westendorp et al. 1997). IL-10 -/- mice have been shown to develop chronic enterocolitis (Berg et al. 1996). In human studies elevated IL-10 levels have been found in autoimmune diseases including systemic lupus erythematosus (SLE), Sjögren syndrome and RA patients (Llorente et al. 1995, Hulkkonen et al. 2001). IL-10 has also been associated with many other diseases like EBV infection, psoriasis, cancer, diabetes and graft-versus-host disease (Moore et al. 2001).

Associations between atopic phenotypes and IL-10 have also been found. In animal studies Treg cells have been shown to inhibit allergen specific IgE response in mice and this effect was at least partly mediated by IL-10 (Cottrez et al. 2000). Treg cells have also been shown to regulate airway hyperreactivity in TGF-β and IL-10 dependent manner in mice (Joetham et al. 2007). In addition, diminished IL-10 concentrations in the lungs and genetically determined low IL-10 production have been associated with asthma in humans (Borish et al. 1996, Lim et al. 1998). Allergen specific immunotherapy has been related with increased IL-10 production *in vitro* in many studies (Jutel et al. 2003, Savolainen et al. 2004, Hawrylowicz et al. 2005, von Hertzen et al. 2009).

IL-10 has been considered for therapeutic use because of its anti-inflammatory functions. The early studies of recombinant IL-10 treatment showed promising results, but in larger studies the results have been contradictory and side effects have also been reported (Moore et al. 2001, O'Garra et al. 2008) Other ways of using IL-10 for treatment are under investigation. For example, gene therapy approaches to target local expression of IL-10 by adenoviral vector expressing IL-10 have been studied in animal models (Scumpia et al. 2005, O'Garra et al. 2008).

Figure 2. Examples of functions and cytokines of T helper (Th) 1, Th1 and T regulatory cells (Treg). Modified from Akdis et al. (Akdis et al. 2009)



2.1.3. IL10 gene polymorphisms

The *IL10* gene is located on chromosome 1q31-q32 and is composed of five exons and four introns. It seems that *IL10* gene is in some degree constitutively transcribed and that IL-10 production is mostly regulated at the transcriptional level (Reuss 2002). Twin and family studies have suggested that 50-75% of the variation of IL-10 production is genetically determined (Westendorp et al. 1997, Reuss et al. 2002).

IL10 gene is polymorphic and at the moment 73 SNPs are listed in the *IL10* gene region determined as in the NCBI SNP database (http://www.ncbi.nlm.nih.gov). However, some of these SNPs have only one genotype or the genotypes are not determined suggesting that at least some of them could be artefacts of the database. Three single base-exchange polymorphisms located at -1082G>A (rs 1800896), -819C>T (rs 1800871) and -592C>A (rs 1800872) form haplotypes of which four forms have been described in Caucasian population (GCC, ACC, ATA, GTA). The haplotype GTA seems to be extremely rare in Caucasian populations (0.6%) and in some studies it has not been found at all (Crawley et al. 1999, Eskdale et al. 1999, Hulkkonen et al. 2001). However, in Chinese SLE patients GTA haplotype has been more common with a prevalence of 4% (Mok et al. 1998).

These *IL10* promoter haplotypes seem to be functionally relevant, because they have been associated with IL-10 production. The ATA haplotype has been associated with lower transcriptional activity than GCC haplotype and the ATA/ATA haplotype combination with lower IL-10 production in LPS stimulated whole blood cultures compared to other haplotypes (Crawley et al. 1999). The GCC haplotype has been shown to have 20% higher transcriptional activity compared to ACC and ATA haplotype in luciferase reporter gene assay (Reuss et al. 2002). *In vivo* the GCC haplotype has been associated with elevated plasma IL-10 levels in primary Sjögren's syndrome patients (Hulkkonen et al. 2001). However, there are also controversial results and ATA haplotype carriers have been reported to have higher IL-10 levels than non-carriers among healthy adults (Kilpinen et al. 2002).

It has been postulated that *IL10*-1082, *IL10*-819 and *IL10*-592 polymorphisms are located within important regulatory regions and may alter the structure of transcription factor binding sites. The *IL10*-1082 polymorphism is located within E26 transformation specific (ETS) transcription factor-binding site and allele A has been shown to confer a higher binding affinity to the transcription factor PU.1, which inhibits gene expression and leads to decreased IL-10 expression (Reuss et al. 2002). In addition, the *IL10*-1082 allele A carriers have been associated with lower IL-10 production compared to allele A non-carriers (i.e. GG genotype) in Con A stimulated PBMC cultures (Turner et al. 1997). The *IL10*-819 is located within a putative positive regulatory region and the *IL10*-592 polymorphism is situated within a possible STAT3 binding site and a negative regulatory region, but the exact transcription factors have not been found (Kube et al. 1995, Reuss et al. 2002).

Almost one hundred reports on an association between *IL10* promoter haplotype -1082/-819/-592 and diseases have been published. For example, the putatively low producing haplotype ATA has been found to be associated with asthma severity, SLE with renal disease, susceptibility to herpes zoster, a severe form of malaria, aggressive periodontitis and susceptibility to melanoma yet at the same time with better survival in advanced melanoma (Lim et al. 1998, Mok et al. 1998, Haanpää et al. 2002, Vuoristo 2007, Ouma et al. 2008, Reichert et al. 2008). The possibly high producing haplotype GCC has in turn been associated with Sjögren's syndrome, poor response to IFN-α therapy in hepatits C and SLE (Edwards-Smith et al. 1999, Hulkkonen et al. 2001, Rosado et al. 2008). In some studies no associations between diseases and *IL10* promoter haplotype have been found (Hollegaard et al. 2006).

2.2. IL10 promoter haplotype and EBV

2.2.1. EBV infection

EBV belongs to the human herpes virus family. EBV infection is very common and approximately only 5% of adults are seronegative for EBV. The primary EBV infection usually occurs within the first years of life, when the symptoms are mild or the infection is asymptomatic. In industrialized countries with a high standard of living many children are protected from early infection and usually contract EBV infection during adolescence and even in adulthood, when up to 30-50% of EBV infections can be presented as acute infectious mononucleosis (IM) with fever, tonsillitis, lymphadenopathy, hepatitis and splenomegaly. In some cases IM has potentially life-threatening manifestations like meningo-encephalitis, myocarditis and pneumonia. EBV has also been associated with many malignancies including Burkitt's lymphoma, Hodgkin's disease, nasopharyngeal carcinoma and gastric carcinoma (Crawford 2001).

EBV is mainly transmitted through the salivary contact. After oral transmission, EBV replicates in a permissive cell type in the oropharynx. These cells are probably specialized epithelial cells that bind virus directly or acquire virus by transfer from the surface of adjacent B cells. The virus infects mucosal B cells and initiates a latent infection simultaneously (Shannon-Lowe et al. 2006). Cell mediated immunity and cytokines seem to be crucial to the host's defense against infection. Immune responses are able to control the primary infection, but they do not eliminate the virus. The EBV infected B cells constitute the site of latency and after the primary infection the virus remains in the body for life (Crawford 2001).

EBV infection seldom occurs after 20 years of age and 85-95% of 20-year olds are already EBV seropositive. The reason why some people remain seronegative for EBV is not clear, but it seems that seronegative adults differ from seropositive adults in some immunologic functions. Higher percentage of monocytes in the peripheral blood and increased IFN-α and IL-6 levels in culture supernatants of seronegative adults have been reported. However, the expression levels of the EBV receptor CD21 on peripheral B cells have not differed between EBV negative and positive subjects (Jabs et al. 1996, Jabs et al. 1999). It has also been hypothesized that EBV seronegative individuals may have immunogenetic differences compared to seropositive (Helminen et al. 1999).

2.2.2. Role of IL-10 in Epstein-Barr virus infection

IL-10 plays a central role in the establishment and persistence of EBV infection. Elevated levels of circulating IL-10 have been found in people with acute and chronic acute EBV infection and it has been speculated that IL-10 may contribute to disease pathogenesis by inhibiting host immunity and allowing the development of latency (Taga et al. 1995, Kanegane et al. 1997). During EBV

latency EBV gene products are able to enhance *humanIL10 (hIL10)* transcription and production of hIL-10, thus supporting the persistence of latent infection (Marshall et al. 2003).

EBV codes for a cellular homolog of IL-10 called viral IL10 (ebvIL-10, later vIL-10). The amino acid sequences of hIL-10 and vIL-10 are 84% identical and vIL-10 shares similar properties with human IL-10 including both cell proliferative and anti-immune functions. The effect of vIL-10 may depend on the duration of exposure, because vIL-10 has been shown to have a stimulatory effect on T cells after long-term vIL-10 secretion, whereas short exposure to vIL-10 has shown inhibitory effects (Müller et al. 1999). vIL-10 is 3 to 10-fold less potent than hIL-10 and has at least 100 to 1000-fold lower affinity to IL-10 receptor than hIL-10 (Moore et al. 2001). Both vIL-10 and hIL-10 have been able to induce expression of EBV latent membrane protein 1 in EBV infected B or NK cells and thus IL-10 may have a role in the establishment of latency (Kis et al. 2006). *ebvIL10* gene (BCRF1) seems to be well conserved among the EBV strains, which emphasizes the importance of vIL-10 in EBV infection (Kanai et al. 2007).

2.2.3. Associations between IL10 gene promoter polymorphisms and Epstein-Barr virus

Associations between *IL10* gene polymorphisms and EBV or EBV associated diseases have been investigated in many studies. High *IL10* gene expression has been reported among the *IL10*-1082 allele G carriers in EBV-transformed lymphoblastoid cells lines of full-term healthy infants (Capasso et al. 2007). *IL10*-1082 polymorphism has also been shown to have an effect on both the susceptibility and severity of EBV infection in Finnish adults (Helminen et al. 1999). *IL10*-1082 allele G carriers were more often seronegative for EBV whereas *IL10*-1082 allele A carriers had more severe EBV infection leading to hospitalization. *IL10*-1082 allele G has been associated with higher IL-10 producing capability and therefore it has been speculated that the possibly lower producing capability associated with *IL10*-1082 allele A makes people more susceptible to severe EBV infection (Helminen et al. 1999). In addition, *IL10*-819 CC genotype has been associated with increased risk of elevated EBV IgG antibody titers in Japanese women (Yasui et al. 2008). However, there is only one report concerning an association between *IL10* promoter haplotype 1082G>A/-819C>T/-592C>A and EBV infection. In this study ATA haplotype was associated with diminished risk of early EBV infection (Helminen et al. 2001).

EBV has been associated with several malignancies including gastric carcinoma, nasopharyngeal carcinoma and Hodgkin's lymphoma. Therefore the relationships between *IL10* promoter polymorphisms and these diseases have been investigated. *IL10*-1082 allele G frequency has been increased in EVB negative gastric carcinoma patients compared to controls (Wu et al. 2002). However, *IL10*-1082 allele G was extremely rare, especially among EBV

seropositive gastric carcinoma patients, so the results cannot be generalized. A parallel association has been found in patients with undifferentiated carcinoma of nasopharyngeal type (UCNT) (Pratesi et al. 2006). In this study *IL10* -1082/-819/-592 haplotype was not related to UCNT, but the *IL10*-1082 allele G was associated with EBV-negative UCNT (Pratesi et al. 2006). In addition, the possibly high producing *IL10*-1082 GG genotype has been associated with EBV-positive Hodgkin's lymphoma development, whereas *IL10*-819 and *IL10*-592 polymorphisms were not related to this disease. The three-locus haplotype was not analyzed in this study (da Silva et al. 2007). The *IL10*-1082 GG genotype has also been associated with decreased risk of late-onset EBV-associated post-transplant lymphoproliferative disorder in solid organ recipients (Babel et al. 2007). However, this association has not been seen in children (Lee TC et al. 2006).

3. *Interleukin -4* promoter polymorphism and atopy

3.1. Interleukin-4

3.1.1. Function of IL-4

Interleukin-4 is a pleiotropic cytokine produced by activated T cells, mast cells and basophils. It was already functionally characterized in 1982 as a T cell-derived B cell growth factor distinct from IL-2 (Howard et al. 1982).

IL-4 plays an important role in the regulation of B and T cell mediated immune reactions. It promotes immunoglobulin synthesis and directs the immunoglobulin class switching into the synthesis of IgE and IgG4 in activated B lymphocytes (Pene et al. 1988). It also enhances the antigen presenting capacity of B cells and up-regulates IgE receptors on B lymphocytes, mast cells and basophils thereby enhancing the activation of these cells during allergic challenge. Differentiation of precursor Th cells into the Th2 subset is also regulated by IL-4. IL-4 stimulates the production of Th2 cytokines including IL-5, IL-9, IL-13 and IL4 itself. IL-4 also has anti-inflammatory effects by inhibiting the production of pro-inflammatory cytokines like IL-1, TNF-α and IL-6 and stimulating IL-1Ra production. IL-4 also activates the expression of adhesion molecules like vascular cell adhesion molecule 1 and chemokines like eotaxin and thereby directing the migration of cells to the inflammatory site and promoting eosinophilic inflammation (Paul 1991, Romagnani 2004, Andrews et al. 2006).

IL-4 exerts its biological effects by binding to IL-4 receptor (IL-4R) complex, which consists of two subunits: IL-4Rα, which is a high affinity IL-4 binding site shared with IL-13, and γc-chain (type I receptor) or IL-13Rα1 (type II receptor). Type I receptor binds only IL-4 and requires IL-4Rα for assembly

with γc-chain whereas type II receptors binds both IL-4 and IL-13 and require assembly of IL-4Rα with IL-13Rα1subunit. Because IL-4 and IL-13 share the same receptor they have many similar functions. IL-4Rs are expressed on both hematopoietic and nonhematopoietic cells like epithelial, endothelial, muscle and liver cells (Kelly-Welch et al. 2003, Steinke 2004). There is also a soluble form of IL-4R (sIL-4R) (Andrews et al. 2006).

Due to its many functions IL-4 has been associated with various diseases, especially with atopy, which will be discussed in detail later. IL-4 has also shown a potent antitumor activity (Paul 1991). However, the results of recombinant human IL-4 (rhIL-4) in cancer treatment have been disappointing, because rhIL-4 has shown only low antitumor activity (Vokes et al. 1998, Whitehead et al. 2002). Furthermore, autocrine IL-4 production has been seen in many kinds of cancer cells and it seems that colon cancer stem cells resist apoptosis by producing IL-4 (Todaro et al. 2007).

Th2 cells are important in immune reactions against many parasites. High levels of IL-4 have been found in parasite infected mice (Paul 1991). IL-4 seems to be important in host protection against parasites like *Trichinella spiralis* (Finkelman et al. 2004). In bacterial infections the role of IL-4 is somewhat confusing. In mouse models IL-4 has been shown to enhance pulmonary clearance of *Pseudomonas aeruginosa*, but in case of *Staphylococcus aureus* IL-4 has been associated with increased risk of septic arthritis (Hultgren et al. 1998, Jain-Vora et al. 1998).

3.1.2. IL4 gene polymorphisms

IL4 gene is located on chromosome 5q31, which in many studies has been linked with atopic phenotypes (Marsh et al. 1994, Palmer et al. 1998, Ober et al. 2000). Many other atopy related genes like *IL5*, *IL9*, *IL13* and *CD14* are located in the adjacent area as seen in Figure 2. *IL4* gene has 4 exons and 3 introns and is about 9 kb in length. At the moment 104 SNPs have been listed in *IL4* gene region defined by NCBI (http://www.ncbi.nlm.nih.gov). However, Sakagami and co-workers sequenced 25.6 kb genomic region including both *IL13* and *IL4* genes and found 45 SNPs in *IL4* gene region (from -600 to +8500 bps from transcription start site) (Sakagami et al. 2004). Sakagami and colleagues classified 14 of these 45 SNPs in *IL4* gene as common SNPs (minor allele frequency at least 0.10 in two populations), which were used for further analysis. A strong linkage disequilibrium across the *IL4* gene was found. Two major haplotypes accounted for >80% of haplotypes in European Americans and Japanese. However, the haplotype frequencies differed substantially between these populations and therefore it was speculated that natural selection has acted differently on *IL4* haplotypes in separate populations (Sakagami et al. 2004).

This dissertation focuses on *IL4* gene promoter base-exchange polymorphism C to T at position -590 from the open reading frame (rs 2243250). This polymorphism was first described in 1994 and belongs to the most studied polymorphisms of *IL4* gene (Borish et al. 1994). The *IL4*-590 allele T has been

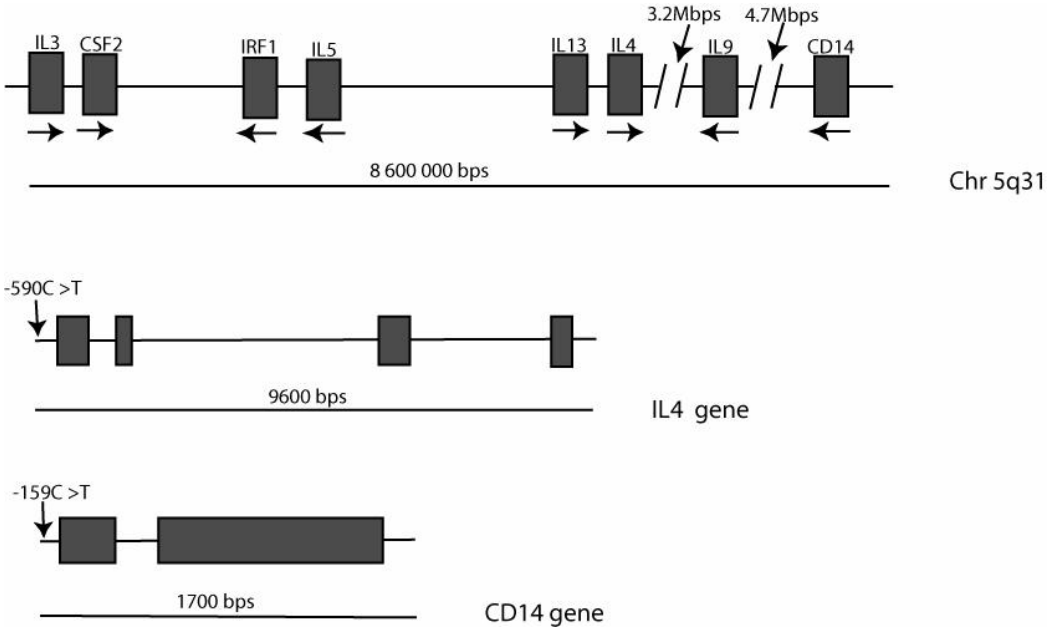
associated with stronger transcription of *IL4* and has also shown a greater binding activity to nuclear transcription factors than allele C (Rosenwasser et al. 1995, Nakashima et al. 2002).

The *IL4*-590 polymorphism seems to be functional and therefore its role in diseases has been studied extensively in recent years. The *IL4*-590 allele T has been associated, for example, with human immunodefiency virus 1 (HIV-1) syncytium-inducing phenotype, elevated antibody levels against malaria, severity of respiratory syncytial virus infection and *H.pylori cagA* positive infections (Nakayama EE et al. 2000, Luoni et al. 2001, Hoebee et al. 2003, Zambon et al. 2008). An association between *IL4*-590 polymorphism and diphtheria and tetanus vaccine responses in Australian children has also been reported. This association was modified by parental tobacco smoking: among children unexposed to parental tobacco smoking the *IL4*-590 allele T carriers had more stronger diphtheria and tetanus antibody responses than did allele T non-carriers whereas in exposed children tetanus antibody responses were decreased in allele T carriers (Baynam et al. 2007). *IL4*-590 polymorphism has also been associated with many atopic phenotypes, which will be discussed in more detail later.

The role of the *IL4*-590 allele T seems to vary in different diseases. It has been associated, for example, with susceptibility to subacute sclerosing panencephalitis in Japanese children, RA in Columbian patients and Crohn's disease in Caucasian population (Klein et al. 2001, Inoue et al. 2002, Moreno et al. 2007). However, the *IL4*-590 allele T has also been associated with diminished risk of autoimmune thyroid diseases, myocardial infarction at young age, severity of Sjögren's syndrome, survival in colorectal cancer in Caucasian populations and in minimal change nephritic syndrome in Japanese children (Hunt et al. 2000, Kobayashi et al. 2003, Pertovaara et al. 2006, Paffen et al. 2008, Wilkening et al. 2008).

Figure 3. *IL4* and *CD14* gene regions

IL4 and *CD14* genes are located on chromosome 5 at chromosome band 5q31. This area also contains many other atopy associated genes like *IL5*, *IL9* and *IL13* as seen in the diagram. The arrows under the gene symbol indicate the direction of transcription. The exon - intron organization of common *IL4* and common *CD14* gene transcripts (ENST00000302014 and ENST00000231449 respectively) are indicated in the lower part of the picture. Exons are shown as boxes. The sites of *IL4*-590C>T and *CD14*-159C>T polymorphisms are marked in the diagram. Information is based on Ensemble Database (www.ensembl.org). The diagram is not to scale.



3.2. IL4-590C>T polymorphism and atopy

3.2.1. Atopy

Atopy is defined by the European Academy of Allergology and Clinical Immunology as a personal or familial tendency to produce IgE antibodies in response to low dose of allergens and to develop typical symptoms such as asthma, rhinoconjunctivitis or eczema/dermatitis (Johansson et al. 2001). The presence of specific IgE is usually studied by either skin prick tests (SPT) or serum assay. The results of SPTs are generally in line with anamnestic data of atopic symptoms, but not all sensitized individuals develop an atopic disease. Elevated serum total IgE levels have also been associated with atopic phenotypes (Burrows et al. 1989). However, many patients suffering from atopic diseases do not have allergen specific IgE and their serum total IgE levels are normal (Cookson 2004).

Atopic diseases run in families and, for example, asthma heritability has been estimated in twin studies to vary from 36% to 87% (Nieminen et al. 1991, Laitinen et al. 1998). In addition to genetics, many environmental factors seem to have a role in the etiology of atopy. Epidemiological evidence has shown that childhood infectious diseases, especially gastrointestinal infections such as *Helicobacter pylori*, *Toxoplasma gondii* and hepatitis A associated with poor level of hygiene, have been associated with decreased risk of atopic diseases (Matricardi et al. 2000, Seiskari et al. 2007). Other environmental factors associated with atopy and serum total IgE include, for example, growing up on a farm, number of siblings, tobacco smoke and socioeconomic status (von Mutius 2000).

The prevalence of atopic diseases has increased markedly in developed countries in recent decades. This change has been explained, at least partly, by decrease of infections due to improved standard of living and better hygiene. This so called “hygiene hypothesis” was first introduced by Strachan in 1989 (Strachan 1989). This theory has evolved a great deal in recent years and there are at least four dimensions that need be considered when assessing this hypothesis: extensive variety of allergic phenotypes, diversity of environmental exposures, timing of the exposure and the individual’s genetic susceptibility to react to these exposures (von Mutius 2007). In light of many studies it seems that infections occurring during the first years of life could be the most important, because they may have an effect on the maturation of immune system and the development of Th1/Th2 balance (Matricardi et al. 2000, von Mutius 2007).

The hygiene hypothesis has also been explained on the cellular level. Th cells have been classified at least as Th1 and Th2 cells according to their main functions and cytokine production (Mosmann et al. 1989). Th1 cells are mainly responsible for cell-mediated immunity while Th2 cells participate in humoral

immune reactions, especially in IgE formation. Th1 cells produce IL-2, IFN-γ and TNF-β whereas Th2 cells produce IL-4, IL-5, IL-9 and IL-13 (Mosmann et al. 1989). The differentiation of Th cells is known to be modulated by microbial factors and by the cytokines they induce. For example, in the presence of LPS the differentiation of Th cells tends towards Th1 whereas Th2 direction is favored in the absence of this kind of stimulus. This immune deviation theory has been widely accepted (Romagnani 2004). However, it is coming clear that Treg cells and IL-10 and TGF-β have an important role in the balance between peripheral tolerance and allergy. In atopy disturbed balance between Treg and Th2 cells has been shown in both animal and human studies (Cottrez et al. 2000, Akdis et al. 2004, Ling et al. 2004).

The role of allergen exposure in the development of atopic disease is still under debate. It has been postulated that the dose, type and timing of allergen exposure may be critical. For example, in asthmatic children early exposure to cat allergen has been shown to increase sensitization to cat, but it had no effect on asthma risk, whereas exposure to dog did not sensitize to dog allergen but protected against asthma and sensitization to airborne allergens (Almqvist et al. 2003). The negative association between pet exposure and sensitization has also been reported in other studies (Karjalainen et al. 2005).

3.2.2. IL-4 and atopy

IL-4 is considered to be a key cytokine in allergic inflammation because, for example, it induces IgE synthesis and promotes Th cell differentiation in Th2 direction (Romagnani 2004). The role of IL-4 in atopy has been widely studied in both animal models and human.

In IL-4 knockout mice sensitization, development of bronchial hyperresponsiveness and anaphylactic shock did not occur and similar effects have been seen in mice missing a functional IL-4 receptor (Brusselle et al. 1995, Grunewald et al. 1998). IL-4 has also been shown to induce mucin gene expression and hypersecretion of mucus in the airways of mice (Dabbagh et al. 1999).

In allergic individuals serum IL-4 levels have been increased, likewise the number of IL-4 messenger RNA positive cells in bronchoalveolar lavage (Robinson et al. 1992, Daher et al. 1995). Indivuduals with atopic dermatitis have been reported to have more IL-4 producing T cells than non-atopic individuals (Chan et al. 1996). Inhaled rhIL-4 has shown increased airway hyperresponsiveness associated with eosinophilia in sputum (Shi et al. 1998). However, there is also evidence that genetic risk for atopy could be associated with decreased production of both Th1 and Th2 cytokines, because the cord blood IL-4 and IFN-γ have been inversely associated with the development of asthma and atopy and TNF-α inversely associated with atopy at age 6 (Macaubas et al. 2003).

Because IL-4 seems to have an important role in atopy, blocking the effects of IL-4 has been investigated for treatment of atopic diseases. In animal models

sIL-4R has efficiently inhibited IgE production and therefore recombinant sIL-4Rα has been evaluated as asthma treatment, however in larger studies it was not clinically efficient (Steinke 2004). IL-4R antagonist (IL-4 mutein), anti-IL-4 monoclonal antibody and anti-IL-4R monoclonal antibody are under investigation for asthma treatment, but the results have not been too promising so far (Steinke 2004, Andrews et al. 2006).

3.2.3. IL4-590C>T polymorphism, Helicobacter pylori and atopy

Associations between *IL4*-590C>T polymorphism and various atopic phenotypes have been studied widely and over 50 investigations had been published by January 2009 according to the Pubmed Database (www.pubmed.com). *IL4*-590 polymorphism has been associated with many atopic phenotypes like asthma, serum total IgE, atopic dermatitis, rhinitis and sensitization determined by skin prick test or specific IgE (Rosenwasser et al. 1995, Burchard et al. 1999, Zhu et al. 2000, Söderhall et al. 2002, Liu et al. 2004, Chiang et al. 2007, Li et al. 2008). However, the results have been inconsistent, and in many studies no association between *IL4*-590 polymorphism and atopic phenotypes has been found (Walley et al. 1996, Dizier et al. 1999, Elliott et al. 2001).

H. pylori is one example of environmental factors which have been associated with decreased risk of atopic phenotypes in many studies (Kosunen et al. 2002, McCune et al. 2003, von Hertzen et al. 2006, Seiskari et al. 2007, Konturek et al. 2008). However, this association has not been seen in every study (Bodner et al. 2000, Uter et al. 2003, Law et al. 2005). In addition, a positive association between food allergy and *H.pylori* has even been reported (Corrado et al. 1998). *H.pylori* is a common gastrointestinal pathogen infecting gastric mucosa usually early in life and often leading to life-long chronic gastritis (Brown 2000, Suerbaum et al. 2002). The prevalence of *H.pylori* is strongly related with socioeconomic status. *H.pylori* is positively associated with low socioeconomic class, poor living conditions including absence of fixed hot water supply, contaminated water and household overcrowding (Mendall et al. 1992, Malaty et al. 1994, McCallion et al. 1996). *H.pylori* infection has been suggested to influence the development of the immune system by LPS binding with the CD14 receptor which results in increased production of IL-12 and IFN-γ. This could drive the immune responses towards the Th1 pathway and thereby have a protective effect against the development Th2 polarized diseases like atopy (von Mutius 2000). It has also been proposed that negative association between *H.pylori* and atopy may be due to increased IL-10 expression associated with this infection in some studies (Bontems et al. 2003, Maciorkowska et al. 2005, Oderda et al. 2007), because IL-10 is considered to be crucial in the development and maintenance of immunological homeostasis in allergy (Hawrylowicz et al. 2005).

In animal models IL-4 has been shown to limit *H.pylori* associated gastric inflammation (Smythies et al. 2000). In children with *H.pylori* infection elevated IL-4 levels have been found in gastric and duodenal mucosa (Bontems et al.

2003, Maciorkowska et al. 2005). Because *IL4*-590 polymorphism may have an effect on IL-4 production, associations between this polymorphism and *H.pylori* have been investigated. The *IL4*-590 allele T has been shown to enhance the risk for *cagA* positive *H.pylori* infection in gastric cancer patients (Zambon et al. 2008). However, in another study no association between *IL4*-590 polymorphism and *H.pylori* was found (García-González et al. 2007).

There is increasing evidence that gene-gene and gene-environment interactions may have an effect on atopic phenotypes. Several reports of gene-gene interactions including *IL4*-590 polymorphism have been published. For example, the *IL4*-590 allele T has been shown to increase the risk of asthma in Finnish females when combined with the *TLR4*+896 allele G. However, this combination was not associated with atopy defined by SPT (Ådjers et al. 2005). The *IL4*-590 allele T has also been associated with development of atopic dermatitis and SPT positivity at age of 24 months when combined with the *IL13*Arg130Gln allele 130Gln. However, this gene-gene interaction was not associated with probable asthma diagnosis or rhinitis (He et al. 2003).

There are only few reports of gene-environment interactions on atopy including *IL4*-590 polymorphism and the results have been contradictory. Interaction between exposure to environmental tobacco smoke (ETS) and *IL4*-590 polymorphism has been associated with wheezing without cold in the first year of life in African Americans, but not in non-African Americans (Smith et al. 2008). Additionally, in Australian children no interaction between *IL4*-590 polymorphism and ETS on atopy defined as SPT positivity was found (Baynam et al. 2007). In another study the effect of *IL4*-590 polymorphism on sensitization to mite analyzed by specific IgE has been shown to be modulated by *Dermatophagoides pteronyssinus* (Der p 1) allergen levels. In this study the *IL4*-590 allele T was a risk for mite senzitisation only when the Der p 1 allergen levels were high (Liu et al. 2004).

4. *CD14* promoter polymorphism and IgE

4.1. CD14

4.1.1. Function of CD14

CD14 is an important innate immunity receptor belonging to so-called pattern recognition receptors, which participate in starting the innate immune responses. CD14 acts as an accessory receptor for many TLRs including TLR2, TLR3, TLR4 (Schmitz et al. 2002, Lee HK et al. 2006). cDNA of CD14 was described in 1988 and the functional role of CD14 in LPS recognition was found in 1990 (Ferrero et al. 1988, Wright et al. 1990).

CD14 recognizes a vast variety of microbial ligands, for example, LPS of gram-negative bacteria, lipoteichoic acids of gram-positive bacteria, mycobacterial glycolipids and mannans from yeast (Vercelli 2002). CD14 has an important role in endotoxin signaling by facilitating endotoxin responses through TLR4-MD2 system, which leads to the activation of innate host-defense mechanisms by releasing cytokines, such as IL-12, which are regarded as obligatory signals for the differentiation of naïve T cells into Th1 cells. Thereby CD14 also has a role in directing adaptive immunity reactions (Vercelli et al. 2001, Martinez 2007). In addition to microbe recognition, CD14 has also been shown to have an important role in the recognition and clearance of apoptotic cells by macrophages (Devitt et al. 1998). There is also evidence that sCD14 may regulate T and B lymphocyte activation and function (Rey Nores et al. 1999, Arias et al. 2000). sCD14 also contributes to lipid metabolism as sCD14 transports lipids and LPS to high density lipoprotein (HDL) (Wurfel et al. 1995).

CD14 is expressed on the surfaces of monocytes, macrophages and neutrophils as membrane bound molecule (mCD14). There is also a soluble form in the sera (sCD14), which is recognized, for example, by dendritic cells when it is coupled with LPS (LeVan et al. 2001). sCD14 is thought to be derived from both protease-dependent shedding of mCD14 on myeloid cells and protease-independent release from intracellular compartments. There is accumulating evidence that sCD14 is also produced by hepatinocytes (LeVan et al. 2001, Zhao et al. 2007).

Expression of CD14 is regulated by many factors. Bacterial cell wall components including LPS have been shown to up-regulate CD14 (Landmann et al. 1996). In addition, maternal exposure to a farming environment rich in microbial compounds has led to increased up-regulation of innate immune receptors, including CD14, in childhood (Ege et al. 2006). IL-4 and IL-13 have been shown to down-regulate the expression of *CD14* (Lauener et al. 1990, Cosentino et al. 1995). In addition to microbial products many other environmental factors like alcohol consumption and use of non-steroidal anti-inflammatory analgetics, have been associated with increased sCD14 levels (Karhukorpi et al. 2002, Campos et al. 2005).

Innate immunity receptors, including CD14, participate in the host defense against harmful pathogens and at the same time influence the etiology of several diseases. CD14 has been shown to be an important inflammatory mediator in gram-negative sepsis and increased sCD14 levels in the beginning of this disease have been associated with high mortality (Landmann et al. 1995). Consistent with this finding, CD14 knockout mice have been more resistant to LPS induced shock than wild type controls (Haziot et al. 1996). Elevated sCD14 levels have also been associated with many other diseases including HIV, malaria, atopic dermatitis and extensive tissue damage in trauma and severe burns (LeVan et al. 2001). Anti-CD14 therapies for sepsis treatment are under investigation, but so far they have achieved limited success (Axtelle et al. 2003)

4.1.2. CD14 gene polymorphisms

CD14 gene is located on chromosome 5q31 near *IL5*, *IL9*, *IL13* and *IL4* genes as seen in Figure 2. This area has been connected with the regulation of serum total IgE and atopy in many linkage studies as mentioned earlier (Marsh et al. 1994, Palmer et al. 1998, Ober et al. 2000, Xu et al. 2000). *CD14* gene is about 1.6 kb in length and has 2 exons. *CD14* encodes two protein forms: mCD14 and sCD14, which lacks the anchor of mCD14 (LeVan et al. 2001).

There are 21 SNPs reported in *CD14* gene area defined by NCBI (http://www.ncbi.nlm.nih.gov). In a recent study *CD14* genomic area from -6000 up till +2500 bps from the transcription site was sequenced and 17 SNPs were found. 15 of these 17 SNPs were reported to have minor allele frequency >10% (LeVan et al. 2008). This dissertation focuses on one of the most studied *CD14* promoter region single base-exchange polymorphisms C to T at position -159 from transcription start site (-260 from the translation start site, rs 2569190), which was first described in 1999 (Baldini et al. 1999). The *CD14*-159 allele T has been associated with decreased affinity of DNA/ protein interactions at a GC box containing binding sites for transcription factors Sp1, Sp2, and Sp3. An increase in *CD14* gene expression has been seen in *CD14*-159 C to T change so that allele T is transcriptionally more active (LeVan et al. 2001, Zhao et al. 2007). The *CD14*-159 allele T has also been associated with elevated sCD14 levels in many studies (Zhang et al. 2008). The *CD14*-159 TT genotype has been associated with increased IL-10 and IL-1β levels after endotoxin stimulation and lower IL-4 levels after concanavalin A stimulation (Keskin et al. 2006).

Associations between *CD14*-159 polymorphism and diseases have been studied abundantly and over 150 reports had been published by January 2009. Many of these studies investigated the relationship between *CD14*-159 polymorphism and serum total IgE or other atopic phenotypes, which will be discussed in more detail later. A wide variety of other diseases, like IBD, coronary artery disease, type I diabetes, severity of *Streptococcus pneumoniae* infection and *H.pylori* related gastric carcinoma have also been associated with *CD14*-159 polymorphism (Arroyo-Espliguero et al. 2005, Baumgart et al. 2007, Zhao et al. 2007, Dezsöfi et al. 2008, Yuan et al. 2008).

4.2. CD14-159C>T polymorphism and serum total IgE

4.2.1. IgE

IgE is an important mediator in allergic reactions and in immune defense against parasites. IgE was discovered as early as 1960's (Ishizaka et al. 1966, Johansson 1967). The total serum IgE levels have been shown to be markedly heritable in different populations whereas it seems that specific IgE response is mainly determined by environmental factors (Hopp et al. 1984, Hanson et al. 1991, Miller et al. 2005).

IgE is produced by B cells after the immunoglobulin isotype switching to IgE synthesis mediated by IL-4, IL-13 and co-stimulatory signals from $CD4^+$ T cells (Vercelli 2001). IgE acts through two receptors, high affinity IgE receptor FcεRI, and low affinity IgE receptor FRcεII also called CD23. FcεRI is mainly expressed in mast cells and basophils. FRcεII has two forms: FRcεIIa, which is normally expressed on B cells and FRcεIIb, which is inducible on T cells, B cells, monocytes and macrophages by IL-4 and IL-13 (Corry et al. 1999). It seems that IgE up-regulates the expression of FcεRI at least on basophils by interacting through FcεRI (MacGlashan et al. 1998). Exposure to allergen in a sensitized individual leads to cross linking of IgE/FcεRI complexes, which causes mast-cell degranulation and the initiation of allergic inflammation (Cookson 1999).

IgE production is regulated by stimulatory signals, such as IL-4, IL-5, IL-9 and IL-13, provided by Th2 cells and inhibitory signals like IL-2 and IFN-γ produced by Th1 cells (Pene et al. 1988, Nakanishi et al. 1995, Corry et al. 1999). IL-12 and IL-18 have also been shown to suppress IgE secretion probably through IFN-γ induction (Yoshimoto et al. 1998). However, IL-18 has also been shown to increase IgE production (Yoshimoto et al. 2000). IL-10 seems to influence the IL-4 -induced isotype switching from IgE to IgG_4 which may promote tolerance instead of allergic reactions (Jeannin et al. 1998). At very high IgE levels CD23 may have an inhibitory effect on IgE synthesis (Corry et al. 1999). The role of sCD14 in regulation of IgE is not clear, which will be discussed more in the next chapter.

Parasites, especially heminths, are known to up-regulate IgE production (Ramaswamy et al. 1994). Smoking, alcohol consumption and exposure to diesel exhaust have also been associated with increased serum total IgE (Beeh et al. 2000, Campos et al. 2006). Many other factors like age, gender, ethnicity and socioeconomic status have been found to affect serum total IgE, but the results have been inconsistent and in some studies no associations have been found (Burrows et al. 1989, Beeh et al. 2000, Litonjua et al. 2005b).

Elevated serum total IgE levels have been strongly associated with bronchial hyperresponsiveness, sensitization, atopic dermatitis and both atopic and non-atopic asthma (Burrows et al. 1989, Beeh et al. 2000, Matricardi et al. 2000, Gern et al. 2004). In addition to atopic phenotypes, IgE has been suggested to be linked with chronic urticaria, myeloma, IBD, HIV infection and Job's syndrome (hyper IgE syndrome), malaria and helminth infections (Ramaswamy et al. 1994, Beeh et al. 2000, Johansson et al. 2001, Seka-Seka et al. 2004, Vonakis et al. 2008). Because IgE has a key role in atopic reactions, anti-IgE therapy has been developed for the treatment of allergic disease. It has shown efficacy in asthma and allergic rhinitis patients (Holgate et al. 2005). There are also promising results of anti-IgE therapy in atopic eczema (Belloni et al. 2007).

4.2.2. CD14 and IgE

CD14 has been called a bridge between innate immunity and adaptive immune responses because CD14 seems to influence the effects of bacterial components on the development of Th1/Th2 balance and thereby possibly affect regulation of IgE (Vercelli et al. 2001). Therefore genetic alterations in CD14 production may have an effect on serum total IgE (Vercelli et al. 2001).

However, the relationship between CD14 and IgE is not clear. In some studies CD14 has been shown to have an inhibitory effect on IgE production. Interaction between sCD14 and B cells has resulted in higher levels of IgG1 and decreased IgE production and inhibition of IL-4 and IL-6 secretion (Arias et al. 2000). sCD14 has also been associated with IL-4 inhibition in other studies (Baldini et al. 1999, Rey Nores et al. 1999). However, the effect of sCD14 on Th1 cytokine IFN-γ has differed from inhibition to stimulation (Baldini et al. 1999, Rey Nores et al. 1999). In addition, the CD14 mediated stimulus on monocytes/macrophages has resulted in the secretion of cytokines, such as IL-6, that potently amplify IgE synthesis (Jabara et al. 1994). Thus activation of the CD14 pathway could have an inhibitory or enhancing effect on IgE expression (Vercelli 2003). In human studies, reverse correlation between high serum sCD14 levels and low serum total IgE have been reported (Baldini et al. 1999, Tan et al. 2006). However, this association has not been repeated consistently (Kabesch et al. 2004).

4.2.3. CD14-159C>T polymorphism, Helicobacter pylori and serum total IgE

Baldini and colleagues first reported an association between *CD14*-159 polymorphism and serum total IgE. In their study atopic children with the TT genotype had higher sCD14 levels and lower serum IgE levels than allele C-carriers (Baldini et al. 1999). This finding has received a lot of attention and over fifty studies concerning associations between *CD14*-159 polymorphism and serum total IgE or atopic phenotypes has been published between the first report and January 2009.

The original finding of Baldini and colleagues has been repeated in many studies in different ethnic groups (Gao et al. 1999, Koppelman et al. 2001, Leung et al. 2003). However, there are also opposite findings. The *CD14*-159 allele T has been associated, for example, with atopy in a rural population, eczema and elevated serum total IgE in children and food allergy and nonatopic asthma in adults (Ober et al. 2000, Woo et al. 2003, Litonjua et al. 2005a). In several studies no association has been found (Sengler et al. 2003, Kabesch et al. 2004, Liang et al. 2006, Nishimura et al. 2006, Zhang et al. 2008). These conflicting results have been explained, for instance, by ethnic differences, lack of power in some studies and gene-gene interactions (Zhang et al. 2008).

The suggested mechanism by which *CD14*-159 polymorphism could modify serum total IgE is via CD14. A genetically determined increase in CD14 expression could result in enhanced responsiveness to pathogen products in early

life and modify immune reactions into Th1 direction thus protecting against atopy (Vercelli 2003, Martinez 2007). The carriers of the transcriptionally more active *CD14*-159 allele T have had higher sCD14 levels than allele T non-carriers in many studies (Baldini et al. 1999, Leung et al. 2003, Kabesch et al. 2004). However, in some studies no association was found (Liang et al. 2006, Zhang et al. 2008).

Many environmental factors, including *H.pylori*, have been associated with serum total IgE and atopy as already discussed in Chapter 3. The exact mechanism behind association between *H.pylori* and atopy is not known. However, LPS of *H.pylori* is able to bind to mCD14 on monocytes and to stimulate monocytes to secrete cytokines and chemokines (Bliss et al. 1998). Therefore genetically defined changes in *H.pylori* LPS responses through CD14 could have an effect on the Th1/Th2 balance and atopy risk. In Finnish adults increased sCD14 levels have been found to be associated with *H.pylori* infection. This association was modified by *CD14*-159 polymorphism so that sCD14 levels differed significantly more between *H.pylori* positive and negative individuals with the *CD14*-159 CC homozygotes compared to other genotypes (Karhukorpi et al. 2002). However, not every study found an association between *H.pylori* and *CD14*-159 polymorphism (Park et al. 2006).

4.2.4. Effect of gene-environment interactions on serum total IgE and atopy

The results of association studies concerning *CD14*-159 polymorphism and serum total IgE and other atopic phenotypes are contradictory, as discussed earlier. It is well known that serum total IgE is regulated by both environmental and genetic factors and therefore gene-environment interactions may be more relevant than *CD14*-159 polymorphism alone in the regulation of serum total IgE.

The concomitant effect of endotoxin and *CD14*-159 polymorphism on IgE and atopy has been investigated in many studies. In five studies the *CD14*-159 CC genotype has been associated with diminished risk of atopy and high serum total/specific IgE when exposure to endotoxin has been high, whereas at low endotoxin levels this genotype has been an increased risk for these phenotypes (Eder et al. 2005, Zambelli-Weiner et al. 2005, Simpson et al. 2006, Williams et al. 2006, Williams et al. 2008). Marginally significant interactions between endotoxin exposure and *CD14*-159 polymorphism on serum total IgE or atopic sensitization were found in four of these five studies (Eder et al. 2005, Simpson et al. 2006, Williams et al. 2006, Williams et al. 2008).

Other environmental factors have also been included in interaction studies. Eder and colleagues found a significant interaction between *CD14*-159 polymorphism and animal exposure related to specific and total serum IgE: the *CD14*-159 allele C was associated with lower levels of both total and specific IgE in children with regular contact with stable animals, whereas this allele was a risk factor for elevated IgE levels in children with regular contact with furry pets (Eder et al. 2005). Similar interactions between *CD14*-159 polymorphism

and pet exposure associated with serum total IgE, sensitization and atopic dermatitis have been reported in children (Gern et al. 2004, Bottema et al. 2008). However, conflicting results concerning exposure to farming environment including stable animals has been reported (Leynaert et al. 2006). Alcohol and ETS have been reported to have interaction with *CD14*-159 polymorphism on IgE or atopy (Choudhry et al. 2005, Campos et al. 2006). However, in some other studies no interaction between ETS and *CD14*-159 polymorphism on atopy phenotypes has been found (Simpson et al. 2006, Bottema et al. 2008).

The exact mechanisms behind these interactions are not known. According to the endotoxin switch model environmental endotoxin modulates the Th1/Th2 balance so that low and very high endotoxin exposure directs this balance in Th2 direction and with intermediate endotoxin exposure Th1 responses will develop (Vercelli 2003). Polymorphisms of innate immunity genes, like *CD14* crucial in host/environment interface, may modulate immune responses by changing the endotoxin switch so that the carriers of the probably high producing *CD14*-159 allele T require less environmental endotoxin to switch in Th1 responses, and that thereby this the *CD14*-159 allele T could protect from allergy (Vercelli 2003). However, when the effects of *CD14*-159 polymorphism on serum total IgE and atopy have been concomitantly investigated with environmental endotoxin exposure, the *CD14*-159 allele C has been suggested to be a risk factor for atopic phenotypes at low levels of endotoxin or microbial exposure, whereas the *CD14*-159 allele T seemed to be a risk factor at high levels of exposure (Martinez 2007). It has also been speculated that there could be a difference in constitutive and induced CD14 synthesis so that constitutive CD14 expression may be higher in *CD14*-159 TT homozygotes than in other genotypes, whereas induced synthesis of CD14 could be higher in the C*D14*-159 allele C carries when the doses of CD14 agonist, like LPS, are high (Martinez 2007).

Aims of the study

The present study was undertaken in order to:

I Investigate whether pro-inflammatory cytokine *IL1B* gene promoter polymorphism is associated with febrile seizures.

II Study possible associations between pro- and anti-inflammatory cytokines and febrile seizures.

III Study whether the *IL10* gene promoter haplotype is associated with primary EBV infection.

IV Analyze whether there is a gene-environment interaction between *IL4* promoter polymorphism and *Helicobacter pylori* infection that might have an effect on atopy.

V Examine gene-environment interaction between *CD14* promoter polymorphism and *Helicobacter pylori* on serum total IgE.

Subjects and methods

1. Subjects

1.1. Studies I and II

For studies I and II blood samples were obtained from 55 children with FS treated in the Department of Pediatrics at the Tampere University Hospital between October 1997 and March 2000. The inclusion criteria for FS patients were age 6 months to 5 years, no other identifiable cause for the seizure like encephalitis, meningitis, raised intracranial pressure, disturbance in electrolytic equilibrium, epilepsy, poisoning or trauma, and temperature at least 38.5ºC. Data regarding the family history for FSs, earlier FSs, duration of the seizure, and duration of fever before seizure were obtained from the parents by questionnaire. Family history was regarded positive when seizure was reported in a first-degree relative. Whole blood was available only from 35 patients out of 55 (64%) and plasma from all 55 children. CSF samples were obtained from 16 out of 55 (29%) children based on the clinical judgment of the attending pediatrician. For statistical analysis infections were divided into viral and bacterial infections. Viral infection was defined as an infection with fever, low C-reactive protein and no need for antibiotic treatment. Bacterial infections were treated with antibiotics and included both focal and septic infections defined as bacterial growth in blood sample. The blood and CSF samples were taken immediately upon arrival at the hospital.

In Study II plasma samples were obtained from 20 control children treated at the pediatric department of Tampere University Hospital between February 1999 and March 2000 with febrile illness without convulsions and no history of FS according to parents and medical records. The control group was matched for age and temperature with the FS group. Laboratory and clinical data were obtained from the medical records in both the FS and the control group.

Healthy blood donors were used as controls in Study I because the likelihood of febrile seizure in this group is small (2-6%). Blood samples (buffy coats) from these 400 healthy adults (18-60 years old) were obtained from the Finnish Red Cross Blood Transfusion Centre, Tampere. The blood donors did not have any blood-transmitted diseases or any signs of other infections during a 2-week period prior to the blood donation according to information acquired by questionnaire. The ethnic background of the patients and controls was Finnish Caucasian. Characteristics of study subjects are presented in Table 1.

1.2. Study III

The blood samples were obtained from 116 children aged between 9 months and 15 years, attending to the Tampere University Hospital for pediatric consultation between November 1999 and May 2000. Cord blood was collected from the umbilical veins of 50 healthy, full-term newborns after normal vaginal delivery in Tampere University Hospital. The 400 healthy adult blood donors from Study I were used as controls in Study III. All cases and controls were of the same ethnic origin, Finnish Caucasian. Characteristics of study subjects are presented in Table 1.

1.3. Study IV

In Study IV 245 asthmatic and 405 control subjects were participants in a Finnish population based case-control study aimed at identifying risk factors and predictors of the outcome of adult asthma. Inclusion criteria for asthmatic subjects were entitlement to reimbursement for asthma medication from the Social Insurance Institution of Finland and age over 30 years. The entitlement to asthma medication was granted during the course of the study if the subject fulfilled the criteria for persistent asthma confirmed by a chest specialist. Typical history, clinical features and course of asthma needed to be documented. At least one of the following physiological criteria was required for diagnosis: a variation of $\geq$ 20% in diurnal peak expiratory flow rate (PEF) recording (reference to maximal value) or an increase of $\geq$ 15% in PEF or forced expiratory volume in one second (FEV_1) with β_2- agonist or a decrease of $\geq$ 15% in PEF or FEV_1 in exercise test. A period of at least 6 months of continuing use of anti-asthmatic drugs must have elapsed by the time of the decision. This method of case ascertainment has been described in detail and evaluated elsewhere (Karjalainen et al. 2001). Per study case one or two controls with no asthma or chronic obstructive pulmonary disease were initially selected from a registry covering the whole population of Finland. Controls were matched with subjects for age, sex and area of residence. The ethnic background of the patients and controls was Finnish Caucasian. Characteristics of study subjects are presented in Table 1.

1.4. Study V

In Study V the cohort of 266 Russian Karelian children from our earlier study was used as a study population (Seiskari et al. 2007). The Finnish child cohort from the earlier study was excluded from this study, because seropositivities for *H.pylori* and *T.gondii* were too rare for statistical analyses among Finnish schoolchildren (5% and 2% respectively).

The Russian Karelian child population was recruited as a part of the type 1 diabetes-related EPIVIR Project (EU INCO-Copernicus Programme, contract

number IC15-CT98-0316, Coordinator Professor Hyöty). In the EPIVIR Project whole blood and serum samples were collected from a total of 1998 randomly selected schoolchildren in Russian Karelia during the period 1997-1999. From this group all children with both parents of Finnish or Karelian ethnicity were included in Study V (n=266). However, two of these children were excluded from the study because their DNA samples were accidentally confused in the laboratory so the final number of subjects was 264 in Study V. The study children were not selected according to possible allergic or other diseases. Characteristics of study subjects are presented in Table 1.

Table 1. Characteristics of study groups (Studies I-V).

	Populations	Age: mean±SD (range)	Ethnic background	Investigated variables
Study I	35 FS patients 400 adult blood donors	19 ± 9 months (6 - 36 months) (18 - 60 years)	Finnish Caucasian Finnish Caucasian	*IL1B*-511 polymorphism
Study II	55 FS patients 20 children with febrile illness without FS	25 ± 12 months (6 – 56 months) 22 ±12 months (7 – 42 months)	Finnish Caucasian Finnish Caucasian	Plasma and CSF IL-1β, IL-1RA, IL-6, IL-10 levels
Study III	116 children 50 neonates 400 adult blood donors	7 ± 5 years (9 months - 15 years) newborn (18 - 60 years)	Finnish Caucasian Finnish Caucasian Finnish Caucasian	*IL10*-1082/-819/-592 haplotype, plasma IL-10 levels and EBV serology
Study IV	245 asthmatic adults 405 non-asthmatic adults	59 ± 11 years (31-84 years) 60 ± 11 years (31-89 years)	Finnish Caucasian Finnish Caucasian	*IL4*-590 polymorphism, *H.pylori* IgG antibodies, SPT and serum total IgE
Study V	264 children	11 ± 2 years (7 – 15 years)	Russian Karelian	*CD14*-159 and *TLR4*+896 polymorphisms, *H.pylori* and *T.gondii* IgG antibodies and serum total IgE

FS=febrile seizure, EBV= Epstein-Barr virus, SPT=skin prick test, CSF=cerebrospinal fluid

2. Methods

2.1. Measurement of cytokine plasma levels (Studies II, III)

In Studies II and III IL-10 cytokine plasma levels were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit according to manufacturer's instructions (ELISA; CLB, PeliKine Compact human IL-10 ELISA kit, Amsterdam, The Netherlands). The detection limit of the assay was 1.2 pg /ml. In Study II IL-1β, IL-1RA, IL-6 and TNF-α cytokine plasma and CSF levels were measured using commercially available ELISA kits according to manufacturer's instructions (ELISA; CLB, PeliKine Compact human IL-1β, IL-6 and TNF-α ELISA kits, Amsterdam, The Netherlands and for IL-1RA RD systems Quantikine kit, Minneapolis, MN, U.S.A.). The detection limits of the assays were 0.4 pg/ml for IL-1β, 46.9 pg/ml for IL-1Ra, 0.6 pg/ml for IL-6 and 1.4 pg/ml for TNF-α.

2.2. EBV, H.pylori and T.gondii serology (Studies I, IV, V)

In Study III EBV antibodies were measured by enzyme immunoassay according to manufacturer's instructions (Enzygnost anti-EBV/IgG, Behring, Marburg, Germany). *H. pylori* IgG antibodies were measured by Pyloriset EIA-G III, (Orion Diagnostica, Espoo, Finland) in Study IV and by Enzygnost Anti-Helicobacter pylori/IgG Assay (Dade Behring, Marburg, Germany) in Study V. Both assays were used according to manufacturer's instructions. *T. gondii* IgG antibodies were measured by Enzygnost Toxoplasmosis IgG Assay (Dade Behring, Marburg, Germany) according to manufacturer's instructions in Study V.

2.3. Analysis of IL1B, IL4, IL10, CD14 and TLR4 gene polymorphisms (Studies I, III, IV, V)

Genomic deoxyribonucleic acid (DNA) was extracted from buffy coats or whole blood using the salting out method described earlier (Miller et al. 1988) (Studies I, III and IV) or the QIAamp DNA blood Mini Kit (QIAGEN Inc., USA) (Study V).

IL1B promoter region single base-exchange C to T polymorphism at position -511 (rs 16944) was amplified by polymerase chain reaction (PCR) in 50 µl reaction containing 100 ng of template DNA, 20 pmol of each primer, 0.1mM dNTPmix (Pharmacia Biotech), 1 mM $MgCl_2$, 1x PCR buffer for DyNAzyme (Finnzymes, Espoo, Finland) and 1U of DyNAzyme polymerase (Finnzymes,

Espoo, Finland) using the primers earlier described (di Giovine et al. 1992). The PCR conditions were as follows: 95 ºC for 2 min, then 36 cycles of 95 ºC for 1 min, 55 ºC for 1 min and 74 ºC for 1 min, and finally 74 ºC for 4 min. After amplification the PCR products were digested for 3 hours in +37 ºC with *AvaI* restriction enzyme (New England Biolabs inc., Boston, USA) in 50 µl reaction containing 25µl of the PCR product, 6U of *AvaI* and 1x NEbuffer 4 (New England Biolabs inc., Boston, USA). After digestion the fragments were separated by electrophoresis in 9% polyacrylamide gel (PAGE) and visualized with ethidium bromide staining under ultraviolet (UV) light.

Detection of the *IL4* gene promoter region polymorphic site at position -590 (rs 2243250) was done by PCR and restriction fragment length polymorphism (RFLP) using primers and restriction enzyme *AvaII* as earlier described (Noguchi et al. 2001). The PCR reaction mix of total volume 50 µl contained 400 ng of template DNA, 20 pmol of each primer, 0.1mM dNTPmix (Pharmacia Biotech), 20 mM PCR buffer of $(NH_4)_2SO_4$ and 0.1% Tween 20, 5mM $MgCl_2$, 2% DMSO and 1,25U of *Taq* DNA polymerase (Fermentas, International Inc., Burlington, Canada). The PCR conditions were as follows: 94 ºC for 2 min, then 35 cycles of 94 ºC for 40 sec, 58 ºC for 40 sec and 72 ºC for 50 sec, and finally 72 ºC for 10 min. After amplification the PCR products were digested overnight in +37 ºC with *AvaII* restriction enzyme (Fermentas, International Inc., Burlington, Canada) in 25 µl reaction containing 12.5 µl of the PCR product, 5U of *AvaII* and 1x BufferR^+ (Fermentas, International Inc., Burlington, Canada). Fragments were analyzed by electrophoresis on 3.5% agarose gel stained with ethidium bromide under UV light.

The *IL10* promoter region polymorphisms at positions -592 (rs 1800872) and -819 (rs 1800871) were detected by PCR and RFLP using primers and restriction enzymes as described earlier (Mok et al. 1998, Edwards-Smith et al. 1999). The composition of PCR mixture contained 100 ng template DNA, 20 pmol of each primer, 0.1 mM dNTP mix (Pharmacia Biotech), 1x PCR buffer for DyNAzyme (Finnzymes, Espoo, Finland), 2.5 mM $MgCl_2$ and 1U of DNA Polymerase (Finnzymes, Espoo, Finland) in total volume 50 µl. The PCR conditions used were as follows: : 94 ºC for 2 min, then 35 cycles of 94 ºC for 30 sec, 60 ºC for 45 sec and 72 ºC for 1 min, and finally 74 ºC for 10 min. In case of the *IL10*-592 polymorphism, the PCR product was digested for 3 hours at 37 ºC in a 50 µl reaction containing 30 µl of the PCR product, 1x BufferY^+/TANGO^{TM} (Fermentas, International Inc., Burlington, Canada) and 5U of *RsaI* restriction enzyme (Fermentas, International Inc., Burlington, Canada). In case of the *IL10*-819 polymorphism, the PCR product was digested for 3 hours at 55 ºC in a 50 µl reaction containing 15 µl of the PCR product, 1x Mae III buffer (Roche Diagnostics, GmbH, Mannheim, Germany) and 2U MaeIII restriction enzyme (Roche Diagnostics, GmbH, Mannheim, Germany). After digestion the fragments were separated by electrophoresis in 9% PAGE and visualized with ethidium bromide staining under UV light.

Amplification of the IL10 promoter polymorphism at position -1082 (rs 1800896) was performed using the HotStarTaq kit (Qiagen, Melbourne, Australia), and 20 pmol of each primer and Q-solution was included in the PCR

mixture as earlier described (Edwards-Smith et al. 1999). The PCR conditions were 15 min at 95 ºC followed by 35 cycles of 1 min at 94 ºC, 1 min at 60 ºC and 1 min at 72 ºC and last 7 min at 72 ºC. The PCR products were digested for 3 hours at 37 ºC in 50 µl solution containing 30 µl of the PCR product,1x Buffer G^+ (Fermentas, International Inc., Burlington, Canada), 1mM BSA and 10U of *MmlI* restriction enzyme (Fermentas, International Inc., Burlington, Canada). The fragments were visualized on 3% agarose gel in UV light after ethidium bromide staining.

The *CD14* gene polymorphism at position -159 (rs 2569190) and the *TLR4* gene polymorphism at position +896 (rs 4986790) were genotyped with TaqMan® chemistry using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, CA, USA) for both PCR and allelic discrimination. Designed unlabeled PCR primers and fluorogenic TaqMan® minor groove binding (MGB) probes were used (Assay by Design, ABI, CA, USA). The universal PCR thermal cycling conditions from ABI were followed: first 50 ºC for 2 min and 95 ºC for 10 min, and then 40 cycles at 95 ºC for 15 sec and 60 ºC for 1 min. The PCR reaction was done in 25 µl reaction containing TaqMan® Universal PCR Master Mix with AmpErase® UNG (ABI, CA, USA), 1x Assay Mix (primers and probes, ABI, CA, USA) and 10-100ng of template DNA. The genotypes were selected manually from the allelic discrimination tab.

2.4. Skin prick test (Study IV)

Skin prick tests (SPT) were performed by specially trained nurses with a panel of 22 common allergen extracts (ALK A7S, Copenhagen, Denmark) in Study IV. The allergens used were birch, alder, timothy grass, meadow foxtail, mugworth, dog, cat, horse, cow dander, *Dermatophagoides farina, Dermatophagoides pteronyssinus, Acarus siro, Tyrophagus putrescentiae, Lepidoglyphus destructor, Aterbaria alernata, Cladosporium herbarum, Aspergillus fumigates,* oats, barley, barley flour, wheat flour and rye flour. The test sites were placed on the volar side of the arm. A test was considered to be positive when the diameter of the weal was at least 3 mm larger than the negative control (saline). These procedures were done in line with Position Paper of European Academy of Allergology and Clinical Immunology (EAACI 1989). The patient was considered prick test positive if at least one allergen gave a positive result. Allergy testing by the skin prick method was carried out on 99.1% of the asthmatic and 99.3% of control subjects. Positive reactions to specific allergens and differences between asthmatics and controls have been published earlier (Karjalainen et al. 2002).

2.5. Measurement of serum total IgE (Studies IV-V)

In Study IV serum total IgE determinations were carried out by the immunoluminometric method (Ciba Corning Diagnostics, Halsted, U.K.)

according to manufacturer's instructions. Serum total IgE was measured using the ImmunoCAP® fluoroenzyme immunoassay (Phadia Diagnostics, Uppsala, Sweden) according to manufacturer's instructions in Study V.

2.6. Statistical analyses (Studies I-V)

For skewed continuous variables non-parametric statistics (Mann-Whitney U-test, Kruskall-Wallis test) were used. Frequencies were compared using the chi-square test. Student's t-test was used to analyze differences in mean values in Study III. Logistic regression analysis was used to define relationships between factors in Studies II and III. Logistic regression was also used to analyze interaction between *H.pylori* serology and *IL4* genetics on the risk of atopy and asthma in Study IV. Multiway ANOVA was used for modeling gene-environment interactions on serum total IgE levels, which were logarithmically transformed, in Studies IV and V. Bonferroni correction was used for p-values of plasma cytokine levels in study II and for association analyses with serum total IgE in Study V. Statistical calculations were carried out on Statistica software (ver Win 5.1D, StatSoft, Tulsa, OK, USA) and SPSS software (ver. 6.0, 9.0, 10.1., 11.5, 12.0 and 14.0, SPSS inc., Chicago, IL, USA). To test the fit of genotype frequencies with the Hardy-Weinberg equilibrium the Arlequin program (ver. 1.1 and 2.0, Genetics and Biometry Laboratory, Geneva, Switzerland) was used. In Study IV odds ratios and 95% confidence intervals were calculated using CIA software (ver 1.1., copyrighted by M.J. Gardner and British Medical Journal 1989). P values <0.05 were considered statistically significant.

2.7. Ethics (Studies I-V)

The ethical committee of the Tampere University Hospital approved the study plans for Studies I-IV. The ethics board of the Finnish Red Cross Blood Transfusion Centre gave their approval for human blood use in Studies I and III. The study protocol for Study V was approved by the Ministry of Health, Karelian Republic Russia and the collection of samples was organized by the Department of Pediatrics, University of Petrozavodsk. Written informed consent was obtained from subjects' parents for Studies I-III, from the participants in Study IV and from both parents and participants in Study V.

Results

1. Effect of *IL1B*-511 gene polymorphism on febrile seizures (Study I)

Interleukin-1β is a well known inducer of fever, which is a risk for FSs (Berg AT et al. 1995). *IL1B*-511 polymorphism seems to be functional possibly affecting IL-1β production as discussed in the review of the literature. Therefore we wanted to ascertain whether there is an association between FSs and *IL1B*-511 polymorphism.

Thirty-five out of 55 FS patients with DNA samples obtained were included in this study. The mean age of the 35 FS patients was 19.2 months (range 6 months to 36 months). Seventeen (49%) patients were under 18 months old. Forty-nine percent of the children were male (n= 17). The mean temperature was 39.6 ºC (95% CI 39.4-39.9) when hospitalized. Twenty-three (66%) children had seizure duration less than 5 minutes and only two children (5.5%) had seizures lasting over 15 minutes. Nine children (26%) had a positive family history of FS in first-degree relatives and 4 children (11%) had a previous history of FS. Most of the children had no signs of bacterial infection and their diseases were classified as viral infections (n=23).

The genotype frequencies of *IL1B*-511 polymorphism did not significantly differ between FS patients and healthy blood donors (p=0.1). However, the frequency and carriage of allele T (=allele 2) of *IL1B*-511 was significantly increased in FS patients compared to controls (0.54 vs. 0.41, p = 0.03, 0.80 vs. 0.64, p=0.05 respectively). The genotype frequencies followed the Hardy-Weinberg equation.

Allele frequencies and allele T carrier status of *IL1B*-511 did not differ between children with or without positive family history of FS. Type of infection (viral vs. bacterial) was not associated with *IL1B*-511 allele frequencies or the allele T carrier status in FS children. Due to the small number of FS children, analyses of genotype distributions according to family history of FS and type of infection could not be conducted.

2. Plasma and cerebrospinal fluid cytokines and febrile seizures (Study II)

The balance between pro- and anti-inflammatory cytokines is crucial in the pathogenesis of many diseases and, it for example, has a role in the regulation of fever (Dinarello 2005). Therefore disturbances in this balance may have an effect on FSs. In Study II we wanted to investigate whether pro- and anti-inflammatory cytokines and the balance between them have an effect on FSs.

Fifty-five FS patients were included in this study. Fifteen (27%) children had a positive family history of FSs in first-degree relatives and eight (15%) had a positive history of FSs. Thirty-seven (67%) children had seizure duration less than 5 minutes and only two (3.6%) children had FS lasting over 15 minutes. Children with FSs did not differ from the twenty control children with febrile illness without convulsions and without history of FS by gender, age, type of infection, duration of fever before the blood sample, fever when hospitalized or laboratory data.

2.1. Plasma cytokines and febrile seizures

FS patients had significantly higher plasma IL-1Ra levels, plasma IL-1Ra/IL-1β ratio and plasma IL-6 levels than control children, as shown in Table 3. There was a trend for lower plasma IL-1β levels in FS children compared to controls. However, when multiple testing was taken into account by Bonferroni correction (=multiplying by the number of cytokines measures (n=5)), this difference was not statistically significant (P=0.1). Differences in IL-1Ra and IL-6 levels were so remarkable that they remained significant even after Bonferroni correction. There was no difference in plasma IL-10 or plasma TNF-α levels between FS and control patients. The cytokine plasma levels and the plasma IL-1Ra/IL-1β ratio are presented in Table 3.

Logistic regression analysis was used to identify the most significant factors associated with FSs. Cytokine plasma levels, age and fever at the time of hospitalization were divided into two groups using median value as a cut-off point. In the univariate logistic regression analysis we included the plasma cytokines studied, age, sex, type of infection, fever at the time of hospitalization and duration of fever before the blood sample. Significant associations were found between FSs and high plasma IL-1Ra levels (odds ratio (OR) 6.5, 95% confidence interval (CI) 1.9-22.0), FSs and high plasma IL-1Ra/IL-1β ratio (OR 36.0, 95% CI 4.5-289.9) and FSs and high plasma IL-6 levels (OR 4.2, 95% CI 1.4-13.8). These significant variables were included in the multivariate logistic regression analysis. In this analysis the high plasma IL-1RA/IL-1β ratio was the most significant factor associated with FSs (OR 41.5, 95% CI 4.9-352.8). High plasma IL-6 levels were also significantly associated with FSs (OR 5.3, 95% CI 1.4-20.3), but in this model plasma IL-1Ra did not have a discrete role.

2.2. Cerebrospinal fluid cytokines in febrile seizures

CSF samples were available of 16 FS patients. The CSF samples had cell count < 5/µl and normal protein level 0.24 - 0.5 g/l. Unfortunately, the volume of some samples was not enough for all cytokine analyses. IL-6 levels were detectable in all 16 FS children studied (median 9.4 pg/ml, interquartile range 5.7-16.2 pg/ml), IL-10 levels in 10 of 16 (median 7.2 pg/ml, interquartile range 0-19.5 pg/ml) and IL-1Ra levels in nine of 12 FS children (median 170.0 pg/ml interquartile range 0-213.0 pg/ml). Only one out of 10 children had measurable IL-1β levels and TNF-α was undetectable in all the 15 children studied. No significant correlations between IL-1Ra, IL-6 and IL-10 plasma and CSF levels were found among FS children (unpublished data).

2.3. IL1B-511C>T polymorphism and plasma cytokines in febrile seizures

Plasma IL-1β levels were not associated with *IL1B*-511 genotype (p=0.7) or allele T carrier status (p=0.4) in Finnish FS children, nor were there any associations between IL-1Ra, IL-6, TNF-α, IL-10 levels and *IL1B*-511 polymorphism in this population (unpublished data).

Table 2. Plasma cytokine levels in febrile seizure and control children.

Cytokine	FS patients Median pg/ml (25-75%)	Control children Median pg/ml (25-75%)	P-value
IL-1Ra	8450 (4875.0-11075.0)	2860 (1485.0-6110.0)	0.0001
IL-1β	10.1 (5.9- 18.9)	24.9 (7.5-118.4)	0.02
IL-1Ra/ IL-1β	790.0 (319.8-1592.4)	105.0 (42.4-257.6)	<0.0001
IL-6	19.6 (13.6-37.0)	10.5 (5.7-16.4)	0.001
IL-10	14.8 (10.1.-28.6)	21.4 (8.3-32.6)	0.5
TNF-α	0 (0.0-3.04)	0.8 (0.0-0-3.4)	0.5

* Mann-Whitney U-test

3. Effect of *IL10* promoter haplotype on Epstein-Barr virus infection (Study III)

IL10 promoter polymorphism at position -1082 has been associated with susceptibility to EBV infection (Helminen et al. 1999). In Study III we wanted to ascertain, whether *IL10* promoter haplotype -1082/-819/-592 influences EBV seroconversion. Mean age of the 116 study children was 6.7±4.9 years ranging from 1 year to 15 years. Forty-five (39%) children were under 2 years of age, 41 (35%) children between 2 and 10 years and 30 (26%) children over 10 years of age. Thirty-eight percent (44/116) of the children were seropositive for EBV. As expected, seropositivity was more common among older children: 13.3 % (n=6) of the children under 2 years old, 46.3% (n=19) of the children between 2 and 10 years and 63.3% (n=19) of the children over 10 years were seropositive for EBV. Among adult blood donors 95% (380/400) were seropositive for EBV.

The *IL10* -1082/-819/-592 promoter haplotype frequencies did not differ between study populations and were GCC 0.44, ACC 0.36, ATA 0.20 in children, GCC 0.51, ACC 0.32 and ATA 0.17 in neonates and GCC 0.43, ACC 0.35 and ATA 0.22 in blood donors. Among children *IL10* haplotype ATA was more common in EBV seronegative compared to seropositive subjects (44% vs. 25%, p=0.035). In further analysis by logistic regression, ATA positivity was significantly associated with EBV seronegativity when controlled by age (OR 2.6, 95% CI 1.04-6.7, P=0.04). No significant association between carriage of two other haplotypes GCC and ACC and EBV seropositivity was found.

ACC haplotype carriage was significantly more common in the EBV seropositive adults compared to seronegative adults (61% vs. 25% respectively, p=0,004). In blood donors neither GCC nor ATA haplotype carrier status was associated with EBV seropositivity. However, the seronegative adults were more often homozygous for the GCC haplotype (GCC/GCC) compared to seropositive (55% vs. 17% respectively, p<0.01).

In both adults and neonates the *IL10* haplotype ATA carriers had higher plasma IL-10 levels than non-carriers. In adults the ACC and the GCC haplotype carrier statuses were not associated with plasma IL-10 levels, whereas in neonates GCC haplotype carriers had significantly lower IL-10 levels than GCC haplotype non-carriers (p=0.001). In neonates ACC haplotype did not have effect on plasma IL-10 levels.

4. Effect of *IL4*-590 C>T polymorphism and *Helicobacter pylori* on skin prick test positivity (Study IV)

4.1. Associations

H. pylori and *IL4*-590C>T polymorphism have both been associated with atopy. IL-4, which induces IgE production, has also been associated with *H.pylori* infection (Smythies et al. 2000). However, no studies investigating the interaction between these two factors on atopy risk are available. Thus we wanted to investigate in Study IV whether *H.pylori* and *IL4*-590 polymorphism have interactions that have an effect on sensitization defined by SPT.

The 245 asthmatics and 405 non-asthmatic controls did not differ by age, gender or history of smoking. Fifty-five percent of asthmatics had at least one positive reaction in SPTs whereas only 38% of controls had positive SPT results. This difference was significant, as reported earlier ($p<0.001$) (Karjalainen et al. 2002). Controls were more often seropositive for *H.pylori* than asthmatics (60% vs. 47%), but this difference was not statistically significant $p=0.3$). Six asthmatics and 9 controls were excluded from further analysis due to lack of results from all measurements needed (=SPT, *H.pylori* antibodies and *IL4*-590 polymorphism).

An association between *H.pylori* seropositivity and SPT positivity was found in Finnish adults. *H.pylori* seronegativity was associated with increased SPT positivity in both asthmatic and control groups (OR 2.28, 95%CI 1.35-3.85 and OR 1.59, 95%CI 1.06-2.39 respectively). Most of the SPT positive subjects in both asthmatic and control groups had more than one positive reaction (78.4% and 66.2% respectively). When SPT positive subjects were divided into monosensitized (=one positive SPT reaction) and plurisensitized (=more than one positive SPT reaction), *H.pylori* seropositivity was associated with dimished SPT positivity only in plurisensitized subjects in both asthmatics ($p=0.0005$) and controls ($p=0.004$).

IL4-590 genotype frequencies and allele T carrier status did not differ between asthmatics and controls ($p=0.3$ and $p=0.1$ respectively). When the subjects were divided into subgroups according to *H.pylori* seropositivity, *IL4*-590 allele T carrier status was associated with asthma only in the *H.pylori* seronegative group ($p=0.03$). This association was not seen in *H.pylori* seropositive subjects ($p=0.9$). Neither *IL4*-590 genotypes nor the *IL4*-590 allele T carrier status was statistically associated with SPT positivity even when SPT positive subjects were divided into mono- and plurisensitized. However, the asthmatic *IL4*-590 allele T carriers had decreased risk for *H.pylori* seropositivity (OR 0.49, 95%CI 0.29-0.82). This effect was independent of atopic status and was not seen in controls. *IL4*-590 genotype frequencies followed the Hardy-Weinberg equation in both asthmatics and controls.

4.2. Interactions

Interaction between *IL4*-590 polymorphism and *H.pylori* seropositivity on SPT positivity was analyzed in the logistic regression model, but no interaction was found in either asthmatics or controls, nor was there any interaction between *H.pylori* and *IL4*-590C>T polymorphism on serum total IgE studied by multiway ANOVA model. In these study populations serum total IgE was not associated with *IL4*-590 polymorphism or *H.pylori* seropositivity (unpublished data).

5. Effect of *CD14*-159 C>T polymorphism and *Helicobacter pylori* on serum total IgE (Study V)

5.1. Associations

CD14-159C>T polymorphism has been associated in many studies with serum total IgE levels, but the results have been inconsistent. There is some evidence that environmental factors like exposure to endotoxin, tobacco smoke, farming environment and animals may modulate this association (Choudhry et al. 2005, Eder et al. 2005, Leynaert et al. 2006, Williams et al. 2006). However, the effect of microbes on this association has not been studied. Therefore we wanted to investigate in Study V if there were interactions between *CD14*-159 polymorphism and *H.pylori* or between *CD14*-159 polymorphism and *T.gondii* affecting serum total IgE in Russian Karelian children. TLR4 has an important role in the same functional pathway in endotoxin response as CD14 and therefore we included asthma associated *TLR4* polymorphism +896A>G, in this study (Fagerås Böttcher et al. 2004).

The Russian Karelian children were 7.1-15.0 years old (mean age 11.4 years) and 43% (n=114) of them were male. The median of serum total IgE was 76.1 IU/L (interquartile range 30.9-236.0 IU/L). Sex did not have an effect on serum total IgE levels (p=0.8). In these Russian Karelian children seropositivity for *H.pylori* and *T.gondii* was common: 73% (n=193) of children were seropositive for *H.pylori*, 24% (n=63) for *T.gondii* and 20% for both these microbes.

T.gondii seropositive children had significantly higher serum total IgE levels than seronegative (median 114.0 IU/L, interquartile range 44.0-393.0 vs median 68.5 IU/L, interquartile range 28.3-174.3 respectively, p=0.009), as we have reported earlier (Seiskari et al. 2007). This difference was statistically significant even when multiple testing was taken into account using Bonferroni correction (p=0.036). *H.pylori* seropositivity did not have an effect on serum total IgE (in the seropositive group IgE median 76.8 IU/L and in seronegative median 68.2 IU/L, p=0.86) as reported earlier (Seiskari et al. 2007). Because most of the *T.gondii* seropositive subjects were also *H.pylori* seropositive further analysis between *H.pylori* and *T.gondii* seropositive and negative subgroups was done by Kruskall-Wallis ANOVA. A significant difference between groups was found

such that *T.gondii* seropositive but *H.pylori* seronegative children had the highest serum total IgE levels compared to other groups. The association between subgroups and serum total IgE is presented in Table 3**.**

CD14-159 genotype and allele T carrier status were not associated with serum total IgE or with *H.pylori* or with *T.gondii*, nor were there associations between *TLR4*+896 genotype or the allele G carrier status with serum total IgE or with *H.pylori* or with *T.gondii*. The genotype distributions of both polymorphisms followed the Hardy-Weinberg equilibrium.

Table 3. Serum total IgE levels in *Helicobacter pylori* and *Toxoplasma gondii* seropositive and seronegative Russian Karelian children.

		Serum total IgE	
Seropositivity	**N (%)**	**median (interquartile range)**	**P-value***
H.pylori and *T.gondii* negative	61 (23)	59.3 (28.7-166.0)	
H.pylori positive and *T.gondii* negative	139 (53)	75.8 (27.3-184)	
T.gondii positive and *H.pylori* negative	10 (4)	429.0 (37.6-1086.0)	
H.pylori and *T.gondii* positive	53 (20)	84.4 (44.9-292.5)	0.043

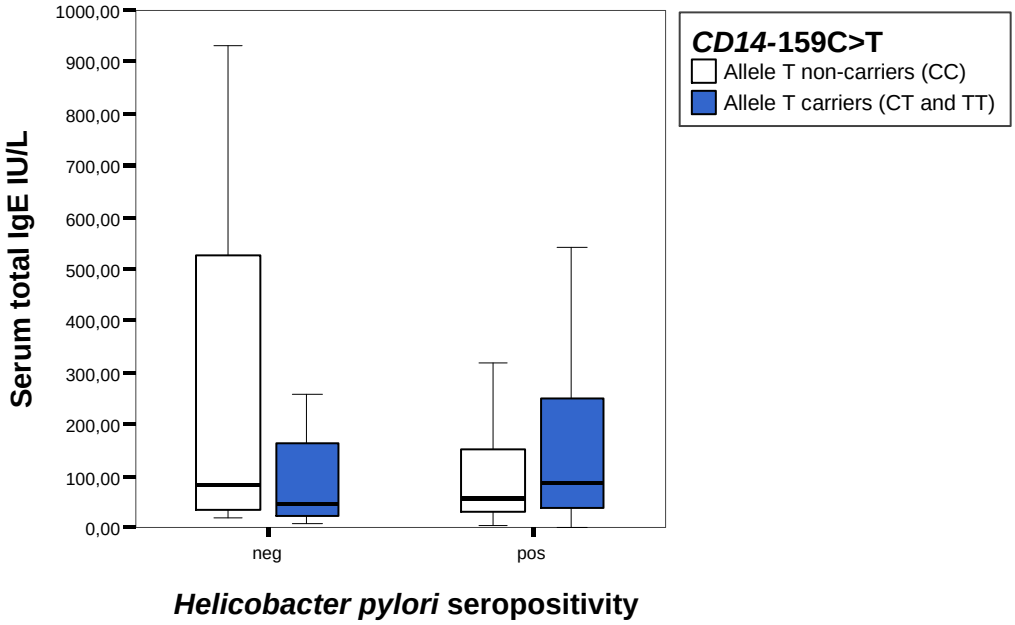
*Kruskall-Wallis ANOVA

5.2. Interactions

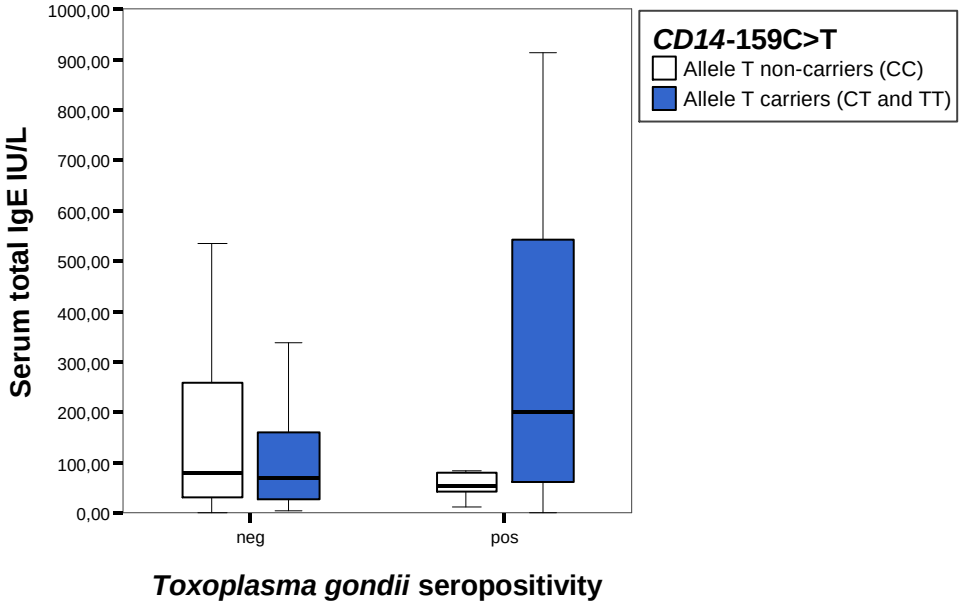
Gene-environment and gene-gene interactions on serum total IgE were analyzed by a multiway ANOVA model. A statistically significant interaction between *H.pylori* seropositivity and the *CD14*-159 allele T carrier status on serum total IgE was found (p=0.004). Those *H.pylori* seronegative children who were *CD14*-159 allele T non-carriers (i.e. genotype CC) had higher serum total IgE levels than allele T carriers (i.e. genotypes CT and TT). However, in *H.pylori* seropositive children allele T non-carriers had lower IgE levels than allele T carriers (Figure 3). There was a trend for interaction between *T.gondii* seropositivity and the *CD14*-159 allele T carrier status on serum total IgE, but this interaction was not statistically significant (Figure 3). There were no statistically significant interactions between *TLR4*+896 and *H.pylori* or *TLR4*+896 and *T.gondii* on serum total IgE in this population. No statistically significant interaction between *CD14*-159 allele T carrier status and *TLR4*+896 allele G carrier status on serum total IgE was found. Moreover, there was no interaction between *T.gondii* and *H.pylori* on serum total IgE.

Figure 4.

A) Effect of *CD14*-159 allele T carrier status and *Helicobacter pylori* seropositivity on serum total IgE in Russian Karelian children (p=0.004, multiway ANOVA). Medians and interquartile ranges are shown in the picture.



B) Effect of *CD14*-159 allele T carrier status and *Toxoplasma gondii* seropositivity on serum total IgE in Russian Karelian children (p=0.06, multiway ANOVA). Medians and interquartile ranges are shown in the picture.



Discussion

1. IL-1β and febrile seizures

1.1. Association between IL1B-511C>T polymorphism and febrile seizures

An association between the *IL1B*-511C>T allele T and FSs was reported for the first time in our study (Study I). This finding has only been replicated in one Japanese study, in which *IL1B*-511 allele T and *IL1B*-31 allele C were associated with sporadic simple FSs (Kira et al. 2005). In another Japanese study prolonged FSs strengthened the association between the *IL1B*-511 allele T and TLE+HS, but there was no association between FSs and *IL1B*-511 polymorphism in this study (Kanemoto et al. 2003). In addition, no association between FSs and *IL1B*-511 polymorphism was found in German, Taiwanese, Turkish and Japanese studies (Tilgen et al. 2002, Chou et al. 2003, Haspolat et al. 2005, Matsuo et al. 2006). Some of the contradictory results might be explained by differences in sampling, definition of FSs and ethnic backgrounds. Another significant reason behind these inconsistent results could be the small sample size in every study, which causes low power to detect genetic effects that most probably are small in multifactorial diseases like FSs. In a recent meta-analysis no statistically significant relationship between *IL1B*-511 polymorphism and FS or TLE risk was found. However, TLE+HS risk was found to be increased among *IL1B*-511 allele T homozygous individuals in this meta-analysis, suggesting that this polymorphism may play some role in convulsions (Kauffman et al. 2008).

The major limitation of Study I was the small sample size, which increases the risk of positive findings by chance**.** It is also possible that some other polymorphisms which are in linkage disequilibrium with *IL1B*-511, like *IL1B*-31, are actually more relevant in the pathogenesis of FSs than *IL1B*-511 polymorphism. Because simple and complex FSs seem to have different genetic background (Baulac et al. 2004, Waruiru et al. 2004), it would have been interesting to compare *IL1B*-511 genotype and allele frequencies between simple and complex FSs, but it was not statistically possible due to the small number of study subjects in Study I.

1.2. Associations between cytokines and febrile seizures

The relationship between IL-1β and FSs has been investigated in recent years because IL-1β is a well known pyrogen and may therefore play a role in FSs. In some studies an association has been found (Helminen et al. 1990, Tütüncüoglu et al. 2001, Haspolat et al. 2005, Matsuo et al. 2006). However, the results have been inconsistent and in many studies no association between IL-1β and FSs has been seen (Lahat et al. 1997, Ichiyama et al. 1998, Tomoum et al. 2007). In Finnish children plasma IL-1Ra and IL-6 levels as well as IL-1Ra/IL-1β ratio were higher in FS patients than in children with febrile illness without convulsions (Study II). Plasma IL-1β levels did not differ between FS and control children in Study II. However, high IL-1Ra plasma levels in FS patients may indicate preceding high IL-1β production at the beginning of the infection, because production of IL-1Ra is stimulated by IL-1β and a strong initial IL-1β response could lead to high IL-1Ra levels later during infection (Dinarello 1996a).

Plasma IL-1Ra/IL-1β ratio was the most significant factor associated with FSs in Finnish children (Study II). The mechanism behind this finding is not known. However, inflammatory reactions, including fever, are regulated by both pro- and anti-inflammatory cytokines (Mackowiak et al. 1997, Dinarello 2005). Therefore the balance between IL-1Ra and IL-1β could be more important than a single cytokine in regulating fever during infection. It could be speculated that the vast excess of IL-1Ra seen in FS patients could be produced to neutralize the effect of IL-1β produced earlier. However, increased IL-1Ra levels in FS patients could also be a consequence of seizure (Eriksson et al. 1998).

In earlier studies the authors have speculated that the intrathecal presence of cytokines could be used to differentiate FSs from CNS infections (Azuma et al. 1997, Ichiyama et al. 1998). According to our results, detectable IL-6, IL-10 or IL-1RA CSF levels cannot be used for this purpose, because these cytokines were also found in FS children without any signs of CNS infection. However, the sample size was too small to permit firm conclusions so larger studies are needed to ascertain the role of CSF cytokines in differentiation of CNS infections from harmless FSs.

2. Association between *IL10* promoter haplotype and EBV infection

The *IL10* promoter haplotype ATA consisting of three SNPs at positions -1082 (A>G), -819 (C>T) and -592 (A>C) was associated with EBV seronegativity in Finnish children, but not in adult blood donors, among whom increased frequency of GCC/GCC haplotype was seen in EBV seronegative subjects instead (Study III). According to these results it could be speculated that even though ATA haplotype seems to be associated with delayed primary EVB

infection it does not seem to be associated with EBV seronegativity itself. It would have been interesting to investigate whether this haplotype is associated with IM, because *IL10*-1082 allele A involved in ATA haplotype has been associated with more severe forms of IM in Finnish adults (Helminen et al. 1999). However, the study population was not suitable for this investigation.

The *IL10* ATA haplotype was associated with increased plasma IL-10 levels in healthy Finnish blood donors and neonates. Even though IL-10 is considered an anti-inflammatory cytokine, it has been shown to enhance the activation of NK cells by EBV transformed B cells, to increase NK cell activity and decrease viral replication and thereby to enhance immune response against EBV (Stewart et al. 1992, Kurilla et al. 1993). Therefore it could be speculated that the high spontaneous IL-10 plasma levels seen in *IL10* ATA haplotype carriers could result in a strong initial antiviral effect thus delaying the onset age of primary EBV infection. However, ATA haplotype has also been associated with decreased IL-10 levels or IL-10 production in some studies, which does not support this hypothesis (Crawley et al. 1999, Edwards-Smith et al. 1999, Hulkkonen et al. 2001). It would have been interesting to analyze whether association between IL-10 levels and *IL10* haplotype is influenced by acute EBV infection, but it was not possible because samples were not taken during acute illness.

The most significant weakness of this study was the relatively small study population for purpose of genetic analyses. It should also be noted that socioeconomic and other environmental aspects were not investigated in this study, because the subgroups would have been too small for statistical analysis. However, it is well known that these factors influence the onset age of EBV infection (Crawford 2001, Junker 2005).

3. Association between *IL4*-590C>T polymorphism, *Helicobacter pylori* and skin prick test

In Finnish asthmatic and non-asthmatic adults *H.pylori* seropositivity was associated with SPT positivity so that among *H.pylori* seropositive subjects SPT positivity to more than one allergen (=plurisensitizatoin) was decreased (Study IV). This finding is in line with other studies, in which *H.pylori* has been shown to be associated with decreased risk of atopy (McCune et al. 2003, von Hertzen et al. 2006, Seiskari et al. 2007, Konturek et al. 2008). This result also supports the hygiene hypothesis according to which exposure to pathogens may reduce the risk of atopy (Strachan 1989, von Mutius 2007). However, an association between *H.pylori* and decreased risk of atopic phenotypes was not seen in every study (Bodner et al. 2000, Uter et al. 2003, Law et al. 2005).

The exact mechanism by which *H.pylori* may affect the risk of atopy is not known, but it has been speculated that it could be related to the increased Th1 activity associated with *H.pylori* infection (von Mutius 2000). Increased IL-10 expression associated with *H.pylori* in some studies has been suggested to be

another possible mechanism by which *H.pylori* might be related to reduced risk of atopy, because there is mounting evidence that IL-10 has an important role in down-regulation of atopic response (von Hertzen et al. 2006, Oderda et al. 2007, von Hertzen et al. 2009). However, because *H.pylori* has been associated with growing up in a rural area, low socioeconomic status and poor living conditions like absence of fixed hot water supply, contaminated water and domestic overcrowding in childhood, it is possible that *H.pylori* is only a surrogate marker of environmental factors associated with poor hygiene and low socioeconomic status (Mendall et al. 1992, Malaty et al. 1994, Brown 2000). Therefore this association between *H.pylori* and reduced risk of atopy may, at least partly, be mediated by other infectious agents, like parasites, or by other environmental factors associated with low socioeconomic status mentioned above.

The *IL4*-590 allele T associated with increased IL-4 production has been related to atopic asthma in a current meta-analysis (Li et al. 2008). The *IL4*-590 allele T has also been associated with other atopic phenotypes like elevated serum total IgE, atopic dermatitis, rhinitis and sensitization defined by SPT positivity (Rosenwasser et al. 1995, Zhu et al. 2000, Söderhall et al. 2002). However, in many studies, including our study on Finnish asthmatic and non-asthmatic adults (Study IV), *IL4*-590 polymorphism has not been associated with atopy (Walley et al. 1996, Elliott et al. 2001). However, when the Finnish adults were further divided into subgroups according to *H.pylori* seropositivity, the *IL4*-590 allele T was positively associated with asthma, but only in *H.pylori* seronegative subjects. According to these results it could be speculated that the association between *IL4*-590 polymorphism and atopic phenotypes may be so modest that the association is only seen when environmental factors affecting susceptibility are absent.

In addition, in Finnish asthmatics the *IL4*-590 allele T was associated with decreased *H.pylori* seropositivity. However, this association was not seen in the non-asthmatic group. The significance of this finding is not known and it is inconsistent with other studies in which the *IL4*-590 allele T has been shown to increase the risk of *cagA* positive *H.pylori* infection or in which no association between *H.pylori* and *IL4*-590 polymorphism has been found (García-González et al. 2007, Zambon et al. 2008). However, it could be speculated that *IL4*-590 polymorphism could modify the relationship between infectious agent and atopy by affecting the host susceptibility to microbes, which for one might modulate the susceptibility to atopic diseases. Nevertheless, the association between the *IL4*-590 allele T and *H.pylori* seropositivy seen in Study IV could also be a false positive finding.

Gene-environment interactions may be more relevant than a single SNP in atopy because many environmental factors, like infections, seem to be associated with atopy risk in addition to genetics (Beghé et al. 2003). However, in Finnish adults no interaction between *IL4*-590 polymorphism and *H.pylori* seropositivity on SPT positivity was found, suggesting that association between *H.pylori* and SPT positivity is not influenced by this polymorphism.

4. Association between *CD14*-159C>T polymorphism, *Helicobacter pylori* and serum total IgE

Serum total IgE is regulated by both genetic and environmental factors. Among Russian Karelian children neither *CD14*-159 polymorphism nor *H.pylori* seropositivity was associated with serum total IgE (Study V). The results of earlier studies analyzing associations between *CD14*-159C>T polymorphism and serum total IgE and atopy have been conflicting because the same genetic variants have been associated with both increased and decreased risk of atopy in different environments (Baldini et al. 1999, Ober et al. 2000, Eder et al. 2005). It has been speculated that the effect of *CD14*-159 polymorphism on atopy is quite modest and dependent on co-existing environmental risk factors. Thus recently investigated gene-environment interactions could be more relevant and explain some of the inconsistent results (Martinez 2007, Zhang et al. 2008).

Endotoxin exposure, contact with animals in childhood, exposure to ETS and farming environment in early life have been reported to interact with *CD14*-159 polymorphism in the modulation of serum total IgE (Choudhry et al. 2005, Eder et al. 2005, Leynaert et al. 2006, Williams et al. 2006). In Russian Karelian children a statistically significant interaction between the *CD14*-159 allele T carrier status and *H.pylori* seropositivity associated with serum total IgE was found (Study V). This was the first report of gene-environment interaction between specific microbe and *CD14*-159 polymorphism on serum total IgE. In this population *H.pylori* seronegative *CD14*-159 allele T non-carriers had higher serum total IgE than allele T carriers. Among *H.pylori* seropositive children allele T non-carriers had lower IgE levels than allele T carriers. This result supports the hypothesis that the same genotype may increase, decrease or have no effect on risk of a certain phenotype, in this case serum total IgE, depending on the environmental factors, such as microbes, to which the population is exposed (Martinez 2007). Our result emphasizes the role of gene-environment interaction in the regulation of serum total IgE and suggests that *H.pylori* may be one of the microbes that modulate the genetic regulation of serum total IgE. However, caution should be exercised when interpreting interactions between microbes and *CD14*-159 polymorphism on serum total IgE, because, for example, *H.pylori* may be only a surrogate marker for poor hygiene and large microbial load as discussed in the preceding chapter. Therefore the interactions detected could either reflect interaction between socioeconomic factors associated with *H.pylori* or the whole microbial burden and *CD14*-159 polymorphism as well as the interaction between a specific microbe and the polymorphism.

A limitation of this study was that specific IgE could not be used in gene-environment interaction analyses because the number of atopic subjects in the study population was too small for statistical analyses (n=16). Many factors like allergens and parasite infections influence serum total IgE levels and therefore the interpretation of the results is difficult. In our earlier study we found that serum total IgE levels were significantly higher in Russian Karelian children

than in Finnish children even though the sensitization to common allergens measured by allergen specific IgE was lower in Russian Karelian children. This suggests that other factors, like parasite infections, could modulate serum total IgE in this population (Seiskari et al. 2007).

Multiple testing also complicates the interpretation of the results. However, the interaction between the *CD14*-159 allele T carrier status and *H.pylori* seropositivity on serum total IgE was so significant that it most probably would not disappear even though multiple testing were done (i.e. remaining significant after Bonferroni correction). The major limitation of our study was the small number of study subjects; therefore these gene-environment analyses should be repeated in larger populations.

5. Candidate gene studies

Studies I, III, IV andV are based on the candidate gene approach. This approach has been widely used to identify alleles, which may have a role in the pathogenesis of different diseases even though this approach has many limitations. Four criteria for candidate gene studies of complex diseases have been suggested: consistent results, location of the gene in a chromosomal area of linkage, change in protein level or function by the mutation and biological plausibility of the gene for the disease (Hall 1999). Similar issues have been emphasized in other articles (NatureGenetics 1999, Tabor et al. 2002). However, these criteria are rarely fulfilled. The main problem with candidate gene studies seems to be conflicting and unreplicable results. In addition, the variety of phenotype definitions and ethnic differences between the populations makes interpretation very challenging in the candidate gene studies. Another difficulty in the candidate gene approach is that a single gene or SNP usually makes only a small contribution to the susceptibility or severity of multifactorial disease (Zhang et al. 2008).

Considering our results according to these recommendations, some reservations are called for. All the genotypes *IL1B*-511C>T, *IL4*-590C>T, *CD14*-159C>T and the *IL10* promoter haplotype -1082/-819/-592 studied have been shown to modulate protein production. However, the data of the exact functional changes that these polymorphisms cause is still quite modest. There are biologically plausible relationships between inflammatory mediators and the clinical conditions investigated in this study as seen in the review of the literature. In addition, the roles of IL-10 in EBV infection, IL-1 in FSs, IL-4 and CD14 in atopy have been seen in many studies. Therefore functional polymorphisms in their genes may be associated with these diseases. However, the results of association studies concerning the polymorphisms and clinical conditions investigated in this dissertation are not unequivocal, as discussed earlier. The small sample size in Studies I, III, IV and V also challenges the interpretation of the results, because the small study population increases the risk of both positive findings by chance and on the other hand diminishes the power

of the study to identify alleles with small effects (Tabor et al. 2002). The results of this study should be considered taking these shortcomings into account.

Several ways to improve the candidate gene approach have been proposed. For example, gene-gene and gene-environment interactions might be more relevant than simple candidate gene in the pathogenesis of complex diseases (Martinez 2007). Therefore the scope of investigations in this dissertation was expanded from a candidate gene approach into gene-environment interactions in Studies IV and V. However, even though gene-environment interactions may explain some of the inconsistent results of the simple candidate gene approach studies, the interpretation of these results is even more difficult because of the complex nature of gene-environment interactions. For example, it has been proposed that disease phenotypes are a result of genetically determined inadequate responses to a complex variety of environmental exposures and that the genes and environmental factors may predispose to disease, protect against disease or be neutral depending the context of the interaction (Martinez 2007). Therefore further research is needed to ascertain the mechanisms behind these interactions.

Conclusions

IL-1β is a well known pyrogen and therefore it has been speculated that *IL1B* gene polymorphisms may be associated with the susceptibility or severity of FS. In Finnish children an association between *IL1B*-511 polymorphism and FSs was observed. However, in light of recent investigations it seems unlikely that this polymorphism plays a role in the etiology of FSs.

Plasma IL-1Ra, IL-6 and IL-1Ra/IL-1β ratio were increased in children with FS compared to children with febrile illness without seizure. This finding emphasizes the relationship between inflammatory cytokines and FSs reported in other studies. However, it was not possible to ascertain whether these cytokines were predisposing factors for FSs or more likely a consequence of seizure activity.

The *IL10* promoter -1082/-819/-592 haplotype ATA was associated with increased frequency of EBV seronegativity in childhood, but not in adult blood donors, suggesting that this haplotype may be more likely associated with delayed EBV infection rather than with EBV seronegativity.

In Finnish asthmatic and non-asthmatic adults *H.pylori* seropositivity was associated with decreased risk of SPT positivity, but *IL4*-590 polymorhism was not associated with SPT. In addition, no interaction between *H.pylori* seropositivy and *IL4*-590 polymorhism having an effect on SPT positivity was found. According to this result it might be speculated that the association between *H.pylori* and sensitization measured by SPT is not influenced by this polymorphism, at least in Finns.

In Russian Karelian children neither *H.pylori* seropositivity nor *CD14*-159 polymorphism were associated with serum total IgE alone. However, a significant interaction between *H.pylori* seropositivity and *CD14*-159 on serum total IgE was found, which further emphasizes the role of gene-environment interaction in the regulation of serum total IgE.

Acknowledgements

This work was carried out at the Medical School, University of Tampere, at the Department of Microbiology and Immunology, during the years 1998-2008. The study was financially supported by Tampere Graduate School in Biomedicine and Biotechnology (TGSBB), the Medical Research Fund of the Tampere University Hospital, the Tampere Tuberculosis Foundation, the Finnish Anti-Tuberculosis Association Foundation and the Väinö and Laina Kivi Foundation. They all are gratefully acknowledged.

I express my deepest gratitude to my supervisor, Professor Mikko Hurme, MD, PhD, for granting me the opportunity to prepare this dissertation in his group. His patient guidance has been a great support throughout these years.

I am deeply grateful to my other supervisor Merja Helminen, MD, PhD for clinical and scientific guidance and support during this project. Thank you for believing in me at the times when I did not believe in my own ability to complete this dissertation.

My sincere thanks go to Docent Riitta Karttunen, MD, PhD and Docent Johannes Savolainen, MD, PhD for their evaluation and constructive comments of this manuscript.

I wish to thank my co-authors Sanna Kilpinen, Tanja Pessi, Kati Ådjers, Jussi Karjalainen, Hilpi Rautelin, Timo Kosunen, Tapio Seiskari, Anita Kondrashova, Mikael Knip and Heikki Hyöty for their valuable contribution to the original communications. I want especially thank Sanna and Tanja for their help and support.

I express my sincere thanks to Heini Huhtala, MSc, for advising me with the statistical matters and making them more understandable.

I am grateful to Virginia Mattila, M.A. for careful revision of the English language of the manuscript.

I wish to thank my post and present colleagues at the Department of Microbiology and Immunology: Nina Lahdenpohja, Janne Hulkkonen, Carita Eklund, Tanja Pessi, Annika Raitala, Kati Ådjers, Marja Pertovaara, Maarit Oikarinen, Leena Teräväinen, Juulia Jylhävä, Atte Haarala, Petri Niinisalo, Tapio Kotipelto, Anita Vuorenmaa and Minna Vittaniemi. I want especially

thank Janne and Sanna for statistical advice and guidance at the beginning of this dissertation process, Carita for interesting discussions and support during the hardest times in writing this dissertation. In addition, my warmest thanks go to Sinikka Repo-Koskinen and Eija Spåre for excellent technical laboratory assistance and good sense of humor; we have shared many cheerful moments during past years.

I wish to thank my friends and colleagues Miia, Hanna, Marika, Karita, Teea, Anniina, Kirsi, Noora, Aino and Anna-Katriina for the opportunity to exchange views on scientific work, clinical work and also other aspects of life; it has meant a great deal to me.

My warmest thanks go to my dear childhood friends Hanna, Kati, Virve, Rita and Satu and their families. We have shared many things during the past twenty seven years. Thank you for standing by me throughout this dissertation, I know that it has not always been easy. Special thanks to Hanna, who always had time to listen to my troubles. I also want to thank all my other friends for their support.

I warmly thank my grandmother Sirkka Laakso, godmother Sirpa Laakso-Varis and her husband Aatos Varis, Aunt Riitta Paajanen and her family and Aunt Tiina Laakso. You have offered me encouragement during this project and tried to stop me from doing too many things at the same time.

My warmest thanks go to Jarmo, who has shared my happy moments and the deepest despair during the writing of this dissertation. Thank you for your patience and positive attitude!

I want to express my deepest gratitude to my parents Irma and Kari Virta. You have always believed in me and loved me as I am with or without academic achievements. This thesis would never have been completed without your support. Thank you!

Tampere, August 2009

Miia Virta

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Original publications

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ELSEVIER

Increased Frequency of Interleukin-1β (−511) Allele 2 in Febrile Seizures

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Febrile seizures can be the first sign of epilepsy. In a recent study, patients with temporal lobe epilepsy were reported to carry the interleukin-1β allele 2 at position −511 more often than healthy control subjects. Because pro-inflammatory cytokines, such as interleukin-1, are well-known inducers of fever and therefore could play an important part in the pathogenesis of febrile seizures, we have, in this study, analyzed the cytokine gene polymorphism of interleukin-1β at position −511 in children with febrile seizures and control subjects. We found a statistically significant increase in the frequency and the carriage of interleukin-1β (−511) allele 2 in children with febrile seizures (n = 35) compared with healthy blood donors (n = 400) ($P = 0.03$ and $P = 0.05$, respectively). In previous studies, this allele has been connected to increased in vitro production of interleukin-1. Children with febrile seizures may therefore have an increased pro-inflammatory reaction during fever. This pro-inflammatory reaction may also predispose some children to the development of epilepsy.

Virta M, Hurme M, Helminen M. Increased frequency of interleukin-1β (−511) allele 2 in febrile seizures. Pediatr Neurol 2002;26:192-195.

Introduction

Three to five percent of children between 6 months and 5 years of age have febrile seizures [1]. The seizures have a tendency to run in the family, and 20-30% of children with febrile seizures have a positive family history in first-degree relatives [1,2]. Genetic analysis of certain families has suggested that there are several genes that might be involved in the pathogenesis of febrile seizure [2,3]. However, in the majority of patients the pathogenesis is not known. Besides family history, rapid rise of fever and high temperature have been connected to increased risk of febrile seizures [1]. The genetic predisposition to febrile seizures and their association to high fever suggest that the genetics of inflammatory mediators, cytokines, might be involved in the pathogenesis of this syndrome.

Cytokines are important regulators of the immune system during infection and inflammation [4]. The effects observed during infection result from a delicate balance between pro- and anti-inflammatory cytokines. Pro-inflammatory cytokines, such as interleukin-1, are well-known inducers of fever [4]. Cytokines are also important immunomodulators in the central nervous system and are under active research in several psychiatric and neurologic disorders [5]. In experimental animal models studying epilepsy, active cytokine production has been demonstrated [6,7]. In central nervous system infections, such as encephalitis and bacterial meningitis, increased levels of both interleukin-1 and tumor necrosis factor-α have been present [8,9].

In febrile seizures, there are some conflicting reports of cytokine production [10-13]. The high fever observed in febrile seizures suggests that cytokines may play an important role in the pathogenesis of this syndrome. However, the short half-life of cytokines makes the analysis of individual cytokine levels less reliable [4]. Cytokine genes have several polymorphic sites, and there is evidence that these polymorphisms may have effect on the amount of the cytokine produced [4,14]. Therefore polymorphisms of cytokine genes could influence the pathogenesis of febrile seizures. In this study, we have analyzed the genetic polymorphisms of the interleukin-1 gene complex in children with febrile seizures and in healthy blood donors.

Patients and Methods

This study was performed between October 1997 and January 1999 at the Tampere University Hospital and University of Tampere Medical School. The study was approved by the ethical committee of the hospital.

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Received May 22, 2001; accepted September 20, 2001.

PII S0887-8994(01)00380-0 • 0887-8994/02/$—see front matter

Table 1. Clinical characteristics of febrile seizure patients

		No of Cases (%)	Mean (95% CI)
Age (mo)	<18	17 (49)	19.2 (16.3-22.2)
	≥18	18 (51)	
Temperature (°C)	38.5-38.9	7 (20)	39.6 (39.4-39.9)
	39.0-39.5	4 (11)	
	≥39.5	24 (69)	
Sex	Male	17 (49)	
	Female	18 (51)	
Duration of the febrile seizure (min)	≤5	23 (66)	
	5-15	10 (28.5)	
	>15	2 (5.5)	
Family history of febrile seizure	Yes	9 (26)	
	No	25 (71)	
	Unknown	1 (3)	
Previous history of febrile seizure	Yes	4 (11)	
	No	31 (89)	

Informed consent was obtained from the parents. Blood samples were obtained from the following two groups of patients: children with diagnosis of febrile seizures (n = 35) and healthy blood donors (n = 400). Samples from the blood donors were obtained from the Finnish Red Cross Blood Transfusion Center, Tampere, Finland. Adult blood donors were used as control subjects because the likelihood of febrile seizure in this group is below 5% [1]. Inclusion criteria for febrile seizure patients were an age of 6 months to 5 years, no other identifiable cause for the seizure, and temperature at least 38.5°C when hospitalized. Hospitalization for one night is common practice in Finland. Data regarding the family history, earlier febrile seizures, and duration of the seizure were obtained from the parents of febrile seizure patients by using a questionnaire. Family history was regarded positive when seizure was reported in first-degree relatives.

Amplification of genomic DNA by polymerase chain reaction and detection of interleukin-1β gene base exchange polymorphisms at positions −511 (C/T) were performed as described earlier [15].

Data Analysis

The chi-square test was used to compare allele frequencies between febrile seizure patients and healthy blood donors. Statistical calculations were performed using Statistica software (StatSoft Inc., Tulsa, OK).

Results

Thirty-five patients were included in the study. The mean age of the patients was 19.2 months (16.3-22.2) (Table 1). All the children had fever above 38.5°C, and 80% had seizure duration less than 15 minutes. The results of the analysis of the genetic polymorphism of interleukin-1β at position −511 are presented in Table 2. The frequency and the carriage of allele 2 of interleukin-1β (−511) gene were significantly increased in febrile seizure patients compared with healthy control subjects (0.54 vs 0.41, $P = 0.03$, 0.8 vs 0.64, $P = 0.05$, respectively). The amount of homozygous individuals for allele 2 did not significantly differ between febrile seizure patients and control subjects (0.29 vs 0.18, $P = 0.1$) When children with positive family history for febrile seizure were compared with children without such history, we could demonstrate no difference in the distribution of interleukin-1β alleles at position −511 (data not shown).

Discussion

Immune response is regulated by both pro- and anti-inflammatory cytokines. The most important pro-inflammatory cytokines are interleukin-1, tumor necrosis factor-α, and interleukin-6 [4]. In addition to pro-inflammatory activities, interleukin-1 and tumor necrosis factor-α also seem to have neuromodulatory functions in normal brain [5]. They are known inducers of sleep and anorexia during infection but also seem to influence the electrophysiology of neurons [4,5].

Febrile seizures are caused by a variety of infectious agents. Common features to typical febrile seizures include age between 6 months and 5 years, the occurrence of seizure at the rapid onset of high fever, and benign outcome [1]. In infection the function of pro-inflammatory cytokines include induction of the acute phase response including fever [4]. This finding suggests that pro-inflammatory cytokines may play a role in the pathogenesis of febrile seizures. In previous studies the results of the

Table 2. Genetic polymorphism of interleukin-1β in febrile seizure patients and healthy blood donors

	Febrile Seizure Patients (n = 35)	Healthy Blood Donors (n = 400)	*P*-value
Interleukin-1β(−511) genotype			
1.1	7 (20%)	146 (37%)	
1.2	18 (51%)	182 (45%)	
2.2	10 (29%)	72 (18%)	
Interleukin-1β(−511) allele frequency			
Allele 1	46%	59%	
Allele 2	54%	41%	0.03
Interleukin-1β(−511) allele 2 carriage			
Carrier of allele 2	80%	64%	
Noncarrier of allele 2	20%	36%	0.05

importance of fever-inducing cytokines in febrile seizures have been conflicting. Helminen and Vesikari [10] and, recently, Straussberg et al. [11] have demonstrated that interleukin-1 production of lipopolysaccharide-stimulated mononuclear cells isolated from patients with febrile seizures is increased compared with control subjects. Lahat et al. [12] found no difference in cerebrospinal fluid and blood interleukin-1β levels between patients and control subjects when measured with enzyme-linked immunoassay. Ichiyama et al. [13] were able to demonstrate increased levels of tumor necrosis factor-α, interleukin-1β, and interleukin-6 in cerebrospinal fluid from children with acute encephalitis/encephalopathy but not from children with febrile seizures. The discrepancy in these studies may be explained by differing study methods. Because of the short half-life and numerous interactions of the cytokines, individual measurements also may not reflect the true situation [4]. The genetic make-up of the individual may be more relevant.

The purpose of this study was to analyze the importance of cytokine gene polymorphism in febrile seizures. The data indicate increased frequency and carriage of the interleukin-1β (−511) allele 2 in children with febrile seizures compared with healthy blood donors. Interleukin-1 induces fever by stimulating local production of prostaglandins which in turn raise the set point of the thermoregulatory center [16]. There are at least two polymorphic sites within the interleukin-1β gene: at position −511 in the promoter region and at position +3953 in the fifth exon [14,15]. In previous studies, interleukin-1β allele 2 at position −511 has been connected to increased production of this cytokine [17]. Therefore the increased occurrence of allele 2 observed in this study may suggest that interleukin-1 production is elevated in febrile seizures. The elevated production of interleukin-1 during febrile seizures likely explains the high fever typically observed at the onset of seizure. However, this study cannot distinguish the impact of interleukin-1 genetics on fever and seizure activity independently. Also, interleukin-1 genetics cannot be the only determinant factor in febrile seizures.

The importance of interleukin-1 and its family in epilepsy has been reported previously in experimental studies. Increased mRNA levels of interleukin-1β, interleukin-1 receptor antagonist, and interleukin-1 receptor expression have been observed by in situ hybridization in kainic acid-induced seizures [6,7]. Whether the supposedly increased production of interleukin-1 that was observed in this study is connected to the seizure or simply reflects the high fever typically connected to febrile seizures is unknown. However, approximately 30% of patients suffering from temporal lobe epilepsy with hippocampal sclerosis have febrile seizures in their childhood [18]. In a recent study, Kanemoto et al. [19] demonstrated that patients with temporal lobe epilepsy and hippocampal sclerosis were more often homozygotes for interleukin-1β allele 2 at position −511 than temporal lobe epilepsy patients without hippocampal sclerosis or healthy control subjects, suggesting that interleukin-1β may be relevant in the pathogenesis of this entity. The increased carriage of interleukin-1β allele 2 at position −511 observed in the study of Kanemoto et al. in temporal lobe epilepsy patients and in this study in children with febrile seizures supports the theory that febrile seizures connected to increased interleukin-1β production may predispose to the development of epilepsy. This finding may help neurologists to find the children with febrile seizures that are at risk of developing future seizures or epilepsy.

This work was supported by a grant from The Research Fund of Tampere University Hospital. The authors would like to thank Ms Mervi Salomäki for expert technical assistance.

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CONCISE COMMUNICATION

Susceptibility to Primary Epstein-Barr Virus Infection Is Associated with Interleukin-10 Gene Promoter Polymorphism

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In total, 116 children were investigated to determine whether the interleukin (IL)–10 polymorphism influences the age at primary Epstein-Barr virus (EBV) infection. The promoter of IL-10 is polymorphic, with 3 known single base substitutions (G/A at −1082, C/T at −819, and C/A at −592), which form 3 haplotypes: GCC, ACC, and ATA. This study found that carriage of the ATA haplotype protects against early EBV infection. The presence of the ATA haplotype was associated with EBV seronegativity (odds ratio, 2.6; 95% confidence interval, 1.04–6.7; $P = .04$), when controlled by age. To examine the effect of haplotypes on IL-10 production, IL-10 plasma levels were measured in 50 newborns and 400 adults and were correlated with the IL-10 haplotype. The IL-10 levels were significantly higher in the ATA carriers than in the noncarriers. These data suggest that the IL-10 ATA haplotype confers protection against primary EBV infection and that the effect is mediated by high IL-10 levels.

Primary Epstein-Barr virus (EBV) infection usually occurs within the first years of life. At an early age, the infection is usually asymptomatic, whereas, during adolescence and adulthood, it can present as acute infectious mononucleosis (IM). The infection is extremely common, and >90% of adults are seropositive for EBV. The infection is spread through salivary contact, and the mucosal epithelium of the oropharynx is considered to be the first site of infection and replication. From the oropharynx, the virus is transmitted to locally infiltrating B cells, where it persists for a person's life [1].

The clinical picture of acute IM is believed to result from the host's immune response against the invading virus. This response includes cytotoxic T cells and NK cells, which provide initial control of the infection. Cytokines also seem to be important mediators in the immune response against EBV [2]. EBV infection of B cells induces the proliferation of lymphocytes and production of interleukin (IL)–10 [3]. The EBV itself codes for a cellular homologue of IL-10, viral IL-10, which shares properties similar to cellular IL-10 [4]. Cellular IL-10 is considered to be an anti-inflammatory cytokine that induces the proliferation of B cells and inhibits the antigen-specific activation of T cells and the production of proinflammatory cytokines. It is produced by B cells and by monocytes and T cells [4].

The interindividual variation seen in IL-10 production is genetically determined. The promoter region of IL-10 contains 3 base substitutions at −1082 G/A, −819 C/T, and −592 C/A, which are related to IL-10 protein production in vitro [5]. These alleles combine as 3 possible haplotypes, GCC, ACC, and ATA, which participate in the regulation of IL-10 gene transcription [5]. We previously showed that IL-10 genetics influence the clinical picture of EBV infection [6]. In this study, we analyzed whether IL-10 genetics influence the age when EBV seroconversion occurs. We also measured IL-10 plasma levels in neonates and in healthy adults and correlated these to the IL-10 genotype.

Patients, Materials, and Methods

Patients. The study was done at the Tampere University Hospital and University of Tampere (Tampere, Finland). Blood samples were obtained from 116 children, 9 months to 15 years old, which were obtained for pediatric consultation between November 1999 and May 2000. Umbilical cord blood samples were obtained from 50 healthy neonates. Blood samples from 400 healthy blood donors were obtained from the Finnish Red Cross Blood Transfusion Center (Tampere).

EBV serology. EBV serology was measured by EIA, according to the manufacturer's instructions (Enzygnost anti-EBV/IgG; Behring). The assay's detection limit is 1.0 pg/mL.

IL-10 gene promoter polymorphism. Genomic DNA was isolated from the blood samples. The region of the IL-10 promoter from −1120 to −533 was amplified by polymerase chain reaction, and single nucleotide polymorphisms (G/A at −1082, C/T at −819, and C/A at −592) were analyzed by restriction fragment length polymorphism [6].

Received 12 March 2001; revised 29 May 2001; electronically published 7 August 2001.

The study was approved by the Tampere University Hospital ethics committee and was conducted according to the hospital's guidelines. Informed consent was obtained from parents before study enrollment.

Financial support: Tampere University Hospital research fund.

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The Journal of Infectious Diseases 2001; 184:777–80

0022-1899/2001/18406-0015$02.00

IL-10 plasma levels. IL-10 plasma levels were measured by EIA, according to the manufacturer's instructions (Central Laboratory of The Netherlands Red Cross Blood Transfusion Service, Amsterdam).

Statistical analysis. We analyzed differences between the seropositive and the seronegative children by means of the Student's *t* test and the significance between the differences in the genotype and haplotype carrier frequencies by means of the χ^2 test. Logistic regression analysis was used to define relationships among IL-10 haplotype, age, and EBV serologic status. Because the probability of EBV infection was not linear, age was categorized into 3 groups based on the probability of infection: <2, 2–10, and >10 years. We compared IL-10 plasma levels by using the Mann-Whitney *U* test and the Kruskal-Wallis analysis of variance. $P < .05$ was considered to be significant. Statistical calculations were done with SPSS for Windows, version 6.1.

Results

Study children. The mean age of the 116 study children was 6.7 ± 4.9 years (median, 7.8 years). We found that 45 (13.3%) of children <2 years old were EBV seropositive, as were 41 (46.3%) of those 2–10 years old and 30 (63.3%) of those >10 years old.

IL-10 haplotype frequencies in children. There were no significant differences in the frequencies of IL-10 genotypes between seronegative and seropositive children. However, the ATA haplotype carriage was more common in seronegative than in seropositive children (44% vs. 25%, respectively; $P = .035$). By use of logistic regression analysis, ATA positivity was associated significantly with EBV seronegativity (odds ratio [OR], 2.6; 95% confidence interval [CI], 1.04–6.7; $P = .04$) when controlled by age. No significant association was found with carriage of the GCC or ACC haplotypes and EBV status (OR, 0.8; 95% CI, 0.3–2.0 and OR, 0.9; 95% CI, 0.4–2.2, respectively; table 1).

IL-10 haplotype frequencies in adults. The ACC haplotype carriage was significantly more common in EBV-seropositive adults than in EBV-seronegative adults (61% vs. 25%, respectively; $P = .004$). No difference was detected in the overall carriage of GCC or ATA haplotype between seropositive and seronegative adults (table 1). However, seronegative adults were significantly more often homozygous for the GCC haplotype than were seropositive adults (55% vs. 17%, respectively; $P = .000$).

IL-10 plasma levels. The IL-10 genotype status had no effect on IL-10 plasma levels in adults or in neonates ($P = .10$ and $P = .11$, respectively; figure 1*A* and 1*B*, respectively). IL-10 plasma levels were significantly higher in adults with ATA carrier status than in the noncarriers ($P = .03$). Median values and quartiles were 1.80 pg/mL (0–3.45 pg/mL) and 1.46 pg/mL (0–2.62 pg/mL), respectively. The ACC or the GCC carrier status had no effect on plasma IL-10 levels (1.60 [0–3.35] vs. 1.48 pg/mL [0–2.70]; $P = .35$; and 1.50 [0–2.87] vs. 1.71 pg/mL [0–3.55]; $P = .18$, respectively; figure 1*C*). Also, in the group of healthy neonates, the ATA carriers had increased IL-10 plasma levels, compared with those in noncarriers; median values and quartiles were 3.18 pg/mL (2.35–5.56 pg/mL) and 2.14 pg/mL (1.38–3.42 pg/mL), respectively ($P = .01$). In neonates, the GCC carriers had significantly decreased IL-10 levels, compared with levels in noncarriers (1.89 [1.41–3.24] vs. 3.18 pg/mL [2.42–5.60], respectively; $P = .01$). The ACC carrier status did not have effect on plasma IL-10 levels when carriers and noncarriers were compared (2.42 [1.44–3.96] vs. 2.54 pg/mL [1.56–3.53]; $P = .94$; figure 1*D*).

Table 1. Interleukin (IL)–10 genotypes and haplotype carrier rates and frequencies in Epstein-Barr virus (EBV)–seropositive (EBV^+; $n = 380$) and –seronegative (EBV^-; $n = 20$) adults and EBV^+ ($n = 44$) and EBV^- ($n = 72$) children.

Genotype and haplotype carriers	Adults			Children		
	EBV^+	EBV^-	P^a	EBV^+	EBV^-	P^a
Genotype						
GCC/ACC	124 (33)	2 (10)	.061	17 (39)	19 (27)	.167
GCC/GCC	66 (17)	11 (55)	.000	9 (20)	13 (18)	.749
GCC/ATA	60 (16)	3 (15)	.826	5 (11)	17 (24)	.103
ACC/ACC	46 (12)	0	.196	7 (16)	8 (12)	.455
ACC/ATA	60 (16)	3 (15)	.826	5 (11)	13 (18)	.334
ATA/ATA	24 (6)	1 (5)	.843	1 (1)	2 (1)	.868
Haplotype carriers						
GCC	250 (66)	16 (80)	.544	31 (70)	49 (68)	.312
ACC	230 (61)	5 (25)	.004	29 (66)	40 (56)	.836
ATA	144 (38)	7 (35)	.981	11 (25)	32 (44)	.035

NOTE. With the exception of *P* values, data are no. (%) of study subjects.
[a] χ^2 Test.

Discussion

Advances in molecular biology and increased interest in genetics have greatly facilitated research in basic mechanisms of immune response regulation in various autoimmune disorders and infectious diseases. This study shows that cytokine genetics influence a person's susceptibility to infection. EBV is contracted by nearly everyone during childhood or adolescence, so that only ~5% of adults remain uninfected [7]. A study by Jabs et al. [7] previously showed that adults who remain seronegative are immunologically different from seropositive persons [7]. During childhood, EBV infection is mainly asymptomatic but, if contracted during adolescence or adulthood, can present as IM with fever, lymphadenopathy, and hepatitis [1]. Acute EBV infection is controlled mainly by the cell-mediated immune system, including NK and T cells. It is considered likely that symptomatic infection is caused by the host's immune response and that the replicating virus plays a minor role [1].

We previously showed that IL-10 genetic polymorphism at position −1082 influences both susceptibility to the infection and the clinical picture [6]. EBV-seronegative adults are more often carriers of the base G at this position than are seropositive adults. The study also showed that the base A was connected to a more severe clinical picture that leads more often to hos-

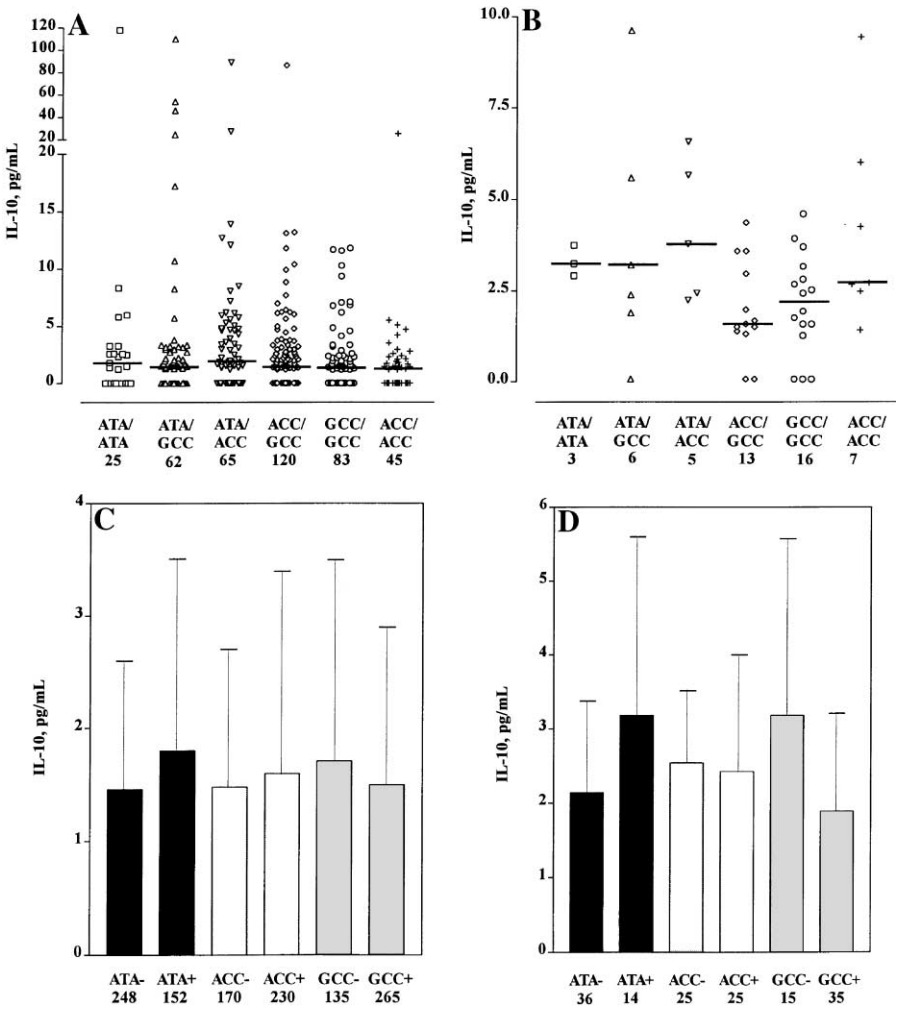

Figure 1. Interleukin (IL)–10 plasma levels in 400 adults *(A)* and 50 neonates *(B)* grouped by IL-10 genotype. Each type of symbol in panels *A* and *B* represents 1 sample; bars indicate group medians. IL-10 plasma levels in 400 adults *(C)* and 50 neonates *(D)* by IL-10 haplotype are shown. Columns represent group medians with 75% quartiles.

pitalization [6]. In this study, we extended this analysis by genotyping the other known base-pair exchanges in the promoter region (C/T at position −819 and C/A at position −592) and analyzed the presence of the haplotypes that they form in seropositive and seronegative adults. Only 3 of the possible haplotypes are found in white persons, GCC, ACC, and ATA [5]. Our results show that the previously observed difference at position −1082 was due to variation in the frequencies of the GCC and ACC haplotypes. The seronegative adults were more often homozygous for the GCC haplotype than were the seropositive adults, and ACC haplotype carriage was less common in seronegative than in seropositive adults.

We also studied the haplotype frequencies in children of various ages to determine whether the IL-10 promoter haplotype influences the age of EBV infection. The ATA haplotype was found to protect against early infection and was more common in seronegative than seropositive children when controlled for age. In a previous study, the ATA haplotype was connected to low IL-10 production [5]. In the present study, the finding was the opposite with higher IL-10 plasma levels in ATA haplotype carriers. However, in the study by Turner et al. [10], which suggested that ATA is connected to low IL-10 levels, IL-10 production was measured in in vitro–stimulated peripheral blood lymphocytes [5]. We measured spontaneous IL-10 plasma levels. Our findings show that carriage of the ATA haplotype is connected to both high spontaneous IL-10 plasma levels and to late age of primary EBV infection.

Previous studies have yielded conflicting data about IL-10 and its effect on EBV infection. Acute primary EBV infection is characterized by a rapid immune response, including initially NK cells and a rapidly ensuing cytotoxic T cell reaction [2]. IL-10, however, is usually considered to be an anti-inflamma-

tory cytokine that inhibits NK and T cell activity and thus is believed to control the strong inflammatory response after primary infection [8]. In 1992, Stewart and Rooney [9] showed that IL-10 enhances the activation of NK cells by EBV-transformed B cells and that it may actually enhance immune response against EBV. In 1993, Kurilla et al. [10], by using an SCID-mouse model, found that IL-10 actually increased NK cell activity and decreased viral replication [10]. Thus, the late age of infection in ATA haplotype carriers could be explained by the high spontaneous IL-10 plasma levels that could result in a strong initial antiviral effect postponing the age of primary EBV infection.

In children, the ATA haplotype confers protection against early infection, whereas seronegative adults are more likely to be GCC haplotype carriers. It is generally believed that the few adults who remain uninfected probably remain so for life and that they are immunologically different from the rest of the population [7]. The seronegative children probably benefit from the IL-10 ATA haplotype, so that EBV infection is postponed to later childhood or adolescence, but this haplotype does not prevent the infection. However, the GCC haplotype homozygosity or the absence of the ACC haplotype may prevent the infection from ever taking place. A large number of study children for follow-up would be needed to verify this hypothesis.

If EBV is contracted during adolescence or early adulthood, it is more often symptomatic. EBV infection symptoms are thought to be caused by the host's immune response. This study suggests that the ATA haplotype of IL-10 increases the age of primary infection and most likely also the risk for symptomatic disease. Previous studies concerning septic infections have suggested that the balance between pro- and anti-inflammatory cytokines determines the clinical picture during infection [11]. The role IL-10 plays in immune response during acute EBV infection clearly needs to be studied further. In some studies, IL-10 polymorphism was connected to differential clinical expression of various diseases, such as asthma and systemic lupus erythematosus [12]. Previous studies also suggest that EBV infection or the age of primary EBV infection may play a role in the pathogenesis of these diseases [13, 14]. These areas clearly need further study.

Acknowledgment

We thank Heini Huhtala for expert advice concerning statistical analysis of the data.

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Original Paper

International Archives of Allergy and Immunology

Int Arch Allergy Immunol 2005;137:282–288
DOI: 10.1159/000086421

Received: December 21, 2004
Accepted after revision: April 7, 2005
Published online: June 17, 2005

Genetic and Environmental Factors in the Immunopathogenesis of Atopy: Interaction of *Helicobacter pylori* Infection and *IL4* Genetics

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Key Words

Helicobacter pylori · Interleukin-4 · Genotype · Polymorphism · Atopy · Skin prick test

Abstract

Background: Both genetic and environmental factors, e.g. early childhood infections, have a role in the pathogenesis of atopic diseases. ***Objective:*** To examine simultaneously the strength and possible interactions of two known such factors, *IL4* genetics and *Helicobacter pylori* infection, on the risk of atopy and asthma. ***Methods:*** Gene polymorphism analyses and skin prick tests (SPT) were determined in 245 adult asthmatics and 405 non-asthmatic controls of population-based case-control study. SPTs were used as an indicator of atopy. *H. pylori* infection was verified by detecting anti-*H. pylori* IgG antibodies in sera. ***Results:*** A significant negative association was seen between the presence *H. pylori* antibodies and SPT positivity (≥1 positive reactions) in both asthmatics and controls (p = 0.002 and p = 0.025, respectively) but the effect of *IL-4* polymorphism (SNP –590C/T) was nonsignificant in both groups (p = 0.071 and p = 0.072, respectively). However, *IL4* genetics had an effect on susceptibility to *H. pylori*: asthmatics carrying the *IL4* –590 allele T had a diminished risk to be *H. pylori* infected (OR 0.485 95%CI 0.287–0.819). This effect was not seen in controls. Logistic regression analysis indicated that *H. pylori* and *IL4* effects on atopy risk are not interdependent. ***Conclusions:*** This study showed that the effect of *H. pylori* infection on atopy risk is stronger than that of *IL4* genetics. There is no interaction between these factors on the pathogenesis of atopy suggesting that these factors have distinct immunopathogenetic mechanisms. However, the genetic effect may modify the role of infective agents by effecting on susceptibility to disease.

Introduction

Both genetic and environmental factors are known to have an effect on susceptibility to atopic diseases (such as atopic eczema, rhinoconjunctivitis and asthma). Of the environmental factors, childhood infections have an important role: there is epidemiological evidence showing that infectious diseases (especially gastrointestinal infections) during early childhood decrease the risk of development of atopic diseases in later life, and vice ver-

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sa, the absence of infectious diseases increases the risk, i.e. the factor which probably explains the rapid increase of atopic diseases in the developed countries during the last decades (reviewed in [1]). This 'hygiene hypothesis' can be explained also at the cellular level. The T helper (Th) cells are divided into two different functional subsets, Th1 and Th2, based on the cytokine pattern they produce (reviewed in [2]). Th1 cells are mainly responsible for the cell-mediated immunity, while Th2 cells help the antibody formation, especially of the IgE class. Consequently, the Th1/Th2 balance seems to be shifted towards the Th2 direction in atopic diseases. Differentiation of the Th cells is strongly influenced by factors derived from infectious agents (e.g. bacterial lipopolysaccharide, LPS) and by the 'cytokine milieu' induced, the presence of these factors strongly favoring differentiation towards the Th1 direction. In the absence of these stimuli, the differentiation of the Th2 subset is preferred (reviewed in [3]).

The Th2 cytokines (interleukin (IL)-4, IL-5, IL-9, IL-10 and IL-13) have a decisive role in the pathogenesis of atopic diseases [4]. IL-4 is a major cytokine responsible for induction of IgE synthesis and promotion of differentiation to Th2 cells [5]. The gene encoding *IL4* is located on chromosome 5q31–33 that has been shown to be in linkage with atopic diseases in several studies [6–10]. A common single nucleotide polymorphism (SNP) consisting of a C to T exchange at position –590 in the promoter of *IL4* gene (*IL4* –590C/T) has been associated with asthma, skin prick test (SPT) positivity and elevated levels of total IgE [7–10]. Moreover, the same polymorphism has also been linked to pathogenesis of severe infections, e.g. progression of HIV infection [11] and protection against malaria [12].

Helicobacter pylori is the most common infectious gastrointestinal pathogen that infects gastric mucosa in early life and often leads to a life-long chronic gastritis (reviewed in [13]). It induces a vigorous Th1 mediated inflammatory response [14]. Several studies [15–17] have demonstrated that *H. pylori* infection is associated with lower prevalence of atopy.

Very little is known about the interactions of genetics and environmental factors in the pathogenesis of atopy and asthma. We now analyzed the effect of two known factors, *IL4* –590C/T and *H. pylori* infection, on the prevalence of atopy and asthma in cohort of 245 adult asthmatics and in their 405 controls. Atopy was defined by more than one positive skin prick test and *H. pylori* infection by the presence of IgG anti-*H. pylori* antibodies.

Table 1. Characteristics of study groups

Variable		Asthmatics (n = 245)	Controls (n = 405)
Age, years	mean ± SD	59 ± 11	60 ± 11
Gender	female/male	152/93	254/151
Smoking	non/ex/current	123/80/42	237/89/79
Atopy[1]	yes	139	154
H. pylori antibodies	yes	115	205

[1] One or more positive reactions in skin prick test.

Methods

Subjects

Asthmatic and control subjects were participants in a Finnish population-based case-control study aimed at identifying risk factors and predictors of the outcome of adult asthma. Inclusion criteria for asthmatic subjects were age over 30 years and entitlement to special reimbursement for asthma medication from the Social Insurance Institution of Finland. The entitlement is granted if the criteria for persistent asthma are fulfilled as certified by a chest specialist. Typical history, clinical features and course of asthma must be documented. At least one of the following physiological criteria is required for diagnosis: (a) a variation of ≥20% in diurnal PEF recording (reference to maximal value); (b) an increase of ≥15% in PEF or FEV_1 with β_2-agonist; (c) a decrease of ≥15% in PEF or FEV_1 in exercise testing. Moreover, at least a 6-month period of continuing regular use of anti-asthmatic medication must have elapsed by the time of the decision. This method of case ascertainment has been described in detail and evaluated [18]. One to two controls matched for age, gender and area of residence not suffering from asthma or chronic obstructive pulmonary disease were initially selected for each subject through a register covering the entire population. The ethnic origin of patients and controls was the same (Finnish Caucasian). The basic characteristics of patient and control groups are presented in table 1. Approval for this study was obtained from the ethical committee at Tampere University Hospital. All subjects gave informed consent to participate.

Skin Prick Tests

Skin prick tests were performed by specially trained nurses with a panel of 22 common allergen extracts, including dog, cat, birch, cow dander, horse, mugwort, alder, meadow foxtail, timothy, barley, oats, wheat, rye, *Alternaria alternata*, *Acarus siro*, *Aspergillus fumigatus*, *Cladosporium herbarum*, *Der. farinae*, *Der. pteronyssinus*, *Lepidoglyphus destructor*, *Tyrophagus putrescentiae* and to both a negative control (saline) and a positive control histamine (ALK-Abello, Hørsholm, Denmark). The patient was considered prick test positive if at least one allergen elicited a weal with a diameter at least 3 mm larger than that of the negative control. Allergy testing by the skin prick method was carried out on 99.1% of asthmatic (93 males and 150 females) and 99.3% of control subjects (150 males and 252 females). Positive reactions to specific allergens and differences between asthmatics and nonasthmatic controls have been published earlier [19].

Table 2. Effect of *H. pylori* serology and *IL4* –590C/T allele carrier status on skin prick test positivity (SPT+) in asthmatics and controls

	SPT+		SPT–		p	OR	95% CI
	n	%	n	%			
H. pylori serology							
Asthmatics							
Seronegative	82	60.7	42	40.4	0.002	2.28	1.35–3.85
Seropositive	53	39.3	62	59.6		1	
Controls							
Seronegative	84	55.6	108	44.1	0.025	1.59	1.06–2.39
Seropositive	67	44.4	137	55.9		1	
IL4 genetics							
Asthmatics							
IL4 allele T noncarrier	48	35.5	49	47.1	0.071	1	
IL4 allele T carrier	87	64.5	55	52.9		1.61	0.96–2.72
Controls							
IL4 allele T noncarrier	63	41.7	125	51.0	0.072	1	
IL4 allele T carrier	88	58.3	120	49.0		1.46	0.97–2.19

SPT+ = One or more positive skin prick test reaction; allele T noncarrier = CC genotype; allele T carrier = CT and TT genotypes.

p value calculated in 2 × 2 table by χ^2 test.

Serum Samples and H. pylori *Serology*

Serum samples and citrated whole-blood samples were collected from each patient. Samples were stored at –70°C until testing. The sera were assayed for helicobacter IgG antibodies. *Helicobacter* IgG antibodies were measured by using the enzyme-linked immunosorbent assay (Pyloriset EIA-G III, Orion Diagnostica, Espoo, Finland). Titres of 30 or higher were considered positive for *H. pylori* antibodies. With this cut-off value, a sensitivity of 99% and a specificity of 90% were demonstrated in a separate series of 16- to 91-year-old dyspeptic patients (gastroscopied at primary care level in Vammala, Finland, n = 561, median age 56 years, *H. pylori* infection prevalence 32.3%) used for the validation of the test (culture and histology as reference methods; data not shown). *Helicobacter* IgG antibody measurement was carried out on 98.8% of asthmatic (92 males and 150 females) and 98.8% of control subjects (151 males and 249 females).

DNA Extraction and Genotyping

DNA specimens from citrated whole-blood samples were prepared using standard methods. The region which contains the *Ava*II polymorphic site at position –590 (C to T base exchange) of the *IL4* gene was amplified by PCR [8]. The oligonucleotides 5′ TAA ACT TGG GAG AAC ATG GT 3′ and 5′ TGG GGA AAG ATA GAG TAA TA 3′ were used as primers. Fragments were analyzed by electrophoresis on 3% agarose stained with ethidium bromide. The genotype distribution of the *IL4* gene studied followed the Hardy-Weinberg equilibrium. *IL4* genotyping was carried out on 99.2% of asthmatic (92 males and 151 females) and 99.0% of control subjects (151 males and 250 females).

Statistical Analysis

To test the fit of genotype frequencies with the Hardy-Weinberg equation the exact test using Markov chain algorithm in Arlequin software was used (Arlequin program, ver. 2.0. A software for population genetics data analysis, Schneider S, Roessli D, Excoffier L, Genetics and Biometry Laboratory, Geneva, Switzerland). χ^2 test calculations were carried out with Statistica software (ver. Win.5.1D, StatSoft, Tulsa, OK). Odds ratios (OR) with 95% CI were calculated by CIA software (ver. 1.1, copyrighted by M.J. Gardner and the *British Medical Journal*, 1989). Binary logistic regression analysis of SPSS was used to analyze the interaction between *H. pylori* infection and *IL4* genetics as well as simultaneously significance of these factor on atopy risk. Findings were considered statistically significant at $p < 0.05$. Altogether 97.6% of asthmatics and 97.8% of controls had all required measurements (skin prick tests, serological and genotype results) and were used in statistical calculations.

Results

The effects of *H. pylori* serology and *IL4* genetics on SPT positivity were tested. Our data confirmed the earlier data [15–17] on the association of SPT positivity rates and *H. pylori* serology. In both asthmatics and controls SPT positivity rate was lower in subjects with *H. pylori* antibodies (table 2). A trend of association was observed between *IL4* gene polymorphism and SPT positivity rates

in asthmatics and controls (p = 0.071 and p = 0.072, respectively; χ^2 test, d.f. = 1). Allele T carriers (i.e. subjects having genotypes CT or TT) had slightly increased the risk of SPT positivity (table 2).

These two factors, *IL4* gene polymorphism and *H. pylori* serology, were then put in a logistic regression model to evaluate simultaneous significance and independency of these factors. The model showed that *H. pylori* serology was more significant predictor of SPT positivity than *IL4* polymorphism in both asthmatics and controls (table 3). These factors were independent. There was no interaction between seropositivity and *IL4* gene polymorphisms on SPT results in asthmatics and controls (p = 0.685 and 0.709, respectively).

To evaluate the effect of *H. pylori* serology and *IL4* genetics on plurisensitization to allergens SPT-positive patients were divided into monosensitized (= one positive SPT reaction) and plurisensitized (= more than one positive SPT reaction) subgroups. As seen in table 4, in both asthmatics and controls the number of plurisensitizated subjects was diminished in seropositive group (p = 0.0005 and p = 0.004, respectively; χ^2 test, d.f. = 1).

Table 3. Simultaneous effect of *H. pylori* serology and *IL4* –590C/T allele carrier status on skin prick test positivity (SPT+) in asthmatics and controls

	n	OR	95% CI	p
Asthmatics				
H. pylori				0.004
Seronegativity	124	2.16	1.272–3.668	
Seropositivity	115	1		
IL4 polymorphism				0.195
IL-4 allele T noncarrier	97	1		
IL-4 allele T carrier	142	1.43	0.834–2.440	
Controls				
H. pylori				0.033
Seronegativity	192	1.56	1.036–2.353	
Seropositivity	204	1		
IL4 polymorphism				0.094
IL-4 allele T noncarrier	188	1		
IL-4 allele T carrier	208	1.42	0.941–2.145	

Factors put into a logistic regression model. Allele T noncarrier = CC genotype; allele T carrier = CT and TT genotypes.

Table 4. Effect of *H. pylori* serology and *IL4* –590C/T allele carrier status on mono [SPT+(m)] and plurisensitization [SPT+(p)] to allergens in asthmatics and controls

	SPT+ (p)		SPT+ (m)		SPT–		SPT– vs. SPT+ (m), p	SPT– vs. SPT+ (p), p
	n	%	n	%	n	%		
(a) *H. pylori* serology								
Asthmatics								
Seronegative	70	64.2	12	46.2	42	40.4	0.59	0.0005
Seropositive	39	35.8	14	53.8	62	59.6		
Controls								
Seronegative	61	61.0	23	45.1	108	44.1	0.89	0.004
Seropositive	39	39.0	28	54.9	137	55.9		
(b) *IL4* genetics								
Asthmatics								
IL4 allele T noncarrier	38	34.9	10	38.5	49	47.1	0.43	0.069
IL4 allele T carrier	71	65.1	16	61.5	55	52.9		
Controls								
IL4 allele T noncarrier	41	41.0	22	43.1	125	51.0	0.306	0.091
IL4 allele T carrier	59	59.0	29	56.9	120	49.0		

SPT + (m) = One positive skin prick test reaction; SPT + (p) = more than one positive skin prick test reaction; allele T noncarrier = CC genotype; allele T carrier = CT and TT genotypes. p value calculated in 2 × 2 table by χ^2 test.

Table 5. Effect of *IL4* allele carrier status on *H. pylori* seropositivity in asthmatics and in controls

	Seropositive		Seronegative		p	OR	95% CI
	n	%	n	%			
Asthmatics							
IL4 allele T noncarrier	57	49.6	40	32.3	0.007	1	
IL4 allele T carrier	58	50.4	84	67.7		0.485	0.287–0.819
Controls							
IL4 allele T noncarrier	103	50.5	85	44.3	0.220	1	
IL4 allele T carrier	101	49.5	107	55.7		0.779	0.525–1.16

Allele T noncarrier = CC genotype; allele T carrier = CT and TT genotypes.
p value calculated in 2 × 2 table by χ^2 test.

Whereas there was only a trend of association between *IL4* gene polymorphism and plurisensitization in asthmatics and controls (p = 0.069 and p = 0.091, respectively; χ^2 test, d.f. = 1).

There was an association (p = 0.007; χ^2 test, d.f. = 1) between *IL4* gene polymorphism and *H. pylori* seropositivity among asthmatics (table 5). Allele T carriers had diminished risk for *H. pylori* infection (OR 0.485 95% CI 0.287–0.819). The effect was independent on atopic status of the subject (data not shown). In controls, the association between *IL4* gene polymorphism and *H. pylori* seropositivity was not significant (p = 0.220; χ^2 test, d.f. = 1).

The effect of these two factors on asthma risk was also evaluated. *H. pylori* seropositivity rates were similar in asthmatics and controls (p = 0.370, χ^2 test, d.f. = 1, see table 1). For the asthmatics, the frequencies were 0.41 for allele T noncarriers and 0.59 for allele T carriers at –590 of the *IL4* gene. For controls respective values were 0.48 and 0.52. No difference in carrier frequencies was observed between asthmatics and controls (p = 0.093; χ^2 test, d.f. = 1).

Discussion

In here interaction of two known atopy risk factors (*H. pylori* infection and *IL4* genetics) was studied. The result showed that the protective effect of *H. pylori* was not dependent on *IL4* genetics. Recently McIntire et al. [20] observed that hepatitis A infection protects individuals from atopy only if they carry a certain allele of the *TIM1* gene (a gene expressed on activated Th cells during differentiation to Th2 direction and serving as a receptor for the virus). This allele did not have any effect on the infection rate, but obviously modified the effect of the virus on the Th cell differentiation. In here, *IL4* genetics and *H. pylori* infection were independent risk factors. The atopy protective effect of *H. pylori* infection observed here may be explained by the increased Th1 activity [21, 22]. *IL4* –590 T allele has been shown to be associated with a stronger transcription of *IL4* than allele C [11, 23], and consequently, the presence of this allele leads to preferential differentiation of the Th cells towards the Th2 direction. Our data suggest that *IL4* polymorphism and *H. pylori* infection have distinct pathways to regulate the pathogenesis of atopy.

Our study showed that *IL4* genetics had effect on infection rate: absence of allele T was a risk factor for this infection. IL-4 has a central role in the antibody production. *H. pylori* infection was evaluated by measuring specific IgG antibodies. One possibility is that *IL4* allele C, low IL4-producing allele, is involved in total IgG antibody production, contrast to IgE promoting allele T [11, 23]. Moreover, our observation is line with the associations observed with *IL4* –590C/T and other infectious agents (such as malaria antigens and HIV) [11, 12], explained by this increased Th1 activity.

Rockman et al. [24] have recently observed that allele frequency variation of the *IL4* SNP –590 in different populations is too great to be explained by simple genetic drift. They suggested that natural selection, e.g. various infections, have had an effect on the allele frequencies. In here the *IL4* –590 polymorphism was associated with susceptibility to a bacterial infection as well as there was a trend of association to atopy risk. This does not give di-

rect evidence for hypothesis that allergies are just by-products of the effective immune defense against microbes, a capacity which has had a positive selective advantage during evolution.

IL4 allele T has been linked to asthma in many studies [6–10]. However, contradictory results have been reported as well [25, 26]. Our study showed a weak association of *IL4* genetics and asthma risk but only in seronegative patients ($p = 0.03$; table 5). This might indicate that genetic risk is stronger in the absence of certain environmental factors. Obviously, more data are needed on the interactions of these genetic and environmental factors.

We observed that the protective effect of *IL4* polymorphism on *H. pylori* infection was seen in asthmatics but not in controls (table 5). The reason to this can only be speculated, but obviously asthmatics have some other factors modulating the effect of the *H. pylori* infection. One candidate is the anti-inflammatory cytokine IL-10. It has been observed that the severity of asthma is associated with the presence of a low IL10 producing haplotype [27], which then allows uninhibited production of pro-inflammatory cytokines and thus a more efficient shift of the Th1/2 balance towards the protective Th1 direction.

In this study, presence of *H. pylori* antibodies had a clear inverse association to the incidence of atopy. This finding is in line with previous reports from our country [15] and from elsewhere [16, 17], thus confirming the role of *H. pylori* infection as a strong atopy-risk modulating factor (according to the hygiene hypothesis, see introduction). The effect of *H. pylori* seropositivity was strikingly different in subjects with one positive SPT (monoallergic) vs. several positive SPTs (pluriallergic), i.e. *H. pylori* infection only protected against plurisensitization. This might indicate that *H. pylori* infection does not affect the susceptibility per se but rather on the severity of the atopic disease. However, as *H. pylori* exposure is stronger in poor hygienic conditions [28, 29], we cannot exclude the possibility that *H. pylori* is just a surrogate marker of, e.g. a general bacterial LPS exposure.

Acknowledgements

The authors would like to thank Prof. Markku M Nieminen, Prof. Arpo Aromaa and Docent Timo Klaukka for taking part in collection of the patient material. We also thank Mrs. Heini Huhtala, MSc for statistical consultation as well as Mrs. Eija Spåre, Sinikka Repo-Koskinen and Pirjo Kosonen for expert technical assistance as well as Orion Diagnostica, Espoo, Finland, for the Pylori-set kits.

Funding /Support

The study was financially supported by the Academy of Finland (grant no 205653), Rehabilitation Funds of the Finnish Social Insurance Institution, the Tampere Tuberculosis Foundation, the Medical Research Fund of Tampere University Hospital and the Research Funds of the University of Helsinki. The sponsors of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

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doi: 10.1111/j.1365-2222.2008.03103.x
Clinical and Experimental Allergy, **38**, 1929–1934

ORIGINAL PAPER **Epidemiology of Allergic Disease**

Interaction between *CD14* −159C>T polymorphism and *Helicobacter pylori* is associated with serum total immunoglobulin E

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Cite this as: M. Virta, T. Pessi, M. Helminen, T. Seiskari, A. Kondrashova, M. Knip, H. Hyöty and M. Hurme, *Clinical and Experimental Allergy*, 2008 (38) 1929–1934.

Summary

Background Total serum IgE is regulated by both environmental and genetic factors. Association and linkage studies have suggested a role of *CD14* −159C>T polymorphism in the regulation of serum total IgE, but the results have been contradictory. It seems that gene–environment interactions are involved in this regulation.

Objective The aim of this study was to examine the possible gene–environment interactions among *Toxoplasma gondii*, *Helicobacter pylori*, *CD14* −159C>T and Toll-like receptor *(TLR) 4* +896A>G polymorphism on serum total IgE. For this study, we expanded the scope of our earlier comparison of allergic sensitization and microbial load between Finland and Russian Karelia by studying the *CD14* −159C>T and *TLR4* +896A>G polymorphism in a cohort of Russian Karelian children.

Methods For this study, *CD14* −159C>T and *TLR4* +896A>G polymorphisms were analysed in 264 healthy Russian Karelian children. Serum total IgE levels and *H. pylori* and *T. gondii* antibodies were also measured.

Results We constructed a multiway ANOVA model to analyse the gene–environment interactions among *T. gondii* seropositivity, *H. pylori* seropositivity, *CD14* −159C>T and *TLR4* +896A>G polymorphisms on serum total IgE. The model showed that there was an interaction between the *CD14* −159 allele T carrier status and *H. pylori* antibodies on serum total IgE ($P = 0.004$). No other interactions were found.

Conclusion Our results further emphasize the role of gene–environment interaction in the regulation of serum total IgE.

Keywords *CD14* polymorphism, gene–environment interaction, *Helicobacter pylori*, IgE, *Toxoplasma gondii*
Submitted 7 February 2008; revised 18 June 2008, 8 August 2008; accepted 11 August 2008

Introduction

IgE is an important mediator in allergic reactions and also contributes to the immune defence against parasites. Serum total IgE is regulated by both environmental and genetic factors, and interaction between the environment and the genetic background of the host seems to be important [1–4]. Environmental factors associated with atopy and serum total IgE include, for example, *Helicobacter pylori* and *Toxoplasma gondii* infections, endotoxin, tobacco smoke, number of siblings, regular contact with stable animals and socio-economic status, which could reflect the microbial load the population is facing [2–7].

Many linkage studies have indicated that one or more loci on chromosome 5q may control total serum IgE [8–11]. One gene mapping to this area is *CD14*, which is a component of a multi-ligand pattern recognition receptor complex involved in innate immunity reactions [12]. CD14 is also essential for the T-helper1 (Th1)/Th2 balance and coordinates the adaptive immune responses [13]. CD14 recognizes, for example, lipopolysaccharide (LPS), lipoteichoic acids, mycobacterial glycolipids and mannans from yeast and enhances the function of Toll-like

receptors (TLR) 2 and 4, which are involved in immune defence against *T. gondii* and *H. pylori* [12–15].

CD14 gene promoter region polymorphism −159C>T has been associated with serum total IgE and atopy, but the results have been contradictory. The same allele has been shown to have opposite effects on IgE [11, 16–21]. The gene–environment interactions in the regulation of serum total IgE detected in recent studies have been postulated to be one explanation for the inconsistent results [1]. Although CD14 is an important molecule for the endotoxin signalling, other molecules such as TLR4 are needed for signal transduction [13]. A single A>G base-exchange polymorphism in the *TLR4* gene at the position +896 in the fourth exon has been associated with atopic asthma, but there are also negative findings [22–24].

In our earlier study, we observed that serum total IgE levels were significantly higher in children living in Russian Karelia than in children living in Finland, even though sensitization to allergens was clearly less frequent among Russian Karelian children [6]. The exact reason for this phenomenon is not known, but one explanation for the higher IgE levels could be parasite infections, which are more common in Russian Karelia than in Finland [6, 25]. In this study, we wanted to expand our earlier investigation and analyse possible gene–environment interactions among microbes, *CD14* −159C>T and *TLR4* +896A>G polymorphisms on serum total IgE in a child population from Russian Karelia.

Materials and methods

Study population

Altogether 266 schoolchildren from Petrozavodsk, Russian Karelia, having both parents of either Finnish or Karelian ethnicity participated in the present study. Two children were excluded, because their whole blood samples were accidentally confused in the laboratory. The study population has been described earlier in detail [6]. Finnish schoolchildren from the earlier study were not included in this study due to the low prevalence of seropositive subjects for *H. pylori* and *T. gondii* (5% and 2%, respectively). The collection of samples from Russian Karelia was done as a part of the EU INCO-Copernicus program (contract number IC15-CT98-0316, coordinator Professor Hyöty) during the years 1997–1999. Collection of samples was organized by the Department of Pediatrics, University of Petrozavodsk, and the study protocol was approved by the Ministry of Health in Russian Karelia. There was no local ethics board in Karelia to apply to, and the accepted route for ethical approval for the research studies was via the Ministry of Health. Written consent was obtained from all the children who took part in the study and from their parents. Whole blood and serum samples were collected from each child.

Immunoglobulin E and microbial antibodies

Serum total IgE was measured using the ImmunoCAP® fluoroenzyme immunoassay (Phadia Diagnostics, Uppsala, Sweden). *T. gondii* IgG antibodies were measured by Enzygnost Toxoplasmosis IgG Assay and *H. pylori* IgG antibodies by Enzygnost Anti-*H. pylori*/IgG Assay according to the manufacturer's instructions (Dade Behring, Marburg, Germany). Behring ELISA Processor III (Dade Behring, Marburg, Germany) was used for further processing of the tests and for the calculation of the antibody levels. Measurement of *T. gondii* antibodies succeeded in 263 children of 264 (99.5%), whereas *H. pylori* antibodies were measured among all the 264 (100%) study children.

Genotype analyses

DNA was extracted using standard techniques. Genotyping of *CD14* −159C>T polymorphism (rs 2569190) and *TLR4* +896A>G (rs 4986790) was performed using the ABI PRISM 7000 Sequence Detection System for both PCR and allelic discrimination. The oligonucleotides 5′-CCC TTC CTT TCC TGG AAA TAT TGC A-3′ and 5′-CTA GAT GCC CTG CAG AAT CCT T-3′ were used as primers for CD14 −159C>T polymorphism, and the oligonucleotides 5′-TGA CCA TTG AAG AAT TCC GAT TAG CA-3′ and 5′-ACA CTC ACC AGG GAA AAT GAA GAA-3′ for TLR4 +896A>G polymorphism.

Statistics

To detect a possible deviation of the genotype frequencies from the Hardy–Weinberg equation, the exact test using the Markov chain algorithm was used (Arlequin program, ver. 2.0. A software for population genetics data analysis; Schneider S, Roessli D, Excoffier L; Genetics and Biometry Laboratory, Geneva, Switzerland). Other statistical analyses were performed with SPSS for Windows version 14.0 (SPSS Inc., Chigago, IL, USA). The statistical method selected is presented in 'Results'. Serum total IgE levels were not normally distributed and therefore non-parametric tests were used when serum total IgE levels were analysed. Serum total IgE values were logarithmically transformed for multiway ANOVA which was used for modelling gene–environment interactions among *CD14* −159 allele T carrier status, *TLR4* +896 allele G carrier status, *T. gondii* seropositivity and *H. pylori* seropositivity on serum total IgE levels. For statistical analysis, we grouped *CD14* −159 genotypes according to the allele T carrier status, because this allele has been shown to be transcriptionally more active in monocytic cell line than allele C [26]. *TLR4* +896 genotypes were grouped according to the allele G carrier status, because the number of GG genotype was too small ($n = 3$) for grouping

according to allele A. *P*-values <0.05 were considered to be significant.

Results

The study cohort of 264 children included 114 boys (43%). The mean age of the children was 11.4 years (range 7.1–15.0). The median total IgE in sera was 76.1 IU/L (interquartile range 30.9–236.0). There were no significant differences in total IgE levels between boys and girls (median 75.8 and 76.4 IU/L, respectively, $P=0.8$, Mann–Whitney *U*-test). Among the study children, 193 out of 264 (73%) were seropositive for *H. pylori* and 63 out of 263 (24%) for *T. gondii*. Fifty-two (20%) children were seropositive for both the microbes. Only 16% ($n=10$) of *T. gondii* seropositive children were *H. pylori* seronegative, whereas 72% ($n=139$) of *H. pylori* seropositive children were *T. gondii* seronegative. Serum total IgE levels were significantly higher in *T. gondii* seropositive children ($P=0.009$, Mann–Whitney *U*-test), whereas *H. pylori* seropositivity did not have any effect on serum total IgE (Table 1), as reported earlier [6]. Association between *T. gondii* seropositivity and serum total IgE remained statistically significant after Bonferroni correction ($P=0.036$).

The genotype frequencies of *CD14* −159C>T and *TLR4* +896A>G polymorphisms as well as allele T carrier status of *CD14* −159C>T and allele G carrier status of *TLR4* +896A>G are shown in Table 1. *CD14* −159C>T genotype or allele T carrier status did not show any direct association with serum total IgE, and there was no association between *TLR4* +896A>G genotype or allele G carrier status and serum total IgE (Table 1). Neither of these polymorphisms was associated with seropositivity for *H. pylori* or *T. gondii* (data not shown). The genotype distributions followed the Hardy–Weinberg equilibrium.

We constructed a multiway ANOVA model to investigate gene–environment interactions among *CD14* −159C>T allele T carrier status, *TLR4* +896A>G allele G carrier status, *H. pylori* seropositivity and *T. gondii* seropositivity on serum total IgE levels. In this model, we found an interaction between *H. pylori* seropositivity and *CD14* −159 allele T carrier status on serum total IgE ($P=0.004$, multiway ANOVA, Table 2a). The *H. pylori* seronegative children who were allele T non-carriers (i.e. genotype CC) had higher serum total IgE than allele T carriers (i.e. genotypes CT and TT). Among *H. pylori* seropositive children, allele T non-carriers had lower IgE levels than allele T carriers (Table 2a). There was a trend for interaction between *T. gondii* seropositivity and *CD14* −159 allele T carrier status on serum total IgE, but this interaction was not statistically significant (Table 2b). No statistically significant interactions between *TLR4* +896A>G allele G carrier status and *H. pylori* or *TLR4* +896A>G allele G carrier status and *T. gondii* on serum total IgE was found ($P=0.95$ and 0.3, respectively, multiway ANOVA). We also analysed gene–gene interaction between *CD14* −159C>T allele T carrier status and *TLR4* +896A>G allele G carrier status on serum total IgE, but no

Table 1. Associations between *Toxoplasma gondii* serology, *Helicobacter pylori* serology, *CD14* −159C>T polymorphism, *TLR4* +896A>G polymorphism and serum total IgE

	N (%)	IgE median (quartiles)	*P*-value	*P*-value*
T. gondii				
Seropositive	63 (24)	114.0 (44.0–393.0)		
Seronegative	200 (76)	68.5 (28.3–174.3)	0.009†	0.036
H. pylori				
Seropositive	193 (73)	76.8 (32.7–234.0)		
Seronegative	71 (27)	68.2 (30.0–257.0)	0.86†	NS
CD14 −159C>T				
CC	88 (33)	66.4 (32.0–238.5)		
CT	139 (53)	80.9 (33.1–203.5)		
TT	37 (14)	62.3 (23.8–262.0)	0.9‡	NS
Allele T carrier (CT and TT)	176 (67)	79.2 (30.8–229.5)		
Allele T non-carrier (CC)	88 (33)	66.4 (32.0–238.5)	0.8†	NS
TLR4 +896A>G				
AA	223 (85)	76.4 (34.1–217.0)		
AG	38 (14)	62.0 (21.9–266.0)		
GG	3 (1)	107.0 (14.1–171.0)	0.8‡	NS
Allele G carrier (AG and GG)	41 (15)	67.5 (21.6–262.0)		
Allele G non-carrier (AA)	223 (85)	76.4 (34.1–217.9)	0.5†	NS

*Bonferroni correction.

†Mann–Whitney *U*-test.

‡Kruskall–Wallis ANOVA.

NS, non-significant.

Table 2. (a) Interaction between *Helicobacter pylori* serology and *CD14* −159C>T allele T carrier status on serum total IgE ($P = 0.004$, multiway ANOVA). (b) Interaction between *Toxoplasma gondii* serology and *CD14* −159C>T allele T carrier status on serum total IgE ($P = 0.06$, multiway ANOVA)

	Seropositive		Seronegative	
CD14 −159 C>T	IgE median (quartiles)	*N*	IgE median (quartiles)	*N*
(a) *H. pylori*				
Allele T carrier (CT and TT)	87.4 (36.9–253.8)	130	44.1 (24.2–162.5)	46
Allele T non carrier (CC)	56.0 (29.5–154.0)	63	84.7 (34.1–530.5)	25
(b)*T. gondii*				
Allele T carrier (CT and TT)	202.5 (59.4–550.8)	42	67.5 (26.8–160.5)	133
Allele T non carrier (CC)	55.3 (39.1–81.7)	21	79.7 (29.5–272.0)	67

interaction was seen ($P = 0.7$, multiway ANOVA). Furthermore, there was no interaction between the environmental factors *T. gondii* and *H. pylori* on serum total IgE ($P = 0.2$, multiway ANOVA).

Discussion

Gene–environment interactions are very complex. It has been speculated that the same genetic variants may be associated with different phenotypes in different environments, but so far, only few human studies have been published [2, 3, 27]. *H. pylori*, *T. gondii* and *CD14* −159C>T polymorphisms have all been independently associated with atopic markers and IgE. In this study, we wanted to further analyse whether there are gene–environment interactions among *H. pylori*, *T. gondii*, *CD14* and *TLR4* polymorphisms that have an effect on serum total IgE. To increase the sensitivity and detect possible interactions, we carried out the study in an environment that is characterized by a high exposure rate to these two microbes.

CD14 is part of the TLR signalling complex that facilitates endotoxin responses through TLR4-MD2 and can also bind with a variety of microbial TLR ligands. *CD14* gene (MIM158120) is located on chromosome 5 (5q31). In many linkage studies, this area has been connected with the regulation of serum total IgE and atopy [8–11]. *CD14* promoter region single base-exchange polymorphism C to T at position −159 has been extensively studied, because it has been associated with the level of protein production allele T being transcriptionally more active in monocytic cell line [17, 26, 28]. An association between *CD14* −159C>T polymorphism and total IgE and other atopic markers has been shown in the earlier studies, but the results have been contradictory. Baldini et al. [17] reported that atopic children homozygous for allele T (i.e. TT genotype) had higher sCD14 levels and lower IgE levels in sera than allele C carriers. An association between high IgE levels or other atopic markers and the *CD14* −159CC genotype has been reported in many other studies [16, 19, 20]. An opposite association has been reported by Ober et al. [11], who showed that the *CD14* −159 allele T was associated with atopy in a rural population. In some studies, no associations have been found [18, 21].

Environmental differences between study populations may in part explain these conflicting results. In recent studies, exposure to endotoxin and other environmental factors has been studied concomitantly with *CD14* −159C>T polymorphism. Eder et al.[2] reported that the *CD14* −159C>T allele C was associated with lower levels of both total and specific IgE in children in regular contact with stable animals, whereas the result was the opposite in children with regular contact with furry pets. They speculated that one explanation for this difference could be the exposure to different kinds of microbial products [2]. Williams et al. [3] found in an adult female population that the association between *CD14* −159C>T polymorphism and serum total IgE was modified by the level of endotoxin exposure. At lower levels of endotoxin exposure, the CC genotype was associated with the highest serum total IgE levels, whereas at the highest tertile of endotoxin exposure, individuals carrying the TT genotype had the highest IgE levels [3]. On the other hand, in a French study, early-life exposure to a farming environment and the *CD14* −159 TT genotype together significantly reduced the risk of nasal allergy and atopy. In addition, there was a trend among these subjects for lower risk of having increased serum total IgE levels [27].

Eder et al. [2] speculated that the effect of *CD14* −159C>T polymorphism on IgE might be dependent on microbial exposure, but they did not study the specific microbes. In the present study, we found that interaction between *CD14* −159C>T polymorphism and *H. pylori* serology had an effect on serum total IgE. Among *H. pylori* seronegative children, *CD14* −159 allele T non-carriers (i.e. CC genotype) had higher serum total IgE than allele T-carriers (i.e. CT and TT genotypes), whereas among *H. pylori* seropositive children, serum total IgE was lower in allele T non-carriers. There was also a trend of gene–environment interaction between *T. gondii* seropositivity and *CD14* −159C>T polymorphism. Because of our result, one may speculate that *H. pylori*, and possibly also *T. gondii*, could be among the microbes that influence the genetic regulation of serum total IgE, but the exact mechanism is not known.

H. pylori infection has been suggested to influence the development of the immune system by LPS binding with the CD14 receptor which results in increased production of IL-12. This could drive the immune responses towards the Th1 pathway and thereby have a protective effect against the development of Th2 polarized diseases such as atopy [29]. *CD14* −159 allele T carriers have been reported to have higher sCD14 levels than allele T non-carriers and these high sCD14 levels have been associated with low serum total IgE [17, 28]. The exact mechanism is not known, but interaction between sCD14 and B cells has resulted in higher levels of IgG1 and lower levels of IgE production [17, 30]. Karhukorpi et al. [28] have reported that *H. pylori* seropositivity has an effect on sCD14 levels in such a way that *H. pylori* seropositive individuals have higher levels than seronegative subjects, especially if they carry the *CD14* −159 CC genotype. One may speculate that the CC genotype is especially sensitive to environmental factors.

TLR4 plays a role in the same functional pathway as CD14, and both these molecules are needed for endotoxin responsiveness. A single A>G base-exchange polymorphism in the *TLR4* gene at position +896 has been associated with atopic asthma, but the results have been contradictory [22–24]. This base exchange induces an amino acid substitution of glysine for asparagine, which results in a reduction in cell surface expression of TLR4 and subsequent disruption of LPS-mediated signalling [31]. In the present study, *TLR4* +896A>G polymorphism was not associated with serum total IgE and there was no interaction between this polymorphism and *H. pylori* or *T. gondii* on serum total IgE. It has been speculated that the combined effect of the genetic variants of CD14 and TLR4 could strengthen the associations found earlier, but no interaction between *CD14* −159C>T and TLR4 +896A>G polymorphisms was seen in our study, which is in line with previous findings of Sackesen et al. [24].

The major limitation of our study is the small number of study subjects and therefore these gene–environment analyses should be repeated in a larger population. Another restriction was that we could not use specific IgE in gene–environment interaction analyses, because the number of atopic subjects in the study population ($n = 16$) was too small for statistical analyses. The problem with serum total IgE is that many factors such as allergens and parasite infections influence its levels and therefore the interpretation of the results is challenging. Multiple testing also complicates the interpretation of our results. However, the interaction between *CD14* −159C>T allele T carrier status and *H. pylori* seropositivity on serum total IgE was so significant that it most probably does not disappear even though multiple testings are done (i.e. remaining significant after Bonferroni correction). Caution should also be exercised when interpreting interactions between microbes and *CD14* −159C>T polymorphism on serum total IgE, because both *H. pylori* and *T. gondii* could be surrogate markers for poor hygiene and large microbe load. Therefore, the interactions detected could either reflect interaction between the whole microbe load and *CD14* −159 polymorphism or the interaction between specific microbe and the polymorphism.

Our study supports the hypothesis that the same genotype may increase, decrease or have no effect on total IgE depending on the environmental factors, such as microbes, that the population is facing.

Acknowledgements

This study was supported by grants from the Medical Research Fund of the Tampere University Hospital, the Tampere Tuberculosis Foundation, the Finnish Anti-Tuberculosis Association Foundation, the Väinö and Laina Kivi Foundation, the Päivikki and Sakari Sohlberg Foundation, the Academy of Finland and the EC as a part of the EPIVIR project (INCO-Copernicus Programme, contract number IC15-CT98-0316).

The authors thank Ms Eija Spåre and Ms Sinikka Repo-Koskinen for their expert technical assistance and Heini Huhtala MSc for expert advice on the statistical analysis of the data.

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