SANNA KILPINEN

Inflammatory Cytokines and Their Promoter Polymorphisms

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ACADEMIC DISSERTATION

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to my parents

CONTENTS 4

LIST OF ORIGINAL COMMUNICATIONS 6

ABBREVIATIONS 7

INTRODUCTION 9

REVIEW OF THE LITERATURE 11
1. Interleukin-1α 11
1.1. IL-1 cytokine family 11
1.2. Production of IL-1α 12
1.3. IL-1 signal transduction 12
1.4. IL-1α as a proinflammatory cytokine 13
1.5. IL-1α as an adjuvant 15
1.6. IL-1α gene and polymorphic sites 15
1.7. Association studies on the IL-1α -889 polymorphism 16

2. Interleukin-6 19
2.1. Production of IL-6 19
2.2. IL-6 protein and IL-6 receptors 19
2.3. IL-6 as a proinflammatory cytokine 20
2.4. IL-6 transgenic and gene knockout mice models 22
2.5. IL-6 in stress models 23
2.6. IL-6 gene and polymorphic sites 24
2.7. Association studies on the IL-6 -174 polymorphism 27

3. Interleukin-10 30
3.1. Production of IL-10 30
3.2. IL-10 protein and IL-10 receptors 30
3.3. IL-10 as an anti-inflammatory cytokine 31
3.4. IL-10 and Epstein-Barr virus-infection 33
3.5. IL-10 gene and polymorphic sites 34
3.6. Association studies on the IL-10 promoter polymorphisms 36

AIMS OF THE PRESENT STUDY 38

MATERIALS AND METHODS 39
1. Subjects and samples 39
1.1 Studies I, II and III 39
1.2 Study IV 39
1.3 Study V 40

2. Methods 40
2.1 Measurement of cytokine plasma levels (Studies I - IV) 40
2.2 Measurement of IL-6 production (Study I) 40
2.3 EBV serology (Study II) 40
2.4 Analysis of antibodies to 60 kD heat-shock proteins (Study IV) 41
2.5 Measurement of plasma immunoglobulins (Study V) 41

2.6 Analysis of IL-1α, IL-6 and IL-10 gene polymorphisms (Studies I - V) 41
2.7 Statistical analyses 42
2.8 Ethics 43

RESULTS 44
1. The IL-6 -174 promoter polymorphism and IL-6 responses *in vivo* and *in vitro* (Study I) 44

2. The IL-10 (-1082, -819, -592) promoter polymorphisms, primary EBV infection and IL-10 plasma levels (Study II) 45

3. The IL-1α -889 and the IL-10 (-1082, -819, -592) promoter polymorphisms and IL-10 plasma levels (Study III) 46

4. The IL-6 -174 promoter polymorphism, anti-hsp60 antibody levels and IL-6 plasma levels (Study IV) 48

5. The IL-1α -889 promoter polymorphism and immunoglobulin plasma levels (Study V) 49

DISCUSSION 51
1. The allele and haplotype frequencies of cytokine promoter polymorphisms 51
2. The IL-6 -174 polymorphism and IL-6 responses 52
3. The promoter polymorphisms and humoral responses 53

SUMMARY AND CONCLUSIONS 56

ACKNOWLEDGEMENTS 58

REFERENCES 60

ORIGINAL COMMUNICATIONS 79

LIST OF ORIGINAL COMMUNICATIONS

This dissertation is based on the following original communications, which are referred to by their Roman numerals.

I Kilpinen S, Hulkkonen J, Wang X-Y, Hurme M: The promoter polymorphism of the IL-6 gene regulates IL-6 production in neonates but not in adults. *Eur Cytokine Netw 12: 62-68, 2001*

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

III Kilpinen S, Huhtala H, Hurme M: The combination of IL-1α -889 genotype and IL-10 ATA haplotype is associated with increased IL-10 plasma levels in healthy individuals. *Eur Cytokine Netw 13: 66-71, 2002*

IV Veres A, Prohaszka Z, Kilpinen S, Singh M, Füst G, Hurme M: The promoter polymorphism of the IL-6 gene is associated with levels of antibodies to 60 kD heat-shock proteins. *Immunogenetics 53: 851-856, 2002*

V Kilpinen S, Laine S, Hulkkonen J, Hurme M: Immunoglobulin G3 and immunoglobulin M isotype plasma levels are influenced by interleukin-1α genotype. *Scand J Immunol 57: 296-302, 2003*

In addition to the above-mentioned communications, this thesis contains unpublished data.

ABBREVIATIONS

Ab, antibody

Ag, antigen

APC, antigen presenting cell

bp, base pair (only with numbers)

c, cellular (e.g. cIL-10)

CABG, coronary artery bypass graft

CB, cord blood

Con A, Concanavalin A

CRP, C-reactive protein

DNA, deoxyribonucleic acid

EBV, Epstein-Barr virus

ECS, elective caesarean section

EIA, enzyme immune assay

ELISA, enzyme-linked immunosorbent assay

gp, glykoprotein

h, hour (only with numbers)

h, human (e.g. hIL-1β)

HCV, hepatitis C virus

HIV, human immunodeficiency virus

HLA, human leukocyte antigen

hsp, heat-shock protein

Ig, immunoglobulin

IL, interleukin (e.g. IL-1)

i.v., intravenously

kb, kilobase

kDa, kiloDalton

KO, knock-out

LDL, low-density lipoprotein

LPS, lipopolysaccaride

m, mouse (e.g. mIL-1β)

MHC, major histocompatibility complex

mRNA, messenger ribonucleic acid

NK cell, natural killer cell

OR, Odds ratio

PAGE, polyacrylamide gel electrophoresis

PB, peripheral blood

PBMC, peripheral blood mononuclear cell

PBS, phosphate-buffered saline

PCR, polymerase chain reaction

PWM, pokeweed-mitogen

R, receptor (e.g. IL-1R)

r, recombinant (e.g. rIL-1)

RA, rheumatoid arthritis

s.c., subcutaneously

SLE, systemic lupus erythematosus

SNP, single nucleotide polymorphism

SS, Sjögren's syndrome

v, viral (e.g. vIL-10)

VD, vaginal delivery

VLDL, very low-density lipoprotein

VNTRs, variable number of tandem repeats

INTRODUCTION

A polymorphism means a common, inherited variation in the DNA sequence and is distinguished from rare variations in that the least abundant allele is required to have a frequency of 1 % or more. Several types of polymorphisms exist in the genome. Single nucleotide polymorphisms (SNPs) are stable, biallelic, single base pair differences between the DNA sequences of individuals. The deletion or duplication of a single base is another type of variation of DNA. The Variable Number of Tandem Repeats (VNTRs) are characterised by a core sequence, which consists of a number of identical repeated sequences. They can be divided into two categories based on the repeat length; the microsatellites have a repeat length of 2 - 6 bp, and the minisatellites have a repeat length of roughly 20 - 200 bp. HLA-molecules, hemoglobin, receptors of chemokines and immunoglobulins as well as blood group antigens are examples of highly polymorphic molecules in humans. The frequencies of polymorphic alleles may vary between and within populations.

SNPs are highly abundant and are estimated to occur at an average rate of 1 per 1,000 bases in the human genome. It has been estimated that 1 % of all SNPs alter an amino acid in a protein. Thus, most SNPs do not directly affect protein function. Some SNPs have effects on proteins indirectly. They may change the function of regulatory sequences that control gene expression, or they may alter the stability or the processing of the mRNA of the gene (International SNP Map Working Group 2001)

Cytokines are small, soluble protein molecules that transmit information from one cell to another. Many cytokines are produced by more than one cell type and act on a variety of target cells at different stages of cellular proliferation and differentiation. They all bind to specific receptors expressed on the surface of the target cell. They thereby trigger complex intracellular signaling events, which control gene expression required for the cellular response. These mediators usually have an effect on closely adjacent cells, and therefore function in a predominantly paracrine fashion. They may also act at a distance (endocrine), and they may have effects on the cell of origin (autocrine) (Callard et al. 1999). Since each cytokine has many overlapping functions and, furthermore, since each function is potentially mediated by more than one cytokine, it is not simple to classify these molecules. However, functionally

inflammatory cytokines may be grouped into proinflammatory, such as IL-1 and IL-6, and anti-inflammatory, such as IL-10, cytokines. Genes of cytokines and cytokine receptors have also been proven to be polymorphic.

In this thesis, we studied the allele, haplotype and genotype frequencies of certain inflammatory cytokine (IL-1, IL-6 and IL-10) promoter polymorphisms in healthy Finnish populations. In addition, we were interested in whether these various alleles or haplotypes associate with cytokine baseline levels and/or with cytokine responses *in vitro.* Whether the effect of distinct alleles in cytokine promoters could also be seen on inducible, systemic cytokine levels was also examined; normal labour-related stress served as an *in vivo* stimulus. Cytokine SNPs have been analysed to identify potential markers of susceptibility, severity and clinical outcome of human diseases. Here, we used human EBV-infection as a disease model. All cytokines studied appear to play major roles in host defense mechanisms, and one such activity is the ability to mount humoral responses. Thus, we investigated whether the distinct alleles may influence antibody responses in healthy subjects.

REVIEW OF THE LITERATURE

1. Interleukin-1α

1.1. IL-1 cytokine family

The IL-1 cytokine family consists of IL-1α, IL-1β and IL-1 receptor antagonist (IL-1ra). The three known members of the family are structurally related to one another and bind to IL-1 receptors (IL-1R) on cells (Dinarello 1994, Dinarello 1996). IL-1α shares 22 % sequence similarity with mature IL-1β, and 18 %, with IL-1ra, while IL-1β displays 26 % sequence similarity with IL-1ra. Additionally, IL-1α and IL-1β fold in a highly similar manner. It is noteworthy that the sequence similarity of individual IL-1 cytokine familymember is high between mammalian species, ranging from 60 to 80 %. Thus, the amino acid sequences of a given family member retain significantly higher identities across species boundaries than the three family members combined within a single species (Tocci and Schmidt 1997). IL-1α and IL-1β are potent agonists and recognize the same receptor, which is the type I IL-1 receptor (IL-1RI) (Sims et al. 1988). IL-1ra acts as an antagonist to this receptor thereby blocking the biological responses of the agonists. Nearly all of the cell types that produce IL-1α and IL-1β also produce IL-1ra and the genes encoding these members of the IL-1 family map to a cluster on chromosome 2 (Tocci and Schmidt 1997).

IL-18 can also be included in the IL-1 family, since its gene structure and predicted tertiary protein structure are very similar to those of IL-1β and IL-1ra (Bazan et al. 1996). IL-18 binds to its own receptor, and, unlike the other genes of the family, its gene maps to chromosome 11 (Nolan et al. 1998). Recently, six gene sequences, that are predicted to encode six new members of the IL-1 family, have been documented, and the new members have been named from IL-1F5 to IL-1F10 (Barton et al. 2000, Busfield et al. 2000, Debets et al. 2001, Kumar et al. 2000, Lin et al. 2001, Mulero et al. 1999, Pan et al. 2001, Smith et al. 2000). They were discovered mainly by searching for homologs to IL-1 in DNA databases, and so far no other family members have been found in the genome. Most of them are expressed in monocytes,

macrophages and/or dendritic cells, and specific functions for these proteins are currently being sought.

1.2. Production of IL-1α

Various cell types produce IL-1α, including peripheral blood monocytes, normal human B cells and Epstein-Barr virus-transformed B-lymphoblastoid cell lines, cloned T cell lines, helper T lymphocytes, as well as NK cells. Among specialized tissue cells, macrophages, activated mast cells, keratinocytes and chondrocytes can be cellular sources of IL-1α. However, mature tissue macrophages express approximately 10 - times lower levels of IL-1α mRNA than monocytes. Among the group of granulocytes, eosinophils express small amounts of IL-1α (Tocci and Schmidt 1997).

IL-1α is synthesized as a 31-kDa precursor protein. The mRNA for proIL-1α is translated in association with microtubules, and due to the absence of a leader peptide, proIL-1α remains in the cytosol (Dinarello 1996). Immunohistochemical studies, using LPS stimulated human blood monocytes, show a diffuse staining pattern, which suggests that there is no notable accumulation of IL-1α in any specific cell organelle (Andersson et al. 1992). Approximately 10 - 15 % of proIL-1α is myristoylated, and this form is thought to be transported to the cell membrane. Processing proIL-1α into the 17-kDa mature form requires the activation of the calcium-dependent, membrane-associated cysteine protease called calpain. After processing, a 17-kDa mature IL-1α is released into the extracellular compartment, and the IL-1α propiece, containing amino acids 1 - 115, may bind to nuclear DNA. In addition, the intracellular proIL-1α is capable of nuclear binding either by itself, or as a proIL-1α/IL-1RI complex. Thus, it may act as an active precursor. It is also possible that proIL-1α leaks from a dying cell and becomes cleaved by extracellular proteases (Dinarello 1996).

1.3. IL-1 signal transduction

The IL-1 signaling system is critical for innate immunity that involves responses to infectious agents, and it is conserved in plants, insects and mammals. The cytoplasmic domains of both IL-1 receptor subunits (the IL-1 type I receptor and the IL-1 receptor accessory protein) share significant homology with the *Drosophila melanogaster*

receptor, Toll, that is involved both in development and in primitive immune responses (Gay and Keith 1991). The striking conservation across species may be evidence of the efficiency of this signaling system. It has been reported that the IL-1 signal transduction has been observed in primary cells expressing less than 10 type I receptors per cell (Stylianou et al. 1992).

The IL-1 type I receptor (IL-1RI) is the primary signal transducing receptor, since the IL-1 type II receptor (IL-1RII) lacks a signal-transducing cytosolic domain, and thus acts as a "decoy" molecule. The signaling receptor complex consists of the IL-1RI and IL-1 Receptor Accessory Protein (IL-1RAcP). IL-1 binds to the IL-1 receptor complex with high affinity (Dinarello 1996). The Myeloid Differentation factor 88 (MyD88) is a cytoplasmic mediator molecule which links the activated IL-1 receptor complex to the IL-1 Receptor Associated Kinase (IRAK), a serine-threonine kinase. Upon the binding of IL-1 to its receptor, IRAK is phosphorylated. Then it dissociates from the receptor complex and associates with TNF Receptor Associated Factor 6 (TRAF6). The activation and oligomerization of TRAF6 molecules induces the activation of two distinct kinase pathways; the IkappaB kinase (IKK) complex and the p38 mitogen-activated protein kinase (MAPK) family. The IKK complex induces the phosphorylation of IκB, which renders IκB susceptible to being ubiquitinated and degraded. This liberates NF-κB and allows it to translocate into the nucleus where it can induce target gene expression. The activation of MAPK kinases can in turn lead to AP-1 activation through the c-Jun kinase (Jnk). The way in which TRAF6 interacts with these two downstream kinase pathways is not well understood (Akira et al. 2001).

1.4. IL-1α as a proinflammatory cytokine

Although the role of IL-1 as a proinflammatory cytokine in infection immunity and inflammation seems to be quite clear, little is known of the distinction between the roles of IL-1α and IL-β during these processes. Since IL-1α remains primarily cell-associated and is found mainly in the cytosol and on the plasma membranes of cells, its plasma levels are not usually detectable.

Gene knockout models do also not bring any further information, due to the continuing absence of IL-1α gene-deficient mice. The relative contributions of IL-1α and IL-1β in these mouse models have so far been analysed by comparing the responses of an IL-1β deficient mouse to the responses of a IL-1RI knock-out mouse and assuming that the noted differences result from the role of IL-1α. However, studies using methods like *in situ* hybridization, measurement of steady state levels of mRNA, and antibody staining of tissues have tried to clarify the specific role of IL-1α.

In experimental inflammatory bowel disease of rabbits, there is a better correlation between disease severity and colonic tissue levels of IL-1α than between disease severity and levels of IL-1β (Cominelli et al. 1990). A similar distinct and specific role for IL-1α has been found in the induction of intestinal inflammation in the Peyer's patches of mice in response to experimental *Yersinia enterocolitica* infection. Dube et al. reported that a bacterial mutant of *Y. enterocolitica,* which induces normal levels of IL-1β, TNF-α and IFN-γ but not IL-1α expression, causes far less intestinal inflammation than the wild type *Y. enterocolitica,* which, in contrast, does induce IL-1α expression. Accordingly, the depletion of IL-1α using neutralizing antibodies in mice infected with a wild-type *Y. enterocolitica* led to a significantly decreased intestinal inflammation (Dube et al. 2001).

Skin keratinocytes are constitutive producers of IL-1α. The level of IL-1α mRNA in human keratinocytes is equal to or greater than that of IL-1β (Tocci and Schmidt 1997). IL-1α is thought to play an important role in inflammatory skin reactions. It has been shown that mechanical stress to keratinocytes permits the release of large amounts of IL-1α in the absence of cell death (Lee et al. 1997) – perhaps as a first line of defence in the event of traumatic skin injury. An excessive production and discharge of IL-1α by keratinocytes has been linked to psoriasis, bullous diseases and other skin diseases (Mizutani et al. 1999). IL-1α gene expression and protein production have also been observed in human dermal fibroblasts from systemic sclerosis patients which suggests a possible effect of IL-1α on the fibrosis found in this disease (Kawaguchi 1994). In addition, the proinflammatory properties of IL-1α

may be relevant to the development of atherosclerotic vascular disease. Using immunohistochemical staining, IL-1α has been consistently present in 55 occluded bypass grafts with sclerotic histopathologic changes, and absent from internal mammary arteries with normal anatomic structures (Brody et al. 1992).

1.5. IL-1α as an adjuvant

Staruch and Wood were the first to demonstrate the adjuvanticity of interleukin 1. The simultaneous administration of IL-1 and bovine serum albumin (BSA) could increase the secondary antibody response against the protein antigen (Staruch and Wood 1983). However, since its pyrogenic and inflammatory effects were well-known, the entire molecule as such could not be employed as an immunostimulatory agent in humans.

A short peptide in the position 163 - 171 of human IL-1β seemed to be responsible for immunostimulatory activity without inducing inflammatory responses (Antoni et al. 1986). Subsequently, it was demonstrated that the adjuvant effect of hIL-1β on both T helper-dependent and T helper-independent immune responses could be mimicked by this short peptide fragment (Nencioni et al. 1987).

Although studies on the adjuvanticity of IL-1β dominate the literature, studies on the adjuvanticity of IL-1α also exist. Cholera toxin (CT) is a potent mucosal adjuvant, and the results from both the allogenic and the Ag-specific system showed how CT-stimulated production of IL-1α increased APC-function in macrophages leading to two to threefold enhancement in T cell proliferation (Bromander et al. 1991). In addition, recent observations have confirmed the role of IL-1α as a mucosal vaccine adjuvant. IL-1α seemed to be as effective as IL-1β and CT in the induction of Ag-specific serum IgG, vaginal IgG and IgA, systemic delayed-type hypersensitivity and of lymphocyte proliferative responses when intranasally administered with a soluble protein Ag (Staats and Ennis 1999).

1.6. IL-1α gene and polymorphic sites

The IL-1α, IL-1β and IL-1RA proteins are encoded by separate genes; IL1A, IL1B and IL1RA, respectively. The genes are located in a 430-kb region on the long arm of

human chromosome 2 (2q13) (Nicklin et al. 1994). Sequence analyses suggest that the three genes arose by duplication from a common ancestral gene more than 350 million years ago (Tocci and Schmidt 1997). The IL1A gene is approximately 10.2 kb in size and consists of 7 exons and 6 introns (Furutani et al. 1986). The IL-1α promoter region has a poorly defined TATA element (TTACAAA). Previous transfection studies with IL1A gene 5' upstream flanking sequences have suggested that positive regulatory elements might be situated at -47 to -70 bp and at -70 to -103 bp (Tocci and Schmidt 1997).

The IL-1A gene is polymorphic (http://snp.cshl.org), and at least one of the polymorphisms has been identified in the 5' regulatory region of the promoter: a biallelic base exchange (C → T) at position -889 (McDowell et al. 1995). Recent transfection studies showed that the -889 TT construct had significantly higher expression than the -889 CC construct. In the same study, PB mononuclear cells of the carriers of the TT genotype produced increased IL-1α mRNA and protein levels compared to the cells having the CC genotype (Dominici et al. 2002). The presented results suggest that the -889 promoter polymorphism may have a functional significance.

Intron 6 of the IL1A gene contains a variable number of repeats of the 46-bp sequence. DNA samples from 72 unrelated individuals from a Caucasian population were screened, and six alleles were found (5, 8, 9, 12, 15, and 18 repeats). The most frequent allele (0.62) contained 9 repeats, the second most frequent allele (0.23) contained 18 repeats, and the rest of the alleles were much rarer (Bailly et al. 1993). This 46-bp tandem repeat polymorphism may be of significance in the gene function. Each repeat contains three potential binding sites for transcription factors: an SP1 site, a viral enhancer element and a glucocorticoid responsive element (Bailly et al. 1993, Haugen et al. 1989). At least the last-mentioned has site-specific protein binding activity in an electromobility shift assay (Bailly et al. 1993).

1.7. Association studies on the IL-1α -889 polymorphism

Disease association studies have related the -889 position to diseases such as Alzheimer's disease, early-onset pauciarticular juvenile rheumatoid arthritis,

rheumatoid arthritis, severe periodontitis, and schizophrenia (Table 1). In healthy subjects, the 2.2 genotype and the allele 2 have additionally been associated with increased plasma levels of IL-1β and IL-10, respectively (Table 1).

Nicoll et al. provided evidence establishing that the 2.2 genotype was more common in neuropathologically confirmed Alzheimer's disease patients than in nondemented controls, when the subjects were controlled for age and ApoE genotype; the OR was 3.0. The IL-1A (-889) 2.2 genotype together with the IL-1B (+3953) 2.2 genotype conferred even greater risk; the OR was 10.8 (Nicoll et al. 2000). Although the allele 2 or the 2.2 genotype seems to increase the risk for disease onset, it also seems to result in a slower rate of cognitive decline in AD once the disease has become manifest (Table 1). In the study by Murphy et al., 1.1 homozygotes had a more rapid decline in the Mini-Mental State Examination than the others, when the patients were followed for an average of 3.8 years (Murphy et al. 2001).

McDowell et al. reported an increased carrier status of allele 2 in patients with early-onset, pauciarticular juvenile RA (EOPA-JRA) compared to the controls; the OR was 2.1 (McDowell et al. 1995). Within the group of EOPA-JRA patients (N = 103) the association with allele 2 was particularly strong among the patients who developed iridocyclitis, compared to those without chronic iridocyclitis; the OR was 6.2. Interestingly, the allele 2 was also related to the elevation of the erythrocyte sedimentation rate within the same group. However, no association was found between the -889 alleles and oligoarticular juvenile chronic arthritis, nor between the -889 alleles and chronic anterior uveitis in UK patients (Donn et al. 1999).

Few association studies have succeeded in the measurements of IL-1α protein levels. In the study by Shirodaria et al., the IL-1α protein levels were measured by enzyme immunoassay in 46 patients with severe periodontal disease. The carrier status of allele 2 was clearly associated with an almost four fold increase in the IL-1α protein levels (Shirodaria et al. 2000). This is an interesting finding, considering that the same allele in combination with the allele 2 of IL-1B (+3953) has previously been related to severe periodontitis in a Caucasian population (Kornman et al. 1997).

Table 1. Association studies on the IL-1α -889 polymorphism

Disease association/ other association	*Allele/ genotype*	*Study population*	*References*
Early-onset Alzheimer's disease	2.2 genotype	318 patients vs. 335 controls Italy	(Grimaldi et al. 2000)
Early-onset Alzheimer's disease	2.2 genotype	247 patients vs. 187 controls USA	(Rebeck 2000)
Late-onset Alzheimer's disease	A2	259 patients vs. 192 controls USA and Germany	(Du et al. 2000)
Alzheimer's disease	2.2 genotype	232 patients vs. 167 controls the UK and USA	(Nicoll et al. 2000)
More rapid cognitive decline in Alzheimer's disease	1.1 genotype	114 patients USA	(Murphy et al. 2001)
Temporal lobe epilepsy	No	50 patients with HS 53 patients without HS 112 controls Japan	(Kanemoto et al. 2000)
Childhood chronic immune thrombocytopenic purpura	No	37 patients vs. 218 controls Caucasians	(Foster et al. 2001)
Diabetic nephropathy in insulin-dependent diabetes mellitus	No	95 IDDM patients with nephropathy vs. 96 IDDM patients without nephropathy North Ireland	(Loughrey et al. 1998)
Early-onset pauciarticular juvenile rheumatoid arthritis Chronic iridocyclitis in EOPA-JRA	A2 A2	103 patients vs. 99 controls 28 patients with chr. iridocyclitis vs. 75 patients without chr. iridocyclitis Norway	(McDowell et al. 1995)
Oligoarticular juvenile rheumatoid arthritis	No	164 patients vs. 173 controls the UK	(Donn et al. 1999)
Severity of rheumatoid arthritis	A2	51 patients with destructive RA vs. 47 patients with non-destructive RA France	(Jouvenne et al. 1999)
Schizophrenia	A2*	50 patients vs. 400 controls Finland	(Katila et al. 1999)
Severe periodontitis	A2**	49 patients with mild/no disease vs. 43 patients with severe disease Caucasian Northern Europeans	(Kornman et al. 1997)
↑ IL-1α levels in gingival crevicular fluid	A2	46 patients with severe periodontal disease the UK	(Shirodaria et al. 2000)
↑ IL-1β plasma levels	2.2 genotype	400 healthy blood donors Finland	(Hulkkonen et al. 2000)
↑ IL-10 plasma levels	A2	400 healthy blood donors Finland	(Kilpinen et al. 2002)

* IL-1A2 (-889) + IL-1B1 (-511) + IL-1RN1 Intron 2 VNTR genotype, ** IL-1A2 (-889) + IL-1B2 (+3953) genotype, EOPA-JRA = Early-onset pauciarticular juvenile rheumatoid arthritis, HS = hippocampal sclerosis, IDDM = insulin-dependent diabetes mellitus, No = no association was found, RA = rheumatoid arthritis

2. Interleukin-6

2.1. Production of IL-6

IL-6 is produced by a variety of leukocytes: monocytes, macrophages, T and B lymphocytes, polymorphonuclear leukocytes and mast cells. Additionally, many nonimmune cells may be an IL-6 source: endothelial and epithelial cells, keratinocytes, fibroblasts, adipocytes, vascular smooth-muscle cells, osteoblasts, bone marrow stromal cells, synoviocytes, chondrocytes, Leydig cells of the testis, endometrial stromal cells and trophoblasts. Within the central nervous system, IL-6 can be expressed by astrocytes, microglial cells and folliculostellate cells of the pituitary (Cox and Gauldie 1997, Mohamed-Ali et al. 1997). Human cerebrospinal fluid and human milk also contain IL-6 (Hawkes et al. 1999, Jellinger et al. 2001).

2.2. IL-6 protein and IL-6 receptors

IL-6, leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), oncostatin M (OSM), IL-11 and cardiotrophin-1 (CT-1) are called gp130 signaling cytokines. All these cytokines have some features in common: functional redundancy, structural similarity and a signal-transducing component (gp130) in their receptors (Hirano 1998).

Human IL-6 is synthesised as a precursor protein of 212 amino acids. Depending on the site of cleavage of the signal peptide, the mature polypeptide consists of 183 - 185 amino acids (Cox and Gauldie 1997). The first 28 amino acids of the mature human IL-6 molecule can be removed without significantly affecting its biologic activity (Brakenhoff et al. 1989). In contrast, the C-terminus of the molecule seems to be important; the removal of only four amino acids results in complete loss of biological activity (Kruttgen et al. 1990). Different molecular forms of IL-6 with apparent molecular weights of 23 - 30 kDa have been described. Most likely, they reflect post-translational modifications, such as glycosylation and phosphorylation of the protein (Cox and Gauldie 1997). IL-6 is composed of a bundle of four α-helices linked by loops and an additional mini-helix (Somers et al. 1997). In serum, IL-6 is bound to α2-macroglobulin, which protects the cytokine from cleavage by proteases, and possibly functions as a transport protein (Matsuda et al. 1989).

The IL-6 receptor consists of two polypeptides: the α and the β chain. A strong expression of the α subunit is seen on activated B cells and plasma cells; a weaker expression, on most of the leukocytes. The β subunit is expressed on most cell types, and, similarly to the α-chain, the strongest expression is seen on activated B cells and plasma cells. The α chain (CD126) is an 80-kDa transmembrane glycoprotein, and it binds IL-6 with low affinity. Its cytoplasmic domain is not necessary for signal transduction. A soluble form of the IL-6Rα has also been described with a molecular weight of approximately 50 kDa, apparently arising from proteolytic cleavage of the membrane-bound IL-6Rα. It acts in an agonistic rather than an antagonistic manner (Hirano 1998). It has been found that IL-6 activates gp130 through this soluble form, even on cells that lack the IL-6Rα on their membranes (Kishimoto et al. 1994).

The β chain (CD130) is a 130 kDa transmembrane glycoprotein, and its D2 and D3 domains interact with the IL-6/IL-6Rα complex to form a trimer. This trimer as such is not capable of signaling, and the D1 domain of gp130 is required for forming the higher order activation complex (Kurth et al. 1999). Functional studies have indicated that the IL-6 signaling complex is a hexamer composed of IL-6, IL-6Rα and gp130 – two of each three (Paonessa et al. 1995). The sequential assembly of the IL-6 signalling complex results in the activation of the JAK kinases followed by the phosphorylation and nuclear translocation of a member of the STAT family of transcription factors (Hirano 1998).

2.3. IL-6 as a proinflammatory cytokine

IL-6 is a multifunctional cytokine that is involved in the immune response, inflammation, hematopoiesis as well as in the endocrine and nervous systems. IL-6 acts as a B cell differentiation factor responsible for the final maturation of B cells into plasma cells and for an increased production of IgM, IgG and IgA (Cox and Gauldie 1997). The rhIL-6 causes 3 to 10-fold increases in IgG, IgM and IgA antibody production in PWM-stimulated tonsillar and peripheral blood mononuclear cells in a dose-dependent fashion. In addition, anti-IL-6 is able to inhibit this PWM-induced IgG production up to 80 % (Muraguchi et al. 1988). The effect of IL-6 on human IgG subclass production has also been studied using similarly stimulated PBMCs. IL-6 can enhance the production of each subclass, although each of them

requires IL-6 differently. Maximal IgG1 and IgG4 responses occur when IL-6 is present for the entire culture period or for the latter half of the culture period; whereas maximal IgG3 responses require the presence of IL-6 throughout the entire culture period. In contrast, maximal IgG2 responses are seen when the cytokine is present solely during the latter half of the culture period (Kawano et al. 1994). As opposed to B cells, both resting and activated T cells express IL-6R, and a variety of effects on T cells have been detected. IL-6 stimulates the proliferation of thymocytes and peripheral T cells, as well as promotes the activation, proliferation and differentiation of NK cells (Cox and Gauldie 1997).

As a hemopoietic factor, IL-6 induces human hematopoietic stem cells to proliferate and differentiate. For example, human IL-6 stimulates the maturation of megakaryocytes in vitro and increases platelet levels in mice in vivo (Cox and Gauldie 1997). IL-6 is known to be an important cytokine for myeloma cell growth and proliferation. Recent evidence suggests that this cytokine is not only a growth factor, but also a survival factor inhibiting apoptosis of myeloma cells (Frassanito et al. 2001). Autocrine IL-6 production by myeloma cell clones is related to a highly malignant phenotype of myeloma cells and an aggressive clinical disease (Frassanito et al. 2001). Additionally, serum levels of IL-6 and sIL-6R have been described to reflect the disease severity and prognosis of myeloma (DuVillard et al. 1995, Kyrtsonis et al. 1996).

One of the initially known names of IL-6 was “hepatocyte stimulating factor” reflecting its relevant role in the regulation of the acute phase response. Although many cytokines are involved in the synthesis of acute phase proteins, IL-6 is the only cytokine that can stimulate the synthesis of all those involved in the inflammatory response: CRP, serum amyloid A, fibrinogen, α_1-chymotrypsin and haptoglobin (Castell et al. 1989). Various disease models have demonstrated a strong correlation between serum CRP and IL-6 levels (Bataille and Klein 1992, Biasucci et al. 1996). Recent data on human saphenous vein endothelial cells indicate that CRP is also able to induce IL-6 production (Verma et al. 2002). Like other proinflammatory cytokines, IL-6 is also known to be a fever-inducing pyrogen. The release of IL-6 has been

shown to be a necessary component of fever responses induced by LPS, IL-1β and TNF-α (Chai et al. 1996).

2.4. IL-6 transgenic and gene knockout mice models

Data on the in vivo functions of IL-6 have been obtained from studies that have used IL-6 transgenic and IL-6 knock-out mice. IL-6 transgenic mice with excess IL-6 show massive plasmacytosis in the spleen, lymph nodes, thymus, lungs, liver and kidneys that is associated with a pronounced hypergammaglobulinemia, especially of the IgG1 subclass. In some cases, the condition progresses to plasmacytoma (Papanicolaou et al. 1998). These mice exhibit marked megakaryocytosis in the bone marrow and thrombocytosis in serum, but the numbers of white and red blood cells are normal. Furthermore, they have an increase in their levels of acute-phase proteins (Cox and Gauldie 1997). De Benedetti et al. have demonstrated that IL-6 transgenic mice with the human IL-6 gene express high levels of circulating IL-6 soon after birth and present a reduced growth rate, resulting in mice 50 - 70 % of the size of nontransgenic mice (De Benedetti et al. 1997). On the other hand, IL-6 knock-out mice develop almost normally and have normal levels of B cells (Ramsay et al. 1994). Both these mice models suggest that IL-6 can induce overproliferation of B cells, but that it is not essential for the normal development of the mice.

In IL-6 knock-out mice, the circulating immunoglobulin levels are normal, but the mice are unable to mount an IgA response to a local challenge (Kopf et al. 1994, Ramsay et al. 1994). Additionally, the numbers of thymocytes and peripheral T cells are reduced to 60 - 80 % of those of wild type controls. However, the cells express normal patterns of surface markers. The mice have also impaired T cell responses against certain microbes; at least against vaccinia and vesicular stomatitis viruses, as well as against *Listeria monocytogenes*. IL-6 deficient mice do not develop leukocytosis in response to *L. monocytogenes*, and, not surprisingly, their mortality rate of this infection is significantly increased compared to that among normal mice (Dalrymple et al. 1995, Kopf et al. 1994, Kopf et al. 1995).

The generation of the acute-phase response in IL-6 deficient mice varies depending on the stimuli used. The response to turpentine administered s.c. causing sterile tissue

damage and the response to *Listeria monocytogenes* administered i.v. were greatly reduced, whereas the response to LPS administered i.v. was only slightly reduced (Kopf et al. 1994, Kopf et al. 1995). Mice with no IL-6 gene manifest decreased numbers of progenitor cells in the bone marrow (Kopf et al. 1995). The downstream role of IL-6 in LPS, IL-1β and TNF-α-induced fevers was demonstrated by challenging normal and IL-6 knock-out mice with LPS, rmIL-1β and rmTNF-α. None of these pyrogens induced fever in the IL-6 KO mice, even though the mice have normal thermoregulation, as evidenced by identical diurnal rhythms of IL-6 KO and wild-type mice. Intraperitoneal injections of IL-6 were not sufficient to recover the fever response, but instead, the intracerebroventricular injection of IL-6 was sufficient (Chai et al. 1996).

2.5. IL-6 in stress models

IL-6 functions as a stress hormone and participates in a feedback loop of the hypothalamic-pituitary-adrenal axis. It stimulates this axis primarily by acting on the corticotropin-releasing hormone neuron, and, on the other hand, cortisol exerts a negative feedback on IL-6 secretion. IL-6, given subcutaneously to normal human volunteers, firstly results in elevated plasma levels of the adrenocorticotropin hormone (ACTH) and then in elevated plasma levels of cortisol (Papanicolaou et al. 1998). Various stress models, such as trauma, infection, physical exercise, psychological stress and delivery, have indicated that this cytokine is secreted during stress, and that it participates in the stress response.

Elevated plasma levels of IL-6 have been detected both in experimental and clinical sepsis. In normal humans receiving endotoxin i.v., the peak of circulating IL-6 occurs within 2 - 3 h, which is later than that of TNF-α (Ottaway et al. 1998, Santos and Wilmore 1996). When the comparison was made between patients with septic shock, cardiogenic shock and bacterial pneumonia, the highest IL-6 concentration was found in patients with acute septic shock (de Werra et al. 1997). In septic shock, the IL-6 levels can become exceedingly high, and they have been related to mortality. An IL-6 concentration above 1 ng/ml has been found to increase the risk of death significantly (Damas et al. 1997). Strenuous exercise at different intensities and durations has also been shown to induce elevated IL-6 plasma levels, and larger amounts of IL-6 are

produced in response to it than of any other cytokine (Pedersen et al. 2001). A 79-fold increase in circulating IL-6 concentration was observed during a 3 - 5 h running exercise (Camus et al. 1997), whereas a 250-km cycling race lasting about 6.5 h increased the plasma level 45-fold (Gannon et al. 1997). Accordingly, the IL-6 concentration increases as much as 100-fold after a marathon race (Pedersen et al. 2001). Generally speaking, those plasma levels seen under physical activity are lower than those induced in experimental and clinical endotoxemia.

Both animal and human studies have confirmed that psychological stress can also elevate IL-6 plasma levels (Maes et al. 1998, Song et al. 1999, Zhou et al. 1993). Interestingly, it has been reported that after total overnight sleep deprivation, healthy young males had increased daytime plasma IL-6 levels associated with somnolence and fatigue during the next day, whereas a good night's sleep was associated with decreased daytime IL-6 plasma levels and a good sense of wellbeing (Vgontzas et al. 1999). It also seems obvious that normal, labour-related stress is associated with increased circulating IL-6. Higher IL-6 plasma levels have been found in mothers and their newborn infants after normal vaginal delivery (VD) than in mothers and newborn infants after an elective caesarean section (ECS) (Buonocore et al. 1995).

2.6. IL-6 gene and polymorphic sites

The human IL-6 gene is located at chromosome 7p21 (Sehgal et al. 1986) and consists of 5 exons and 4 introns (Zilberstein et al. 1986). Within the gene, the positions of exon/intron boundaries, exon lengths, and location of cysteine residues within exons are conserved across species. The amino acid sequence identity is 96 % between human and macaque, and 39 % between human and mouse IL-6 (Simpson et al. 1997).

The human IL-6 gene is polymorphic, and one of the characterized polymorphisms is a single base change (G $\rightarrow$ C) at the promoter site -174 (Olomolaiye et al. 1998). Functional studies have suggested that the -174 polymorphism may alter the rate of IL-6 gene expression. The –550 - +61 bp constructs transfected into HeLa cells have been compared in reporter gene studies (Fishman et al. 1998). In unstimulated cells, the -174C construct showed a significantly lower expression than the -174G construct.

After LPS or IL-1 stimulation, the expression from the -174G construct increased in comparison to the unstimulated level, but the expression from the -174C construct did not change significantly. Although transcription factor binding analyses of this site are still lacking, the base change from a G to a C at position -174 creates a potential binding site for the transcription factor NF-1. In HeLa cells, this factor has been shown to be a repressor of gene expression (Rein et al. 1995). Furthermore, the region between -225 and -164 has previously been reported to represent a negative regulatory domain (Ray et al. 1990).

In the promoter region at position -373, there is also a polymorphic A_nT_n tract with six alleles (Fishman et al. 1998), and previous data have demonstrated that the -174C allele is usually associated with the A_8T_{12} pattern. In the study by Fishman et al., 19 subjects out of 20 analysed had this combination, and correspondingly, in the study by Terry et al., 39 subjects out of 40 analysed had it (Fishman et al. 1998, Terry et al. 2000). In addition, the genotypes of the -597G→A polymorphism are in strong allelic association with -174 genotypes (Brull et al. 2001).

Terry et al. also examined the effect of the –221 - +13 construct on CAT (chloramphenicol acetyltransferase) expression in HeLa cells and in ECV304 cells for the two alleles at the -174 position (Terry et al. 2000). The results by Fishman et al. (Fishman et al. 1998) could not be confirmed, since there was no significant difference between the two alleles. However, they also studied the functional effects of four polymorphisms (-597G→A, -572G→C, -373A_nT_n, -174G→C) on the IL-6 promoter and found that these different haplotypes may have an important role in determining the transcription levels of the IL-6 gene. Transcription was higher from the GG9/11G haplotype and lower from the AG8/12G haplotype in the ECV304 cell line. Haplotype - specific differences could not be found in the HeLa cell line. Another important finding was the different transcriptional regulation seen in the different cell lines suggesting cell type-specific regulation of IL-6 gene expression (Terry et al. 2000).

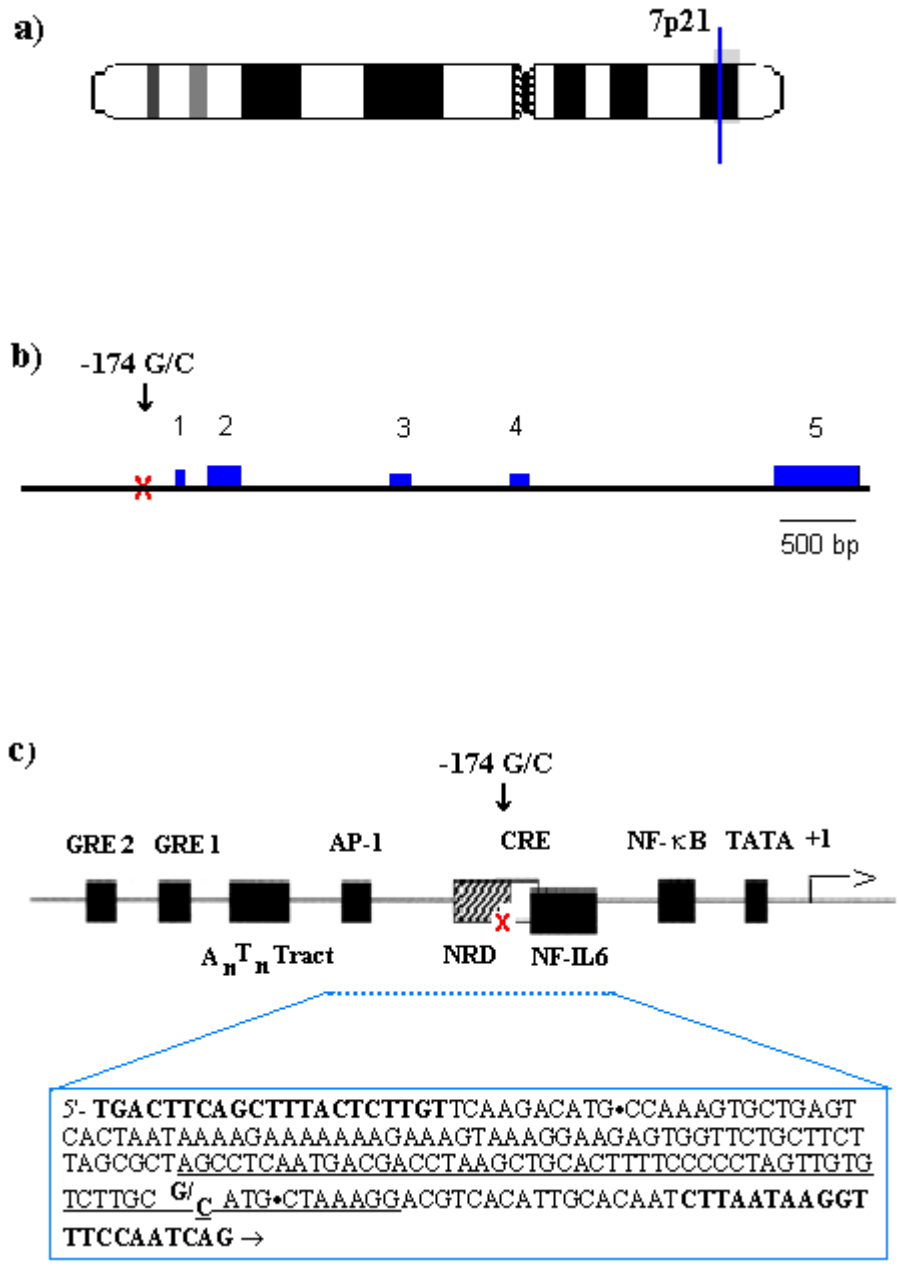

Figure 1 a) The human IL-6 gene is located at chromosome 7p21 (Sehgal et al. 1986) and **b)** consists of 5 exons and 4 introns (Zilberstein et al. 1986).
c) Schematic presentation of the promoter region of the IL-6 gene from -590 to +1 identifying the -174 G/C SNP, transcription factor binding sites and the A_nT_n promoter polymorphism: GRE (glucocorticoid responsive element), AP-1 (activation protein 1), NRD (negative regulatory domain), CRE (cAMP responsive element). The amplified region (from -324 to -126) is shown with a blue dashed line. In the text box below this amplified region is shown as the nucleotide sequence with primers (bold letters), *Nla* III restriction sites (•), negative regulatory domain (NRD, underlined sequence) and the site of -174 G/C SNP ($^{G}/_{C}$). Based to Fishman et al. (1998) and OMIM 2003 database.

2.7. Association studies on the IL-6 -174 polymorphism

In the first report of 102 healthy subjects, the IL-6 -174 CC homozygotes had the lowest IL-6 plasma levels, and the reporter vector analyses in the same study supported this in vivo finding (Fishman et al. 1998). However, the relationship between the IL-6 genotypes and IL-6 plasma levels appears to be more complex. Results from subsequent large-scale studies showed no significant association between IL-6 levels and the -174 polymorphism among healthy subjects (Rauramaa et al. 2000, Kilpinen et al. 2001, Veres et al. 2002). In a group of patients undergoing elective CABG, preoperative IL-6 levels did not differ between genotypes (Brull et al. 2001). Secondly, the C allele and the CC genotype have been related to higher IL-6 levels in subjects with chronic inflammation (Jones et al. 2001), or in subjects after acute severe injury (Brull et al. 2001). On the other hand, the GG genotype has also been associated with elevated plasma levels in primary Sjögren's syndrome patients and in HIV-infected males with Kaposi sarcoma – both disease conditions with chronic inflammation (Table 2).

Inflammatory processes are known to play a role in atherogenesis, and as a key mediator of these responses, IL-6 may have an important role in the development of atherosclerotic changes. Thus, it is not surprising that also the promoter -174G→C polymorphism has been associated with vascular diseases, such as coronary heart disease, lacunar infarction and asymtomatic carotid artery atherosclerosis (Table 2). Furthermore, it has been related to some classic risk factors of atherosclerosis, like blood pressure and lipid abnormalities, as well as to such inflammatory markers as CRP and heat-shock proteins (Table 2).

Several experimental and clinical studies have provided evidence that IL-6 is excessively released into the circulation during sepsis (de Werra et al. 1997, Ottaway et al. 1998, Santos and Wilmore 1996). Clinical data have also shown that IL-6 plasma levels correlate closely with the severity and outcome of sepsis, suggesting a pathogenic role of IL-6 (Damas et al. 1997). In the study by Schluter et al., the genotype distribution and allele frequencies did not differ significantly between patients with (N = 50) or without sepsis (N = 276) and healthy controls (N = 207) (Table 2). The major finding was that there was a significantly different distribution

of genotypes in the 25 septic patients with lethal outcome, when compared with the 25 surviving septic patients. Only 2 out of 25 nonsurvivors were GG homozygotes in comparison to 11 out of 25 survivors. In line with previous reports, a clear correlation was found betwen median systemic IL-6 levels during sepsis and sepsis lethality (Schluter et al. 2002). Interestingly, there was a lack of association between the IL-6 promoter polymorphism and these systemic levels (Table 2).

Table 2 Association studies on the IL-6 -174 polymorphism

Disease association/ other association	*Allele/ genotype*	*Study population*	*References*
↑ IL-6 plasma levels Systemic-onset juvenile chronic arthritis	G	102 healthy subjects 92 patients vs. 383 controls Caucasians	(Fishman et al. 1998)
↑ IL-6 plasma levels in cord blood ↑ IL-6 response of neonatal mononuclear cells to LPS	CC	50 neonates after normal VD 42 neonates after ECS 50 neonates after normal VD Finland	(Kilpinen et al. 2001)
↑ IL-6 plasma levels	C	127 patients after CABG the UK	(Brull et al. 2001)
↑ IL-6 plasma levels ↑ cardiovascular mortality aneurysm growth rate	CC CC No	466 patients with abdominal aortic aneurysm the UK	(Jones et al. 2001)
↑ IL-6 plasma levels	GG	66 primary Sjögren's syndrome patients Finland	(Hulkkonen et al. 2001b)
↓ IL-6 response of whole blood to LPS	GC	89 healthy blood donors Caucasians	(Heesen et al. 2002)
Coronary heart disease ↑ systolic blood pressure ↑ plasma CRP levels	C	2 751 healthy men 494 healthy men the UK	(Humphries et al. 2001)
Lacunar infarction	C	82 patients vs. 82 controls Spain	(Revilla et al. 2002)
Carotid artery atherosclerosis IL-6 plasma levels	GG No	87 healthy men Finland	(Rauramaa et al. 2000)
Lipid abnormalities: ↑ plasma triglycerides ↑ plasma VLDL-triglycerides ↓ HDL cholesterol	G	32 healthy subjects Caucasians	(Fernandez-Real et al. 2000)
↑ plasma CRP levels	C	588 members of 98 families the UK	(Vickers et al. 2002)
↑ hsp-60 Ab levels ↑ hsp-65 Ab levels	GG	176 male blood donors Finland	(Veres et al. 2002)
IgA nephropathy	No	167 patients vs. 400 blood donors Finland	(Syrjänen et al. 2002)
Kaposi sarcoma ↑ IL-6 production	GG	115 HIV-infected men with KS vs. 126 HIV-infected men without KS USA	(Foster et al. 2000)
Multiple myeloma	No	73 patients vs. 129 healthy subjects Sweden	(Zheng et al. 2000)
↓ survival in sepsis incidence of sepsis IL-6 plasma levels of patients	C No No	326 surgical patients vs. 207 healthy subjects German Caucasians	(Schluter et al. 2002)

CABG = coronary artery bypass graft surgery, ECS = elective caesarean section, KS = Kaposi sarcoma
No = no association was found, VD = vaginal delivery

3. Interleukin-10

3.1. Production of IL-10

Both TH_1 and TH_2 type CD4+ T cell clones are able to produce IL-10 in humans, which is in contrast to murine systems where only TH_2 T cell clones produce this cytokine. Human CD8+ T cells and clones also produce this cytokine (Yssel et al. 1992). In addition to peripheral T cells, activated human monocytes, macrophages and B cells are cellular sources of IL-10. Among human B cells, especially those infected with EBV and those within the CD5+ subset are significant producers of IL-10. Furthermore, the cytokine is secreted by human keratinocytes (Powrie et al. 1997).

Kinetic studies have indicated that IL-10 is produced at a relatively late stage following the activation of T cells, monocytes or macrophages. Maximal IL-10 mRNA expression in CD4+ T cell clones and purified peripheral blood T cells is obtained after 24 h, and maximal IL-10 protein synthesis occurs between 24 h and 48 h after activation (Yssel et al. 1992).

3.2. IL-10 protein and IL-10 receptors

The open reading frame of IL-10 gene encodes a 178-amino-acid long peptide. Among different species, these amino acid sequences are rather well conserved; there is 73 % amino acid homology between hIL-10 and mIL-10. The mature protein consists of 160 amino acids, and the molecule primarily exists as a homodimer in the extracellular space. The monomeric form of IL-10 is reported to bind the IL-10 receptor and retain its biological activity, although the affinity is reduced 60-fold and the specific activity is 10 times lower in comparison to the wild-type molecule. When two 160-amino-acid molecules fit together, a compact dimeric structure is formed. Two IL-10 dimers aggregate with four extracellular domains of IL-10R, and this complex forms the activation unit (Moore et al. 2001).

The IL-10 receptor consists of two subunits that are members of the interferon receptor family. Just as IL-10 and IFN-γ are structurally related, an analogy is also seen between their receptors. IL-10R1 is expressed by most hemopoietic cells, although the expression level may vary depending on the cells. There are generally only a few hundred receptors per cell. On B cells, up to 7 000 receptors are found per

cell (Liu et al. 1994). Nonhematopoietic cells express fewer receptors, and on these cells, expression is more often induced rather than constitutive. IL-10R1 binds IL-10 with a high affinity. The 578-amino-acid IL-10R1 molecule is composed of an extracellular domain, a transmembrane segment and a cytoplasmic domain, and has a molecular mass of 90 - 120 kDa. So far no soluble IL-10R1 has been detected in vivo. The hIL-10R1 gene maps to chromosome 11q23.3 (Moore et al. 2001).

IL-10R2 is an accessory subunit of the IL-10R complex. It is constitutively expressed in most cells, and its gene is located in the IFNR gene complex on chromosome 21. IL-10R2 does little to participate in IL-10 binding; its principal role seems to be in the recruitment of a JAK kinase into the IL-10R complex. The JAK family tyrosine kinases JAK1 and TYK2 are constitutively associated with IL-10R1 and IL-10R2. The binding of IL-10 to the receptor complex results in the activation of the JAK - STAT signaling pathway. This occurs at first by the phosphorylation of tyrosine kinases, and then by the activation of STAT-molecules, signal transducers and activators of transcription (Moore et al. 2001).

3.3. IL-10 as an anti-inflammatory cytokine

IL-10 was initially characterized as the cytokine synthesis inhibitory factor (CSIF) in studies on the regulation of functions of mouse T-helper-cell subsets. An activity produced by mouse TH_2 clones was demonstrated to produce a factor that inhibited proliferation and cytokine production by activated TH_1 clones (Fiorentino et al 1989). IL-10 has later on been shown to inhibit the proliferation and cytokine synthesis of normal human T cells as well as IFN-γ production by NK cells. The major explanation for the inhibitory effects of IL-10 is thought to be the inhibition of MHC class II expression on antigen-presenting cells. The CSIF activity of IL-10 also involves downregulation of co-stimulatory molecules for T and NK cell activation. IL-10 is able to inhibit the production of a variety of cytokines, such as IL-1, TNF-α and IL-12, which have been reported as activating cytokines for T and NK cells. Additionally, IL-10 is able to inhibit the expression of CD80 and CD86 surface molecules on APCs, which are ligands for CD28 and CTLA 4 on T cells, and which mediate costimulatory signals. This downregulation of MHC class II and co-

stimulatory molecules leads to reduced antigen presentation and processing in APCs (Moore et al. 2001, Powrie et al. 1997).

IL-10 has been termed as the macrophage-deactivating factor because of its ability to inhibit a number of inflammatory functions of monocytes and macrophages. Many of its anti-inflammatory functions are mediated by inhibiting the synthesis of inflammatory cytokines. These include IL-1α, IL-1β, TNF-α, IL-6, IL-8 and IL-12. Accordingly, IL-10 has also been found to upregulate soluble TNFR-1 and TNFR-2, and thus causes the damping of a TNF-α response. IL-10 has also been shown to inhibit macrophage microbicidal activity by downregulating the production of reactive oxygen and reactive nitrogen intermediates, which are involved in macrophage killing mechanisms (Moore et al. 2001, Powrie et al. 1997).

The anti-inflammatory properties of IL-10 have also been analysed using in vivo mouse models. At the age of 2 - 3 months, IL-10 knock-out mice develop chronic enterocolitis, which mimics many of the features of inflammatory bowel disease in humans (Kuhn et al. 1993). IL-10 deficient mice are very susceptible to LPS-induced endotoxic shock. As little as 5 μg of LPS was a lethal dose for these mice, in comparison to the 200 μg of LPS for control mice. Furthermore, the mice had greatly increased serum levels of pro-inflammatory cytokines, such as IL-1α and TNF-α, compared to control mice (Berg et al. 1995). Accordingly, over 90 % of the IL-10 deficient mice infected with *Toxoplasma gondii* died, whereas less than 5 % of the infected control mice died. It was also shown that the high mortality rate of the knock-out mice was not due to the infection, but to an abnormally strong inflammatory response with high levels of proinflammatory cytokines (Gazzinelli et al. 1996). These results suggest that a major function of IL-10 is to limit harmful immune responses of the host. On the other hand, because of its potent immunosuppressive activities, IL-10 may, under certain circumstances, increase the host's susceptibility to infection. In different mouse models of infection immunity, a low IL-10 level increases resistance and a high IL-10 level increases susceptibility to intracellular pathogens (Moore et al. 2001). The role of IL-10 in the immune responses to infection is rather complex, since there is also evidence that it could have a harmful role only

during the early stages of infection, but that during the later stages it may be needed for host protection (Denis and Ghadirian 1993, Greenberger et al. 1995).

3.4. IL-10 and Epstein-Barr virus-infection

The Epstein-Barr virus belongs to the human herpesvirus family. Most persons acquire the virus during early childhood without symptoms. During adolescence and adulthood, the infection can present itself as acute infectious mononucleosis. An EBV-infection is extremely common, and by the age of 20 years, more than 90 % of adults are seropositive for EBV. The virus is transmitted primarily by saliva, but it is not clear whether mucosal epithelial cells provide the primary target for the virus, or whether the virus must first be amplified in locally infiltrating B cells. Cell-mediated immunity (especially $CD8^+$ CTL and NK cell responses) and cytokines are believed to be crucial to the host's defence against an EBV infection. The importance of the humoral response to the control of the primary infection is uncertain. The infected B cells constitute the site of latency, and from these cells the virus is able to reactivate. Thus, despite the effective cellular immune responses, virus latency within the B cell pool and virus replication in the oropharynx are never completely eliminated (Rickinson and Kieff 1996).

Approximately 5 % of the adult population remain EBV seronegative, but the explanation for this phenomenon is not clear. It has been assumed that persons over 20 years of age have settled down socially, and have been able to avoid EBV-exposure. On the other hand, there is evidence that seronegative adults differ from seropositive adults in some immunologic functions. Seronegative adults have significantly higher percentage of monocytes in the peripheral blood than seropositive adults. Furthermore, elevated levels of IFN-α and IL-6 have been found in culture supernatants of seronegative adults (Jabs et al. 1996). In the same study, serum immunoglobulin levels have been shown to be within the normal range in all study participants, excluding possible hypogammaglobulinemia or selective IgG deficiency as reasons for undetectable EBV antibodies. The expression levels of the EBV receptor CD21 on peripheral B cells did not differ between EBV- (seronegative) and EBV+ (seropositive) individuals (Jabs et al. 1999). Recent data suggest that

immunogenetic differences are possible between EBV seronegative and seropositive adults (Helminen et al. 2001).

An EBV infection induces the production of both cellular IL-10 (cIL-10) and viral IL-10 (vIL-10) in B cells. In human tonsillar B cells infected for 6 h with EBV, vIL-10 expression was detected within a few hours of infection, whereas the expression of cIL-10 followed 20 - 30 hours later (Miyazaki et al. 1993). This viral cytokine, ebvIL-10, is encoded in the genome of EBV by a gene called BCRF-1 (Hsu et al. 1990). The hIL-10 and ebvIL-10 amino acid sequences are 84 % identical (Powrie et al. 1997). The vIL-10 mimics many activities of hIL-10 including CSIF and macrophage-deactivating factor activity on human cells as well as human B cell stimulatory activities (Powrie et al. 1997). However, its specific activity appears to be 3 to 10-fold lower than that of hIL-10, and it binds IL-10R1 with a much lower affinity (1000-fold) than cIL-10 (Moore et al. 2001). A recent report showed that, depending on the kinetics of secretion, ebvIL-10 could have both immunostimulatory and inhibitory effects. A short exposure to ebvIL-10 had inhibitory effects, whereas longer exposure had stimulatory effects on T cell function (Muller et al. 1999). The kind of contribution ebvIL-10 has in viral pathogenesis remains to be elucidated. In any case, because of its conserved structure, vIL-10 is likely to have a number of advantages for EBV in its interaction with the immune system.

3.5. IL-10 gene and polymorphic sites

The hIL-10 gene is located on chromosome 1 at 1q31-32, and it is composed of five exons. Transcription of the IL-10 gene results in an approximately 2-kb mRNA. Several possible transcriptional regulatory sequence elements have been identified in the mIL-10 gene, all of which resemble those found in the IL-6 gene (Moore et al. 2001).

The hIL-10 gene is highly polymorphic. At the promoter region, three single nucleotide polymorphisms at positions -592 (C $\rightarrow$ A), -819 (C $\rightarrow$ T) and -1082 (G $\rightarrow$ A) as well as two CA repeat polymorphisms, IL10.G and IL10.R, approximately at positions -1200 and -4000, have been reported (Eskdale and Gallagher 1995, Eskdale et al. 1996, Turner et al. 1997). Previous studies have demonstrated that strong

associations exist within these microsatellite alleles; for example, between the IL10.R2 and IL10.G13 alleles and the IL10.R3 and IL10.G9 alleles (Eskdale et al. 1996). The SNPs are also in strong linkage disequilibrium forming three haplotypes common in Caucasian populations; GCC, ACC and ATA (Turner et al. 1997). Furthermore, the haplotype GTA is a possible, but an extremely rare combination in Caucasians; 0.6 % in Dutch individuals (Eskdale et al. 1999). The family study has shown that the microsatellite alleles IL10.G and IL10.R as well as -592, -819 and -1082 SNPs together form 29 possible haplotypes, but that they combine to form four major haplotype families at the human IL-10 locus (Eskdale et al. 1999). Recently, new polymorphisms, both at the promoter and at the coding sequence, have been identified (D'Alfonso et al. 2000, Donger et al. 2001, Eskdale et al. 1999).

A difference in IL-10 secretion has been demonstrated between the different IL-10 -1082 alleles. Following Con A stimulation of PB lymphocytes in vitro, carriers of allele G showed significantly higher IL-10 production than carriers of allele A (Turner et al. 1997). When whole blood assays and LPS stimulation were used, the haplotype IL10.R2/IL10.G14 associated with the highest IL-10 secretion, whereas the haplotype IL10.R3/IL10.G7 associated with the lowest IL-10 secretion (Eskdale et al. 1998). The transfection studies have been performed by cloning the –1137 - +25 constructs of the IL-10 5' flanking region from GCC, ACC and ATA homozygous individuals into the luciferase vector and transiently transfecting them into a monocytic cell line. When the relative transcriptional activities of the different constructs were compared, the haplotype ATA had a significantly weaker activity than the GCC haplotype. In the same study, IL-10 production after LPS stimulation in whole blood cultures was also analysed, and, accordingly, the ATA/ATA genotype was associated with the lowest production (Crawley et al. 1999).

It is thought that the reported SNPs at the promoter of IL-10 gene occur within important regulatory regions, and that they may alter the structure of the transcription factor binding sites. These potential transcription factors are still poorly defined. The SNP at position -1082 lies within an ETS-like recognition site and may therefore affect the binding of this factor. The SNP at -819 is situated within a putative positive regulatory region, and the SNP at -592 occurs within a possible STAT 3 binding site and a negative regulatory region (Kube et al. 1995).

3.6. Association studies on the IL-10 promoter polymorphisms

IL-10 promoter polymorphisms (-1082, -819 and -592) have been reported to associate with various rheumatic diseases (Table 3): with a more severe form of rheumatoid arthritis in children and females, with primary Sjögren's syndrome and with different phenotypes of SLE. Additionally, differences in allele/haplotype frequencies have been found in kidney allograft recipients and asthma patients (Table 3).

IL-10 mRNA and protein have been detected in human atherosclerotic plaques (Uyemura et al. 1996, Mallat et al. 1999b), and the results of two previous animal studies suggest its preventive role in the formation of these plaques (Mallat et al. 1999a, Pinderski Oslund et al. 1999). Furthermore, in two recent studies with large study sampling, the promoter SNPs -1082, -819 and -592 were not related to the risk of coronary artery disease or myocardial infarction (Donger et al. 2001, Koch et al. 2001). However, in a study where 300 chronic hemodialysis patients were followed for approximately 2 years for the development of cardiac, cerebrovascular or peripheral artery disease complications, the -1082A allele predicted a higher cardiovascular morbidity than the -1082G allele. The former allele was also associated with signs of systemic inflammation in dialysis patients (Table 3).

Few studies exist on the relationships between promoter SNPs and infectious diseases. Different IL-10 haplotypes are associated with EBV seronegativity in Finnish children and adults (Table 3). The ATA haplotype, viral genotype 3a, a low viral load and little fibrosis on a liver biopsy predicted a good response of an HCV infection to IFN-α therapy (Table 3). In a human endotoxemia model, the -1082 SNP did not associate with systemic IL-10 levels (Fijen et al. 2001).

Table 3. Association studies on the IL-10 promoter (-592, -819, -1082) polymorphisms

Disease association/ other association	*Allele/ genotype/ haplotype*	*Study population*	*References*
↑ IL-10 response of PB lymphocytes to ConA	-1082G	37 healthy controls the UK	(Turner et al. 1997)
↑ IL-10 response of whole blood to LPS	-1082A	158 healthy controls The Netherlands	(Huizinga et al. 2000)
IL-10 plasma levels in a 24-h period after LPS infusion	No	12 healthy controls The Netherlands	(Fijen et al. 2001)
Severe asthma	ATA	113 patients with severe asthma 82 patients with mild asthma 241 healthy controls the UK	(Lim et al. 1998)
↑ Cardiovascular morbidity ↑ CRP and fibrinogen ↓ albumin	-1082A	300 chronic hemodialysis patients Germany	(Girndt et al. 2001)
Coronary heart disease	No	1107 patients vs. 1082 controls France and the UK	(Donger et al. 2001)
EBV seronegativity ↑ IL-10 plasma levels	ATA GCC/GCC ATA	116 healthy children 400 healthy adults 50 healthy neonates + 400 healthy adults Finland	(Helminen et al. 2001)
HCV infection Response to IFN-α treatment	No ATA	53 patients vs. 37 healthy controls 24 responders vs. 29 nonresponders the UK	(Edwards-Smith et al. 1999)
Extended oligoarthritis ↓ IL-10 response of whole blood to LPS	ATA ATA/ATA	78 juvenile RA children with extended oligoarthritis vs. 86 juvenile RA children with oligoarthritis 45 healthy controls the UK	(Crawley et al. 1999)
↑ joint destruction	-1082GG	91 female RA patients The Netherlands	(Huizinga et al. 2000)
Reactive arthritis	No	85 patients vs. 62 controls Finland	(Kaluza et al. 2001)
Primary Sjögren's syndrome ↑ IL-10 plasma levels	GCC GCC	62 patients vs. 400 healthy controls 62 patients with primary SS Finland	(Hulkkonen et al. 2001a)
SLE Ro autoantibodies	No GCC	76 patients and 119 healthy controls 76 patients the UK	(Lazarus et al. 1997)
SLE Lupus nephritis	No ATA	88 patients vs. 83 healthy controls 38 SLE patients with renal disease China	(Mok et al. 1998)
SLE Neuropsychiatric SLE	No ATA	92 patients vs. 162 healthy controls 42 patients The Netherlands	(Rood et al. 1999)
Renal transplantation rejection	-1082G	115 kidney allograft recipients the UK	(Sankaran et al. 1999)

AIMS OF THE PRESENT STUDY

1. To investigate the allele, haplotype and genotype frequencies of the promoter polymorphisms of inflammatory cytokines

2. To investigate whether certain alleles or combinations of distinct alleles on the promoter of inflammatory cytokines associate with cytokine plasma levels and/or with cytokine responses *in vitro*

3. To investigate whether certain alleles or combinations of distinct alleles on the promoter of inflammatory cytokines protect against primary EBV infection

4. To investigate whether certain alleles on the promoter of inflammatory cytokines may influence antibody responses (anti-hsp60, IgM, IgG and subclasses) in healthy subjects

MATERIALS AND METHODS

The subjects and methods are described in detail in the original publications I - V.

1. Subjects and samples

1.1 Studies I, II and III

For studies I and II, cord blood was collected from the umbilical veins of 50 healthy, full-term newborns after normal vaginal delivery. Furthermore, for study I, cord blood was collected from the umbilical veins of 42 healthy, fullterm neonates after elective caesarean section. An informed consent was obtained from the parents. For study I, 450 adult blood samples were obtained from the Finnish Red Cross Blood Transfusion Centre, Tampere. Of these, 400 adult blood samples were also analysed in studies II and III. The donors were adults (18 - 60 years old), and, according to the information acquired using questionnaires, they did not have any blood-transmitted diseases or any signs of other infections during a 2-week period prior to the blood donation. The samples were collected into plastic tubes containing citrate. From the blood samples, plasma was separated, aliquoted and stored at -20° C until further use. From the remaining blood, the mononuclear cells were isolated by Ficoll-Isopaque centrifugation (Pharmacia, Uppsala, Sweden).

The blood samples of patients in study II were obtained from 116 children aged between 9 months and 15 years who had arrived for pediatric consultation between November, 1999 and May, 2000. The mean age ± SD of the children was 6.7 ± 4.9 years. The study was carried out at the Tampere University Hospital and at the University of Tampere. From the blood samples, plasma and mononuclear cells were isolated as described above.

1.2 Study IV

The blood samples of 176 healthy adult males were obtained from The Finnish Red Cross Blood Transfusion Centre, Tampere. The mean age ± SD was 44 ± 10.6 (range 21 - 63) years. From the blood samples, plasma and mononuclear cells were isolated as described above.

1.3 Study V

400 blood samples were obtained from the Finnish Red Cross Blood Transfusion Centre, Tampere. The study population consisted of 182 (46 %) Finnish females and 218 (54 %) Finnish males, and the mean age (range) of the females was 42 (19 - 65) years and of the males, 46 (21 - 64) years. They had no blood-transmitted diseases, nor did they show any signs of other infections during a 2-week period prior to their blood donation. From the blood samples, DNA and plasma were isolated, extracted and stored at -20° C until further use.

2. Methods

2.1 Measurement of cytokine plasma levels (Studies I - IV)

Cytokine plasma levels (IL-1β, IL-6 and IL-10) were measured using available commercial enzyme-linked immunosorbent assays (ELISA; CLB, Pelikine Compact human IL-1β, IL-6 and IL-10 ELISA kit, Amsterdam, The Netherlands). The lower detection limit of the IL-1β assay was 0.4 pg/ml; of the IL-6 assay, 0.2 - 0.4 pg/ml; and of the IL-10 assay, 1.0 pg/ml. The assays were performed according to the manufacturer's instructions. The optical density of individual wells was determined with a Multiscan Biochromatic 348 spectrophotometer (Labsystems, Helsinki, Finland).

2.2 Measurement of IL-6 production (Study I)

The IL-6 production in vitro was analyzed in 50 cord blood and in 50 adult blood samples. Unstimulated and LPS-stimulated mononuclear cells (10^6 cells/ml and 1 ml/well) were incubated at 37° C for 24 h. LPS was used at a final concentration of 1 μg/ml. The cells were then harvested and the supernatants were collected and stored at -20° C. IL-6 levels were measured as described above.

2.3 EBV serology (Study II)

EBV antibodies were measured using an enzyme immunoassay according to the manufacturer's instructions (Enzygnost anti-EBV/IgG, Behring, Marburg, Germany).

2.4 Analysis of antibodies to 60 kD heat-shock proteins (Study IV)

The amounts of IgG-type antibodies reacting with proteins of the chaperonin 60 family (recombinant human hsp60, recombinant *M. bovis* hsp65 [batch MA14] Lionex GmbH, Braunschweig, Germany) were assessed by ELISA as described previously (Prohaszka et al. 1999). Plates were coated with 0.05 μg/well human hsp60 or *M. bovis* hsp65. After washing and blocking (PBS, 0.5 % gelatine), the wells were incubated with 50 μl of serum samples diluted 1:100 in PBS containing 0.5 % gelatine and 0.05 % Tween 20. The binding of anti-hsp antibodies was determined using γ-chain specific anti-human IgG peroxidase-labeled antibodies (Sigma, St. Louis, USA) and an o-phenylene-diamine (Sigma) detection system. The optical density was measured at 490 nm (reference at 620 nm), and the means of duplicate wells were calculated. A serial dilution of a high positive human serum was used as standard. Data obtained as optical density values were calculated to arbitrary unit/ml values related to the standard.

2.5 Measurement of plasma immunoglobulins (Study V)

Plasma immunoglobulins were measured in the Laboratory of Clinical Microbiology of Tampere University Hospital using conventional methods. Plasma concentrations of IgM, IgG and IgG subclasses were determined using a Behring Nephelometer (Behringwerke AG, Marburg, Germany) according to the manufacturer's instructions.

2.6 Analysis of IL-1α, IL-6 and IL-10 gene polymorphisms (Studies I - V)

Genomic DNA was isolated from mononuclear cells using the salting out method (Miller et al. 1988), or from blood using the QIAamp^{R} DNA mini kit (QIAGEN).

Amplification of the *IL-1α -889 polymorphism* (McDowell et al. 1995) was performed in a volume of 50 μl containing 200 ng of template DNA, 40 pmol of each primer (Table 4), 10 mM dNTP mix (Pharmacia Biotech), PCR buffer (Finnzymes) and 1U of DNA Polymerase (DyNAzyme, Finnzymes). The PCR Conditions used were as follows: a denaturing step of 96° C for 1 min and then 40 cycles of 94° C for 1 min, 51° C for 1 min, 72° C for 1 min, and finally 72° C for 4 min and 55° C for 5 min.

The composition of the PCR mixture for the amplification of the *IL-6 -174 polymorphism* (Fishman et al. 1998) was the same as described above, except the fact that 20 pmol of each primer (Table 4) and 2.5 mM MgCl were used. The PCR conditions used were as follows: five cycles of 96° C for 9 minutes, 55° C for 1 minute and 72° C for 3 minutes; followed by 30 cycles of 95° C for 1 minute, 55° C for 1 minute and 72° C for 1 minute; with a final incubation at 72° C for 10 minutes.

The IL-10 -592 and -819 polymorphisms (Edwards-Smith et al. 1999, Mok et al. 1998) were detected by PCR using primers that amplified a short fragment of DNA containing the polymorphism (Table 4). The composition of the PCR mixture was the same as described for IL-1α -889, except the fact that 20 pmol of each primer and 2.5 mM MgCl were used. The PCR conditions used were as follows: a denaturation step at 94° C for 2 minutes and then 35 cycles of denaturation at 94° C for 30 seconds; annealing at 60° C for 45 seconds; and extension at 72° C for 1 minute. This was followed by final extension at 72° C for 10 minutes.

Amplification of *the IL-10 -1082 polymorphism* (Edwards-Smith et al. 1999) was performed using the HotStarTaq kit (Qiagen), and 20 pmol of each primer and Q-Solution were included in the PCR mixture. The PCR conditions used were as follows: a denaturation step at 95° C for 15 minutes and then 35 cycles of denaturation at 94° C for 1 minute; annealing at 60° C for 1 minute and; extension at 72° C for 1 minute. This was followed by final extension at 72° C for 7 minutes.

Identification of the 2 alleles at each polymorphic site was performed by incubating the PCR product with a restriction enzyme chosen to cut 1 of the 2 alleles (Table 4), followed by electrophoresis on 9 % PAGE (IL-1α -889, IL-6 -174, IL-10 -592 and IL-10 -819) or on 3 % agarose gel (IL-10 -1082).

2.7 Statistical analyses

If the variables were not normally distributed, statistical methods appropriate for non-parametric data were used. Differences between two groups were analysed using the Mann-Whitney U-test, and differences between three or more groups, using the Kruskal-Wallis test. Correlations were estimated using the Pearson or Spearman

correlation coefficient tests, and before the Pearson test, the variables that were not normally distributed were square-root transformed. Frequencies were compared using the chi-square test. The odds ratios were calculated using the logistic regression analysis. Statistical calculations were performed using SPSS for Windows, version 6.1 or 10.1 (SPSS Inc., Chicago, Illinois). The Hardy-Weinberg equations were calculated using the Arlequin program, version 1.1 (Genetics and Biometry Lab, University of Geneva, Switzerland).

2.8 Ethics

The ethics board of the Finnish Red Cross Blood Transfusion Centre gave their approval for human blood use. The ethical committee of the hospital approved the study plan for study II.

Table 4. Technical data for the analysis of IL-1α, IL-6 and IL-10 gene polymorphisms

Polymorphic site	Primer sequences	Resulting fragment (bp)	Restriction enzyme digestion time	Restriction pattern (bp)
IL-1α -889	5' AAG CTT GTT CTA CCA CCT GAA CTA GGC 3' 5' TTA CAT ATG AGC CTT CCA TG-3'	99	*NcoI* 3h	C: 16 + 83 T: 99
IL-6 -174	5' TGA CTT CAG CTT TAC TCT TGT 3' 5' CTG ATT GGA AAC CTT ATT AAG 3'	168	*NlaIII* 24h	G: 168 C: 49 + 119
IL-10 -592	5' CCT AGG TCA CAG TGA CGT GG 3' 5' GGT GAG CAC TAC CTG ACT AGC 3'	412	*RsaI* 3h	C: 412 A: 176 + 236
IL-10 -819	5' TAA ATA TCC TCA AAG TTC C 3' 5' ATC CAA GAC AAC ACT ACT AA 3'	588	*MaeIII* 3h	C: 79 + 217 + 292 T: 79 + 509
IL-10 -1082	5' TCT TAC CTA TCC CTA CTT CC 3' 5' CTC GCT GCA ACC CAA CTG GC 3'	139	*MnlI* 3h	A: 139 G: 33 + 106

RESULTS

1. The IL-6 -174 promoter polymorphism and IL-6 responses *in vivo* and *in vitro* (Study I)

Results by Fishman et al. suggested that the -174 SNP in the promoter region of the IL-6 gene could affect the transcription rate of the IL-6 gene and the IL-6 plasma levels of healthy subjects (Fishman et al 1998). To analyse the biological significance of the polymorphism, we examined the IL-6 plasma levels in cord blood and the IL-6 production by neonatal cells after LPS-stimulation in relation to the presence of the IL-6G and IL-6C alleles. We hypothesized that since healthy neonates lack a previous exposure to exogenous antigens, their cytokine production could be genetically regulated. We also assumed that normal labour-related stress could provide a physiological stimulus for IL-6 production. We were also interested in whether IL-6 plasma levels of healthy, adult subjects vary according to these dictinct alleles.

The frequencies of the C allele in neonates born by vaginal delivery (N = 50) and in neonates born by elective caesarean section (N = 42) were comparable with the frequencies of two adult groups (N = 400 and N = 50). The respective values were 0.52, 0.61, 0.55 and 0.54

The IL-6 plasma levels in both groups of neonates were significantly higher than those in healthy adults. In addition, the cord blood IL-6 levels after VD differed from those after ECS. The median value for neonates born by VD was 11.4 pg/ml (4.5 - 45.9), for neonates born by ECS, 2.9 pg/ml (1.9 - 6.4) and for adults, 1.2 pg/ml (0.7 - 2.0). According to this finding, normal labour-related stress could increase IL-6 plasma levels in newborn infants. It was noted that the carriers of the CC genotype in both groups of neonates had increased IL-6 plasma levels compared to the carriers of the G allele. The respective median IL-6 values for newborns born by VD were 21.4 pg/ml (9.5 - 81.3) and 9.6 pg/ml (3.5 - 36.2) and for newborns born by ECS, 6.3 pg/ml (2.2 - 12.9) and 2.7 pg/ml (1.7 - 4.1). No association was found between IL-6 plasma levels and the different IL-6 genotypes in the adult controls.

In vitro experiments confirmed our in vivo results. After LPS stimulation, the mononuclear cells of the neonates of the CC genotype secreted more IL-6 than those of the carriers of the G allele; the respective median values were 17,425 pg/ml (11,400 - 33,900) and 6,980 pg/ml (4,175 - 16,800). There was no difference in the IL-6 production of adult cells between the carriers and the non-carriers of allele G. The IL-6 production of unstimulated neonatal or adult cells did not associate with the IL-6 -174 polymorphism.

2. The IL-10 (-1082, -819, -592) promoter polymorphisms, primary EBV infection and IL-10 plasma levels (Study II)

In the study by Turner et al., the IL-10 -1082 SNP was related to IL-10 protein production in vitro (Turner et al. 1997). Subsequently, the three possible haplotypes (GCC, ACC and ATA) were reported to affect the transcription rate of the IL-10 gene (Crawley et al. 1999). Furthermore, it has been previously shown that the IL-10 -1082 SNP influences both the susceptibility to the EBV infection and the clinical picture of the infection (Helminen et al. 1999). In this study, we wanted to analyse whether the promoter SNPs of the IL-10 gene influence the age at which EBV seroconversion occurs. We also wanted to measure IL-10 plasma levels in newborn children and healthy adults and to associate them to the haplotypes.

13.3 % of the children < 2 years (N = 45), 46.3 % of the children 2 - 10 years (N = 41) and 63.3 % of the children over 10 years (N = 30) were seropositive for EBV. The IL-10 haplotype frequencies in children were GCC 0.44, ACC 0.36 and ATA 0.20. The respective frequencies in the group of healthy adults (N = 400) are described in section 3.

The ATA haplotype carriage was more common in EBV seronegative than in EBV seropositive children (44 % vs. 25 %, respectively). In addition, using logistic regression analysis, ATA positivity was found to associate significantly with EBV seronegativity (OR= 2.6, CI 95 % 1.04 - 6.7) when controlled by age. No significant association was found between the carriage of the GCC or ACC haplotypes and the EBV status. There were also no significant differences in the frequencies of the different IL-10 genotypes between EBV seronegative and seropositive children.

In EBV seropositive adults, the ACC haplotype carriage was significantly more common than in seronegative adults; 230 individuals out of 380 studied (61 %) vs. 5 individuals out of 20 studied (25 %), respectively. No difference was detected in the overall carriage of GCC or ATA haplotypes between seropositive and seronegative adults. However, seronegative adults were significantly more often homozygous for the GCC haplotype than seropositive adults; 11 individuals out of 20 (55 %) studied vs. 66 individuals out of 380 studied (17 %), respectively.

In the group of healthy neonates (N = 50), the ATA carriers had higher IL-10 plasma levels than the noncarriers. The respective median values and quartiles were 3.18 pg/ml (2.35 - 5.56) and 2.14 pg/ml (1.38 - 3.42). The GCC carriers had significantly lower IL-10 levels than the noncarriers (1.89 pg/ml [1.41 - 3.24] vs. 3.18 pg/ml [2.42 - 5.60], respectively). The ACC carrier status did not have any effect on the plasma IL-10 levels, when carriers and noncarriers were compared. The IL-10 genotype status by itself did not have any effect on the IL-10 plasma levels in neonates.

3. The IL-1α -889 and the IL-10 (-1082, -819, -592) promoter polymorphisms and IL-10 plasma levels (Study III)

Family studies have demostrated striking differences between individuals in their ability to produce IL-10 following lipopolysaccaride (LPS) stimulation of whole blood cultures in vitro, which suggests that differences in IL-10 production involve a considerable hereditary component (Westendorp et al. 1997). In this study, we wanted to analyse the possible effect of the IL-10 genotypes and the haplotypes on IL-10 plasma levels in healthy blood donors. As previous reports have demonstrated that endogenously produced IL-1 induces LPS-stimulated IL-10 production (Foey et al. 1998) and that IL-10 inhibits synthesis of IL-1 in human monocytes (de Waal Malefyt et al. 1991), it is apparent that these two cytokines form an autoregulatory feedback loop. Thus, we were interested in whether any relationship could be found between IL-10 and IL-1β in vivo. To examine this, the influence of the IL-1α -889 genotype on IL-10 plasma levels as well as a putative correlation between IL-10 and IL-1β were analysed.

The study group consists of 400 healthy adult blood donors. The frequency of IL-1A1 (C) was 0.67 and of IL-1A2 (T), 0.33. The IL-10 haplotype frequencies were GCC 0.44, ACC 0.34 and ATA 0.22. The median IL-10 plasma value was 1.52 pg/ml (0 - 3.00 pg/ml), and in 252 of 397 samples (63 %), the concentrations were above the detection limit of the assay.

The IL-10 genotype status by itself did not have any effect on IL-10 levels. However, when the levels were compared according to the IL-10 haplotypes, it was obvious that the ATA carriers had significantly higher IL-10 plasma levels than the noncarriers; the respective median values were 1.8 pg/ml (0.0 - 3.5) and 1.5 pg/ml (0.0 - 2.6). The ACC or GCC carrier status did not have any effect on the plasma IL-10 level. It was also noted that individuals with the IL-1α 2.2 and 1.2 genotypes had significantly higher IL-10 plasma levels than those with the genotype 1.1; the respective median values were 1.8 (0.0 - 5.8), 1.7 (0.0 - 3.1) and 1.3 (0.0 - 2.7). This finding was also confirmed, when a comparison was made according to the carrier status. The carriers of allele 2 secreted significantly more IL-10 than the noncarriers; 1.7 (0.0 - 3.3) and 1.3 (0.0 - 2.7). Neither the genotype IL-1β -511 nor the genotype IL-1RA VNTR had an effect on IL-10 levels.

Since both the carriers of the IL-1α -889 allele 2 and the carriers of the IL-10 ATA haplotype had increased IL-10 plasma levels, we wanted to analyse a possible influence of the allele combination on IL-10 levels. This combination, IL-1A2+/ATA+, was found in 93 subjects out of 400 analysed (23 %) and associated with significantly high IL-10 plasma levels. The respective group median was 2.11 pg/ml (1.22 - 4.73) for IL-1A2+/ATA+, 1.32 pg/ml (0.00 - 2.73) for IL-1A2-/ATA+ , 1.48 pg/ml (0.00 - 2.48) for IL-1A2+/ATA- and 1.40 pg/ml (0.00 - 2.89) for IL-1A2-/ATA- .

The subjects were additionally grouped into three groups according to the 25 % and 75 % quartiles of IL-10 values: nonproducers (0.00 - 0.00 pg/ml, N = 145), moderate producers (1.20 - 2.97 pg/ml, N = 152) and high producers (3.00 - 118.00 pg/ml, N = 100). It was evident that among high producers, the frequency of the IL-1A2+/ATA+ genotype combination was clearly higher than that among nonproducers or among

moderate producers; 37/100 (37 %) versus 22/145 (15.2 %) or 34/152 (22.4 %), respectively. Accordingly, the IL-1A2+/ATA+ combination was more likely present among the high producers than among the nonproducers of IL-10 (OR = 3.3, 95 % CI 1.8 - 6.0) or among the moderate producers of IL-10 (OR = 2.0, 95 % CI 1.2 - 3.6).

The possible relationship between IL-10 and IL-1β plasma levels in study subjects with measureable IL-10 plasma levels (range 1.20 pg/ml - 118.00 pg/ml) was analysed, and a moderate correlation was found; r = 0.6. This result could indicate that the production of IL-1β may induce the synthesis of IL-10 in vivo, and that these levels may be genetically determined.

4. The IL-6 -174 promoter polymorphism, anti-hsp60 antibody levels and IL-6 plasma levels (Study IV)

Elevated IL-6 plasma and anti-hsp60 antibody levels have been associated with atherosclerosis (Ridker et al. 2000, Zhu et al. 2001). The -174 SNP in the promoter region of the IL-6 gene has been demonstrated to affect the transcription rate of the IL-6 gene and the IL-6 plasma levels of healthy subjects (Fishman et al. 1998). In this study, we wanted to investigate the possible associations between these factors. We were interested in whether the -174 SNP could have an effect on anti-hsp60 antibody and/or IL-6 plasma levels in apparently healthy men. Also, a putative correlation between anti-hsp60 antibody and IL-6 plasma levels was analysed.

Antibody levels to human hsp60 and IL-6 plasma levels were measured in the samples of 176 healthy male blood donors. The G allele frequency was 0.47 and the C allele frequency was 0.53.

The IL-6 SNP at position -174 was associated with human hsp60 antibody levels. The CC homozygotes had the lowest antibody level (25.20 [12.57 - 44.24] AU/ml; median [quartiles] AU/ml), and the GG homozygotes had the highest antibody level (44.11 [22.08 - 75.77] AU/ml). The GC heterozygotes showed medium antibody levels. This observation was confirmed, when the analysis was done according to carrier status. The GG homozygotes had almost 2 times higher anti-hsp60Ab levels than the carriers

of allele C (44.11 [22.08 - 75.77] AU/ml vs. 22.32 [13.01 - 49.44] AU/ml, respectively).

The IL-6 levels were low in this cohort of healthy subjects; the median value with quartiles was 0.87 pg/ml (0.00 - 1.35). In 52/176 (30 %) subjects no detectable IL-6 was present. Individuals with detectable or non-detectable serum IL-6 levels had no different hsp60 antibody titres. IL-6 plasma levels did not associate with different IL-6 genotypes in this cohort of healthy men. There was no significant correlation between IL-6 and the hsp60 antibody levels.

5. The IL-1α -889 promoter polymorphism and immunoglobulin plasma levels (Study V)

The immunoglobulin plasma levels are known to be, at least partially, genetically regulated, but all the genes involved are not known (Kohler et al. 1985). IL-1 is a potent proinflammatory cytokine able to serve as an adjuvant for immune responses (Staats and Ennis 1999). IL-1α gene is polymorphic, and at least one of the polymorphisms has been identified in the 5' regulatory region of the promoter; a biallelic base exchange (C → T) at position -889 (McDowell et al. 1995). We set out to study whether the IL-1α genotype might contribute to the genetic component seen in the steady-state antibody levels of healthy individuals.

The frequencies of allele 1 (C) and allele 2 (T) of IL-1α -889 SNP were 0.69 and 0.31. The allele frequencies did not differ significantly between males (N = 218) and females (N = 181); the respective frequencies for males were 0.68 and 0.32 and for females, 0.70 and 0.30.

An association was found between IgG3 plasma levels and IL-1α -889 SNP. Male 1.1 homozygotes had higher IgG3 levels than male 1.2 heterozygotes, but no such difference was found between 1.1 and 2.2 homozygotes, or between 1.2 heterozygotes and 2.2 homozygotes. Accordingly, in males, the IL-1α 1.1 homozygosity was more likely related to high IgG3 plasma levels (0.68 - 0.21) than to low plasma levels (0.20 - 0.04); OR 2.5, 95 % CI 1.5 - 4.4. Female IL-1α 1.1 homozygotes also had increased IgG3 plasma levels compared to female 1.2 heterozygotes. Identically with the results

on males, no such difference was found, when 1.1 and 2.2 homozygotes or 1.2 heterozygotes and 2.2 homozygotes were compared.

Age did not influence the IgG3 plasma levels, nor did IgG3 levels vary significantly between males and females in the different age groups (19 - 38, 39 - 49 and 50 - 65-year-olds). However, when comparisons were made according to the IL-1A2 carrier status, the most prominent finding was made among the 50 - 65-year-old males; the IL-1α 1.1 homozygotes had higher IgG3 concentrations than the carriers of allele 2. No significant difference could be found in the other age groups of men nor in women.

A weak but significant association was also found between IgM plasma levels and the IL-1α -889 SNP in men. There was a significant difference between 1.1 and 2.2 homozygotes and a nearly significant difference between 1.1 homozygotes and 1.2 heterozygotes. Such a difference was not found between male 1.2 heterozygotes and 2.2 homozygotes or in females. When IgM concentrations were compared between three different age groups, the levels did not vary significantly. As expected, females had significantly higher IgM levels than males in every age group.

Discussion

1. The allele and haplotype frequencies of cytokine promoter polymorphisms

Studies on various ethnic groups will be important to control population variation of allele frequencies in different populations. The IL-1α -889 allele frequencies seem to vary fairly little among Caucasians. Among healthy Finnish adult blood donors, the IL-1A2 (T) frequency was 0.33 or 0.31 (Study III and V). Accordingly, in a healthy Norwegian population, the frequency was 0.30 (McDowell et al. 1995). Similar frequencies have been found in other populations; 0.28 in the USA, 0.29 in a population drawn from the USA and the United Kingdom, and 0.33 in an Italian population (Rebeck 2000). Healthy blood donors from Sheffield and Manchester had an IL-1A2 frequency of 0.29 (Cox et al. 1998). However, Kanemoto et al. have interestingly reported that the IL-1A2 is more than 50 % rarer in a Japanese population than in the populations summarized here (Kanemoto et al. 2000).

There is also considerable interethnic variation in the allelic distribution of the IL-6 -174 (G→C) polymorphism. In the first report on a random population of healthy Caucasian blood donors, the C allele frequency was 0.41 (Olomolaiye et al. 1998). Fishman et al. described the C allele as being considerably rarer among Gujarati Indians (0.15) and Afro-Caribbeans (0.05) than among UK Caucasians (0.40) (Fishman et al. 1998). Fishman's data on the British population have subsequently been confirmed in a large study (N = 2,751) on middle-aged healthy British men, whose C allele frequency was 0.43 (Humphries et al. 2001). A similar value has been found in a control population from Germany (Schluter et al. 2002). The C allele seems to be rarer in Spanish and Italian populations; 0.31 and 0.24, respectively (Bonafe et al. 2001, Revilla et al. 2002). In the Spanish study, the -174 SNP was determined using the methodology by Fishman et al. In Sweden and Finland, the C allele appears to be more common than in the UK or Germany. Healthy blood donors from Sweden had a C allele frequency of 0.47 (Zheng et al. 2000). In our studies, the frequency varied between 0.53 and 0.55 (Kilpinen et al. 2001, Veres et al. 2002). In another Finnish study including 87 men living in the Kuopio area, Eastern Finland, the allele frequency was even higher (0.56) (Rauramaa et al. 2000). Interestingly, DNA samples of primates have also been examined, and in the study the primates (N = 9) all proved

to be GG homozygotes (Fishman et al. 1998). The finding suggests that the G allele is ancestral and that the C allele represents a relatively recent change in the IL-6 promoter region.

The allele, genotype and haplotype frequencies of the IL-10 promoter polymorphisms found in our studies are remarkably similar to those found in Caucasian populations. The ATA haplotype frequency was 0.20 when 116 healthy Finnish children, and 0.22 when 400 healthy Finnish adults were studied (Study III). Frequencies of 0.21 - 0.23 have been described in populations from the UK and Poland (Crawley et al. 1999, Lim et al. 1998, Turner et al. 1997, Koss et al. 2000). A somewhat lower frequency of the ATA haplotype (0.16 - 0.18) is present in Dutch populations (Eskdale et al. 1999, Rood et al. 1999). However, the genotype and haplotype frequencies found in the Chinese differ significantly from those described in the Caucasian populations. The ATA haplotype frequency was extremely high, 0.64, in healthy individuals from Southern China (Mok et al. 1998). The haplotype frequencies of the Japanese also differ from those of Caucasians and even from those of the Southern Chinese individuals; the frequency of ATA was 0.71 (Yamazaki et al. 2001).

If these promoter polymorphisms prove to have some functional significance, any differences in frequencies between populations could partially explain the interethnic variation found in some disease states, such as in autoimmune and infectious diseases. However, more functional data and also data on these frequencies in different ethnical groups will be required before such conclusions can be made.

2. The IL-6 -174 polymorphism and IL-6 responses

The cord blood IL-6 plasma levels and IL-6 production by neonatal mononuclear cells associated with the promoter polymorphism, whereas *in vivo* and *in vitro* responses of adult controls did not (Study I). This lack of association between systemic IL-6 levels and -174 SNP was later confirmed in study IV, which included 176 healthy Finnish males. One could speculate that the significantly lower IL-6 concentrations seen in adult plasma could explain the result and that the SNP effect on IL-6 levels of healthy adults could only be seen after some kind of induction (Table 2). However, as our *in vitro* results demonstrate, the LPS-stimulated cells of adults produced IL-6 in amounts comparable to the cells of neonates, but even then the SNP effect was found

only in neonatal cells, not in adult cells. The underlying mechanism for this difference is not clear, but it seems obvious that IL-6 secretion is regulated in a cell-type and stimulation-specific manner. Various cell types are responsible for producing IL-6 into blood, and it is likely that the responses of the repeatedly stimulated adult cells differ from the responses of the naive neonatal cells. In accordance, various factors during a normal pregnancy and labour may induce IL-6 plasma levels in cord blood; high levels of IL-6 could be caused by an inflammation-like process at normal term (Austgulen et al. 1994, Steinborn et al. 1996), by the exposure of newborn infants to a variety of bacteria after membrane rupture (Romero et al. 1993) and/or by the physical strain of labour (Buonocore et al. 1995).

Previous studies suggest that the -174 SNP is of functional significance, but that there is cell-type and stimulus-specific regulation of IL-6 expression. It has been shown using allele specific constructs in transfected Hela cells that the -174C construct (–550 - +61 bp) has lower basal expression and also much lower expression after LPS or IL-1 stimulation than the -174G construct (Fishman et al. 1998). The haplotype-specific constructs, including -174 SNP, have been compared in transfected Hela cells (epithelial-like) and ECV304 cells (endothelial-like), and the haplotype-specific functional differences were found in ECV304 cells but not in Hela cells (Terry et al. 2000). In line with the above, an association between the IL-6 promoter polymorphism and *in vitro* protein production was evident after anti-CD3/CD28, but not after concanavalin-A stimulation of PB lymphocytes (Hoffmann et al. 2001).

3. The promoter polymorphisms and humoral responses

Heat shock proteins are intracellular proteins produced in response to stress stimuli, such as viral/bacterial infection, high temperature, cytokine stimulation, free radicals and mechanical stress. Most of these proteins are synthesized under normal conditions, but their levels multiply under stress conditions. Hsp60 has been found in all species analysed so far. It exhibits a remarkable sequence homology with its counterparts in plants, bacteria and animals (Zugel and Kaufmann 1999). The hsp60 of many bacteria and parasites is a major target of T cell and antibody responses during infections. Healthy subjects also have T cells that can recognize both self-hsp60 and bacterial hsp60 (Lamb et al. 1989). This finding suggests its possible role as an autoantigen. Immune responses to HSPs have also been reported during the

development of autoimmune diseases. Hsp60 has been implicated as an autoantigen in rheumatoid arthritis and in insulin-dependent diabetes mellitus (Cohen 1991). Furthermore, the presence of serum antibodies to hsp65 has been associated with carotid atherosclerosis (Xu et al. 1993). It is not clear whether the immune reactivity against stress proteins is simply a consequence of these diseases, or whether such responses may contribute to disease pathology.

Our results show an association between IL-6 -174 SNP and anti-hsp60 antibodies in healthy males. Antibody levels did not correlate with systemic IL-6 levels, nor did the IL-6 levels associate with the -174 SNP. However, in a subgroup of blood donors with high autoantibody levels, both anti-hsp60 antibody and plasma CRP levels were found to be related to this polymorphic site (unpublished observation). In line with the above, two studygroups have earlier found the association between -174 SNP and plasma CRP levels in healthy subjects (Humphries et al. 2001, Vickers et al. 2002). Since -174 SNP has been demonstrated to be functional, and since IL-6 is able to induce both the production of acute phase proteins and hsps, this finding seems reasonable. Autoantibody association with a cytokine polymorphism has not previously been described in healthy subjects but in SLE patients; the IL-10 haplotype GCC was increased in SLE patients possessing Ro autoantibodies (Lazarus et al. 1997).

A strong correlation between anti-hsp65 antibodies and carotid atherosclerosis has been found previously. Interestingly, the GG genotype, which associated with elevated anti-hsp60 Ab levels in healthy Finnish males (study IV), also associated with asymptomatic carotid artery atherosclerosis in another Finnish study where 87 healthy males were analysed (Rauramaa et al. 2000). In either study, no association was found between systemic IL-6 levels and the -174 SNP.

The immune reactivity against stress proteins also seems to be involved in the pathogenesis of juvenile chronic arthritis. In children with JCA, proliferative responses have been obtained in T cells taken from both peripheral blood and synovial compartment. The most significant responses were seen by stimulating the cells with the self-antigen human hsp60 (De Graeff-Meeder et al. 1991). The immunogenetic factors may regulate the strenght of these responses, since the G allele of the -174

SNP increased the risk of JCA (Fishman et al. 1998), and since the GG genotype resulted in higher anti-hsp60 antibody levels in our IV study.

Summary and Conclusions

A polymorphism is a common, inherited variation in the DNA sequence, and several types of polymorphism exist in the genome. HLA-molecules and blood group antigens are good examples of highly polymorphic molecules in humans. Single nucleotide polymorphisms (SNPs) are stable, biallelic, single-base-pair differences between the DNA sequences of individuals. SNPs are highly abundant and are estimated to occur at an average rate of 1 per 1,000 bases in the human genome. It has been estimated that 1 % of all SNPs alter an amino acid in a protein encoded by a gene. Thus, most of them do not directly affect protein function. SNPs in the coding regions of genes that alter the function or structure of the encoded proteins are necessary and a sufficient cause of most of the known recessively or dominantly inherited monogenic disorders. These SNPs are routinely analysed for diagnostic purposes. Some SNPs have an indirect effect on protein function. They may change the function of regulatory sequences that control gene expression, or they may alter the stability or the processing of the mRNA of the gene. However, most SNPs are located in non-coding regions of the genome and have no known impact on the phenotype of an individual. These SNPs are useful markers in population genetics and evolutionary studies.

Cytokines are small, soluble proteins that transmit information from one cell to another. Many cytokines are produced by more than one cell type and act on a variety of target cells at different stages of cellular proliferation and differentiation. They all bind to specific receptors expressed on the surface of the target cell, thereby triggering complex intracellular signaling events, which control gene expression required for the cellular response. Since each cytokine has many overlapping functions, and since, at the same time, each function is potentially mediated by more than one cytokine, it is not easy to classify these molecules. Functionally, cytokines may, however, be grouped into proinflammatory, such as IL-1 and IL-6, and anti-inflammatory, such as IL-10, cytokines. Genes of cytokines and cytokine receptors have also proved to be polymorphic.

The aim of this study was to investigate the allele, haplotype and genotype frequencies of promoter SNPs of inflammatory cytokines (IL-1α, IL-6 and IL-10). In

this study, the effects of these SNPs on protein plasma levels and/or on protein responses *in vitro* were analysed. In addition, possible associations between the SNPs and levels of different types of antibodies (EBV-IgG Ab, anti-hsp60 Ab and plasma immunoglobulins) were investigated in healthy subjects. All adult samples were obtained from healthy blood donors. Cord blood was collected from umbilical veins. Blood samples of 116 children who arrived for pediatric consultation were also analysed. The polymorphic sites were investigated using PCR and gel electrophoresis. Cytokine plasma levels and production, EBV-IgG Abs and anti-hsp60 Abs were measured using ELISA. Plasma concentrations of immunoglobulins were determined using a Behring Nephelometer.

The allele or haplotype frequencies of the SNPs studied did not differ from those present in Caucasian populations. Cytokine levels (IL-6 and IL-10) in cord blood and IL-6 production of stimulated neonatal mononuclear cells were associated with their respective SNPs (IL-6 -174 and IL-10 ATA haplotype) on the promoter region. Accordingly, IL-10 plasma levels and the IL-10 ATA haplotype were found to be related in healthy adults. A specific combination of SNPs, IL-1A2^{+}/ATA^{+}, associated with increased IL-10 plasma levels in healthy adults. The GG genotype of the IL-6 -174 SNP associated with high hsp60 autoantibody levels in healthy males. When plasma immunoglobulin levels were analysed, low IgG3 and IgM concentrations were found in male IL-1α -889 heterozygotes (C/T) aged 50 - 65 years. No similar finding could be demonstrated in women. When the immunogenetic characteristics of EBV seronegative children and adults were studied, the ATA haplotype was more common in seronegative than in seropositive children, and the GCC/GCC genotype was more common in seronegative than in seropositive adults.

The associations observed between the promoter SNPs of cytokine genes and cytokine production may indicate that the SNPs are functional and important in gene expression. Additionally, they may influence humoral responses seen in healthy subjects. However, our study sampling consisted of healthy individuals, and it is thus likely that the differences observed do not have pathological significance. Furthermore, information from uncontrolled association studies is always limited, and such studies are prone to confounding factors.

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