Functional analysis of the -1087 single nucleotide polymorphism in the IL-10 promoter region

Lena Larsson

Department of Periodontology
Institute of Odontology
Sahlgrenska Academy
University of Gothenburg

UNIVERSITY OF GOTHENBURG

2010

Printed at Intellecta infolog AB, Kållered, Sweden 2010.

ISBN 978-91-628-8166-5

Cover illustration: B cells expressing Sp1 (red).

To Anders, Linnea and Amanda.

Table of contents

Abstract

Functional analysis of the -1087 single nucleotide polymorphism in the IL-10 promoter region.

Lena Larsson

Department of Periodontology, Institute of Odontology, the Sahlgrenska Academy at University of Gothenburg

Interleukin (IL) 10 is recognized as a pro-inflammatory cytokine that promotes B cell proliferation. The objectives of the present series of studies were to analyze (i) differences in transcription factor binding to the -1087 IL-10 gene polymorphism in B cells, (ii) the influence of the A to G nucleotide transition on IL-10 and Sp1 gene expression in B cells, (iii) differences in Sp1 expression in periodontitis lesions from GG and AA genotype subjects and (iv) epigenetic modifications around the -1087 site and their influence on IL-10 gene expression. Using B cells from subjects with GG and AA genotypes it was demonstrated that PU.1 and Spi-B bound to both G- and A-alleles, whereas Sp1 only bound to the G-allele at -1087. LPS-stimulation resulted in a larger increase in IL-10 and Sp1 gene expression in B cells with GG than in B cells with AA genotypes (study I and II). Sp1 was present in B cells in periodontitis lesions and subjects with the GG genotype exhibited larger proportions of Sp1-positive cells and expressed larger amounts of Sp1 mRNA and protein in the lesion than AA genotype subjects (study III). Epigenetic modifications influenced IL-10 gene expression and differences in epigenetic modifications in the promoter region were found between GG and AA genotypes (study IV). The results found in the present series indicate that Sp1 is important in the regulation of IL-10.

Key words: IL-10, B cells, Sp1, transcription factors, gene expression.

ISBN 978-91-628-8166-5

Preface

The present thesis is based on the following publications, which will be referred to in the text by their Roman numerals.

I. Larsson L, Johansson P, Jansson A, Donati M, Rymo L & Berglundh T (2009). The Sp1 transcription factor binds to the G-allele of the -1087 IL-10 gene polymorphism and enhances transcriptional activation. *Genes and Immunity* 10:280-284.

II. Larsson L, Rymo L & Berglundh T (2010). Sp1 binds to the G allele of the -1087 polymorphism in the IL-10 promoter and promotes IL-10 mRNA transcription and protein production. *Genes and Immunity* 11:181-187.

III. Larsson L, Thorbert-Mros S, Rymo L & Berglundh T (2010). IL-10 genotypes of the -1087 single nucleotide polymorphism influence Sp1 expression in periodontitis lesions. Submitted.

IV. Larsson L, Thorbert-Mros S, Rymo L & Berglundh T (2010). Influence of epigenetic modifications in the IL-10 promoter on IL-10 gene expression. Submitted.

List of abbreviations

APC	antigen presenting cells
AP1	activator protein 1
5-aza	5-aza-deoxycytidine
BC	bone crest
BCR	B cell receptor
bp	base pair
CD	cluster of differentiation
cDNA	complementary DNA
C/EBP	CCAAT-Enhancer-Binding Protein
CEJ	cemento-enamel junction
ChIP	chromatin immunoprecipitation
cMaf	c-maf musculoaponeurotic fibrosarcoma oncogen homolog
CpG	cytosine and guanine separated by a phosphate
Ct	threshold cycle
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphates
ddNTP	dideoxyribonucleoside triphosphates
EBV	Epstein-Barr virus
ELISA	enzyme-linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
ERK	extracellular signal-regulated kinase
ETS	E-twenty-six specific
FACS	fluorescence activated cell sorting
HAT	histone acetyltransferase
HDAC	histone deacetylase
HDACi	HDAC inhibitor
HRM	high resolution melt curve analysis
HRP	horseradish peroxidase
HSC	hematopoietic stem cell
IFN	interferon
Ig	immunoglobulin
IHC	immunohistochemistry
IL	interleukin
JNK	c-Jun NH_2-terminal kinase
kb	kilo base pair
LBP	lipopolysaccharide-binding protein

LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MD2	myeloid differentiation-2 protein
MHC II	Major histocompatibility complex II
MMP	matrix metalloproteinase
mRNA	messenger RNA
NFκB	nuclear factor κB
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PLA	proximity ligation assay
PMN	polymorphonuclear
PU.1	Purine-rich box 1
RCA	rolling circle amplification
RCP	rolling circle product
RNA	ribonucleic acid
RNA pol II	RNA polymerase II
RNAi	RNA interference
siRNA	short interfering RNA
SLE	systemic lupus erythematosus
SL2	Schneider's *Drosophila* line 2
SNP	single nucleotide polymorphism
Sp1	SV40 promoter factor 1/ specificity protein 1
Spi-B	Spleen focus-forming virus integration site
Stat	Signal transducer and activator of transcription
TBP	TATA-box binding protein
Th	T helper cells
TLR	Toll like receptor
TNF	tumor necrosis factor
TSA	trichostatin A
VPA	valproic acid
XKLF	Krüppel-like factor

Introduction

The immune system is a complex process involving several cell types and cytokines. The host response to bacteria or viruses is regulated by different signaling pathways, which in turn is influenced by genetic and also epigenetic factors. This may explain differences in susceptibility to a disease between subjects and, hence identifying target molecules that promote susceptibility is important. Research on the immune response to periodontal pathogens has identified B cells and the cytokine Interleukin (IL) 10 as important components in the perpetuation process of the chronic inflammatory lesion. The aim of this thesis was to analyze the influence of genetic and epigenetic factors on the regulation of B cell-derived IL-10 gene expression.

Periodontitis

Periodontitis is an infectious disease caused by microorganisms colonizing the tooth surface at or below the gingival margin (Socransky & Haffajee 2003). As a result an inflammatory cell infiltrate develops in gingival tissues and in susceptible individuals this inflammatory lesion may progress and lead to loss of connective tissue attachment and alveolar bone (Page & Kornman 1997). Chronic periodontitis is a common disorder and severe forms of the disease occur in about 10% of an adult population (Hugoson *et al.* 2008). Several morphological changes occur in the tissues during the development of the periodontitis lesion. A central part is the apical migration of the junctional epithelium and the formation of the pocket epithelium (Bartold & Narayanan 2006). The periodontitis lesion contains large proportions of inflammatory cells and vascular structures.

The host response to pathogens involves reactions of the innate and the adaptive immune systems. The innate host response is the first line defense against pathogens and consists of the epithelial barriers, phagocytic cells and proteins, such as the complement system and cytokines, which regulate the functions of the cells. In contrast to the innate immune system the adaptive host response develops over time and has the capacity to recognize and react specifically to different pathogens. The cells of the adaptive response are primarily lymphocytes and antigen presenting cells (APCs) (Abbas & Lichtman 2005). In periodontitis lesions several cells serve as antigen presenting cells; langerhans cells, dendritic cells, macrophages and B cells

(Berglundh & Donati 2005). Interaction between activated T cells and B cells lead to B cell proliferation and differentiation into plasma cells or memory cells. B cells and macrophages act to maintain the inflammatory reaction (Abbas & Lichtman 2005). Activation of immune cells results in an increase in the production of pro- and anti-inflammatory cytokines. Cytokines influence the immune response either by direct action or by initiating the production of other cytokines. They also influence the signaling of proteins and transcription factors in the regulation of gene expression and cellular functions (Bartold & Narayanan 2006).

The most common cell types in periodontitis lesions are B cells and their subset plasma cells; together they represent about 68% of the cells in the lesion (Fig 1). The proportions of plasma cells and B cells in periodontitis lesions appear to correlate to the level of disease severity (Berglundh & Donati 2005).

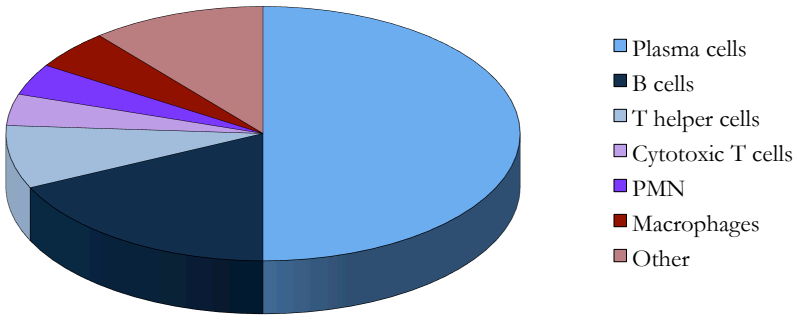

Fig 1. Distribution of cells in periodontitis lesions; plasma cells, B cells, T helper cells, Cytotoxic T cells, polymorphonuclear (PMN) cells, macrophages and other cell types (e.g. mast cells, fibroblasts). (Adapted from Berglundh & Donati 2005).

B cells

B cells serve as antigen presenting cells and express MHC class II antigen upon stimulation. They are part of antigen recognition and early response mechanisms in host defense. B cells are suggested to be the major type of APC in periodontitis lesions (Gemmell *et al.* 2003). B cells develop from pluripotent hematopoietic stem cells (HSC) into immature B cells in the bone marrow (Fig 2). Cytokine signaling and different transcription factors, including PU.1, are essential in the differentiation of HSC into B cells and in the regulation of early B cell lymphopoiesis (Welner *et al.* 2008; Busslinger 2004). The immature B cells go through a tolerance checkpoint where cells expressing autoreactive B cells receptors (BCR) will be subjected to apoptosis or gene rearrangement of the BCR in order to change specificity. Cells that have passed this checkpoint are termed mature B cells and leave the bone marrow. The B cells continue to develop in secondary lymphoid organs, in which they go through a second tolerance checkpoint (Loder *et al.* 1999; Rajewsky 1996; Wang *et al.* 2007). Surviving B cells circulate between follicles in the spleen, lymph nodes and the bone marrow. When they meet a BCR specific antigen they mature into memory B cells or plasma cells.

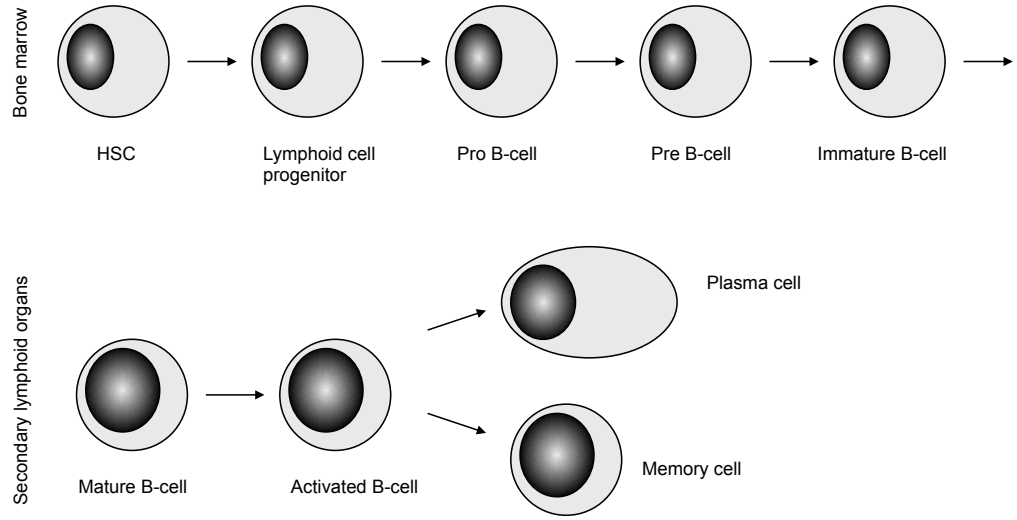

Fig 2. Maturation of B cells from hematopoietic stem cells.

Conventional B cells that mature in the bone marrow are termed B-2 cells. They are part of the adaptive immune response including the long-term T cell dependent antigen response and isotype switch and differentiate into high affinity memory B cells. Another phenotype of B cells is the B-1 cells. These cells are also part of the innate immune response and produce low affinity IgM antibodies that recognize bacterial antigen and self antigen (Porakishvili *et al.* 2001; Welner *et al.* 2008). B-1 cells develop from peritoneal precursor cells and are further divided into CD5 positive (+) B-1a cells and CD5 negative (-) B-1b cells. The CD5 molecule function is a regulator of BCR signaling by preventing incorrect activation of autoreactive B-1a cells (Bertland & Wortis 2002). Auto-antibodies produced by B-1a cells react to nuclear antigens, cell surface molecules and intracellular matrix proteins. This may, in turn, affect tolerance against self-antigens and lead to a dysfunction of the immune system (Klinman & Steinberg 1987).

Large numbers of B-1a cells are found in peripheral blood from patients with autoimmune diseases, e.g. rheumatoid arthritis and Sjögren's syndrome (Burastero *et al.* 1988; Youinou *et al.* 1988). Large amount of B-1a cells were also detected in lesions of chronic periodontitis patients. Subjects with periodontitis exhibit a larger fraction of circulating and local autoreactive B cells than periodontally healthy subjects (Berglundh *et al.* 2002; Sugawara *et al.* 1992; Aramaki *et al.* 1998). In a study by Berglundh and co-workers (1999) it was found that the amount of circulating B-1a cells did not decrease after successful non-surgical therapy in subjects with chronic periodontitis. It was suggested that the level of B-1a cells may be a marker for susceptibility rather than an indicator of the presence of disease.

IL-10

Interleukin (IL)-10 is a cytokine that is produced by many cells among which B cells and monocytes are the primary sources (Mocellin *et al.* 2004). The main cellular source of IL-10 may, however, vary in different tissues and also during acute and chronic stages of an infection (Couper *et al.* 2008). IL-10 is encoded by five exons on chromosome 1q31-32 (Eskdale *et al.* 1997; Mocellin *et al.* 2004; Mosser & Zhang 2008). It was originally described as a cytokine mainly produced by Th2 cells and the function of which was to inhibit the production of pro-inflammatory cytokines (IL-1β, IL-6, IL-8, IL-12 and TNF-α) (Borish 1998; Fiorentino *et al.* 1989). IL-10 is also recognized as a pro-inflammatory cytokine that promotes a Th2 response as well as B cell proliferation and antibody production (Moore *et al.* 2001; Rousset *et al.* 1992). The main source of B cell-derived IL-10 is autoreactive B-1 cells. Auto-antigens and/or

bacterial antigens promote expression of IL-10, which may act as an autocrine growth factor for B-1 cells (O'Garra *et al.* 1992).

While the anti-inflammatory function of IL-10 is considered important in the regulation of the inflammatory response to microbial infection (Moore *et al.* 2001), IL-10 may also contribute to the development of different diseases. High levels of IL-10 were found in patients with meningitis that had a poor or fatal outcome (Lehmann *et al.* 1995; Westendorp *et al.* 1997). Strong correlations were found between IL-10 plasma/tissue levels and chronic infectious diseases e.g. malaria, leprosy and AIDS (Mocellin *et al.* 2004). IL-10 can be either cancer-promoting due to its immunosuppressive features or cancer inhibiting through its anti-angiogenic characteristics (Howell & Rose-Zerilli 2007). B cells were found to produce high levels of IL-10 in several autoimmune diseases, e.g. Sjögren's syndrome, rheumatoid arthritis and systemic lupus erythematosus (SLE) (Llorente *et al.* 1993; Llorente *et al.* 1994; Mongan *et al.* 1997). SLE is characterized by polyclonal B cell activation and high levels of serum auto-antibodies. Treatment with anti-IL-10 monoclonal antibodies resulted in a reduction of pathological conditions of the disease (Mocellin *et al.* 2004).

The presence of IL-10 in periodontitis lesions was reported in several studies. Aramaki and co-workers (1998) found a larger amount of IL-10 in inflamed gingival tissue than in peripheral blood. Higher levels of IL-10 in periodontitis lesions than in gingivitis lesions were also demonstrated (Nakajima *et al.* 2005). In a study by Yamazaki and co-workers (1997) the authors reported that the amount of IL-10 mRNA was higher in gingival tissues than in peripheral blood mononuclear cells in periodontitis patients. Stimulation of peripheral blood mononuclear cells with *Porphyromonas gingivalis* lipopolysaccharide (LPS) showed an association between high percentage of B-1a cells and high IL-10 production (Stein *et al.* 1997). These findings indicate that periodontal pathogens may induce an IL-10 response resulting in a proliferation of B-1a cells and a production of auto-antibodies.

Genetic components

Subjects with periodontitis exhibit a similar clinical phenotype but the biological mechanisms and factors resulting in this phenotype may differ between subjects. Periodontitis is a complex disease associated with variations in multiple genes. It has been estimated that 10 to 20 genes may contribute to the disease. The immune response to a pathogen is influenced by different factors, genetic as well as epigenetic

modifications were proposed to be key components in the expression of periodontitis (Loos *et al.* 2008; Offenbacher *et al.* 2008).

Genetic modifications can occur in the DNA resulting in variations in the nucleotide sequence. The most common change in the genome is a single nucleotide base pair substitution called a single nucleotide polymorphism (SNP). This results in a shift in base pair from a G-C (guanine-cytosine) to an A-T (adenine-thymine) base pair. A part of a chromosome or several SNPs on a segment of a chromosome that are inherited together is called a haplotype (Snustad & Simmons 2010). SNPs are mostly located in coding regions of the gene resulting in a change in the amino acid sequence of the protein and, hence the function of the protein. If a SNP occurs in the promoter of a gene this may alter the binding of regulatory proteins to the DNA and thereby influencing gene transcription. Several gene polymorphisms in various genes have been studied to investigate their role in periodontitis, e.g. CD14, IL-1α, IL-1β, IL-4, IL-6, MMP 1,3 and 9, TLR2, TLR4 and also IL-10 (Loos *et al.* 2008). The association of the –1087 IL-10 single nucleotide polymorphism and periodontitis was studied in different populations (Berglundh *et al.* 2003; Scarel-Carminaga *et al.* 2004; Yamazaki *et al.* 2001). It was suggested that the -1087 IL-10 polymorphism in the IL-10 promoter was associated with severe chronic periodontitis in Caucasian subjects (Berglundh *et al.* 2003).

In summary, the expression of a gene is either regulated at the transcriptional level through influences by SNPs and haplotypes, or post-transcriptionally during the translation process of converting mRNA into protein. Furthermore, a second level of regulation is the chromatin status of the DNA. This process is termed epigenetic and is not encoded in the DNA. In the present series the focus was on the transcriptional regulation of B cell-derived IL-10 and the influence of epigenetic modifications of the chromatin in the IL-10 promoter region. The principles of these two mechanisms are described below.

Transcriptional regulation

Transcription is the process in which genetic information in the DNA is transferred to RNA (Snustad & Simmons 2010). Most of the regulation of genes occurs at the initiation of the transcription process. In protein coding genes the transcription is performed by RNA polymerase II (RNA pol II). There are two regulatory DNA elements: the promoter and distal regulatory elements, which can be either enhancers

or silencers. The core promoter is located upstream from the transcription start point and serves as the point of assembly for the basal transcriptional machinery and initiation of transcription (Maston *et al.* 2006; Snustad & Simmons 2010).

Transcription factors are cellular components that bind to the DNA and regulate gene expression. Such factors can be expressed in either most tissues having a broad function or in one or two tissues indicating a specific function. Transcription factors influence the promoter in different ways depending on the stimuli or tissue type, and will induce a higher transcription activity compared to the basal transcription (Farnham 2009; Maston *et al.* 2006) (Fig 3).

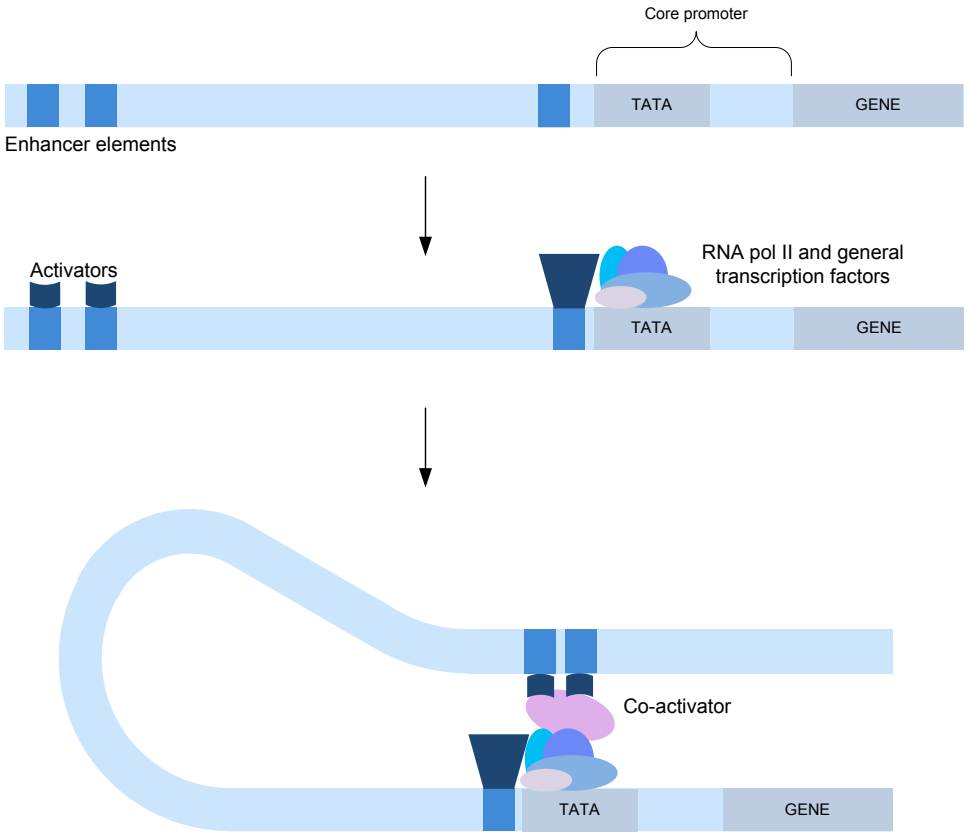

Fig 3. A model for transcriptional regulation. The RNA pol II and general transcription factors bind to the proximal promoter. Activators bind to distal enhancer elements and by protein-protein interactions with co-activators a bending of DNA (DNA looping) occurs (Vilar & Saiz 2005). Transcription factors binding to distal promoter regions may thereby influence the transcription machinery and regulate transcription. (Modified from Farnham 2009).

In the present thesis the transcription factors PU.1 (Spi-1), Spi-B and Sp1, and their roles in the regulation of IL-10 were investigated. PU.1 and Spi-B are members of the ETS transcription factor family. They share a 43% overall amino acid sequence identity and a 67% identity in the DNA binding domain (Ray *et al.* 1992). The ETS transcription factors recognize and bind to DNA sequences containing the core motif 5'-GGAA/T-3'. The sequence of nucleotides flanking this core motif is specific for the different members of the ETS family (Gallant & Gilkeson 2006). PU.1 is expressed in B cells, mast cells, megakaryocytes, macrophages and neutrophils, while Spi-B is only present in the lymphoid lineage (Su *et al.* 1996). These transcription factors are essential in the regulation of the immune response and the lymphocytic development (Gallant & Gilkeson 2006; Su *et al.* 1996). PU.1 and Spi-B can bind to identical DNA-binding sequences and activate the same target genes *in vitro* (Ray *et al.* 1992; Su *et al.* 1996). Sp1 is a member of the Sp/XKLF (specificity protein /Krüppel-like factor) family of transcription factors (Fig 4). These transcription factors recognize and bind to GC-(GGGGCGGGG) boxes. Sp1 regulates transcription through interactions with the basal transcriptional machinery by binding to the TATA-box binding protein (TBP) or by binding to distal enhancer regions (Bouwman & Philipsen 2002; Courey & Tjian 1988; Emili *et al.* 1994). Sp1 is involved in cell cycle regulation, chromatin remodeling and maintenance of methylation free islands (Brandeis *et al.* 1994; Li *et al.* 2004; Macleod *et al.* 1994).

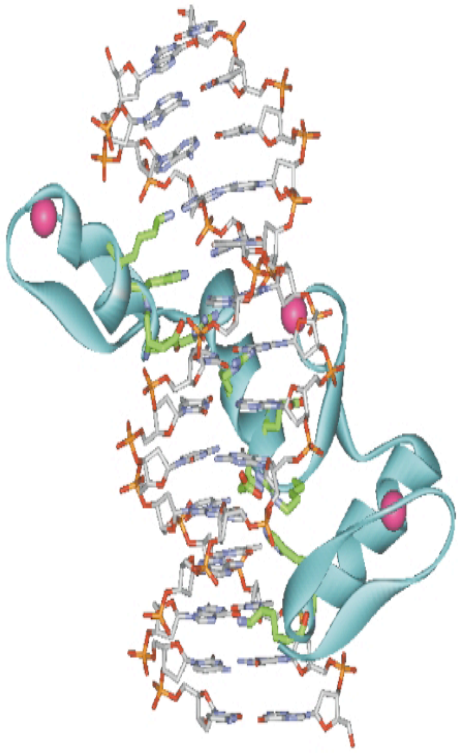

Fig 4. A 3D model of the DNA binding domain of Sp1 binding to DNA published by Marco et al (2003). Reprinted with the perimission from Elsevier Science Ltd, © 2003.

Sp1 binding to several sites in a promoter is characterized by multiple Sp1 complexes through protein-protein interactions forming a loop between distally and proximally bound Sp1 molecules. This multimer complex interacts with other DNA binding proteins, transcriptional regulators and chromatin remodeling factors (Li *et al.* 2004; Su

et al. 1991). Sp1 has also been shown to influence the gene expression through binding to ETS transcription factors (Dittmer *et al.* 1997; Lu *et al.* 2006) and by interaction with the p65 subunit of NFκB. The p65-Sp1 complex recruits TBP associated factors and polymerase II that results in an increase in gene expression (Perkins *et al.* 1994).

Epigenetic modifications

The term epigenetics relates to mitotic and/or meiotic inherited changes in gene expression that are not encoded in the DNA sequence itself (Bird 2002). Epigenetic modifications include chemical alterations of the DNA and its associated proteins. Such alterations lead to remodeling of the chromatin and activation or inactivation of a gene. DNA methylation and histone acetylation and methylation are the major epigenetic modifications. They occur and contribute to the development of cancer, autoimmune and inflammatory diseases. Epigenetic alterations of a gene can be induced by environmental factors (Gomez *et al.* 2009; Wilson 2008).

DNA is stored in the nucleus as a chromatin complex (Fig 5). Nucleosomes, which are the basal structure components of chromatin, consist of 146 bp DNA and a core histone complex including two copies each of histone H2A, H2B, H3 and H4 and the linker histone H1 that connects the nucleosomes (Fitzpatrick & Wilson 2003; Workman & Kingston 1998).

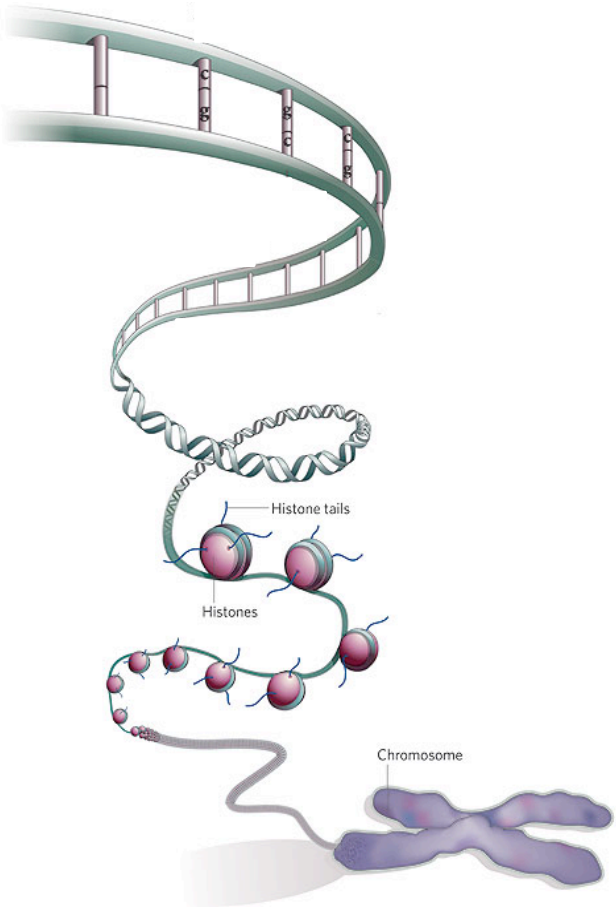

Fig 5. The organisation of DNA within the chromatin structure. Adapted from Qiu 2006. Reprinted with permisson from Macmillan Publishers Ltd: Nature, © 2006.

Histone acetylation is regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs). Histone deacetylases remove acetyl groups, which lead to alterations in the packing of DNA around histones (Bäckdahl *et al.* 2009). Hyperacetylation is associated with

transcriptional active chromatin, while hypoacetylation is associated with inactivated regions of the chromatin (Jenuwein & Allis 2001; Turner 2000).

Methylation occurs at different lysine molecules on histones H3 and H4, and is important in the formation of the chromatin. The complex mechanism of this process is currently a main research area with focus on evaluating the influence of the different possible lysine methylation patterns. Histone lysine methylation can result in both active and inactive chromatin depending on the type of lysine molecule (Martin & Zhang 2005). Furthermore, certain lysine molecules can be mono-, di- or trimethylated (Farnham 2009; Green *et al.* 2006; Pinskaya & Morillon 2009). Recent studies indicate that certain transcription factors have affinity for specific histone modifications and that the combination of transcription factors and co-activators are responsible for the specific histone code that is associated with certain genes. The resulting combinations of histone modifications may promote unique cellular responses (Jenuwein & Allis 2001; Turner 2000).

The DNA itself can be modified by the addition of methyl groups to a cytosine situated next to a guanine, so called CpG islands or CpG sites. Such sequences are associated with promoter regions but also 5' ends of many genes (Bird 2002; Robertson & Wolffe 2000). Methylation of the DNA results in silencing or down-regulation of a gene, whereas transcriptionally active genes are associated with low levels of DNA methylation. DNA methylation inhibits gene expression either by inhibiting the binding of transcription factors, or by recruitment and binding of regulatory proteins that contains a methyl-CpG binding domain to the DNA. These proteins assemble a complex containing co-repressors and histone deacetylases, which make the chromatin inaccessible for transcription factor binding (Fitzpatric & Wilson 2003). While acetylation of histones is a transient mechanism, methylation of the DNA is stable and can sustain through cell division (Bäckdahl *et al.* 2009). It has been suggested that DNA methylation and histone modifications are linked (Robertson & Wolffe 2000) (Fig 6).

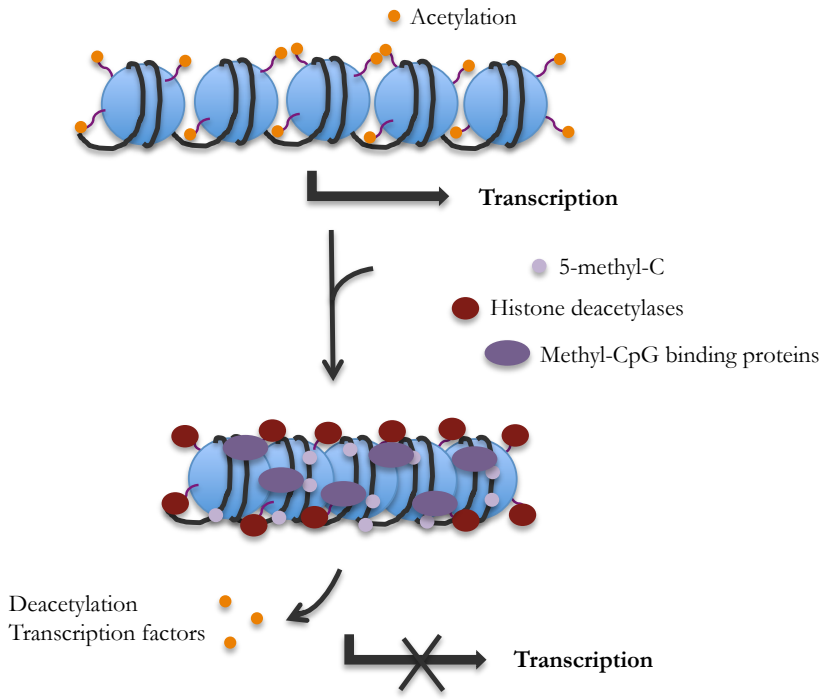

Fig 6. The mechanism for gene silencing by DNA methylation and histone deacetylation. A transcriptional active region is characterized by histone acetylation and an open chromatin structure. Methylation of the cytosine molecules in the DNA results in the recruitment of methyl-CpG-binding proteins and histone deacetylases. The deacetylated chromatin forms a tightly packed structure that inhibits the binding of transcription factors. (Adapted from Robertson & Wolffe 2000).

In a recent review by Bäckdahl et al. (2009) the authors suggested that hypo-acetylated histones and hyper-methylated CpGs in chronic inflammatory diseases may result in persistent inflammation. DNA methylation may also be promoted by chronic inflammation and bacterial infection (Bobetsi *et al.* 2007). Epigenetic modifications of a certain part of a gene can differ between cell types and may result in a different local and systemic expression of a gene. Research on epigenetic modifications in combination with genetic analysis may provide further evidence to inter-individual differences in local expression of genes associated with inflammation.

Transcriptional and epigenetic regulation of IL-10 gene expression

About 75% of the IL-10 production in blood cells following LPS-stimulation is determined by genetic factors (Westendorp *et al.* 1997). The inter-individual genetic variability in IL-10 production was suggested to be a result of differences in the transcriptional regulation and the activity of the promoter for IL-10 (Rees *et al.* 2002). The IL-10 gene promoter was sequenced in 1995 by Kube and co-workers in EBV-positive Burkitt's lymphoma cells (Kube *et al.* 1995). Several regulatory regions has then been identified in the 5' flanking region, including two dinucleotide repeats (microsatellites) located 1.2 kb (IL10.G) and 4 kb (IL10.R) upstream of the transcription start site. A positive regulatory sequence was identified in the promoter region between –1100/-800 (Eskdale *et al.* 1995,1996,1997; Kube *et al.* 1995). Three linked single nucleotide polymorphism (SNP) are present in the IL-10 promoter; -1087 (G/A), -824 (C/T) and -597 (C/A) which together form the haplotypes ATA, ACC and GCC associated with low, medium and high levels of IL-10 production (Hoffmann *et al.* 2001). Eskdale et al. (1999) combined the microsatellites and the haplotypes for the three SNPs and investigated the distribution of the alleles. Four core haplotype families were constructed:

IL10.01: R3-(IL10.G).G-C-C

IL10.02: R2-(IL10.G)-A-C-C

IL10.03: R2-(IL10.G)-G-C-C

IL10.04: R2-(IL10.G)-A-T-A

The results showed a higher IL-10 protein expression for the IL10.02 haplotype than the IL10.03 haplotype when the IL10.G allele 13 was present. Haplotypes containing the IL10.R3 allele were found to produce lower amounts of IL-10 than IL10.R3 negative alleles (Eskdale *et al.* 1998).

The –1087 G/A SNP (sometimes referred to as -1082) was associated with differences in IL-10 promoter activity and IL-10 production. The results on the influence of the –1087 SNP on the IL-10 production in different cell types are, however, conflicting. Thus, the presence of an A was shown to result in an increase in the IL-10 promoter activity (Rees *et al.* 2002). Conversely, other reports have suggested that the G-allele of –1087 was associated with an increase in the IL-10 promoter activity (Crawley *et al.* 1999; Kremer *et al.* 2007; Reuss *et al.* 2002, Turner *et al.* 1997). The -1087 SNP was also associated with different diseases, e.g. joint destruction (Huizinga *et al.* 2000), susceptibility to EBV infection (Helminen *et al.* 1999), inflammatory bowl diseases and

Ulcerative colitis (Tagore *et al.* 1999). A correlation between IL-10 haplotypes and progression of periodontitis was reported. Haplotypes negative for the -1087 G-allele experienced 20% fewer sites with probing depth of ≥4 mm during a 5-year period (Cullinan *et al.* 2008).

The –1087 SNP lies in a GGAA/G motif and the transcription factor PU.1 was found to bind to the –1087 site in a human monocytic cell line (Reuss *et al.* 2002). Recognition sequences for a number of other transcription factors have also been identified in the IL-10 promoter, including AP1, Sp1 (Kube *et al.* 1995), cMaf (Cao *et al.* 2005), C/EBP (Liu *et al.* 2003) and Stat (Staples *et al.* 2007). Although all these transcription factors influence IL-10 gene expression, Sp1 in particular was found to be an important factor in IL-10 gene expression. The Sp1 binding site at -89 to -77 bp is essential for IL-10 promoter activity in T cells and macrophages in both humans and mice (Brightbill *et al.* 2000; Tone *et al.* 2000). An additional Sp1 binding site has been located upstream of the -571 site (Steinke *et al.* 2004).

The transcriptional regulation of IL-10 is complex and the chromatin may be differently modified in different cell types. Several studies on the regulation on IL-10 gene expression emphasize the important role of Sp1 in the transcription of IL-10, and different mechanisms for this activation have been proposed. Sp1 induced IL-10 transcription is activated by LPS binding to the LPS binding protein (LBP) and this complex interacts with the CD14 receptor on the cell surface. CD14 then transfers LPS to the transmembrane signaling receptor Toll-like receptor 4 (TLR4) and its associated receptor myeloid differentiation-2 protein (MD2) (Miyake 2004). This process activates signaling through three MAPK pathways (ERK, JNK and p38), which in turn activates and up-regulate nuclear translocation of Sp1 (Chanteux *et al.* 2007). Inhibition of ERK activation prevents histone modifications and decreases Sp1 binding to the IL-10 promoter. Activation of ERK results in transient modifications in the chromatin at the IL-10 locus, which make the IL-10 promoter accessible to transcription factors leading to an increase in IL-10 expression (Zhang *et al.* 2006).

Additional mechanisms for Sp1 induced IL-10 production have been reported. The transcription factor NFκB regulates the expression of several genes involved in inflammatory processes. An interaction between NFκB and Sp1 has been shown to result in an increase in promoter activity. However, a report on NFκB and IL-10 transcription suggested that Sp1 may be the key regulator of IL-10 expression and that

NFκB is not necessary for the IL-10 expression (Brightbill *et al.* 2000). IL-10 transcription can also be induced by the human immunodeficiency virus (HIV) trans-activator (Tat) protein. The IL-10 promoter contains a Tat-responsive sequence located at -625 to -610. Ets1 and Sp1 have been identified to bind to this sequence and to be activated by p38 (Li & Lau 2007). Chiang and co-workers (2006) used mouse macrophage cells and showed that Sp1 interacts with C/EBPδ after LPS-stimulation and thereby co-activates IL-10 gene expression. Two models for the complex formation and gene activation were presented. The first model suggested that the C/EBPδ-Sp1 complex is formed in the nucleus and binds to the IL-10 promoter. An alternative model was that Sp1 binds to the recognition site in the promoter and then C/EBPδ binds to the Sp1.

In addition to different transcription factors participating in and influencing the transcriptional regulation of IL-10 described above, DNA methylation of CpG sites in the IL-10 promoter may also regulate gene transcription. However, tissue specific genes such as IL-10 have low numbers of CpG sites. In the IL-10 promoter there are 8 CpG sites in the proximal 700 bp promoter region (Szalmás *et al.* 2008; Tsuji-Takayama *et al.* 2008). Research on the methylation pattern showed differences between IL-10 producing cells and IL-10 non-producing cells in methylation of the two proximal CpG sites at positions -185 and -110 in the promoter (Szalmás *et al.* 2008). Treatment with 5-aza, an inhibitor of DNA methyltransferases, resulted in an increase in IL-10 production. This indicates that the IL-10 gene expression can be regulated by DNA methylation (Dong *et al.* 2007).

Histone modifications near the IL-10 promoter region are associated with IL-10 gene silencing. The transcriptional active promoter in an IL-10 producing cell exhibits an open chromatin configuration and a high degree of acetylated histone H3 and histone H4 in the proximal part of the promoter. IL-10 producing Th cells were found to be hyperacetylated on histone H4 even prior to stimulation. Such cells showed a basally low expression of IL-10 and a rapid induce of IL-10 mRNA after stimulation (Im *et al.* 2004).

Even though several studies on epigenetic modifications of IL-10 promoter indicate the importance of the epigenetic status of the proximal part, there are reports suggesting that HDAC proteins in the distal part may induce changes in the three dimensional chromatin structure resulting in a bending of the distal region towards the

proximal region (Villagra *et al.* 2009). Regulatory factors bound to the distal part of the promoter could thereby regulate the proximal region, through changes in histone acetylation and transcription factor binding needed for the activation of IL-10.

Sp1 is known to affect DNA methylation but data on the influence of methylation on Sp1 binding are conflicting. DNA methylation of a CpG site in an Sp1 recognition sequence does not interfere with binding of Sp1. It was suggested that Sp1 prevents methylation and is important in keeping CpG sites unmethylated. However, methylation of the cytosine outside or nearby CpG sites inhibits Sp1 binding (Clark *et al.* 1997; Zhu *et al.* 2003). The absence of methylation at methylation-free islands in housekeeping genes was suggested to be a consequence of the constitutive binding of Sp1 at these sites (Höller *et al.* 1988). In the study referred to, the results showed that Sp1 can bind to both methylated and unmethylated sites, and that CpG methylation at these sites does not inhibit transcription.

The regulation of IL-10 is a key mechanism in keeping the immune balance in vivo. Even though a lot of research has been performed on the different regulatory mechanisms of IL-10 gene expression, the regulation of IL-10 is still poorly understood. The -1087 SNP is associated with high IL-10 production and has also been associated with disease. However, limited information on the function of this SNP in the regulation of IL-10, and in particular in B cells, is available. We thus, analyzed the binding pattern of transcription factors to this site in the IL-10 promoter (study I and II). We also investigated the result of an alteration in binding pattern to this site on the promoter activity and gene expression (study I and II). The identification of transcription factor Sp1 binding to -1087 and the correlation between IL-10 and Sp1 mRNA transcription prompted the analysis of Sp1 in periodontitis lesions (study III). The presence of transcription factors in most cell types and the tissue specific expression of the IL-10 gene suggested a second level of regulation, i.e. epigenetic modifications of the DNA and histones (study IV).

In conclusion, the transcriptional regulation of IL-10 is complex and may be influenced by both genetic and epigenetic factors. Research on the regulation of IL-10 suggests specific mechanisms related to cell type and stimuli. Different transcription factors and signaling pathways are most probably involved in the IL-10 gene expression with Sp1 as a key factor (Fig 7). The association of the high IL-10 producing -1087 GG genotype and periodontitis indicates a possible difference in the

IL-10 promoter regulation between subjects. These differences may be genetic and/or epigenetic. Therefore, research on genetic and epigenetic modifications may provide new information on pathological processes and suggest new treatment models, not only for periodontitis but also for other diseases characterized by a chronic inflammation.

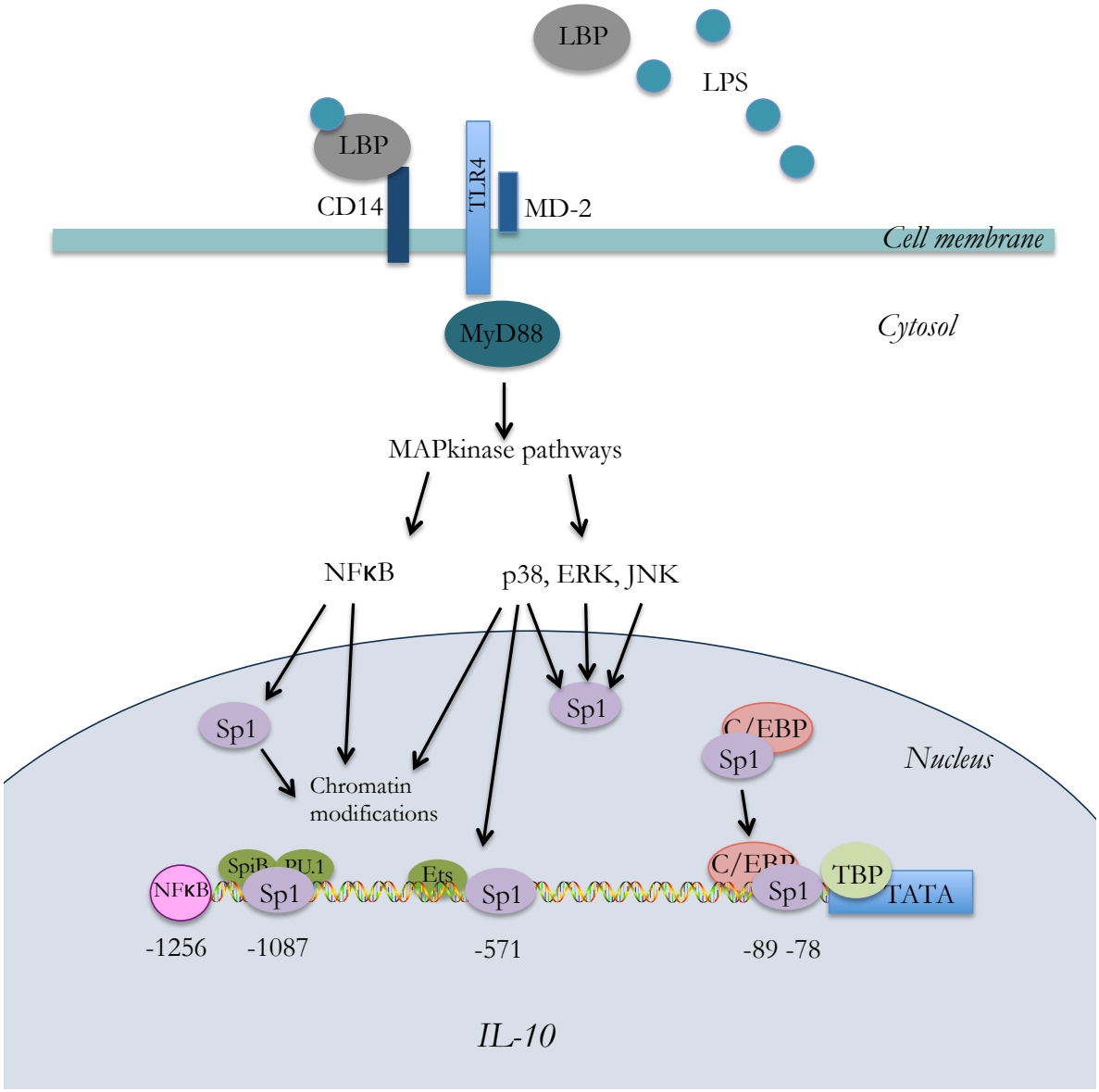

Fig 7. A model of the different Sp1 signaling pathways involved in the regulation of IL-10 gene expression.

Aims

Overall aim

IL-10 is an important cytokine in the regulation of the inflammatory response in a number of diseases. The variation in IL-10 production between subjects is genetically determined and the regulation of IL-10 is predominantly on the transcriptional level. The IL-10 promoter contains a number of regulatory elements and the -1087 single nucleotide polymorphism is found to be important for IL-10 gene expression. However, there is limited knowledge on the function of this SNP. The overall aim of the present series of studies was to examine functional aspects of the -1087 IL-10 gene polymorphism.

Specific aims:

- To analyze differences in transcription factor binding to the -1087 position in the IL-10 promoter region in B cells.

- To study the influence of the -1087 A/G nucleotide transition on IL-10 and Sp1 gene expression.

- To analyze differences in Sp1 expression in periodontitis lesions in GG and AA genotype subjects.

- To study epigenetic modifications around the -1087 site and their influence on IL-10 gene expression

Material and methods

Study population (study II, III and IV)

The study protocols were approved by the local human review board and prior to enrollment all subjects received information about the study and signed an informed consent. In study II periodontally healthy subjects were recruited. The subjects demonstrated normal radiographic bone levels, i.e. a distance of <3 mm between the cemento-enamel junction (CEJ) and the most coronal level of bone crest (BC) at >95% of the proximal tooth sites. In study III and IV subjects with severe, generalized chronic periodontitis were recruited at the Clinic of periodontics, Gothenburg.

Genotyping (study II, III and IV)

In study II, III and IV blood samples were collected from subjects with severe, generalized chronic periodontitis and from periodontally healthy subjects. Genotyping was performed to identify subjects with either GG or AA genotypes for the -1087 IL-10 gene polymorphism. DNA was extracted and genotyped for the -1087 IL-10 gene polymorphism rs1800896 (Berglundh *et al.* 2003; Padyukov *et al.* 2001).

Cell lines (study I, II and IV)

The cell lines used in these studies were the Schneider's *Drosophila* line 2 (SL2) (study I), the DG75 B cell line (study I) and B cell lines from subjects with either GG or AA genotypes (study II and IV). SL2 is a *Drosophila* cell line that does not express the Sp1 transcription factor endogenously (Courey & Tjian 1988). The SL2 cells were grown at 28°C in Schneider's *Drosophila* medium supplemented with 10% fetal bovine serum, 100 U penicillin ml^{-1} and 100 μg streptomycin ml^{-1}. DG75 is an Epstein Barr (EBV)-negative Burkitt's lymphoma B cell line (Ben-Bassat *et al.* 1977). In order to study the influence of the -1087 gene polymorphism on IL-10 gene expression B cell lines were established from subjects with GG and AA genotypes for the -1087 IL-10 gene polymorphism. Blood samples were collected and leukocytes were separated using density gradient centrifugation. Immortalization of the cells was done by infecting the B cells with the B95-8 strain of the EBV. The B cell lines were maintained as suspension cultures in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U penicillin ml^{-1}, and 100 μg streptomycin ml^{-1}. The B cell lines were grown at 37°C in a humidified atmosphere containing 5% CO_2.

Isolation of B cells (study II and IV)

B cells were purified from peripheral blood samples collected from subjects with GG and AA genotypes for the -1087 IL-10 gene polymorphism using the MACS magnetic cell sorting technique and anti-CD19 microbeads (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany) (study II and IV). The purity of the B cells was about 96% as confirmed by FACS. The B cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U penicillin ml^{-1} and 100 μg streptomycin ml^{-1} at 37°C in a humidified atmosphere containing 5% CO_2.

Tissue samples (study III and IV)

In study III and IV gingival biopsies were obtained from subjects with severe, generalized chronic periodontitis and with either the GG or the AA genotype. The gingival biopsies were collected from diseased sites demonstrating bone loss ≥50%, probing pocket depth ≥6 mm and bleeding on probing. The biopsies were fixed in 4% formalin for minimum 24h, dehydrated and embedded in paraffin. In study III five-micrometer sections were cut at a microtome, dried at 37°C for 24 h and stored at -70°C. In study III additional sections were obtained for RNA extraction, while in study IV sections were obtained for DNA extraction.

Electrophoretic Mobility Shift Assay (study I)

The Electrophoretic Mobility Shift Assay (EMSA) is a sensitive method for studying interactions between proteins and DNA (Garner & Revzin 1981). The protein-DNA binding reaction was performed *in vitro* by mixing nuclear extract with a labeled double stranded DNA probe. In study I double stranded synthetic ^{32}P-labeled oligonucleotides corresponding to the sequence from -1099 to -1074 of the IL-10 promoter, with either G or A at the -1087 position and nuclear extracts from DG75 cells were used. The protein-DNA complexes were separated from free DNA by non-denaturing polyacrylamide gel electrophoresis (PAGE), based on differences in charge, conformation and size. When a protein bound to the DNA it caused the complex to move slower and this resulted in a shifted band on the electrophoresis gel. To identify the proteins binding to the DNA, antibodies recognizing the target proteins were added to the reaction. When the antibody bound to the protein the size of the complex changed and resulted in a so-called supershift. To further verify the specificity of a band competition experiments were performed. In these experiments an excess of unlabelled probe was added to the reaction, band that disappeared were considered specific complexes shifted by target proteins.

The antibodies for the supershift experiments were Sp1 (sc-59 X), PU.1 (sc-352 X) and Spi-B (sc-5944 X) (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA). In addition to unlabeled oligonucleotides corresponding to the sequence in the IL-10 promoter consensus oligonucleotides for Sp1, ETS and C/EBP were also used.

Transfection assays (study I)

Two different methods for transient transfections were used in study I. Transient transfections of DG75 cells were performed using electroporation, while the SL2 cells were transfected using the Lipofectamine ™ LTX kit (Invitrogen Corp, Carlsbad, CA, USA). The –1111 bp to +1 bp sequence of the human IL-10 promoter was fused into a plasmid in front of a luciferase reporter gene (Kremer *et al.* 2007). The two reporter plasmids were referred to as ACC and GCC as defined by the G or A nucleotide at –1082, C at –819 and C at the –592 position. The cells were transfected with the reporter plasmids and assayed for luciferase activity using the Luciferase Assay System. In addition, the SL2 cells were co-transfected with the reporter plasmids together with an Sp1 expression vector. The result of the assay was an indirect measurement of the promoter activity.

Chromatin Immunoprecipitation Assay (study II and IV)

In study II the Fast chromatin immunoprecipitation (ChIP) assay was done according to methods modified from those described by Nelson et al. (2006). EBV-immortalized B cells were incubated for 24h with or without LPS. The cells were crosslinked by adding 37% formaldehyde and incubated for 7 minutes at room temperature, followed by the addition of glycine to stop the reaction. The cells were lysed in 100 µl/10^6 cells buffer A on ice (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, supplemented with proteinas inhibitors). After 10 minutes incubation, the nuclei were recovered by centrifugation. Chromatin was extracted in 100 µl SDS lysis buffer per 2 x 10^6 cells for 10 minutes on ice (1% Triton X-100, 0.1% SDS, 10 mM EDTA, 50 mM Tris pH 8.0, supplemented with protease inhibitors). The DNA was sonicated on ice into fragments of 500 to 1000 bp in size. The sheared chromatin was incubated with antibodies over night at 4°C. The antibodies used for ChIP were Sp1 (sc-14047 X), PU.1 (sc-352) and Spi-B (sc-5944) (Santa Cruz Biotechnology Inc.). A rabbit IgG antibody was used as a negative control and an anti-acetylated histone H3 antibody was used as a positive control (Millipore SAS, Molsheim, France). The immunocomplexes were captured using Protein A sepharose 4 fast flow, washed and precipitated. DNA fragments were isolated using 10% Chelex-100.

In study IV we used the ChIP-IT express enzymatic shearing kit and protein G-coated magnetic beads (Active Motif, Carlsbad, CA, USA) to examine histone modifications of the IL-10 promoter region around the -1087 site. The antibodies used in this study were anti-acetyl-histone H3 (06-599), anti-acetyl-histone H4 (06-866) and anti-mono/di/trimethyl-histone H3 lys4 (04-791) antibodies (Millipore S.A.S, Molsheim, F).

RNA analysis and real-time PCR (study II, III and IV)

To study the IL-10 and Sp1 gene expression, RNA was extracted from cells (study II and IV) or gingival tissues (study III), converted into cDNA and analyzed using real-time PCR. The relative gene expression of IL-10 and Sp1 were calculated by normalizing the Ct values of the target genes to the Ct values of two or three reference genes according to the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001; Pfaffl 2001). For stability comparisons of suitable reference genes the software geNorm (Vandesompele *et al.* 2002) was used. In addition, the software NormFinder (Andersen *et al.* 2004) was used in study II.

ELISA (study II)

Enzyme-linked immunosorbent assay (ELISA) is a method for detection and quantification of antibodies or antigens. In study II the concentration of IL-10 protein were assayed using the IL-10 human Easy ELISA kit (GE Healthcare Bio-sciences AB, Uppsala, Sweden). The supernatant from the cell cultures was added to a 96-well plate coated with anti-IL-10 antibodies. Following incubation with a detection antibody and a subsequent addition of a streptavidin-HRP complex, a substrate reactive with HRP was added to the reaction. This resulted in a colored product, which was formed in proportion to the amount of IL-10 present in the sample. The absorbance was measured in a spectrophotometer at 450 nm.

RNA interference (study II)

RNA interference (RNAi) is a process where single-stranded RNA molecules bind to mRNA in a sequence–specific manner, resulting in RNA degradation and gene silencing. A double-stranded RNA called a short interfering RNA (siRNA) is introduced into the cell and enters the RNAi pathway of the cell. In study II the influence of Sp1 knockdown on IL-10 gene expression was analyzed by transfecting Sp1 siRNA into B cell lines from subjects with either GG or AA genotypes. The Sp1 siRNA and the pEGFP-C3 expression vector were transfected into the cells using electroporation. In some experiments, LPS was added to the cells 48h after the

electroporation. After 72h the cells were harvested and RNA was extracted. The decrease in IL-10 mRNA was measured and compared to the IL-10 mRNA transcription in cells transfected with a negative control siRNA.

in situ Proximity Ligation Assay (study III)

in situ Proximity Ligation Assay (*in situ* PLA) was used in study III for detection and quantification of Sp1 positive cells in periodontitis lesions. The *in situ* PLA was done using an Sp1 primary antibody (sc-420 Santa Cruz Biotechnology Inc.), the Duolink single recognition system and the Nova Red detection system (OlinkBioscience, Uppsala, Sweden). Briefly, after incubation with the primary antibody, the two secondary PLA probes conjugated to antibodies were added and the oligos hybridized and ligated to form circular DNA strands. Signal amplification was done by replicating the DNA circles via rolling circle amplification (RCA) using DNA polymerase (Jarvius *et al.* 2007). After amplification detection was performed using a complementary HRP-labeled probe. Quantification of the Sp1 positive cells and Sp1 protein molecules was made using the BlobFinder software (Allalou & Wählby 2009).

Immunohistochemistry (study III)

A double immunohistochemistry (IHC) staining protocol for detection of Sp1 positive B cells were used in study III. CD20-positive B cells were detected using the MACH2 alkaline phosphatase system and the Vulcan Fast Red substrate (Biocare Medical, Concord, CA, USA). Sp1 positive cells were visualized by a second incubation with the MACH2 system and the Ferengi Blue substrate (Biocare Medical). The antibodies used were CD20 (M0755 DakoCytomation A/S, Glostrup, DK) and Sp1 (sc-420 Santa Cruz Biotechnology Inc.). The density of labeled cells was assessed using a point counting procedure. A lattice comprising 400 points was superimposed over the tissue area. Cross points that indicated the positive cell markers in the compartment to be examined were counted and related to the total counts for the entire inflammatory cell infiltrate.

Analysis of epigenetic modifications (study IV)

To analyze the influence of DNA methylation on the IL-10 promoter B cells were treated with 5-aza. This drug induces DNA de-methylation, which may reactivate silenced genes and increase gene transcription. The cells were incubated with 5-aza for 72h. Histones in transcriptionally silent regions are usually deacetylated. Treatment with Histone deacetylase inhibitors (HDACi) changes the conformation of the nucleosome and thereby activates the gene transcription. In order to study the

influence of histone modifications on the IL-10 gene transcription, B cells were treated with the HDAC inhibitors TSA, VPA or Butyrate for 48h. The IL-10 mRNA expression after treatment with these substances were measured and compared to un-treated cells.

Bisulfite modification analysis (study IV)

Methylated cytosines cannot be detected using conventional sequencing. Thus, in study IV the sodium-bisulfite DNA modification technique (Herman *et al.* 1996) was used. DNA from peripheral blood was treated with sodium bisulfite, which converted all unmethylated cytosines into uracils. This modified DNA was then amplified using PCR and the methylation pattern of the three CpGs distal of the -1087 SNP was analyzed using DNA sequencing modified from those described by Sanger (Sanger 1977). The components were DNA, heat resistant DNA polymerase, 4 dNTPs, 4 ddNTP's (dideoxy terminator nucleotides) fluorescently labeled with four different dyes and buffer containing MG^{2+} and K^{+}. The primer bound to the complementary DNA strand and was extended in a linear mode until a ddNTP was incorporated. The dideoxy-configuration prevented the polymerase from adding any other base to the fragment and the extension was terminated. Thus, at the end of the cycles numerous fragments with different lengths and one labeled nucleotide were generated. After post sequencing reaction cleanup, the samples were sequenced and the fluorescent signal translated into a nucleotide sequence. The sequencing analysis was performed at Eurofins MWG Operon (Ebersberg, Germany)

In addition, DNA from blood and tissue samples was analyzed using High resolution melt curve analysis (HRM) and acrylamide electrophoresis. In the HRM analysis a HRM melting curve was performed after the PCR amplification. The unmethylated and methylated controls have different contents of methylcytosine, which resulted in different melting temperatures and thereby different HRM melting curves. The melting curves of the DNA samples were then compared to those of the controls. For the acrylamide electrophoresis PCR amplicons were incubated with the restriction enzyme Hph1 and the fragments were separated on a 20% acrylamide gel.

Data analysis (study I, II and III)

In study I differences in luciferase reporter activity were analyzed using analysis of variance (ANOVA), the Student's t-test and the Student-Newman-Keuls test. In study II differences in IL-10 mRNA transcription and IL-10 protein production were analyzed using the Mann-Whitney test. The two sample non-parametric Wilcoxon test was used in study III for evaluation of differences in Sp1 and IL-10 mRNA expression, as well as differences in cell densities of Sp1 positive cells, Sp1 positive B cells and Sp1 protein molecules. In all studies p-values <0.05 were considered significant.

Results

Binding of transcription factors to the -1087 position in the IL-10 promoter (study I and II)

The EMSA analysis performed in study I resulted in three specific complexes using the 1087G probe and two specific complexes using the 1087A probe. It was demonstrated that PU.1 and Spi-B bound to the region around -1087 in both the G- and the A-allele. The transcription factor Sp1, however, only bound when there was a G at -1087. A 4th complex was identified for both probes and the results indicated that it was ETS related. In study II chromatin immunoprecipitation assay was used to investigate differences in binding of PU.1, Spi-B and Sp1 to the -1087 position in the *in vivo* situation in B cell lines from subjects with GG or AA genotypes. The analysis revealed a larger increase of PU.1 and Spi-B for the GG genotype (IgG fold 7.5 and 4.7 respectively) than for the AA genotype (1.8 and 0.5). These differences were statistically significant (PU.1; p=0.034, Spi-B; p=0.046). The corresponding results for the transcription factor Sp1 were 6.0 for GG genotype cells and 0 for the AA genotype cells (p=0.028).

Influence of the -1087 IL-10 gene polymorphism on IL-10 gene expression (study I and II)

We evaluated the relation between the IL-10 promoter function and the presence of A or G at -1087 using transient transfection. DG75 B cells were transfected with reporter constructs with G or A at -1087. After 24h of LPS-stimulation cells transfected with the GCC reporter plasmid showed a 15-fold increase in promoter activity, whereas the untreated cells showed a 5-fold increase. For the cells transfected with the ACC reporter plasmid, the fold increase was sixfold for the stimulated cells and threefold for the un-stimulated cells. Moreover, co-transfection of SL2 cells with increasing amount of an Sp1 reporter plasmid resulted in a corresponding increase in promoter activity. Transfection with 0.5 μg of Sp1 expression vector resulted in a fourfold activation for the GCC reporter plasmid, whereas transfection with 1μg of Sp1 vector resulted in 10-fold increase in promoter activity. For the ACC reporter plasmid, the corresponding fold activation was two and four, respectively. This difference was statistically significant when using 1 μg of Sp1 expression vector (p=0.0043) (study I).

In study II, we also analyzed the IL-10 gene expression by measuring the amount of IL-10 and Sp1 mRNA in B cells from subjects with either AA or GG genotypes. Following LPS-stimulation GG genotype cells exhibited a larger increase in both IL-10 and Sp1 mRNA transcription (1.6 and 1.2 fold, respectively) than AA genotype cells (1.0 and 0.8). The difference in Sp1 mRNA expression was statistically significant ($p=0.041$). LPS-stimulation of purified B cells revealed a larger fold increase in IL-10 mRNA for the GG genotype cells than for the AA genotype cells (3.3 and 1.0, respectively). The corresponding increase in Sp1 mRNA transcription was 1.8 for the GG and 1.2 for the AA genotype cells.

In order to investigate whether the increase in IL-10 mRNA expression after LPS-stimulation resulted in a corresponding increase in IL-10 protein production we analyzed the supernatants from the cell cultures using the ELISA technique. The analysis showed that LPS treatment of GG genotype cells resulted in a concentration of 125 pg/ml of IL-10 compared to 85 pg/ml for the AA genotype cells. The corresponding numbers for the untreated cells were 81 vs. 59 pg/ml. For the purified B cells LPS-stimulation resulted in 173 pg/ml for the GG genotype and 12 pg/ml for the AA genotype cells. Untreated purified B cells from GG genotype subject had a 131 pg/ml IL-10 production compared to 12 pg/ml for the AA genotype cells.

The significance of Sp1 on IL-10 gene expression was further studied using RNA interference. These experiments resulted in a decrease in both Sp1 and IL-10 mRNA for cells transfected with an Sp1 siRNA in relation to cells transfected with the control siRNA. In LPS-stimulated GG genotype cells the reduction in Sp1 mRNA was 40%±23% in the LPS-stimulated cells and 36%± 9% in the un-stimulated cells. The corresponding numbers for the AA genotype were 37%±13% in the LPS stimulated and 41%±29% in the un-stimulated cells. The remaining IL-10 expression after transfection with Sp1 siRNAs was in the LPS-stimulated B cells 58% for the GG genotype and 72% for the AA genotype. In non-stimulated cells the remaining IL-10 expression was 66% for the GG genotype and 77% for the AA genotype.

Expression of Sp1 in the periodontitis lesion (study III)

The results in study II showed a larger Sp1 mRNA expression in GG genotype cells compared to AA genotype cells. Thus, we evaluated the possibility of differences in Sp1 expression in periodontitis lesions from subjects with either GG or AA genotypes (study III). 25 subjects with chronic periodontitis were genotyped for the -1087 IL-10 gene polymorphism. Among these subjects 32% were GG, 40% were AG and 28% were AA genotypes. Quantification of Sp1 mRNA in the periodontitis

lesions revealed a 4-fold increase in Sp1 mRNA in GG genotype subjects compared to AA genotype subjects. The GG genotype subjects also had a 4 fold increase of IL-10 mRNA in the lesion compared to the AA genotype subjects. In periodontitis lesions from GG genotype subjects 64% (SD 13%) of the cells were Sp1 positive compared to 35% (SD19%) for the AA genotype subjects ($p<0.005$). Using double-staining immunohistochemistry it was shown that 79% (SD17%) of the B cells in periodontitis lesions from subjects with the GG genotype were Sp1 positive while in subjects with the AA genotype 61% (SD 26%) of the B cells were Sp1 positive. This difference was statistically significant ($p=0.05$). Moreover, there was a larger number of Sp1 protein molecules in periodontitis lesions from subjects with the GG genotype (2679, SD 1211 RCPs) compared to subjects with the AA genotype (1687, SD 781 RCPs).

Influence of epigenetic modifications on the IL-10 gene expression (study IV)

Gene expression is not only regulated by genetic factors but is also influenced by epigenetic modifications of the chromatin. We therefore investigated the influence of histone modifications and DNA methylation on B cell-derived IL-10 transcription (study IV). Furthermore, since binding of Sp1 may alter the chromatin structure of a promoter we hypothesized that there may be a difference in chromatin modifications between the GG and AA genotype. In study IV treatment of B cells with HDACi resulted in an increase in IL-10 mRNA and this increase was somewhat larger for AA than GG genotype cells. Treatment with Butyrate and VPA resulted in a 4- and 3-fold increase in IL-10 transcription for the AA genotype cells compared to the GG genotype cells (fold increase 2 and 2). No increase in IL-10 mRNA transcription was found after treatment of the cells with TSA. Treatment with 5-aza, an inhibitor of DNA methyltransferases, resulted in a 5-fold increase in IL-10 transcription for AA genotype cells compared to a 4-fold increase for the GG genotype cells. These experiments were performed to demonstrate a possible influence of histone acetylation and DNA methylation on the regulation of IL-10 gene expression.

To distinguish active chromatin from inactive chromatin in the IL-10 promoter region around the -1087 SNP we used the ChIP assay and antibodies for histone acetylation and methylation. The level of acetylation of histone H4 and methylation of histone H3 were found to be more pronounced in LPS-stimulated B cells from subjects with the GG genotype cells than in AA genotype cells (38%±5% and 40%±11%, respectively). The AA genotype had a somewhat larger (7%±5%) increase in acetylation of histone H3 compared to the GG genotype. For the un-stimulated cells the corresponding numbers of fold increase for the GG genotype cells compared

to AA genotype cells were 88%±4% for acetylation of H3 and 66%±11% for methylation of H3. For the detection of acetylated histone H4 the AA genotype had a 36%±19% higher fold increase compared to the GG genotype. Overall, LPS-stimulation resulted in an 89%±1% fold increase for all antibodies.

Three CpG sites upstream of the -1087 SNP were identified. Sequencing of the amplicon confirmed the previously identified sequence of the IL-10 promoter region. Bisulfite sequencing, HRM and gel electrophoresis analysis of the three CpGs distal of the -1087 site indicated that these CpGs were methylated in DNA from peripheral blood and gingival tissue samples from both GG and AA genotype subjects.

Main findings

The transcription factors PU.1 and Spi-B bound to the region around the -1087 position in the IL-10 promoter, while the Sp1 transcription factor only bound to the G-allele of the -1087 SNP.

LPS-stimulation resulted in a larger increase in IL-10 and Sp1 gene expression in B cells with GG than in B cells with AA genotypes of the -1087 SNP.

Sp1 was present in B cells in periodontitis lesions and subjects with the GG genotype exhibited larger proportions of Sp1-positive cells and expressed larger amounts of Sp1 mRNA and protein in the lesion than AA genotype subjects.

Epigenetic modifications influenced IL-10 gene expression and differences in epigenetic modifications in the promoter region were found between GG and AA genotype subjects.

Concluding remarks

In the present thesis functional aspects on the -1087 A to G nucleotide transition in the IL-10 promoter were studied. In particular, the identification of transcription factors, differences in transcription factor binding and the influence of transcription factor binding on the IL-10 gene expression were assessed. In addition, the presence of Sp1 in periodontitis lesions and epigenetic modifications of the IL-10 promoter were evaluated.

In study I we identified three transcription factors binding to the region around -1087 in the IL-10 promoter *in vitro*. The transcription factors PU.1 and Spi-B bound at -1087 in both the G- and the A-allele, while Sp1 only bound when there was a G at -1087. To our knowledge, this was the first report on Sp1 binding to -1087 in the human IL-10 promoter. Previous research has shown that the transcription factor PU.1 is able to bind at this site in human monocytic THP1 cells (Reuss *et al.* 2002). The -1087 A-allele matches the PU.1 core motif, while the -1087 G-allele differs. The authors therefore suggested that PU.1 has an optimal binding affinity for the A-allele and that this binding inhibits gene expression. However, the results in the EMSAs in Study I indicated that Sp1 had a higher binding affinity for this site than PU.1 and Spi-B. It may therefore be suggested that Sp1 rather than PU.1 and Spi-B is the key factor for the regulation of promoter activity for the GG genotype. These findings were further supported by the results of the co-transfection experiments in the SL2 cells where transfection with increasing amount of Sp1 expression vector resulted in a corresponding increase in IL-10 promoter activity. This increase was larger for the G-allele than the A-allele. Using the B cell line DG75 we showed that transfection with the GCC construct after LPS-stimulation resulted in a higher promoter activity than transfection with the ACC construct. LPS-stimulation of THP1 cells has previously been shown to result in a larger promoter activity for the GCC promoter construct compared to the ACC reporter construct (Reuss *et al.* 2002). However, a study on transfection of an un-stimulated EBV infected B cell line resulted in a larger transcriptional activity for the A-allele compared to the G-allele (Rees *et al.* 2002). Taken together, these results indicate that the -1087 SNP is important in the regulation of LPS-induced IL-10 gene expression.

In study II, we further investigated the difference in transcription factor binding *in vivo* in B cell lines established from subjects with either the GG or AA genotype for the

-1087 SNP. The results confirmed our previous findings that Sp1 only bound to the -1087 G-allele.

Stimulation of B cells with LPS resulted in a larger increase in IL-10 and Sp1 mRNA transcription for the GG than AA genotype cells. The -1087 A/G polymorphism has previously been shown to influence the regulation of IL-10 gene expression but the results were inconclusive. Turner et al. (1997) reported that subjects negative for the A-allele had a higher IL-10 production than subjects positive for the A-allele. Similar results were presented by Hoffmann et al. (2001) and in study II of the present series. However, in a study by Yilmaz et al. (2005) LPS-stimulation of peripheral blood mononuclear cells revealed no difference in IL-10 production between AA and GG genotype subjects. Eskdale et al. (1999) compared the IL-10 secretion in relation to haplotypes and found that the A-allele was associated with higher IL-10 production than the G-allele. The authors suggested that the reason for the conflicting results between studies may relate to the experimental model including types of cells and stimuli.

LPS-associated IL-10 expression was previously demonstrated to be associated with larger inter-individual variations compared to EBV-infection of the cells and stimulation using dibutyryl-cAMP (Mörmann *et al.* 2004). It was suggested that intracellular infectious agents, like EBV, regulate IL-10 through a pathway independent of the low- and high producer haplotypes. EBV infects primary B cells in the mucosal lymphoid tissue, and this infection induces the B cell to produce high amounts of IL-10. This IL-10 production may contribute to the immortalization of the B cells *in vitro* (Babcock 1998; Burdin *et al.* 1993).

The decrease in IL-10 expression following Sp1 siRNA transfection in study II is in accordance with results presented by Chanteux et al. (2007). They reported that treatment of cells with the Sp1 inhibitor Mithramycin resulted in a decrease in nuclear Sp1 activation and an almost complete inhibition of LPS-induced IL-10 production at mRNA and protein level. The decrease in IL-10 transcription in study II was somewhat larger for the GG genotype cells compared to the AA genotype cells. It may be suggested that activation of MAPkinase pathways and the subsequent activation of Sp1 is essential in LPS-induced IL-10 production.

In summary, the results presented in study I and II indicate that Sp1 has an important function in the regulation of IL-10 during the response to inflammatory signals and

that the -1087 A/G SNP influences IL-10 gene expression by altering the binding pattern of transcription factors.

The reported findings on high levels of IL-10 and B cells in periodontitis lesions, together with the observations made in study II on a corresponding increase in Sp1 and IL-10 gene expression in B cells after LPS-stimulation, suggest a possible influence of Sp1 in periodontitis lesions. Thus, in study III we assessed the amount of Sp1 in lesions from subjects with severe, generalized periodontitis and found a larger amount of Sp1 in lesions from GG genotype subjects compared to AA genotype subjects. Reports on the presence of Sp1 in tissues are predominantly from the field of cancer research. It was reported in several studies that Sp1 was strongly expressed, or even over-expressed, in tumor tissues, while in normal tissues Sp1 was absent (Jiang *et al.* 2008; Zhang *et al.* 2005). Using the threshold for Sp1 over-expression as defined by Jiang et al. (2008), the results presented in study II show an over-expression of Sp1 for both the A-allele and the G-allele for -1087. It has been demonstrated that Sp1 is an important factor in inflammation and the regulatory function of Sp1 does not only involve IL-10. The LPS-induced NFκB binding to the IL-6 promoter is enhanced by Sp1 and the result is a more rapid response to inflammatory stimuli (Kang *et al.* 1996). Sp1 is also involved in the regulation of the TNF-α induction of protein A20 (Ainbinder *et al.* 2002), and in the maintenance of the basal TLR2 promoter activity in human epithelial cells (Furuta *et al.* 2008). In addition, the periodontal pathogen *P.gingivalis* is recognized by TLR2/1 and CD14. This interaction results in an activation of ERK1/2 (Krauss *et al.* 2010), providing an additional role of Sp1 in periodontitis.

The results in study III also revealed a larger amount of IL-10 mRNA in periodontitis lesions from GG genotype subjects than in AA genotype subjects. The presence of IL-10 in periodontitis lesions has been reported in several studies (Aramaki *et al.* 1998; Lappin *et al.* 2001; Yamazaki *et al.* 1997), and a recent study demonstrated that periodontitis subjects with the GG genotype for the -1087 polymorphism had a larger amount of IL-10 positive cells in periodontitis lesions than subjects with the AA genotype (Donati *et al.* 2008). The larger IL-10 mRNA expression in study III corresponded to a similar larger Sp1 mRNA for GG than AA genotypes. The reason for this association of larger mRNA expression for the -1087 IL-10 GG genotype is presently not understood.

In summary, study III is to our knowledge, the first report on the presence of Sp1 in periodontitis lesions. While the results indicate a possible role for Sp1 in periodontitis

further research on the Sp1 signaling pathways is needed to elucidate the role of Sp1 in the inflammatory processes of periodontitis.

In study IV, it was demonstrated that different epigenetic modifications of DNA and histones influence IL-10 gene expression. Differences between genotypes were found for acetylation of both histone H3 and H4, and for methylation of histone H3.

To investigate whether epigenetic mechanisms can regulate IL-10 gene expression, B cells from subjects with either the GG or AA genotype were treated with HDAC inhibitors and the DNA methylation inhibitor 5-aza. Although TSA did not affect IL-10 expression, the increase in IL-10 expression after treatment with VPA, Butyrat and 5-aza indicated that epigenetic modifications influence IL-10 gene expression in B cells. The effect of Butyrate and 5-aza is in accordance with previous studies (Dong *et al.* 2007; Park *et al.* 2007; Säemann *et al.* 2000). Little is known of the effect of VPA on IL-10 gene expression. In a study on autism a decrease in IFN-γ/IL-10 ratio in mice exposed to VPA was found (Schneider *et al.* 2008). The reason for the larger IL-10 increase after treatment with HDACi and 5-aza for the AA genotype compared to the GG genotype found in study IV is not clear. Vincent et al. (2008) reported that the expression of the human mucin gene MUC4 was found to increase after TSA and 5-aza treatments in non-producing and low producing cells. While TSA inhibited expression in high producing cells, 5-aza was found to have no affect on high producing cells.

In study IV, the acetylation of histones around -1087 was investigated using the ChIP assay and antibodies against acetylation of histone H3 and H4, respectively. Un-stimulated cells that produced small amounts of IL-10 were acetylated to a smaller degree at both H3 and H4 than LPS-stimulated cells, in which the IL-10 promoter and gene expression were more active. The increase in methylation of histone H3 lys 4 was associated with a more open chromatin in the -1087 region, which indicates a more active transcription. Since differences were found between the GG and AA genotype cells it may be suggested that the -1087 SNP influences the chromatin structure by alterations in transcription factor binding.

The effect of methylation on Sp1 binding may be gene specific and depend on the strength of the Sp1-DNA interaction and on the sequence of the Sp1 site in the promoter (Vincent *et al.* 2008). It was reported that Sp1 binding to DNA could influence both acetylation and DNA methylation. Studies have shown that Sp1 can

bind and activate transcription even when the site is methylated (Höller *et al.* 1988). Therefore, we wanted to investigate if the binding of Sp1 influenced the methylation pattern at the -1087 site in the IL-10 promoter in GG and AA genotypes. We identified three CpG sites distal of the -1087 SNP and evaluated their methylation pattern in DNA from blood cells and gingival tissue. The results showed no differences in methylation of the CpGs between genotypes or between blood and tissue samples. Sp1 binding may be inhibited by methylation of adjacent CpG sites or methylation of the outer C (Clark *et al.* 1997; Zhu *et al.* 2003). However, since the three CpGs identified in the present series were located at a certain distance from the -1087 SNP (-1194, -1197 and -1227) it is unlikely that the G to A transition at -1087 influences the methylation of these CpGs. Previous research on DNA methylation of the IL-10 promoter revealed 8 CpG sites in the proximal part of the promoter. It was demonstrated that the two most proximal CpGs at -185 and -110 differ most between IL-10 producing and non-producing cell lines (Szalmás *et al.* 2008). These findings show that the degree of methylation differs between CpGs in a promoter and that the promoter activity may be affected differently. In the study by Ehrlich et al. (1982) the authors reported on differences in DNA methylation between different cell types and tissues. The DNA methylation in a specific tissue did not differ between individuals and the DNA methylation did not always correlate with the level of transcription.

The IL-10 promoter does not contain any CpG islands and only a few CpG sites. The bisulfite modification changes the unmethylated cytosines into uracils, which during the PCR are amplified as thymines. In the IL-10 promoter region investigated in the present series of studies the absence of CpG islands resulted in an AT-rich sequence after PCR amplification. The AC repeat downstream presented additional limitation for the primer design. This resulted in a short amplicon of 59 bp and sensitive sequencing methods were therefore required. Since the 3 identified CpG sites were not part of a CpG island, difficulties in the interpretation of the controls were obvious. Additional research is needed on the methylation level of these CpGs in regards to genotypes and tissue types.

In summary, in study IV epigenetic modifications were found to influence IL-10 gene expression. Differences were found between the GG and AA genotypes, indicating a possible influence of the -1087 SNP on epigenetic modifications in the IL-10 promoter region.

The present series of studies has shown that the GG genotype for the -1087 IL-10 gene polymorphism is associated with a higher IL-10 production than the AA genotype and that the binding of Sp1 to the G-allele influences the IL-10 gene expression. The results presented indicate an important role for Sp1 in the regulation of IL-10. The presence of Sp1 in periodontitis lesions suggests that Sp1 may influence the regulation of immune signaling in periodontitis. Further studies on the interaction between Sp1 and other factors involved in IL-10 regulation are needed to elucidate the role of Sp1 in the transcription process and in the regulation of other pro- and anti-inflammatory cytokines.

References

Abbas KA & Lichtman AH (2005). Cellular and Molecular Immunology, fifth edition, pp 3-15. Philadelphia: Elsevier Saunders.

Ainbinder E, Revach M, Wolstein O, Moshonov S, Diamant N & Dikstein R (2002). Mechanism of rapid transcriptional induction of tumor necrosis factor alpha-responsive genes by NF-kappaB. *Mol Cell Biol* 22: 6354-6362.

Allalou A & Wählby C (2009). BlobFinder, a tool for fluorescence microscopy image cytometry. *Comput Methods Programs Biomed* 94: 58-65.

Andersen LC, Jensen JL & Orntoft TF (2004). Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res* 64: 5245-5250.

Aramaki M, Nagasawa T, Koseki T & Ishikawa I (1998). Presence of activated B-1 cells in chronic inflamed gingival tissue. *J Clin Immunol* 18: 421-429.

Babcock GJ, Decker LL, Volk M & Thorley-Lawson DA (1998). EBV persistence in memory B cells in vivo. *Immunity* 9: 395-404.

Bartold PM & Narayanan AS (2006). Molecular and cell biology of healthy and diseased periodontal tissues. *Periodontol 2000* 40: 29-49.

Ben-Bassat H, Goldblum N, Mitrani S, Goldblum T, Yoffey JM, Cohen MM, Bentwich Z, Ramot B, Klein E & Klein G (1977). Establishment in continuous culture of a new type of lymphocyte from a "Burkitt like" malignant lymphoma (line D.G.-75). *Int J Cancer* 19: 27-33.

Berglundh T, Liljenberg B & Lindhe J (1999). Some effects of periodontal therapy on local and systemic immunological parameters. *J Clin Periodontol* 26: 91-98.

Berglundh T, Liljenberg B, Tarkowski A & Lindhe J (2002). The presence of local and circulating autoreactive B cells in patients with advanced periodontitis. *J Clin Periodontol* 29: 281-286.

Berglundh T, Donati M, Hahn-Zoric M, Hanson LA & Padyukov L (2003). Association of the -1087 IL 10 gene polymorphism with severe chronic periodontitis in Swedish Caucasians. *J Clin Periodontol* 30: 249-254.

Berglundh T & Donati M (2005). Aspects of adaptive host response in periodontitis. *J Clin Periodontol* 32 (Suppl. 6): 87-107.

Berland R & Wortis HH (2002). Origins and functions of B-1 cells with notes on the role of CD5. *Annu Rev Immunol* 20: 253-300.

Bird AP (2002). DNA methylation patterns and epigenetic memory. *Genes Dev* 16: 6-21.

Bobetsi YA, Barros SP, Lin DM, Weidman JR, Dolinoy DC, Jirtle RL, Boggess KA, Beck JD & Offenbacher S (2007). Bacterial infection promotes DNA hypermethylation. *J Dent Res* 86: 169-174.

Borish L (1998). IL-10: evolving concepts. *J Allergy Clin Immunol* 101: 293-297.

Bouwman P & Philipsen S (2002). Regulation of the activity of Sp1-related transcription factors. *Mol Cell Endocrinol* 195: 27-38.

Brandeis M, Frank D, Keshet I, Siegfried Z, Mendelsohn M, Nemes A, Temper V, Razin A & Cedar H (1994). Sp1 elements protect a CpG island from de novo methylation. *Nature* 371: 435-438.

Brightbill HD, Plevy SE, Modlin RL & Smale ST (2000). A prominent role for Sp1 during lipopolysaccharide-mediated induction of the IL-10 promoter in macrophages. *J Immunol* 164: 1940-1951.

Burastero SE, Casali P, Wilder RL & Notkins AL (1988). Monoreactive high affinity and polyreactive low affinity rheumatoid factors are produced by CD5+ B cells from patients with rheumatoid arthritis. *J Exp Med* 168: 1979-1992.

Burdin N, Péronne C, Banchereau J & Rousset F (1993). Epstein-Barr virus transformation induces B lymphocytes to produce human Interleukin 10. *J Exp. Med* 177: 295-304.

Busslinger M (2004). Transcriptional control of early B cell development. *Annu Rev Immunol* 22: 55-79.

Bäckdahl L, Bushell A & Beck S (2009). Inflammatory signalling as mediator of epigenetic modulation in tissue-specific chronic inflammation. *Int J Biochem Cell Biol* 41: 176-184.

Cao S, Liu J, Song L & Ma X (2005). The protooncogene c-Maf is an essential transcription factor for IL-10 gene expression in macrophages. *J Immunol* 174: 3484-3492.

Chanteux H, Guisset AC, Pilette C & Sibille Y (2007). LPS induces IL-10 production by human alveolar macrophages via MAPKinases- and Sp1-dependent mechanisms. *Respir Res* 8: 71-81.

Chiang B-T, Liu Y-W, Chen B-K, Wang J-M & Chang W-C (2006). Direct interaction of C/EBPdelta and Sp1 at the GC-enriched promoter region synergizes the IL-10 gene transcription in mouse macrophage. *J Biomed Sci* 13: 621-635. .

Clark SJ, Harrison J & Molloy PL (1997). Sp1 binding is inhibited by (m)Cp(m)CpG methylation. *Gene* 195: 67-71.

Couper KN, Blount DG & Riley EM (2008). IL-10: the master regulator of immunity to infection. *J Immunol* 180: 5771-5777.

Courey AJ & Tjian R (1988). Analysis of Sp1 in vivo reveals multiple transcriptional domains, including a novel glutamine-rich activation motif. *Cell* 55: 887-898.

Crawley E, Kay R, Sillibourne J, Patel P, Hutchinson I & Woo P (1999). Polymorphic haplotypes of the interleukin-10 5' flanking region determine variable interleukin-10 transcription and are associated with particular phenotypes of juvenile rheumatoid arthritis. *Arthritis Rheum* 42: 1101-1108.

Cullinan MP, Westerman B, Hamlet SM, Palmer JE, Faddy MJ, Seymour GJ, Middleton PG & Taylor JJ (2008). Progression of periodontal disease and interleukin-10 gene polymorphism. *J Periodont Res* 43: 328-333.

Dittmer J, Pise-Masison CA, Clemens KE, Choi KS & Brady JN (1997). Interaction of human T-cell lymphotropic virus type I Tax, Ets1, and Sp1 in transactivation of the PTHrP P2 promoter. *J Biol Chem* 272: 4953-4958.

Donati M, Liljenberg B, Padyukov L & Berglundh T (2008). Local expression of interleukin-10 and mCD14 in relation to the -1087 IL-10 and -159 CD14 gene polymorphisms in chronic periodontitis. *J Periodontol* 79: 517-524.

Dong J, Ivascu C, Chang H-D, Wu P, Angeli R, Maggi L, Eckhardt F, Tykocinski L, Haefliger C, Möwes B, Sieper J, Radbruch A, Annunziato F & Thiel A (2007). IL-10 is excluded from the functional cytokine memory of human CD4+ memory T lymphocytes. *J Immunol* 179: 2389-2396.

Ehrlich M, Gama-Sosa MA, Huang L-H, Midgett RM, Kuo KC, McCune RA & Gehrke C 1982). Amount and distribution of 5-methylcytosine in human DNA from different types of tissues or cells. *Nucleic Acids Res* 10: 2709-2721.

Emili A, Greenblatt J & Ingles CJ (1994). Species-specific interaction of the glutamine-rich activation domains of Sp1 with the TATA box-binding protein. *Mol Cell Biol* 14: 1582-1593.

Eskdale J & Gallagher G (1995). A polymorphic dinucleotide repeat in the human IL-10 promoter. *Immunogenetics* 42: 444-445.

Eskdale J, Kube K & Gallagher G (1996). A second polymorphic dinucleotide repeat in the 5' flanking region of the human IL10 gene. *Immunogenetics* 45: 82-83.

Eskdale J, Kube D, Tesch H & Gallagher G (1997). Mapping of the human IL10 gene and further characterization of the 5' flanking sequence. *Immunogenetics* 46: 120-128.

Eskdale J, Gallagher G, Verweij CL, Keijser V, Westendorp RGJ & Huizinga TWJ (1998). Interleukin 10 secretion in relation to human IL-10 locus haplotypes. *Proc Natl Acad Sci USA* 95: 9465-9470.

Eskdale J, Keijsers V, Huizinga T & Gallagher G (1999). Microsatellite alleles and single nucleotide polymorphisms (SNP) combine to form four major haplotype families at the human interleukin-10 (IL-10) locus. *Genes Immun* 1: 151-155.

Farnham PJ (2009). Insights from genomic profiling of transcription factors. *Nat Rev Genet* 10: 605-616.

Fiorentino DF, Bond MW & Mosmann TR (1989). Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J Exp Med* 170: 2081-2095.

Fitzpatrick DR & Wilson CB (2003). Methylation and demethylation in the regulation of genes, cells, and responses in the immune system. *Clin Immunol* 109: 37-45.

Furuta T, Shuto T, Shimasaki S, Ohira Y, Suico MA, Gruenert DC & Kai H (2008). DNA demethylation-dependent enhancement of toll-like receptor-2 gene expression in cystic fibrosis epithelial cells involves SP1-activated transcription. *BMC Mol Biol* 9: 39-54.

Gallant S & Gilkeson G (2006). ETS transcription factors and regulation of immunity. *Arch Immunol Ther Exp* 54: 149-163.

Garner MM & Revzin A (1981). A gel electrophoresis method for quantifying the binding of proteins to specific DNA regions: application to components of the Escherichia coli lactose operon regulatory system. *Nucleic Acids Res* 9: 3047-3060.

Gemmell E, Carter CL, Hart DNJ, Drysdale KE & Seymour GJ (2003). Antigen-presenting cells in human periodontal disease tissues. *Oral Microbiol Immunol* 18: 388-393.

Gomez RS, Dutra WO & Moreira PR (2009). Epigenetics and periodontal disease: future perspectives. *Inflamm Res* 58: 625-629.

Green MR, Yoon H & Boss JM (2006). Epigenetic regulation during B cell differentiation controls CIITA promoter accessibility. *J Immunol* 177: 3865-3873.

Helminen M, Lahdenpohja N & Hurme M (1999). Polymorphism of the Interleukin-10 gene is associated with susceptibility to Epstein-Barr virus infection. *J Inf diseas* 180: 496-499.

Herman JG, Graff JR, Myöhänen S, Nelkin BD & Baylin SB (1996). Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci USA* 93: 9821-9826.

Hoffmann SC, Stanley EM, Cox DE, Craighead N, DiMercurio BS, Koziol DE, Harlan DM, Kirk AD & Blair PJ (2001). Association of cytokine polymorphic inheritance and in vitro cytokine production in anti-CD3/CD28-stimulated peripheral blood lymphocytes. *Transplantation* 72: 1444-1450.

Howell WM & Rose-Zerilli MJ (2007). Cytokine gene polymorphisms, cancer susceptibility, and prognosis. *J Nutr* 137: 194S-199S.

Hugoson A, Sjödin B & Norderyd O (2008). Trends over 30 years, 1973-2003, in the prevalence and severity of periodontal disease. *J Clin Periodontol* 35: 405-414.

Huizinga TWJ, Keijser V, Yanni G, Hall M, Ramage W, Lanchbury J, Pitzalis C, Drossaers-Bakker WK, Westendorp RGJ, Breedveld FC, Panayi G & Verweij CL (2000). Are differences in interleukin 10 production associated with joint damage? *Rheumatol* 39: 1180-1188.

Höller M, Westin G, Jiricny J & Schaffner W (1988). Sp1 transcription factor binds DNA and activates transcription even when the binding site is CpG methylated. *Genes Dev* 2: 1127-1135.

Im S, Hueber A, Monticelli S, Kang K & Rao A (2004). Chromatin-level regulation of the IL10 gene in T cells. *J Biol Chem* 279: 46818-46825.

Jarvius M, Paulsson J, Weibrecht I, Leuchowius K-J, Andersson A-C, Wählby C, Gullberg M, Botling J, Sjöblom T, Markova B, Östman A, Landegren U & Söderberg O (2007). In situ detection of phosphorylated platelet-derived growth factor receptor beta using a generalized proximity ligation method. *Mol Cell Proteomics* 6: 1500-1509.

Jenuwein T & Allis CD (2001). Translating the histone code. *Science* 293: 1074-1080.

Jiang NY, Woda BA, Banner BF, Whalen GF, Dresser KA & Lu D (2008). Sp1, a new biomarker that identifies a subset of aggressive pancreatic ductal adenocarcinoma. *Cancer Epidemiol Biomarkers Prev* 17: 1648-1652.

Kang SH, Brown DA, Kitajima I, Xu X, Heidenreich O, Gryaznov S & Nerenberg M (1996). Binding and functional effects of transcriptional factor Sp1 on the murine interleukin-6 promotor. *J Biol Chem* 271: 7330-7335.

Klinman DM & Steinberg AD (1987). Systemic autoimmune disease arises from polyclonal B cell activation. *J Exp Med* 165: 1755-1760.

Krauss JL, Potempa J, Lambris JD & Hajishengallis G (2010). Complementary Tolls in the periodontium: how periodontal bacteria modify complement and Toll-like receptor responses to prevail in the host. *Periodontol 2000* 52: 141-162.

Kremer KN, Kumar A & Hedin KE (2007). Haplotype-independent costimulation of IL-10 secretion by SDF-1/CXCL12 proceeds via AP-1 binding to the human IL-10 promoter. *J Immunol* 178: 1581-1588.

Kube D, Platzer C, von Knethen A, Straub H, Bohlen H, Hafner M & Tesch H (1995). Isolation of the human interleukin 10 promoter. Characterization of the promoter activity in Burkitt's lymphoma cell lines. *Cytokine* 7: 1-7.

Lappin DF, MacLeod CP, Kerr A, Mitchell T & Kinane DF (2001). Anti-inflammatory cytokine IL-10 and T cell cytokine profile in periodontitis granulation tissue. *Clin Exp Immunol* 123: 294-300.

Lehmann AK, Halstensen A, Sornes S, Rokke O & Waage A (1995). High levels of Interleukin 10 in serum are associated with fatality in meningococcal disease. *Infect Immun* 63: 2109-2112.

Li JCB & Lau ASY (2007). A role for mitogen-activated protein kinase and Ets-1 in the induction of interleukin-10 transcription by human immunodeficiency virus-1 Tat. *Immunology* 121: 337-348.

Li L, He S, Sun JM & Davie JR (2004). Gene regulation by Sp1 and Sp3. *Biochem Cell Biol* 82: 460-471.

Liu Y-W, Tseng H-P, Chen L-C, Chen B-K & Chang W-C (2003). Functional cooperation of simian virus 40 promoter factor 1 and CCAAT/enhancer-binding protein beta and delta in lipopolysaccharide-induced gene activation of IL-10 in mouse macrophages. *J Immunol* 171: 821-828.

Livak KJ & Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25: 402-408.

Llorente L, Richaud-Patin Y, Wijdenes J, Alcocer-Varela J, Maillot MC, Durand-Gasselin I, Fourrier BM, Galanaud P & Emilie D (1993). Spontaneous production of interleukin-10 by B lymphocytes and monocytes in systemic lupus erythematosus. *Eur Cytokine Netw* 4: 421-427.

Llorente L, Richaud-Patin Y, Fior R, Alcocer-Varela J, Wijdenes J, Fourrier BM, Galanaud P & Emilie D (1994). In vivo production of interleukin-10 by non-T cells in rheumatoid arthritis, Sjogren's syndrome, and systemic lupus erythematosus. A potential mechanism of B lymphocyte hyperactivity and autoimmunity. *Arthritis Rheum* 37: 1647-1655.

Loder F, Mutschler B, Ray RJ, Paige CJ, Sideras P, Torres R, Lamers MC & Carsetti R (1999). B cell development in the spleen takes place in discrete steps and is determined by the quality of B cell receptor-derived signals. *J Exp Med* 190: 75-89.

Loos BG, van der Velden U & Laine ML (2008). Susceptibility. In: J Lindhe, NP Lang and T Karring (ed.) Clinical periodontology and implant dentistry, 5th edition, pp. 328-346. Oxford: Blackswell Munksgaard.

Lu N, Heuchel R, Barczyk M, Zhang W-M & Gullberg D (2006). Tandem Sp1/Sp3 sites together with an Ets-1 site cooperate to mediate alpha11 integrin chain expression in mesenchymal cells. *Matrix Biol* 25: 118-129.

Macleod D, Charlton J, Mullins J & Bird AP (1994). Sp1 sites in the mouse aprt gene promoter are required to prevent methylation of the CpG island. *Genes Dev* 8: 2282-2292.

Marco E, García-Nieto R & Gago F (2003). Assessment by molecular dynamics simulations of the structural determinants of DNA-binding specificity for transcription factor Sp1. *J Mol Biol* 328: 9-32.

Martin C & Zhang Y (2005). The diverse functions of histone lysine methylation. *Nat Rev Mol Cell Biol* 6: 838-849.

Maston GA, Evans SK & Green MR (2006). Transcriptional regulatory elements in the human genome. *Annu Rev Genomics Hum Genet* 7: 29-59.

Miyake K (2004). Innate recognition of lipopolysaccharide by Toll-like receptor 4-MD-2. *Trends Microbiol* 12: 186-192.

Mocellin S, Marincola F, Rossi CR, Nitti D & Lise M (2004). The multifaceted relationship between IL-10 and adaptive immunity: putting together the pieces of a puzzle. *Cytokine Growth Factor Rev* 15: 61-76.

Mongan AE, Ramdahin S & Warrington RJ (1997). Interleukin-10 response abnormalities in systemic lupus erythematosus. *Scand J Immunol* 46: 406-412.

Moore KW, de Waal Malefyt R, Coffman RL & O'Garra A (2001). Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* 19: 683-765.

Mosser DM & Zhang X (2008). Interleukin-10: new perspectives on an old cytokine. *Immunol Rev* 226: 205-218.

Mörmann M, Rieth H & Hua TD, Assohou C, Roupelieva M, Hu SL, Kremsner PG, Lufy AJF & Kube D (2004). Mosaics of gene variations in the Interleukin-10 gene promoter affect interleukin-10 production depending on the stimulation used. *Genes Immun* 5: 246-255.

Nakajima T, Ueki-Maruyama K, Oda T, Ohsawa Y, Ito H, Seymour GJ & Yamazaki K (2005). Regulatory T-cells infiltrate periodontal disease tissues. *J Dent Res* 84: 639-643.

Nelson JD, Denisenko O, Sova P & Bomsztyk K (2006). Fast chromatin immunoprecipitation assay. *Nucleic Acids Res* 34(1):e2.

O'Garra A, Chang R, Go N, Hastings R, Haughton G & Howard M (1992). Ly-1 B (B-1) cells are the main source of B cell-derived interleukin 10. *Eur J Immunol* 22: 711-717.

Offenbacher S, Barros SP & Beck JD (2008). Rethinking periodontal inflammation. *J Periodontol* 79: 1577-1584.

Padyukov L, Hahn-Zoric M, Lau YL & Hanson LÅ (2001). Different allelic frequencies of several cytokine genes in Hong Kong Chinese and Swedish Caucasioans. *Genes Immun* 2: 280-283.

Page RC & Kornman KS (1997). The pathogenesis of human periodontitis: an introduction. *Periodontol 2000* 14: 9-11.

Park J-S, Lee E-J, Lee J-C, Kim W-K & Kim H-S (2007). Anti-inflammatory effects of short chain fatty acids in IFN-g-stimulated RAW 264.7 murine macrophage cells: involvement of NF-kB and ERK signaling pathways. *Int immunopharmacol* 7: 70-77.

Perkins ND, Agranoff AB, Pascal E & Nabel GJ (1994). An interaction between the DNA-binding domains of RelA(p65) and Sp1 mediates human immunodeficiency virus gene activation. *Mol Cell Biol* 14: 6570-6583.

Pfaffl MW (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29: 2002-2007.

Pinskaya M & Morillon A (2009). Histone H3 lysine 4 di-methylation: a novel mark for transcriptional fidelity? *Epigenetics* 4: 302-306.

Porakishvili N, Mageed R, Jamin C, Pers J-O, Kulikova N, Renaudineau Y, Lydyard PM & Youinou P (2001). Recent progress in the understanding of B-cell functions in autoimmunity. *Scand J Immunol* 54: 30-38.

Qiu J (2006). Epigenetics: unfinished symphony. *Nature* 441: 143-145.

Rajewsky K (1996). Clonal selection and learning in the antibody system. *Nature* 381: 751-758.

Ray D, Bosselut R, Ghysdael J, Mattei MG, Tavitian A & Moreau-Gachelin F (1992). Characterization of Spi-B, a transcription factor related to the putative oncoprotein Spi-1/PU.1. *Mol Cell Biol* 12: 4297-4304.

Rees LE, Wood NA, Gillespie KM, Lai KN, Gaston K & Mathieson PW (2002). The interleukin-10-1082 G/A polymorphism: allele frequency in different populations and functional significance. *Cell Mol Life Sci* 59: 560-569.

Reuss E, Fimmers R, Kruger A, Becker C, Rittner C & Hohler T (2002). Differential regulation of interleukin-10 production by genetic and environmental factors - a twin study. *Genes Immun* 3: 407-413.

Robertson KD & Wolffe AP (2000). DNA methylation in health and disease. *Nat Rev Genet* 1: 11-19.

Rousset F, Garcia E, Defrance T, Péronne C, Vezzio N, Hsu D-H, Kastelein R, Moore KW & Banchereau J (1992). Interleukin 10 is a potent growth and differentiation factor for activated human B lymphocytes. *Proc Natl Acad Sci USA* 89: 1890-1893.

Sanger F, Nicklen S & Coulson AR (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* 74: 5463-5467.

Scarel-Caminaga RM, Trevilatto PC, Souza AP, Brito RB, Camargo LE & Line SR (2004). Interleukin 10 gene promoter polymorphisms are associated with chronic periodontitis. *J Clin Periodontol* 31: 443-448.

Schneider T, Roman A, Basta-Kaim A, Kubera M, Budziszewska B, Schneider K & Przewtocki R (2008). Gender-specific behavioral and immunological alterations in an animal model of autism induced by prenatal exposure to valproic acid. *Psychoneuroendocrinol* 33: 728-740.

Snustad DP & Simmons MJ (2010). Principles of genetics, fifth edition; pp 282-299. Hoboken: John Wiley & Sons, Inc.

Socransky S & Haffajee AD (2003). Microbiology of periodontal disease. In: J Lindhe, T Karring and NP Lang (ed.) Clinical periodontology and implant dentistry, fourth edition, pp. 106-149. Oxford: Blackswell Munksgaard.

Staples KJ, Smallie T, Williams LM, Foey A, Burke B, Foxwell BMJ & Ziegler-Heitbrock L (2007). IL-10 induces IL-10 in primary human monocyte-derived macrophages via the transcription factor Stat3. *J Immunol* 178: 4779-4785.

Stein SH, Hart TE, Hoffman WH, Hendrix CL, Guske CJ & Watson SC (1997). Interleukin-10 promotes anti-collagen antibody production in type I diabetic peripheral B lymphocytes. *J Periodontal Res* 32: 189-195.

Steinke JW, Barekzi E, Hagman J & Borish L (2004). Functional analysis of -571 IL-10 promoter polymorphism reveals a repressor element controlled by sp1. *J Immunol* 173: 3215-3222.

Su GH, Ip HS, Cobb BS, Lu MM, Chen HM & Simon MC (1996). The Ets protein Spi-B is expressed exclusively in B cells and T cells during development. *J Exp Med* 184: 203-214.

Su W, Jackson SM, Tjian R & Echols H (1991). DNA looping between sites for transcriptional activation: self-association of DNA-bound Sp1. *Genes Dev* 5: 820-826.

Sugawara M, Yamashita K, Yoshie H & Hara K (1992). Detection of, and anti-collagen antibody produced by, CD5-positive B cells in inflamed gingival tissues. *J Periodontal Res* 27: 489-498.

Szalmás A, Bánáti F, Koroknai A, László B, Fehér E, Salamon D, Gergely L, Minárovits J & Kónya J (2008). Lineage-specific silencing of human IL-10 gene expression by promoter methylation in cervical cancer cells. *Eur J Cancer* 44: 1030-1038.

Säemann MD, Böhmig GA, Österreicher CH, Burtscher H, Parolini O, Diakos C,Stöckl J, Hörl WH & Zlabinger GJ (2000). Anti-inflammatory effects of sodium butyrate on human monocytes: potent inhibition of IL-12 and up-regulation of IL-10 production. *FASEB J* 14: 2380-2382.

Tagore A, Gonsalkorale WM, Pravica V, Hajeer AH, McMahon R, Whorwell PJ, Sinnott PJ & Hutchinson IV (1999). Interleukin-10 (IL-10) genotypes in inflammatory bowel disease. *Tissue antigen* 54: 386-390.

Tone M, Powell MJ, Tone Y, Thompson SA & Waldmann H (2000). IL-10 gene expression is controlled by the transcription factors Sp1 and Sp3. *J Immunol* 165: 286-291.

Tsuji-Takayama K, Suzuki M, Yamamote M, Harashima A, Okochi A, Otani T, Inoue T, Sugimoto A, Toraya T, Takeuchi M, Yamasaki F, Nakamura S & Kibata M (2008). The production of IL-10 by human regulatory T cells is enhanced by IL-2 through a STAT5-responsive intronic enhancer in the *IL-10* locus. *J Immunol* 181: 3897-3905.

Turner BM (2000). Histone acetylation and an epigenetic code. *Bioessays* 22: 836-845.

Turner DM, Williams DM, Sankaran D, Lazarus M, Sinnott PJ & Hutchinson IV (1997). An investigation of polymorphism in the interleukin-10 gene promoter. *Eur J Immunogenet* 24:1-8.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A & Speleman F (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 3: 34.1-34.11.

Wang H, Feng J, Qi C-F, Li Z, Morse HC & Clarke SH (2007). Transitional B cells lose their ability to receptor edit but retain their potential for positive and negative selection. *J Immunol* 179: 7544-7552.

Welner RS, Pelayo R & Kincade PW (2008). Evolving views on the genealogy of B cells. *Nature rev immunol* 8: 95-106.

Westendorp RG, Langermans JA, Huizinga TW, Elouali AH, Verweij CL, Boomsma DI & Vandenbrouke JP (1997). Genetic influence on cytokine production and fatal meningococcal disease. *Lancet* 349: 170-173.

Vilar JMG & Saiz L (2005). DNA looping in gene regulation: from the assembly of macromolecular complexes to the control of transcriptional noise. *Curr Opin Genet Dev* 15: 136-144.

Villagra A, Cheng F, Wang H-W, Suarez I, Glozak M, Maurin M, Nguyen D, Wright KL, Atadja PW, Bhalla K, Pinilla-Ibarz J, Seto E & Sotomayor EM (2009). The histone deacetylase HDAC11 regulates the expression of interleukin 10 and immune tolerance. *Nat Immunol* 10:92-100.

Wilson AG (2008). Epigenetic regulation of gene expression in the inflammatory response and relevance to common diseases. *J Periodontol* 79: 1514-1519.

Vincent A, Ducourouble M-P & Van Seuningen I (2008). Epigenetic regulation of the human mucin gene MUC4 in epithelial cancer cell lines involves both DNA methylation and histone modifications mediated by DNA methyltransferases and histone deacetylases. *FASEB J* 22: 3035-3045.

Workman JL & Kingston RE (1998). Alteration of nucleosome structure as a mechanism of transcriptional regulation. *Annu Rev Biochem* 67: 545-579.

Yamazaki K, Nakajima T, Kubota Y, Gemmell E, Seymour GJ & Hara K (1997). Cytokine messenger RNA expression in chronic inflammatory periodontal disease. *Oral Microbiol Immunol* 12: 281-287.

Yamazaki K, Tabeta K, Nakajima T, Ohsawa Y, Ueki K, Itoh H & Yoshie H (2001). Interleukin-10 gene promoter polymorphism in Japanese patients with adult and early-onset periodontitis. *J Clin Periodontol* 28: 828-832.

Yilmaz V, Yentür SP & Saruhan-Direskeneli G (2005). IL-12 and IL-10 polymorphisms and their effects on cytokine production. *Cytokine* 30: 188-194.

Youinou P, Mackenzie L, le Masson G, Papadopoulos NM, Jouquan J, Pennec YL, Angelidis P, Katsikis P, Moutsopoulos HM & Lydyard PM (1988). CD5-expressing B lymphocytes in the blood and salivary glands of patients with primary Sjogren's syndrome. *J Autoimmun* 1: 185-194.

Zhang X, Edwards JP & Mosser DM (2006). Dynamic and transient remodelling of the macrophage IL-10 promoter during transcription. *J Immunol* 177: 1282-1288.

Zhang Z, Zhu Z-G, Ji J, Yuan F, Yu Y-Y, Liu B-Y & Lin Y-Z (2005). Transcription factor Sp1 expression in gastric cancer and its relationship to long-term prognosis. *World J Gasteroenterol* 11: 2213-2217.

Zhu W-G, Srinivasan K, Dai Z, Duan W, Druhan LJ, Ding H, Yee L, Villalona-Calero MA, Plass C & Otterson GA (2003). Methylation of adjacent CpG sites affects Sp1/Sp3 binding and activity in the p21(Cip1) promoter. *Mol Cell Biol* 23: 4056-4065.