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Inflammatory Cytokines and Cytokine Gene Polymorphisms in Chronic Lymphocytic Leukaemia, in Primary Sjögren's Syndrome and in Healthy Subjects

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ACADEMIC DISSERTATION

To be presented, with the permission of
the Faculty of Medicine of the University of Tampere,
for public discussion in the auditorium of Finn-Medi 1,
Lenkkeilijänkatu 6, Tampere, on May 8th, 2002, at 12 o'clock.

Acta Universitatis Tamperensis 864
University of Tampere
Tampere 2002

ACADEMIC DISSERTATION
University of Tampere, Medical School
Tampere University Hospital
Finland

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http://granum.uta.fi

Cover design by
Juha Siro

Printed dissertation
Acta Universitatis Tamperensis 864
ISBN 951-44-5329-8
ISSN 1455-1616

Electronic dissertation
Acta Electronica Universitatis Tamperensis 170
ISBN 951-44-5330-1
ISSN 1456-954X
http://acta.uta.fi

Tampereen yliopistopaino Oy Juvenes Print
Tampere 2002

CONTENTS

ABBREVIATIONS

Ab, antibody
APP, acute phase protein
bp, base pair (only with numbers)
CLL, chronic lymphocytic leukaemia
Con A, Concanavalin A
DNA, deoxyribonucleic acid
DIC, disseminated intravascular coagulation
EBV, Epstein-Barr virus
EIA, enzyme immune assay
ELISA, enzyme-linked immunosorbent assay
ELISPOT, enzyme-linked immunospot assay
ESR, erythrocyte sedimentation rate
FITC, fluorescein isothiocyanate
h, hour (only with numbers)
HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
IFN, interferon (e.g., IFN-α)
Ig, immunoglobulin
IL, interleukin (e.g., IL-2)
IRMA, immunoradiometric assay
kDa, kiloDalton
LIF, leukaemia inhibitory factor
LDH, lactate dehydrogenase
LPS, lipopolysaccharide
mAb, monoclonal antibody
mRNA, messenger ribonucleic acid
MS, multiple sclerosis
n, number in study or group
p, probability
PAGE, polyacrylamide gel electrophoresis
PBMC, peripheral blood mononuclear cell
PBS, phosphate-buffered saline
PNS, peripheral nervous system
PCR, polymerase chain reaction
PE, phycoerythrin
PHA, phytohemagglutinin
PMA, phorbol 12-myristate 13-acetate
PPD, purified protein derivative (tuberculin)
pSS, primary Sjögren's syndrome
r, recombinant, (e.g., rIFN-)
R, receptor (e.g., IL-2R)
RA, rheumatoid arthritis
RFLP, restriction fragment length polymorphism
RIA, radioimmunoassay
SAC, staphylococcus aureus Cowan I strain
SD, standard deviation
SLE, systemic lupus erythematosus
SNP, single nucleotide polymorphism
SS, Sjögren's syndrome
TGF, tumour growth factor
TPA, 12-O-tetradecanoylphorbol 13-acetate
U, unit
VNTRs, variable number of tandem repeats

LIST OF ORIGINAL COMMUNICATIONS

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

I Hulkkonen J, Vilpo J, Vilpo L, Hurme M: Diminished production of interleukin-6 in chronic lymphocytic leukaemia (B-CLL) cells from patients at advanced stages of disease. *Br J Haematol 100: 478-483, 1998*

II Hulkkonen J, Vilpo J, Vilpo L, Hurme M: Interleukin-1 beta (IL-1β), interleukin-1 receptor antagonist (IL-1Ra) and interleukin-6 (IL-6) plasma levels and cytokine gene polymorphism in chronic lymphocytic leukaemia (B-CLL): Correlation to the prognostic parameters. *Haematologica 85: 600-6, 2000.*

III Hulkkonen J, Laippala P, Hurme M: A rare allele combination of the interleukin-1 gene complex is associated with high interleukin-1 beta plasma levels in healthy individuals. *Eur Cytokine Netw 11:251-6, 2000*

IV Hulkkonen J, Pertovaara M, Antonen J, Pasternack A, Hurme M. Elevated interleukin-6 (IL-6) plasma levels are regulated by promoter region polymorphism of the IL-6 gene in the primary Sjögren's syndrome and correlate to the severity of the disease. *Rheumatology 40: 656-61, 2001*

V Hulkkonen J, Pertovaara M, Antonen J, Lahdenpohja N, Pasternack A, Hurme M: Genetic association between IL-10 promoter region polymorphism and primary Sjögren's syndrome. *Arthritis Rheum 44:176-9, 2001*

In addition, this thesis contains unpublished data.

1. INTRODUCTION

Maintaining the physiological balance or homeostasis is of vital importance for any living organism. Various forms of endogenous and exogenous stress constantly interfere with this homeostasis. As a result of genetically determined adaptive potential, the effect of these stress factors is normally compensated, and a new level of homeostasis is usually rapidly achieved. The adaptive potential between the species and between individuals of the same species has a great variation. One of the most important population genetic mechanisms explaining this natural diversity is genetic polymorphism. By having slight deviations in DNA the population will eventually be rich in variation, which is essential for the survival of the species while the sources of stress vary during evolution. It is evident that under selection pressure the advantage of a single polymorphic feature is largely dependent on environmental circumstances. Alleles encoding useful characteristics to given circumstances will enrich in a population in the course of time, and those which are harmful will disappear from the population.

Inflammatory stress is a stress form which is constantly present. Cytokines are a group of small soluble or cell-membrane-bound protein or glycoprotein messenger molecules with high potential in the regulation of inflammatory responses. The balance of proinflammatory and anti-inflammatory cytokines is essential for normal cellular function. Like most human genes, the cytokine genes are also polymorphic. Some alleles of polymorphic cytokine genes have been shown to be associated with variation in cytokine production capacity. Moreover, allelic imbalance of several cytokine genes has been described in a number of diseases. These complementary biological and pathological associations of cytokine gene polymorphism make them an interesting subject of research in autoimmune disorders.

In this thesis we studied a group of inflammatory cytokines and their genetic polymorphisms in healthy Finnish blood donors and in patients with two aetiologically unknown disorders, namely B cell chronic lymphocytic leukaemia and primary Sjögren's syndrome. Such data from genetic and cytokine association studies may be used when new diagnostic markers or targets for immunomodulatory treatments are sought, or when disease associated risk factors are explored. Clinically oriented genetic association studies are of great value as genetic risk-assessment based treatment strategies will become applicable in clinical practice in the near future.

2. REVIEW OF THE LITERATURE

2.1 Cytokines

Cytokines are a group of small soluble or cell-membrane-bound protein or glycoprotein messenger molecules that convey information from one cell to another. More than 200 cytokines have been identified, and these are generally divided into subgroups of interleukins, growth factors, chemokines, interferons and colony stimulating factors. Traditionally cytokines have been also divided by their inflammatory activity into pro-inflammatory (e.g. IL-1, IL-6, TNF-α, TGF-β) and anti-inflammatory (e.g. IL-1Ra, IL-10) subgroups. Cytokine functions are cell-specifically mediated by cytokine receptors located on the surface of the target cells. As soluble mediators, the function of cytokines may be autocrine, paracrine or systemic (hormone-like). Some cytokines have soluble forms of their receptors working agonistically with the given cytokine. Cytokine mediated proinflammatory effector functions may be inhibited by anti-inflammatory cytokines or cytokine receptor specific antagonism. The balance of these excitatory and inhibitory factors is essential for normal cellular function, and it is quite logical that unbalanced cytokine production has been observed in a number of diseases. These disturbances in net cytokine production may be caused at several levels, including synthesis, secretion or catabolism. However, as cytokines have an effect on the expression of other inflammatory factors and on each other, and as these functional relationships are non-linear, the causal relationships of cytokines and diseases are very complicated (Callard et al. 1999). Constant genetic markers reflecting cytokine production capacity are actively sought and the knowledge in this field is increasing rapidly (Bidwell et al. 1999).

2.2 Measurement of cytokines

The first widely applied measurement tools used to measure soluble cytokines were based on bioassays, i.e. measurement of specific proliferative responses of cytokine responsive cells by the use of radioactive tracers (thymidine). Since in hybridoma technology made monoclonal antibodies easily available, breakthroughs in cytokine research also took place. Based on the monoclonal antibodies it was possible to set up easy, rapid, reliable and inexpensive EIA-based measurement tools. EIA based cytokine measurements can be done in cell cultures (cell lines, whole blood cultures, peripheral blood mononuclear cells and purified subtypes of leukocytes) *in vitro* or directly from circulating blood (plasma or sera). During the last few decades, knowledge of cytokine gene sequences has enabled the measurement of mRNA expression by sequence specific techniques such as Northern blot and RT-PCR *in vitro*. The major drawback of *in vitro* assays is that quite often the

spontaneous cytokine production in cell cultures is undetectable, and artificial cell stimulants have to be used in order to activate cells to produce cytokines. Moreover, the purification steps needed to enrich leukocyte populations may activate these cells non-specifically and the observed cytokine profile does not reflect the situation *in vivo.* In order to circumvent these drawbacks, direct *in situ* methods applying cytokine-specific and cell-specific labelled antibodies and fluorescent, colorimetric or radioactive detection have been developed. Although the measurement of cytokines by modern methods is quite reliable, reproducible and easy, the value of information concerning single cytokine production is limited (Whiteside 1994). The complex cytokine interactions seem to be related to each other in a non-linear fashion and the net effect of single cytokine at the level of the whole cytokine network is so far unpredictable (Callard et al. 1999). These implications of long time cytokine defects or overexpression *in vivo* have successfully been studied using gene knock-out or transgenetic animals (see below). However, these approaches applying genetic manipulation have not yet extended to human.

2.3 IL-1 family of cytokines (Studies II, III)

2.3.1 Physiological role of IL-1

Interleukin-1 is a prototype of pleiotropic cytokine. The first studies concerning IL-1 were conducted as early as the 1940s, when the factors causing fever were sought. Later on, IL-1 was originally discovered independently in several institutes and this small group of proteins was subsequently named by their discrete biological activities (Tocci and Schmidt 1997). The former names endogenous pyrogen, leukocyte endogenous mediator, lymphocyte-activating factor, B cell activating factor, osteoclast activating factor, epidermal cell derived thymocyte activating factor, hemopoietin-1 and mononuclear cell factor describe the major biological roles of IL-1 (Dinarello 1996; Tocci and Schmidt 1997). In addition to fever, IL-1 family cytokines have important roles in endocrinology and in the regulation of responses associated with inflammatory stress. The effects of systemic IL-1 are reflected e.g. in the regulation of basic metabolic rate, blood glucose levels, blood pressure, iron metabolism and bone remodelling. In addition, IL-1 augments bone marrow responses to inflammation, induces gene expression of inflammatory prostanoids and of several colony-stimulating factors (Bagby 1989; Dinarello 1996). In the 1970s these activities were shown to be shared by two proteins with molecular masses of 18 kDA and 38 kDa. In 1984 the two different cDNAs encoding proteins IL-1 alpha (IL-1α) and IL-1 beta (IL-1β), were identified (Tocci and Schmidt 1997). The natural inhibitor of IL-1, known as IL-1 receptor antagonist (IL-1Ra), was first identified in the mid-1980s (Arend 1993).

2.3.2 IL-1a and IL-1b

There are two forms of IL-1. The IL-1α and IL-1β proteins share a similar profile of functions. IL-1α remains primarily cell associated and is found largely in the cytosol and on the plasma membrane of cells. IL-1β is the mainly secreted form of IL-1 (Dinarello 1994). The synthesised 31kDa precursor of IL-1α remains in the cytosol until it is myristoylated and translocated to the cell membrane. Approximately 10 to 15 % of the IL-1α is myristoylated (Kurt-Jones et al. 1985; Stevenson et al. 1993). IL-1α may be found in the circulation when being released from dying cells, or alternatively after proteolytic cleavage of myristoylated IL-1α by calpainin in the process in which 17 kDa form of IL-1α is formed (Dinarello 1996). The IL-1β is also primarily synthesised as an immature 31 kDa protein called pro-IL-1β. This pro IL-1β remains cytosolic until converted to mature (17.5 kDa) IL-1β by a proteolytic cleavage with interleukin-1 converting enzyme (ICE). A small amount of pro-IL-1β can be exported outside the cell by an unknown mechanism, and this amount of it is increased when ICE activity is blocked (Thornberry et al. 1992). On the other hand, the biological activity of pro-IL-1β is marginal (Dinarello 1996). Interestingly, the overexpression of ICE activity has been found to potentiate Fas-mediated cell death and inhibition of ICE activity seems to prevent this kind of apoptosis (Los et al. 1995). The principal cellular sources of IL-1α and IL-1β are monocytes, specialised tissue macrophages such as alveolar and synovial macrophages, Langerhans cells, chondrocytes, endothelial cells, mast cells and fibroblasts (Tocci and Schmidt 1997).

2.3.3 IL-1 receptor

Two forms of cell bound IL-1 receptors, one receptor accessory protein and their soluble counterparts have been identified. The IL-1 receptor I (IL-1RI) is the molecule which is responsible for mediating the biological responses of IL-1 to target cells. Binding of IL-1 with IL-1RI induces a heterodimerization of IL-1RI and IL-1 receptor accessory protein (IL-1RacP) initiating signal transduction events. The IL-1 receptor II (IL-1RII), has an antagonistic role by binding IL-1 protein without inducing signal transduction events (Colotta et al. 1994; Dinarello 1996). Also soluble IL-1RII (and to a lesser extent soluble IL-1RI) work antagonistically by binding IL-1β in a liquid phase. The role of soluble IL-1 receptor accessory protein is unclear (Dinarello 1996).

2.3.4 IL-1Ra

IL-1Ra is present in secreted (sIL-1Ra) and two intracellular (icIL-1Ra I and icIL-1Ra II) forms. All forms of IL-1RA share similar physiological activities. The antagonist activity of IL-1Ra is based on its capability to bind IL-1RI with nearly the same affinity as IL-1α and IL-1β thus blocking the

formation of IL-1RI/IL-1RacP heterodimer and interrupting the outset of signal transduction. Although the high IL-1Ra binding affinity to IL-1 receptor I, 10-100 fold molar excess is needed to inhibit IL-1 activity (Arend et al. 1990; Dinarello 1996). The principal cellular sources of IL-1Ra are monocytes, alveolar and synovial macrophages, neutrophils, keratinocytes, fibroblasts and chondrocytes. In contrast to IL-1, endothelial cells do not produce IL-1Ra (Tocci and Schmidt 1997).

2.3.5 IL-1 gene complex

The genes for IL-1 are presumably located on chromosome 2q14.2, in close linkage with the IL-1Ra gene in a region that spans more than 430 kilobases (Figure 1) (Steinkasserer et al. 1992). The IL-1α and IL-1β genes both have 7 exons (Figures 2 and 3) (Nicklin et al. 1994). The IL-1Ra gene (termed as IL-1RN), contains 6 exons and encodes all three forms of IL-1RA (Figure 4) (Eisenberg et al. 1991; Nicklin et al. 1994). In secreted IL-1Ra the first exon encodes the hydrophobic secretory leader, while in intracellular forms of IL-1Ra this signal sequence is replaced by a short stretch of nonhydrophobic residues. The promoter region for icIL-1Ra is 9.6 kb upstream of the promoter region of sIL-1RA (Figure 4). These variable forms of IL-1RA generated by alternative splicing and distinct promoter region usage alternatively result in 152 amino acid secreted IL-1RA (22kDa), 155 amino-acid intracellular IL-1Ra I (22 kDA) or 176 amino-acid (25 kDa) intracellular IL-1RA II (Figure 4) (Muzio et al. 1995; Tocci and Schmidt 1997). The 63 bp sequence coding additional 21 amino acids for type II icIL-1Ra lies between the first and second exon of icIL-1RA (Tocci and Schmidt 1997).

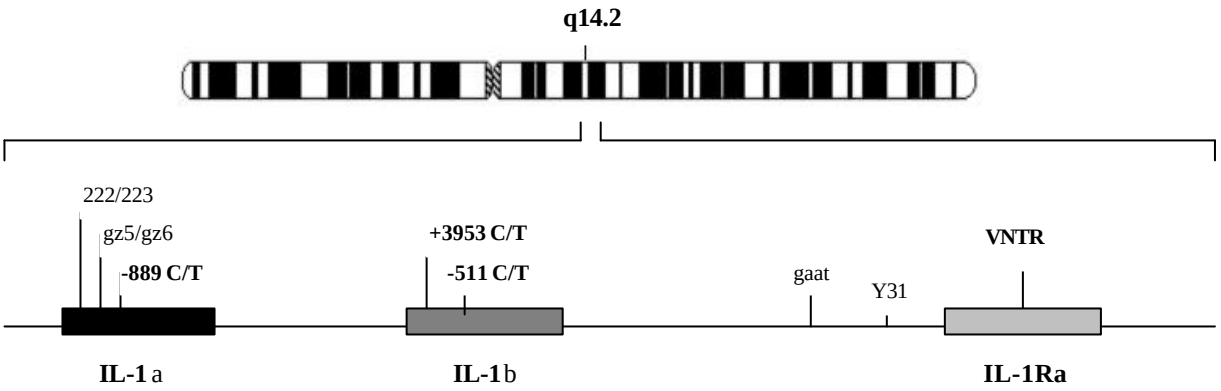


Figure 1. Schematic presentation of IL-1 gene family in chromosome 2 with known polymorphic loci. The polymorphisms studied in this thesis are shown in bold letters. Based to Cox et al. (1998).

The IL-1 gene complex is polymorphic in at least 8 sites (Figure 1). Three of these polymorphic sites have been located at the IL-1α gene (Figure 2). The C (allele 1) to T (allele 2) base exchange polymorphism studied in this thesis is located at the (-889) in the promoter region of IL-1α gene (McDowell et al. 1995). In addition, two microsatelite polymorphisms termed "222/223" (ten alleles) and "gz5/gz6" (four alleles) were recently located in the IL-1α gene (Cox et al. 1998). The IL-1β gene has two base exchange polymorphic sites which have been located at the promoter region site (-511) and at the fifth exon site (+3953) of the IL-1β gene. Both of these polymorphisms are caused by C (-511 allele 1, +3953 allele 2) to T (-511 allele 2, +3953 allele 1) transitions (Figure 3) (di Giovine et al. 1992; Pociot et al. 1992). The polymorphism at the IL-1RN is caused by variable numbers of an 86 bp tandem repeat located in the second intron of the gene (Figure 4). The most common allele has been termed allele one (4 repeats), allele two having 2 repeats, allele three 5 repeats, allele four 3 repeats and allele five 6 repeats (Steinkasserer et al. 1991; Tarlow et al. 1993). In addition, there are two other microsatelite polymorphism termed "gaat" (four alleles) and "Y31" (12 alleles) located between the IL-1β and IL-1RN genes (Figure 1) (Cox et al. 1998; Spur et al. 1996). These 8 polymorphisms are in linkage disequilibrium. The Cox et al (1998) tested 212 unrelated healthy volunteers, and found that the most common haplotype was formed by a combination of "222/223" allele 4, "gz5/gz6" allele 4, IL-1α -889 allele 1, IL-1β (+3953) allele 1, IL-1β (-511) allele 2, "gaat" allele 3, "Y31" allele 3 and IL-1RN tandem repeat allele 2 (in chromosomal order 4-4-1-1-2-3-3-2). This haplotype was seven times more frequent than expected. The second haplotype was (in similar order as above) formed from alleles 3-3-2-2-1-4-6-1, and it was four times more frequent than expected. This shows that IL-1α allele 1, IL-1β +3953 allele 1, IL-1β -511 allele 2 and IL-1RN allele 2 belong to the same haplotype. This result could also be confirmed in a Finnish population (Study III).

The (+3953) polymorphism of IL-1β gene and IL-1RN alleles have been shown to have biological relevance in the regulation of IL-1 and IL-1Ra production. Pociot et al (1992) studied monocyte cultures after LPS stimulation in a mixture of diabetic and healthy male subjects in respect to IL-1β (+3953) polymorphism. In this study the IL-1β (+3953) the allele 1/1 (i.e. T/T) homozygous subjects had higher IL-1β production than the 1/2 heterozygous subjects, who in turn had had higher production than the 2/2 (i.e. C/C) homozygous subjects. It is noteworthy that this difference was not observed if cultures were stimulated with PHA or PPD. In a similar way the IL-1RN allele 2 has been linked with high secreted (but not cell associated) IL-1Ra production in GM-CSF, TGF-β or GM-CSF+INF-γ-stimulated monocytes *in vitro* (Danis et al. 1995). Moreover, this allele was shown to be associated with low cell bound and total IL-1α production. The IL-1RN allele 2

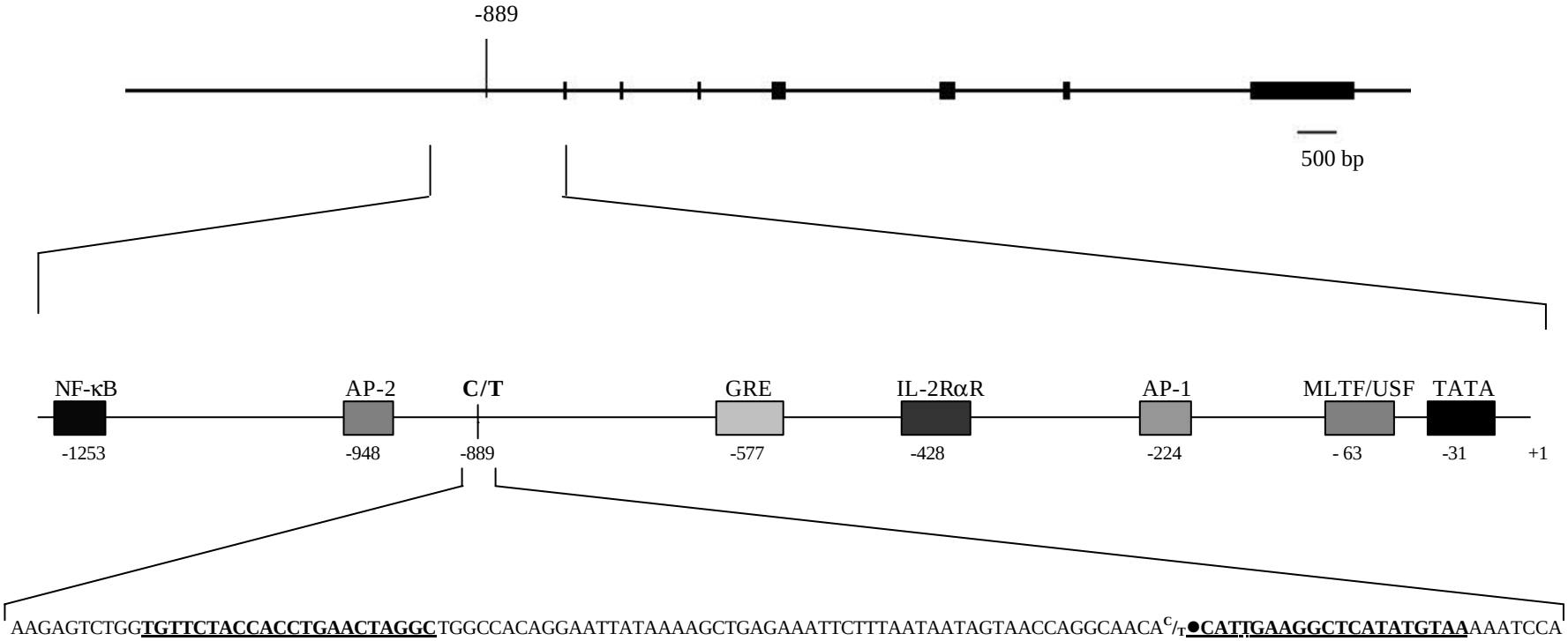
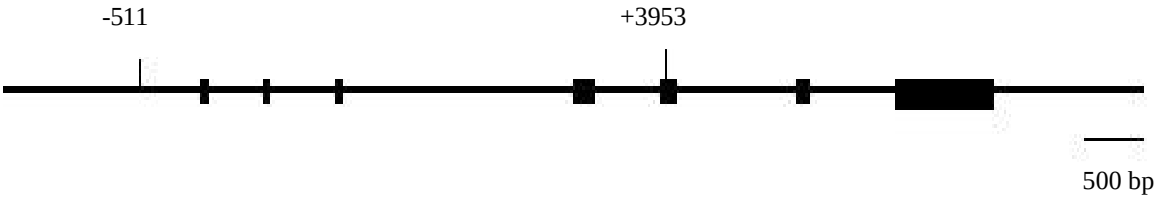


Figure 2. Structure of IL-1α gene. black boxes in the upper figure represent exons. In the middle, components of the promoter region from -1437 to +1. In the text panel below, the region from -978 to -851 showing the binding sites for IL-1α primers (underlined sequences), C/T base exchange polymorphism with the Nco I polymorphic restriction site (**●**). Mismatch base (C instead of A, shown with dashed underline) was used in downstream primer in order to generate Nco I spesific restriction site. Based to McDowell et al. (1995) and a genbank clone X03833 (Furutani et al. 1986). NF-κB; Nuclear factor kappa binding site. AP-1 and AP-2; activation protein 1 and 2 binding sites. GRE; Glucocorticoid responsive element. IL-2RαR; IL-2 receptor α repressor binding site. MLTF/USF; Major late transcription factor/upstream stimulatory factor binding sites. TATA; TATA-box.



A)
AGAGCTCATC**<u>TGGCATTGATCTGGTTCATC</u>**CATGAGATTGGCTAGGGTAACAGCACCTGGTCTTGCAGGGTTGTGTGAGCTTATCTC
CAGGGTTGCCCCAACTCCGTCAGGAGCCTGAACCCTGCATACCGTATGTTCTCTGCCCCAGCCAAGAAAGGTCAATTTTCTCCTCAGA
GGCTCCTGCAATTGACAGAGAGCT$^{C}/_{T}$•CCGAGGCAGAGAACAGCACCCAAGGTAGAGACCCACACCCTCAATACAGACAGGGAGGGC
TATTGGCCCTTCATTGTACCCATTTATCCATCTGT **<u>AAGTGGGAAGATTCCTAAAC</u>**TTAA

B)
CCCTACTGGT **<u>GTTGTCATCAGACTTTGACC</u>**GTATATGCTCAGGTGTCCTCCAAGAAATCAAATTTTGCCACCTCGCCTCACGAGGCCT
GCCCTTCTGATTTTATACCTAAACAACATGTGCTCCACATTTCAGAACCTATCTTCT$^{T}/_{C}$♦CGACACATGGGATAACGAGGCTTATGTGC
ACGATGCACCTGTACGATCACTGAACTGCACGCTCCGGGACTCACAGCAAAAAAGCTTGGTGATG**<u>TCTGGTCCATATGAACTGAA</u>**A
GCTCTCCACCTCCAGGGAC

Figure 3. Structure of IL-1β gene. Black boxes represent exons. In the text panel A the sequence around IL-1β promoter region -511 C/T polymorphic site with the corresponding primer bindings sites (underlined) and Ava I restriction site (•). In the text panel B, the sequence around IL-1β C/T +3953 polymorphic site in the fifth exon of the gene, binding sites for primers (underlined) and Taq I restriction site (♦). Figure and sequences are based to genbank clones M15840 and X04500 by Bensi et al. 1987 and Clark et al. 1986.

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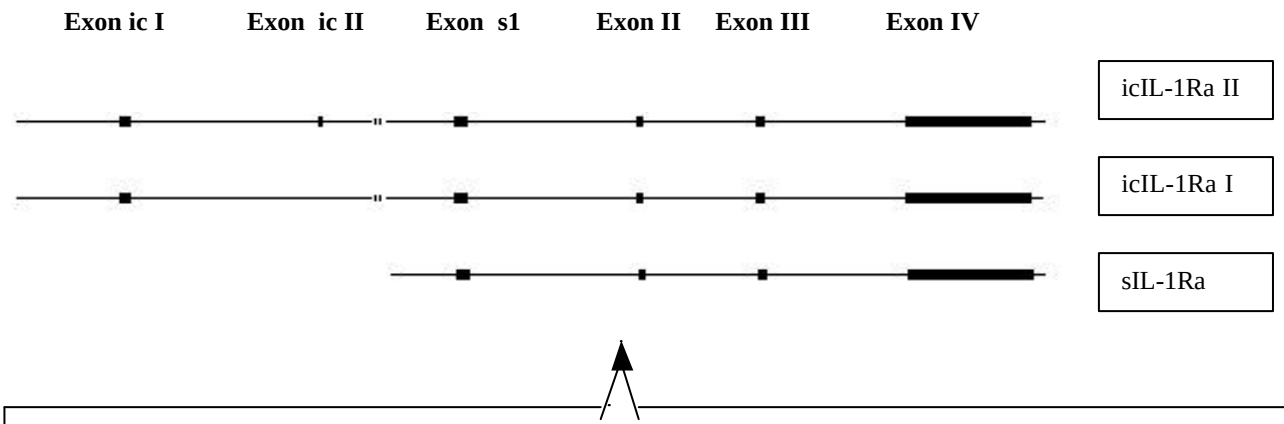
carrier state has been shown to be associated with high plasma IL-1Ra and this IL-1RN*2 effect was enhanced by the presence of IL-1β (-511) allele 2 and absence of IL-1β (+3953) allele 2 (Hurme and Santtila 1998). IL-1RN*2 has also shown to be associated with high IL-1β production in phorbol ester and calcium ionophore stimulated PBMC cultures. This result was independent of IL-1β allelelic status (Santtila et al. 1998). The roles of IL-1β (-511) allele and IL-1α (-889) alleles in respect to protein production are not known for sure. These factors were investigated in Study III of this thesis. The IL-1α (-889), IL-1β (-511), IL-1β (+3953) and IL-1Ra VNTR polymorphisms have been associated with several diseases. The relevant examples of these associations are presented in Table 1. It should be noted, that some of these associations, e.g. particularly in respect to IL-1RN alleles in multiple sclerosis, inflammatory bowel disease and SLE are in dispute.



CTCAGCAACACTCCTATTGACCTGGAGCACAGGTATCCTGGGGAAAGTGAGGGAAATATGGACATCACATGGAACAACATCCAGGA
GACTCAGGCCTCTAGGAGTAACTGGGTAGTGTGCATCCTGGGGAAAGTGAGGGAAATATGGACATCACATGGAACAACATCCAGGAG
ACTCAGGCCTCTAGGAGTAACTGGGTAGTGTGCATCCTGGGGAAAGTGAGGGAAATATGGACATCACATGGAACAACATCCAGGAGA
CTCAGGCCTCTAGGAGTAACTGGGTAGTGTGCATCCTGGGGAAAGTGAGGGAAATATGGACATCACATGGAACAACATCCAGGAGAC
TCAGGCCTCTAGGAGTAACTGGGTAGTGTGCTTGGTTTAATCTTCTAT**TTACCTGCAGACCAGGA**

Figure 4. The gene for IL-1Ra consist of 6 exons, of which 4 to 6 are used when alternative forms of IL-1Ra are formed. This regulation is achieved by alternative splicing and variable promoter region usage. Upper line art picture is showing exon (black boxes) usage, when icIL-1Ra II, icIL-1Ra I or sIL-1Ra are synthesized. For convience, the 7 kb region after the second exon of icIL-1RA II is not shown . In the text panel below, the polymorphic sequence in intron 2 (arrow) with four 86 bp repeats and primer binding sites (underlined). based to Arend et al. (1993), Eisenberg et al. (1991) and a genbank clones U655590 and X64532 by Jenkins et al. (1997) and Lennard et al. (1992).

Table 1. Diseases associated with IL-1 gene family polymorphisms

Polymorphism	Disease	Study populations	Reference
IL-1α (-889)	Juvenile chronic arthritis	269 patients with JRA 99 control subjects	McDowell et al. 1995
IL-1α (-889)	Alzheimer's disease	259 patients with Alzheimer's disease 192 nondemented control subjects	Du et al. 2000
IL-1β (-511)	Inflammatory bowel disease	96 patients with ulcerative colitis 97 patients with Crohn's disease, 132 healthy subjects	Nemetz et al. 1999
IL-1β (-511)	Schizophrenia	50 patients 400 healthy subjects	Katila et al. 1999
IL-1β (+3953)	Type I diabetes	59 IDDM patients 90 healthy subjects	Pociot et al. 1992
IL-1β (+3953)	Rheumatoid arthritis	108 RA patients 128 healthy subjects	Cantagrel et al. 1999
IL-1β (+3953)	Ulcerative colitis	107 patients 82 healthy subjects	Stokkers et al. 1998
IL-1β (+3953)	Squamous intraepithelial cervical lesions	147 patients 100 healthy subjects	Majeed et al. 1999
IL-1β (+3953) IL-1Ra VNTR	Myasthenia gravis	107 patients with myasthenia gravis 82 healthy subjects	Huang et al. 1998
IL-1Ra VNTR	Inflammatory bowel disease	113 patients with ulcerative colitis 78 patients with Crohn disease 261 control subjects	Mansfield et al. 1994
IL-1Ra VNTR	Type I diabetes mellitus	57 IDDM patients 110 control subjects	Pociot et al. 1994
IL-1Ra VNTR	Multiple sclerosis	57 MS patients 65 healthy subjects	Crusius et al. 1995
IL-1Ra VNTR	Grave's disease	100patients 261 control subjects	Blakemore et al. 1995
IL-1Ra VNTR	Sjögren's syndrome	36 patients with pSS 100 healthy subjects	Perrier et al. 1998
IL-1Ra VNTR	Single vessel coronary heart disease	426+248 subjects in two independent cohorts with coronary heart disese 130+102+827 control subjects	Francis et al. 1999
IL-1Ra VNTR	SLE	81 SLE patients 261 control subjects	Blakemore et al. 1994
IL-1Ra VNTR	Ulcerative colitis	236 patients with ulcerative colitis 196 patients with Crohn's disease 338 healthy subjects	Tountas et al. 1999
IL-1Ra VNTR	Osteoporosis	389 patients with osteoporosis 207 normal control subjects	Langdahl et al. 2000
IL-1Ra VNTR	Henoch-Schonlein nephritis	43 patients 98 normal control subjects	Liu et al. 1997
IL-1Ra VNTR	Lichen sclerosis	78 patients 261 control subjects	Clay et al. 1994
IL-1Ra VNTR	Tuberculous pleurisy	89 patients with tuberculosis 114 healthy subjects	Wilkinson et al. 1999
IL-1Ra VNTR	Vulvar vestibulitis	68 patients with vulvar vestibulitis 343 women with no history of vulvodynia	Jeremias et al. 2000

2.4 Interleukin-6 (Studies I, II, IV)

2.4.1 Physiological role of IL-6

Interleukin-6 is a pleiotropic cytokine with proinflammatory, anti-inflammatory and endocrine functions. IL-6 was originally identified as hepatocyte-stimulating factor (HSF) (Ritchie and Fuller 1983). Later on (1985) it was found that the HSF was similar to a B-cell differentiation factor (BSF-2 or interferon β-2) (Gauldie et al. 1987; Hirano et al. 1985) It is now known that IL-6 is a cytokine primarily responsible for the regulation of production of the acute-phase proteins (APPs, e.g. C-reactive protein, serum amyloid A protein, albumin, haptoglobulin). IL-6 stimulates the hypothalamic-pituitary-adrenal axis by promoting CRH release, adrenocorticotrophic hormone (ACTH) synthesis and corticosteroid production (Naitoh et al. 1988; Tsigos et al. 1997). In addition to inflammatory functions, IL-6 promotes megakaryocyte maturation and B cell differentiation as well as IgG, IgM and IgA synthesis (Baatout 1996; Kishimoto et al. 1995; Muraguchi et al. 1988). IL-6 has also been shown to play a role in bone remodelling and in cardiac hypertrophy (Papanicolaou et al. 1998; Wollert and Chien 1997). Most nucleated cells have been shown to express and synthesize IL-6 *in vitro*, but the most prominent source of IL-6 appears to be stimulated monocyte/macrophage lineage cells, cytokine stimulated stromal cells (fibroblasts) and endothelial cells (Kato et al. 1990). In monocytes lipopolysaccaharide (LPS) is the most potent stimulator of IL-6 gene with phorbol ester (TPA) also being prominent (Cox and Gauldie 1997). Although IL-6 has a role in the B cell maturation, the studies on IL-6 gene knock-out mice have shown that IL-6 is not essential for B cell development and IL-6 defect is most prominently demonstrated in impaired mucosal IgA responses as well as impaired acute phase response (Fattori et al. 1994a; Kopf et al. 1994; Libert et al. 1994; Ramsay et al. 1994). IL-6 overexpression studies on transgenic mice have shown that the excess of IL-6 results in massive plasmacytosis in the spleen, lymph nodes, thymus, lung, liver and kidney and that it is associated with hypergammaglobulinemia especially of the IgG1 subclass (Brandt et al. 1990; Campbell et al. 1993; Fattori et al. 1994b; Katsume et al. 1997; Suematsu et al. 1989; Suematsu et al. 1992; Woodroofe et al. 1992). IL-6 transgenic mice also exhibit thrombocytosis (and an increase of mature megakaryocytes in the bone marrow) and leukocytosis as well as decrease in red blood cell count and haemoglobin concentration (Katsume et al. 1997). The persistent overexpression of IL-6 in these mice leads to a syndrome similar to Castleman syndrome in human (hypergammaglobulinemia, lymph node enlargement, and increased APP synthesis), which in some cases progresses to myeloma (Brandt et al. 1990). The long-term overexpression of IL-6 may also result in a development of lymphomas in mice (Woodroofe et al. 1992). Administration of IL-6 to human results in fever, anorexia, fatigue and influenza-like

symptoms as well as anaemia, leukocytosis, thrombocytosis and induction of an acute phase response (Papanicolaou et al. 1998; Schuler et al. 1998).

2.4.2 IL-6 receptor

IL-6 effects are mediated by IL-6 receptor (IL-6R). IL-6R consists of two chains. The major chain or the "body" of the receptor, 130-kD protein, or glycoprotein 130 is not ligand-specific as it is used also by other cytokines, such as leukaemia inhibitory factor (LIF), Oncostatin M (OSM), interleukin-11 (IL-11), ciliary neurotrophic factor (CNTF) and cardiotrophin-1 (CT-1) (Peters et al. 1998). Glycoprotein 130 is therefore also called by the term "common cytokine signal transducer". The human ligand-binding IL-6 receptor is called glycoprotein 80 or CD126 (Hibi et al. 1996). Gp 80 is a member of immunoglobulin gene superfamily and it is mainly present on monocytes, hepatocytes, T cells and activated (but not resting) B cells (Cox and Gauldie 1997). Interaction of IL-6 and IL-6R induces homodimerization of gp 130 and activation of signal transduction cascades on cell membrane which lead to the activation of various tyrosine kinases and transcription factors, namely JAK/STAT, Ras/Raf, and Src-family of kinases (Hallek et al. 1997b; Kishimoto et al. 1995).

2.4.3 Soluble IL-6 receptor

The soluble IL-6 receptor (sIL-6R, 55 kDa form) is generated by cleavage of extracellular domain of transmembrane IL-6 receptor by a transmembrane metalloproteinase, or alternatively via translation from alternatively spliced mRNA (Peters et al. 1998). The sIL-6R has an agonistic function to IL-6 protein (Olsson et al. 1993). Normally cells that have gp130 but do not have specific IL-6 receptor on their surface are not able to respond to IL-6. Such cells may obtain IL-6 responsiveness when a sIL-6R+IL-6 protein complex forms a homodimer with the cell surface IL-6R (gp130), which then mediates the specific IL-6 response to the target cell (Peters et al. 1998). Studies on transgenic mice also suggest, that the presence of sIL-6R prolongs plasma half-life of IL-6, which further enhances the IL-6 responses (Peters et al. 1998).

2.4.4 Viral IL-6

IL-6 has also a viral counterpart. This vIL-6 shows a 25 % amino acid identity to human IL-6 and has been shown to have a functional role (Aoki et al. 1999; Burger et al. 1998). The history of viral IL-6 is interesting. It was noticed that antisense oligonucleotides to IL-6 can inhibit the proliferation of Kaposi's sarcoma cells (Miles et al. 1990). Later on this disease was shown to be associated with human herpes virus 8 (HHV-8) which codes for a homologue to human IL-6 (Iscovich et al. 2000).

2.4.5 IL-6 gene

The IL-6 gene is a single copy gene located on chromosome 7p15-p21 for human (Bowcock et al. 1988; Hirano et al. 1986). The gene consists of five exons and four introns (Yasukawa et al. 1987). The IL-6 gene is polymorphic in both 5' and 3' flanking regions (Bowcock et al. 1989; Fishman et al. 1998). The promoter region -174 single G/C base exchange polymorphism was recently demonstrated to have biological function (Figure 5)(Fishman et al. 1998; Olomolaiye et al. 1998). Using luciferase reporter vector transiently transfected to HeLa cells Fishman et al. (1998) showed that the allele G construct was associated with higher spontaneous IL-6 gene transcriptional activity and higher inducible IL-6 transcriptional responses to LPS or IL-1 stimulus than the allele C construct. Their observation that allele G homozygotes and allele G/C heterozygotes had higher plasma IL-6 levels than allele C homozygotes was perfectly in line with these data. An imbalance of this polymorphism was observed in 92 patients with systemic-onset juvenile rheumatoid arthritis when compared with 383 healthy controls. This result was principally caused by a reduced frequency of the potentially protective CC ("low IL-6 producer") genotype among patients with an early-onset form of the disease (onset < 5 years of age). Subsequently, the allele G ("high IL-6 producer" genotype) has been found to be associated with unfavourable plasma lipid profile (high triglyserides, very low-density lipoproteins and free fatty acids, low high density lipoprotein) (Fernandez-Real et al. 2000). The exact mechanism for these phenotypic differences linked to IL6 alleles is not so far known, but as the promoter region of IL-6 gene has binding sites for several transcription factors (e.g. AP-1, NF-IL-6, and NF-κB), it is likely that differences in IL-6 production are caused by different transcription factor binding capacity (Figure 5) (Matsusaka et al. 1993). In addition to -174 polymorphism, the promoter region of IL-6 gene also contains $A_{(n)}T_{(n)}$ polymorphic region with at least six alleles ($A_{(8)}T_{(12)}$, $A_{(9)}T_{(11)}$, $A_{(10)}T_{(10)}$, $A_{(9)}T_{(10)}$, $A_{(10)}T_{(9)}$, $A_{(10)}T_{(11)}$) (Fishman et al. 1998). Moreover, Msp I and Bgl II RFLP polymorphism in the 5 ' flanking region have also been described, but neither biological function nor any disease association to these has been indicated (Blankenstein et al. 1989; Fugger et al. 1989a; Fugger et al. 1989b; Fugger et al. 1989c). Moreover, the 3' untranslated region of IL-6 gene contains a VNTR polymorphic site, which is caused by variable numbers of AT tandem repeats resulting at least 8 different alleles (Bowcock et al. 1989; Linker-Israeli et al. 1996). A higher than normal degree of this length polymorphism (which is also detectable with Xba I restriction enzyme) has been shown to be associated with SLE (Linker-Israeli et al. 1996). This polymorphism may have a biological role as EBV-B lymphoblastoid cell lines derived from SLE patients with SLE associated IL-6 alleles produced more IL-6 than similar cell lines from patients with "normal" IL-6 alleles.

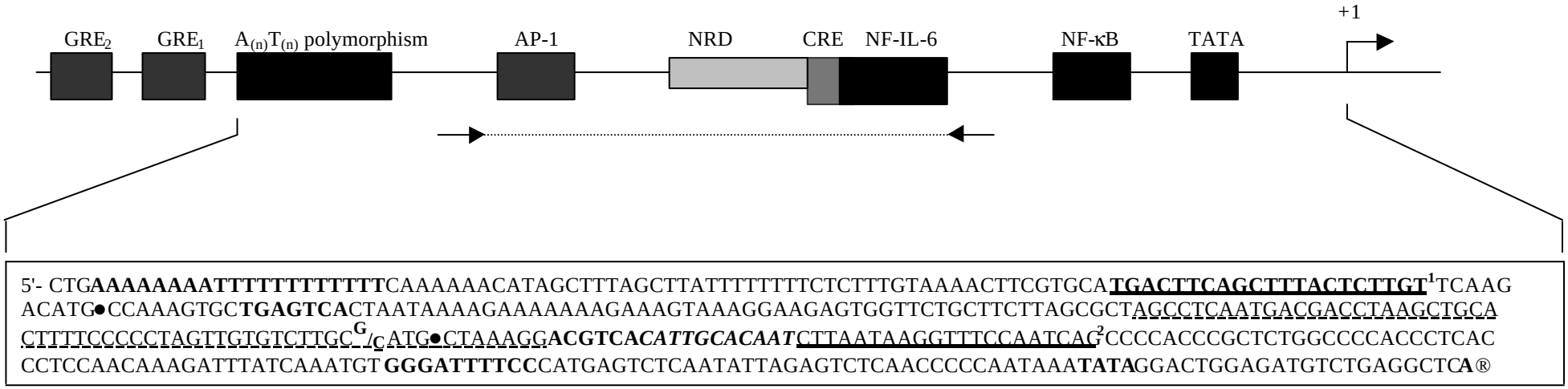


Figure 5. Schematic presentation of promoter region of IL-6 gene from -590 to +1. The Amplified region is showed with a dashed line between the arrows. In the text box below, the nucleotide sequence of the interleukin-6 gene promoter region from -398 to +1 with the $A_{(n)}T_{(n)}$ promoter region polymorphism, the binding site for IL-6 5' primer (underlined sequence 1), Nla III restriction sites (●), AP-1 binding site (TGAGTCA); Negative regulatory domain (NRD, dashed underline), the site of -174 G/C polymorphism ($^{G}/_{C}$); CAMP responsive element (CRE, ACGTCA), NF-IL-6 binding site (*CATTGCACAAT*), the binding site for IL-6 3' primer (underlined sequence 2), NF- κB binding site (GGGATTTTCC), TATA-box and a major transcription start site (®). GRE, Glucocorticoid responsive element. Based to Yasukawa et al. (1987), Isshiki et al. (1990), Ray et al. (1990) and Fishman et al. (1998).

Some of these SLE-associated 3' alleles have also been shown to be associated with increased IL-6 mRNA stability and low bone mineral density (Linker-Israeli et al. 1999; Murray et al. 1997). Analysis of 3' variation has also been used as an aid in population studies (Titenko et al. 1991). Additionally, IL-6 gene locus polymorphism with a 13 to 18 CA dinucleotide repeat variation has been recently described, and the 18 repeat form of it was shown to be associated with low bone mineral density (Tsukamoto et al. 1998; Tsukamoto et al. 1999).

2.5 Interleukin-10 (Study V)

2.5.1 Physiological roles of IL-10

IL-10 is a pleiotropic cytokine first demonstrated in 1991 (Vieira et al. 1991). IL-10 is usually considered to have a role in the downregulation of cell-mediated and cytotoxic inflammatory responses, thus being a potent anti-inflammatory mediator. These anti-inflammatory properties of IL-10 are partly mediated by its ability to downregulate HLA II expression and antigen presentation of monocyte-macrophage lineage cells (Bogdan et al. 1991; de Waal Malefyt et al. 1991; Powrie et al. 1997). In addition, IL-10 inhibits the proliferation and cytokine production of T cells responding to antigen as well as IFN-γ production by NK cells (Powrie et al. 1997). IL-10 also has some proinflammatory and haematological functions. IL-10 promotes B cell activation and differentiation and induces immunoglobulin synthesis and autoantibody production (Llorente et al. 1995; Rousset et al. 1992). IL-10 has been shown to stimulate secretion of IgG1 and IgG3 isotypes as well as B cell proliferation after activation with CD40, suggesting a potential role as a switch factor in immunoglobulin synthesis (Briere et al. 1994). Studies on gene knock-out mice suggest that IL-10 has a protective function against antigen driven inflammatory responses (Rennick et al. 1997). In these studies IL-10 -/- mice developed chronic enterocolitis with marked mononuclear cell infiltration and high IL-1, IL-6, TNF-α and nitric oxide levels in colonic lesions (Berg et al. 1996; Kuhn et al. 1993). The protective role of IL-10 has also been observed in experimental endotoxin shock as the lethal dose of endotoxin was 40 times lower in IL-10 -/- mice compared to control mice (Berg et al. 1995). On the other hand the blockade of circulating IL-10 with neutralising antibodies in SLE prone mice delays the onset of the disease and increases survival, suggesting that IL-10 promotes the development SLE in the mouse (Ishida et al. 1994). This autoimmune link of IL-10 has been suggested also to exist in human SLE, rheumatoid arthritis and Sjögren's syndrome in which the levels of circulating IL-10 are elevated (Llorente et al. 1994). Due to its anti-inflammatory characteristics IL-10 has been considered for therapeutic use. IL-10 administration to humans even at high doses (up to 100 μg/kg) is quite safe and has been shown to suppress the production of proinflammatory cytokines such as IL-1, IL-6 and TNF-α after LPS stimulation in

whole blood or PBMC cultures (Chernoff et al. 1995; Huhn et al. 1996; Powrie et al. 1997). The natural cellular sources of IL-10 in human are T-helper 1 and T helper 2 CD4+ cells, activated monocytes, B cells (especially CD5+ or EBV infected B cells) (Powrie et al. 1997)

2.5.2 IL-10 receptor

The IL-10 receptor is composed of at least two subunits, the ligand binding IL-10R1 and an accessory subunit IL-10R2 (Moore et al. 2001). The IL-10 receptor is mainly detected on haematopoietic cells (Liu et al. 1994). The human IL-10 receptor gene maps to a gene rich area at chromosome 11q23.3 (Taniyama et al. 1995).

2.5.3 Viral IL-10

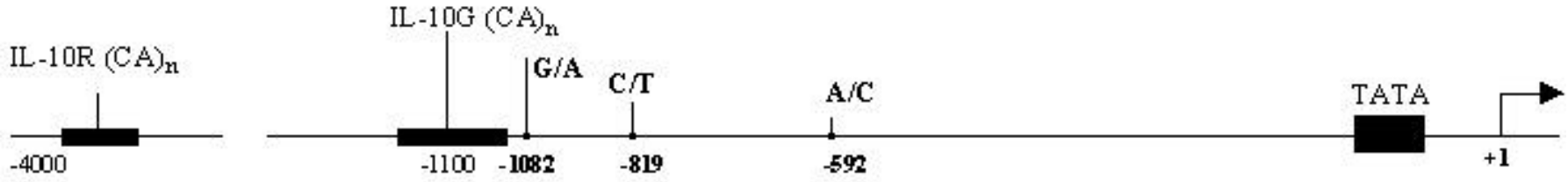
The human IL-10 exhibits strong DNA and amino acid sequence homology to an open reading frame in the Epstein-Barr virus (Moore et al. 1990; Vieira et al. 1991). The human IL-10 and EBV viral IL-10 mature protein sequences are 84 % identical (Powrie et al. 1997). Viral IL-10 is 3 to 10-fold less potent than the cellular cytokines in those systems where it is active and has at least 100- to 1000-fold lower affinity than human IL-10 for the cloned IL-10 receptor (Liu et al. 1994; Powrie et al. 1997).

2.5.4 IL-10 gene

IL-10 gene is located at chromosome 1q31-q32 (Eskdale et al. 1997a; Kim et al. 1992). The gene is composed of five exons (Powrie et al. 1997). Several possible transcriptional regulatory sequence elements were identified in the mouse IL-10 gene, but fairly little is known about the regulation of IL-10 transcription in humans (Kim et al. 1992; Kube et al. 1995; Moore et al. 2001). On the other hand, twin studies and family studies have suggested that approximately 75 % of the variation in IL-10 production is genetically determined (Westendorp et al. 1997). Is seems that IL-10 production is mainly controlled at transcriptional level and the genetic polymorphism of the 5' flanking region may partly explain this variation (Eskdale et al. 1998a; Turner et al. 1997). Schematic presentation of the major IL-10 polymorphisms observed in Caucasian test populations is given in Figure 6.

Three single base exchange polymorphisms located at positions -1082 (G to A), -819 (C to T) and -592 (C to A) form haplotypes, of which only three forms (GCC, ACC and ATA) have been described in Caucasian populations (Figure 6) (Turner et al. 1997). In addition, the presence of a rare GTA haplotype has been described recently in southern Chinese SLE patients (Mok et al. 1998). These IL-10 polymorphisms have been linked to IL-10 production. The IL-10 GCC haplotype has been found to be associated with high IL-10 production in Con A stimulated

Figure 6. Schematic presentation of promoter region of IL-10 gene from -4000 to +1. The base exchange polymorphism studied in this theses are marked with bold letters. In the text box below, the nucleotide sequence from -1100 to +1 with the $CA_{(n)}$ nucleotide repeat polymorphism (Eskdale and Gallagher 1995), the binding sites for primers used in analyses (underlined sequences), the -1082 $^{A}/_{G}$ the -819 $^{C}/_{T}$ and the -592 $^{A}/_{C}$ polymorphisms and corresponding restriction sites for Mnl I (■), Mae III (·) and Rsa I (¨), TATA box and transcription start site, putative sp1 **(CCCCGCC)** and ets binding site (**TGTAGGAAG).** Nucleotide sequence in text box is based to genbank clone X78437.1 by Kube et al. (1995) and Hobbs et al. (1998). The numbers after sequencies refer to corresponding primer pairs in table 2



A**CTCGCCGCAACCCAACTGGC**[1]TCCCCTTACCTTCTA*CACACACACACACACACACACACACACACACACACACACACACACACACAA***ATCCAAGACAACACTACTAA***[2]GGCTTC■
TTTGGG$^{A}/_{G}$AGG**GGAAGTAGGGATAGGTAAGA**[1]GGAAAGTAAGGGACCTCCTATCCAGCCTCCATGGAATCCTGACTTCTTTTCCTTGTTATTTCAACTTCTTCCACC
CCATCTTTTAAACTTTAGACTCCAGCCACAGAAGCTTACAACTAAAAGAAACTCTAAGGCCAATTTAATCCAAGGTTTCATT**CTATGTGCTGG**AGATGGTGT[4]ACAG
TAGGGTGAGGAAACCAAATTCTCAGTTGGCACTGGTGTACCCTTGTACAGGTGAT· GTAA$^{C}/_{T}$ATCTCTGTGCCTCAGTTTGCTCACTATAAAATAGAGACGGTAGGG
GTCAT**GGTGAGCACTACCTGACTAGC**[3]ATATAAGAAGCTTTCAGCAAGTGCAGACTACTCTTACCCACTTCCCCCAAGCACAGTTGGGGTGGGGGACAGCTGAAG
AGGTGGAAACATGTGCCTGAGAATCCTAATGAAATCGGGGTAAAGGAGCCTGGAACACATCCT · GTGA **CCCCGCC**TGT ¨ $^{A}/_{C}$C**TGTAGGAAG**CCAGTCTCTGGAA
AGTAAAATGGAAGGGCTGCTTG**GGAACTTTGAGGATATTTA**[2]GCCCACCCCCTCATTTTTACTTGGGGAAACTAAGGCCCAGAGACCTAAGGTGACTGCCTAAGTT
AGCAAGGAGAAGTCTTGGGTATTCATCCCAGGTTGGGGGGACCCAATTATTTCTCAATCCCATTGTATTCTGGAATGGGCAATTTGT**CCACGTCACTGTGACCTAG**
G[3]AACACGCGAATGAGAACCCACAGCTGAGGGCCTCTGCGCACAGAACAGCTGTTCTCCCCAGGAAATCAACTTTTTTTAATTGAGAAGCTAAAAAATTATTCTAA
GAGAGGTAGCCCATCCTAAAAATAGCTGTAATGCAGAAGTTCATGTTCAACCAATCATTTTTGCTTACGATGCAAAAATTGAAAACTAAGTTTATTAGAGAGGTTA
GAGAAGGAGGAGCTCTAAGGAGAAAAAATCCTGTGCCGGGAAACCTTGATTGTGGCTTTTTAATGAATGAAGAGGCCTCCCTGAGCTTACAA**TATA**AAAGGGGGA
CAGAGAGGTGAAGGTCTACACATCAGGGGCTTGCTCTTGC**A**®

peripheral blood mononuclear cell cultures (Turner et al. 1997). The ATA haplotype has been associated with lower transcriptional activity than GCC haplotype and ATA/ATA genotype with lower IL-10 production in LPS-stimulated whole blood cultures than other genotypes (Crawley et al. 1999b). Edwards-Smith et al. (1999) confirmed these *in vitro* results in a small number (n=10) of healthy subjects and divided the IL-10 haplotypes into high producers (GCC/GCC), intermediate producers (GCC/ACC, GCC/ATA) and low producers (ATA/ATA, ACC/ATA, ACC/ACC). The frequencies of different IL-10 haplotypes have been shown to vary with distinctive SLE phenotypes. The frequency of GCC haplotype seems to be increased in SLE patients with renal involvement and SSA-ab (Ro) positivity, potentially explaining the high IL-10 concentrations observed in these patients (Lazarus et al. 1997). Surprisingly, the ATA, "IL-10 low-producer haplotype" has also been found to be associated with renal disease in southern Chinese SLE patients. (Mok et al. 1998). In a later study no association with ATA haplotype and renal forms of SLE was found, but the frequency of ATA haplotype was increased in SLE patients with neuropsychiatric manifestations (Rood et al. 1999). Other controversial findings of ATA and renal involvement as well as GCC and SSA-ab positivity in SLE have also subsequently been published (Crawley et al. 1999a). During the last few years, the ATA-haplotype has been found to be associated with severe asthma, a severe form of rheumatoid arthritis and with severe juvenile rheumatoid arthritis (Crawley et al. 1999b; Hajeer et al. 1998; Lim et al. 1998). Moreover, the allele A at -592 (located in some studies at -571) has been associated with increased total IgE levels in asthmatic subjects (Hobbs et al. 1998). These IL-10 polymorphisms have been suggested to have therapeutic significance in chronic hepatitis C as the patients having the GCC ("high-producer") haplotype had a poor response to IFN-α therapy (Edwards-Smith et al. 1999). These results suggest that individual clinical strategy could be applicable for patients depending on their IL-10 genetics.

In addition to the above-mentioned SNPs, there are alleles at 2 microsatelite loci formed by variable numbers of dinucleotide CA repeats in the 4 kb and 1.1 kb upstream of the human IL-10 transcription initiation site (Figure 6) (Eskdale and Gallagher 1995; Eskdale et al. 1996). At least 16 alleles at this 1.1 kb site have been described, causing a 2 to 32 base length variation of the promoter region (Eskdale and Gallagher 1995). Similarly to the SNPs, these microsatelite polymorphisms have been shown to be associated with variable LPS stimulated IL-10 secretion in whole blood cultures. The haplotypes at -4000 containing the allele IL10.R3 were associated with lower IL-10 production than other IL10.R alleles. When this analysis was carried out together with IL10.G polymorphism located at -1100, the haplotype of IL10.R2/IL10.G14 was associated with the highest IL-10 production, whereas the haplotype IL10.R3/IL-10.G7 was associated with the lowest IL-10 production (Eskdale et al. 1998a). The above-mentioned 1.1 kb microsatelite polymorphism

has been found to be associated with SLE (Eskdale et al. 1997b). This result has since been confirmed, as the -1.0 kb polymorphic site shown to be associated with SLE in a later study by Mehrian et al. (1998) is exactly the same as the 1.1 kb site described by Eskdale et al. (1997b). In a recent multicenter study, the IL10.R3 allele was stated to be underrepresented in rheumatoid arthritis, regardless of the ethnic background of the patients (Eskdale et al. 1998b). The complementary results of GCC haplotype and microsatelite polymorphisms are likely to be caused by linkage disequilibrium between these polymorphisms (Eskdale et al. 1999). In addition to the above-mentioned polymorphisms, novel base exchange polymorphisms at -1354 (G to A) and at -3538 (T to A) have recently been described (Eskdale et al. 1999).

2.6 Tumour necrosis factor a (Study I)

2.6.1 Physiological roles of TNF-a

Tumour necrosis factor alpha (TNF-α) was first identified in 1975 as a macrophage-derived factor capable of necrotizing tumours in mice (Tracey 1997). In normal concentrations TNF is of benefit with its potency to promote tissue remodelling and repair, inflammation, cytotoxic reactions and anti-tumoural immunity (Sidhu and Bollon 1993). On the other hand, high serum levels of TNF that are produced acutely lead to shock and tissue damage, catabolic hormone release, vascular leakage syndrome, adult respiratory distress syndrome, gastrointestinal necrosis, acute renal tubular necrosis, adrenal haemorrhage, DIC and fever (Papadakis and Targan 2000). Chronic low-dose exposure to TNF leads to weight loss, anorexia, protein catabolism, lipid depletion, hepatosplenomegaly, subendocardial inflammation, insulin resistance and APP release (Papadakis and Targan 2000). With its crucial role in inducing inflammation, TNF has been shown to be potentially harmful in several autoimmune diseases such as RA, MS, SLE, diabetes, asthma and Crohn's disease and also in cancer (Papadakis and Targan 2000; Ruuls and Sedgwick 1999; Sidhu and Bollon 1993; Tracey 1997). TNF plays a pivotal role in cell proliferation, differentiation and apoptosis. At the single cell level TNF may have a cytotoxic, cytostatic or growth stimulatory effect, depending on cell type and growth environment (Sidhu and Bollon 1993).

Human TNF-α has membrane-bound (26 kDa) and secreted forms, both of which are biologically active (Tracey 1997). The membrane bound form of TNF is cleaved by a metalloprotease disintegrin (TNF-α converting enzyme) to a secreted (17 kDa) monomer, three of which then associate, forming the biologically active secreted form of TNF (Ruuls and Sedgwick 1999; Tracey 1997). Activated monocyte-macrophage lineage cells are the major origin of local and circulating TNF-α. In addition, the local production of TNF in brain (glial cells) and heart (cardiac myocytes) has great physiological significance (Ruuls and Sedgwick 1999; Tracey 1997). Many leukaemic B

cell lines as well as human lymphoblastoid cell lines have been shown to produce TNF spontaneously (Sidhu and Bollon 1993). The principal stimulus to TNF production in monocyte-macrophage lineage cells is bacterial endotoxin (LPS), phorbol esters also being effective (Sidhu and Bollon 1993).

Because of its antitumour effects, TNF has been considered for therapeutic purposes. Unfortunately TNF-α bears systemic toxicity and administration of TNF-α to humans is dangerous even in low doses causing fever, myalgia, diarrhoea, headache, nausea, (severe) hypotension, vascular leakage syndrome and accelerated protein degradation (Sidhu and Bollon 1993; Tracey 1997). The serum half-life of TNF-α in humans ranges from 11-30 min at doses under maximum tolerated dose (200 ug/m^2) (Sidhu and Bollon 1993). Although the trials using TNF-α as a drug have been disappointing, anti-TNF-α therapy has been under investigation and has achieved some success. The *in vivo* TNF blockade with anti-TNF fusion proteins in Crohn's disease and TNFRI-Fc fusion protein in RA have induced a clear therapeutic benefit (Feldmann et al. 1998; Hodgson 1999).

2.6.2 TNF-a-receptor

There are two membrane bound TNF receptors namely TNF-R1 (60 kDa) and TNF-R2 (80 kDa) (Ruuls and Sedgwick 1999). Signalling occurs when 2 to 3 receptors are linked by a single TNF trimer (Tracey 1997). The majority of mammalian cells carries these receptors (Papadakis and Targan 2000; Sidhu and Bollon 1993). Interestingly, the mutations in the extracellular domain of human TNF-R1 are linked to dominantly inherited periodic fever syndromes (McDermott et al. 1999; Papadakis and Targan 2000; Ruuls and Sedgwick 1999).

2.6.3 TNF-a gene

The TNF-α gene consists of four exons and three introns (Wilson et al. 1995). The gene is mapped to chromosome 6p21.3. This gene has several allelic variants, which have been associated with susceptibility to a number of complex diseases (OMIM 2001; Ruuls and Sedgwick 1999; Tracey 1997). However, as the TNF-α gene is located within the MCH between the HLA-DR and HLA-A genes the interpretation of these associations is very complicated because of the linkage of HLA susceptibility genes and TNF "risk" alleles (Ruuls and Sedgwick 1999). For example, the presence of the rare allele TNF2 (adenosine at -308) has been shown to increase transcription 6-7 fold in a reporter gene assay (Wilson et al. 1997). A strong association between TNF2 homozygosity and death from cerebral malaria has been reported (McGuire et al. 1994). However, the TNF2 allele is strongly associated with the HLA-A1-B8-DR3-DQ2 haplotype (Wilson et al. 1993; Wilson et al. 1995; Wilson et al. 1997). The fact that the HLA-DR3 and DR4 have also correlated with high rates

of TNF-production as compared to HLA-DR2 makes the situation complex (Ruuls and Sedgwick 1999; Tracey 1997).

2.7 Cytokines in chronic lymphocytic B cell leukaemia

2.7.1 B-CLL

Chronic lymphocytic leukaemia of the B-cell type (B-CLL) is a disease characterised by expansion of mature-appearing monoclonal CD5+ B-lymphocytes in the peripheral blood and bone marrow (Rozman and Montserrat 1995). B-CLL is the most common form of leukaemia in western societies with an annual incidence of approximately 120 in Finland (Finnish Cancer Registry 1998). The disease occurs most commonly in males (male to female ratio 2:1) at median age between 60 and 70 years (Brittinger et al. 1997). So far the diagnostic criteria for B-CLL are as follows: 1) Persistent elevation of blood lymphocytes above 5 x 10^9/L, 2) disease specific immunophenotype (CD5+, CD23+, FMC7-, weak CD22, monoclonal κ/λ light chain, and weak surface IgM or/and IgD) and typical CLL lymphocyte morphology, 3) monoclonal B cell infiltration (>30 %) in bone marrow (Brittinger et al. 1997; Hamblin and Oscier 1997; Matutes et al. 1994; Rozman and Montserrat 1995). The clinical course of the disease is extremely variable, with a survival time of individual patients ranging from a few months to 20 years, median survival being around 9 years (Hallek et al. 1997a; Rozman and Montserrat 1995). The most important prognostic factors of the disease include clinical stage (Binet A, approximately 60 % cases, median survival 9 years, Binet B ~ 30 % cases, median survival 5 years, Binet C ~ 10 % cases, median survival 2 years), lymphocyte morphology (atypical morphology precedes poor prognosis), lymphocyte doubling time (if <12 months, precedes poor prognosis), bone marrow infiltration patterns (diffuse patterns predict poor prognosis), serum β2-microglobulin, serum lactate dehydrogenase, serum thymidine kinase and chromosomal aberrations (Foon et al. 1990; Hallek et al. 1997a; Wierda and Kipps 1999). The aetiology of the disease is unknown and there is no curative therapy for B-CLL (Rozman and Montserrat 1995; Wierda and Kipps 1999). Up to 82 % of B-CLL patients have chromosomal abnormalities, such as 13q deletions (55 %, median survival 133 months), trisomy of 12q (16 %, median survival 114 months), 11q deletions (18 %, median survival 79 months), deletion in 17p (7 %, median survival 32 months) and deletion in 6q (7 %) (Dohner et al. 2000; Dohner et al. 1999). Patients with normal karyotype have a median survival of 111 months. CLL is characterised by several autoimmune phenomena and immunological complications (Hamblin and Oscier 1997; Jurlander 1998; Zaknoen and Kay 1990). Autoimmune haemolytic anaemia is quite common, affecting 10-25 % of the patients during the disease (Rozman and Montserrat 1995). Defects in cell mediated and humoral immunity are very often seen in B-CLL (Hamblin and Oscier 1997;

Jurlander 1998; Molica 1994; Zaknoen and Kay 1990). The major cause of morbidity in B-CLL is bacterial infection such as pneumonia or septicaemia, the incidence of which is influenced by hypogammaglobulinemia, a common complication affecting up to 50 % of patients (Molica 1994). It is now generally believed that the proliferative component in B-CLL pathogenesis is low and the accumulation of neoplastic B cells is caused by extended cell survival rather than proliferation (Jurlander 1998; Meinhardt et al. 1999; Wierda and Kipps 1999). As the course of the disease is extremely variable, and as the low risk patients are not treated, one of the major challenges in B-CLL is how to identify the individual high risk patients who would benefit from early intensive treatment. As mentioned above, this risk assessment is based on clinical stage, interphase cytogenetics, but also on serum markers of tumour mass such as soluble CD23, β2-microglobulin, and thymidine kinase (Wierda and Kipps 1999).

2.7.2 Overview of cytokine studies in B-CLL

The B-CLL cells spontaneously and rapidly undergo apoptosis when placed in culture, which led to the theory that these cells are dependent on soluble exogenous stimulus *in vivo* (Collins et al. 1989). Several cytokines have been shown to either induce or inhibit leukaemic B cell apoptosis, or induce or inhibit the proliferation of the malign clone (Orsini and Foa 2001). The B-CLL cells have been shown to express receptors at least for IL-1β, IL-2, IL-6, IL-7, IL-8, IL-10, IFN-γ, TGF-β and in contrast to other leukaemias also for IL-4 (Digel et al. 1990; Jurlander 1998; Zola et al. 1994). B-CLL cells express and produce several cytokines such as IL-1β, IL-6, IL-7, IL-8, IL-10, TNF-α, IFN-γ, TGF-β (Jurlander 1998; Meinhardt et al. 1999; Plate et al. 1993). Some of the cytokines such as the IL-6, IL-8 and TNF-α are produced constitutively in B-CLL, as some such as IL-7 lost their expression rapidly in culture (Biondi et al. 1989; di Celle et al. 1994; Foa et al. 1990; Long et al. 1995). B-CLL cells do not produce IL-2 or IL-4 (Jurlander 1998). Levels of several cytokines such as IL-6, IL-8, IL-10, IFN-γ, TNF-β and have been found to be increased in the serum of B-CLL patients (Jurlander 1998; Meinhardt et al. 1999). Some of these circulating proteins such as IL-6, IL-10, TNF-α and have been shown to be associated with B-CLL in a stage dependent manner (Jurlander 1998; Meinhardt et al. 1999). There are only preliminary but promising results concerning the prognostic value of serum cytokines in B-CLL. Binet stage A patients with increased (>4.5 pg/ml) serum IL-8 have been suggested to progress more rapidly to an advanced clinical stage than those with lower IL-8 serum concentrations (Molica et al. 1999).

2.7.3 TNF-a in B-CLL (Study I)

TNF-α was one of the first cytokines to be proposed to play a role in the activation, growth and apoptosis of leukaemic B-cells. The observations that TNF-α is constitutively expressed by B-CLL cells, B-CLL cells express TNF receptor, and exogenous TNF-α increase thymidine incorporation in neoplastic clones led to theory that TNF-α is an autocrine/paracrine growth factor for these cells (Cordingley et al. 1988; Digel et al. 1989; Foa et al. 1990). However, the interactions of TNF-α in B-CLL are complicated. The cellular responses to TNF-α show interindividual variation as proliferative patterns differ from inducing or neutral to reducing (Digel et al. 1989; Foa et al. 1990; Reittie et al. 1996; van Kooten et al. 1992). In one study even the anti-TNF-α antibody was capable of increasing thymidine-intake in cell cultures of individual B-CLL cases (Foa et al. 1990). Moreover, there is conflicting data concerning the role of TNF-α in B-CLL cell death. TNF-α may or may not protect B-CLL cells from apoptosis (Cordingley et al. 1988; Dancescu et al. 1992; Tangye and Raison 1997). It has been suggested that TNF-α mediates its effect by inducing other cytokines e.g. IL-6, which could partly explain some of the heterogeneity observed in TNF-α responses (Jurlander 1998). TNF-α may still have some prognostic significance, as the cellular release of TNF-α decreases progressively in relation to disease stage (Foa et al. 1990). This stage-dependence *in vitro* may be influenced by culture conditions and by the presence of accessory cells, as in LPS stimulated whole blood cultures no association with TNF-α and disease stage was found (Dahlke et al. 1995). In whole blood cultures the production of TNF-α (and IL-6) was shown to be decreased in B-CLL patients compared to healthy controls (Dahlke et al. 1995). In contrast to *in vitro* studies, the studies concerning the circulating TNF-α are in a good concordance. Several studies have shown that the levels of circulating TNF-α are elevated compared to normal controls and the concentrations of TNF-α increase significantly in relation to disease stage (Adami et al. 1994; Aguilar-Santelises et al. 1999; Foa et al. 1990).

2.7.4 IL-6 in B-CLL (Studies I and II)

Neoplastic cells from the majority (i.e. 18/26 studied patients) of B-CLL cases have been shown to express IL-6R (gp 80) on the cell surface (Lavabre-Bertrand et al. 1995). As IL-6 seems to decrease TNF-α induced proliferation in B-CLL cells , it has been suggested to have a growth inhibitory effect in B-CLL (Aderka et al. 1993; Orsini and Foa 2001; Reittie et al. 1996; van Kooten et al. 1992). However, in contrast to inhibitory impact on TNF-induced responses, exogenous IL-6 seems to have diverse effects on spontaneous B-CLL cell proliferation. Recombinant IL-6 has either induced spontaneous proliferation (in 4/6 of cases) (Reittie et al. 1996) or has had no effect on neoplastic clone proliferation (van Kooten et al. 1992; van Kooten et al. 1993). Reports are also

contradictory in respect of IL-6 and B-CLL cell apoptosis. IL-6 has been reported to have either protective or more frequently no effect on neoplastic cell apoptosis (Bussing et al. 1999; Castejon et al. 1999; Dancescu et al. 1992; Mainou-Fowler and Prentice 1996; Reittie et al. 1996). The studies concerning the cellular source of constitutively produced IL-6 in B-CLL are contradictory. Biondi et al (1989) detected spontaneous IL-6 mRNA expression in 6/11 cases and protein expression 3/4 cases of B-CLL. Patients with progressive disease have been shown to spontaneously produce less IL-6 protein *in vitro* compared to non-progressive patients (Aguilar-Santelises et al. 1991). However, Aguilar-Santelises et al. (1991) failed to detect spontaneous IL-6 production in neoplastic cells by immunohistochemistry or flow cytometry. Aderka et al. (1993) fractionated B cells and monocytes of 5 B-CLL cases and suggested that the cellular origin of spontaneous IL-6 release in B-CLL is probably the monocyte fraction. Even though there is a wide variation in *in vitro* study approaches, collectively these data suggest that there may be a great interindividual variation in IL-6 production and IL-6 responsiveness.

As was the case with studies concerning circulating TNF-α, the studies concerning circulating IL-6 are more homogenous than the *in vitro* studies. Plasma levels of IL-6 have been shown to be elevated in B-CLL in a stage-dependent manner. In a study by Reittie et al. (1996) the authors used immunoradiometric assay (IRMA) to detect IL-6 serum levels in 27 out of 50 patients studied, the mean levels being significantly higher in Rai III-IV stage cases compared to Rai 0-II cases. In Study II of this dissertation this result was confirmed with 35 patients, and we moreover showed that IL-6 levels were higher in B-CLL patients compared to normal values from 400 healthy controls. Callea et al. (1996) studied 47 cases of B-CLL and found that serum IL-6 concentrations were significantly higher in patients with an advanced/progressive disease (n=37) than in patients having a "smouldering" (i.e. stage A, stable) B-CLL (n=10). All of these studies are in agreement with the recent study by Fayad et al. (2001), in which the authors found that serum IL-6 levels were higher in CLL patients (n=151) than normal controls (n=55) and that the patients with elevated serum IL-6 had poorer median and 3-year survival with unfavourable prognostic characteristics such as elevated β2-microglobulin or LDH and Rai stage III or IV.

Only a few studies disagree with the increased serum IL-6 in B-CLL. Du Villard et al. (1995) compared 7 CLL patients and 26 controls by IL-6 RIA and did not observe any difference between the patients and the control group. In a similar way, Denizot et al. (1995) compared IL-6 serum concentrations by ELISA in various lymphomas and found that IL-6 serum levels obtained from 6 B-CLL cases did not deviate from the values obtained from 41 normal controls. However, the small patient cohorts diminish the statistical reliability of these studies. Only one study disagrees with the

stage-dependence of serum IL-6. Aderka et al. (1993) studied IL-6 production in 76 B-CLL cases and 25 controls by ELISA and found in dichotomous analysis (i.e. IL-6 present/not present) that IL-6 was more frequently present in serum with mild disease forms than in patients with severe disease. On the other hand, in this study the serum levels of soluble IL-6 receptor were elevated in B-CLL and correlated to disease stage. These sIL-6R results have subsequently been partly confirmed as concentrations of the soluble IL-6R were found to be higher in B-CLL (170 ± 12.6 ng/ml) than in age-matched controls (100 ± 5.6 ng/ml), being highest in stage B patients (Lavabre-Bertrand et al. 1995).

2.7.5 IL-1 family cytokines in B-CLL (Study II)

B-CLL cells have been demonstrated to express IL-1β receptors and constitutively produce IL-1β mRNA and protein (di Celle et al. 1994; Pistoia et al. 1986; Plate et al. 1993; Schena et al. 1992; Uggla et al. 1987). This constitutive IL-1β expression is heterogeneous in B-CLL and has been shown to be restricted to B-CLL clones, which are positive for myelomonocytic antigen CD14 (di Celle et al. 1994; Morabito et al. 1987). Most B-CLL lines have been demonstrated also to express IL-1α and variably also membrane IL-1α (Aguilar-Santelises et al. 1991). The cellular source of IL-1β in B-CLL is controversial. IL-1β protein could not be detected in neoplastic cells by immunohistochemistry or flow cytometry (Aguilar-Santelises et al. 1991). However, IL-1β mRNA has been found to be present in CD5+/CD19+ leukaemic cells when the transcript was analysed from a cell population obtained by fluorescence activated cell sorting (Plate et al. 1993).

The studies concerning the biological effect of recombinant IL-1β in B-CLL are controversial. Mainou-Fowler et al (1995) demonstrated that some B-CLL clones responded to IL-1 by proliferation, but the variability among B-CLL cases was great. In a study by Takeuchi et al. (1994) few (2/7) B-CLL cases reacted to exogenous IL-1 by increasing cell surface immunoglobulin. In most studies, however, exogenous IL-1 has not had any direct effect on B-CLL proliferation (Carlsson et al. 1989; Ghaderi et al. 1988; van Kooten et al. 1992). Moreover, several studies have shown that exogenous IL-1 has no direct effect on apoptosis in B-CLL (Dancescu et al. 1992; Mainou-Fowler et al. 1995; Mainou-Fowler and Prentice 1996). However, it is possible that IL-1 has an indirect role in the pathogenesis of B-CLL and its complications. The cellular production of IL-1β protein and mRNA has been shown to be decreased in progressive B-CLL compared to non-progressive disease and the overall serum IL-1α has shown to be slightly increased in B-CLL patients (Aguilar-Santelises et al. 1992; Aguilar-Santelises et al. 1991). Membrane-bound IL-1α has been been shown to be higher in patients with benign forms of B-CLL (Aguilar-Santelises et al.

1993). Interestingly, in a recent large-scale cDNA microarray analysis in which the expressions of 1024 selected genes were analysed in 54 B-CLL cases the reduced expression of IL-1β gene was among those which significantly correlated with reduced patient survival and advanced clinical stage of the patients (Stratowa et al. 2001).

Very little is known about IL-1Ra in B-CLL. In the study bu Duensing et al. (1998) high IL-1Ra plasma levels in B-CLL were associated with decreased cellular expression of BCL-2 oncoprotein, which is known to have antiapoptotic function.

2.7.6 Cytokine gene polymorphism in B-CLL

As mentioned above, several cytokines have been found to be associated with progression, disease severity or pathogenesis of B-CLL. However, only few studies concerning cytokine gene polymorphism in B-CLL have been published. Demeter et al. (1996) studied the VNTR polymorphism within the second intron of the IL-1Ra gene in patients with CLL, hairy cell leukaemia, multiple myeloma, acute myeloid leukaemia, chronic myeloid leukaemia and Hodgkin's disease and found that only in AML was the frequency of the IL-1RN*4 allele significantly increased compared to normal controls. This negative finding concerning the IL-1RN polymorphism in B-CLL could be confirmed in Study II of this thesis.

The few studies concerning tumour necrosis factor polymorphism in B-CLL are contradictory. Demeter et al. (1997) suggested that the frequency of allele 1 (G at -308 of TNF-α gene) was significantly increased in German B-CLL patients (n=73) compared to controls (n=117). Wihlborg et al. (1999) could not confirm this difference in a group of Swedish B-CLL patients (n=49) and controls (n=51). Demeter et al. (1997) also studied Nco I polymorphism of TNF-β gene in B-CLL patients and controls. No differences in the allele frequencies of this gene were observed between patients and controls, but the frequency of the TNFB*2 allele was increased in CLL patients with advanced clinical stage. In a very recent study of this field, the allele frequencies of neither TNF-α nor TNF-β differed from normal among B-CLL patients, non-Hodgkin's lymphoma and Hodgkin's disease (Mainou-Fowler et al. 2000). We can confirm this negative finding concerning TNF-α (-308) alleles, as no differences in allele frequencies were found among 35 Finnish B-CLL patients and 400 controls (unpublished observation).

Except for study II of this dissertation, no other studies concerning the IL-1α (-889), IL-1β (-511), IL-1β (+3953) or IL-6 (-174) gene polymorphisms in B-CLL have been published.

2.8 Cytokines in primary Sjögren's syndrome

2.8.1 pSS

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disorder that primarily affects the salivary and lacrimal glands. With a prevalence between 1-3 % the pSS (along with RA), is the most common rheumatic disease (Dawson et al. 2000; Price and Venables 1995). 90 % of patients are women of mean age of 48-52 (Kelly et al. 1991; Pease et al. 1993; Price and Venables 1995). Predisposing factors for SS include HLA DR3, B8 and heterozygosity for DQ1 and DQ2 in Caucasian population (Fox et al. 1998; Pease et al. 1993; Price and Venables 1995). The most important manifestations of the disease are keratoconjunctivitis sicca and oral dryness caused by diminished lacrimal and salivary gland secretory activity. Classically this glandular dysfunction is associated with progressive lymphocytic infiltration, which leads to the destruction of acinar and ductal epithelial cells and loss of glandular parenchyma (Fox et al. 1998). Extraglandular (or nonexocrine/systemic) manifestations of the disease affect e.g. the skin, joints, muscles, peripheral and central nervous system, kidneys and lungs (Moutsopoulos 1994; Oxholm and Asmussen 1996). Sjögren's syndrome patients often have other autoimmune diseases such as autoimmune thyroiditis, primary biliary cirrhosis and coeliac disease (Fox et al. 1998). Raynaud's phenomenon is also very common in pSS, affecting approximately 25-80 % of patients (Asmussen et al. 1996; Kelly et al. 1991; Pease et al. 1993). Polyclonal B lymphocyte hyperactivity is one of the principal features of pSS (Oxholm and Asmussen 1996). Probably partly due to this and partly due to other factors, hypergammaglobulinemia is very common in pSS, affecting approximately 40-60 % of patients (Kelly et al. 1991; Pease et al. 1993). Antibodies against ribonucleoproteins (SS-A/Ro and SS-B/La) as well as rheumatoid factors are frequently present in the sera of SS patients (Price and Venables 1995). The risk for non-Hodgkin's lymphoma is increased 20-40 fold in pSS patients compared with normal population (Fox et al. 1998; Gannot et al. 2000). The diagnosis of pSS is based to objective keratoconjuctivitis sicca, xerostomia and either positive minor salivary gland biopsy or characteristic autoantibodies (i.e. SS-A and/or SS-B) and exclusion of diseases causing secondary sicca symptoms, such as hepatitis C, HIV, HTLV infections as well as SLE (Fox et al. 1998). The Aetiology of pSS is unknown and there is no curative treatment for the disease (Dawson et al. 2000; Fox et al. 1998). There are only few longitudinal studies concerning the prognostic factors for pSS. The presence of SS-A antibodies has been associated with more severe, systemic disease (Kelly et al. 1991). Moreover, also the presence of SS-B antibodies, HLA DR3, lymphocytopaenia, vasculitis, hypergammaglobulinemia or Raynaud's phenomenon are also in association with extraglandular disease (Pease et al. 1993). The degree of lymphocytic infiltration (or "focus score") in salivary gland biopsy correlates (to a certain extent) with extraglandular

disease manifestations such as pulmonary fibrosis, purpura and leukocytopenia (Asmussen et al. 1996).

2.8.2 Overview of cytokine studies in pSS

Several cytokines such as IL-1, IL-1 receptor antagonist, IL-2, IL-6, IL-10, TNF-α, TGF-β and interferon-γ have been proposed to play a role in the pathogenesis of the Sjögren's syndrome (Dubost et al. 1996; Fox et al. 1994; Price and Venables 1995). Messenger RNA expression of IL-1α, IL-2, IL-6, IL-10, TNF-α, TGF-β have been detected in salivary gland epithelial (i.e. acinar and ductal) cells in normal subjects and pSS patients (Fox et al. 1994; Sun et al. 1998). There are stoichiometric differences in the mRNA expression of these cytokines in SS. Salivary gland epithelial cells from SS patients have been shown to produce significantly more (>40-fold) IL-1α, IL-6 and TNF-α mRNA than epithelial cells from histologically normal salivary glands (Fox et al. 1994). In addition to epithelial cells, salivary gland CD4+ T cells also produce over 40-fold more IL-2, INF-γ and IL-10 mRNA than peripheral blood CD4+ cells from the same patients or from normal controls (Fox et al. 1994). In contrast to normal, acinar cells from pSS patients also produce INF-γ mRNA (Sun et al. 1998). The presence of INF-γ has been demonstrated to correlate with HLA-DR expression and La (SS-B) antigen expression by epithelial cells in pSS (Brookes et al. 1995). The number of peripheral blood mononuclear cells spontaneously secreting IL-2 and INF-γ has been shown to be significantly (6-10-fold) lower in pSS than in healthy controls by ELISPOT assay (Hagiwara et al. 1998). Moreover, patients with extraglandular symptoms had significantly lower frequency of IFN-γ secreting cells than patients without these symptoms (Hagiwara et al. 1998). Increased levels of IL-1α, IL-6, IL-10, TNF-α, INF-γ have been found in the saliva of SS patients (Fox et al. 1994). In contrast to these cytokines and especially to IL-1α, the concentrations of IL-1Ra have been shown to be decreased in the saliva of pSS patients compared with age-matched control subjects (Dubost et al. 1996; Perrier et al. 1998). Moreover, Perrier et al. (1998) demonstrated that serum levels of IL-1Ra were elevated in pSS. The studies concerning IL-6 and IL-10 in SS are reviewed in detail below.

2.8.3 IL-6 in pSS

Several reports suggest that IL-6 activity is high in pSS. Pettersson et al. (1992) observed that serum IL-6 (by RIA) was significantly (3.3-fold) higher in patients with pSS (n=22) than in control subjects (n=32). This has been subsequently confirmed by Grisius et al. (1997) in a study with 31 pSS patients and 14 healthy controls and by Carcia-Carrasco et al. (2001) with 62 patients and 28 controls. As mentioned above, Fox et al. (1994) found that salivary gland epithelial cells produced

markedly more IL-6 mRNA than these cells from healthy controls, and among other cytokines, increase in salivary IL-6 was observed in SS. Grisius et al. (1997) also measured salivary IL-6 in pSS and observed an over 20-fold increase in salivary IL-6, correlation of salivary IL-6 with serum IgG and with ESR. Likewise Tishler et al. (1998) observed that tear fluid IL-6 was elevated in pSS patients (n=12) compared to controls (n=12) and that the tear levels of IL-6 correlated with lymphocyte infiltration (i.e. focus score) of lip biopsies in pSS. Furthermore, in a recent study high serum IL-6 was found to be associated with cryoglobulinemia, hypocomplementemia and liver involvement in pSS (Garcia-Carrasco et al. 2001). There are only a few *in vitro* studies concerning PBMC and IL-6 in pSS. Applying ELISPOT assays Halse et al. (1999) found that the mean number of IL-6 secreting peripheral blood mononuclear cells was elevated in pSS (33 patients) compared with healthy controls (n=12). However, this is in contrast to an earlier and quite similar study by Hagivara et al. (1998) in which no differences in the numbers of IL-6 secreting cells were detected between pSS patients (n=20) and controls (n=20).

2.8.4 IL-10 in pSS

Salivary gland CD4+ T cells have been shown to express significantly more IL-10 mRNA than peripheral blood CD4+ T cells from the same patients or from normal controls (Fox et al. 1994). In contrast to IL-6, no differences in the salivary gland epithelial cell IL-10 mRNA expression were found in SS and controls (Fox et al. 1994). This CD4+ T cell derived overexpression of IL-10 is also seen at the protein level, as salivary gland derived CD4+ clones have been found to produce (15-fold) more IL-10 protein than peripheral blood derived CD4+ clones of the same pSS patients (Brookes et al. 1996). Spontaneous IL-10 mRNA expression and protein production have been shown to be increased in PBMCs of pSS patients (n=10) compared with PBMCs from healthy controls (n=15) (Llorente et al. 1994). Surprisingly, the major cellular sources of IL-10 in pSS seem to be B cells and monocytes, and T cells contribute very little to (*in vitro*) IL-10 production in this disease (Llorente et al. 1994). As was the case with IL-6, the mean number of IL-10 secreting PBMCs has been shown to be increased in ELISPOT assays from pSS patients (n=33) compared to assays from healthy controls (n=12) (Halse et al. 1999). In contrast to this, no difference in the number of cells spontaneously secreting IL-10 was observed by Hagiwara et al. (1998) in a similar experimental setting. High, but not statistically significant circulating IL-10 concentrations were measured in a recent study in pSS (Garcia-Carrasco et al. 2001). Moreover, as was the case with IL-6, high serum IL-10 have shown to be associated with liver involvement in pSS (Garcia-Carrasco et al. 2001).

2.8.5 Cytokine gene polymorphism in pSS

So far there have been very few studies concerning cytokine gene polymorphism in pSS. The first and so far the only published study concerning the polymorphism of IL-1 family genes in pSS was published in 1998. Perrier et al. (1998) studied 36 patients with pSS and found that IL-1RN allele 2 was significantly more frequent in the definite form than in the possible form of pSS suggesting that allele 2 is a marker of severe SS. Moreover, in this study IL-1Ra protein production was shown to be differently regulated in saliva and in serum, as patients with IL-1RN allele 2 had lower salivary IL-1Ra and higher serum IL-1Ra than patients without this allele. Fugger et al. (1998d) suggested that the frequency of 5.5 kb fragment representing Nco I RFLP polymorphism of TNF-α gene is increased in pSS. Subsequently it was discovered, that this Nco I RFLP polymorphism was located at the first intron of TNF-β gene, and 5.5 kb RFLP fragment represents TNFB*1 allele instead of TNF-α polymorphism (Messer et al. 1991; Wilson et al. 1995). As TNFB*1 allele is in linkage disequilibrium with TNF2 and both of these with HLA DR3 and DQ2 haplotypes which on the other hand are risk factors for pSS, it is possible that these results are explicable on the whole by the linkage disequilibrium of these HLA-genes and TNF-locus genes (Fugger et al. 1989d; Fugger et al. 1989e; Keso et al. 2001; Price and Venables 1995; Wilson et al. 1993). In addition to TGF-β polymorphism, TNF microsatelite polymorphism in pSS has been studied (Jean et al. 1998). When analysed alone, there were no differences in the allelic frequencies of these microsatelite alleles in patients (n=45) and in controls (n=200). However, when a haplotypic analysis of microsatelite polymorphisms with HLA genes was performed, the TNF2 allele was more frequent in DRB1*0301 positive pSS patients than in DRB1*0301 positive control subjects (Jean et al. 1998). Rischmueller et al. (2000) studied IL-10 polymorphism in pSS and found that in contrast to SLE, the polymorphism of this gene is not associated with anti-Ro autoantibodies in pSS (Rischmueller et al. 2000). This observation concerning anti-Ro antibodies which was published at the very same time as Study V of this dissertation is in concordance with our results. However, in contrast to us, Rischmueller et al. (2000) did not find any association between pSS and IL-10 haplotypes in an Australian population.

3. AIMS OF THE STUDY

The aims of the present study were

1) To investigate IL-6 and TNF-α production in B-CLL cells in order to determine possible connections between these cytokines and prognostic parameters.

2) To investigate circulating IL-1β, IL-1Ra and IL-6 protein levels and polymorphism of these genes in order to determine whether these factors predispose patients to B-CLL, or whether they are associated with disease characteristics of B-CLL.

3) To analyse the effect of IL-1 family gene polymorphism in the regulation of circulating IL-1β levels in healthy Finnish population.

4) To investigate circulating IL-6 and promoter region polymorphism of IL-6 gene in primary Sjögren's syndrome in order to determine whether these factors predispose patients to pSS, or whether they are associated with disease characteristics.

5) To investigate circulating IL-10 and haplotypes of IL-10 gene promoter region polymorphism in primary Sjögren's syndrome in order to determine whether these factors predispose patients to pSS, or whether they are associated to disease characteristics.

4. SUBJECTS AND METHODS

4.1 Subjects

4.1.1 Studies I and II

Patients. Clinical specimens were obtained after informed consent from consecutive patients referred to the CLL out-patient clinic of the Tampere University Hospital. The diagnosis and staging of CLL were based on standard clinical, morphologic and immunophenotypic criteria (see review of the literature). All patients had B-CLL phenotype. The 24 patients investigated in Study I overlapped with the 36 patients explored in Study II. The normal control subjects studied in Study II were the same as in Study III (below)

4.1.2 Study III

Subjects. Blood samples were obtained from the Finnish Red Cross Blood Transfusion Centre, Tampere. The donors were healthy adults (18-60 years old) and they had not had any sign of infection during a 2-week period before the blood donation. Plasma was separated within two hours after the blood donation and kept frozen until tested.

4.1.3 Studies IV and V

Patients. All patients fulfilling three or more modified Californian criteria (referred to in manuscript IV) for pSS were selected from the records of patients with sicca symptoms examined in the Section for Rheumatology of the Department of Internal Medicine, Tampere University Hospital, Finland, during the years 1977-1992 (n=111). Histological findings were graded on the Chisholm-Mason scale, grades 3 and 4 were regarded as diagnostic. All patients also fulfilled the European criteria for pSS. Those patients who were still alive were invited for cytokine and cytokine gene polymorphism determinations and specimens were obtained after informed consent from 66 pSS patients.

4.2 Methods

4.2.1 Cell cultures (Study I)

Peripheral blood mononuclear cells were isolated from heparinized (Noparin, Nova Nordisk, Dagsvaerd, Denmarks) blood samples by centrifugation over a Lymphoprep layer (Nycomed, Oslo, Norway) with a density of 1.077 g/ml (400 g, 40 min). The cells were washed twice with PBS (pH 7.4) and once with complete medium consisting of RPMI 1640, 20 mM Hepes (ICN Biomedical,

Costa Mesa, Calif.), 10 % heat-inactivated fetal calf serum (Gibco BRL, Eggenstein, Germany), 2 mM L-glutamine, and antibiotics (Gibco; 50 units penicillin/ml and 50 ug streptomycin/ml). Cell counting was performed using Technicon H*1 or H*2 analysers (Bayer Diagnostica). Cell viability was determined by trypan blue dye exclusion.

4.2.2 ELISAs (Studies I, II, III, IV, V)

Interleukin-1β, IL-1Ra, IL-6, IL-10 and TNF-α concentrations were determined using commercially available ELISA kits (Pelikine Compact human IL-1β, IL-6, IL-10 and TNF-α ELISA kits, CLB, Amsterdam and Quantikine human IL-1Ra immunoassay, R&D Systems, Mp, USA), following the manufacturer's instructions. The optical density of individual wells was determined with a Multiscan Biochromatic 348 (Labsystems, Helsinki, Finland) spectrophotometer. The detection limits of the assays were 0.4 pg/ml for IL-1β, 0.6 pg/ml for IL-6, 1.2 pg/ml for IL-10, 46.9 pg/ml for IL-1Ra and 1.4 pg/ml for TNF-α.

4.2.3 Immunophenotyping (Studies I and II)

Immunophenotyping was originally performed by flow cytometry (EPICS C, Coulter Electronics, Hialeah, CA, USA) using commercial mouse monoclonal antibodies and corresponding immunoglobulin isotype controls as recommended by the manufacturers. The antibodies were as follows: FITC/PE labelled Simultest Isotype control IgG_1/IgG_{2a} (X40/X39), LeucoGATE CD45/14 (anti-Hle-1/Leu-3M), anti-CD4/CD8 (leu-3a/ leu-2a), anti- κ/anti-λ (TB28-2/1-155-2), FITC labelled anti-CD2 (leu-5b), CD22 (Leu-14), anti-CD5 (Leu-1), anti-CD25 (Anti-IL-2R), PE-labelled CD20 (Leu-16), CD19 (Leu-12), anti- CD23 (Leu-20); all from Becton Dickinson (Mountain View, CA, USA). FITC-labelled anti-FMC7 was purchased from Dako (Glostrup, Denmark) and PE-labelled anti-CD2 from Immunotech (Marseille, France).

4.2.4 Intracellular cytokine detection (Study I)

Expression of intracellular IL-6 protein was studied by flow cytometry (Becton Dickinson Immunocytometry Systems, Palo Alto, CA, USA). Leukaemic cells (2×10^6/ml) were stimulated with PMA (1 ng/ml). After 6, 24 and 72 hours in culture, the cells were stained using commercial cytostain kit (Cytofix/Cytoperm kit, Pharmingen, San Diego, CA, USA) following the manufacturer's protocol. In order to block secretion of synthesised proteins, the protein transport inhibitor Brefeldin-A (BFA: Sigma, 10 ug/ml) was added to the cell cultures 6 hours prior to staining. To demonstrate specificity of staining, an IL-6 ligand block control was prepared by pre-incubating FITC-conjugated rat anti-human IL-6 monoclonal antibody (clone MQ2-6A3, Pharmingen) with a molar excess of IL-6 protein (recombinant human IL-6, CLB). Quadrant

markers for bivariate dot blots were set based on PE-labelled non-specific surface isotype control (IgG1, Becton Dickinson) and ligand-blocked FITC-anti-IL-6 mAb.

4.2.5 Polymorphism analyses (Studies II, III, IV, V)

IL-1 α, IL-1β, IL-1 Ra and IL-6 and IL-10 polymorphisms were analysed using PCR-based approaches. Primers, lengths of the amplified PCR fragments, restriction enzymes, and restriction patterns for each polymorphism are given in Table 2. The adjacent sequences and restriction sites for each polymorphism are illustrated in Figure 2 (IL-1α), Figure 3 (IL-1β -511 and IL-1β +3953), Figure 4 (IL-1RA), Figure 5 (IL-6) and Figure 6 (IL-10). DNA for the studies was isolated from mononuclear cells using the salting out method (Miller et al. 1988). Base exchange at position -889 of the IL-1α gene was analysed as previously described (McDowell et al. 1995). The region that contains the *Ava I* polymorphic locus at position -511 of the IL-1β gene was amplified by PCR as described by (di Giovine et al. 1992). The *Taq I* polymorphic region at exon 5, position +3953 of the IL-1β gene was amplified by PCR as described by (Pociot et al. 1993). The *Nla III* polymorphic site at position -174 of the IL-6 gene was amplified by PCR according to Fishman et al. (1998). After restriction enzyme digestion the products of the PCR analyses were identified by electrophoresis (on a 9% PAGE) and ethidium bromide staining. The IL-1Ra exon 2 polymorphism caused by 86-bp tandem repeats was analysed as described previously (Tarlow et al. 1993), and 2% agarose gel was used for electrophoresis and detection of amplified DNA products. PCR amplification conditions for the primer pairs were analogous to those published previously (references above). The G/A base exchange polymorphism at –1082 of the IL-10 gene was analysed by PCR and cycle sequencing as described (Helminen et al. 1999). The –819 C/T polymorphism and –592 C/A polymorphism of the IL-10 gene were analysed by PCR and RFLP (Edwards-Smith et al. 1999; Mok et al. 1998). The RFLP-based IL-10 methodologies were validated by also analysing 70 samples by sequencing.

4.2.6 Statistical analysis

Mean values were compared by using either Student's t-test, Mann-Whitney U-test or one- and two-way ANOVA. Kruskall-Wallis ANOVA was used in the preparation phase of Study V. Correlations were estimated using Spearman Rank Order correlation test. Calculations were carried out using Statistica (Statsoft inc., Tulsa, USA). Odds ratios and 95 % confidence intervals were calculated using CIA software (ver 1.1, copyrighted by MJ Gardner and British Medical Journal, 1989).

Table 2. Technical data for cytokine gene polymorphism detection methods.

Polymorphism	Primer sequences	Resulting fragment bp	Restriction enzyme	Restriction pattern bp
IL-1α -889	5’ AAGCTTGTTCTACCACCTGAACTAGGC 3’	99	Nco I	a1 (C): 16 + 83
	5’ TTACATATGAGCCTTCCATG 3’			a2 (T): 99
IL-1β -511	5’ TGGCATTGATCTGGTTCATC 3’	304	Ava I	a1 (C): 114 + 190
	5’ GTTTAGGAATCTTCCCACTT 3’			a2 (T): 304
IL-1β +3953	5’ GTTGTCATCAGACTTTGACC 3’	249	Taq I	a1 (T): 114 + 135
	5’ TTCAGTTCATATGGACCAGA 3’			a2 (C): 249
IL-1Ra	5’ CTCAGCAACACTCCTAT 3’	240-595	not used	a1: 410 (4rep)
	5’ TCCTGGTCTGCAGGTAA 3’			a2: 240 (2rep)
				a3: 325 (3rep)
				a4: 500 (5rep)
				a5: 595 (6rep)
TNF-α	5' AGGCAATAGGTTTTGAGGGCCAT 3'	107	NcoI	a1(G): 20 + 87
	5' TCCTCCCTGCTCCGATTCCG 3'			a2(A): 107
IL-6	5’ TGACTTCAGCTTTACTCTTGT 3’	198	Nla III	G: 31 + 167
	5’ CTGATTGGAAACCTTATTAAG 3’			C: 31 + 45 + 122
IL-10 -1082*	5' ACACCATCTCCAGCACATAG 3' (4)	134	Mnl I	A: 134 (when 20 CA rep)
	5' TCTTACCTATCCCTACTTCC 3' (1)			G: 30 + 104
	5' CTCGCTGCAACCCAACTGGC 3' (1)			
IL-10 -819*	5' TAAATATCCTCAAAGTTCC 3' (2)	592	Mae III	C: 79 + 217 + 292
	5' ATCCAAGACAACACTACTAA 3' (2)			T: 79 + 509
IL-10 -592*	5' CCTAGGTCACAGTGACGTGG 3' (3)	412	Rsa I	C : 412
	5' GGTGAGCACTACCTGACTAGC 3' (3)			A: 236 + 176

*Numbers in parenthesis refer to respective primer binding sites in IL-10 gene at figure 6.

4.2.7 Ethics

The study plan for B-CLL study and for the primary Sjögren's syndrome study was accepted by the ethical committee of the Tampere University Hospital. The collection of control samples was authorised by ethical committee of the Finnish Red Cross Transfusion Service.

5. RESULTS

5.1 In vitro production of interleukin-6 and TNF-a in B-CLL (Study I)

5.1.1 IL-6 production in vitro

In almost all cases (21/24) low spontaneous IL-6 production was observed in unstimulated cell cultures. After stimulation with PMA the cells of Binet C stage patients showed significantly lower IL-6 production compared with cells of Binet class A or Binet class B patients. Co-stimulatory experiments with PMA and IL-2 produced similar results. With PMA stimulation, IL-6 production was 28-fold in the Binet A group compared with group C and 16-fold in Binet group B compared with group C. A statistically insignificant, 2-fold difference in IL-6 production was found between cells of Binet stages A and B.

5.1.2 TNF-a production in vitro

With one exception (1/24), TNF-α concentrations were below the assay limit in unstimulated cell cultures. After mitogenic activation with PMA, low TNF-α concentrations were observed in samples of Binet class A and Binet class B patients. None of the Binet class C patients responded to PMA stimulus alone. In co-stimulatory experiments with PMA+IL-2 a clear increasing trend in TNF-α levels took place in all groups. However, when TNF-α concentrations were compared against Binet staging, none of the differences reached statistical significance.

5.1.3 Intracellular IL-6 detection

In order to determine the cellular source of IL-6 protein one index patient was selected for intracytoplasmic cytokine detection studies. After PMA stimulus the most distinct IL-6 response was seen at a time point of 6 hours in cells representing CD19-positive leukaemic cells. Percentages of IL-6 producing CD19 cells were 37.2 % in 6 hour culture, 17.5 % in 24 h culture and 15.6 % in 72 h culture respectively. Percentages of IL-6 positive CD2-cells were under 1.8 % at all time points.

5.2. IL-6, IL-1b, IL-1Ra and their genetic polymorphism in B-CLL (Study II)

5.2.1 Interleukin-1b plasma levels

In 3 of the B-CLL patients (n = 34) and in 9 of the controls (n = 400) the interleukin-1β plasma levels were below the assay limit. The IL-1β plasma levels were lower in B-CLL patients than in

the healthy controls. The IL-1β levels varied with the immunophenotype score. The patients who had immunophenotype score 3 (indicating atypical immunophenotype) had higher plasma IL-1β than patients with score 4 or 5 (indicating more typical immunophenotype). No single immunophenotypic marker was found to be associated with this result. The IL-1β levels were closer to normal in those patients who had a non-progressive disease compared with the group of progressive (intermediate and fast) disease. There were no statistically significant differences in IL-1β production among B-CLL patients when the Binet stage of the disease, FAB-classification or treatments previously given were used as categorising parameters.

5.2.2 Interleukin-1Ra plasma levels

In 4 of the B-CLL patients (n = 34), IL-1Ra plasma levels were below the assay limit. The IL-1Ra plasma concentrations of the healthy controls (n = 200) were all measurable with the methodology applied. As was the case with IL-1β, the observed IL-1 Ra plasma levels were clearly lower in the B-CLL patients than in the normal controls. In contrast to the IL-1β results, IL-1Ra levels were lower in the non-progressive group compared with the fast plus intermediate progression group, although the difference was not statistically significant. No significant differences in IL-1Ra plasma levels were found within the B-CLL group when cell immunophenotypic score, Binet stage, FAB classification or treatments previously given were used to categorise the patients.

5.2.3 Interleukin-6 plasma levels

In 5 (n = 36) of the B-CLL patients and 57 (n = 400) of the controls IL-6 plasma levels were below the assay limit. The plasma levels of IL-6 were elevated in B-CLL patients compared with the normal controls. The IL-6 levels were higher in Binet C patients than in Binet B or Binet A patients. The IL-6 plasma levels correlated negatively (R = -0.53) with the haemoglobin levels of the B-CLL patients. Plasma IL-6 was higher in B-CLL patients having anaemia compared to non-anaemic B-CLL patients. This trend was seen independently at all Binet stages of the disease. A haemoglobin concentration of < 130 g/L for male and < 120 g/L for female subjects was used as the criterion for anaemia. Plasma IL-6 levels correlated positively with the ESR (R = 0.41) in B-CLL patients. As was the case with IL-1Ra, no correlation between cell immunophenotypic score or treatment, and IL-6 production was found.

5.2.4 IL-1Ra/IL-1b ratio

No statistically significant difference was observed in the mean IL-1Ra/IL-1β concentration ratios between normal controls and all B-CLL patients. However, this ratio was markedly lower (32.8 ±

20.8) in the patient group having non-progressive disease compared with patients representing fast and intermediate progressive disease forms (256 ± 449). The difference in IL-1Ra/IL-1β ratios reached statistical significance between the normal controls and the non-progression B-CLL patients.

5.2.5 Allele frequencies

The distributions of the IL-1α, IL-1β (-511), IL-1β (+3953) and IL-1Ra alleles were similar in the patient samples and the controls. The same was true when the frequencies of allele combinations formed on the basis of IL-1α, IL-1β and IL-1Ra genes were compared between patients and controls. No differences in allele frequencies were seen when the progression grade or Binet stage of the disease was applied as a grouping parameter. As expected, the IL-1β and IL-1Ra plasma levels varied depending on the allelic state of the subjects. In the normal controls, IL-1β plasma levels were significantly higher among IL-1β (-511) allele 2 carriers than among non-carriers, this effect was studied thoroughly and reported in Study III of this thesis (below). The same trend was seen in the plasma levels of IL-1β in IL-1β (+3953) allele 2 carriers and non-carriers and in IL-1Ra plasma levels between IL-1Ra allele 2 carriers and non-carriers. Taking into account the fact that the baseline plasma concentrations of IL-1β and IL-1Ra were distinct, the allele-dependent variations followed similar profiles in both the B-CLL patients and the normal controls. There were no significant differences in the IL-6 plasma levels of subjects with different alleles of the IL-6 gene.

5.3 The effect of IL-1 and IL-1Ra alleles on plasma IL-1b (Study III)

5.3.1 Allele frequencies

DNA from blood samples from 400 blood donors was purified and their genotype of the IL-1 complex was analysed. This data demonstrated that the allele frequencies of IL-1α (C to T exchange at -889), IL-1β (C to T exchange at -511) and IL-1 Ra (penta-allelic VNTR in the second intron) correspond to those originally published. As expected, these alleles are significantly associated. It has recently been calculated that of the indicator polymorphisms used here, IL-1α allele 1, IL-1β allele 2 and IL-1Ra allele 2 belong to the most frequent IL-1 gene cluster haplotype (see the review of the literature)

5.3.2 Plasma IL-1b

IL-1β concentrations of the plasma samples derived from these 400 blood donors were analysed using an ELISA method. In 392/400 samples the concentrations were above the detection limit of the assay. When the IL-1β levels were categorised on the basis of IL-1α, IL-1β and IL-1Ra genotypes, the IL-1α genotype had a marked effect on IL-1β plasma levels. The IL-1α 2.2 homozygous donors had significantly higher IL-1β levels than the 1.2 heterozygous or 1.1 homozygous donors. By contrast, the IL-1β or IL-1Ra genotypes did not have statistically significant, independent effect on the plasma IL-1β levels. To examine possible effects of allele combinations, a two-way ANOVA analysis was performed. This analysis showed a trend for the IL-1α genotype and IL-1β allele 2 carrier state to have an interactive effect on IL-1β plasma levels. Accordingly, post-hoc comparisons were carried out and this complementary interaction was shown to be a consequence of distinctive IL-1β production in IL-1α 2.2 homozygous/ IL-1β allele 2 positive subjects. No interaction was seen when the combined effects of IL-1α genotype and IL-1Ra alleles or IL-1β genotype and IL-1Ra alleles were studied.

5.4 Plasma IL-6 and the polymorphism of the IL-6 gene in pSS (Study IV)

5.4.1 Interleukin-6 plasma levels

The IL-6 plasma levels were elevated in the pSS patients compared to the IL-6 plasma levels in the healthy controls. Plasma IL-6 levels were significantly higher among the pSS patients with coeliac disease, in patients with PNS symptoms and in patients with pulmonary fibrosis or alveolitis as compared to the pSS patients without these findings. None of the patients had all three manifestations, and only five patients had two simultaneous manifestations. Thus the observed high IL-6 levels in these discrete disease groups were not caused by a subgroup of patients with all of these manifestations. Plasma IL-6 levels increased in parallel with increasing grade in minor salivary gland biopsy, and were significantly higher in patients with grades 3-4 than in patients with grades 0-2. Furthermore, the plasma IL-6 levels were higher in patients with definite pSS (four Californian criteria) than in those with possible pSS (three criteria). Among the pSS groups, the plasma IL-6 levels were lower in patients with purpura than in patients without purpura. SS-A antibody-positive patients had lower IL-6 concentrations than SS-A antibody-negative patients. No correlation between IL-6 plasma levels and age, disease duration, SS-A or SS-B antibody titers were found.

5.4.2 IL-6 (-174) G/C base exchange polymorphisms

The allele frequencies were similar in pSS patients and in control subjects. The IL-6 plasma levels were higher in pSS patients with allele G/G than in those with G/C or C/C. No significant differences were observed in the control group when the subjects were categorised on the basis of IL-6 genotypes. When the categorisation was made by the allele G carrier state, the allele G positive control subjects had a slightly higher plasma IL-6 than allele G negative subjects. Although some of the pSS sub-groups were too small for reliable frequency analysis, the frequency of allele G seemed to be increased in subgroups with coeliac disease, PNS manifestation and pulmonary manifestation compared to groups without these manifestations. Decreasing trends in allele G frequencies were seen in sub-groups with purpura or SSA-antibodies compared to non-purpura and SSA-ab negative groups. Thrombocyte counts were higher in patients with allele G than in allele G negative patients.

5.5 Plasma IL-10 and the IL-10 gene polymorphism in pSS (Study V)

5.5.1 IL-10 base exchange polymorphisms

After determining the single base exchange polymorphism of -1082, -819 and -562 for each subject, the genotype distribution, the haplotype carrier rate and the haplotype frequencies in pSS patients and control subjects were analysed. In the haplotypic analysis the GCC haplotype frequency was significantly higher among pSS patients than among healthy controls. The ACC haplotype frequency was significantly lower in pSS patients than in healthy subjects. The ATA haplotype frequencies were similar in pSS patients and in control subjects, but the proportion of GCC/ATA genotype was higher in pSS than in normal controls. No correlation with extraglandular symptoms and IL-10 haplotypes was found.

5.5.2 Interleukin-10 plasma levels

The IL-10 plasma levels were higher in the pSS patients than in the healthy controls, although this difference has to be considered as a trend. The patients were then classified according to their genotypes and haplotype carrier state and the effect of these on the plasma IL-10 levels were analysed. The plasma IL-10 levels were significantly higher among the pSS patients carrying the GCC haplotype compared to GCC haplotype negative patients. The patients who were carriers of ACC haplotype had slightly, but not significantly lower IL-10 plasma levels than ACC negative patients. The ATA carrier status did not have any effect on plasma IL-10. No significant differences in IL-10 plasma levels among the healthy controls were seen when they were categorised on the basis of IL-10 haplotypes.

6. DISCUSSION

6.1 Cytokines and cytokine gene polymorphism in B-CLL

6.1.1 Methodology

As may be seen in the review of the literature, *in vitro* studies concerning B-CLL are rarely concordant. The intensive purification procedures that are carried out in order to enrich the proportion of malign cells in cultures may partly explain the discordance in results. The most often used purification steps are based on density gradient centrifugation, T cell depletion by one or two rounds of sheep erythrocyte rosetting and monocyte depletion by plastic adherence or carbonyl iron absorption (Aderka et al. 1993; Aguilar-Santelises et al. 1991; Biondi et al. 1989; Castejon et al. 1999; Cordingley et al. 1988; Dancescu et al. 1992; di Celle et al. 1994; Digel et al. 1989; Foa et al. 1990; Morabito et al. 1987; Reittie et al. 1996). In some studies negative or positive selection with immunomagnetic beads has been used (van Kooten et al. 1992). A few studies have applied fluorescence activated cell sorting in the analyses (Lavabre-Bertrand et al. 1995; Plate et al. 1993). These purification steps are problematic as the selection procedure may enrich certain malign cell subtypes more than others, which may result in incoherent cell populations (Vilpo et al. 1998). As the B-CLL clones are heterogeneous in respect to their cell surface antigen expression, these drawbacks are also evident when antibody- or affinity-based purification measures are applied (Sembries et al. 1999). Moreover, there is direct evidence that intensive purification procedures non-specifically activate B CLL cells. Rambaldi et al. (1993) observed that T cell depletion by rosetting and monocyte depletion by plastic adherence induced IL-6 transcription and increased the accumulation of IL-1β, IL-6 and TNF-α mRNA in the B-CLL samples. In addition to purification procedures, the concentrations of phorbol esters, SAC, LPS or cytokines vary from one study to another and the cytokine measurement techniques are very diverse. The major problem in B-CLL *in vitro* studies, however, is the small patient cohorts. This leads to error when generalised concepts are formed on the basis of one-sided approaches. These factors have to be taken carefully into account when evaluating these studies.

6.1.2 IL-6 in B-CLL (Studies I and II)

In these studies we observed that cellular release of IL-6 (and to a lesser extend TNF-α) is decreased at Binet C stage patients (Study I), who in contrast have the highest circulating IL-6 levels (Study II). We also found that overall plasma IL-6 is increased in B-CLL compared with normal levels. In addition, plasma IL-6 was shown to be associated with anaemia, haemoglobin levels and with ESRs in B-CLL. Moreover, the polymorphisms of IL-1 family genes and IL-6 gene

were studied and the allele distributions of these polymorphisms were observed to be similar in B-CLL patients and in control subjects.

We do not know the exact mechanism of inferior cellular IL-6 (and TNF-α) production among Binet C stage B-CLL. It has been shown that the heterogeneity in the spontaneous IL-1β, IL-6 and TNF-α production *in vitro* is caused neither by different transcription rate nor differences in the stability of mRNA in B-CLL (Rambaldi et al. 1993). Although mRNA levels of these cytokines have interindividual variation in B-CLL when analysed by Northern blot analysis, the mRNA transcription by nuclear run-on analysis has been demonstrated to be very similar among B-CLL patients (Rambaldi et al. 1993). The similar transcription rate among patients (to a certain extent) rules out the possibility that the diverse DNA methylation of these cytokine genes could explain the individual cytokine mRNA expression (and protein production) in B-CLL. In contrast to others (see the review of the literature), we were able to demonstrate IL-6 production in malign B cells. This was achieved by using PMA stimulus and brefeldin-A protein transport inhibitor. However, it is evident that B-CLL clones are heterogeneous in their antigen expression, and probably also in respect to their development stage (Sembries et al. 1999). Thus it is probable that cytokine production capacity and on the other hand the responses to exogenous stimuli are different depending on the developmental stage of the malign clone. If so, the heterogeneity observed in the IL-6 production in culture may reflect the heterogeneity in the cellular origins of the disease. It is also possible that there are signal transduction defects, which lead to deficient cell activation and deficient IL-6 production in patients with severe disease. Moreover, it has been demonstrated that IL-6 production follows the IL-1β production in B-CLL (Aguilar-Santelises et al. 1991). Thus the interrelationships between IL-1, TNF and IL-6 and other cytokines may partly explain our results (Dinarello 1996; Jurlander 1998). Nevertheless, the molecular mechanisms leading to decreased secretion or increased degradation of IL-6 in cell cultures in severe B-CLL are so far unidentified.

We also found that plasma IL-6 elevated in B-CLL compared to levels of normal controls. Plasma IL-6 was highest in patients with Binet C disease. This association of plasma IL-6 to the diseage stage is confirmatory to others (Callea et al. 1996; Reittie et al. 1996). All of these studies are in agreement with the recent study by Fayad et al. (2001), in which the authors found that serum IL-6 levels were higher in CLL patients (n=151) than in normal controls (n=55) and the patients with elevated serum IL-6 had poorer median and 3-year survival with unfavourable prognostic characteristics such as elevated β2-microglobulin or LDH and Rai stage III or IV. As the distribution of IL-6 alleles was normal in our study, and as alleles were not associated with disease stage in B-CLL, other mechanisms than the allelic imbalance of this polymorphic site are causing

the distinct IL-6 profile in B-CLL. The normal and stage-independent IL-6 allele distributions in B-CLL also exclude the possibility that this polymorphic locus could be used in the risk-assessment marker of B-CLL. Thus other factors than allelic imbalance of IL-6 gene are causing the increased synthesis (or decreased degradation) of circulating IL-6 in patients with an aggressive form of B-CLL.

The interindividual variation of IL-6 receptor expression as well as the agonistic role of soluble IL-6 receptor in B-CLL cells makes the evaluation of the paracrine and autocrine effector functions of IL-6 in B-CLL complex (Lavabre-Bertrand et al. 1995). If the IL-6 has the growth inhibitory effect in B-CLL as suggested (Orsini and Foa 2001), the paracrine lack of IL-6 could lead to expansion of the malign clone. This could explain why the low cellular IL-6 release is associated with severe (Binet C) B-CLL. When considering these aspects it should be noted that cellular release of IL-6 is at its lowest, and circulating IL-6 at its highest in Binet C stage patients. This fact suggests that paracrine/autocrine mechanisms for IL-6 production are very different from mechanisms responsible for systemic production and balance of IL-6. This theory is supported by the observation that although we were able to detect IL-6 in malign B-CLL cells, we have not found any correlation with the plasma IL-6 and IL-6 production *in vitro* cultures (data not shown). Even if the cellular source of plasma IL-6 is not definitely known, it can be concluded from this data that the majority of the circulating IL-6 is derived from the non-CLL cells (e.g. endothelial cells or monocytes). Thus it is possible that the increase of circulating IL-6 observed in Binet C disease reflects tumour immunology, in which accessory cells are providing feedback inhibition by secreting excess IL-6. From this point of view, the high IL-6 production capacity may also be protective in B-CLL.

Because of the cross-sectional nature of our study, we can only speculate what the consequences of diminished cellular and increased plasma IL-6 production in B-CLL are. Plasma IL-6 was higher in patients with anaemia, and correlated negatively with haemoglobin levels and positively with ESRs in B-CLL patients. IL-6 induced anaemia is known to be caused by haemodilution, but whether this or other mechanisms are causing the association of anemia, ESR and IL-6 in B-CLL remains to be studied. When considering *in vitro* and *in vivo* IL-6 results on the whole, all these above mentioned findings support the role of IL-6 in the evolution of the disease. The association of inferior cellular IL-6 release and high plasma IL-6 with disease stage suggests that these factors are potential surrogate markers for an inferior prognosis. As mentioned above in the recent study by Fayad et al. (2001), serum IL-6 levels showed significant corretion with the overall survival. In this study the authors suggested that measurements of serum IL-6 levels could be used to identify patients whose

disease is beginning to evolve into a more aggressive form even though morphological evidence of transformation is not yet evident. However, more extensive longitudinal studies are needed in order to explore the causality of IL-6 in disease evolution and pathogenesis. We cannot rule out whether the decrease in cellular IL-6 and increase in plasma IL-6 production are kinetic processes. It is possible that originally high cellular IL-6 production capacity in patients with mild disease forms is lost if their disease progresses to severe Binet C disease. It is also possible that the low cellular or high systemic IL-6 production is a basic property of those patients who are prone to develop a more severe disease. These aspects have to be elucidated before the potential use of IL-6 as a surrogate marker for disease severity is considered.

6.1.3 IL-1b and IL-1Ra in B-CLL (Study II)

We found that the baseline plasma concentrations of IL-1β and IL-1Ra are diminished in B-CLL. Overall increase in serum IL-1α but in contrast to us, no statistically significant decrease in serum IL-1β has been found in B-CLL by others (Aguilar-Santelises et al. 1992). On the other hand, our observation that high circulating IL-1β is associated with non-progressive disease is in concordance with *in vitro* studies done by others on B-CLL. B-CLL cells from patients with indolent disease have been shown to produce more IL-1β mRNA and IL-1β protein than cells from patients with progressive disease (Aguilar-Santelises et al. 1991). The basal membrane IL-1α has also been shown to be higher in patients with benign (Rai stage 0, nonprogressive) B-CLL than in patients with more severe/progressive disease (Aguilar-Santelises et al. 1993). The stage-dependence of IL-1β seems to be a unique phenomenon, as in a recent large-scale cDNA microarray analysis in which the expressions of 1024 selected genes were analysed in 54 B-CLL cases the reduced expression of IL-1β gene was among those few which significantly correlated with reduced patient survival and high clinical stage of the patients (Stratowa et al. 2001). We were first to show that the IL-1Ra/IL-1β ratio is strikingly low in the serum of patients with non-progressive B-CLL. This finding indicates that high IL-1β activity has a protective role in the disease. Moreover, these results suggest that low IL-1Ra/IL-1β ratio is a candidate marker of good prognosis in B-CLL.

In spite of increasing evidence of disturbed IL-1 production in B-CLL, no explanation for deficient IL-1β production in severe B-CLL has been published. The normal allelic distribution and stage-independence of IL-1 family gene polymorphism in B-CLL excludes the possibility that allelic imbalance causes these disturbances. The normal allelic distribution of IL-1 family genes in B-CLL also excludes the possibility that these polymorphic loci could be used in the risk-assessment of B-CLL. As this IL-1β defect has been found at the mRNA level as in plasma, it is possible that the

cause of reduced IL-1β is deficient synthesis or deficient activation (i.e. anergy or signal transduction defect) of IL-1β gene in severe B-CLL. However, as was the case with IL-6, the post-transcriptional mechanisms have to be taken into account as a potential cause for the variation of IL-1β mRNA levels in B-CLL (Rambaldi et al. 1993). When considering plasma IL-1β, we do not know how much from the circulating IL-1β is derived from the leukaemic clone and how much is produced by other cells *in vivo* in B-CLL. As the IL-1β receptor is expressed in malign B-CLL clone, the mechanism for decreased IL-1β in severe B-CLL may be explained by the increased receptor binding (i.e."decay") of this protein. This could also explain why the decrease in IL-1β is observed to be associated with high tumour mass (i.e. severe disease).

In addition to mRNA synthesis or post-transcriptional modification of IL-1β mRNA, it is also possible that the secretion of IL-1β protein is disturbed. Secretion of mature IL-1β is dependent on cleavage by ICE, and if ICE is inhibited so is the secretion of mature IL-1β (Dinarello 1996; Thornberry et al. 1992). On the other hand ICE is in a key role in Fas-mediated apoptosis, also in B-CLL. The inhibitors of ICE inhibit spontaneous and glucocorticoid-induced apoptosis in B-CLL cells (Bellosillo et al. 1997; Chandra et al. 1997). In Study II lowest plasma IL-1β was observed in patients with short lymphocyte doubling time. Thus, in theory it is possible that a high accumulation of malignant cells in progressive disease may be caused by low ICE activity, which in turn is reflected as low plasma IL-1β in these patients. It is worth mentioning that the gene for ICE is located at chromosome region 11q22.2-q22.3, which is frequently affected in B-CLL and associated with poor prognosis. Whether the deficient IL-1β production may serve as a surrogate marker for the disturbed ICE-mediated apoptosis in B-CLL remains to be studied.

As was the case with IL-1β, the biological causes for decrease in overall circulating IL-1Ra in B-CLL is not known. In fact, very little at all is known about IL-1Ra in B-CLL. Thus it is possible, that either decreased synthesis or secretion, or increased IL-1 receptor mediated degradation (decay) are cause the decreased circulating IL-1Ra in B-CLL. No mRNA studies or *in vitro* studies concerning IL-1Ra in B-CLL have so far published, so the synthesis of this protein remains to be studied in B-CLL. As the IL-1β is secreted by an ICE-mediated process while the IL-1Ra is not, the low IL-1Ra/IL-1β plasma ratio in non-progressive disease may be explained partly by high ICE expression, which in theory may have a protective role in the disease.

We also found that the B-CLL patients with low immunophenotypic scores had higher IL-1β plasma levels than the patients with higher scores. To some extent the immunophenotypic scoring

system predicts atypical cell morphology in blood films (Matutes et al. 1994). These atypical cases of B-CLL have been shown to have distinct immunophenotypic, cytogenetic, morphological and prognostic profiles (Criel et al. 1997). Our findings suggest that the atypical forms of B-CLL could also be immunologically divergent from the typical forms of the disease. No systematic study concerning the immunophenotype score and its clinical implications on immune responses or prognosis in B-CLL has been done. Therefore the clinical relevance of this finding has to be confirmed with a larger number of patients.

6.2 Cytokine gene polymorphism and plasma cytokines in healthy adults

6.2.1 Combined effect of IL-1 family alleles on circulating IL-1b (Study III)

In the Study III we demonstrated that the baseline plasma levels of IL-1β are genetically regulated, but this genetic effect could be observed only in those persons who were homozygous for the allele 2 of the IL-1α gene. The effect of IL-1α (-889) allele 2 homozygosity was influenced by the presence of IL-1β (-511) allele 2, suggesting that the products of these two loci interactively regulate the production of the IL-1β, or this rare haplotype is in linkage disequilibrium with a still unidentified genetic locus which causes the spontaneous high production of IL-1β.

As described in the review of the literature, allele 2 of the IL-1α -889 and allele 2 of IL-1β +3953 belong to the same haplotype in the IL-1 gene cluster (Cox et al. 1998). IL-1β +3953 allele 2 has been associated with high LPS-stimulated IL-1β responses *in vitro* cultured monocytes (Pociot et al. 1992). Based on this fact it was possible that the high plasma IL-1β in IL-1α 2.2 homozygous subjects was affected by genetic linkage of IL-1α (-889) and IL-1β (+3953) alleles. As it was essential to rule out this possibility, we extended our previously published IL-1β (+3953) analyses of 200 subjects (Hurme and Santtila 1998) to a cohort of 400 subjects studied here. Thus the IL-1β (+3953) polymorphism was originally tested in the same analytic model as the IL-1α (-889) polymorphism, IL-1β (-511) polymorphism and IL-1Ra polymorphism. In these preliminary analyses the combination of IL-1α -889 allele 2 homozygosity with IL-1β +3953 allele 2 carrier state was found in 26 subjects out of 400. No association with IL-1β (+3953) polymorphism and IL-1β plasma levels were found (p=0.612 in one-way ANOVA) and IL-1β (+3953) was omitted from further analyses.

Our study group has previously stated that the plasma levels of IL-1Ra in healthy subjects (n=200) were higher in IL-1Ra allele 2 carriers than in non-carriers, but this effect was restricted to

individuals who were also carriers of the IL-1β allele 2 (T at the position -511) (Hurme and Santtila 1998). As was the case with IL-1β (+3953), these 200 subjects are included in the cohort of 400 investigated in Study III of this dissertation. In the preparation phase of Study III we re-analysed these previously published data on plasma IL-1Ra retrospectively also in respect to IL-1α (-889) alleles, and IL-1α -889 polymorphism did not change previously published regulatory IL-1Ra associations further (data not shown).

As IL-1β (-511) allele 2 had a cumulative effect on the IL-1α allele 2.2 homozygosity-associated high IL-1β, it could be speculated that this allele contains or codes for a regulative element enhancing the transcription of itself and the adjacent genes. The effect of the IL-1α alleles on the circulating IL-1α is not known. If it is assumed that 2.2 homozygosity is associated with a high IL-1α transcription, these high levels would then effectively induce IL-1β production only in the presence of the "permissive" IL-1β allele 2.

There is not very much data about the clinical significance of the base exchange polymorphism at the position -889 of the IL-1α gene. McDowell et al. (1995) observed that the number of carriers of allele 2 was increased in patients with early-onset, pauciarticular juvenile rheumatoid arthritis. Our group has recently demonstrated that the number of IL-1α 2.2 homozygotes is significantly greater in schizophrenic patients and that the IL-1β plasma levels are elevated in acute, non-treated patients (Katila et al. 1994; Katila et al. 1999). Thus, the present findings are perfectly in line with these earlier observations and provide a plausible explanation for the increased levels of IL-1β in schizophrenic patients.

6.2.1 IL-6 and IL-10 polymorphism in healthy adults (Studies IV and V)

We found no statistically significant differences in plasma IL-10 when healthy controls (n=202) were categorized on the basis of IL-10 haplotypes (Study IV). Likewise the IL-6 genotype had no effect upon IL-6 plasma levels in healthy controls (n=400, Study V). As these polymorphisms are located on the promoter region of respective genes it is possible that IL-10 and IL-6 polymorphisms regulate primarily inducible cytokine responses. It is also possible that more sensitive methods are needed to detect the genotype effect on basal cytokine production. Moreover, the lack of clear genotypic profiles in healthy population also suggests the basal and induced responses of these cytokines are differently regulated.

6.3 Cytokine gene polymorphism and plasma cytokines in pSS

6.3.1 IL-6 genetic polymorphism and plasma IL-6 in pSS (Study IV)

In Study IV we showed that the plasma levels of IL-6 are elevated in primary Sjögren's syndrome patients, which is in agreement with results by Pettersson et al. (1992) and Grisius et al. (1996) and Garcia-Carrasco et al. (2001). We also found that high plasma IL-6 was associated with specific extraglandular manifestations of pSS. Moreover, we analysed the promoter region polymorphism of the IL-6 gene and found that plasma IL-6 concentrations were dependent on IL-6 allelic state in the pSS. The allelic distribution of IL-6 –174 polymorphism was similar in patients and controls.

We observed almost linear correlation between the lymphocyte infiltration grade and plasma IL-6 levels as well as a correlation between the number of pSS criteria and plasma IL-6 concentrations. The degree of lymphocyte infiltration of the salivary tissue has been found to correlate with the early onset of the disease, total number of extraglandular features, presence of specific extraglandular features such as Raynaud's phenomenon, presence of vasculitis and lymph node or spleen enlargement and leukopenia (Asmussen et al. 1996; Gerli et al. 1997). These complementary histological and clinical findings of ours strongly suggest that IL-6 may be used as a surrogate marker for some of the extraglandular manifestations and the severity of pSS. High IL-6 also suggests that IL-6 may be involved in the development of more severe forms of pSS and some forms of extraglandular disease.

However, as was the case in B-CLL, the cross sectional nature of this study limits the possibilities to establish direct causality between high plasma IL-6 and the extraglandular manifestations of pSS. It is also possible that increase in the plasma IL-6 is a kinetic process in pSS. The normal IL-6 allele distribution in pSS excludes the possibility that allelic imbalance of this gene is a primary cause for the overall increase of plasma IL-6 in pSS. Thus other factors, such as increased number of IL-6 secreting cells in the peripheral blood of these patients are causing the increased synthesis of this protein in pSS (Halse et al. 1999). Moreover, it cannot be ruled out that decreased degradation of IL-6 cause the overall increase in circulating IL-6. Interestingly, as mentioned in the review of the literature, tear fluid IL-6 has also been found to be correlated with lymphocyte infiltration in pSS (Tishler et al. 1998). Moreover, salivary IL-6 has been suggested to reflect increased inflammatory activity in pSS, as the increased salivary IL-6 concentrations are associated with ESR and IgG in the disease (Grisius et al. 1997). However, no correlation between serum and salivary IL-6 has been found, suggesting that the secretion of IL-6 in circulating blood and saliva is differently regulated (Grisius et al. 1997).

The highest IL-6 plasma levels were observed in pSS patients with coeliac disease. In the development of coeliac disease the pathological features of gluten sensivity are associated with local and systemic increase of IL-6 and other proinflammatory cytokines. Taking into account the significant role of IL-6 in mucosal immunity and immunoglobulin synthesis (see the review of the literature), it is possible that increased IL-6 promotes the autoimmune process and mucosal inflammation leading to villous atrophy in a subgroup of pSS patients. Thus excess IL-6 may promote the process leading to coeliac disease in a small proportion of pSS patients who are prone to develop this kind of complication. On the other hand, it is also possible that high IL-6 concentrations are caused primarily by other factors known to be associated with coeliac disease process, such as increased Th0 cell responses to gliadin or glutein (Nilsen et al. 1996; O'Keeffe et al. 1999).

As was the case with high IL-6 in the coeliac disease subgroup in pSS, only speculations can be provided on the role of increased IL-6 in the development of PNS symptoms in pSS. Whether the systemic increase in IL-6 has an effect on neurotransmission, or is inducing neurodegenerative demyelination as the locally produced or exogenously added IL-6 in animal models remains to be studied in pSS (Deretzi et al. 1999; Marz et al. 1998; Xia et al. 1999). The fact is, however, that the role of IL-6 in the development of peripheral nervous system symptoms in autoimmune diseases is not known. From this point of view one interesting question is, whether IL-6 has an effect on autoantibody production against muscarinic acetylcholine receptors, which have been suggested play a role in the pathogenesis of pSS (Hakala and Niemelä 2000).

It is probable that the mechanisms causing pulmonary fibrosis and alveolitis in pSS resemble those which are responsible for the development of reactive fibrosis in other autoimmune diseases. A similar association of high IL-6 and fibrosis to that described here has also been found in systemic sclerosis, where the elevated serum IL-6 is related to the occurrence of pulmonary fibrosis and decline of vital capacity (Hasegawa et al. 1998). In addition, IL-6 has been suggested to play a role in alveolar fibroblast proliferation and fibrogenesis in patients with diffuse interstitial fibrosis (Shahar et al. 1996). Confirmatory to us, Garcia-Garrasco et al. (2001) observed increased circulating IL-6 in a subgroup of pSS patients with pulmonary involvement when demonstrated by the clinical picture, altered chest radiography or spirometry.

The mechanism of purpura in pSS is generally believed to be nonthrombocytopenic, but we speculate that the low IL-6 production observed in pSS patients with purpura could partly predispose some patients to this complication as IL-6 has a role in megakaryopoiesis (see review of

the literature). The effect of IL-6 on megakaryopoiesis was also seen on the genetic level, as thrombocyte counts were lower among allele G-negative pSS patients. The confirmatory finding of IL-6 allele association with platelet count was reported recently, as healthy subjects with allele G had significantly higher ($240 \pm 44 \times 10^9$/L, n=44) platelet count than allele C homozygous subjects ($205 \pm 60 \times 10^9$/L, n=15) (Fernandez-Real et al. 2001).

In addition to purpura, low plasma IL-6 was observed in patients with SS-A antibodies. It has been stated that SS-A positivity or combined SS-A/SS-B antibody positivity are more common in pSS patients with a more severe disease (Gerli et al. 1997; Kelly et al. 1991; Pease et al. 1993). In the patients in our study no such associations were observed, and no correlation between SS-A or SS-B antibody titers and plasma IL-6 concentrations was found. We do not know the reason for this discrepancy, but it is obvious that the detection methods of SS-A antibodies are widely variable. It has been shown that only the SS-A antibody with specificity against the 60-kDa component of the Ro antigen correlates with the quantity of extraglandular manifestations. Moreover, the patients in our study were selected by Californian criteria, which are stricter than European criteria. The use of these criteria probably excludes some of the patients with the mildest forms of the disease (and increases the proportion of patients with extraglandular disease).

Increase in circulating IL-6 has been shown to be associated with cryoglobulinemia, hypocomplementemia and liver involvement (Garcia-Carrasco et al. 2001). Unfortunately, these factors were not explored in our study. The association with IL-6 to these parameters is interesting, however, as complement factors are mainly synthesized in liver, and IL-6 is a strong hepatocyte stimulating factor (see the review of the literature).

Although no statistically significant differences in IL-6 allele frequencies were observed in subgroups of patients with extraglandular manifestations, the presence of allele G seemed to be logically associated with high plasma IL-6 in the disease subgroups. As the plasma IL-6 was regulated by this allele in pSS, the presence of allele G may be a risk factor for some extraglandular manifestations associated with high IL-6 in pSS. This study raised some interesting questions, e.g. what is the role of the IL-6 polymorphism in primary diseases which were here studied only as "manifestations" of pSS. No systematic studies concerning the IL-6 polymorphism in primary coeliac disease or diffuse pulmonary fibrosis have so far been published. As the IL-6 plays a role in B cell differentiation and immunoglobulin synthesis, the role of increased IL-6 in the development of hypergammaglobulinemia in pSS should be studied. These potential roles of IL-6 and IL-10 in local and systemic B cell hyperreactivity and hypergammaglobulinemia are discussed below.

6.3.2 IL-10 genetic polymorphism and plasma IL-10 in pSS (Study V)

In this study we analyzed the polymorphism of the IL-10 gene and found that the frequency of GCC haplotype is increased and the frequency of ACC haplotype is decreased in pSS patients. Moreover, we measured the levels of circulating IL-10 and found that GCC haplotype is associated with high plasma IL-10 levels in pSS, which is in agreement with the results of Turner et al. (1997) for mononuclear cell cultures.

As was the case with IL-6, an increased number of IL-10 secreting cells was found in the peripheral blood mononuclear fraction in pSS (Halse et al. 1999). Moreover, as was the case here, high but not statistically significant circulating IL-10 concentrations have been found in the circulating blood of pSS patients (Garcia-Carrasco et al. 2001). Our study is the first one where the haplotypes formed on the basis of the IL-10 gene (-1082, -819 and -592 sites) alleles have been demonstrated to be related to susceptibility to pSS. Furthermore, for the first time the IL-10 haplotypes have been found to regulate levels of circulating IL-10. In contrast to us, however, Rischmueller et al. (2000) did not find any association between pSS and IL-10 haplotypes in an Australian population (Rischmueller et al. 2000). This observation was published simultaneously with our results reporting a positive finding in respect of IL-10 haplotypes and pSS. There are several obvious reasons for this discrepancy, e.g. different statistical approach, different population and most importantly, different diagnostic criteria for the disease.

The abnormal distribution of IL-10 haplotypes suggests that some of these haplotypes may predispose patients to pSS. Moreover, the increase of GCC, IL-10 "high-producer" haplotype in our study may at least partly explain the high circulating IL-10 in pSS observed by ourselves and others. It has been shown that the cellular source of increased IL-10 in PBMC cultures is B-cell and monocyte fraction , and that T cells contribute very little to IL-10 production *in vitro* (Llorente et al. 1994). The cellular source of circulating IL-10 in pSS is not definitely known, but it is possible that increase in circulating IL-10 is partly enhanced by an increased proportion of autoreactive CD5+ B lymphocytes producing IL-10 in the peripheral blood of pSS patients (O'Garra et al. 1992; Powrie et al. 1997). We did not perform any *in vitro* studies on pSS. It remains to be determined whether the IL-10 haplotypes regulate IL-10 production in various cellular compartments of pSS, and whether the increased frequency of GCC genotype explains the local T-cell derived IL-10 overexpression observed by others (Brookes et al. 1996; Fox et al. 1994). This issue is important, as recent studies with IL-10 transgenic mice have indicated that local increase in IL-10 is a necessary and sufficient factor for the development of autoimmune exocrinopathies, such as SS (Saito 2000). In these studies locally introduced increase in IL-10 caused glandular tissue destruction by IL-10 induced

and Fas/Fas ligand mediated apoptosis. The data from other animal studies also suggest that IL-10 may have an essential role in the autoimmune pathogenesis in general, e.g. in the development of SLE (Ishida et al. 1994).

In contrast to IL-6, we did not observe any correlation with the level of plasma IL-10 and lymphocyte focus score, extraglandular manifestations or any other marker of disease activity in pSS. Moreover, no association between circulating IL-10 and SS-A or SS-B was observed, which is confirmatory to *in vitro* studies by others (Llorente et al. 1994). No direct correlation with IL-10 haplotypes and SS-A or SS-B antibodies or antinuclear antibodies were found in this study, which was in agreement with Rischmueller et al. (2000).

Although no direct associations of IL-10 and autoantibody status and extraglandular manifestation were observed in our cross-sectional study, the increased IL-10 together with increased IL-6 may still have several consequences for the pathogenesis of pSS. These cytokines are known to promote the maturation of plasma cells and activation of immunoglobulin synthesis (see the review of the literature). IL-10 stimulates the secretion of IgG1 and IgG3 immunoglobulin isotypes (Briere et al. 1994). As mentioned earlier, pSS is characterized by B cell hyperactivity and hypergammaglobulinemia. Selective polyclonal increase in IgG_1 is one symptom of incipient SS (Hay et al. 1990). In a recent article Perrier et al. (2000) reported that circulating IL-10 correlates with the polyclonal increase of IgG1 in pSS (Perrier et al. 2000). On this basis it is quite probable that the increased levels of IL-10 contribute to the development of hypergammaglobulinemia and promote autoantibody production associated with pSS. However, it is evident that increased IL-10 alone is not sufficient for the development of autoantibodies in pSS and other still unrecognized "necessary factors" are needed for the outbreak of these autoantibodies in pSS. Whether the IL-10 haplotypes have an effect on the IgG isotype levels in pSS is not yet known.

It is also known that patients with SS have an increased risk of developing lymphomas, and it is possible that persistently high levels of IL-10 together with increased IL-6 may contribute to these conditions (Levy and Brouet 1994; Woodroofe et al. 1992). Due to the subtle nature of allelic effects larger cohorts and longitudinal studies are needed to detect these kinds of phenotypic differences.

6.4 Potential pathways for allele effects

6.4.1 Genetic location of polymorphisms

Mutations in the coding region may change the amino acid sequence of a protein. However, the IL-1β +3953 polymorphism which is the only polymorphism located in the exon region of those studied here, is a conservative or "silent" mutation. This polymorphism does not cause change in the amino acid sequence and thus the structure of the corresponding protein is the same independent of the alleles. In theory mutations in exon may influence protein expression by causing alternations in mRNA splicing or by affecting the rate of mRNA decay. There are some examples in the literature suggesting that this hypothesis of mRNA half-life is true, e.g. certain alleles of IL-6 gene 3'-polymorphisms have been found to be associated with increased mRNA stability (Linker-Israeli et al. 1999). It is also possible, that exon polymorphisms result in changes of DNA conformation, which then affects transcription. These topics, however, are not yet very well known. It is probable that associations with most of these"silent" exon polymorphisms and protein productions are caused by the linkage disequilibrium of other, regulatory sites in the corresponding gene.

The VNTR polymorphism of IL-1RN gene is located at intron region (intron 2) of this gene. As was the case with exon polymorphisms above, in theory the phenotypic differences in IL-1Ra protein production may be caused by alternative mRNA splicing, linkage disequilibrium or conformational changes in DNA. Introns may also contain binding sites for proteins with regulatory function in mRNA synthesis (Bidwell et al. 1999). Tarlow et al. (1992) identified three potential protein binding sites in the IL-1RN VNTR polymorphic region, namely α-interferon silencer A, β-interferon silencer B and acute phase responsive element, which have been associated with viral responses. It is also possible that the IL-1RN alleles are in linkage disequilibrium with still unrecognized sites related to alternative splicing of IL-1RA mRNA, or sites regulating the promoter usage of IL-1RN. These steps are in the key role in the production of intracellular IL-1Ra and secreted IL-1Ra (see Figure 4).

Most of the polymorphisms studied in this dissertation i.e. IL-1α (-889), IL-1β (-511), IL-10 (-1082; -819;-592) and IL-6 (-174) are located in the promoter regions of the corresponding genes. Mutations in promoter regions can alter transcription factor binding sites, thus enhancing or decreasing the affinity of transcription factor binding. The binding of these regulatory proteins to these regulatory sequences may enhance or decrease the transcription rate of the genes, or have an effect on the duration of the induced transcription. Several putative transcription factor binding sites

which are in the key role in the cytokine responses are located at or adjacent to the these polymorphic loci (see Figures 2,3,5 and 6).

6.4.2 Effects of target tissue and activation pathways

There are several preliminary examples suggesting that the net effects of cytokine gene polymorphisms are tissue and/or stimulus specific. Stimulus dependent responses were seen by Pociot et al. (1992), when the investigators established that IL-1β secretion is associated with IL-1β (+3953) alleles in LPS stimulated cultures. However, no difference was observed when the PPD (tuberculin) or PHA was used as an stimulant. Moreover, as mentioned earlier, Perrier et al. (1998) demonstrated that in SS the IL1RN*2 allele is associated with high serum levels of IL-1Ra, but paradoxically with low IL-1Ra levels in saliva. The IL-10 microsatelite and IL-10 locus polymorphism have also been suggested to have separate roles in the regulation of IL-10 secretion. The IL-10 microsatelite polymorphisms have been suggested to regulate LPS activated responses in monocytes, as Con A activated responses are mainly regulated by -1082 polymorphism in T cells (Eskdale et al. 1998a; Turner et al. 1997). Such theories are interesting, as the signal transduction and transcription factor activation cascades are cell and tissue specific. We conclude that the biological associations of cytokine polymorphism should thus be studied in a variety of cell types.

SUMMARY AND CONCLUSIONS

In this dissertation we studied two aetiologically unknown disorders (B cell chronic lymphocytic leukaemia and primary Sjögren's syndrome) with imbalanced cytokine production in order to find out whether these imbalances have an effect on disease phenotype or severity. Several cytokine gene polymorphisms were studied in these patients in order to find out whether the allelic imbalance of these genes predisposes patients to disease, or whether disturbed cytokine profiles are caused by these genetic factors. Moreover, circulating cytokines and polymorphisms of corresponding genes were studied in a cohort of healthy subjects.

The role of *in vitro* IL-6 and TNF-α production was studied in 24 patients with B-CLL. The role of circulating IL-1β, IL-1Ra and IL-6 and several polymorphic sites of corresponding genes were studied in 36 patients with B-CLL. Circulating IL-6 and IL-10 and the genetic polymorphism of these genes were studied in cohorts of 66 and 62 patients with pSS. The cytokine gene polymorphisms of IL-1α, IL-1β, IL-1Ra, IL-6 and IL-10 were studied in a cohort of 400 healthy Finnish Red Cross Transfusion Service blood donors, who also served as controls for allele frequency analyses and cytokine production studies.

The *in vitro* and *in vivo* production of IL-6 was stage dependent in B-CLL. The cellular release of IL-6 (and to a lesser extent TNF-α) was decreased in Binet C stage patients, who by contrast have the highest circulating IL-6 levels. The overall plasma IL-6 was increased in B-CLL compared to levels of normal subjects. Plasma IL-6 was shown to be associated with anaemia, haemoglobin levels and with ESRs in B-CLL patients. In contrast to IL-6, baseline plasma concentrations of IL-1β and IL-1Ra were diminished in B-CLL. High circulating IL-1β was associated with non-progressive disease and the plasma IL-1Ra/IL-1β ratio was strikingly low in the patients with non-progressive B-CLL. High plasma IL-1β was associated with atypical B-CLL immunophenotype in B-CLL. The allele distributions of IL-1 family genes and IL-6 gene were similar in B-CLL patients and in healthy control subjects.

These data suggest that other mechanisms than allelic imbalance cause the distinct IL-1β, IL-1Ra and IL-6 profiles in B-CLL. The normal allelic distribution of corresponding genes in B-CLL exclude the possibility that the polymorphic loci of these genes could be used in the risk-assessment of B-CLL. The association of high IL-1β activity in non-progressive disease indicates that high IL-1β has a protective role in the disease. These results also suggest that high plasma IL-6 is a good

potential candidate marker for inferior prognosis in B-CLL. Moreover, high plasma IL-1β and low plasma IL-1Ra/IL-1β ratios are potential candidate markers for non-progressive B-CLL. These preliminary results emphasize the need for longitudinal studies concerning the role of these proteins in B-CLL.

The plasma levels of IL-6 were increased in pSS and high plasma IL-6 was associated with lymphocyte focus score and specific extraglandular manifestations such as coeliac disease, pleuritis and peripheral nervous system symptoms of the disease. The circulating IL-10 levels were also higher in pSS patients than in control subjects, but this difference was not statistically significant. The plasma IL-6 and IL-10 concentrations were dependent on the IL-6 or IL-10 allelic status of the pSS patients. The allelic distribution of IL-6 –174 polymorphism was similar in patients and controls. In contrast to IL-6 alleles, the frequency of IL-10 gene GCC haplotype increased, and the frequency of ACC haplotype decreased in pSS when compared with the control population. Plasma IL-10 was not associated with disease phenotype or disease activity markers in pSS.

The complementary histological and clinical findings concerning IL-6 strongly suggest that IL-6 may be used as a surrogate marker for severity of pSS. These findings also suggest that IL-6 may be involved in the development of some of extraglandular disease manifestations in pSS. The normal IL-6 allele distribution in pSS excludes the possibility that allelic imbalance of this gene is a primary cause for the overall increase of plasma IL-6 in pSS. In contrast to IL-6 polymorphism, polymorphism of the IL-10 gene seems to be a predisposing factor for pSS. The increase of GCC, IL-10 "high producer" haplotype may partly explain increased IL-10 observed earlier in pSS. Due to the subtle nature of allelic effects, larger cohorts and longitudinal studies are needed to find phenotypic effects of allelic imbalance in IL-10 gene.

We also demonstrated that the baseline plasma levels of IL-1β are genetically regulated in healthy subjects by IL-1α polymorphism. This genetic effect could be observed only in those subjects who were homozygous for allele 2 of the IL-1α gene. Moreover, IL-1β (-511) allele 2 had a cumulative effect on the IL-1α allele 2.2 homozygosity-associated high IL-1β. These results suggest that IL-1α allele 2 regulates IL-1β production indirectly by IL-1α production, or that this allele contains or codes for a regulative element enhancing the transcription of itself and the adjacent genes. No statistically significant differences in plasma IL-6 or IL-10 were observed, when healthy controls were categorized on the basis of IL-10 haplotypes. This suggests that IL-10 and IL-6 polymorphisms regulate primarily inducible cytokine responses or that basal and induced responses of these cytokines are differently regulated.

YHTEENVETO

Sytokiinit ovat joukko liukoisia tulehdusvälittäjäaineita, joiden keskinäinen tasapaino on häiriintynyt monissa sairauksissa. Sytokiinien geenit ovat polymorfisia, ja tämän geneettisen vaihtelun on kuvattu liittyvän vaihteluun yksittäisten sytokiinien tuotantokyvyssä. Tässä poikkileikkaustutkimuksessa tutkittiin sytokiinituotannon häiriöiden ja sytokiinigeenien polymorfismin osuutta kahteen etiologialtaan tuntemattomaan sairauteen, krooniseen lymfaattiseen B-soluleukemiaan (B-KLL) ja primääriin Sjögrenin syndroomaan (pSS). Lisäksi sytokiinigeenien jakautumia ja vaikutuksia terveessä väestössä tutkittiin 400 SPR:n verenluovuttajan aineistossa.

Tutkimuksessa havaittiin, että interleukiini-6 (IL-6) proteiinin plasmapitoisuudet olivat koholla sekä B-KLL:ssa että pSS:ssa. Plasman IL-6 pitoisuudet olivat riippuvaisia B-KLL:n vaikeusasteesta siten, että korkeimmat pitoisuudet tavattiin potilailla joilla vaikeusasteluokittelun mukaan oli lyhin ennuste. Korkea plasman IL-6 liittyi potilailla esiintyvään anemiaan, mataliin hemoglobiinitasoihin sekä korkeaan laskoon. Toisaalta neoplastisten solujen kyky tuottaa IL-6 proteiinia forboliesteristimulaation jälkeen *in vitro* oli alentunut vaikeimmassa tautimuodossa, viitaten IL-6 parakriinisen ja systeemisen IL-6 tuotannon olevan eri tavoin säädelty. IL-6:sta poiketen interleukiini-1 perheen sytokiinien IL-1β ja IL-1Ra plasmatasot olivat madaltuneet B-KLL:ssä. Korkea plasman IL-1β taso ja matala IL-1Ra/IL-1β suhde liittyivät B-KLL:ssa non-progressiiviseen tautimuotoon, joten korkealla IL-1β aktiivisuudella saattaa olla taudin etenemiseltä suojaava vaikutus. Kuten B-KLL:ssa, myös primaarissä Sjögrenin syndroomassa plasman IL-6 tasot vaihtelivat riippuen taudin eri manifestaatioista ja taudin vaikeusasteesta. Korkeat plasman IL-6 pitoisuudet korreloivat lähes lineaarisesti pienten sylkirauhasten histopatologiseen lymfosyytti-infiltraatioon, jota on pidetty taudin vaikeusasteen yhtenä tärkeimpänä mittarina. Lisäksi korkea plasman IL-6 pitoisuus liittyi pSS-potilailla erityisiin taudin manifestaatioihin, kuten pleuriittiin, perifeerisen hermoston oireisiin ja keliakiaan.

IL-1 geeniperheen ja IL-6 geenin alleelijakaumat eivätkä alleelien vaikutukset ko.sytokiinien tuotantoihin poikenneet B-CLL-potilaiden ja normaaliväestön välillä toisistaan. IL-1 geeniperheen alleelivaikutus IL-1β plasmatasoihin selittyi normaaliväestössä tämän geeniperheen eri haplotyyppien yhteisvaikutuksen kautta, ja harvinaisimman IL-1 haplotyypin omaavilla kohdehenkilöillä tavattiin ko. proteiinin korkeimmat plasmatasot. IL-6 geenin promoottorialueen polymorfia sääteli IL-6 tasoja pSS:ssä, mutta tämän polymorfian alleelijakauma ei poikennut pSS potilaiden ja normaaliväestön välillä. IL-10 geenin promoottorialueen ns. GCC- haplotyypin ja GCC/ATA genotyypin esiintymistiheys oli lisääntynyt ja ACC haplotyypin esiintymistiheys

alentunut pSS potilailla normaaliväestöön verrattuna. GCC haplotyyppi liittyi myös pSS-potilailla tavattaviin korkeisiin IL-10 plasmatasoihin. IL-6:sta poiketen IL-10 proteiinin plasmatasot eivät liittyneet taudin esiintymismuotoihin.

Yllä kuvatut ennakkotulokset viittaavat siihen, että IL-6 plasmatasojen mittausta voitaisiin käyttää apuna B-CLL:n ja pSS:n vaikeusasteen määrittämisessä ja plasman IL-1Ra/IL-1β suhteen määritystä B-CLL:n etenemisnopeutta ennustavana laboratorioparametrina. IL-1 geeniperheen ja IL-6 geenin promoottorialueen polymorfioitten normaalisuus B-CLL:ssä poissulkee mahdollisuuden, että näiden geenien alleelinen epätasapaino aiheuttaisi ko.taudissa tavattavat sytokiinituotannon häiriöt ja että tutkittuja polymorfioita voitaisiin käyttää B-CLL:n riskinarvioinnissa. Tämä negatiivinen tulos pätee myös IL-6 geenin polymorfiaan pSS:ssä. IL-10 geenin tiettyjen haplotyyppien jakautumien poikkeavuus pSS viittaa siihen, että tämän geenin tietyt haplotyypit tai näihin haplotyyppeihin kytköksissä olevat muut läheiset geenit altistavat potilaita primaarille Sjögrenin syndroomalle. IL-10 haplotyyppien ja IL-10 proteiinituotannon välisten yhteyksien vuoksi on varsin todennäköistä, että geneettinen alttiusvaikutus ainakin osin välittyy lisääntyneen IL-10 tuotannon kautta. Työssämme tehdyt havainnot vahvistavat ennakkotuloksia em. sytokiinien soveltuvuudesta pSS:n ja B-CLL:n ennusteen ja riskin arviointiin. Poikkileikkaustutkimuksen antama informaatio tässä suhteessa on kuitenkin rajallinen, ja kontrolloitujen pitkäaikaistutkimusten tarve on ilmeinen.

ACKNOWLEDGEMENTS

The present study was carried out at the Department of Microbiology and Immunology, University of Tampere Medical School and at the Institute of Medical Technology, University of Tampere.

I wish to express my deepest gratitude to my supervisor Professor Mikko Hurme, M.D., Ph.D., for his support with this study.

I wish to thank my principal co-authors Professor Juhani Vilpo, M.D., Ph.D. and Leena Vilpo, M.Sc. for their ideological and technical support during this process. I especially acknowledge Jussi's inspiring ideas in matters of life and science.

My warmest thanks to Docent Kati Hakkarainen, M.D., Ph.D., Sanna Kilpinen, M.D. and Matti Karppelin, M.D. for their encouraging and collegial support during all these years with me first as a Medical School student and subsequently, as a researcher.

My sincere thanks to Marja Pertovaara, M.D., Ph.D., Docent Jaakko Antonen, M.D., Ph.D., Professor Amos Pasternack, M.D., Ph.D., Professor Pekka Laippala, Ph.D., Nina Lahdenpohja, Ph.D., and Tomi Koski, M.D. for their kind and expertise collaboration during these, our previous and subsequent studies.

I am truly grateful to all my co-workers in our laboratory, especially warm thanks to technicians Sinikka Repo-Koskinen, Mervi Salomäki, Leena Pankko, Eija Spåre, Merja Suoranta and Eeva Tolvanen for their skilfull assistance.

I am most thankful to Virginia Mattila, M.A., for her prompt and careful revision of the language of this dissertation.

Most of all, I thank my wife Satu for sharing the life with me.

This study was supported by grants from the Medical Research Fund of Tampere University Hospital, Pirkanmaa Cancer Society, The Academy of Finland, The Finnish Society of Hematology, Finnish Society of Rheumatology, The Finnish Medical Society Duodecim Foundation, Pirkanmaa Cultural Foundation, The Science Foundation of Farmos and the Science Fund of the City of Tampere.

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