GENE POLYMORPHISMS AND RELATED CELL MARKERS IN PERIODONTITIS LESIONS

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To my father and my mother

A Giorgio e Carla

Contents

Abstract

Gene polymorphisms and related cell markers in periodontitis lesions

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Inflammatory and immune reactions to microbial plaque are the predominant features of periodontitis. Epidemiological studies revealed that differences in periodontitis among individuals could not be explained by differences in oral hygiene alone and that not everybody is equally susceptible. Periodontitis is considered to be a multifactorial disease where the interaction of multiple genetic and environmental components results into disease expression. The objectives of the present series of studies were (i) to investigate the association of gene polymorphisms related to some immune regulation components (*IL10, TNFA, IL4RA CD14*) with severe chronic periodontitis (studies I-II), (ii) to study the local expression of some immune regulation components in relation to gene polymorphisms in subjects with chronic periodontitis (study III), (iii) to study the correlation between inflammatory cells and functional markers in gingival lesions obtained from subjects with severe chronic periodontitis (study IV) (iv) and to study the reaction of B-1a cells to *de novo* plaque formation in subjects who were treated for severe chronic periodontitis (study V).

It was demonstrated that the proportion of subjects that exhibited the −1087 *IL10* GG genotype was significantly larger in the group with severe periodontitis than in the group of healthy controls (study I), that the proportion of subjects that exhibited the −159 *CD14* TT genotype was significantly smaller in the group of subjects with severe periodontitis than in the periodontally healthy group (study II) and that the proportion of IL-10 positive cells in the peripheral area of periodontitis lesions was significantly larger in subjects with the −1087 *IL10* GG genotype than in subjects with AG or AA genotypes (study III).
It was also observed that B cells (B-1a cells and B-2 cells) occurred in larger proportions than T cells, plasma cells and neutrophils in periodontitis lesions and a significant correlation was found between percentages of B-1a cells and plasma cells and between densities of B-lymphocytes and plasma cells (study IV). Further, biopsies retrieved after 3 weeks of plaque accumulation contained larger proportions of B-1a cells than biopsies representing healthy sites (study V).

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Preface

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

I.   Berglundh T, Donati M, Hahn-Zoric M, Hanson LÅ, Padyukov L. (2003) Association of the -1087 *IL-10* gene polymorphism with severe chronic periodontitis in Swedish Caucasians. *Journal of Clinical Periodontology* **30**: 249-254.

II.   Donati M, Berglundh T, Hytönen AM, Hahn-Zoric M, Hanson LÅ, Padyukov L. (2005) Association of the -159 CD14 polymorphism and lack of association of the -308 TNFA and Q551R IL-4RA polymorphisms with severe chronic periodontitis in Swedish Caucasians. *Journal of Clinical Periodontology* **32**: 474-479

III. 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. *Journal of Periodontology* **79**: 517-524

IV.   Donati M, Liljenberg B, Zitzmann NU, Berglundh T. (2009) B-1a cells and plasma cells in periodontitis lesions. *Journal of Periodontal Research* (Accepted for publication)

V.   Donati M, Liljenberg B, Zitzmann NU, Berglundh T. (2009) B-1a cells in experimental gingivitis in man. *Journal of Periodontology* (Accepted for publication)

Abbreviations

ABC = avidin-labelled biotin complex

ACP = Antigen presenting cell

B220 = Isoform of the CD45

BC = Bone crest

BCR = B cell receptor

BoP = Bleeding on probing

CD = Cluster of differentiation

CEJ = cementoenamel junction

CpG = Unmethylated cytosine–guanine sequences of microbial DNA

DAB = Diaminobenzidine

DAPI = 4,6 diamino-2 phenylindole

DC = Dendritic cell

DNA = Deoxyribonucleic acid

EDTA = ethylenediaminetetraacetic acid disodium salt dihydrate

GC = Germinal center

GI = Gingival index

HLA = Human leukocyte antigen

IFNγ = Interferon γ

Ig = Immunoglobulin

IL = Interleukin

IL-4R = Interleukin-4 receptor

ICT = inflammatory cell lesion in the connective tissue

LPS = Lipopolysaccharide

MHC-II = Major histocompatibility complex II

mRNA = messenger ribonucleic acid

MZ = Marginal zone

PAGE = Polyacrylamide gel electrophoresis

PBMC = Peripheral blood mononuclear cell

PDI = Periodontal disease index

PI = Plaque index

PMN = Polymorphonuclear leukocyte

PPD = Probing pocket depth

SDS = Sodium dodecyl sulfate

SLE = Systemic Lupus Erythematosus

SNP = Single nucleotide polymorphism

SRP = Scaling and root planning

STRP = Short tandem repeat polymorphism

TCR = T cell receptor

Th1, Th2 = classes of CD4 effector cells

TLR = Toll like receptor

TNF = Tumor necrosis factor

VNTR = Variable number tandem repeat

Introduction

Findings from epidemiological studies have revealed that chronic periodontitis is a common disorder and severe forms of the disease that may lead to early and considerable amounts of bone loss and tooth mortality appear in 8-10% of adults. In subjects who are susceptible to periodontitis, the response to a subgingival microbial biofilm results in an inflammatory process in the periodontal tissues that mediates destruction of connective tissue attachment and alveolar bone. Leukocytes and/or their products, which are associated with tissue destruction, are controlled by immune-regulatory mechanisms and the equilibrium or imbalance established between the biofilm and the inflammatory process determines the severity of the periodontitis lesion.

Mechanisms of the host response in the periodontal tissues are complex and involve the so-called *innate* (non-specific) and *adaptive* (specific) responses. The *innate* host response is the inborn biological response to the microbial challenge that requires no prior learning or experience and constitutes the early step of the immune response. This defense system includes the physical barriers of the oral mucosal epithelial surfaces and vascular and cellular aspects of the inflammatory response. The *adaptive* host response is a specifically "tailored" defense system against the microbial challenge. This system of defense includes the *humoral* immune response based on the production of specific antibodies by B-lymphocytes directed against particular oral microorganisms and the *cell mediated* immune response that involves cytotoxic T-lymphocytes to protect against virus- or bacteria-infected cells. Parts of the *adaptive* host response in periodontitis are outlined in Fig. 1.

Fig.1 Schematic outline of regulatory components of adaptive host response in periodontitis (Adapted from Berglundh & Donati 2005)
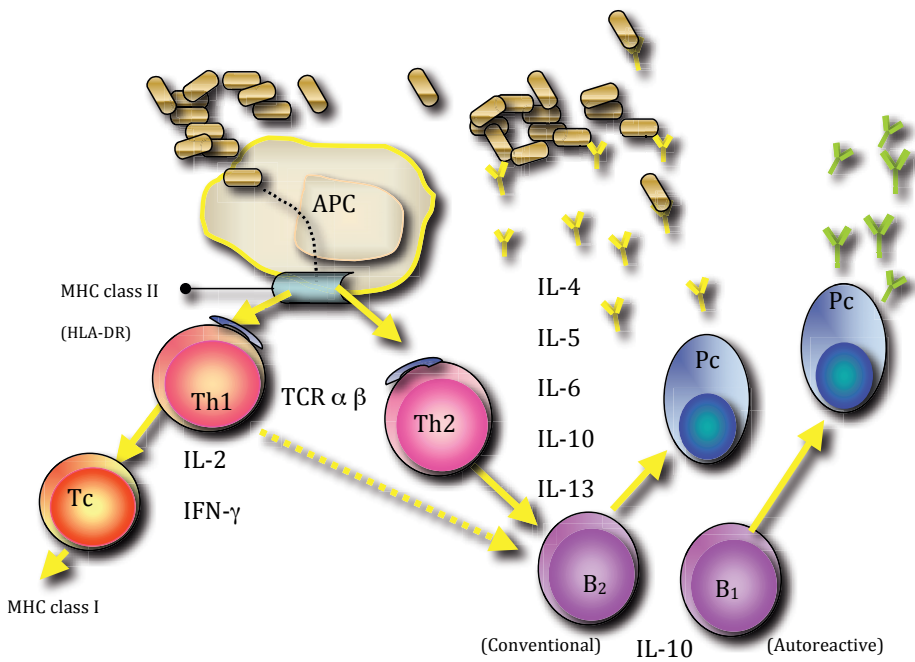
Periodontitis lesion

Page & Schroder (1976) classified the progression of gingival and periodontal inflammation on the basis of the available information gained from animal biopsy material. They divided the progressing lesion in four phases: *initial, early, established* and *advanced* lesions. According to their view, the initial and early lesions described the histopathology of the early stages of gingivitis, while the established lesion reflected the histopathology of more "chronic" gingivitis. However, these descriptions do not properly reflect the

histopathology in humans where elements of acute and chronic inflammation co-exist in early, established and advanced lesions. Kinane et al. (2003) proposed a new classification utilizing the Page & Schroder system as a framework to outline the histopathogenesis of periodontal disease. In this classification the clinical conditions (i.e. normal healthy gingiva, early gingivitis, established gingivitis and periodontitis) are categorized according to the histopathologic condition. Thus, established gingivitis is described as a lesion with neither bone loss nor apical epithelial migration and with a plasma cell density varying between 10% and 30%. Periodontitis is described as a lesion with bone loss and epithelial migration apical of the cemento-enamel junction and with a plasma cell density of more than 50%.

The relevance of plasma cells and B-lymphocytes has been highlighted in several reports on the composition inflammatory cells in periodontitis lesions. In a review on aspects of adaptive host response in periodontitis a meta-analysis was made with regard to the cell composition in periodontitis lesions (Berglundh & Donati 2005). Studies, describing the distribution of cells based on the morphological and phenotypic characteristics are presented in Table 1.

Table 1. Phenotypic characteristics of cells in the periodontitis lesion. (Adapted from Berglundh & Donati 2005)

Reference	Periodontal diagnosis	Sample	Technique	Results
Mackler et al. 1977	Gingival biopsies from areas characterized as clinically normal, mild gingivitis, periodontitis (PDI Ramfjord 1959)	Specimens of each category from 5 patients of various age and sex.	Immunofluorescence	Higher concentration of lymphocytes and plasma cells (IgG & IgM) in periodontitis biopsies compared to normal healthy biopsies.
Seymour & Greenspan 1979	Patients with chronic periodontal disease.	Twelve biopsies. Sites scheduled for surgery with PPD 4-8mm and BoP+	Immunohistochemistry Immunofluorescence	Majority of the lymphocytes had the phenotype of B cells and were positive for IgM and IgG.
Lindhe et al. 1980	Twenty-two patients with advanced periodontal tissue destruction, with PPD≥8 and 50% bone loss	In each patient six sites were selected: two sites with advanced disease, two sites with established gingivitis, two sites with "healthy" gingiva.	Morphometric analysis. For each biopsy numerical density (Nv) and volumetric density (Vv) of cells were calculated.	Periodontitis lesion: 31% Plasma cells, 5-10% Lymphocytes, 5% Fibroblasts, 1.3% Macrophages, 1.3% Neutrophils G., 11% collagen. In the gingivitis lesion the ratio lymphocytes-plasma cells was 1:1, in the periodontitis was 1:3
Charon et al. 1981	Fifteen patients 30-88 year-old with advanced periodontal disease	Biopsies from both diseased and healthy sites and blood samples from each patient.	Immunohistochemistry	The plasma cell predominance, the presence of T-cells and activated macrophages indicated both humoral and cell-mediated responses are operative in human chronic periodontitis

Okada et al. 1983	Patients with advanced periodontitis	Biopsies from sites with PPD≥5mm and bone loss	Immunohistochemistry	Only few PMNs. Plasma cells dominated in the central portion of the lesion.
Gillett at al. 1986	Three groups: Childhood Gingivitis, Juvenile Periodontitis, Chronic Adult Periodontitis	2 biopsies Childhood Gingivitis, 12 Chronic Adult Periodontitis, 6 Juvenile Periodontitis	Immunohistochemistry, monoclonal antibody-HLADr	In Childhood lesions most of the cells were small lymphocytes, in Juvenile Periodontitis >50% of the cells were plasma cells. The Chronic Adult Periodontitis showed a composition of this two extremes, with lymphocytes HLADr+ (B cells) and plasma cells dominating the lesions.
Passo et al. 1988	Nine patients with advanced chronic periodontitis	33 bleeding suppurating (S) and 23 bleeding non-suppurating (NS) interproximal biopsies	Immunohistochemistry and morphometric analysis.	In both (S) and (NS) biopsies, the majority of lymphocytes were positive for B cell surface antigen. The vast majority of T cells were of T-helper, with few T-cytotoxic/suppressor cells. At the apical portion of ICT, B cells and plasma cell predominated.
Reinhardt et al. 1988	Thirteen adult periodontal maintenance patients	Biopsies from "active" sites. Clinically similar but "stable" or healthy sites were obtained from each patient.	Immunohistochemistry.	Pan B cells were significantly more prevalent in infiltrates from active sites than in stable than or healthy sites. The T/B cell ratio was also significantly lower in active than in stable biopsies.

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Cobb et al. 1989	8 subjects each group: healthy gingiva, chronic gingivitis and chronic adult periodontitis.	Healthy gingiva: PI=0, GI =0,gingival sulcus 0-2mm; Chronic gingivitis: PI<1.0, GI at least 2.0, gingival sulcus 0-3mm; Chronic periodontitis GI at least 2.0 PPD $\geq$ 6mm	Immunohistochemistry.	The T- and B-lymphocyte populations increased approximately 20 x progressing from healthy to gingivitis to periodontitis specimens.
Joachim et al. 1990	Biopsies from 20 patients and 3 control volunteers: 5 with treated adult periodontitis (AP), 5 with untreated AP, 5 with treated juvenile periodontitis (JP) and 5 with untreated JP.	5 untreated AP with mean PPD = 6.0; 5 with treated AP with mean PPD =5.0; 5 with treated JP with maen PPD = 6.3; 5 with untreated JP mean PPD = 8.5 3 controls with mean PPD =2.3.	Electron microscopy and quantification of plasma cells by ultrastructural classification at magnification of x 1400 and x 5000	Plasma cell (PC) counts increased significantly (p less than 0.05) with lesion severity. They were absent from epithelium and sparse in the clinically healthy control specimens.
Zappa et al. 1991	10 systemically healthy adult patients with untreated advanced periodontitis were monitored during a period of 10 months.	Site had lost 2 mm or more attachment within the previous month (P), and the controlateral site had not (C).	The cells were identified and quantified by light microscopic evaluation of nuclear and cytoplasmatic staining at a magnification of x 1250	In P-sites, the numbers of macrophages, plasma cells, lymphocytes and total inflammatory cells were significantly higher as compared to C-sites.

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Celenligil et al. 1993	Biopsies were obtained from 16 patients between 22 and 33 years of age.	Gingival biopsies were obtained from the sites with advanced bone destruction, PPD $\geq$ 7mm in each of the 16 patients.	The tissue samples were processed for both histopathological and immunohistochemical examinations.	CD3+ cells were mainly located beneath the pocket epithelium. CD4+ and CD8+ cells were evenly distributed within this T-cell infiltrate. IgG+ plasma cells dominated lesion with equal participation of both T-cell subpopulations.
Yamazaki et al. 1993	Nineteen patients with moderate to advanced adult periodontitis and 5 gingivitis patients as control.	Periodontitis biopsies were obtained with PPD $\geq$5mm. The five gingivitis specimens with PPD <4.	Immunohistochemistry.	The percentage of CD23+ and CD25+, CD19+ B-cells which were identified in 13 out of 19 samples from periodontitis varied significantly in spite of similar clinical status. The frequency of B-cells in the gingivitis was much lower than that of periodontitis.
Liljenberg et al. 1994	Eight subjects (test) with advanced periodontal disease, with >2mm of attachment loss at 3 or more sites during the first and the second 12-month interval and eleven subjects (control) were with non-progressive sites.	From the 8 test subjects, $\geq$1 interproximal site with disease activity (progressive disease active PDA) and controlateral sites without disease progression (progressive disease inactive PDI). 11 sites non-progressive disease (NPD).	Morphometric analysis of the ICT and immunohistochemical examination by monoclonal antibody towards CD3, CD4, CD8, CD14, CD19 and CD22.	The progressive disease sites (PDA) comprised of a larger relative volume of plasma cells, a higher % number of plasma cells and monocytes/macrophages than corresponding sites in the non-progressive disease (NPD) group. Both T cell markers (CD3 and CD4) and B cells markers (CD22) were significantly elevated in the PDA compared to the PDI lesions.

Berglundh et al. 1999	16 individuals with advanced periodontal.	The 3 deepest interproximal sites. Following periodontal therapy (SRP) 1 biopsy was collected at 12-month and at 24-month among the selected sites. Blood samples at the 24-month re-examination.	Immunohistochemical analysis by monoclonal antibody towards CD3 and CD19 and flow cytometry	The improved clinical condition following SRP was, in addition, accompanied by a substantial reduction in the size of the inflammatory lesion (P-ICT). Following therapy both the density of CD19 positive cells and the proportion of CD3 positive cells expressing TCR Vbeta genes were reduced in the P-ICT, but apparently failed to affect the relative distribution of lymphocyte subsets in peripheral blood.
Lappin et al. 1999	Biopsies from 9 adult periodontitis (AP), from 10 patients with early onset periodontitis (EOP).	The PPD of the biopsies sites ranged from 6.0-9.0 mm in patients with AP and 5.8-9.6 mm in EOP.	Immunohistochemistry.	The relative numbers of B cells (CD 20) T cells (CD 3) and macrophages (CD 68) were expressed as a percentage of total for each group. The % of B-lymphocytes was greater in AP sections than in EOP.
Orima et al. 1999	Fourteen patients with moderate to advanced adult periodontitis (AP).	Gingival biopsies were obtained with mean probing pocket depth (PPD) of the biopsy sites: 7.8 ± 2.8	Immunohistochemistry.	In CD3/CD19 double-stained sections, CD19+ cells were the dominant infiltrating cell type. CD80 and CD86 were extensively expressed on the infiltrating cells in the connective tissues beneath the pocket epithelium. The distribution of CD80 and CD86 was consistent with that of CD19+ B cells.

Berglundh et al. 2001	11 children with signs of periodontal disease (LPP group) 21 adults with advanced periodontal disease: (AP) group.	Gingival biopsies and a sample of peripheral blood	The biopsies were prepared for morphometrical and immunohistochemical analysis and the blood samples prepared for immunohistochemical analysis.	The cellular infiltrates in the biopsies of the LPP group contained a larger proportion of lymphocytes and, in particular B cells, than was the case in the AP group. The TCR Valpha/Vbeta gene expression in the lesions in the AP group was dominated by Vbeta 17 and in the LPP group by Valpha2.
Gemmell et al. 2001	Biopsies from 21 healthy/gingivitis and 26 periodontitis subjects.	21 biopsies PPD of<4 mm Bop+. 26 biopsies classified as a periodontitis group with PPD>4 mm..	An immunoperoxidase technique was used to examine CD28, CD152, CD80 and CD86 positive cells in gingival biopsies	The higher percent CD86+ cells suggests a predominance of Th2 responses in both healthy/gingivitis and periodontitis tissues. The mean percent CD80+ and CD86+ B cells and macrophages was 1-7% and 8-16%, respectively.
Hillmann et al. 2001	Ten patients with rapidly progressive periodontitis (RPP) and 5 adult periodontitis (AP).	Biopsies from sites of teeth for extraction and from sites for SRP and then surgery (biopsy).	Immunohistological methods by means of monoclonal antibody.	The biopsies of patients with RPP revealed more B cells and plasma cells than treated sites at baseline and after the initial therapy.
Kleinfelder et al. 2001	Ten subjects EO periodontitis harboring Aa (EOP-Aa) and 10 without Aa (EOP-nonAa). Gingival biopsies before and after SRP.	At baseline 2 biopsies and 2 biopsies form a second quadrant after study subjects had received a full-mouth SRP.	Immunohistochemistry	The CD30-positive cells (Plasma cells) were the largest phenotype in both the subject-groups. Before therapy, mean counts of all cells were higher in the EOP-Aa group than EOP-non Aa. Following SRP, the numbers of all phenotypes decreased in both groups.

Gemmell et al. 2002	Gingival biopsies from 21 healthy or gingivitis and 25 periodontitis subjects.	The samples were divided into three groups according to the size of infiltrate: group 1, small infiltrates; group 2, medium infiltrates; group 3, extensive infiltrates.	An immunoperoxidase technique.	B cells were the predominant APC in group 2 and 3 tissues. The percentage of B cells in group 3 periodontitis lesions was increased in comparison with group 1 periodontitis tissues and also in comparison with group 3 healthy/gingivitis sections.
Younes et al. 2009	8 Healthy control, 10 gingivitis, 10 moderate periodontitis, 10 severe periodontitis	Biopsies from sites of teeth selected for extraction.	Immunohistochemistry	In progressive lesions of periodontal disease, CD-3+ and CD-8+ cell numbers were increased in early stages within the connective tissue, while CD-20+ cell numbers were increased only in late stages.

In the review it was reported that plasma cells and B cells together comprise about 68% of cells in periodontitis lesions. The proportion of B cells was found to be larger than that of all T cells and T helper cells occured in larger numbers than T cytotoxic cells. PMN cells and macrophages were found in fractions of less than 5 % of all cells (Fig. 2).

Fig.2 Distribution of cell proportions in periodontitis lesions calculated from studies reported in Table 1. Plasma cells, B cells, T helper, T cytotoxic, polymorphonuclear (PMN) cells, macrophages and other cells (e.g. mast cells, fibroblast, unidentified cells). (Adapted from Berglundh & Donati 2005)
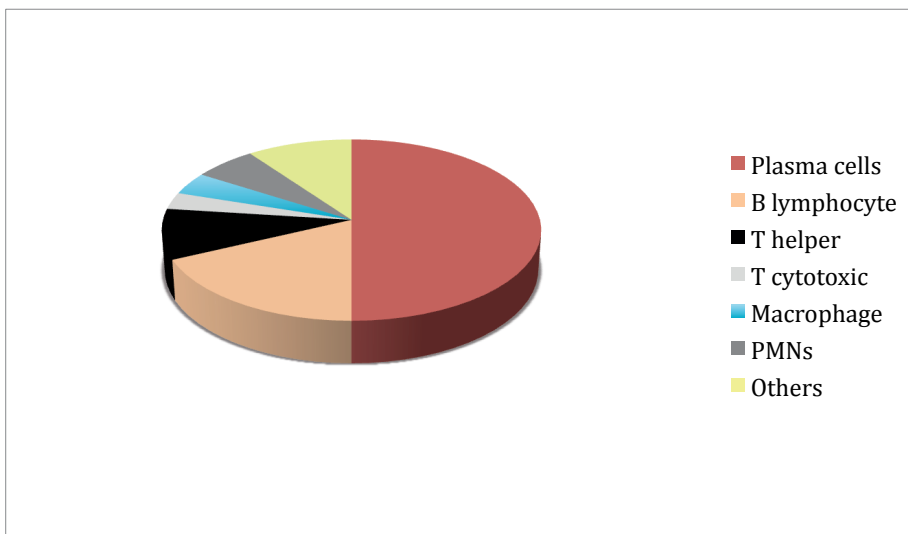
In the review (Berglundh & Donati 2005) it was further observed that lesions in aggressive and chronic forms of periodontitis exhibit similar features with respect to cellular composition. Differences in disease severity, however, may in both forms of periodontitis affect plasma cell and B cell densities. Thus, the proportions of plasma cells and B cells appear to be larger in lesions obtained

from sites representing severe periodontitis than in lesions from areas with moderate or mild periodontitis. The fact that plasma cells develop from B cells and that these two groups of cells dominate in periodontitis lesions calls for the specific attention of the role of B cells in periodontitis.

According to traditional concepts in immunology, B cells serve as a well-controlled part of the adaptive host response and act on systems regulated by T cells. Host defense mechanisms of B cells include the transformation into plasma cells and the production of immunoglobulines, which identify and bind to antigens. Recent reports (Lund et al. 2005; Porakishvili et al. 2001), however, indicate that B cells also participate in several other aspects of the host response and contribute to the activation of the immune system. B cells exhibit important immune-regulatory functions, which include direct and indirect effects of other cells through antigen presentation and production of cytokines.

B-lymphocytes subsets

B cells develop from hematopoietic stem cells initially in the fetal liver before birth and subsequently in bone marrow (Hardy & Hayakawa 2001). Among peripheral mature B cells there are 3 recognized subsets as identified in mouse models and to some extent in humans. First, B-1 cells, or un-conventional B-lineage cells, are divided into B-1a and B-1b. The B-1a cells express the surface marker CD5, while B-1b cells do not. Second, B-2 cells, i.e. conventional B cells, are the traditional and representative B cells of the adaptive immune system. Third, marginal zone (MZ) B cells which are non-circulating mature B cells that segregate anatomically in the marginal zone of the spleen (Martin & Kearney 2000, Lopes-Carvalho & Kearney 2004). The B-2 cells participate in T-dependent germinal center (GC) reactions and yield

isotype-switched, high-affinity memory cells and long-lived plasma cells. On the other hand, B-1 and MZ B cells are residing in specific anatomical compartments (e.g. peritoneal or pleural cavities and splenic MZ respectively) and are responsible for early antibody responses, which may be T cell-independent as well as frequently polyreactive and with low-affinity.

B cells as antigen-presenting cells

The function of B cells in antigen presentation differs to some extent from that of other, so called professional antigen-presenting cells (APCs), e.g. Langerhans cells, macrophages and dendritic cells. Thus, other APCs take up antigens through pinocytosis or internalization of receptors for immune complexes, while B cells internalize antigens by an immunoglobulin-receptor (BCR) in the cell membrane. The antigen is degraded into peptides and subsequently attached to class II molecules of the major histocompatibility complex (MHC II). Finally, the processed antigen is transported to the B cell membrane for presentation to helper T cells (CD4+). B-1a cells may also serve as APCs in the development of an immune response to self-antigens (Liang & Mamula 2000, Porakishvili et al. 2001).

The function of B cells as APCs reported above has also been described in studies on periodontitis. Thus, B cells express class-II antigens upon stimulation and use the capacity of their memory systems in antigen presentation in periodontitis lesions. Mahanonda et al. (2002), who observed a significant upregulation of CD86 and the dendritic cell-marker CD83 on B cells in periodontitis lesions, suggested that B cells may serve as potent APCs in the host response of periodontal disease. Additional evidence on the role of B cells as APCs in periodontitis was provided in study on gingival biopsies obtained from 26 subjects with periodontitis (Gemmel et al. 2002). The

analysis revealed that the B cell group was the predominant type of APCs in the biopsies containing the largest lesions.

Development and function of B-1a cells

B-1a cells are distinguished from conventional B cells (B-2) by their developmental origin, their surface marker expression and their functions. Compared to B-2 cells, B-1a are long-lived and self-renewing, with reduced immunoglobulin receptor (BCR) diversity and affinity (Kantor et al. 1991). B-1a cells also differ from B-2 by the expression levels of several surface markers, including IgM, IgD, CD5, CD43 and B220 (Berland & Wortis 2002).

It has been suggested that immunoglobulin receptor (BCR) signaling is critical for B-1a cell development. Different damage or mutations that may disrupt immunoglobulin receptor signaling result in considerable reduction in B-1a subsets, while minor effects are seen in B-2 cells. On the other hand, mutations that enhance immunoglobulin receptor signaling result in an increased B-1a population (Hardy & Hayakawa 2001, Berland & Wortis 2002). In other words, B-1a cells require an immunoglobulin receptor-generated signal for development, survival, expansion or phenotype expression. In a recent study it was demonstrated that the expression of the marker CD5 makes B-1a cells surviving following the immunoglobulin receptor interaction (Gary-Gouy et al. 2002). It was suggested that CD5 promotes down regulation of the early immunoglobulin receptor reactions and, hence, prevents B-1a cells from immunoglobulin receptor-mediated cell death. In addition, CD5 also enhances the autocrine production of IL-10 and thereby provides a positive regulatory survival "loop" factor for B-1a cells.

B-1a cells generate substantial amounts of natural IgM. This antibody is in general considered to be both polyreactive and weakly autoreactive and responds to many common pathogen-associated carbohydrate antigens (Carroll & Prodeus 1998, Duan & Morel 2006). Although B-1a cells mainly produce natural IgM antibodies that participate in the adaptive immune response (innate-like immune function) in the protection towards bacterial infection and autoimmunity (Gommerman & Carroll 2000, Kruetzmann et al. 2003, Viau & Zouali 2005, Milner et al. 2005) results from recent studies also demonstrated that B-1a cells have the ability to produce antibodies of the IgA isotype in intestinal tissues. Bos and co-workers (2000) suggested that B-1a derived IgA helps to maintain commensal bacteria in the gut, whereas B-2 derived IgA, due to recognition of different epitopes and/or binding with a higher affinity, contributes to the elimination of pathogenic bacteria.

B-1a cells, which have the capacity to produce auto-antibodies with varying degree of affinity, are found in large numbers in peripheral blood of subjects with e.g. Sjögren's syndrome and rheumatoid arthritis (Burastero et al. 1988, Dauphinee et al. 1988, Youinou et al. 1990). It has been demonstrated that B-1a cells, following activation by T cells, undergo class switch and somatic mutation which result in changes from production of low-affinity auto-antibodies to IgG auto-antibodies with high affinity (Taki et al. 1992, Ebeling et al. 1993). Recent studies have also shown that CpG microbial DNA released during infections may exacerbate autoimmunity by stimulating autoreactive B cells to switch from IgM to a more pathogenic IgG isotype independent from T cells (He et al. 2004). In addition, other characteristics, such as the production of high level of IL-10 (O'Garra et al.1992, Stein et al. 1997, Gary-Gouy et al. 2002, Mocellin et al. 2004) and enhanced antigen presentation capacities (Mohan et al.1998, Liang & Mamula 2000,

Porakishvili et al. 2001), as already discussed, have implicated B-1a cells in autoimmunity.

B-1a cells and periodontitis

The presence of B-1a cells in periodontitis patients has been demonstrated in several reports. Afar et al. (1992) collected blood samples from 18 patients with varying severity of periodontitis and from 16 healthy control subjects. Flow cytometry analysis revealed that B-1a cells occurred in significantly larger amounts in periodontitis patients than in controls. Similar results were reported by Berglundh et al. (2002). They analyzed B-1a cells in peripheral blood of 3 different groups of subjects; 22 subjects with severe generalized chronic periodontitis, 7 children with localized aggressive periodontitis and 26 healthy controls. The proportions of B-1a cells were 5-6 times greater in the periodontitis groups than in the controls and it was stated that up to 40-50 % of all circulating B (CD19+) cells were positive to the additional marker of CD5, i.e. the characteristics of B-1a cells. In this context it is interesting to note that systemic levels of B-1a cells seem to be a marker of susceptibility to periodontitis rather than an indicator of the presence of the disease (Berglundh et al. 1999). Berglundh et al. (1999) in a study on local and systemic features of host response in chronic periodontitis before and after non-surgical periodontal therapy, found that the elevated numbers of B-1a cells did not decline after therapy despite the sufficient clinical signs of healing.

Large amounts of B-1a cells were detected in the periodontitis lesions of chronic periodontitis patients in the study by Berglundh et al. (2002). This observation was in agreement with earlier findings by Sugawara et al. (1992) and Aramaki et al. (1998). They found larger amounts of B-1a cells in the

gingival lesions than in peripheral blood and suggested that B-1a cells are activated in periodontitis lesions.

Interleukin-10 and periodontitis

Interleukin-10 is a multifunctional cytokine with diverse effect on most hemopoietic cell types. It is produced by several cell sources such as monocytes/macrophages, dendritic cells (DC), B-lymphocytes (particularly the CD5+ B cells), various subsets of CD4+ and CD8+ T cells (Moore et al. 2001, Mocellin et al. 2004) and also by human keratinocytes (Powrie et al. 1997). The biological activities of IL-10 were historically confined to the suppression of pro-inflammatory cytokines produced by e.g. Th-1 cells (IL-2, INF-gamma, TNF) (Fiorentino et al. 1989), but today it is known that IL-10 has several effects in immunoregulation and inflammation. Thus, IL-10 inhibits MHC class II and co-stimulatory molecule expression on monocytes and macrophages and inhibit macrophage microbicidal activity by downregulating the production of reactive oxygen and reactive nitrogen intermediates, which are involved in macrophage killing mechanisms (Moore et al. 2001). Other reports suggest that IL-10 may not only exhibit inhibitory and anti-inflammtory properties (Lalami et al 1997, Borish 1998). Thus, IL-10 stimulates B cell proliferation, differentiation, antibody production and isotype switch (Beebe et 2002). IL-10 enhances survival of normal human B cells (depending on their activation state), which correlates with increased expression of the anti-apoptotic protein bcl-2 (Levy & Brouet 1994) Furthermore, IL-10 promotes proliferation of autoreactive B cells and auto-antibody production. Indeed, several autoimmune diseases have been associated with increased levels of IL-10.

The presence of IL-10 in periodontitis lesions has been reported previously. Yamazaki and co-workers (1997) applied a reverse transcription polymerase chain reaction technique to determine the level of mRNA for interferon gamma (γ-IFN), interleukin 4 (IL-4), IL-10, IL-12 and IL-13 in gingival biopsies and peripheral blood mononuclear cells (PBMC) obtained from patients with chronic periodontitis. The mean expression of γ-IFN was significantly higher in PBMC than in gingival tissues, while the expression of IL-10 mRNA was higher in gingival tissues than in PBMC. Aramaki and co-workers (1998) analyzed gingival biopsies from 10 periodontitis patients. It was observed that the proportion of autoreactive B lymphocytes (B-1a cells) and the amount of IL-6 and IL-10 were significantly higher in the inflamed gingival tissues than in peripheral blood from the healthy subjects. Increased levels of IL-10 together with large proportions of B-1a cells was also reported in a study on type 1 diabetics by Stein et al. (1997). It was suggested that periodontal pathogens induce a hyperactive IL-10 response leading to proliferation of B-1a cells and to autoantibody production.

Lappin and co-workers (2001) analyzed the occurrence of different cytokines in periodontitis lesions. Tissue biopsies were retrieved from 10 patients with chronic periodontitis and 10 subjects with aggressive forms of periodontal disease. Polyclonal monospecific antibodies and oligonucleotide probes were used to detect cells expressing IL-2, IL-4, IL-6, IL-10, IL-15, TNF α and γ-IFN and it was reported that the most widely expressed cytokine in tissues was IL-10. Furthermore, Nakajima et al. (2005) reported that the expression of IL-10 was higher in periodontitis than in gingivitis lesions. Thus, IL-10 expression is associated with severe periodontitis and appears to be one important cytokine in the regulation of periodontitis lesions.

CD14 and periodontitis

The CD14 molecule is a glycoprotein expressed primarily on surface of monocytes, macrophages, neutrophils and gingival fibroblast (mCD14) and is involved in the process of cellular response to bacterial lipopolysaccharide (LPS) produced by gram-negative bacteria. A soluble form of CD14 (sCD14) is present in serum and derives both from enzymatically cleaved glycosyl-phosphatidylinositol-anchored mCD14 and from secretion of CD14 (Haziot et 1988, Ulevitch & Tobias 1995).

The presence of both the membrane-bound (mCD14) and the soluble (sCD14) forms of the CD14 receptor has been demonstrated in subjects with periodontal disease. Jin & Darveau (2001) demonstrated that subjects with low levels of sCD14 showed greater mean PD and higher percentages of sites with PD$\geq$ 5.0mm, indicating a negative correlation between PD and sCD14 concentrations. Similar results were obtained in another study on antigen presenting cells (APCs) in gingival tissues of different clinical status and inflammatory infiltrates (Gemmell et al.2002). The percentage of the CD14+ cells was more conspicuous in tissue samples with small infiltrates as compared to those with a large ICT. Jin and co-workers (2004) investigated the in vivo expression profile and levels of mCD14 in healthy and diseased gingival tissues. Immunohistochemistry was applied for the detection of the membrane-bound CD14 receptor in 22 biopsies from chronic periodontitis patients and in 7 periodontally healthy tissues used as controls. The mCD14+ cells were mainly confined to the gingival epithelium-connective tissue interface and the expression levels in periodontally healthy subjects were significantly higher than in subjects with periodontitis. The same research group further evaluated the correlation between the expression of the lipopolysaccharide-binding protein (LBP), the mCD14 receptor and the Toll-like receptors (TLR) 2 and 4 in gingival tissues from 43 subjects with chronic

periodontitis and 15 periodontally healthy controls (Ren et al. 2005). While the LBP was confined to the gingival epithelium, the mCD14 was observed around the epithelium-connective tissue interface. The TLR2 was detected in periodontal diseased tissues both in pocket epithelium and in the connective tissues, whereas the TLR4 was predominantly found in connective tissue. In the healthy tissues a weak expression of TLR2 and no presence of TLR4 was detected. A positive correlation was found between the LBP and the mCD14 in both detection and expression levels. The concentration levels of LBP and mCD14 were again significantly higher in healthy individuals than in subjects with periodontitis as reported previously (Jin et al 2004).

Bcl2 protein and periodontitis

Apoptosis, the process of programmed cell death, plays a central role in the immune system in both the maintenance of self-tolerance and homeostatic control of lymphocyte populations. In lymphocytes, apoptosis is mostly recognized to be induced by two major pathways, one of which is associated with mitochondrial permeability changes that lead to the release of cytochrome *c* activating a cysteine protease called caspase-9 responsible for the DNA cleavage, nuclear fragmentation and eventually cell death. Bcl2, member of the Bcl-protein family specifically prevents this pathway of apoptosis by blocking the release of cytochrome *c* (Marsden & Strasser 2005). Recent publications (Gamonal et al. 2001, Bulut et al. 2006) reported on association between the Bcl2 proteins and forms of chronic and aggressive periodontitis. Gamonal et al. (2001) studied the apoptotic markers in gingival tissue of chronic periodontitis subjects and healthy controls. The expression of Caspase-3, Fas, FasL, Bcl2 and p53 was determined by immunohistochemistry. The results demonstrated that only Bcl2 positive cells were significantly higher in biopsies from periodontitis patients than in

control tissues. This finding was confirmed in a recent study by Bulut et al. (2006). They analyzed clinical features and apoptosis proteins (i.e. p53, Bcl2, Caspase-3) in gingival tissues retrieved from 8 subjects with generalized aggressive periodontitis and 10 controls. The immunohistochemical analysis showed that while the expression of caspase-3 and p53 did not differ between diseased and healthy subjects, the frequency of Bcl2 positive cells was significantly higher in lesions from subjects with generalized aggressive periodontitis than in controls.

Susceptibility to periodontitis

Inflammatory and immune reactions to microbial plaque are the predominant features of periodontitis. The host response operates in the gingival tissues to protect against the local microbial challenge and prevents micro-organisms from spreading or invading into the tissues. Epidemiological studies (Loe et al. 1986, Baelum et al. 1986, van der Velden et al. 2006) revealed that differences in periodontal disease among individuals could not be explained by differences in oral hygiene alone and that not everybody is equally susceptibile to periodontitis. Periodontitis is considered to be a complex multifactorial disease where the interaction of multiple genetic and environmental components results into disease expression. It is recognized that complex diseases, such as periodontitis, are associated with variations in multiple genes, each playing a limited role in the expression of the disease and therefore considered disease-modifying genes (Loos et al 2008). Disease-modifying genes differ from major disease genes, which are strongly associated with the expression of the disease.

Evidence for a genetic influence on periodontitis comes from multiple sources including (i) familial aggregation and formal genetic studies of early-onset

forms of periodontitis (Boughman et al. 1992, Marazita et al. 1994) and (ii) twin studies of adult periodontitis (Michalowicz et al. 1991, Corey et al. 1993, Michalowicz et al 2000). Additional support for a genetic contribution for periodontitis emerged from the identification of certain genetic polymorphisms that correlate with immune response phenotypes in certain groups of periodontitis patients.

Genetic polymorphisms and periodontitis

Genetic polymorphisms have historically been used as genetic markers to locate disease-causing genes through linkage studies. However there is increasing appreciation that they may directly influence complex common diseases via a direct effect on gene function. By definition a genetic polymorphism is a nucleotide sequence at particular position (locus) in the DNA molecules exhibiting at least two variants (alleles) that occur in > 1% of a population (Schork et al. 2000). Thus, if the locus is bi-allelic, the most common variant among them occurs in < 99% of the population at large. There are a number of different types of nucleotide structures in the human genome that fits to the definition of genetic polymorphism i.e. *minisatellite variable number tandem repeat* (VNTR), *microsatellite polymorphisms* (STRP) and *single nucleotide polymorphisms* (SNP). A single nucleotide polymorphism is a variation of a single nucleotide at a particular site in the genome. Since each pair of chromosomes (23 pairs in a normal human) is composed by one from the mother and one from the father, a SNP, in a specific locus, defines two alleles for which there could be three possible combinations (*genotypes*) of the two alleles amongst individuals in a given population. A single nucleotide polymorphism may have important biological functions. For example, a SNP located in the promoter region (the region regulating the transcription activity) of the gene may influence the mRNA

expression and possibly the amount of the related protein resulting in biological effects. A SNP in the coding region (the region that codes for amino acids forming a protein) of the gene may result in substitution of amino acids and thereby alter the protein structure and its function.

In a review on putative genetic risk factors for periodontitis, Loos et al. (2005) reported that researchers have focused on genetic polymorphisms related to different aspects of the host immunity. A summary of candidate genes and related proteins as putative risk factors for periodontitis is presented in Table 4.

Table 4. Candidate genes and related proteins for which gene polymorphisms were examined as putative risk factors for periodontitis. (Adapted from Loos et al. 2005)

Gene	Coded protein	Gene	Coded protein
ACE	Angiotensin-converting enzyme	IL4	Interleukin-4
CARD15 (NOD2)	Caspase recruitment domain-15	IL6	Interleukin-6
CCR5	Chemokine receptor-5	IL10	Interleukin-10
CD14	CD-14	LTA	Lymphotoxin-α
ER2	Estrogen receptor-2	MMP1	Matrix metalloproteinase-1
ET1	Endothelin-1	MMP3	Matrix metalloproteinase-3
FBR	Fibrinogen	MMP9	Matrix metalloproteinase-9
FcγRIIa	Fc γ receptor IIa	MPO	Myeloperoxidase
FcγRIIb	Fc γ receptor IIb	NAT2	N-acetyltransferase-2
FcγRIIIa	Fc γ receptor IIIa	PAI1	Plasminogen-activator-inhibitor-1
FcγRIIIb	Fc γ receptor IIIb	RAGE	Receptor adv. glycation end products
FPR1	N-formilpeptide receptor-1	TGFB	Transforming growth factor-β
IFNGR1	Interferon γ receptor-1	TIMP2	Tissue inhibitor of MMP
IL1A	Interleukin-1α	TLR2	Toll-like receptor-2
IL1B	Interleukin-1β	TLR4	Toll-like receptor-4
IL1RN	Interleukin-1 receptor antagonist	TNFA	Tumor necrosis factor-α
IL2	Interleukin-2	TNFR2	Tumor necrosis factor receptor-2

Results from several reviews (Kinane et al. 2005, Loos et al. 2005, Shapira et al. 2005) on gene polymorphisms associated with periodontitis revealed that data from studies on the same gene polymorphism were not always consistent, which may reflect the complexity and heterogeneity of the genetic influence in periodontitis. Thus, suggestions on how to improve the quality of

such studies were highlighted. Overall findings, in these reviews (Kinane et al. 2005, Loos et al. 2005, Shapira et al. 2005) were that studies on gene polymorphisms associated with periodontitis were underpowered for a proper interpretation, had different patient selection, different diagnostic criteria and heterogeneity in the population. Additionally established co-variates such as age, gender and smoking are often not adequately addressed, while a multivariate statistical analysis may better reveal the relevance of the polymorphisms investigated.

IL10 gene polymorphisms and Periodontitis

The human *IL10* (*hIL10*) gene is located on the chromosome 1 at 1q31-32, and it is composed of five exons. At the promoter region, three single nucleotide polymorphisms at position -592 (C→A), -819 (C→T) and -1082 (G→A) (also recognized -597, -824, -1087 respectively) as well as two microsatellites (CA repeat polymorphisms) IL10.G and IL10.R, approximately at position -1200 and -4000, have been reported (Eskdale and Gallagher 1995, Eskdale et al. 1996, Turner et al 1997). The SNPs are in strong linkage disequilibrium forming three *haplotypes* that are common in Caucasian populations; GCC, ACC and ATA (Turner et al. 1997). A difference in IL-10 secretion has been demonstrated between the different *IL10* -1082 (-1087) alleles. Following concanavalin A (Con A) stimulation of peripheral blood (PB) lymphocytes in vitro, carriers of the allele G showed significantly higher IL-10 production than carriers of allele A (Turner et al. 1997). When the relative transcriptional activities of the different haplotypes were compared, the haplotype ATA had a significantly weaker activity than the GCC haplotype (Crawley et al. 1999). In the same study, IL-10 production after LPS stimulation in whole blood cultures was also analysed, and

accordingly, the ATA/ATA genotype was associated with the lowest production. It is thought that the reported SNPs at the promoter of the *IL10* gene occur within important regulatory regions, and that they may alter the structure of the transcription factor binding sites. Some factor-binding sites that might be involved in the regulation of the IL-10 were identified in the *IL10* promoter region (Kube et al. 1995, Eskdale et al 1997, Kube et al. 2001). In a recent study (Larsson et al. 2008) it was demonstrated that PU.1 and Spi-B transcription factors bound the sequence around the -1082 (-1087) position in the *IL10* promoter region. In the same study it was also observed that the Sp1 transcription factor only bound to the G-allele of this site and that this binding resulted in an increase of the promoter activity following lipopolysaccharide (LPS) stimulation of B cells.

IL10 promoter polymorphisms (-1082, -819 and -592) have been associated with other diseases i.e. severe forms of rheumatoid arthritis, SLE, primary Sjögren's syndrome (Huiziga et al. 2000, Hulkkonen et al. 2001, Nath et al 2005) The association between *IL10* gene polymorphisms and periodontitis was also examined (Table 5). The relevance of investigating the *IL10* gene polymorphisms in periodontitis is related to its biological activities in the inflammatory and immune response, particularly in relation to large proportion of B-lymphocytes in periodontitis lesion.

Table 5 Studies on *IL-10* gene polymorphisms in Periodontitis

Reference	Periodontal diagnosis	*IL-10* gene polymorphism	Sample	Ethnicity of subjects	Results
Kinane et al 1999	General Early Onset Periodontitis	Microsatellite IL10.R IL10.G	77 subjects GEOP 102 controls	Caucasian population	No association
Yamazaki et al. 2001	Chronic perio. (moderate to advanced) & EOP	Haplotype of the -1082, -819, -592 gene polymorphisms	34 CP subjects 18 EOP subjects 52 controls	Japanese population	No association
Gonzales et al. 2002	Chronic & Aggressive periodontitis	SNPs -597, -824	23 CP subjects 18 AP subjects 21 controls	Caucasian population	No association
Scarel-Caminaga et al. 2004	Chronic periodontitis	SNPs -597, -824, -1087 & haplotype	67 CP subjects (31 moderate, 36 severe) 43 controls	Mixed population (77% Caucasians background)	Association with the -597 SNP and the -824 SNP
Sumer et al. 2007	Severe Chronic periodontitis	SNPs -597, -824	75 CP subjects 73 controls	Turkish population	Association with the -597 SNP
Reichert et al. 2008	Chronic & Aggressive Periodontitis	SNPs -597, -824, -1087 & haplotype	27 CP subjects 32 AP subjects 34 controls	Caucasian population	Association with the ATA haplotype
Hu et al. 2009	Chronic & Aggressive periodontitis	SNPs -592, -819, -1082 & haplotype	145 CP subjects 65 AP subjects 126 Controls	Chinese & Taiwan population	Association with the -592 & with ATA haplotype

CD14 gene polymorphism and periodontitis

The *CD14* gene is on the chromosome 5 at the location 5q31.1. It has been demonstrated (Baldini et al. 1999) that C to T transition at position −159 (also called -260) is related to the production of the sCD14. Thus, subjects with the homozygous TT genotype exhibited significantly higher sCD14 levels then carriers of CC and CT genotypes. This finding indicated that variants in the promoter region of the *CD14* gene may change the production of CD14 and, thus, influenced, the activation of Th2- to Th1 type cells in the response to bacterial challenge. The -260 *CD14* gene polymorphism has previously been associated with Crohn's disease (Klein et al 2002). The *CD14* gene polymorphisms have been studied in association also with periodontitis (Table 6).

TNFA gene polymorphism and periodontitis

The *TNFA* gene is located on chromosome 6 within the major histocompatibility complex (MHC) gene cluster at the location 6p21.3. Single nucleotide polymorphisms in the gene encoding TNF-α are manly studied in the promoter region at positions -1031, -863, -367, -308, -238, but also in the coding region in the first intron at position +489. The tumor necrosis factor (TNF) is an important mediator in inflammatory reactions and appears to play a central role in the pathogenesis of severe chronic inflammatory diseases (Beutler 1989). Differences in the rate of production of TNF have been demonstrated and a familial ability to produce higher or lower cytokine levels seems to exist (Pociot et al. 1993). The TNF synthesis may be influenced by the presence of certain gene polymorphisms (Duff 1994, Wilson & di Giovine 1995). Some consistent results on association of *TNFA* gene polymorphisms with diseases are reported for infectious diseases particularly malaria (Beyley

et al. 2004). *TNFA* gene polymorphisms were also investigated in association with periodontitis (Table 7).

IL-4R gene polymorphism and periodontitis

Interleukin-4 (IL-4) is a cytokine that is involved in the proliferation and activation of B-lymphocytes. This cytokine can rescue B-lymphocytes from apoptosis and enhance their survival, thus playing a role in promoting B-lymphocytes mediated autoimmunity (Illera et al. 1993, Mori et al. 2000, Singh 2003). It is produced by T helper 2 cells and binds to the IL-4 receptor (IL-4R) of B cells (for review see Gemmell et al. 1997; Gemmell et al. 2002). The *IL4RA* gene is on chromosome 16 at location 6p12.1-p11.2. Variations in the *IL4RA* gene have been associated with different human disorders (Hershey et al. 1997, Kruse et al. 1999, Mitsuyasu et al. 1999). The gene polymorphism at the position 551 at promoter region was shown to be associated also with SLE and primary Sjögren's syndrome (pSS) (Youn et al. 2000). No data are available concerning the possible association of *IL-4RA* gene polymorphisms and periodontal disease.

Table 6. Studies on *CD14* gene polymorphisms in Periodontitis

Reference	Periodontal diagnosis	*CD14* gene polymorphism	Sample	Ethnicity of subjects	Results
Holla et al 2002	Chronic periodontitis	SNPs -159, -1359	135 CP (36 moderate, 99 severe) 207 controls	Caucasian population	No association with the -159, association with the -1359 (GG genotype)
Yamazaki et al. 2003	Chronic periodontitis	SNP -159	163 CP 104 controls	Japanese population	Different distribution of genotypes between older and younger subjects
Folwaczny et al. 2004	Chronic periodontitis	SNP -159	70 CP 75 controls	Caucasian population	Association with the C allele in female
Laine et al. 2005	Chronic periodontitis	SNP -159	100 severe CP 99 controls	Caucasian population	Association with TT genotype
Tervonen et al. 2007	Chronic periodontitis	SNP -159	51 CP (moderate to severe) 178 controls	Caucasian population	Association with the T allele carriage

Table 7. Studies on *TNFA* gene polymorphisms in Periodontitis

Reference	Periodontal diagnosis	*TNFA* gene polymorphism	Sample	Ethnicity of subjects	Results
Galbraith et al. 1998	Chronic periodontitis	SNPs -238, -308	32 subjects CP 32 controls	Caucasian population	No association
Galbraith et al. 1999	Chronic periodontitis	SNP -308	20 subjects CP 20 Gingivitis 45 controls	Caucasian population	Association with *TNFA* allele1 (CP> Gingivitis subjects)
Endo et al 2001	Generalized EOP	SNPs -238, -308, -1031	64 subjects G-EOP, 64 controls	Japanese population	No association
Craandijk et al. 2002	Chronic periodontitis	SNPs -238, -308, -367, +489	90 subjects CP 264 controls	Caucasian population 81% non-Caucasian 19%	No association
Soga et al. 2003	Aggressive periodontitis	SNPs -1031, -308, -238	64 subjects AP 64 controls	Japanese population	No association
Folwaczny et al. 2004	Chronic periodontitis	SNP -308	81 subjects CP 80 controls	Caucasian population	No association
Maria de Freitas et al 2007	Generalized Aggressive periodontitis	SNP -308	30 subjects GAP 70 controls	Brazilian population	No association

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Aims

The main objectives of the present studies were:

1. to study the association of an interleukin *(IL)10* gene polymorphism (G to A transition at the -1087 position) with severe chronic periodontitis.

2. to study the association of gene polymorphisms related to some immune regulation components (G-308A *TNFA*, Q551R *IL4RA* and C-159T *CD14*) with severe chronic periodontitis.

3. to study the local expression of IL-10 and membrane-bound CD14 (mCD14) in relation to the -1087 *IL10* and -159 *CD14* gene polymorphisms in subjects with chronic periodontitis.

4. to study the correlation between inflammatory cells and functional markers in gingival lesions obtained from subjects with severe chronic periodontitis.

5. to study the reaction of B-1a cells to *de novo* plaque formation in subjects who were treated for severe chronic periodontitis.

Material and Methods

Study population (**study I and II**)

The regional human ethics review board, University of Gothenburg; Sweden approved the protocol of both studies. Prior to enrollment all subjects received information regarding the purpose of the studies and gave their written consent. Two groups of Caucasian subjects were included. The test group consisted of 60 patients (28 females and 32 males, aged 36 – 74 years; mean 54.5 ± 8.5) with generalized, severe chronic periodontitis (Consensus Report, 1999). The patients were recruited from the Clinic of Periodontics, Gothenburg and exhibited bone loss >50% at all teeth. Prior to periodontal therapy they all had probing pocket depths (PPD) >6 mm and bleeding on probing (BoP) at >80% of the proximal sites. Sets of intraoral radiographs were obtained using a standardized parallel technique (Eggen 1969). In the radiographs, the distance between the cemento-enamel junction (CEJ) and the most coronal level of the bone crest (BC) was assessed at the mesial and distal aspects of each tooth. For details regarding the radiographic measurements, see Berglundh et al. (1998).

Thirty-nine periodontally healthy Caucasian subjects (24 females and 15 males) between 35 – 78 years of age (mean 51.0 ± 10.9) were also recruited (Control group). The subjects in this group demonstrated normal radiographic bone levels, i.e. a distance of < 3 mm between the CEJ and BC at > 95% of the proximal tooth sites.

Study population (**study III-V**)

The regional human ethics review boards, University of Gothenburg, Sweden and University of Basel, Switzerland approved the protocols of studies III and IV. The regional human ethics review board, University of Basel approved

the protocol of study V. Prior to enrollment all subjects received information regarding the purpose of the studies and gave their written consent.

In **study III** fifty-three Caucasian subjects (23 males and 20 females; mean age: 53.8 ± 9, range: 42 to 65 years) were recruited from the Clinic of Periodontics, Public Dental Services, Gothenburg, Sweden. Ten patients (6 male and 4 female mean age 55.9± 7, range 43-67 years) were enrolled from the Clinic of Reconstructive Dentistry, University of Basel in Switzerland.

In **study IV** thirty-eight Caucasian subjects volunteered. Twenty-eight subjects were recruited from the Clinic of Periodontics, Public Dental Services Gothenburg (15 male and 13 female mean age 52.2±7.5, range 43-69 years). Ten patients (6 male and 4 female mean age 55.9± 7, range 43-67 years) were enrolled from the Clinic of Reconstructive Dentistry, University of Basel in Switzerland.

In **study V** fifteen Caucasian subjects with generalized, severe chronic periodontitis (10 male and 5 female mean age 56.1± 8.4, range 38-69 years) were enrolled from the Clinic of Reconstructive Dentistry, University of Basel in Switzerland.

All patients (study III, IV, V) had generalized, severe chronic periodontitis and exhibited >50% bone loss at all remaining teeth, probing pocket depth (PPD) >6 mm and bleeding on probing (BoP+) at >80% of the proximal sites prior to periodontal therapy.

Blood sampling, DNA separation and genotype detection (**study I-III**)
Samples of peripheral blood were obtained by venipuncture from the arm vein of each subject. The 20 ml blood sample collected in EDTA tubes was given a code and was stored in −70° until further processing.

DNA was isolated from EDTA blood by modified "salting-out" method without SDS as described by Padyukov et al. (2001). Final preparations were stored at -20° C until used as template DNA for PCR.

Detection of the -1087 *IL10* polymorphism (**study I**) and Q551R *IL4RA* polymorphism (**study II**) was performed as described previously (Padyukov et al., 2001, Hytonen et al., 2004). Genotyping of -308 *TNFA* and -159 *CD14* was not previously published. For all methods, specific primers for generic PCR amplification were used with subsequent cleavage of the amplicons with restriction endonuclease (Table 8). Polyacrylamide gel electrophoresis (PAGE) was performed in NOVEX Xcell MiniCell in either a 4 -20% gradient or in continuous 20% Tris-Borate-EDTA gels with following developing by silver staining.

Table 8. Description of genetic markers and genotyping procedures

Polymorphism	rs number	Primers	Enzyme
G-1087A *IL10*	rs1800896	5'AAGGCAACACTACTAAGGCTTCCTT3' 5'TAAATATCCTCAAAGTTCC3'	*EcoN*I
G -308A TNFA	rs1800629	3'ACACAAGCATCAAGGATACCCCTCAC3' 5'CTCCCCCTCAACGGACTCAGC3'	*BsmF1I*
Q551R IL4RA	rs1801275	5'TGGCCGCACAGGTGGCAGGTAA3' 5'TCGGCCCCCACCAGTGGCTACC3'	*BsmFI*
C-159T CD14	rs5744433	5'AGGCTTAGGCTCCCGAGTCAA3' 5'TCCTACACAGCGGCACC3'	*HpyCH4 III*

Biopsy sampling (**study III-V**)

In study III and IV a gingival biopsy was obtained from one randomly selected diseased site (probing pocket depth > 6mm and bleeding on probing positive) from each subject.

In study V gingival biopsies were obtained from each patient at two different time points; (i) at baseline (healed sites) after a 6-month period of meticulous

plaque control (PPD<4mm and BoP-) following periodontal surgery and (ii) after a 3-week period of plaque accumulation. The biopsies were retrieved as reported in Zitzmann et al. (2005). Following local anesthesia, two parallel incisions, 4mm apart, were made through the soft-tissue until bone contact was achieved. The two incisions were connected with a perpendicular incision placed about 4mm apical of the gingival margin. The biopsies were carefully removed and prepared for immunohistochemical analysis.

Immunohistochemical processing (**study III-V**)

The tissue samples were rinsed in saline and subsequently embedded (O.C.T. Compound; Tissue Tek®, Miles, Elkhart, IN), snap frozen in liquid nitrogen and stored in −70°C. From each tissue portion, 5µm thick sections were prepared in a cryostat and exposed to immunohistochemical staining. A panel of monoclonal antibodies was used and a standard avidin-biotin peroxidase (ABC) method (Hsu et al. 1981) was applied for the staining (Vectastain® Elite® ABC Kit, Vector® Vector laboratories, Inc.CA). A diaminobenzidine (DAB) peroxidase kit (Vector® Vector laboratories, Inc.CA) was used as chromogene and the sections were counter stained with methyl green (Vector® Vector laboratories, Inc. CA). In each staining performed, negative controls without the primary antibodies were included. Sections of human tonsils were used as positive controls. Immunohistochemical staining for IL-10 was performed according to a method described by Whiteland et al. (1997).

The identification of the autoreactive B cells (**study IV, V**) was performed as previously reported by Berglundh et al. (2002). Texas Red Avidin and FITC Avidin (Fluorescent Avidin Sample Kit® Vector Laboratories, Ca., USA) were used in a double staining to detect CD5 and CD19 positive cells. The sections were dried and mounted with Vectashield® mounting medium with

4,6 diamidino-2 phenylindole (DAPI) (Vector Laboratories, Ca., USA) to preserve fluorescence and to counterstain cell nuclei.

The panel of antibodies used with their isotype and dilutions are presented in Table 9. The CD3 monoclonal antibody was used to identify all T cells, while CD4 and CD8 monoclonal antibodies identified T-helper cells and T-cytotoxic cells, respectively. The CD19 marker detected B-cells and CD138 identified plasma cells. Monoclonal antibodies were also used to identify elastase (Neutrophil protease), CD14 (specific receptor for lipopolysaccharides) and Bcl2 oncoprotein (blocker of apoptotic cell death).

Table 9. Specificity of mouse anti-human monoclonal antibodies used for immunohistochemical analisys.

Antibodies (clone)	Specificity	Dilutions	Isotype	Study
CD3 (T3-4B5)[*]	pan T cells	1:50	IgG1	III
CD4 (MT310)[*]	T-helper cells	1:20	IgG	IV
CD8 (DK25)[*]	cytotoxic T-cells	1:50	IgG1	IV
CD19 (HD37)[*]	B cells	1:25	IgG1	III, IV
CD5 (D23)[*]	T & B-cell subsets	1:50	IgG1	IV
CD138 (B-54)[#]	Plasma cells	1:500	IgG1	IV
Elastase (NP57)[*]	Neutrophils	1:400	IgG1	IV
CD14 (TÜK4)[*]	LPS receptor	1:20	IgG2a	III, IV
Bcl2 (124)[*]	Anti-Apoptosis protein	1:100	IgG1	IV
IL-10 (23738.111)[§]	Interleukin-10	1:100	IgG2b	III

IgG = Immunoglobulin G
[*]DakoCytomation, Glostrup, Denmark
[#]Serotec, Oxford, UK
[§] R&D Systems Europe, Oxon, U.K.

Histological analysis (**study III-V**)

The histological analysis of the inflammatory cell infiltrate was performed using a Leitz DM-RBE microscope equipped with a Leica Q-500 MC® image system (Leica, Wetzlar, Germany). The size of the infiltrated connective tissue (ICT) was measured using a mouse cursor (magnification x50). For the assessment of the density of labelled cells a point counting procedure was applied as previously described (Liljenberg et al. 1994, Fransson et al. 1999, Zitzmann et al. 2005). Thus, a 400-point lattice was superimposed over the tissue area at a magnification x400 and the number of cross points on positive cells was counted. In addition, the number of positive cross-points was related to the total number of points and finally expressed as percentage (%) of the tissue area. The number of grids used to analyse the sections varied between 2 and 8 depending on the size of the ICT. The assessment of the IL-10 positive cells (**study III**) was performed in two compartments: one within the ICT and one zone (~200 μm wide) in the periphery of the ICT (IL-10 per).

A fluorescence microscope (Leica DM-RXA, Wetzlar, Germany) equipped with a COHU CCD camera and connected to a Leica Q-Fish software (Cambridge UK) for microphotography was used to obtain digital images from sections that were double stained for CD5 and CD19 (**study IV, V**). Digitized images from the lesion including cells stained with FITC and Texas Red were captured and composite images were obtained by integrating the Texas Red (CD5) and FITC (CD19) signals. Cells were identified as positive when presenting either the red (CD5) or the green (CD19) marker on their cellular membranes. Cells positive for both markers (CD5 and CD19) were disclosed by the presence of both the green and the red colour (B-1a cells). Cells positive for both markers (CD5+CD19) were related to the overall number of CD19 positive cells and expressed as percentage as previously reported (Berglundh et al 2002).

Data Analysis

In study I and II the distribution of frequencies of genotypes for each group was compared using the Chi-Square (c^2) test, while the frequencies of allele carriage and the allele frequency in the test and control group were compared using the Fisher's Exact Test. All analyses, except for the allele frequency assessment, were performed using the subject as the statistical unit, i.e. n=60 (test; severe chronic periodontitis) and n=39 (control), respectively. Comparisons of allele frequencies were performed using the chromosome as the statistical unit. Relative risks, i.e. case-control estimates and confidence limits were also calculated. All genetic markers in the studies were found to be in Hardy-Weinberg equilibrium.

In study III, IV and V, mean values and standard deviations were calculated for each variable using the subject as the experimental unit. In study III, differences in the proportions (%) of the various positive cells markers in the ICT between the different genotype groups were analyzed using analysis of variance (ANOVA) and the Student-Newman-Keuls test. In study IV, correlations between the different percentages of cell markers were analyzed by pairwise correlation analysis (Pearson correlation coefficients). In study V, differences in cell densities between baseline (healed) sites and sites representing 3 weeks of plaque accumulation were analyzed using the Student's *t*-test for paired observations. In all studies P values <0.05 were considered as significant.

Results

Study I and II

Radiographic observations

The amount of bone loss assessed in the radiographs from the group of subjects with chronic periodontitis varied between 7.7 and 13.6 mm (mean 9.0 ± 1.4 mm). The mean number of remaining teeth was 21.2 ± 4.6 in the periodontitis group and 27.3 ± 1.4 in the control group.

Genotype frequencies

The distribution of genotypes of the gene polymorphisms examined (i.e. G-1087A *IL10*, G-308A *TNFA*, Q551R *IL4RA* and C-159T *CD14*) within the chronic periodontitis group did not differ from that in control group. The distribution of genotypes within the fraction of non-smokers, however, differed between the test and control groups of subjects in relation to the G-1087A *IL10* gene polymorphism (Fig.3). A significantly larger proportion of subjects in the chronic periodontitis group exhibited the homozygous genotype GG than subjects in the control group (61% vs. 21%). The homozygous AA genotype, on the other hand, occurred in a larger fraction among controls than in subjects with chronic periodontitis (10% vs. 35%). In the periodontally healthy group there was a higher proportion (44% vs. 29%) of the heterozygous (GA) genotype than in the diseased group. The analysis of the distribution of the genotypes within the fraction of non-smokers for all other gene polymorphisms (i.e. G-308A *TNFA*, Q551R *IL4RA* and C-159T *CD14*) did not reveal significant differences.

Fig. 3 Distribution of IL-10 genotypes in non-smokers
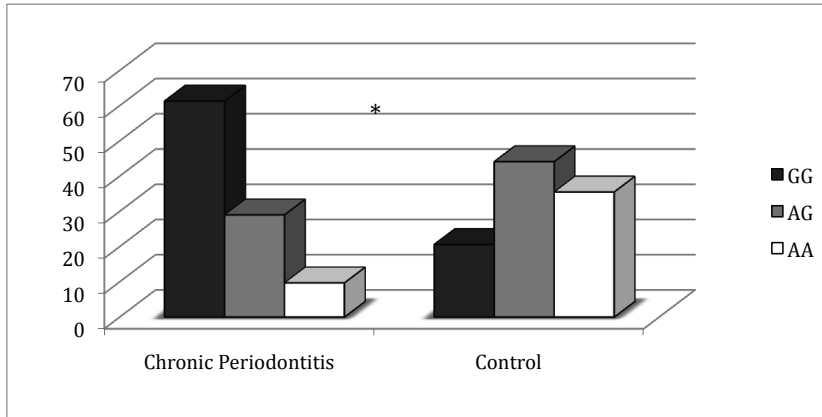
* indicates p<0.05 X^2

Frequencies of the allele carriage

The frequency of the allele carriage, i.e. the proportion of subjects in the two groups carrying one of the alleles was calculated for each gene polymorphism.

While the frequencies of the G allele carriage in study I did not show significant differences between the chronic periodontitis subjects and the healthy controls when evaluating the entire cohort, the analysis of the non-smoking category of subjects revealed that the G allele was found in 90% of the diseased individuals and in 65% of the healthy subjects. This difference was statistically significant (p<0.005 Fisher's Exact test) (Fig.4). Consequently, a significantly larger proportion of subjects with the AA genotype (i.e. no allele G) was found in the control group than in the test group (35% vs. 10%).

Fig.4 *IL10* gene polymorphism. Percentage of subjects carrying any or no allele G in non-smokers of chronic periodontitis group and control group.
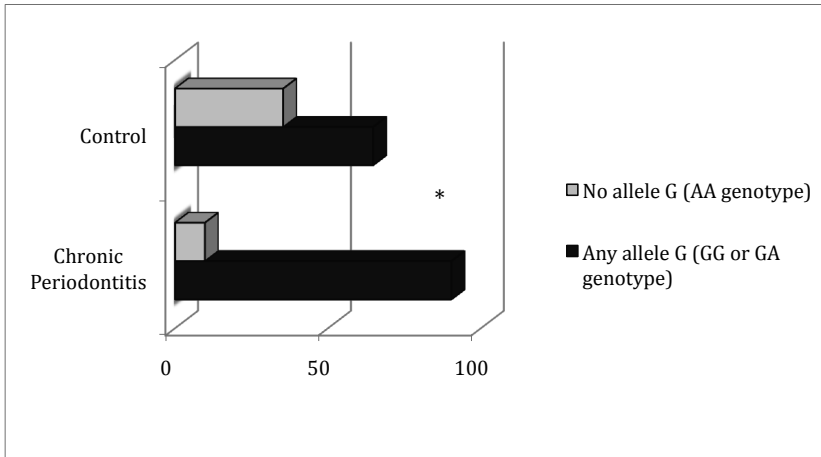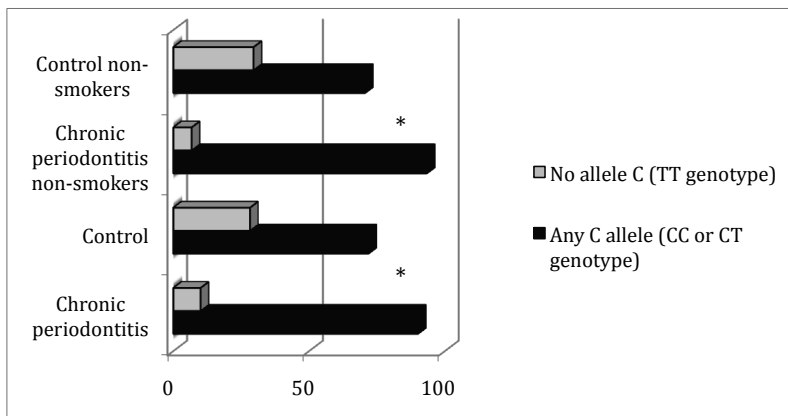

* indicates p<0.05 Fisher's Exact test

The case-control estimates, i.e. the relative risk for the variables GG genotype and G-allele are presented in Table 10. The GG genotype revealed an odds ratio of 2.58 for all subjects in the two groups, while in the non-smoking fraction the ratio reached 6.11. The corresponding estimates for the G allele frequency was for all individuals 1.46 (all subjects) and 5.09 (non-smokers).

Table 10. *IL10* gene polymorphism. The case-control estimate for the variables GG-genotype and G-allele

Variable	p-value	Odds ratio	95% confidence limit
GG-genotype (all subjects; n=99)	0.049	2.58	1.01-6.56
GG-genotype (non-smokers; n=65)	0.001	6.11	2.03-18.37
G-allele carriage (all subjects; n=99)	0.480	1.46	0.59-3.61
G-allele carriage (non-smokers; n=65)	0.019	5.09	1.27-20.29

In study II the analysis of the frequencies of the allele carriage related to the G-308A *TNFA* and Q551R *IL4RA* gene polymorphisms did not reveal significant differences between chronic periodontitis subjects and healthy individuals both for the entire cohort and for the non-smoking fraction of subjects. The analysis of the frequencies of the allele carriage related to the C-159T *CD14* gene polymorphism, however, demonstrated that the proportion of subjects that exhibited the TT genotype was significantly smaller in the group with severe chronic periodontitis than in healthy group ($p < 0.005$ Fisher's Exact test) both in the entire cohort and in the non-smoking fraction of subjects (Fig.5).

Fig.5 *CD14* gene polymorphism. Percentage of subjects carrying any or no allele C in the periodontitis and control groups (all subjects and non-smokers)

* indicates $p < 0.05$ Fisher's Exact test

Study III

Genotype frequencies

The IL10 genotype frequencies assessment revealed that the homozygous GG genotype was detected in 26.4% (14 out of 53) of the subjects, while the homozygous AA genotype was found in 16.9% (9 out of 53). The remaining

56.6% (30 out of 53) of the subjects consequently exhibited the heterozygous genotype (GA). Regarding the CD14 genotype the prevalence of the heterozygous genotype (TC) (52.8%, 28 out of 53) was higher than the two homozygous genotypes CC and TT (26.4% and 20.7%).

Proportion of IL-10+ cells in relation to IL-10 Genotype

The differences in the proportion (%) of IL-10+ cells in the central area of the ICT were not statistically significant between subjects carrying different IL-10 genotypes, i.e. GG, AG and AA. Conversely, in the peripheral area of the ICT (IL-10 per), subjects with the GG genotype showed significantly larger proportions of IL-10+ cells than subjects carrying the AG or the AA genotypes (Table 11). No statistically significant differences were found between the three CD14 genotypes, i.e., CC, TC and TT, with regard to the local expression of the mCD14 receptor.

Table 11. Proportions (%; mean ± SD) of IL-10+ and CD14+ cells in the ICT in relation to the -1087 *IL10* and the -159 *CD14* Genotypes

| *IL10* Genotype	IL-10	IL-10 per	*CD14* Genotype	mCD14
GG	0.60 ± 0.51	1.12 ± 0.47*	**TT**	3.20 ± 2.15
AG	0.45 ± 0.30	0.71 ± 0.37	**TC**	3.51 ± 1.86
AA	0.65 ± 0.43	0.75 ± 0.43	**CC**	3.28 ± 2.43

* indicates p<0.05 between GG and AG and between GG and AA (ANOVA and Student-Newman-Keuls test)

Study IV

Immunohistochemical analysis

The results of the immunohistochemical analysis revealed that B-cells (B-1 and B-2 cells) occurred in larger proportions than T cells and plasma cells. Thus, the percentage of the surface area of the ICT occupied by CD19-positive cells was 8.19 %. The corresponding figures for the B-1a cells (CD19+CD5), T-helper (CD4) and T-cytotoxic (CD8) lymphocytes, plasma cells (CD138) and elastase-positive cells were 5.0 %, 4.04%, 2.16%, 4.36% and 2.56% respectively (Table 2). The density of CD14 and Bcl2 positive cells were 3.65% and 2.96%, respectively. The relative proportion of phenotype markers of the inflammatory cells is presented in Fig. 6.

Fig. 6 Relative proportion of phenotype markers of inflammatory cells in periodontitis lesions.
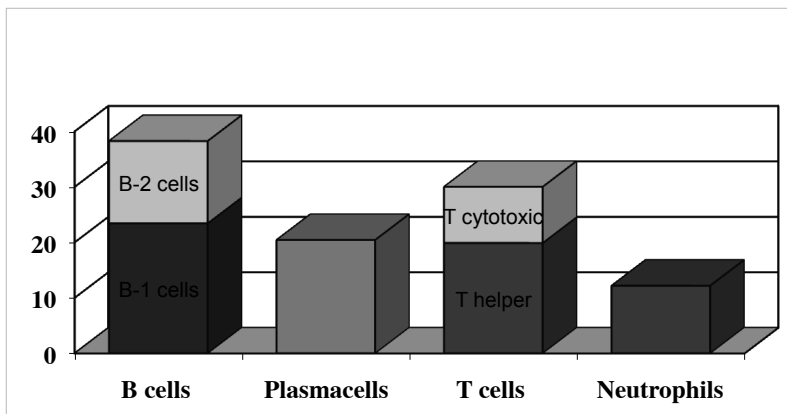
Pairwise correlation analysis

The pairwise correlation analysis revealed a statistically significant correlation between the percentages of B-1a cells (CD5+CD19) and plasma cells (Fig. 7) and between all B-lymphocytes (CD19) and plasma cells. A significant correlation was also observed between the densities of T-helper and T-cytotoxic cells. The proportion of cells that expressed the functional marker CD14 correlated with the fraction of B-lymphocytes, plasma cells, T-helper cells and T-cytotoxic cells. Correlations were also found between Bcl2 positive cells and T-helper and T-cytotoxic cells.

Fig. 7 Correlation between the percentages of B-1a cells and plasma cells
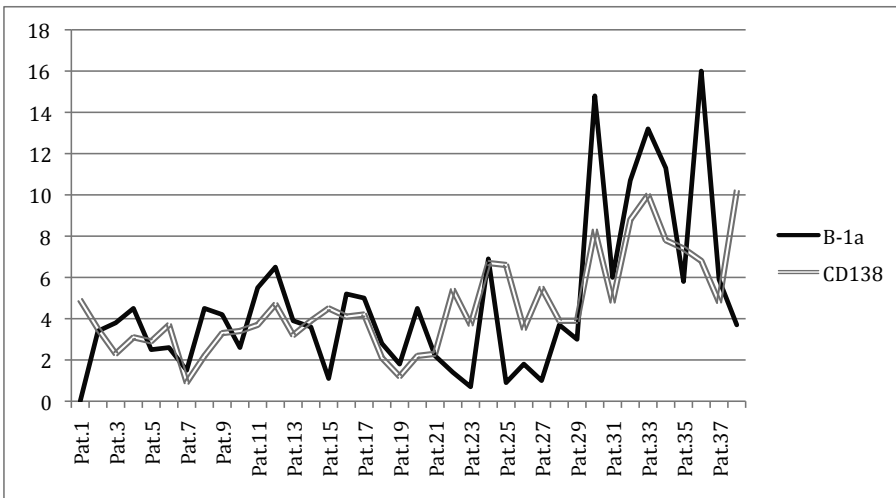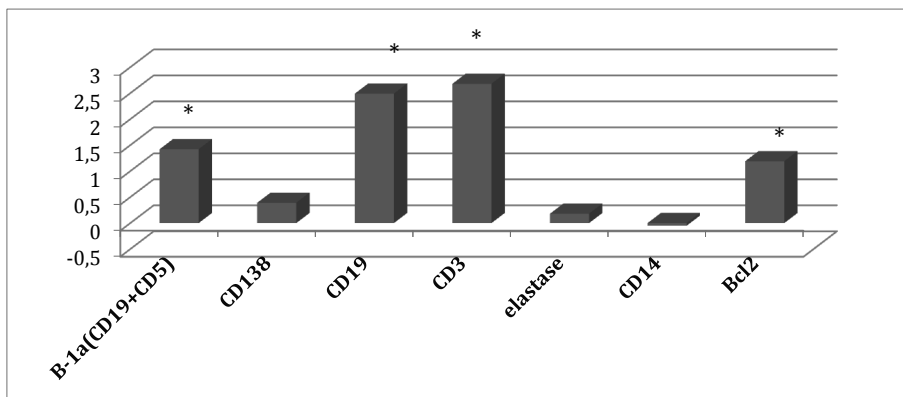

Study V

All specimens sampled at baseline (healed sites) harbored small inflammatory cell infiltrates (ICT), the size of which was 0.13 ± 0.09 mm^2. In sections representing 3 weeks of plaque accumulation the size of the ICT was 0.25 ± 0.18 mm^2. The difference between the two groups of specimens was statistically significant.

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Immunohistochemical analysis

The biopsies retrieved after 3 weeks of plaque accumulation contained larger proportions of CD19+CD5 positive cells than biopsies representing baseline (7.38 ± 2.80 *versus* 5.96 ± 2.48). This difference was statistically significant. The tissue fraction of cells carrying the CD3 and the CD19 markers were significantly larger at sites after 3 weeks of plaque accumulation than at baseline (7.85 ± 2.40% *vs* 5.17 ± 1.74% and 10.09 ± 3.11% *vs* 7.96 ± 2.56%). Although not statistically significant, the proportions of CD138 and elastase positive cells were larger in sections representing 3 weeks of plaque accumulation than in sections representing baseline. While the density of cells positive for the functional marker CD14 was almost similar in sections from baseline (2.03 ± 0.80%) and 3 weeks (1.98 ± 0.55%), the proportion of Bcl2 positive cells was significantly larger in the 3-week than in baseline specimens (3.65 ± 1.62% *vs* 2.46 ± 1.23%).

Fig. 8 Histogram illustrating changes in cell densities between baseline and 3 weeks of experimental gingivitis (* indicates p<0.05 Student's t-test).

Main Findings

The proportion of subjects that exhibited the −1087 *IL10* GG genotype, was significantly larger in the group with severe periodontitis than in the group of healthy controls (**Study I**).

The proportion of subjects that exhibited the −159 *CD14* TT genotype, was significantly smaller in the group with severe periodontitis than in periodontally healthy group (**Study II**).

The proportion of IL-10 positive cells in the peripheral area of the periodontitis lesions was significantly larger in subjects with the −1087 *IL10* GG genotype than in subjects with AG or AA genotypes (**Study III**).

B cells (B-1a cells and B-2 cells) occurred in larger proportions than T cells, plasma cells and neutrophils in periodontitis lesions. A significant correlation was found between percentages of B-1a cells and plasma cells and between densities of B-lymphocytes and plasma cells (**Study IV**).

Biopsies retrieved after 3 weeks of plaque accumulation contained larger proportions of B-1a cells than biopsies representing healthy sites (**Study V**).

Concluding remarks

Study I

In Study I the frequency of -1087 *IL10* alleles in subjects with generalized, severe chronic periodontitis was analyzed. It was demonstrated that the proportion of subjects that exhibited the GG genotype was significantly larger in subjects with severe periodontitis than in periodontally healthy individuals. The difference regarding the occurrence of the GG genotype between the two groups was more conspicuous in non-smokers and yielded an odds ratio of 6.1. The G allele carriage in non-smokers was > 90 % in the periodontitis group and was significantly larger than in healthy controls.

Yamazaki et al. (2001) studied the association of an *IL10* gene haplotype, i.e. the combination of −1082, -819 and −592 (-1087, -824, -597) polymorphisms with periodontitis in a Japanese population. It was demonstrated that none of the haplotypes could be linked to subjects with periodontitis and that the occurrence of a G allele at position −1082 (-1087) in such patients was in relative terms low. Mellati et al. (2007) studied the association of the -1082 (-1087) *IL10* gene polymorpism with subjects suffering from aggressive periodontitis in a population of Iranian Khorasanian. The authors observed no significant differences in terms of genotype frequencies between periodontitis subjects and controls. One reason for the difference between the studies by Yamazaki et al. (2001) and Mellati et al. (2007) on the one hand and Study I of the present thesis on the other, is related to the ethnic origin of the populations examined. In Study I, Caucasians of a north European origin were included, while the participants in the study by Yamazaki et al. (2001) and Mellati et al. (2007) were Japanese and Iranian respectively. Several cytokine gene polymorphisms were analyzed in another oriental population (Hong Kong Chinese) by Padyukov et al. (2001). It was demonstrated that the G

allele in position −1087 was very rare in the Chinese population (allele frequency 0.05 in comparison to 0.53 in Swedish Caucasians). This demonstrates that the interpretation of findings regarding gene polymorphisms also must be related to the ethnic origin of the study population.

The finding of an association between the polymorphism in the *IL10* gene and periodontitis in the Study I is also in contrast to data reported by Brett et al. (2005) and Babel et al (2006). In these studies, subjects of a north European origin were recruited to evaluate the association of the -1082 (-1087) *IL10* gene polymorphisms with chronic periodontitis. Subjects were selected according to the International Workshop for a Classification of Periodontal Diseases and Conditions (Armitage, 1999) but no stratification regarding smoking status of the population was applied. The results of both studies failed to demonstrate an association between the -1082 (-1087) *IL10* gene polymorphism and periodontitis. It should be emphasized, however, that established environmental risk factors for periodontitis, such as smoking, may modulate gene expression and protein production in the periodontium (Cesar-Neto et al. 2007 and Torres de Heens et al. 2009). This finding suggests that established risk factors, such as smoking, should be taken in to consideration when evaluating the influence of genetic polymorphisms in the susceptibility to periodontitis.

The association of a haplotype of three *IL10* gene polymorphisms (-1082 G>A, -819 C>T, -590 C>A) with periodontitis also was investigated. Scarel-Caminaga et al. (2004) and Reichert et al. (2008) observed that the ATA haplotype was more prevalent in the chronic periodontitis group in subjects from the South-eastern of Brazil and in German Caucasian subjects with aggressive periodontitis. Cullinan et al. (2008) demonstrated that individuals

having either the ATA/ACC or the ACC/ACC genotype had around 20% fewer sites with probing depth of ≥ 4 mm than individuals with other genotypes. The difference between the studies referred to (Scarel-Caminaga et al. 2004, Reichert et al. 2008 and Cullinan et al. 2008) and the Study I of the present series may be explained by the selection of periodontitis subjects. In Study I a selection of subjects with severe chronic periodontitis (bone loss >50% at all the teeth and PPD > 6mm and Bop+ at >80% of the proximal sites prior to therapy) was carried out, while in the studies by Scarel-Caminaga et al. (2004), Reichert et al. (2008) and Cullinan et al. (2008) the selection of periodontitis cases included subjects with different disease severity (from mild to severe). The heterogeneity in the selection of periodontitis cases may be considered to be one of the major problems in the interpretation of various studies on to genetic risks factors for periodontitis (Loos et al. 2008).

Study II

In Study II the association of some immune regulation gene polymorphisms (G-308A *TNF A;* Q551R *IL4RA* and C-159T *CD14*) with severe chronic periodontitis was studied. It was demonstrated that while the gene polymorphisms for *TNF A* and *IL-4RA* did not show any association with severe chronic periodontitis, the analysis of the -159 *CD14* gene polymorphism revealed significant differences between test and control groups. Thus, the proportion of subjects that exhibited the TT genotype was significantly smaller in the group with severe periodontitis than in periodontal healthy group. Further, the C allele carriage was 90% in the periodontitis group and significantly higher than in the healthy control group (72%).

Holla et al. (2002) studied two CD14 gene polymorphisms (at position -159 and -1359) in relation to severity of chronic periodontitis in subjects of a Czech ethnicity. It was reported that the distribution of the genotypes did not differ between periodontitis subjects and healthy controls. Holla et al. (2002) further reported that the TT genotype tended to be more frequent in subjects with severe periodontitis than in subjects with moderate periodontitis. This finding is not in agreement with observations made in Study II of the present series. Thus, in Study II a higher frequency of the TT genotype was found in the control group than in the periodontitis group. In other words, a statistically significant difference between test and control subjects regarding the carriage of allele C was found, which indicated a larger proportion of the TT genotype in control subjects (28.2%) than in subjects with severe chronic periodontitis (10.0%).

The reasons for the conflicting results in the Study II and in the study by Holla et al. (2002) are not presently understood but may be related to the inclusion criteria of subjects in the test and control groups as well as differences in genetic structure of the populations. Yamazaki et al. (2003) reported on the occurrence of the -159 *CD14* gene polymorphism in a Japanese population. It was demonstrated that genotype distribution (CT, CC or TT) and the allele frequency did not differ between periodontitis patients and control subjects. Also the results reported by Yamazaki et al. (2003) regarding the -159 *CD14* gene polymorphism are in contrast with the findings made in Study II. In particular, a smaller proportion of the subjects in the test group in the current study exhibited the TT genotype as compared to the test subjects in the study by Yamazaki et al. (2003). While differences may exist with respect to inclusion criteria in test and control groups in the present material and the study by Yamazaki et al. (2003), other reasons, such as ethnic origin of subjects must be considered. Two other studies with Caucasian subjects investigated the -159 *CD14* gene polymorphism in chronic

periodontitis and reported conflicting results. Folwaczny et al. (2004) did not find association between genotypes of the -159*CD14* gene polymoprhism and chronic periodontitis. However, it was shown that the C allele was significantly more prevalent among females with periodontitis than in healthy control subjects. Furthermore, the genotype CC tended to be more prevalent in severe forms of periodontitis than in mild or moderate forms. On the other hand, Laine et al. (2005) demonstrated that the TT genotype of the -159*CD14* gene polymorphism was associated with severe chronic periodontitis in Dutch Caucasians. While the findings by Folwaczny et al. (2004) to some extent were in agreement with the results reported in Study II, the data presented by Laine et al. (2005) were in contrast to those in Study II.

It has been demonstrated that the C to T transition at position −159 is related to the production of the sCD14 (Baldini et al. 1999). Baldini et al. (1999) reported that subjects with the homozygous TT genotype exhibited significantly higher sCD14 levels than individuals with CC and CT genotypes. This finding indicates that variations in the promoter region of the CD14 gene may change the production of CD14 and, also influence the balance between Th2 and Th1 cells in the response to a bacterial challenge. In a series of studies it was demonstrated that higher levels of sCD14 in gingival crevicular fluid were associated with fewer and shallower pockets in periodontitis patients (Jin et al. 2001) and that expression levels of mCD14 in healthy tissues of the controls were significantly higher then those in clinically healthy tissues from periodontitis subjects and, further, that within the patient group, the clinically healthy tissues showed greater levels of mCD14 than tissues from periodontitis sites. (Jin et al. 2004). Taken together, the findings by Baldini et al. 1999, Jin et al. 2001 and Jin et al. 2004 seem to corroborate the results presented in Study II, which revealed that the proportion of subjects that exhibited the TT genotype was significantly

smaller in the group with severe periodontitis than in periodontally healthy group.

Study III

In Study III the expression of IL-10 and mCD14 positive cells in relation to the −1087 *IL10* and the -159 *CD14* gene polymorphisms was analyzed in a group of subjects with severe chronic periodontitis. The results showed that the largest proportion of IL-10 positive cells was identified in gingival biopsies that were retrieved from subjects carrying the GG genotype. In particular, the proportion of IL-10 positive cells in the peripheral area of the periodontitis lesions was significantly larger in subjects with GG genotype than in subjects with AG or AA genotypes. The local expression of the mCD14 receptor, however, did not vary between subjects with different genotypes of the -159 *CD14* gene polymorphism.

The presence of IL-10 in periodonitis lesions has been demonstrated in several reports (Yamazaki et al. 1997; Aramaki et al. 1998; Lappin et al. 2001). In recent publications it was indicated that IL-10 was expressed more intensely in biopsies from chronic periodontitis subjects than in gingival tissue from controls (Garlet et al. 2003) and that the expression of IL-10 was higher in periodontitis than in gingivitis lesions (Nakajima et al. 2005). Thus, IL-10 expression is associated with severe periodontitis and appears to be an important cytokine in the regulation of periodontitis lesions. However, there are no functional studies that evaluated the correlation between IL-10 genotypes and the expression of interleukin-10 in periodontitis lesions. Nevertheless, findings reported in studies from other medical fields suggested that the ability to produce IL-10 is influenced by genetic factors (Turner et al. 1997, Crawley et al. 1999, Koss et al. 2000). In particular, some studies

(Turner et al. 1997, Suarez et al. 2003, Marka et al. 2005, Stanilova et al 2006) emphasized the relevance of the -1082 (-1087) *IL10* gene polymorphism in the expression control of interleukin-10. Suarez and co-workers (2003) evaluated the IL-10 levels and its genetic regulation in a healthy Spanish population. The authors observed that the G allele at position -1082 (-1087) was the most important genetic factor in the regulation of IL-10 mRNA levels. Furthermore, it was also demonstrated that the major production of IL-10 after LPS stimulation corresponded to the −1082 (-1087) GG genotype as demonstrated in previous studies (Turner et al 1997). The findings presented by Marka et al. (2005) and by Stanilova et al. (2006) corroborated earlier results. In fact, while Marka et al. (2005) showed that higher IL-10 plasma levels were found in subjects with the GG genotype than in subjects carrying AA or GA genotypes in a population of Hungarian with Sjögren's syndrome, Stanilova et al. (2006) demonstrated that the AA genotype was associated with lower IL-10 production in stimulated PBMC from healthy subjects and that the carriage of at least one copy of G allele in sepsis patients and in healthy controls resulted in a statistically significant increase in IL-10 production from stimulated PBMC. These findings are in agreement with data presented in Study III in which the GG genotype was associated with larger proportion of IL-10 positive cells in comparison to GA or AA genotypes. Recent findings (Larsson et al. 2008) revealed that the Sp1 transcription factor bound to the -1087 G-allele of the *IL10* promoter region of the gene and that the lipopolysaccharide (LPS) stimulation resulted in a 15-fold increase in promoter activity (e.g. protein production) for the G-allele as compared to the 6-fold increase of the A-allele. The results suggested a role for the Sp1 transcription factor in the activation of the IL-10 through the G-allele of the -1087 *IL-10* gene polymorphism in response to inflammatory signals and highlighted the complexity of the IL-10 regulation and expression.

Study IV

Study IV in the present thesis evaluated proportion of various cell markers in gingival biopsies retrieved from subjects with severe chronic periodontitis. It was observed that B-lymphocytes were found in larger fractions than that of T lymphocytes, plasma cells and neutrophils. Furthermore, about 60% of the B-lymphocytes exhibited both the CD19 and the CD5 markers and, thus, were classified as B-1a cells and the proportion of B-1a cells correlated with that of plasma cells.

A recent meta-analysis of the distribution of leukocytes in periodontitis lesions was presented by Berglundh & Donati (2005). The authors concluded that plasma cells outnumbered other cells in the lesions. This observation was based on findings of the studies included in the analysis. However, these studies used stereological technique/morphological criteria to evaluate the presence of leukocytes in the periodontitis lesions. The results of the Study IV in the present series demonstrated that the proportion of B-lymphocytes was considerably larger than that of plasma cells. The reason for this difference may be explained by the use of a different technique used (i.e. immunohistochemistry) for the detection of inflammatory cells in the periodontitis lesions. In this study it was further observed that almost 2/3 of the B-lymphocytes (CD19) were defined as autoreactive B-lymphocytes (CD19+CD5). Although the presence of such autoreactive B-lymphocytes (B-1a cells) in periodontitis lesions was previously demonstrated (Sugawara et al. 1992, Aramaki et al. 1998, Berglundh et al. 2002) the differences between the percentages of B-1a cells reported in the above mentioned studies are presently not understood and future studies may clarify whether other factors influence the presence of B-1a cells in periodontitis lesions. One of the main objectives of the Study IV of the present series was to evaluate correlations

between different cell proportions in the periodontitis lesion. Significant correlations were found between B-1a cells and plasma cells, and between B-lymphocytes and plasma cells. This observation may thus indicate that plasma cells in periodontitis may develop from both B-1a and B-2 cells and antibodies produced by plasma cells may, consequently, also be auto-antibodies as previously demonstrated (Fits et a. 1986, Hirsh et al. 1988, Jonsson et al. 1991, De Gennaro et al. 2006).

Study V

The reaction of B-1a cells to de novo plaque formation was evaluated in patients who were treated for severe chronic periodontitis. A significant larger proportion of B-1a cells was observed in biopsies retrieved from sites representing 3 weeks of plaque accumulation (inflammation sites) than in biopsies representing baseline (healthy sites).

Previous studies (Fransson et al. 1999, Zitzmann et al. 2001) evaluated the occurrence of the inflammatory cell composition in gingival biopsies after a 3-week period of experimental gingivitis. The results of the immunohistochemical analysis revealed that the specimens retrieved following the experimental gingivitis period harbored significantly higher proportions of B-lymphocytes than the tissue biopsies representing baseline. To our knowledge there are no reports that investigated the reaction of autoreactive B-lymphocytes to *de novo* plaque formation. The contribution of Study V of the present thesis is the novel finding that autoreactive B-lymphocytes (B-1a cells) are involved in the host response to microbial challenge in subjects susceptible to periodontitis. This points to the involvement of autoimmune components in the host response of subjects with chronic periodontitis. Future studies will further elucidate the role of these

cells in the complex mechanisms of the periodontitis lesion in subjects with chronic periodontitis.

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