Ectopic germinal center formation in Sjögren's syndrome

Significance of lymphoid organization

Malin Viktoria Jonsson

Doctor Odontologiae Thesis

University of Bergen
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To my family and friends

SUMMARY

Sjögren's syndrom (SS) is an autoimmune, chronic inflammatory disorder predominantly affecting the salivary and lacrimal glands. The overall aim of this study was to determine clinicopathological features in human and murine disease with regard to organization of ectopic lymphoid tissue, and to explore possible strategies for detection of patients at increased risk for extra-glandular manifestations.

Mononuclear cell infiltrates in the shape of germinal centers (GC) were observed in the salivary glands of approximately 1/4th of patients. Phenotypic markers for GC components such as T and B cells, proliferating cells, follicular dendritic cells and plasma cells confirmed the ectopic GC formation. The pattern and distribution of homing and retentive chemokines CXCL12, CXCL13 and CCL21, and adhesion molecules/integrin pairs ICAM/LFA and VCAM/VLA, was described in various lymphoid organizations in minor salivary glands. In addition, local autoantibody production was detected and correlated with serum levels.

Focal infiltrates and GC could be observed within the same gland, and were separated by altered B and T cell ratios, higher degree of proliferation and the localization of plasma cells in the periphery of infiltrates. Serum levels of BAFF and APRIL were elevated in pSS, and were in part linked to focus score, elevated serum IgG and autoantibody levels.

In a large cohort of pSS, ectopic GC were also associated with higher focus scores, lower mean un-stimulated salivary secretion, Ro/SSA and La/SSB autoantibodies, elevated RF-titres and increased serum IgG. Not all morphological GC could be confirmed by CD21/CD23/CD35 labeling, but clinical features remained comparable.

E-cadherin, an adhesion molecule important for epithelial tissue integrity, was investigated in minor salivary glands. E-cadherin is the ligand of integrin αEβ7/CD103 and lymphocytes expressing this integrin were increased in SS compared to non-SS. E-cadherin+ infiltrating cells were identified as CD68+ macrophages. Serum levels of sE-cadherin were increased in pSS compared to healthy blood donors and most likely mirror the chronic inflammatory state.

The non-obese diabetic (NOD) mouse is an animal model of SS. We observed significant changes in inflammation between 8 and 17 weeks of age, while hyposalivation was first observed between 17 and 24 weeks. In 1/3rd of mice older than 17 weeks, proliferating cells were observed in the focal infiltrates. Significant differences were detected in serum cytokine levels of IL-2, IL-5 and GM-CSF, and IL-4 and TNF-α in saliva. Salivary secretion correlated with IL-4, IFN-γ and TNF-α levels in saliva of NOD mice, but not with inflammatory changes in the salivary glands. Focal sialadenitis preceded hyposalivation, which occurred without a significant change in inflammation in NOD mice. Proliferating inflammatory cells indicate contribution of local factors in progression of SS-like disease.

In conclusion, ectopic germinal centers occur in a sub-group of patients with SS and are characterized by autoantibody production, progressive disease and increased serum IgG. It remains unclear whether the observed GC are a result of long-standing inflammation or indeed have functional properties and thus play an active role in the pathogenesis. A future challenge will be to identify and target trafficking molecules which drive the chronic inflammation in SS, without affecting migration and function of leukocytes required for protective immunity.

TABLE OF CONTENTS

LIST OF PAPERS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. Salomonsson S, **Jonsson MV**, Skarstein K, Hjälmström P, Wahren-Herlenius M, Jonsson R. Cellular basis of ectopic germinal center formation and autoantibody production in the target organ of patients with Sjögren's syndrome. Arthritis & Rheumatism 2003;48(11):3187-201.

II. **Jonsson MV**, Szodoray P, Jellestad S, Jonsson R, Skarstein K. Association between circulating levels of the novel TNF family members APRIL and BAFF and lymphoid organization in primary Sjögren's syndrome. Journal of Clinical Immunology, 2005;25(3):189-201.

III. **Jonsson MV**, Skarstein K, Jonsson R, Brun JG. Germinal centers in primary Sjögren's syndrome indicate a certain clinical immunological phenotype. Submitted.

IV. **Jonsson MV**, Salomonsson S, Gunnvor Øijordsbakken, Skarstein K. Elevated serum levels of soluble E-cadherin in patients with primary Sjögren's syndrome. Scandinavian Journal of Immunology, 2005;62(6):552-9.

V. **Jonsson MV**, Delaleu N, Brokstad KA, Berggreen E, Skarstein K. Impaired salivary gland function in NOD mice – association with changes in cytokine profile but not with salivary gland histopathology. Arthritis & Rheumatism, *in press*.

ABBREVIATIONS

ABC	avidin-biotin-peroxidase complex
ANA	antinuclear antibodies
APC	antigen presenting cell
APRIL	A Proliferation Inducing Ligand
AQP	aquaporin
BAFF/BLyS	B cell Activating Factor/B Lymphocyte Stimulator
BCA-1	B cell Attracting chemokine-1/CXCL13
BCR	B cell receptor
CD	clusters of differentiation
DNA	deoxyribonucleic acid
sE-cadherin	soluble E-cadherin
ELISA	enzyme-linked immunosorbent assay
FasL	Fas ligand
FasR	Fas receptor
FDC	follicular dendritic cell
FI	focal infiltrate
GC	germinal center
GlyCAM-1	glycosylation-dependent cell adhesion molecule-1
H&E	haematoxylin and eosin
HEV	high endothelial venule
HLA	human leukocyte antigen
ICAM-1	intercellular adhesion molecule-1 (CD54)
IDDM	insulin-dependent diabetes mellitus
IFN	interferon
Ig	immunoglobulin
IL	interleukin
kD	kilo Dalton
LFA-1	lymphocyte function associated antigen-1 (CD11a)
MAdCAM-1	mucosal addresin cell adhesion molecule-1
MALT	mucosa associated lymphoid tissue
MHC	major histocompatibility complex
NHBCL	non-Hodgkins B cell lymphoma
PNAd	peripheral node addressin
RNA	ribonucleic acid
NOD	non-obese diabetic
RA	rheumatoid arthritis
RF	rheumatoid factor
SDF-1	stromal cell-derived factor-1 (CXCL12)
SLC	secondary lymphoid tissue chemokine (CCL21)
SLE	systemic lupus erythematosus
SS	Sjögren's syndrome
TCR	T cell receptor
TGF	transforming growth factor
TNF	tumor necrosis factor
TUNEL	terminal deoxynucleotidyl transferase mediated dUTP digoxigenin nick end labelling
VCAM-1	vascular cell adhesion molecule-1 (CD106)
VLA-4	very late activation antigen-4 (CD49d)

INTRODUCTION

Sjögren's syndrome (SS) is a systemic autoimmune disease with organ-specific features. Autoimmune diseases are thought to result from a loss of immunological self-tolerance, leading to immune reactions directed against self by auto-reactive T cells and autoantibody-producing B cells. How and why certain individuals get SS, and why some patients develop a more severe disease than others, remains to be further investigated.

In the present study, minor salivary gland material and serum from patients with SS, and a relevant murine model have been investigated. An overall objective has been to determine clinicopathological features in human and murine disease with regard to organization of lymphoid tissue, and to explore possible strategies for detection of patients at increased risk for extra-glandular manifestations.

GENERAL BACKGROUND

Immunology of mucosa

The immune system can be divided into various anatomical fields, out of which the two most important are the peripheral lymphatic system, consisting of the spleen and lymph nodes, and the mucosal immune system. The lymphocytes ability to recognize foreign antigens or substances sustains a separate entity of lymphocytes in each of these areas. The total mucosal area in the body, approximately 400 m^2, is constantly subject to environmental triggers and therefore in need of an advanced innate (non-specific) and adaptive (specific) immune response. In contrast to the peripheral lymph nodes and blood, the type and distribution of T cells in the gut mucosa contains a large number of γδ T cells. Hence, the adaptive immune response in mucosa associated lymphoid tissue (MALT) differs from the rest of the peripheral lymphatic system (Janeway *et al.*, 2005).

The main type of antibody actively transported to the mucosal surface is the secretory polymeric IgA, with the two subclasses IgA_1 and IgA_2 in humans. The mucosal immune system is continuously exposed to a great number of foreign antigens from food, commensal bacteria in the gut and from pathogenic microorganisms and parasites. Food antigens do not normally elicit an immune response. Meanwhile, soluble antigens from the oral cavity may elicit antigen-specific tolerance or antigen-specific suppression, and pathogenic microorganisms may cause strongly preventive T_H1 responses. Consequently, an important challenge for the immune system is to differentiate between these contradictions (Janeway *et al.*, 2005).

The main difference between tolerance and an adaptive immune response in the mucosal immune system is determined by how peptide antigen is presented to T lymphocytes. In absence of inflammation, peptides are presented to T cells by MHC molecules on the surface of antigen-presenting cells (APCs) without co-stimulation. In the opposite situation, pathogenic microorganisms induce an inflammatory response in the tissue, stimulating maturation and expression of co-stimulatory molecules on the APCs. This form of antigen-presentation to T cells contributes to the development of T_H1 protection (Janeway *et al.*, 2005).

Mediators of the immune system

Cytokines

Cytokines are small proteins (~ 25 kDa) secreted from many cell types as a response to an activating stimulus. Activation induces growth, differentiation and/or function, through binding to specific receptors. Cytokines may have an autocrine, paracrine or endocrine effect (Figure 1) and may have stimulatory/pro-inflammatory effects such as IL-1β, IL-6, GM-CSF and TNF-α, or inhibitory/anti-inflammatory effects, such as IL-4, TGF-β and IL-10.

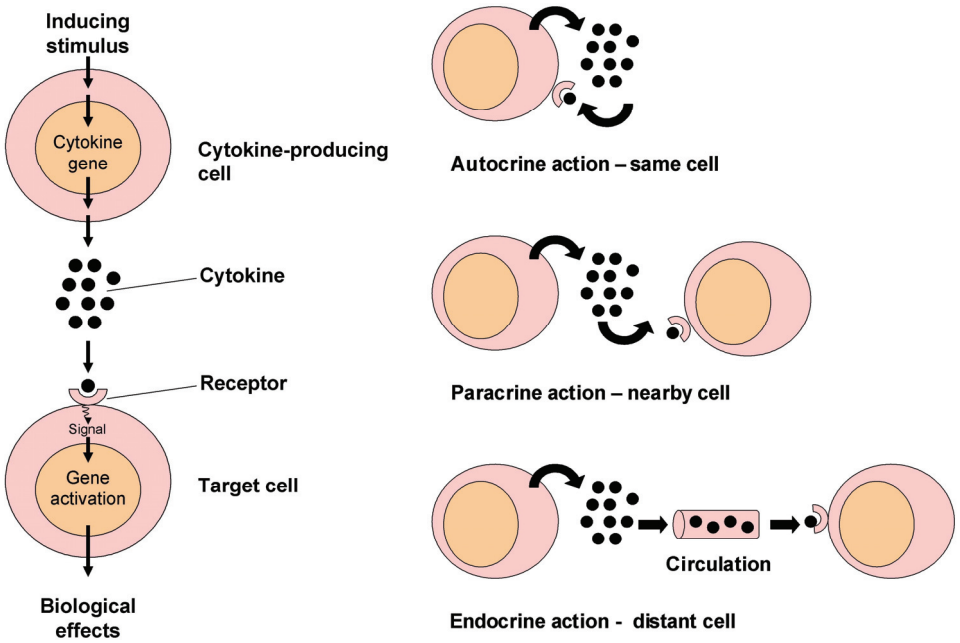


Figure 1. Activation induces a response in the cytokine-producing cell. Autocrine cytokines stimulate the same cell that synthesizes and secretes the cytokine, while paracrine cytokines act on cells in close proximity. Some cytokines may even have an endocrine effect, but this is strongly dependant on the cytokines ability to enter circulation, and the particular cytokines half time.

The term interleukin (IL) was suggested to provide a comprehensive nomenclature related to leukocytes. However, increasing numbers of novel cytokines of different sources, structures and effects, warranted for a more extensive system. Three larger, structural families now define cytokines, the haematopoietin family with growth hormones and many ILs with a role in both innate and adaptive immunity, the TNF family with functions in both adaptive and innate immunity, and the chemokine family (Janeway *et al.*, 2005; Steinke and Borish, 2006).

Cytokines produced by T cells can currently be divided into three entities, the T_H1, T_H2 and T_H3/T_R1/T regulatory cells (T_{reg}) (Figure 2). Cytokines produced by monocytes/macrophages as a response to pathogens, is a structurally derived group of molecules and include IL-1β, IL-6, IL-12, TNF-α and the chemokine CXCL8 (IL-8), reviewed in (Steinke and Borish, 2006).

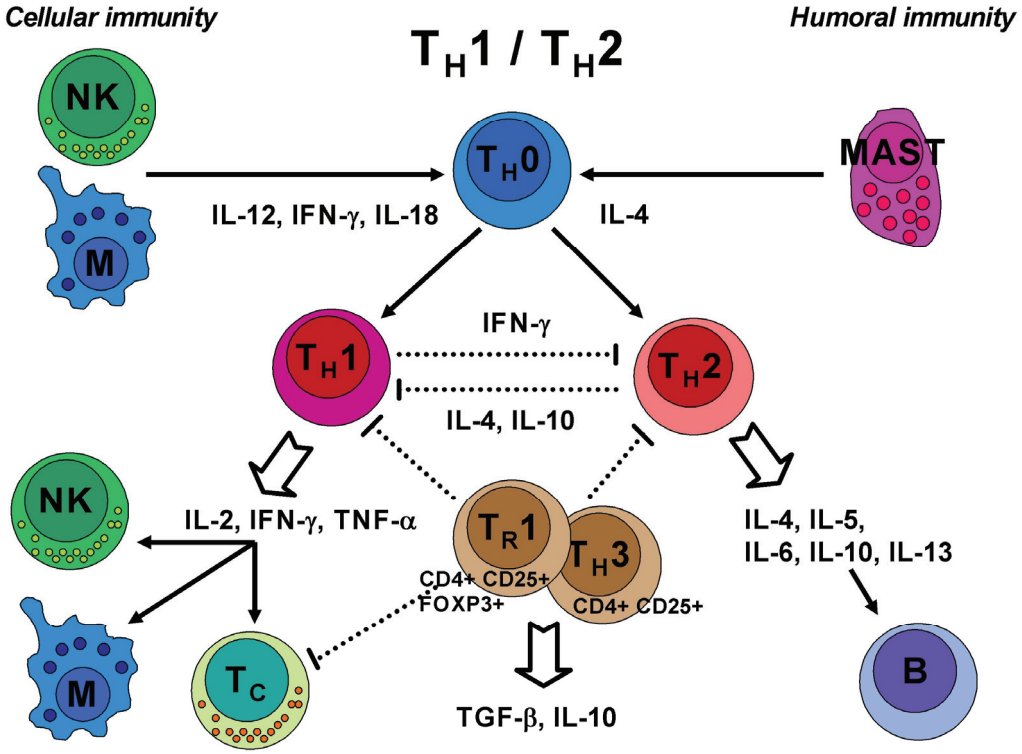


Figure 2. Cytokines produced by CD4 effector T cells. The differentiation into either an armed T_H1 or T_H2 cell determines whether the adaptive immune response will be dominated by macrophage activation (cellular immunity) or antibody production (humoral immunity). NK natural killer cell; M macrophage; MAST mast cell; T_c T cytotoxic cell.

Chemokines

Chemokines are a class of some 50 cytokines with chemo-attractive properties, the ability to induce cells with the appropriate chemokine-receptors to migrate in the direction of the source of the chemokine. In case of an infection, chemokines secreted from phagocytic cells recruit and direct monocytes, neutrophils and other effector cells to the site of antigenic stimulus (Janeway *et al.*, 2005). The exact localization of the B cells, T cells and dendritic cells in peripheral lymphoid organs is conducted by chemokines (Cyster, 1999; Cyster, 2003).

Chemokines can be divided into two major groups – the CC chemokines with two cysteins close to the amino terminal part, and CXC chemokines where the two cysteins are divided by another amino acid. CC chemokines (e.g. CCL21) bind to CC chemokine receptors (e.g. CCR7). CXC chemokines (e.g. CXCL13) bind to CXC receptors (e.g. CXCR5) (Janeway *et al.*, 2005).

Adhesion molecules

The recruitment of effector cells during an inflammatory response is mediated by locally induced adhesion molecules on the surface of vascular endothelial cells and leukocytes. Three families of adhesion molecules, the selectins, intercellular adhesion molecules (ICAMs), and integrins, are important for the specific recruitment (homing) of leukocytes (Janeway *et al.*, 2005).

Selectins are membrane glycoproteins with a distal lectin-like area which binds to specific carbohydrates. These adhesion molecules can be expressed on leukocytes (L-selectin) and vascular endothelium (P- and E-selectin), and are important for precise homing. L-selectin is expressed on circulating T cells and guides their exit from the circulation into tissue. P- and E-selectin are induced on vascular endothelium at sites of infection and recruit effector cells to initiate endothelium-leukocyte interactions. In mucosal endothelium, L-selectin binds to mucosal addressin cell adhesion molecule-1 (MAdCAM-1), gaining entry into mucosal lymphoid tissues, while in lymph nodes, L-selectin binds to CD34 and (Glycosylation-dependent cell adhesion molecule-1) GlyCAM-1 (Janeway *et al.*, 2005) (Figure 3).

ICAMs such as ICAM and VCAM are members of the immunoglobulin super-family (IgSF), and mediate closer adhesion between endothelium and leukocytes. The IgSF also includes the antigen receptors of T and B cells, the co-receptors of CD4, CD8, and CD19, and the invariant domains of MHC molecules. Several of the IgSF members are important in T cell activation, such as ICAM-1-3 which all binds to the T cell integrin lymphocyte function-

associated antigen-1 (LFA-1). ICAM-1 and ICAM-2 are expressed on endothelium as well as on APCs, enabling lymphocyte migration through blood vessels (Janeway *et al.*, 2005).

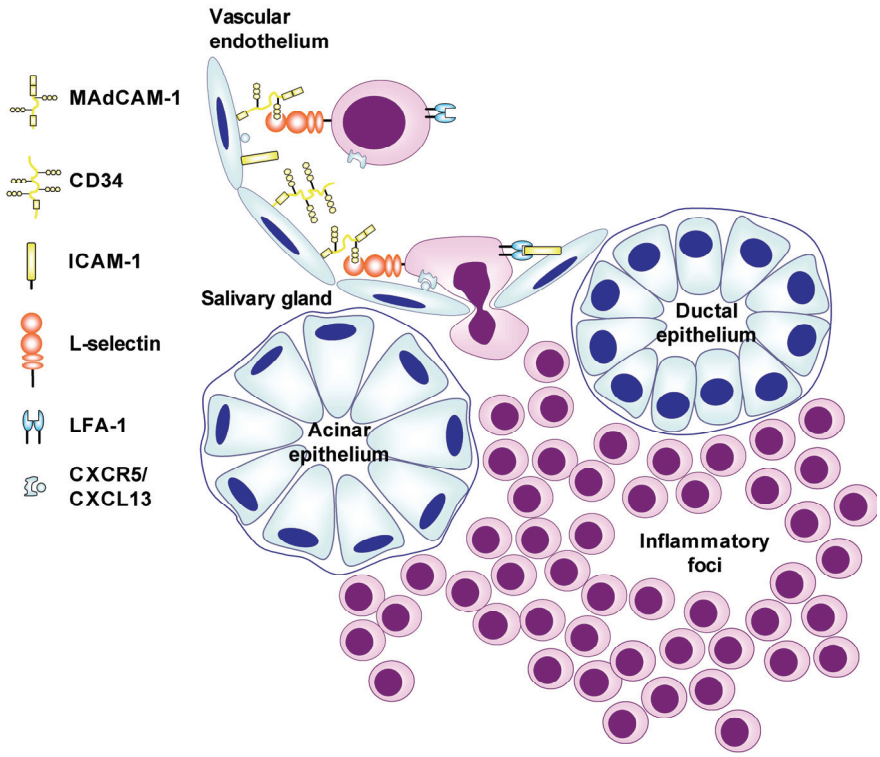


Figure 3. Possible mechanisms of inflammatory cell migration from the circulation into salivary gland tissue. Access is gained by crossing the walls of high endothelial venules/vascular endothelium. The first step is binding of L-selectin (on the lymphocyte) to MAdCAM-1 or CD34 on the endothelium, allowing a rolling interaction. Local expression of chemokine CXCL13 activates LFA-1 on the lymphocyte, allowing tight adhesion to ICAM-1 on the endothelial cell, and the lymphocytes can migrate into the salivary gland tissue. Adapted and modified from (Janeway *et al.*, 2005).

Integrins are heterodimeric proteins on activated leukocytes. All T cells express the β_2 integrin, also known as LFA-1. In addition, LFA-1 is found on macrophages and neutrophils, mediating recruitment of inflammatory cells to sites of infection. Expression of integrin β_1 increases at late stages of T cell activation and is called very late activation antigens (VLA). VLAs are important in directing armed effector T cells to sites of inflammation. Depending on the type of adhesion molecules, cytokines and chemokines that are expressed, a selective and specific homing occurs (Janeway *et al.*, 2005).

Autoimmunity

Autoimmune diseases are characterized by an abnormal state in which the immune system elicits an inflammatory response against the own cells, tissues and proteins. The direct mechanism behind autoimmunity remains undetermined, but several possibilities such as defects in the inflammatory cell control regime have been suggested. In addition, there also seems to be a familiar distribution of autoimmune diseases, suggesting genetic susceptibility (Bolstad and Jonsson, 2002; Kumar *et al.*, 2003; Ollier and Symmons, 1992).

Immunological tolerance

Self-tolerance is the lack of immune-responsiveness to own tissues, and is regulated by central and peripheral tolerance (Figure 4). ***Central tolerance*** is the deletion of self-reactive T and B lymphocytes during maturation in central organs. For T cells this occurs in the thymus, and for B cells in the bone marrow. T cells expressing a receptor for self-antigen are negatively selected and deleted by apoptosis, resulting in a T cell pool depleted of auto-reactive T cells. Unfortunately, not all self-antigens are present in the thymus, and auto-reactive T cells may to some degree escape this control regime. ***Peripheral tolerance*** can be mediated by anergy, Fas/FasL mediated cell death, or suppression by T_{regs} (Kumar *et al.*, 2003).

Anergy, functional unresponsiveness without cell death, is mediated by a prolonged or irreversible unresponsiveness of the lymphocyte. In order to become activated, T cells depend on two signals, the recognition of peptide antigen in association with self-MHC molecules on APCs, and the expression of co-stimulatory molecules, such as B7 molecules, provided by the APCs. If the second co-stimulatory signal is missing, the T cell becomes anergic, and will remain so even if the relevant antigen is presented by competent APCs and the appropriate co-stimulation. Most normal tissues lack strong expression of co-stimulatory molecules, and encounter between auto-reactive T cells and their specific self-antigens frequently results in anergy (Kumar *et al.*, 2003).

B cells may also become anergic if they encounter antigen in absence of specific helper T cells. This kind of anergy is mediated by Fas and FasL. In cases where Fas and FasL are co-expressed on the same cohort of activated T cells, the immune response may be suppressed by apoptosis of these cells. In theory, activation-induced cell death may also cause peripheral deletion of auto-reactive T cells, wherein repeated and persistent stimulation of auto-reactive T cells by abundant self-antigens in the periphery eventually lead to their elimination via Fas-mediated apoptosis.

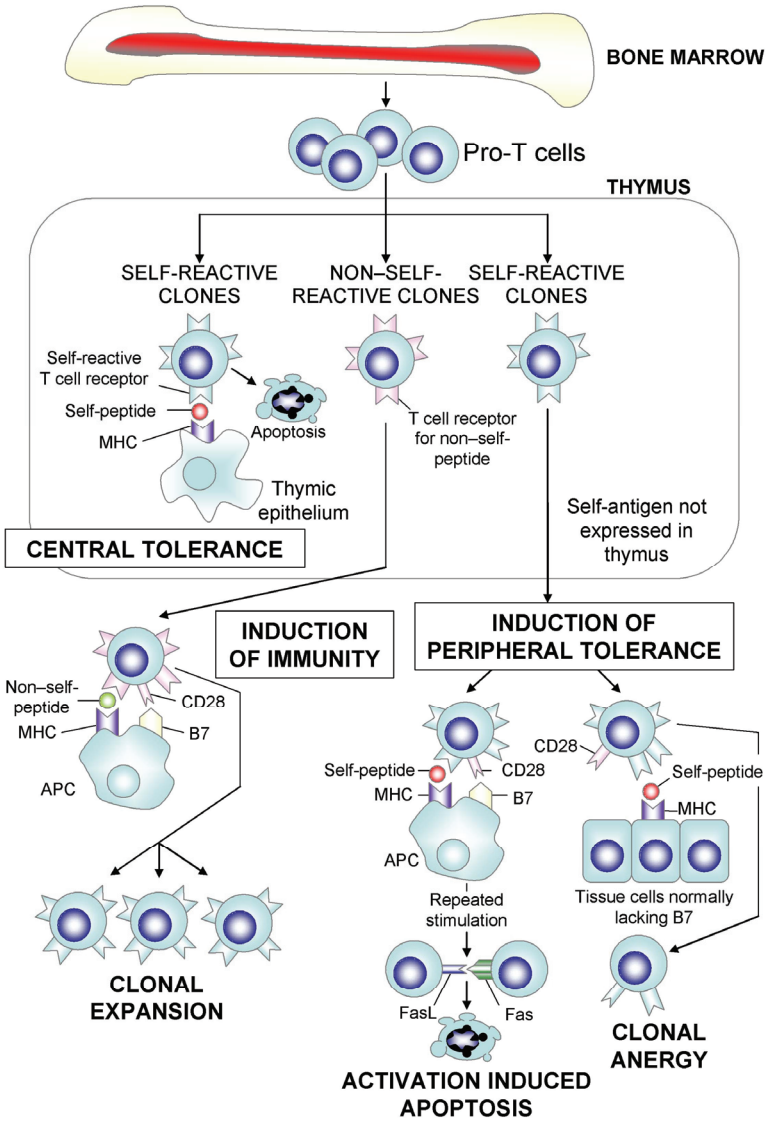


Figure 4. Mechanisms involved in central and peripheral tolerance of T cells, adapted from (Kumar *et al.*, 2003).

The third means of peripheral self-tolerance is suppression by T_{regs}, previously mentioned in the section on cytokines and Figure 2. By secretion of IL-10 and TGF-β, the CD4+CD25+ T_{regs} modulate a variety of responses to autoantigen, reviewed in (Steinke and Borish, 2006). Individuals lacking CD4+CD25+ T cells in the thymus develop autoimmune disease at a young age (Ramsdell, 2003).

Mechanisms of autoimmunity

Breakdown of one or more of the mechanisms of self-tolerance, as described above, can lead to an immunological attack on own tissues and eventually to the development of autoimmune diseases. Most likely the tissue injury is mediated by immunocompetent cells, but the precise

influences that cause the reaction against self are not known. Tolerance can be bypassed in a number of ways, and autoimmune diseases cannot be explained by one single mechanism. The defects vary from disease to disease, and more than one defect may be present in each disease. The breakdown of tolerance and initiation of autoimmunity involves a complex interaction of immunologic, genetic and microbial factors (Kumar *et al.*, 2003). Possible etiological factors are summarized in Figure 5.

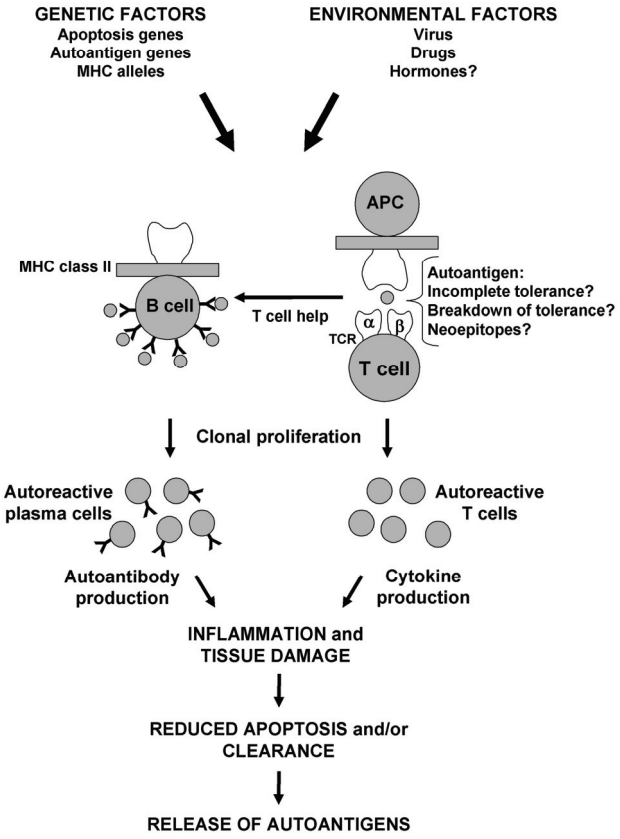


Figure 5. Possible etiopathogenesis of autoimmune diseases.

The clinical aspect of autoimmunity

Autoimmune diseases may be restricted to specific organs of the body – *organ-specific autoimmune diseases*, or may affect several tissues – *systemic autoimmune diseases*. Organ-specific autoimmune diseases may for instance affect the insulin-producing cells in the pancreas in Type I diabetes mellitus, the myelin sheath of nerves in multiple sclerosis (MS), or the red blood cells in autoimmune pernicious anemia (Figure 6).

In systemic autoimmune diseases, the auto-antigen is a constituent of many organs, such as the synovium in rheumatoid arthritis (RA), connective tissue in scleroderma, DNA

and other nuclear components in systemic lupus erythematosus (SLE), or the salivary and lacrimal glands in primary SS (Figure 6). Systemic autoimmune disorders, such as RA, SS and SLE, have a number of autoantibodies and HLA haplotypes in common, reviewed in (Jonsson *et al.*, 2005).

Autoantibodies against receptors may also cause disease, by stimulating or blocking receptor function, such as antibodies targeting the acetylcholine receptors in myasthenia gravis (Vincent, 2002), and the M3R in SS (Waterman *et al.*, 2000).

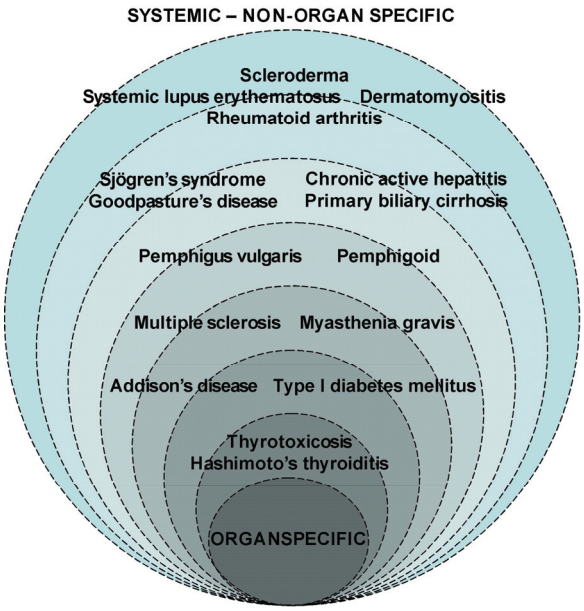


Figure 6. Overview of various organ specific and non-organ specific (systemic) autoimmune diseases. Modified from (Ollier and Symmons, 1992).

SJÖGREN'S SYNDROME

Disease manifestations

Clincical symptoms

Sjögren's syndrome is an autoimmune chronic inflammatory disease which predominantly affects the salivary and lacrimal glands, giving rise to clinical symptoms such as oral and ocular dryness (xerostomia and keratoconjunctivitis sicca, respectively). SS predominantly affects post-menopausal women, in a 9:1 ratio compared to men. Younger individuals and children may also be affected (Jonsson *et al.*, 2005). Common complaints are dryness of the mouth, difficulties to talk, taste and swallow (Abbas, 2005). In addition, patients with SS may also have signs of systemic autoimmune disease with musculoskeletal, pulmonary, gastric, hematological, dermatological, renal and neurological manifestations (Jonsson *et al.*, 2005; Manthorpe *et al.*, 1998).

Patients with pSS have reduced secretion of saliva, higher dental caries activity, and lower salivary pH and buffer capacity compared to healthy controls (Pedersen *et al.*, 2005). Interestingly, levels of potassium, total calcium, total protein and amylase activity in whole and parotid saliva, or the concentration of statherin and proline-rich proteins in stimulated parotid saliva did not differ from healthy controls. These findings indicate that despite lymphocytic infiltration and structural alterations within the salivary glands, the remaining secretory acini are indeed functional and capable of secreting saliva with normal composition (Mandel and Baurmash, 1976; Pedersen *et al.*, 2005).

Proteolytic activity of matrix metalloproteinases (MMPs) has been shown to be increased in pSS minor salivary glands (Goicovich *et al.*, 2003; Perez *et al.*, 2000), and may implicate structural changes in the glandular tissue and thus the quantity and quality of secreted mucins from these glands. The lack of mucins may offer an explanation to the sensation of dry mouth in the early stages of disease, where the submandibular and sublingual glands are more affected than the serous salivary glands (Atkinson, 1993; Lindvall and Jonsson, 1986).

Histopathology of the target organ

Histopathologically, SS is manifested by focal mononuclear inflammatory cell infiltrates of the affected organs, the salivary and lacrimal glands. The pathological designation is lymphoepithelial lesion (Seifert and Sobin, 1991). Intact acinar and ductal epithelium surrounds the lymphocytic infiltrates (Chisholm and Mason, 1968; Greenspan *et al.*, 1974),

but acinar epithelial atrophy and proliferation of ductal epithelium may also be noted (Jonsson *et al.*, 2005).

Traditionally, the infiltrates are an accepted sign of the compromised salivary and lacrimal function (Figure 7), but a direct association between the degree of lymphoid infiltration and exocrine dysfunction is not always obvious (Humphreys-Beher *et al.*, 1999). Immunohistochemical investigations have shown reduced density/lack of immune-reactive nerve fibers in the central areas of large lymphocytic infiltrates, while in the periphery, innervation is as abundant as in healthy controls (Fox and Stern, 2002; Pedersen *et al.*, 2000), indicating that glandular atrophy and inflammation is a consequence of functional inhibition of the nervous control of the gland, rather than the reason for it.

Serology

In a recent study, autoantibodies to the Ro/SSA and La/SSB protein were detected in 71% and 56% of patients with pSS, Ro/SSA and La/SSB, respectively (Garberg *et al.*, 2005). Although serum titers of Ro/SSA and La/SSB of the IgA isotype are reported to correlate with sicca symptoms (Pourmand *et al.*, 1999), and hypergammaglobulinemia and persisting levels of autoantigen-specific IgM were demonstrated in patients with SS (Wahren *et al.*, 1994a), the exact role of the Ro/SSA and La/SSB autoantibodies is not yet determined. Titres of SLE and RA associated autoantibdodies ANA and RF may also be elevated in SS, even in absence of another connective tissue disease (Jonsson *et al.*, 2005).

A variety of criteria have been proposed in the diagnosis of SS, such as the Californian/San Diego criteria (Fox *et al.*, 1986), Greek criteria (Skopouli *et al.*, 1986), the Japanese criteria (Homma *et al.*, 1986), the Copenhagen criteria (Manthorpe *et al.*, 1986), and the European criteria (Vitali *et al.*, 1993). According to the American European Consensus group Criteria (AECC) (Vitali *et al.*, 2002) applied in this study, the diagnosis of SS is based on four objective and two subjective items. At least four items must be fulfilled. One of these must be either focal periductal mononuclear cell infiltration, as demonstrated by light microscopy of H&E stained minor submucosal salivary gland biopsies, or the presence of serum autoantibodies to Ro/SSA and/or La/SSB (Figure 7).

Etiology and pathogenesis

Early inflammatory events

Different factors such as Epstein-Barr virus (Wen *et al.*, 1996) and coxsackievirus (Triantafyllopoulou and Moutsopoulos, 2005), certain HLA genes (Gottenberg *et al.*, 2003;

Nakken *et al.*, 2001) and influence of sex steroid hormones (Brennan *et al.*, 2003) have been implied in the pathogenesis of SS. Transfer experiments suggested that SS is a T cell mediated disease (Skarstein *et al.*, 1997), but recent findings indicate an associated homing of B cells, making up approximately 20% of infiltrating lymphocytes in the salivary glands (Jonsson *et al.*, 2005; Larsson *et al.*, 2005) (Figure 7).

Epithelial cells produce several pro-inflammatory cytokines (Fox *et al.*, 1994) and circulating levels of IL-1β, IL-12p40, TNF-α, and IL-6 were significantly different when comparing patients with SS to healthy controls (Szodoray *et al.*, 2004) (Figure 7). The degree of glandular infiltration and glandular tissue destruction has been related to the expression of T_H1 cytokines, whereas hyposalivation to some extent has been linked to humoral immune reactions promoted by T_H2 cytokines (Mitsias *et al.*, 2002).

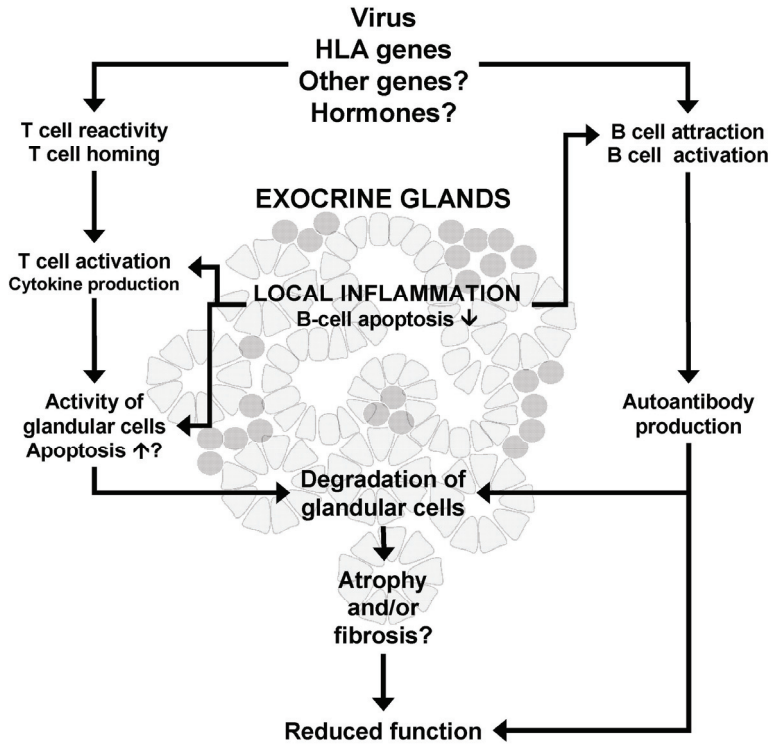


Figure 7. Possible etiological factors of SS. Adapted from (Delaleu *et al.*, 2004; Jonsson *et al.*, 2005).

The homotypic adhesion between epithelial cells is, among others, mediated by the adhesion molecule E-cadherin. E-cadherin is the ligand of integrin αEβ7/CD103, which is normally expressed on CD8+ intraepithelial lymphocytes in the gut mucsoa (Cepek *et al.*, 1993; Cepek *et al.*, 1994). The interaction between E-cadherin and αEβ7/CD103 may participate in the specific homing of inflammatory cells to the salivary glands (Figure 7). E-

cadherin is also an important regulator of cell growth and mobility, tissue morphogenesis and apoptosis, during both embryogenesis and maturation, reviewed in (Gumbiner, 2000).

The novel TNF family member B cell activating factor BAFF (also called BLyS, TALL-1, THANK, and zTNF4), A proliferation inducing ligand APRIL (also called TNFSF 13a) and their receptors BAFF-R, BCMA and TACI (Gross *et al.*, 2000; Moore *et al.*, 1999; Mukhopadhyay *et al.*, 1999; Schneider *et al.*, 1999; Shu *et al.*, 1999) have been investigated in order to determine their possible role in SS pathogenesis (Figure 8). BAFF contributes to disturbances in B cell survival, and this may contribute to autoimmunity and pathogenic autoantibodies (Mackay and Browning, 2002).

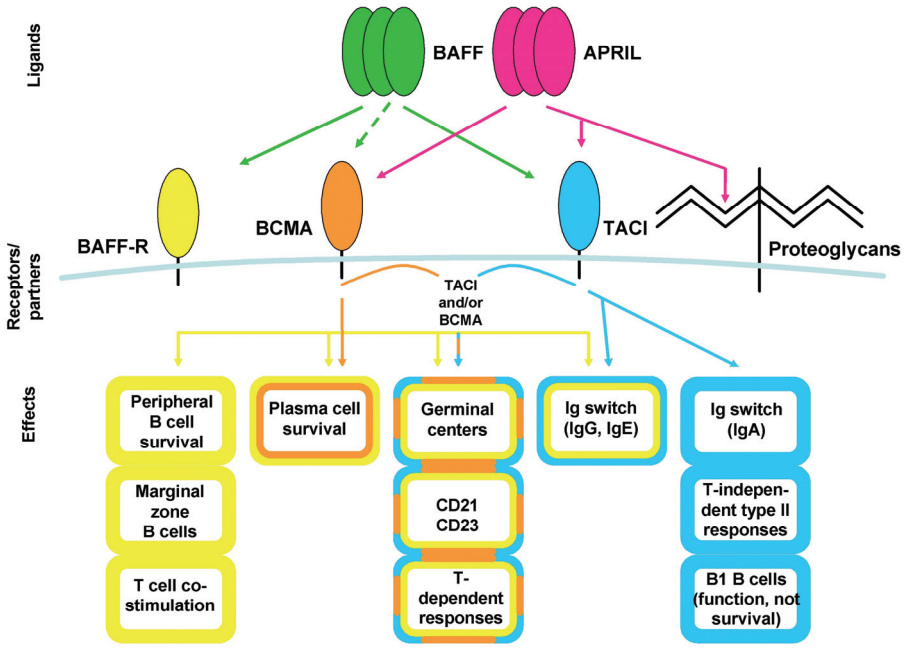


Figure 8. Ligand specificity and function assignment of BAFF and APRIL receptors in the immune system. The interactions of BAFF and APRIL with their receptors (BAFF-R, BCMA and TACI) are shown in the upper part, and the phenotypic and functional outcomes of BAFF and/or APRIL signaling at the bottom. Colors indicate the tentative involvement of one or more receptors in mediating a particular effect. Figure adapted from (Schneider, 2005).

Analyses of serum from patients with SS showed elevated levels of sBAFF (Pers *et al.*, 2005), correlating with autoantibody levels (Groom *et al.*, 2002; Mariette *et al.*, 2003). BAFF expressing cells have been described in SS salivary glands (Lavie *et al.*, 2004) and associated with attenuated apoptosis (Szodoray *et al.*, 2003). Mice transgenic for BAFF develop SLE-like disease, followed by secondary SS-like disease (Groom *et al.*, 2002). BAFF also plays a role in physiological processes but depending on the host, excess BAFF may

induce accumulation of self-reactive B cells by providing help to escape from BCR-mediated apoptosis (Mackay and Browning, 2002).

Ectopic germinal centers

Germinal centers are formed in the B cell follicles of secondary lymphoid tissues during T cell dependant antibody responses. Rapidly proliferating cells are called centroblasts and are localized in the dark zone of the GC. As the centroblasts mature they move into the light zone of the GC, and are now called centrocytes. In the light zone the centrocytes make contact with a rich network of follicular dendritic cells which present antigen to the centrocytes. Strict control regimes ensure that autoreactive cells die by apoptosis, and the surviving cells become either memory B cells or antibody-producing plasma cells (Janeway *et al.*, 2005). Lymphoid neogenesis with formation of ectopic "tertiary" lymphoid follicles in chronic inflammatory diseases (Figure 9) is a complex process regulated by expression of an array of cytokines, chemokines and adhesion molecules (Hjelmström, 2001; Weyand and Goronzy, 2003).

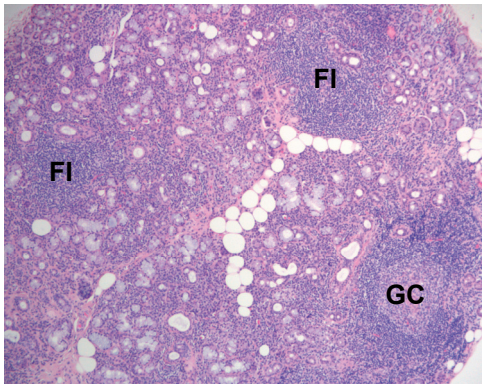


Figure 9. Three periductal inflammatory cell infiltrates: two focal infiltrates (FI) and one ectopic germinal center (GC) in a minor salivary gland from a patient with pSS. Note normal salivary gland tissue surrounding the inflammatory cell foci, and some adipose tissue.

Lymphoid malignancy

In SS, polyclonal B cell activation can develop into an oligo- or monoclonal B cell expansion during disease progression, potentially culminating in lymphoid malignancy, predominantly low-grade marginal zone (MZ) lymphomas (Theander *et al.*, 2005; Voulgarelis *et al.*, 1999). The incidence of lymphoma was reported higher in pSS than in sSS or RA (Kauppi *et al.*, 1997), and the estimated prevalence of malignant lymphoma in pSS was considered 44 times higher than in the general population (Voulgarelis *et al.*, 1999), with a possible relation to ectopic GC formation (Amft and Bowman, 2001; Voulgarelis *et al.*, 1999). In a more recent study, however, the risk of malignant transformation was found to be 16 times (Theander *et al.*, 2005).

Animal models of SS

Clinical symptoms such as dry eyes and mouth appear rather late in SS pathogensis, making early diagnosis difficult. By use of animal models it is possible to study different stages of the disease development. Ideally, a model for SS should have oral and ocular dryness, chronic inflammation in the salivary and lacrimal glands, and systemic deviations such as hypergammaglobulinemia, antinuclear antibodies and autoantibodies to extractable nuclear antigens such as Ro/SSA and La/SSB. In the present study, the non-obese diabetic (NOD) mouse was selected and used to investigate chronological events in SS-like disease progression.

NOD

The NOD mouse is an animal model which spontaneously develops both autoimmune insulin-dependant diabetes mellitus (IDDM) and SS. Progressive salivary gland inflammation may be noted as early as 8 weeks of age and is characterized by focal infiltrates of mononuclear cells. In addition, the NOD mouse has impaired salivary secretion from the major glands, a phenomenon which has not been observed in any other animal models for SS (Humphreys-Beher *et al.*, 2002; van Blokland and Versnel, 2002).

NOD mice in which genes for various cytokines such as IL-4 and IFN-γ were knocked out have also been studied in relation to SS-like disease. NOD IL-$4^{-/-}$ have mononuclear cell infiltration, but normal salivary secretion (Brayer *et al.*, 2001), while NOD IFN-$\gamma^{-/-}$ and NOD IFN-$\gamma R^{-/-}$ have no infiltration and normal salivary secretion (Cha *et al.*, 2004). Despite similar glandular infiltration compared to the NOD/LtJ or NOD.B10-$H2^b$ mice, the NOD.B10-$H2^b$.IL-$4^{-/-}$ and NOD IL-$4^{-/-}$ mice had comparable salivary secretion to 4 week-old mice of the same strain (Gao *et al.*, 2006).

AIMS OF THE STUDY

The general objective of this study was to determine clinicopathological features in human and murine disease with regard to organization of lymphoid tissue, and to explore possible strategies for detection of patients at increased risk for extra-glandular manifestations.

The specific aims were

I. To analyze the functional properties of ectopic germinal centers in SS and to determine at which frequency such structures develop

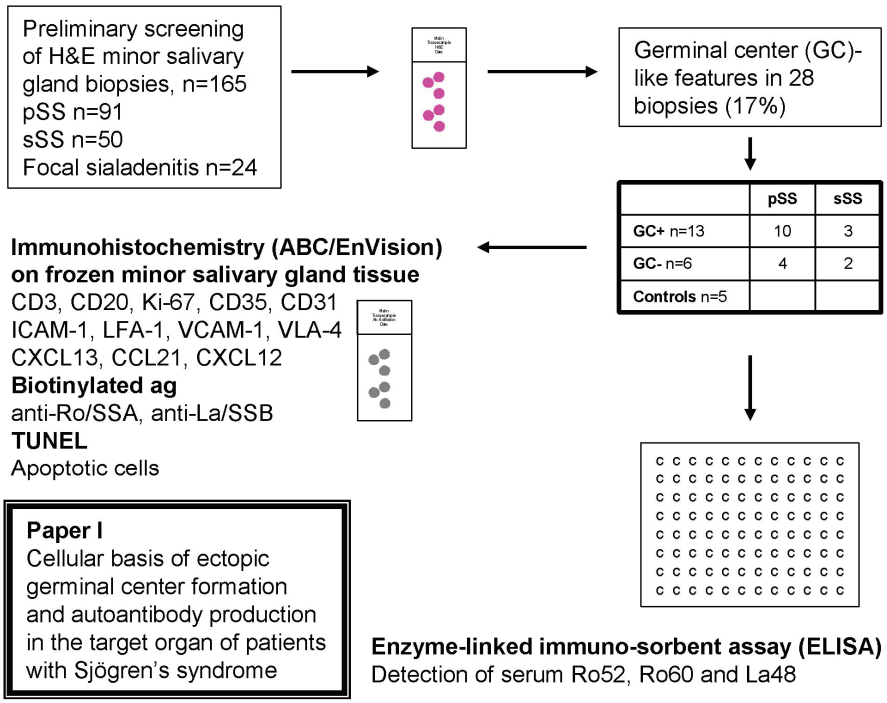
II. To investigate the possible association between circulating levels of the novel TNF family members APRIL and BAFF and lymphoid organization in pSS

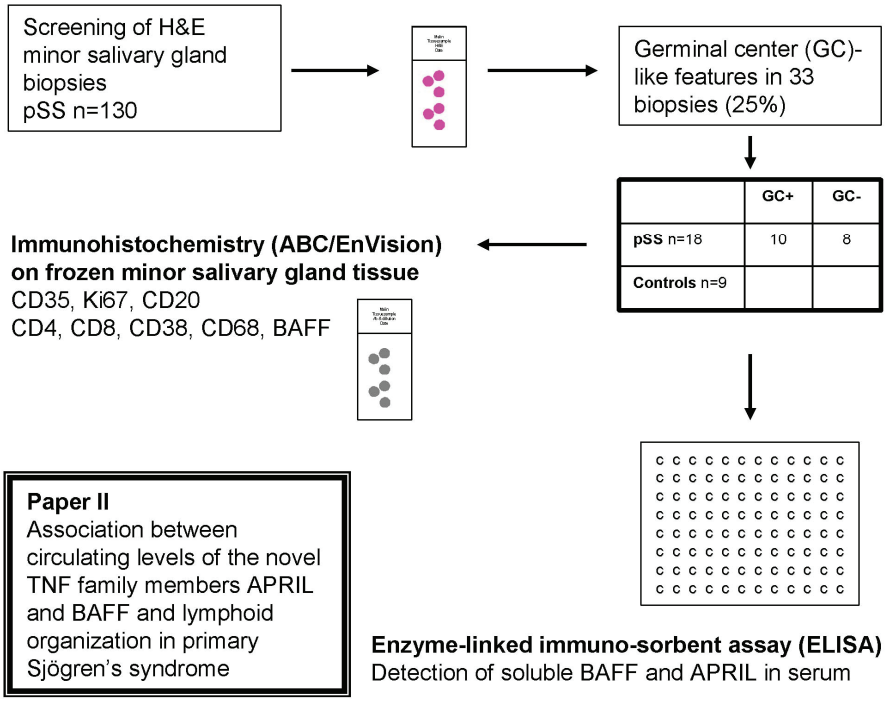
III. To explore features of ectopic germinal centers in pSS, to determine if GC identified by phenotypic analysis correlate with morphological GC, and if such structures define a distinct clinical immunological phenotype

IV. To determine serum levels of soluble E-cadherin (sE-cadherin) and to describe the expression of E-cadherin and αEβ7 on glandular epithelial cells and mononuclear cells in salivary gland epithelium in patients with SS

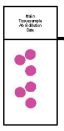
V. To stratify the chronological disease course and characterize possible inter-relationships between salivary gland inflammation, hyposalivation and cytokine levels in NOD mice, a model for SS

OVERVIEW OF PAPERS I-IV





Retrospective study of biopsies and medical records
pSS n=169
sSS n=38
Focal sialadenitis n=65

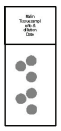


GC-like features in 47/169 biopsies (28%)

	GC+	GC-
pSS n=60	11	49

Immunohistochemistry on formalin-fixed paraffin-embedded salivary gland tissue

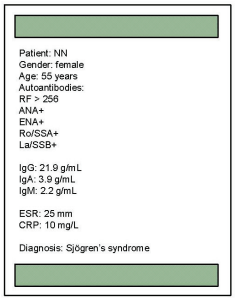
CD21
CD23
CD35 } FDC networks
IgD → Mature B cells



B cell/FDC staining-patterns

0) No positive cells
1) Few, dispersed positive cells
2) Network-like staining
3) Small networks/aggregates
4) Organized ectopic follicles
5) Ectopic follicles with GC

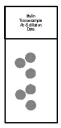
Various lymphocytic organization



GC+	GC-
18	42

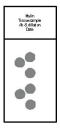
Paper III
Germinal centers in primary Sjögren's syndrome indicate a certain clinical immunological phenotype

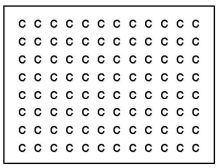
	pSS	sSS
GC+ n=22	12	10
GC- n=5	3	2
Controls n=8		



Immunohistochemistry (EnVision) on frozen minor salivary gland tissue
E-cadherin, αEβ7/CD103

Double-labelling (EnVision) on frozen minor salivary gland tissue
E-cadherin and CD68/CD35





Enzyme-linked immuno-sorbent assay (ELISA)
Detection of soluble E-cadherin in serum

Paper IV
Elevated serum levels of soluble E-cadherin in patients with primary Sjögren's syndrome

MATERIALS AND METHODS

Overviews of Papers I-V are illustrated on pp 23, 24 and 26.

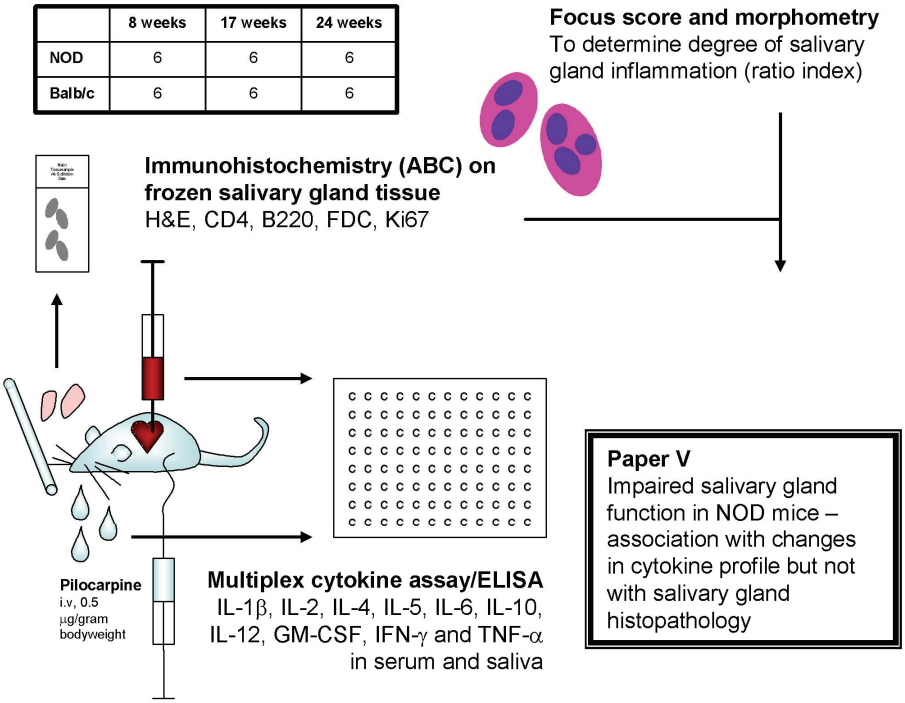
Human tissue material

Patients and controls (Paper I-IV)

Minor submucosal labial salivary glands and serum samples were obtained from patients fulfilling the revised American-European classification criteria (Vitali *et al.*, 2002) for primary or secondary SS. Patients were recruited from the Departments of Rheumatology and Otolaryngology/Head and Neck Surgery, Haukeland University Hospital, Bergen, Norway, from 1989-2005. Some of the minor salivary gland biopsy material had been divided in two portions. One portion was formalin fixed and used for routine histopathological evaluation, and the other portion transported in Histocon (HistoLab Products AB, Gothenburg, Sweden), and snap frozen in iso-pentane by liquid nitrogen and stored at -80°C.

Medical records were obtained from all patients, dated as close to the time of the minor salivary gland biopsy as possible. For immunohistochemistry, normal controls consisted of individuals evaluated for SS presenting with subjective xerostomia but with sparse infiltration of mononuclear cells/absence of focal inflammation, negative serology, and normal salivary flow. The degree of inflammation had previously been determined by focus scoring of histological, formalin-fixed paraffin-embedded haematoxylin and eosin (H&E) stained sections, by an oral pathologist. Focus score denominates the number of focal mononuclear cell infiltrates (foci) of at least 50 mononuclear cells, per 4 mm^2 (Chisholm and Mason, 1968; Greenspan *et al.*, 1974). For the serological investigations, healthy age- and sex-matched blood donors served as controls. The studies were approved by the Committee of Ethics at the University of Bergen (145/96-44.96).

OVERVIEW OF PAPER V



Murine tissue material

NOD and Balb/c mice (Paper V)

Female NOD and Balb/c mice were purchased from Taconic/Bomholtgård, Denmark. The animals were kept under standard animal housing conditions. Serum glucose levels were measured using Reflotron Plus Glucose test kit, Roche Diagnostics, Canada, and did not differ significantly between or within age-groups in NOD or Balb/c. NOD mice did not decrease in weight between 17 and 24 weeks of age. The experimental protocol was approved by the Committee for research on animals/Forsøksdyrutvalget (79-04/BBB).

Stimulated salivary flow was investigated in all mice. Prior to stimulation, mice were fasted for a minimum of 5 hours with water *ad libitum* and anesthetized using 0.10 ml Ketalar/Domitor per 10 g bodyweight. Whole saliva was collected after stimulation of secretion by pilocarpine in saline (0.5 µg per gram bodyweight, Sigma Chemical Co, St Louis, MO, USA) administered via the femoral artery, to ensure reliable uptake. Saliva was collected with capillary tubes for 10 minutes, the volume determined, and stored at -80º C until analyzed.

Salivary gland tissue was carefully dissected, kept on Histocon and snap-frozen as previously described. The tissue samples were cut by a cryostat, stained with H&E and then evaluated and morphometrically analyzed using a Leica DMLB light microscope connected to

a ColorView III-camera and AnalySIS® software (Soft Imaging System GmbH), to determine the focus score (Jonsson *et al.*, 1987), i.e. the number of foci of 50 or more mononuclear cells per mm^2 of glandular tissue, and the ratio of inflammation to the area of glandular tissue (ratio index) (Skarstein *et al.*, 1997). Both glands were examined in at least two tissue sections. In the majority of cases, histomorphology and degree of inflammation was similar. In case of inconsistencies the tissue block was cut further down and new H&E sections evaluated.

Immunohistochemistry

Frozen tissue

Avidin-Biotin-peroxidase Complex/ABC (Paper I, II, V)

Briefly, frozen salivary gland tissue was cut (five μm sections) with a cryostat (Leica Instruments GmbH, Nussloch, Germany) onto APES treated glass slides. Following stepwise fixation in +4°C 50% acetone for 30 seconds and 100% acetone for 5 minutes, endogenous peroxidase was quenched with 0.3% H_2O_2 in TBS pH 7.6. To avoid background staining from endogenous biotin, sections were treated with Avidin D and Biotin blocking solution (Vector Laboratories, Burlingame, CA, USA) for 15 minutes each. Non-specific binding was blocked by incubation for 30 minutes with normal rabbit serum in antibody diluent. The sections were then incubated with the relevant primary antibody for 60 minutes. The appropriate biotinylated secondary antibody was applied for 30 minutes. Binding of secondary reagents was detected by incubation with Avidin-Biotin solution kit (5 μl Avidin DH + 5 μl Biotin Peroxidase in 625 μl TBS) for 60 minutes.

Diaminobenzidine (DAB+) was used as chromogen. Sections were counterstained with haematoxylin and mounted with aqueous mounting medium (Thermo Shandon Immu-mount, Pittsburgh, PA). Following incubations, sections were washed with TBS pH 7.6 for 2 x 5 minutes. All procedures were performed at room temperature. Unless otherwise indicated, all materials were purchased from Dako A/S, Glostrup, Denmark.

EnVision detection technique (Papers I-IV)

After quenching of endogenous peroxidase activity (see above), sections were incubated with the appropriate primary antibody for 60 minutes and thereafter with EnVision+ (Dako A/S, Glostrup, Denmark), a horseradish peroxidase (HRP) and secondary antibody conjugated (dextrane) polymer, for 30 minutes. DAB+ was used as chromogen and remaining steps were similar to what is described for the ABC-method.

Immunostaining protocol with biotinylated antigens (Paper I)

Recombinant proteins were expressed from the pMAL vector (New England Biolabs, Beverly, MA). Purification (Elagib *et al.*, 1999), biotinylation of proteins and immunostaining were performed as previously described (Tengner *et al.*, 1998). The recombinant fusion partner maltose binding protein (MaBP) was used in parallel. In short, endogenous peroxiadase, avidin and biotin activity were blocked as described above, and non-specific protein binding was blocked by 5% powdered milk and 4% bovine serum albumin (BSA) in TBS for 15 minutes. Sections were then incubated with biotin labelled Ro 52-kD protein, Ro 60-kD protein, La protein, or MaBP for 45 minutes. Avidin and biotin–labelled HRP was used as secondary reagent and sections were incubated for 60 minutes. 3-amino-9-ethyl-carbazole (AEC) was used as a substrate. Sections were counterstained with Harris' hematoxylin. Following each step, except for the blocking step, the slides were rinsed and washed in TBS 2 x 5 minutes.

Detection of apoptotic cells by in situ labelling using the TUNEL method (Paper I)

DNA fragmentation during apoptosis was detected in situ by the TUNEL method as previously optimized (Ohlsson *et al.*, 2001). Cryosections (5 μm) were dried and fixed with 10% paraformaldehyde/phosphate buffered saline (PBS) for 30 minutes. Endogenous peroxidase was quenched by 0.1% H_2O_2 in TBS for 10 minutes. Sections were equilibrated in terminal deoxynucleotidyl transferase (TdT) buffer (0.5M cacodylate [pH 6.8], 1 mM CoCl2, 0.5M dithiothreitol, 0.05% BSA, 0.15M NaCl) at 37°C and thereafter covered with TdT buffer containing 0.1 units/μl TdT (Boehringer, Mannheim, Germany) and 8 nmoles/ml digoxigenin-conjugated dUTP (Boehringer) in a humidified chamber for 60 minutes at 37°C.

The reaction was stopped by washing in TBS supplemented with 5% fetal calf serum (FCS) followed by incubation with sheep anti-digoxigenin IgG (Boehringer) diluted to 5 μg/ml in blocking solution for 60 minutes. After soaking in 10% pooled human sera, 5% rabbit sera, and 2% FCS in TBS, sections were incubated with HRP-conjugated rabbit anti-sheep antibodies diluted 1:100 in blocking solution for 60 minutes. TUNEL-positive cells were visualized by incubation with AEC (see above) for 15 minutes, counterstained in Harris' haematoxylin, and mounted as previously described. Between each step, sections were washed in TBS, and unless otherwise indicated all steps were carried out at room temperature.

Negative control tissue sections from each patient were incubated as described above, omitting dUTP.

Double-labelling (Paper IV)

Freshly cut sections of frozen minor salivary gland tissue were fixed as previously described. Sections were incubated with the first primary antibody for 60 minutes, followed by quenching of endogenous peroxidase by 0.03% H_2O_2 for 5 minutes. Thereafter, sections were incubated with Envision HRP for 30 minutes. DAB+ was used as a chromogen. Following a 5 minute rinse in water, sections were incubated with Doublestain Block for 3 minutes. Incubation with the second primary antibody for 60 minutes was followed by incubation with Envision Alkaline-phosphatase for 30 minutes. After development with New Fuchsin for 12 minutes or Liquid Permanent Red for 7 minutes, sections were counterstained with Haematoxylin, rinsed in tap water, and mounted with an aqueous mounting medium (see above). Between each step, sections were washed in TBS pH 7.6 for 10 minutes. Antibodies were diluted in antibody diluent. For comparison, serial sections were incubated separately with the two first primary antibodies. Unless otherwise indicated, all reagents were purchased from DAKO, Glostrup, Denmark.

Formalin-fixed, paraffin-embedded tissue sections

Heat induced antigen retrieval (Paper III)

Paraffin-embedded salivary gland tissue was cut (4-6 μm sections) with a microtome (Leica Instruments GmbH, Nussloch, Germany) onto SuperFrost® Plus microscope slides (Menzel GmbH & Co KG, Braunschweig, Germany). Following step-wise de-paraffinization and re-hydration by xylene and alcohol, heat-induced antigen retrieval (HIER) by use of Citrate buffer (S1699, Dako, Glostrup, Denmark) was performed. Quenching of endogenous peroxidase, incubations with primary antibodies and detection of antigen by the HRP-conjugated EnVision+ detection system was employed (see above). Following counter-staining by Haematoxylin, sections were de-hydrated and mounted with a non-aqueous mounting medium (Eukitt) prior to analysis. Except for the HIER, all incubations were performed in room temperature.

Double-labelling for CD21/CD23/CD35 and IgD (Paper III)

To visualize the ectopic GC-formation, double-staining was performed. Briefly, paraffin-embedded minor salivary gland tissue was cut and pre-treated as described above. Sections were incubated with monoclonal mouse anti-human CD21 (1:10), CD23 (1:5) or CD35 (1:5) for 60 minutes, followed by quenching of endogenous peroxidase for 5 minutes. Thereafter, sections were incubated with HRP-conjugated Envision for 40 minutes. DAB+ was used as a chromogen for 7 minutes. Following a 5 minute rinse in water, sections were incubated with Doublestain Block for 3 minutes. Incubation with the second primary antibody IgD (1:2.000) for 60 minutes at room temperature and over-night at 4ºC, was followed by incubation with Alkaline-phosphatase (AP)-conjugated Envision for 40 minutes. After development with Liquid Permanent Red for 7-10 minutes, sections were counterstained with haematoxylin, rinsed in tap water, and mounted with aqueous mounting medium. Between each step, sections were washed in TBS for 10 minutes.

Evaluation of staining

Between 3 and 6 minor salivary glands from each patient were evaluated in all stainings performed (*Papers I-IV*). Cells were counted using a light microscope with a grid and a 10x or 40x objective. Positive cells were analyzed within 5-6 randomly selected fields across the whole section (both infiltrates and stroma). In specimens with fewer than 5 infiltrates, more than one area of infiltrating mononuclear cells within the same focal infiltrate was counted. For the adhesion molecules, cells with more than 50% of the cell membrane immunohistochemically stained quantified as positive. In quantifying TUNEL reactivity, only stained nucleus or nuclear fragments that morphologically matched apoptotic cells were counted as positive. Chemokines were mainly expressed by epithelial cells, and positive acinar and ductal epithelial cells in each glandular section were counted and expressed as the mean of 3–6 glands.

In *Paper II*, the morphology of the salivary gland tissue infiltration could be divided into three units: GC, FI and scattered cells/small cell clusters in close relation to acinar or to ductal epithelium. Interstitial infiltration was also included in this paper, as these cells may participate directly in cell and tissue damage. Intensity of staining was not evaluated. For GC and FI the expression of CD4, CD8, CD20, CD35, CD38, CD68, Ki-67 and BAFF was evaluated as the percentage of positive infiltrating inflammatory cells of the total number of infiltrating inflammatory cells. Interstitially, the expression of immune reactive molecules was evaluated as the number of positive cells in close relation to acinar or ductal epithelium

of the total number of acinar or ductal epithelial cells, respectively, hereby providing a number for the density of the interstitial infiltration.

In *Paper III* sections were evaluated based on the appearance of the FDC/B cell networks as presented by CD21, CD23 and CD35, and the distribution and pattern of the IgD staining within chronic inflammatory infiltrates. A scoring system for ectopic germinal center formation – GC-score, was defined.

The staining-pattern of CD21, CD23 and CD35 within chronic inflammatory cell infiltrates, could be divided into six morphological entities numbered, each considered a progression of the previous. IgD positive cells were divided into three entities based on the density of the cell staining. The denomination obtained from the phenotypic staining pattern of CD21, CD23, CD35, and IgD was summarized and provided the basis for a "GC-score" ranging from 0-17. The 30% who presented with highest GC-score (cut-off $\geq$ 12) were defined as GC+, and GC-score < 12 were defined as GC-.

In *Paper IV* the expression of E-cadherin positive cells is presented as the percentage of E-cadherin positive ductal and acinar epithelial cells of the total number of ductal and acinar epithelial cells. The αEβ7/CD103 positive mononuclear cells are presented as the percentage of ductal or acinar units infiltrated by one or more αEβ7/CD103 positive cells and the total number of ductal or acinar units, respectively.

Tissue controls

In the human material (*Papers I-IV*) incubations with isotype and concentration matched controls were performed to ensure specific staining. Tonsil was used as the positive tissue control. Salivary gland tissue from patients evaluated for SS but not fulfilling the revised criteria (focus score < 1, negative serology and normal salivary flow) (Vitali *et al.*, 2002) served as negative tissue controls (*Papers I, II and IV*).

In the murine material (*Paper V*), incubations with antibody diluent was performed to ensure specific staining. Cervical lymph nodes served as the positive tissue control. Salivary gland tissue from age-matched control-mice (Balb/c) served as the negative tissue control.

Enzyme linked immunosorbent assay – ELISA (Papers I, II, IV)

Serum antibodies against Ro52, Ro60 and La (*Paper I*) were detected by an in-house ELISA as previously described (Elagib *et al.*, 1999) with minor modifications. Commercial ELISA kits were used to detect soluble BAFF and APRIL (*Paper II*) and E-cadherin (*Paper IV*) in

serum samples form patients with pSS, and healthy controls. All analyzes were performed according to the manufacturer's instructions.

Cytokine measurements in serum and saliva (Paper V)

Serum and saliva was analyzed using a mouse cytokine ten-plex assay kit (Catalog No LMC0001, BioSource, Nivelles, Belgium), as recommended by the manufacturer, measured on a Luminex 100 (Luminex Corp, Austin, Texas, USA) and analyzed using StarSection software (Applied Cytometry Systems, Dinnington, UK). Mean values of cytokines IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, GM-CSF, IFN-γ and TNF-α present at detectable levels were compared between the different age-groups of mice, and to the age-matched controls.

Statistical analyses

Paper I: Statistical analysis. The Mann-Whitney test was used for statistical analysis of nonparametric data. The Spearman correlation coefficient was calculated for testing correlation of parameters. Chi-square analysis was employed for comparing GC occurrence in primary and secondary SS. P-values less than 0.05 were considered statistically significant.

Paper II: Statistical analyses were performed using the Mann–Whitney test for non-parametric data. Correlation was investigated using Pearson's correlation co-efficient. A p-value of $p < 0.05$ was considered statistically significant.

Paper III: Data were frequently not normally distributed. The Mann-Whitney test was used to study differences between groups and Spearman correlation for relationships between variables. Chi-square analysis was employed for categorical data.

Paper IV: Statistical analyses were performed using the Mann–Whitney test for non-parametric data. Correlation was investigated using Pearson's correlation co-efficient. A p-value of $p < 0.05$ was considered statistically significant.

Paper V: Statistical analyzes were performed using 1-way Anova followed by Bonferroni post-test for selected groups (modified unpaired two-tailed student's t-test optimized for multiple group comparison). To determine the linear relationship between two variables, values were compared using Pearson's correlation test (two-tailed).

Statistical analyses in *Papers I-IV* were preformed using SPSS 13.0 and GraphPad Prism 4.0 in *Paper V*.

Methodological considerations

Minor salivary gland tissue samples

The tissue sections described in Papers I-IV were obtained from patients under investigation for SS at the Department of Rheumatology between 1991 and 2002 (Paper I, n=165), 1989 and 2003 (Paper II, only pSS, n=130), 1989 and 2005 (Paper III, n=272), and 1991 and 2004 (Paper IV, n=27).

		n	GC+ (%)	Paraffin (P) / Frozen (F)
Paper I		165	28 (17)	
	pSS	91	19 (21)	F (n=14)
	sSS	50	7 (14)	F (n=5)
	FS	24	2 (8)	
Paper II	pSS	130	33 (25)	F (n=37)*
Paper III		272	59 (22)	
	pSS	169	47 (28)	P (n=60)
	sSS	38	6 (16)	
	FS	65	6 (9)	
Paper IV		27*	22	
	pSS	22	12	F (n=22)*
	sSS	5	3	F (n=5)*

*partially overlapping with Paper I, supplemental frozen salivary gland tissue added

Routine histopathological assessment for focal inflammation was performed at the Department of Oral Sciences – Oral pathology and Forensic Odontology and/or the Gade Institute – Department of Pathology. The morphological screening for GC-like features was performed by MVJ on the H&E stained paraffin embedded material. In Papers I, II and IV, immunohistochemical investigations were performed on frozen material from tissue sampled at the same time as the paraffin embedded material diagnosis was based on. As a result, similar inflammation and/or morphology may not have been present in the frozen as in the paraffin, and vice versa. There is some overlap concerning frozen minor salivary gland tissue used in Paper I, II and IV, but inflammation and morphology may have changed as the tissue was cut down. In Paper III, immunohistochemical investigations were performed on the same tissue blocks as had been used in the diagnostics, i.e inflammation and morphology was the most similar to the diagnostic background. As could be expected, however, the morphology of these sections changed as well, as a result of being cut down.

In addition, to obtain staining on paraffin embedded material, heat-induced epitope-retrieval was detrimental. Staining is highly dependant on the fixation of tissue (Hayat, 2002).

Frozen tissue was transported in Histocon prior to snap-freezing by iso-pentane and storage in -80°C. Cut sections were stained within three weeks in order not to lose antigen expression. Frozen tissue sections were stored at -80°C and cut paraffin sections at 4°C.

Detection method – EnVision and ABC

When applicable, an EnVision based staining system was utilized in favour of the traditional AvidinBiotinComplex method (Jordan *et al.*, 2002; Marthinussen *et al.*, 2002; Sabattini *et al.*, 1998; Vyberg *et al.*, 2005). The EnVision technique is based on a polymer back-bone conjugated with both secondary antibodies and enzyme (HRP or AP). Several secondary antibodies on the same backbone makes EnVision more sensitive than the ABC method, and it is possible to use higher dilutions of the primary antibody. In addition, the EnVision system does not contain biotin, which is an advantage when working with salivary glands where endogenous biotin may give rise to unwanted background staining. Finally, since the secondary antibody and enzyme are applied simultaneously, the protocol is less time-consuming. Unfortunately, the EnVision system is limited to antibodies made in mouse or rabbit.

Substrate – AEC and DAB+

For most of the immunohistochemical staining, DAB+ was selected before AEC, because DAB+, in our hands, was considered more reactive. The combination of EnVision+ and DAB+ provides an increase in sensitivity and specificity (Marthinussen *et al.*, 2002).

Statistical considerations (Paper V)

To determine if the means between age-groups of NOD and Balb/c mice, or between NOD and age-matched controls differed significantly, all data was analyzed using 1-way Anova followed by Bonferroni post-test for selected groups (modified unpaired two-tailed student's t-test optimized for multiple group comparison). To normalize skewed distributions, cytokine data was log-transformed prior to all statistical analyses. Correlation analyses were restricted to NOD and to datasets where a scientific reason for a causal connection was given from either our results or from previous studies.

SUMMARY OF RESULTS

Paper I

GC-like structures were observed in 28 of 165 patients (17%). When GC were defined as T and B cell aggregates, with proliferating cells, a network of follicular dendritic cells, and activated endothelial cells, such microenvironments were found in all patients in whom structures with GC-like morphology were observed. The defined microenvironments were not found in patients without apparent GC-like structures in the H&E routine biopsy. The GC formed within the target tissue showed functional features with production of autoantibodies (anti-Ro/SSA and anti-La/SSB) and apoptotic events (by TUNEL staining), and the local production of anti-Ro/SSA and anti-La/SSB autoantibodies was significantly increased (P = 0.04) in patients with GC development.

Paper II

The B cell related molecules B cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL) are members of the tumour necrosis factor super-family. Circulating levels of BAFF and APRIL were investigated in relation to serological deviations and lymphoid organization in the salivary glands of SS. Lymphoid organization in the shape of ectopic germinal centers were detected in 33 of 130 consecutive minor salivary gland biopsies and coincided with increased focus score and elevated levels of serum IgG. Follicular dendritic cell networks, significantly increased levels of proliferating cells, increased numbers of B cells and reduced numbers of T cells were detected in GC compared to FI, both when comparing within and between GC+ and GC- glands. BAFF+ cells were detected in similar levels in GC and FI. Interestingly, CD38+ plasma cells were detected in the periphery of GC, albeit at reduced numbers compared to the FI, where plasma cells appeared scattered within the whole infiltrate.

Paper III

Retrospectively, minor salivary gland biopsies (n=272) with focal lymphoid aggregates corresponding to focus score $\geq$ 1 were evaluated for the presence of GC-like morphology. Randomly selected biopsies from patients with pSS (n=60) were further investigated by immunohistochemistry. Based on the reticular network staining-pattern of CD21, CD23 and CD35, and the distribution of IgD positive cells, a GC-score was constructed and biopsies characterized as GC+ or GC-. Relevant clinical information was obtained from medical

records. 169/272 patients fulfilled the American-European criteria for pSS (Vitali *et al.*, 2002). The remaining either fulfilled criteria for sSS, or records were not accessible and it was not possible to ascertain either pSS or sSS. By morphology, GC-like features were observed in 47/169 (28%) biopsies. By immunohistochemistry, ectopic follicles/GC were determined in 18/60 (30%) patients, compared to 11/60 (18%) by the original morphological screening of this random cohort. Morphological and phenotypic GC corresponded in 5/11 biopsies. Irrespective of method to detect GC, mean inflammatory focus score was significantly increased in GC+ compared to GC- ($p<0.05$), and elevated titers of rheumatoid factor, serum autoantibodies and IgG levels were more common in GC+.

Paper IV

Serum levels of sE-cadherin were significantly increased in SS compared to non-SS and non-significantly in GC+ compared to GC– patients. Membrane-bound E-cadherin was detected on the majority of acinar and ductal epithelial cells in both SS and non-SS. αEβ7/CD103-positive cells were found scattered in focal infiltrates and GC, and in small clusters close to ductal and acinar epithelium at an increased level in SS compared to non-SS. Interestingly, E-cadherin-positive cells were detected randomly dispersed in focal lymphocytic infiltrates in 10/21 patients. By double-labelling, the mononuclear cells with the E-cadherin-positive component were identified as CD68+ macrophages.

Paper V

Female NOD mice of different age-groups were used to mimic different disease-stages of SS. Histopathology and cellular composition were compared in 8-, 17- and 24-week old female mice. In addition, cytokines were analyzed in serum and saliva, and compared to age-matched controls. Salivary flow remained unchanged when comparing 8- and 17-week old NOD mice, while a significant decrease took place between 17 and 24 weeks ($p<0.001$). In contrast, the significant changes in histopathology of the salivary glands occurred before 17 weeks of age. Immunohistochemical analyses revealed changes in inflammatory cell organization in the salivary glands, resembling ectopic germinal centers. Regarding cytokines, significant differences were detected in serum levels of IL-2, IL-5 and GM-CSF, and IL-4 and TNF-α in saliva. Correlation analyses revealed a negative association between salivary secretion and IFN-γ and TNF-α levels in saliva. Association of these cytokines in relation to histopathological changes was consistently weak.

GENERAL DISCUSSION

Recruitment of inflammatory cells in SS – attraction and retention

Chronic inflammation in the form of a designated lymphoepithelial lesion (Seifert and Sobin, 1991) is an important issue in the diagnostics of SS (Vitali *et al.*, 2002). From a pathological point of view, the histopathological aspect of SS is based on the presence of focal mononuclear cell infiltrates consisting of at least 50 cells per 4 mm^2 minor salivary gland tissue (Chisholm and Mason, 1968; Greenspan *et al.*, 1974). In approximately $1/4^{th}$ of patients with SS we have discovered GC-like formations within otherwise normal salivary gland tissue. These structures were linked to increased attraction and retention of chronic inflammatory cells and local autoantibody production (Paper I), B cell activation and disease progression (Paper II) and a distinct seroimmunologcial profile characterized by increased IgG, RF and autoantbodies (Paper III).

The inflammatory cell foci interact with the surrounding epithelium as well as contribute to the progressive inflammation, by synthesis and expression of various cytokines, chemokines and adhesion molecules, reviewed in (Delaleu *et al.*, 2004). In chronic inflammatory sites, cells are prevented from dying and leaving the inflamed tissue, a result of inappropriate expression of pro-survival and pro-retention signals by stromal elements. Fibroblasts represent such stromal elements in RA (Buckley *et al.*, 2001; Buckley, 2003). In SS, salivary gland epithelium may represent such stromal elements (Figure 10).

Chemokines choreograph the positioning of cells during an immune response. We have investigated local factors mediating attraction and retention of chronic inflammatory cells, by chemokines CXCL12, CXCL13 and CCL21, and by adhesion molecules ICAM-1/LFA-1 and VCAM-1/VLA-1 (Paper I). The CXCL12-CXCR4 interaction was previously investigated in RA, enabling a switch from migratory to stationary phenotype as leukocytes enter tissue microenvironments, reviewed in (Buckley, 2003). CXCL13 and its receptor CXCR5 has previously been investigated in chronic inflammatory cell infiltrates in salivary glands of patients with SS (Amft *et al.*, 2001; Salomonsson *et al.*, 2002; Xanthou *et al.*, 2001) and RA (Burman *et al.*, 2005; Schmutz *et al.*, 2005; Shi *et al.*, 2001; Takemura *et al.*, 2001). The migration of naïve and activated T cells into lymphoid tissues is mediated by the chemokine SLC/CCL21, expressed on HEVs, stromal cells and dendritic cells in lymphoid tissue, and binds to the CCR7 chemokine receptor on naïve T cells (Buckley, 2003). We have detected high expression of CCL21 (Paper I) in chronic inflammatory cell infiltrates in SS, indicating that SS is a result of increased lymphocyte entry to the salivary glands. Interstingly, CCR7 has also been ascribed in T lymphocyte exit from peripheral tissues such as skin

(Debes *et al.*, 2005). The occurrence of CCR7 was investigated in peripheral blood B cells in pSS {Hansen, 2005 #351} but no significant differences were detected compared to healthy controls. All the same, it is tempting to speculate whether CCR7 expression is altered or impaired upon contact/interaction with salivary gland tissue stroma.

Recent findings indicate that CXCR3, the target of CXCL10, CXCL11 and CXCL19, acts as a chemokine scavenging receptor in normal salivary gland epithelial cells. In salivary gland epithelium from patients with SS, however, the function of CXCR3 was impaired, possibly favouring chemotaxis and increased recruitment of T lymphocytes expressing CXCR3 (Sfriso *et al.*, 2006).

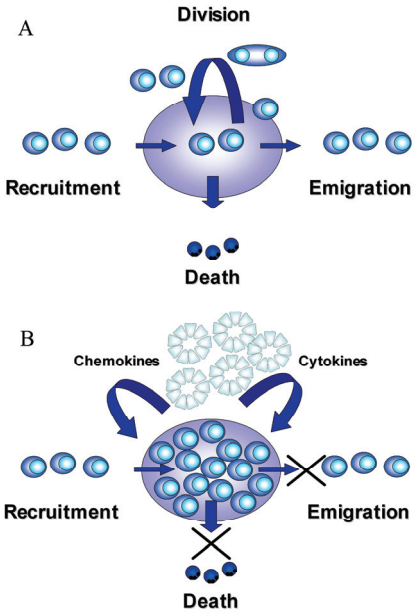


Figure 10. Homeostasis is maintained during a normal inflammatory reaction (A). Cell accumulation in an organ depends on the balance of cell attraction, division (proliferation), emigration and death (apoptosis). In order for the immune reaction to resolve, recruited cells must either die by apoptosis or exit via lymphatic vessels. In a chronic inflammatory reaction (B), local pro-retentive and pro-survival factors (cytokines and chemokines) produced by salivary gland epithelial cells or inflammatory cells, influence the local environment and cause inappropriate accumulation of cells. Adapted and modified from (Buckley *et al.*, 2001).

In NOD mice the attraction and retention of lymphocytes in lacrimal glands depended on expression of integrin αEβ1, L-selectin, and LFA-1 expression on lymphocytes, and VCAM-1 and peripheral node addressin (PNAd) on endothelial cells (Mikulowska-Mennis *et al.*, 2001). We have detected increased levels of αEβ7/CD103+ cells in close relation to E-cadherin+ epithelial cells in the salivary glands of patients with SS. This issue will be further discussed in the section on Epithelial cell adhesion molecules in SS (Paper IV). In contrast, migration to the lacrimal glands seemed to occur independently of MAdCAM and $\alpha_4\beta_7$ (Mikulowska-Mennis *et al.*, 2001). However, these data were based on findings in mice and in lacrimal glands, and in human salivary gland tissue or intestinal mucosa, the situation may be different.

Local and systemic autoantibody production

Although the exact role and function of the autoantigen and Ro/SSA and La/SSB autoantibodies are not yet determined, positive serology of Ro/SSA and La/SSB are associated features of SS (Vitali *et al.*, 2002). Local autoantibody production has been described by immunohistochemistry in the salivary glands of patients with SS (Salomonsson and Wahren-Herlenius, 2003; Tengner *et al.*, 1998) and was demonstrated in association with GC-like structures in the salivary glands from patients with SS (Paper I). Autoantibody producing cells may also reside in other tissues, as demonstrated in the thymus and lymph nodes in addition to salivary glands of sero-positive MRL/*lpr* mice (Wahren *et al.*, 1994b).

In addition to serum and salivary glands, autoantibodies have also been detected in saliva (Horsfall *et al.*, 1989) and ectopic GC-formation with autoantibody production in the salivary glands was thus implicated in the pathogenesis of SS. When the immune system fails to create an efficient immune response against a highly localized antigen, it may prove advantageous to move the lymphoid tissue to the target of the inflammatory response, in SS the salivary glands, as will be further discussed in the section on Clinical implications of ectopic GC formation.

In Paper I, the autoantibody-producing cells were located in the margin of large infiltrates/GC, as well as interstitially. In correlation, CD38+ plasma cells were detected in the periphery of large infiltrates, as well as in cellular accumulations in the salivary gland interstitial area (Paper II). The local production of anti-Ro/SSA and anti-La/SSB in the salivary glands was associated with higher levels of autoantibodies in sera (Paper I). Although absolute titres of Ro/SSA and La/SSB were not determined in Paper II and Paper III, a higher frequency of patients with ectopic GC were seropositive for anti-Ro/SSA and/or anti-La/SSB, confirming findings in Paper I.

Autoantibody-producing cells in large infiltrates were also in part related to apoptotic events, identified by the TUNEL method as previously described (Ohlsson *et al.*, 2001). In accordance to previous findings, moderate levels of apoptotic cells were detected (Ohlsson *et al.*, 2001), somewhat more frequently in the GC+ compared to the GC- salivary glands (Paper I). These findings indicate an attenuation or escape from apoptosis (Ohlsson *et al.*, 2002) possibly mediated by BAFF (Szodoray *et al.*, 2003).

B cell activation and germinal center formation in SS

Germinal center formation was investigated in more detail (Paper II), and the contemporary existence of GC and FI was disclosed. GC were characterized by B and T cell organization,

increased levels of proliferating cells, follicular dendritic cell networks and the localization of plasma cells in a mantel zone-like area. Antigen-presenting cells in the focal infiltrates and germinal centers indicate ongoing antigen-presentation (Paper II and IV). BAFF was expressed on inflammatory cells scattered in both GC and FI, but no particular staining pattern could be determined for the two entities.

BAFF-expressing cells are considered important in the pathogenesis of pSS and have previously been linked to attenuated apoptosis (Groom *et al.*, 2002; Lavie *et al.*, 2004; Szodoray *et al.*, 2003). The attenuation of apoptosis induced by excess levels of BAFF has been suggested to contribute to progression of disease (Mackay and Browning, 2002). The expression of BAFF was comparable in GC and FI, showing a membrane-bound staining on scattered infiltrating cells. Serum levels of sBAFF were increased in pSS, but similar in GC+ and GC- patients (Paper II). This observation indicates that BAFF is not fundamental for the GC-formation, confirming findings in BAFF knock-out mice (Vora *et al.*, 2003). Increased serum levels of sBAFF compared to healthy donors reflect an ideal environment for B cell activation, wherein excess levels of BAFF may "tip the scale" in favour of auto-reactive B cells, supplying them with various survival factors (Figure 11). Elevated levels of sBAFF have also been described to correlate with serum levels of autoantibodies in pSS (Groom *et al.*, 2002; Mariette *et al.*, 2003), although we could only detect a slight increase of sBAFF in the anti-Ro/SSA/La/SSB+ patients (Paper II).

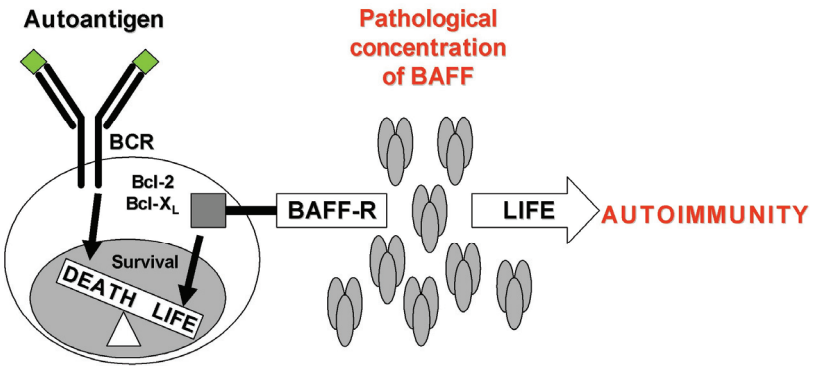


Figure 11. The balance between B cell death and survival is modified by signals generated by interactions between BCR and BAFF–BAFF-R. Physiological levels of BAFF in the presence of low-affinity BCR signals lead to cell maturation, while the encounter of BCR with antigen in physiological circumstances and normal BAFF levels leads to negative selection. Increased BAFF production favours B cell survival, promoted by induction of anti-apoptotic pathways such as Bcl-2 and Bcl-X_L. Figure modified from (Mackay and Browning, 2002).

Inflammatory cells expressing BAFF were detected in close relation to acinar epithelium in both GC+ and GC- patients, indicating that although ectopic GC-formation in pSS occurs independently of BAFF, BAFF may still play a role in the initiation and establishment of chronic inflammatory cell infiltrates. These data were further supported by the correlation between increased levels of sBAFF and increased focus score in the GC+ patients (Paper II). No significant correlations were detected in GC- patients. The response of infiltrating B cells to BAFF may be modified by various cytokines (Szodoray *et al.*, 2005) and this may differ in GC+ and GC- patients.

Salivary gland epithelial cells stimulated by IFN-α and INF-γ are reported to secrete BAFF (Ittah *et al.*, 2006). Type I interferons were recently implicated in the pathogenesis of SS (Hjelmervik *et al.*, 2005), wherein IFN-α promotes the autoimmune process with increased autoantibody production and formation of endogenous IFN-α inducers (Båve *et al.*, 2005) and IFN-γ is reported to inducing expression of T cell attracting chemokine I-TAC/CXCL11 in ductal salivary gland epithelium (Ogawa *et al.*, 2004). Interestingly, TNF-α in combination with IFN-γ was the strongest stimulatory agent for secretion of BAFF by salivary gland epithelial cells (Ittah *et al.*, 2006).

APRIL has previously been reported to have a possible role in T-independent type II antigen responses and T cell survival, but also to induce proliferation/survival of non-lymphoid cells (Mackay and Ambrose, 2003). Interestingly, a correlation was noted between focus score, serum levels of IgG and increased levels of sAPRIL, and sAPRIL levels were significantly increased in the Ro/SSA/La/SSB+ patients with pSS (Paper II). Salivary gland epithelium showed a cytoplasmic staining of APRIL (authors' own unpublished observations).

Clinical implications of ectopic germinal center formation?

In Paper III, all the investigated patients with pSS (n=169) fulfilled the revised European criteria of 2002 (Vitali *et al.*, 2002). By morphology, GC-like structures were detected in 47/169 of patients. Focus score, IgG levels and RF titres were elevated compared to the GC- patients. In the randomly selected cohort (n=60), eleven patients had morphological GC. Morphological GC were ascertained by the CD21/CD23/CD35 phenotype in 5/11 biopsies. If taking morphology into account, this number increased to 7/11. Autoantibodies to Ro/SSA and La/SSB were also more common in GC+ compared to GC- (Paper III). However, sero-positivity for anti-Ro/SSA or anti-La/SSB does not directly implicate disease, as indicated by

a recent report wherein 12/100 healthy blood donors had auto-reactive Ro-52 and 15/100 had auto-reactive La-48 (Garberg *et al.*, 2005).

Mean un-stimulated salivary flow was reduced in the GC+ patients (Paper III). Previous reports indicate that the submandibular and sublingual salivary glands are most severely affected in SS (Lindvall and Jonsson, 1986). As a result, the lubricating saliva from these glands is the first that is lost, while the watery, protein-rich parotid saliva remains seemingly unaffected. Consequently, total salivary secretion can therefore be within normal values, while un-stimulated, resting saliva is affected, giving a low secretion rate. On the other hand, it is debated how objective and reliable the un-stimulated salivary secretion measurement is, and how the un-stimulated secretion from the parotid gland compares to the submandibular and sublingual glands (Atkinson *et al.*, 1990; Atkinson, 1993; Lindvall and Jonsson, 1986).

It is speculated whether the chronic inflammation in the salivary glands in SS is a result of chronic inflammation resulting from retained factors in saliva, or antigen in the salivary gland tissue. Focal infiltrates develop surrounding the ducts, indicating that the ducts either contain chronic triggers, the ductal epithelial cells function as APCs, or the the situation is similar to the tonsil/lymph node where antigen enters the lymphoid tissue via the epithelium. Nevertheless, autoimmune diseases are considered the result of a multi-step process wherein genetic and environmental factors interact over a long period of time. Lymphoid neogenesis within the salivary glands in SS may be a result of chronic local stress, or indeed an important aspect in the chronic nature of the autoimmune response, imposing a possible risk for immune recognition and breakdown of self-tolerance per se (Figure 12).

An association of GC development with increased risk of B cell lymphomas has been proposed (Voulgarelis *et al.*, 1999), wherein formation of proliferating GC were thought to contribute to malignant transformation and development of MALT lymphoma (Figure 12). The estimated life-time risk of lymphoma in SS has been estimated to be 5-10% (Ioannidis *et al.*, 2002; Voulgarelis *et al.*, 1999), but in general mortality is not significantly increased compared to the general population (Theander *et al.*, 2004). Patients with SS and chronic *Helicobacter pylori* infection are at increased risk for developing lymphomas, possibly related to prolonged lymphocytic activation in the target organ(s) of these patients (Raderer *et al.*, 2001; Streubel *et al.*, 2004). In a recent study, significant predictors of lymphoproliferative disease were purpura/skin vasculitis, low levels of complement factors C3/C4, CD4+ T-lymphocytopenia and a low CD4+/CD8+ T-cell ratio (Ioannidis *et al.*, 2002; Theander *et al.*, 2005). Unfortunately, data on C3/C4 levels was only available in 50/165 patients (Paper III).

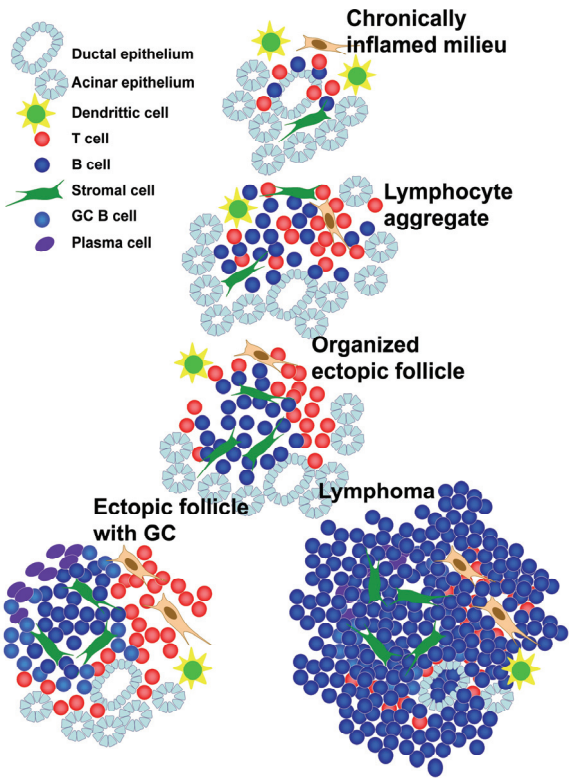


Figure 12. Chronic inflammation in the salivary glands may induce formation of ectopic lymphoid tissue. Hypothetical steps leading to development of GC in salivary gland tissue are illustrated above. Activated T cells, B cells and DC are detected in the salivary and lacrimal glands and preferentially accumulate around ductal epithelium. Activated lymphocytes induce a change of stromal cells into FDCs that produce CXCL13 and CXCL12 leading to attraction of CXCR5 and CXCR4-expressing B and T cells, respectively. Organized aggregates composed of B cells and a network of functionally mature FDCs form and a GC develop. T cells and plasma cells are present at the periphery of the follicle. Long-term, inappropriate cytokine production may lead to prolonged BAFF expression/secretion, in turn possibly promoting illegitimate DNA recombinations and somatic mutations that contribute to neoplastic transformations, reviewed in (Szodoray and Jonsson, 2005). Figure adapted from (Uccelli *et al.*, 2005) and modified.

B cell dysfunction (d'Arbonneau *et al.*, 2006) and disturbances of B cell maturation (Bohnhorst *et al.*, 2001; Bohnhorst *et al.*, 2002) have been demonstrated in patients with SS, implying that over-stimulation and neglected selection may take place in induced GC in the affected glands. In accordance, formation of ectopic lymphoid microstructures in non-lymphoid organs participate in the pathogenesis of organ-specific autoimmune reactions and underscore an essential role for the target organ in inflammatory cell recruitment and disease progression (Paper I-III). Despite inconclusive findings regarding the C3/C4 levels in the present pSS cohort, results from Paper III indicate a certain clinical immunological phenotype for SS patients with ectopic lymphoid organization, which warrant further prospective studies. Whether ectopic GC identifies patients at risk remains to be seen in prospective studies.

Epithelial cell adhesion molecules in SS

E-cadherin is expressed by epithelial cells and infiltrating cells in both germinal centers and focal infiltrates, mediating homo- and heterotypic adhesion between epithelial cells (Gumbiner, 1996) and αEβ7/CD103+ T lymphocytes (Cepek *et al.*, 1994). Altered E-cadherin staining patterns are related to malignant transformation of various cancers, to both metastasis and invasive growth (Davies *et al.*, 2001; Noë *et al.*, 2001). Soluble E-cadherin (sE-cadherin) has, among others, previously been investigated in relation to systemic inflammatory responses (Pittard *et al.*, 1996) and as a pre-therapeutic factor for survival in gastric cancer (Chan *et al.*, 2001; Chan *et al.*, 2003).

The majority of salivary gland epithelial cells expressed E-cadherin. An interesting finding was the observation of E-cadherin positive cells within chronic inflammatory cell infiltrates, as previously described in major salivary glands from NOD mice (Esch *et al.*, 2000). By co-staining, the E-cadherin+ infiltrating cells were identified as CD68+ macrophages (Paper IV).

Clusters of αEβ7/CD103 positive cells were located nearby and occasionally infiltrated E-cadherin expressing ductal and acinar epithelium at significantly increased levels in SS compared to non-SS individuals. These findings and the positive correlation of infiltrated acini and disease progression (focus score) suggest a role for the αEβ7/CD103 and E-cadherin interaction in early homing and retention of inflammatory cells in the salivary glands in SS.

In contrast to previous findings (Fujihara *et al.*, 1999; Kroneld *et al.*, 1998) our study demonstrated αEβ7/CD103 positive cells in various lymphocytic infiltration (GC and FI) and in relation to salivary gland acinar and ductal epithelium from non-SS individuals as well. Fujihara et al did not detect αEβ7/CD103 on lymphocytes adhering to acinar epithelium in any of the non-SS lacrimal glands (Fujihara *et al.*, 1999), while Kroneld et al detected αEβ7/CD103 positive cells in 90% of the SS salivary glands investigated (Kroneld *et al.*, 1998). Factors such as fixation/preparation of tissue specimens, detection methods and substrate may affect the expression and possibility to detect antigen, as considered in the Materials and Methods section.

Elevated serum levels of sE-cadherin were detected in patients with SS, and indicate increased epithelial tissue turnover, possibly related to ongoing tissue regeneration induced by chronic inflammatory changes in the exocrine glands, but a relationship with the expression of membrane-bound E-cadherin on salivary gland epithelial cells could not be detected. Most

likely, the soluble peptide fragment (80 kDa) detected as sE-cadherin is a degradation product of membrane-bound E-cadherin, generated by a Ca^{2+} dependent proteolytic action. *In vitro* experiments indicate that MMPs matrilysin and stromelysin-1 cleave E-cadherin at the cell surface, and that the released fragment inhibits E-cadherin functions in a paracrine way (Noë *et al.*, 2001). MMPs have previously been described in glandular destruction in SS (Perez *et al.*, 2000) by disturbing the extra-cellular matrix (Goicovich *et al.*, 2003).

Serum levels of sE-cadherin were further studied in regard to a potential non-invasive indicator of SS. Other chronic inflammatory diseases such as oral lichen planus (OLP), and RA were included as controls. Serum levels of sE-cadherin were found to be significantly increased compared to healthy donors, but not compared to patients with OLP or RA. Interestingly, one of the highest levels of sE-cadherin measured was in one of the blood donors (authors' own unpublished, preliminary observations).

In comparison to other studies, our assay (Paper V) was purchased from R&D Systems (www.rndsystems.com), and has a different standard/detection limit from the Zymed assay (www.invitrogen.com) used by others (Chan *et al.*, 2001; Chan *et al.*, 2003). Similar trends were noted, but limited the possibility to at least quantitatively compare one study to another.

SS pathogenesis in the NOD mouse

Chronic inflammation and hyposalivation

In NOD major salivary glands, we further investigated cellular composition and lymphoid organization of the chronic inflammatory cell infiltrates. Interestingly, distinct B and T cell areas, and proliferating cells were detected in 4/12 glands from mice > 17 weeks of age (Paper V). These findings indicate that in some mice proliferation of inflammatory cells, in addition to attraction and retention (homing), contributes to the progression of disease. This has previously been suggested in RA synovium (Weyand and Goronzy, 2003) and human minor salivary glands (Amft *et al.*, 2001; Stott *et al.*, 1998; Xanthou *et al.*, 2001) but to our knowledge not been described in NOD salivary glands. As observed in human minor salivary glands (Papers I-III), proliferating cells were associated with progression of disease (higher focus score) compared to litter-mates/peers. No differences in cytokine levels were observed when comparing NOD mice with or without proliferating inflammatory cells in the salivary glands (unpublished data from paper V). The significance of proliferating cells within chronic inflammatory cell infiltrates remains unknown, but may be related to increased B cell activity and survival, as discussed in Paper II.

It has long been debated when the (clinical) loss of salivary secretion occurs and how it is mediated, what comes first and what is the link between glandular inflammation and hyposalivation. Possible suggestions include autoantibodies to the M3R (Waterman *et al.*, 2000) and/or influence of the innervation (Rosignoli *et al.*, 2005). NOD IL-4-/- develop focal inflammation but no loss of salivary secretion unless there is a transfer of T cells, indicating the importance of T and B cells interactions. Following transfer, anti-M3R antibodies can be detected (Brayer *et al.*, 2001; Gao *et al.*, 2006). Systemic and local production of anti-Ro-52 has been described in MRL/*lpr* (Wahren *et al.*, 1994b) but reports on the occurrence of anti-Ro and anti-La in NOD are limited (Skarstein *et al.*, 1995) and anti-Ro-52-positivity in serum did not seem to be related to the inflammatory component. In the NOD mice we observed, loss of secretory function occurred late in the disease course, between 17-24 weeks of age.

Cytokines in serum and saliva

Protein levels of cytokines were measured in serum and saliva. IL-2, IL-5 and GM-CSF presented with the most interesting findings in serum, and IL-4, TNF-α and IFN-γ in saliva (Paper V). These changes in cytokine levels indicate a role for the adaptive immune response in progression of disease.

However, immunological reactions unrelated to the SS-like disease may influence the salivary gland inflammation, hyposalivation and cytokine production, and the NOD strain must be considered a model of general as well as specific immune dysregulation. A major draw-back of using NOD mice to mimic SS is the concomitant development of IDDM – it is not possible to determine whether pathology in the salivary glands is related to the IDDM or the SS-pathogenesis. The mice investigated (Paper V) were purchased from Taconic, and are not used as often as mice obtained from Jackson laboratories, but we believe that the NOD mice in our setting were suitable to model SS. All mice showed characteristic inflammatory changes in the salivary glands, and a significant decrease in salivary secretion compared to younger peers and age-matched Balb/c. In addition to supplier, stalling conditions may influence how prone the mice are to actually develop IDDM – the cleaner facilities, the higher incidence of diabetes.

IL-2 is a T_H2 cytokine which has been reported to play an important role in maintaining natural immunological self-tolerance by promoting growth and suppressor functions of regulatory T cells. Neutralization of circulating IL-2 aggravated diverse autoimmune manifestations (Setoguchi *et al.*, 2005). Recent research indicates that absence of IL-5, another T_H2 cytokine, can induce a shift towards adaptive immune responses. However,

the observed decrease of serum IL-5 (Paper V) is difficult to interpret because little is known about the role of IL-5 in SS. Increased levels of IL-4 in saliva (Paper V) further implied a function of the adaptive immune system. The negative association between IL-4 and salivary secretion are in conformity with findings in NOD.B10-*H2*^{b}.IL-4$^{-/-}$ mice which develop focal salivary gland inflammation but no loss of salivary secretion (Gao *et al.*, 2006). IFN-γ and IL-4 are suggested to antagonize the development of T_H-17 cells (Figure 13), triggering pro-inflammatory responses in autoimmune disease and causing destructive tissue pathology (Harrington *et al.*, 2005; Park *et al.*, 2005; Wynn, 2005). In accordance, NOD.B10-*H2*^{b}.IL-4$^{-/-}$ and NOD IL-4$^{-/-}$ both develop SS-like disease in the salivary glands (Brayer *et al.*, 2001; Gao *et al.*, 2006). NOD IFN-γ$^{-/-}$ and NOD IFN-γR$^{-/-}$ do not display SS-like pathology in the salivary glands, and have normal salivary secretion (Cha *et al.*, 2004).

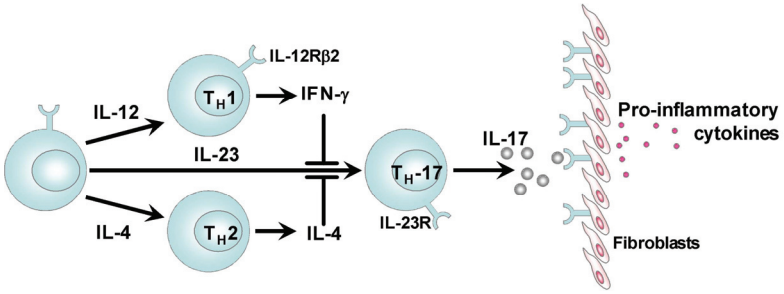


Figure 13. IL-17 acts in cellular immunity. The T_H-17 cells are regarded as a potent pro-inflammatory mediator linked to autoimmune and inflammatory diseases, reviewed in (Kolls and Lindén, 2004), and has recently been suggested to be a separate and early lineage of effector CD4+ T_H cells (Harrington *et al.*, 2005; Park *et al.*, 2005). IL-17 can induce expression of a range of cytokines and chemokines on epithelial cells and vascular endothelial cells, such as IL-6, GM-CSF and CXCL10, and neutrophil-activating IL-6 and CXCL8 (IL-8) secretion by fibroblasts (Steinke and Borish, 2006). Development of IL-17 producing effector CD4+ T cells (T_H-17) by IL-23 is inhibited by IFN-γ and IL-4. Figure adapted from (Wynn, 2005).

GM-CSF is a major regulator of granulocytes and macrophages, and a recent report indicate a role of GM-CSF in inflammation and autoimmunity (Hamilton, 2002). From *in vitro* studies, tissue neutrophil recruitment through the induction of GM-CSF and IL-8 indicate a pro-inflammatory role of IL-17 (Kolls and Lindén, 2004). TNF-α is one of the cytokines capable of inducing GM-CSF production (Leizer *et al.*, 1990) and interestingly, overt stage of disease in NOD was associated with increased saliva levels of TNF-α (Paper V). Little is known on the role of GM-CSF in SS, whereas TNF-α is known to have a wide spectrum of biological activities (Janeway *et al.*, 2005). Amongst other, TNF-α is considered

an essential cytokine in the pathogenesis of rheumatic diseases, and anti-TNF-α therapies were successfully introduced in the treatment of RA (Feldmann, 2002). Initial results in treatment of SS were encouraging (Steinfeld *et al.*, 2002). Unfortunately, subsequent clinical trials failed to confirm these findings (Mariette *et al.*, 2004; Sankar *et al.*, 2004). Autoimmune and inflammatory disorders may be more efficiently treated by targeting the activity of T_H-17 rather than T_H1 or T_H2 cells.

CONCLUSIONS

In analogy with the proposed specific aims, the following conclusions can be drawn:

- Ectopic germinal centers are formed in approximately 25% of patients with pSS
- Ectopic GC formation is characterized by increased levels of local and systemic autoantibody production (Ro/SSA, La/SSB and RF), higher degree of inflammation (focus score) and increased serum levels of IgG
- Soluble E-cadherin is increased in SS compared to non-SS and may be a result of, or an initiating/contributing factor to the chronic inflammation
- In NOD mice, focal sialadenitis precedes hyposalivation, and the loss of salivary secretion is not accompanied by a change in inflammation
- 30% of NOD mice have proliferating cells in the focal infiltrates and these mice have more inflammation (higher focus score) than their peers/littermates

FUTURE PROSPECTIVES

SS is a slowly progressive disease with immunological and clinical implications. Ideally, treatment of a disease should inhibit, or at least prevent progression of disease. At present a number of factors counteract this possibility in SS: 1) initiating factor(s) remain(s) obscure, 2) clinical symptoms develop late and 3) there are no tests for early detection of SS. Pharmacological inhibitors currently available either target other diseases, or have severe side-effects and restricted to the really sick patients. Until the initiating factor in SS is known, and can be "eliminated" treatment should relief symptoms and inhibit, or at least interfere with and modify the pathological gathering of activated T and B cells (Figure 14).

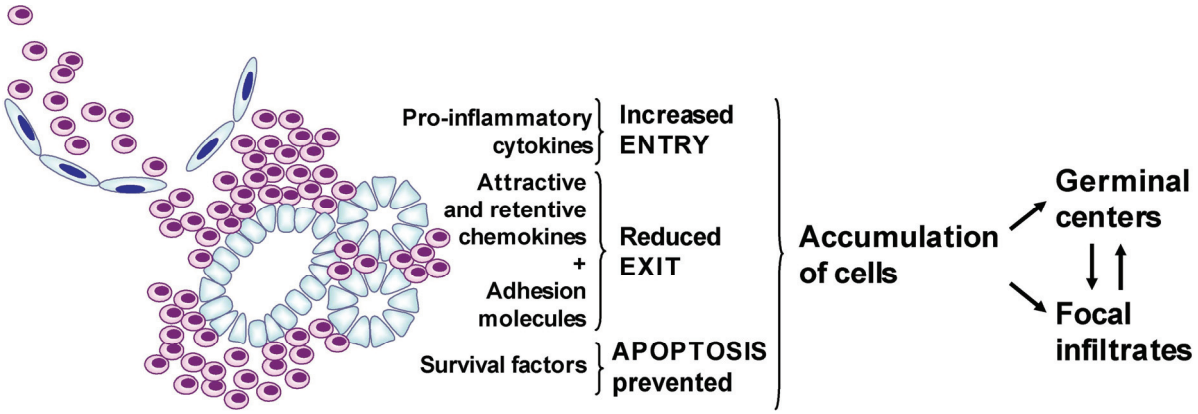


Figure 14. Summary of pathogenic events illustrating possibilities for immune-targeting. As a response to a yet unknown stimulus, salivary gland epithelial cells produce pro-inflammatory cytokines and chemoattractive and retentive chemokines which activate adhesion molecules on salivary gland endothelium and inflammatory cells (Paper I and V). Entrance of inflammatory cells into the target tissue microenvironment most likely alters or modifies both the inflammatory cells, and the target tissue. Reduced apoptosis/increased cell survival is, among others, mediated by BAFF (Paper II). BAFF expression and secretion by salivary gland epithelial cells can be induced by Type I interferons. Serum IL-2 and IL-5, and saliva IL-4, TNF-α and IFN-γ were related to disease progression (Paper V). Reduced exit, possibly resulting from impaired CCR7 signaling, further leads to accumulation of activated T and B cells and ultimately GC-formation with increased local and systemic autoantibody production (Paper I-III), isotype switching and possibly lymphomagenesis. Shedding of epithelial cells and E-cadherin most likely occurs as a result of MMP-mediated tissue destruction (Paper IV) and offer a potential tool for early detection of SS.

In Crohns disease, MS, RA and atherosclerosis, a monoclonal antibody targeting integrin VLA-4/α4β1, an antisense inhibitor of VCAM-1 and a probucol derivative of ICAM-1 are under development, reviewed in (Delaleu *et al.*, 2004). Treatment targeting BAFF in SLE is under development (Baker *et al.*, 2003). Recent findings indicate an additive role of

TNF-α and IFN-γ in inducing BAFF-expression in SS salivary gland epithelium (Ittah *et al.*, 2006), and as discussed above, initial results concerning anti-TNF-α treatment in SS were promising (Steinfeld *et al.*, 2002), but could not be confirmed (Mariette *et al.*, 2004; Sankar *et al.*, 2004). Indeed, a combination therapy – a cocktail – of several cytokine inhibitors may provide the solution?

Initital results targeting B cells by anti-CD20 monoclonal antibodies showed clinical improvement in a few cases of patients with SS presenting with MZ or non-Hodgkins lymphoma (Voulgarelis *et al.*, 2004), and a recent case-report indicated improvement of both clinical symptoms and histopathological changes (Pijpe *et al.*, 2005b), confirmed by an open-label Phase II study concluded that Rituximab is effective in the treatment of SS (Pijpe *et al.*, 2005a). Patients with ectopic GC have increased B cell activity, and may represent a target group in such trials, supporting the attempt in this thesis to sub-categorize patients with pSS.

Multiple cellular responses initiate events that under normal conditions lead to resolution of the inflammatory reaction (Han and Ulevitch, 2005). Thus, breakdown in the regulation of an inflammatory response results in chronic inflammation (Figure 14). Future treatment should aim to inhibit tissue-specific inflammation by regional delivery and without interfering with the migration and function of leukocytes in protective immunity (Luster *et al.*, 2005).

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REFERENCES

Abbas AK (2005). Diseases of immunity. In: Robbins and Cotran Pathologic Basis of Disease. V Kumar, AK Abbas and N Fausto editors. 7th ed. Philadelphia, Pennsylvania: Elsevier Saunders, pp. 193-268.

Amft N, Bowman SJ (2001). Chemokines and cell trafficking in Sjögren's syndrome. *Scand J Immunol* 54(1-2)*:*62-69.

Amft N, Curnow SJ, Scheel-Toellner D, Devadas A, Oates J, Crocker J, Hamburger J, Ainsworth J, Mathews J, Salmon M, Bowman SJ, Buckley CD (2001). Ectopic expression of the B cell-attracting chemokine BCA-1 (CXCL13) on endothelial cells and within lymphoid follicles contributes to the establishment of germinal center-like structures in Sjögren's syndrome. *Arthritis Rheum* 44(11)*:*2633-2641.

Atkinson JC, Travis WD, Pillemer SR, Bermudez D, Wolff A, Fox PC (1990). Major salivary gland function in primary Sjögren's syndrome and its relationship to clinical features. *J Rheumatol* 17(3)*:*318-322.

Atkinson JC (1993). The role of salivary measurements in the diagnosis of salivary autoimmune diseases. *Ann N Y Acad Sci* 694:238-251.

Baker KP, Edwards BM, Main SH, Choi GH, Wager RE, Halpern WG, Lappin PB, Riccobene T, Abramian D, Sekut L, Sturm B, Poortman C, Minter RR, Dobson CL, Williams E, Carmen S, Smith R, Roschke V, Hilbert DM, Vaughan TJ, Albert VR (2003). Generation and characterization of LymphoStat-B, a human monoclonal antibody that antagonizes the bioactivities of B lymphocyte stimulator. *Arthritis Rheum* 48(11)*:*3253-3265.

Bohnhorst JO, Bjørgan MB, Thoen JE, Natvig JB, Thompson KM (2001). Bm1-Bm5 classification of peripheral blood B cells reveals circulating germinal center founder cells in healthy individuals and disturbance in the B cell subpopulations in patients with primary Sjögren's syndrome. *J Immunol* 167(7)*:*3610-3618.

Bohnhorst JO, Bjorgan MB, Thoen JE, Jonsson R, Natvig JB, Thompson KM (2002). Abnormal B cell differentiation in primary Sjögren's syndrome results in a depressed percentage of circulating memory B cells and elevated levels of soluble CD27 that correlate with Serum IgG concentration. *Clin Immunol* 103(1)*:*79-88.

Bolstad AI, Jonsson R (2002). Genetic aspects of Sjögren's syndrome. *Arthritis Res* 4(6)*:*353-359.

Brayer JB, Cha S, Nagashima H, Yasunari U, Lindberg A, Diggs S, Martinez J, Goa J, Humphreys-Beher MG, Peck AB (2001). IL-4-dependent effector phase in autoimmune exocrinopathy as defined by the NOD.IL-4-gene knockout mouse model of Sjögren's syndrome. *Scand J Immunol* 54(1-2)*:*133-140.

Brennan MT, Sankar V, Leakan RA, Grisius MM, Collins MT, Fox PC, Baum BJ, Pillemer SR (2003). Sex steroid hormones in primary Sjögren's syndrome. *J Rheumatol* 30(6)*:*1267-1271.

Buckley CD, Pilling D, Lord JM, Akbar AN, Scheel-Toellner D, Salmon M (2001). Fibroblasts regulate the switch from acute resolving to chronic persistent inflammation. *Trends Immunol* 22(4)*:*199-204.

Buckley CD (2003). Michael Mason prize essay 2003. Why do leucocytes accumulate within chronically inflamed joints? *Rheumatology (Oxford)* 42(12)*:*1433-1444.

Burman A, Haworth O, Bradfield P, Parsonage G, Filer A, Thomas AM, Amft N, Salmon M, Buckley CD (2005). The role of leukocyte-stromal interactions in chronic inflammatory joint disease. *Joint Bone Spine* 72(1)*:*10-16.

Båve U, Nordmark G, Lövgren T, Ronnelid J, Cajander S, Eloranta ML, Alm GV, Rönnblom L (2005). Activation of the type I interferon system in primary Sjögren's syndrome: a possible etiopathogenic mechanism. *Arthritis Rheum* 52(4)*:*1185-1195.

Cepek KL, Parker CM, Madara JL, Brenner MB (1993). Integrin alpha E beta 7 mediates adhesion of T lymphocytes to epithelial cells. *J Immunol* 150(8 Pt 1)*:*3459-3470.

Cepek KL, Shaw SK, Parker CM, Russell GJ, Morrow JS, Rimm DL, Brenner MB (1994). Adhesion between epithelial cells and T lymphocytes mediated by E-cadherin and the alpha E beta 7 integrin. *Nature* 372(6502)*:*190-193.

Cha S, Brayer J, Gao J, Brown V, Killedar S, Yasunari U, Peck AB (2004). A dual role for interferon-gamma in the pathogenesis of Sjögren's syndrome-like autoimmune exocrinopathy in the nonobese diabetic mouse. *Scand J Immunol* 60(6)*:*552-565.

Chan AO-O, Lam SK, Chu KM, Lam CM, Kwok E, Leung SY, Yuen ST, Law SY, Hui WM, Lai KC, Wong CY, Hu HC, Lai CL, Wong J (2001). Soluble E-cadherin is a valid prognostic marker in gastric carcinoma. *Gut* 48(6)*:*808-811.

Chan AO-O, Chu KM, Lam SK, Wong BC-Y, Kwok KF, Law S, Ko S, Hui WM, Yueng YH, Wong J (2003). Soluble E-cadherin is an independent pretherapeutic factor for long-term survival in gastric cancer. *J Clin Oncol* 21(12)*:*2288-2293.

Chisholm DM, Mason DK (1968). Labial salivary gland biopsy in Sjögren's disease. *J Clin Pathol* 21(5)*:*656-660.

Cyster JG (1999). Chemokines and cell migration in secondary lymphoid organs. *Science* 286(5447)*:*2098-2102.

Cyster JG (2003). Lymphoid organ development and cell migration. *Immunol Rev* 195:5-14.

d'Arbonneau F, Pers JO, Devauchelle V, Pennec Y, Saraux A, Youinou P (2006). BAFF-induced changes in B cell antigen receptor-containing lipid rafts in Sjögren's syndrome. *Arthritis Rheum* 54(1)*:*115-126.

Davies G, Jiang WG, Mason MD (2001). Matrilysin mediates extracellular cleavage of E-cadherin from prostate cancer cells: a key mechanism in hepatocyte growth factor/scatter factor-induced cell-cell dissociation and in vitro invasion. *Clin Cancer Res* 7(10)*:*3289-3297.

Debes GF, Arnold CN, Young AJ, Krautwald S, Lipp M, Hay JB, Butcher EC (2005). Chemokine receptor CCR7 required for T lymphocyte exit from peripheral tissues. *Nat Immunol* 6(9)*:*889-894.

Delaleu N, Jonsson MV, Jonsson R (2004). Disease mechanisms of Sjögren's syndrome. *Drug Discovery Today: Disease Mechanisms* 1(3)*:*329-336.

Elagib KE, Tengner P, Levi M, Jonsson R, Thompson KM, Natvig JB, Wahren-Herlenius M (1999). Immunoglobulin variable genes and epitope recognition of human monoclonal anti-Ro 52-kd in primary Sjögren's syndrome. *Arthritis Rheum* 42(11)*:*2471-2481.

Esch TR, Jonsson MV, Levanos VA, Poveromo JD, Sorkin BC (2000). Leukocytes infiltrating the submandibular glands of NOD mice express E-cadherin. *J Autoimmun* 15(4)*:*387-393.

Feldmann M (2002). Development of anti-TNF therapy for rheumatoid arthritis. *Nat Rev Immunol* 2(5)*:*364-371.

Fox RI, Robinson CA, Curd JG, Kozin F, Howell FV (1986). Sjögren's syndrome. Proposed criteria for classification. *Arthritis Rheum* 29(5)*:*577-585.

Fox RI, Kang HI, Ando D, Abrams J, Pisa E (1994). Cytokine mRNA expression in salivary gland biopsies of Sjögren's syndrome. *J Immunol* 152(11)*:*5532-5539.

Fox RI, Stern M (2002). Sjögren's syndrome: mechanisms of pathogenesis involve interaction of immune and neurosecretory systems. *Scand J Rheumatol Suppl* 116*:*3-13.

Fujihara T, Fujita H, Tsubota K, Saito K, Tsuzaka K, Abe T, Takeuchi T (1999). Preferential localization of CD8+ alpha E beta 7+ T cells around acinar epithelial cells with apoptosis in patients with Sjögren's syndrome. *J Immunol* 163(4)*:*2226-2235.

Gao J, Killedar S, Cornelius JG, Nguyen C, Cha S, Peck AB (2006). Sjögren's syndrome in the NOD mouse model is an interleukin-4 time-dependent, antibody isotype-specific autoimmune disease. *J Autoimmun:*90-103.

Garberg H, Jonsson R, Brokstad KA (2005). The serological pattern of autoantibodies to the Ro52, Ro60, and La48 autoantigens in primary Sjögren's syndrome patients and healthy controls. *Scand J Rheumatol* 34(1)*:*49-55.

Goicovich E, Molina C, Perez P, Aguilera S, Fernandez J, Olea N, Alliende C, Leyton C, Romo R, Leyton L, Gonzalez MJ (2003). Enhanced degradation of proteins of the basal lamina and stroma by matrix metalloproteinases from the salivary glands of Sjögren's syndrome patients: correlation with reduced structural integrity of acini and ducts. *Arthritis Rheum* 48(9)*:*2573-2584.

Gottenberg JE, Busson M, Loiseau P, Cohen-Solal J, Lepage V, Charron D, Sibilia J, Mariette X (2003). In primary Sjögren's syndrome, HLA class II is associated exclusively with autoantibody production and spreading of the autoimmune response. *Arthritis Rheum* 48(8)*:*2240-2245.

Greenspan JS, Daniels TE, Talal N, Sylvester RA (1974). The histopathology of Sjögren's syndrome in labial salivary gland biopsies. *Oral Surg Oral Med Oral Pathol* 37(2)*:*217-229.

Groom J, Kalled SL, Cutler AH, Olson C, Woodcock SA, Schneider P, Tschopp J, Cachero TG, Batten M, Wheway J, Mauri D, Cavill D, Gordon TP, Mackay CR, Mackay F (2002). Association of BAFF/BLyS overexpression and altered B cell differentiation with Sjögren's syndrome. *J Clin Invest* 109(1)*:*59-68.

Gross JA, Johnston J, Mudri S, Enselman R, Dillon SR, Madden K, Xu W, Parrish-Novak J, Foster D, Lofton-Day C, Moore M, Littau A, Grossman A, Haugen H, Foley K, Blumberg H, Harrison K, Kindsvogel W, Clegg CH (2000). TACI and BCMA are receptors for a TNF homologue implicated in B-cell autoimmune disease. *Nature* 404(6781)*:*995-999.

Gumbiner BM (1996). Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell* 84(3)*:*345-357.

Gumbiner BM (2000). Regulation of cadherin adhesive activity. *J Cell Biol* 148(3)*:*399-404.

Hamilton JA (2002). GM-CSF in inflammation and autoimmunity. *Trends Immunol* 23(8)*:*403-408.

Han J, Ulevitch RJ (2005). Limiting inflammatory responses during activation of innate immunity. *Nat Immunol* 6(12)*:*1198-1205.

Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, Weaver CT (2005). Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol* 6(11)*:*1123-1132.

Hayat MA (2002). Microscopy, Immunohistochemistry, and Antigen Retrieval Methods. New York, NY: Kluwer Academic/Plenum Publishers.

Hjelmervik TO, Petersen K, Jonassen I, Jonsson R, Bolstad AI (2005). Gene expression profiling of minor salivary glands clearly distinguishes primary Sjögren's syndrome patients from healthy control subjects. *Arthritis Rheum* 52(5)*:*1534-1544.

Hjelmström P (2001). Lymphoid neogenesis: de novo formation of lymphoid tissue in chronic inflammation through expression of homing chemokines. *J Leukoc Biol* 69(3)*:*331-339.

Homma M, Tojo T, Akizuki M, Yamagata H (1986). Criteria for Sjögren's syndrome in Japan. *Scand J Rheumatol Suppl* 61:26-27.

Horsfall AC, Rose LM, Maini RN (1989). Autoantibody synthesis in salivary glands of Sjögren's syndrome patients. *J Autoimmun* 2(4)*:*559-568.

Humphreys-Beher MG, Brayer J, Yamachika S, Peck AB, Jonsson R (1999). An alternative perspective to the immune response in autoimmune exocrinopathy: induction of functional quiescence rather than destructive autoaggression. *Scand J Immunol* 49(1)*:*7-10.

Humphreys-Beher MG, Brayer J, Cha S, Nagashima H, Diggs S, Peck AB (2002). Immunogenetics of autoimmune exocrinopathy in the nod mouse: more than meets the eye. *Adv Exp Med Biol* 506(Pt B)*:*999-1007.

Ioannidis JP, Vassiliou VA, Moutsopoulos HM (2002). Long-term risk of mortality and lymphoproliferative disease and predictive classification of primary Sjögren's syndrome. *Arthritis Rheum* 46(3)*:*741-747.

Ittah M, Miceli-Richard C, Eric Gottenberg J, Lavie F, Lazure T, Ba N, Sellam J, Lepajolec C, Mariette X (2006). B cell-activating factor of the tumor necrosis factor family (BAFF) is expressed under stimulation by interferon in salivary gland epithelial cells in primary Sjögren's syndrome. *Arthritis Res Ther* 8(2)*:*R51.

Janeway CA, Travers P, Walport M, Schlomchik M (2005). Immunobiology: the immunesystem in health and disease. 6th ed. New York: Garland Science Publishing.

Jonsson R, Tarkowski A, Bäckman K, Holmdahl R, Klareskog L (1987). Sialadenitis in the MRL-l mouse: morphological and immunohistochemical characterization of resident and infiltrating cells. *Immunology* 60(4)*:*611-616.

Jonsson R, Bowman SJ, Gordon TP (2005). Sjögren's syndrome. In: Arthritis and Allied Conditions. WJ Koopman, Moorland LW, editors. 15th ed. Philadelphia: Lippincott Williams & Wilkins, pp. 1681-1705.

Jordan RC, Daniels TE, Greenspan JS, Regezi JA (2002). Advanced diagnostic methods in oral and maxillofacial pathology. Part II: immunohistochemical and immunofluorescent methods. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 93(1)*:*56-74.

Kauppi M, Pukkala E, Isomaki H (1997). Elevated incidence of hematologic malignancies in patients with Sjögren's syndrome compared with patients with rheumatoid arthritis (Finland). *Cancer Causes Control* 8(2)*:*201-204.

Kolls JK, Lindén A (2004). Interleukin-17 family members and inflammation. *Immunity* 21(4)*:*467-476.

Kroneld U, Jonsson R, Carlsten H, Bremell T, Johannessen AC, Tarkowski A (1998). Expression of the mucosal lymphocyte integrin alphaEbeta7 and its ligand E-cadherin in salivary glands of patients with Sjögren's syndrome. *Scand J Rheumatol* 27(3)*:*215-218.

Kumar V, Cotran RS, Robbins SL (2003). Diseases of immunity. In: Robbins Basic Pathology. V Kumar, RS Cotran and SL Robbins, editors. 7th ed. Philadelphia, Pennsylvania: W.B Saunders Company, pp. 103-164.

Larsson Å, Bredberg A, Henriksson G, Manthorpe R, Sallmyr A (2005). Immunohistochemistry of the B-cell component in lower lip salivary glands of Sjögren's syndrome and healthy subjects. *Scand J Immunol* 61(1)*:*98-107.

Lavie F, Miceli-Richard C, Quillard J, Roux S, Leclerc P, Mariette X (2004). Expression of BAFF (BLyS) in T cells infiltrating labial salivary glands from patients with Sjögren's syndrome. *J Pathol* 202(4)*:*496-502.

Leizer T, Cebon J, Layton JE, Hamilton JA (1990). Cytokine regulation of colony-stimulating factor production in cultured human synovial fibroblasts: I. Induction of GM-CSF and G-CSF production by interleukin-1 and tumor necrosis factor. *Blood* 76(10)*:*1989-1996.

Lindvall AM, Jonsson R (1986). The salivary gland component of Sjögren's syndrome: an evaluation of diagnostic methods. *Oral Surg Oral Med Oral Pathol* 62(1)*:*32-42.

Luster AD, Alon R, von Andrian UH (2005). Immune cell migration in inflammation: present and future therapeutic targets. *Nat Immunol* 6(12)*:*1182-1190.

Mackay F, Browning JL (2002). BAFF: a fundamental survival factor for B cells. *Nat Rev Immunol* 2(7)*:*465-475.

Mackay F, Ambrose C (2003). The TNF family members BAFF and APRIL: the growing complexity. *Cytokine Growth Factor Rev* 14(3-4)*:*311-324.

Mandel ID, Baurmash H (1976). Sialochemistry in Sjögren's syndrome. *Oral Surg Oral Med Oral Pathol* 41(2)*:*182-187.

Manthorpe R, Oxholm P, Prause JU, Schiödt M (1986). The Copenhagen criteria for Sjögren's syndrome. *Scand J Rheumatol Suppl* 61:19-21.

Manthorpe R, Jacobsson LT, Kirtava Z, Theander E (1998). Epidemiology of Sjögren's syndrome, especially its primary form. *Ann Med Interne (Paris)* 149(1)*:*7-11.

Mariette X, Roux S, Zhang J, Bengoufa D, Lavie F, Zhou T, Kimberly R (2003). The level of BLyS (BAFF) correlates with the titre of autoantibodies in human Sjögren's syndrome. *Ann Rheum Dis* 62(2)*:*168-171.

Mariette X, Ravaud P, Steinfeld S, Baron G, Goetz J, Hachulla E, Combe B, Puechal X, Pennec Y, Sauvezie B, Perdriger A, Hayem G, Janin A, Sibilia J (2004). Inefficacy of infliximab in primary Sjögren's syndrome: results of the randomized, controlled Trial of Remicade in Primary Sjögren's Syndrome (TRIPSS). *Arthritis Rheum* 50(4)*:*1270-1276.

Marthinussen MC, Øijordsbakken G, Johannessen AC, Cionca L, Ganuta N (2002). An Immunohistochemical study of p53 and p21 expression in oral squamous cell carcinoma (OSCC) by using two different methods of detection. 8th International Congress on Oral Cancer, Rio de Janeiro, Brazil: Rajkamal Electric Press.

Mikulowska-Mennis A, Xu B, Berberian JM, Michie SA (2001). Lymphocyte migration to inflamed lacrimal glands is mediated by vascular cell adhesion molecule-1/alpha(4)beta(1) integrin, peripheral node addressin/l-selectin, and lymphocyte function-associated antigen-1 adhesion pathways. *Am J Pathol* 159(2)*:*671-681.

Mitsias DI, Tzioufas AG, Veiopoulou C, Zintzaras E, Tassios IK, Kogopoulou O, Moutsopoulos HM, Thyphronitis G (2002). The Th1/Th2 cytokine balance changes with the progress of the immunopathological lesion of Sjögren's syndrome. *Clin Exp Immunol* 128(3)*:*562-568.

Moore PA, Belvedere O, Orr A, Pieri K, LaFleur DW, Feng P, Soppet D, Charters M, Gentz R, Parmelee D, Li Y, Galperina O, Giri J, Roschke V, Nardelli B, Carrell J, Sosnovtseva S, Greenfield W, Ruben SM, Olsen HS, Fikes J, Hilbert DM (1999). BLyS: member of the tumor necrosis factor family and B lymphocyte stimulator. *Science* 285(5425)*:*260-263.

Mukhopadhyay A, Ni J, Zhai Y, Yu GL, Aggarwal BB (1999). Identification and characterization of a novel cytokine, THANK, a TNF homologue that activates apoptosis, nuclear factor-kappaB, and c-Jun NH2-terminal kinase. *J Biol Chem* 274(23)*:*15978-15981.

Nakken B, Jonsson R, Brokstad KA, Omholt K, Nerland AH, Haga HJ, Halse AK (2001). Associations of MHC class II alleles in Norwegian primary Sjögren's syndrome patients: implications for development of autoantibodies to the Ro52 autoantigen. *Scand J Immunol* 54(4)*:*428-433.

Noë V, Fingleton B, Jacobs K, Crawford HC, Vermeulen S, Steelant W, Bruyneel E, Matrisian LM, Mareel M (2001). Release of an invasion promoter E-cadherin fragment by matrilysin and stromelysin-1. *J Cell Sci* 114(Pt 1)*:*111-118.

Ogawa N, Kawanami T, Shimoyama K, Ping L, Sugai S (2004). Expression of interferon-inducible T cell alpha chemoattractant (CXCL11) in the salivary glands of patients with Sjögren's syndrome. *Clin Immunol* 112(3)*:*235-238.

Ohlsson M, Skarstein K, Bolstad AI, Johannessen AC, Jonsson R (2001). Fas-induced apoptosis is a rare event in Sjögren's syndrome. *Lab Invest* 81(1)*:*95-105.

Ohlsson M, Szodoray P, Loro LL, Johannessen AC, Jonsson R (2002). CD40, CD154, Bax and Bcl-2 expression in Sjögren's syndrome salivary glands: a putative anti-apoptotic role during its effector phases. *Scand J Immunol* 56(6)*:*561-571.

Ollier W, Symmons DPM (1992). Autoimmunity Oxford: BIOS Scientific Publishers Limited.

Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang YH, Wang Y, Hood L, Zhu Z, Tian Q, Dong C (2005). A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol* 6(11)*:*1133-1141.

Pedersen AM, Dissing S, Fahrenkrug J, Hannibal J, Reibel J, Nauntofte B (2000). Innervation pattern and Ca2+ signalling in labial salivary glands of healthy individuals and patients with primary Sjögren's syndrome (pSS). *J Oral Pathol Med* 29(3)*:*97-109.

Pedersen AM, Bardow A, Nauntofte B (2005). Salivary changes and dental caries as potential oral markers of autoimmune salivary gland dysfunction in primary Sjögren's syndrome. *BMC Clin Pathol* 5(1)*:*4.

Perez P, Goicovich E, Alliende C, Aguilera S, Leyton C, Molina C, Pinto R, Romo R, Martinez B, Gonzalez MJ (2000). Differential expression of matrix metalloproteinases in labial salivary glands of patients with primary Sjögren's syndrome. *Arthritis Rheum* 43(12)*:*2807-2817.

Pers JO, Daridon C, Devauchelle V, Jousse S, Saraux A, Jamin C, Youinou P (2005). BAFF overexpression is associated with autoantibody production in autoimmune diseases. *Ann N Y Acad Sci* 1050:34-39.

Pijpe J, van Imhoff GW, Spijkervet FK, Roodenburg JL, Wolbink GJ, Mansour K, Vissink A, Kallenberg CG, Bootsma H (2005a). Rituximab treatment in patients with primary Sjögren's syndrome: an open-label phase II study. *Arthritis Rheum* 52(9)*:*2740-2750.

Pijpe J, van Imhoff GW, Vissink A, van der Wal JE, Kluin PM, Spijkervet FK, Kallenberg CG, Bootsma H (2005b). Changes in salivary gland immunohistology and function after rituximab monotherapy in a patient with Sjögren's syndrome and associated MALT lymphoma. *Ann Rheum Dis* 64(6)*:*958-960.

Pittard AJ, Banks RE, Galley HF, Webster NR (1996). Soluble E-cadherin concentrations in patients with systemic inflammatory response syndrome and multiorgan dysfunction syndrome. *Br J Anaesth* 76(5)*:*629-631.

Pourmand N, Wahren-Herlenius M, Gunnarsson I, Svenungsson E, Lofstrom B, Ioannou Y, Isenberg DA, Magnusson CG (1999). Ro/SSA and La/SSB specific IgA autoantibodies in serum of patients with Sjögren's syndrome and systemic lupus erythematosus. *Ann Rheum Dis* 58(10)*:*623-629.

Raderer M, Osterreicher C, Machold K, Formanek M, Fiebiger W, Penz M, Dragosics B, Chott A (2001). Impaired response of gastric MALT-lymphoma to Helicobacter pylori eradication in patients with autoimmune disease. *Ann Oncol* 12(7)*:*937-939.

Ramsdell F (2003). Foxp3 and natural regulatory T cells: key to a cell lineage? *Immunity* 19(2)*:*165-168.

Rosignoli F, Roca V, Meiss R, Leceta J, Gomariz RP, Perez Leiros C (2005). Defective signalling in salivary glands precedes the autoimmune response in the non-obese diabetic mouse model of sialadenitis. *Clin Exp Immunol* 142(3)*:*411-418.

Sabattini E, Bisgaard K, Ascani S, Poggi S, Piccioli M, Ceccarelli C, Pieri F, Fraternali-Orcioni G, Pileri SA (1998). The EnVision++ system: a new immunohistochemical method for diagnostics and research. Critical comparison with the APAAP, ChemMate, CSA, LABC, and SABC techniques. *J Clin Pathol* 51(7)*:*506-511.

Salomonsson S, Larsson P, Tengner P, Mellquist E, Hjelmström P, Wahren-Herlenius M (2002). Expression of the B cell-attracting chemokine CXCL13 in the target organ and autoantibody production in ectopic lymphoid tissue in the chronic inflammatory disease Sjögren's syndrome. *Scand J Immunol* 55(4)*:*336-342.

Salomonsson S, Wahren-Herlenius M (2003). Local production of Ro/SSA and La/SSB autoantibodies in the target organ coincides with high levels of circulating antibodies in sera of patients with Sjögren's syndrome. *Scand J Rheumatol* 32(2)*:*79-82.

Sankar V, Brennan MT, Kok MR, Leakan RA, Smith JA, Manny J, Baum BJ, Pillemer SR (2004). Etanercept in Sjögren's syndrome: a twelve-week randomized, double-blind, placebo-controlled pilot clinical trial. *Arthritis Rheum* 50(7)*:*2240-2245.

Schmutz C, Hulme A, Burman A, Salmon M, Ashton B, Buckley C, Middleton J (2005). Chemokine receptors in the rheumatoid synovium: upregulation of CXCR5. *Arthritis Res Ther* 7(2)*:*R217-229.

Schneider P, MacKay F, Steiner V, Hofmann K, Bodmer JL, Holler N, Ambrose C, Lawton P, Bixler S, Acha-Orbea H, Valmori D, Romero P, Werner-Favre C, Zubler RH, Browning JL, Tschopp J (1999). BAFF, a novel ligand of the tumor necrosis factor family, stimulates B cell growth. *J Exp Med* 189(11)*:*1747-1756.

Schneider P (2005). The role of APRIL and BAFF in lymphocyte activation. *Curr Opin Immunol* 17(3)*:*282-289.

Seifert G, Sobin LH (1991). Histological typing of salivary gland tumors. 2nd ed. Berlin Heidelberg: Springer-Verlag.

Setoguchi R, Hori S, Takahashi T, Sakaguchi S (2005). Homeostatic maintenance of natural Foxp3(+) CD25(+) CD4(+) regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization. *J Exp Med* 201(5)*:*723-735.

Sfriso P, Oliviero F, Calabrese F, Miorin M, Facco M, Contri A, Cabrelle A, Baesso I, Cozzi F, Andretta M, Cassatella MA, Fiocco U, Todesco S, Konttinen YT, Punzi L, Agostini C (2006). Epithelial CXCR3-B regulates chemokines bioavailability in normal, but not in Sjögren's syndrome, salivary glands. *J Immunol* 176(4)*:*2581-2589.

Shi K, Hayashida K, Kaneko M, Hashimoto J, Tomita T, Lipsky PE, Yoshikawa H, Ochi T (2001). Lymphoid chemokine B cell-attracting chemokine-1 (CXCL13) is expressed in germinal center of ectopic lymphoid follicles within the synovium of chronic arthritis patients. *J Immunol* 166(1)*:*650-655.

Shu HB, Hu WH, Johnson H (1999). TALL-1 is a novel member of the TNF family that is down-regulated by mitogens. *J Leukoc Biol* 65(5)*:*680-683.

Skarstein K, Wahren M, Zaura E, Hattori M, Jonsson R (1995). Characterization of T cell receptor repertoire and anti-Ro/SSA autoantibodies in relation to sialadenitis of NOD mice. *Autoimmunity* 22(1)*:*9-16.

Skarstein K, Johannessen AC, Holmdahl R, Jonsson R (1997). Effects of sialadenitis after cellular transfer in autoimmune MRL/lpr mice. *Clin Immunol Immunopathol* 84(2)*:*177-184.

Skopouli FN, Drosos AA, Papaioannou T, Moutsopoulos HM (1986). Preliminary diagnostic criteria for Sjögren's syndrome. *Scand J Rheumatol Suppl* 61:22-25.

Steinfeld SD, Demols P, Appelboom T (2002). Infliximab in primary Sjögren's syndrome: one-year followup. *Arthritis Rheum* 46(12)*:*3301-3303.

Steinke JW, Borish L (2006). 3. Cytokines and chemokines. *J Allergy Clin Immunol* 117(2 Suppl Mini-Primer)*:*S441-445.

Stott DI, Hiepe F, Hummel M, Steinhauser G, Berek C (1998). Antigen-driven clonal proliferation of B cells within the target tissue of an autoimmune disease. The salivary glands of patients with Sjögren's syndrome. *J Clin Invest* 102(5)*:*938-946.

Streubel B, Huber D, Wohrer S, Chott A, Raderer M (2004). Frequency of chromosomal aberrations involving MALT1 in mucosa-associated lymphoid tissue lymphoma in patients with Sjögren's syndrome. *Clin Cancer Res* 10(2)*:*476-480.

Szodoray P, Jellestad S, Teague MO, Jonsson R (2003). Attenuated apoptosis of B cell activating factor-expressing cells in primary Sjögren's syndrome. *Lab Invest* 83(3)*:*357-365.

Szodoray P, Alex P, Brun JG, Centola M, Jonsson R (2004). Circulating cytokines in primary Sjögren's syndrome determined by a multiplex cytokine array system. *Scand J Immunol* 59(6)*:*592-599.

Szodoray P, Alex P, Jonsson MV, Knowlton N, Dozmorov I, Nakken B, Delaleu N, Jonsson R, Centola M (2005). Distinct profiles of Sjögren's syndrome patients with ectopic salivary gland germinal centers revealed by serum cytokines and BAFF. *Clin Immunol* 117(2)*:*168-176.

Szodoray P, Jonsson R (2005). The BAFF/APRIL system in systemic autoimmune diseases with a special emphasis on Sjögren's syndrome. *Scand J Immunol* 62(5)*:*421-428.

Takemura S, Braun A, Crowson C, Kurtin PJ, Cofield RH, O'Fallon WM, Goronzy JJ, Weyand CM (2001). Lymphoid neogenesis in rheumatoid synovitis. *J Immunol* 167(2)*:*1072-1080.

Tengner P, Halse AK, Haga HJ, Jonsson R, Wahren-Herlenius M (1998). Detection of anti-Ro/SSA and anti-La/SSB autoantibody-producing cells in salivary glands from patients with Sjögren's syndrome. *Arthritis Rheum* 41(12)*:*2238-2248.

Theander E, Manthorpe R, Jacobsson LT (2004). Mortality and causes of death in primary Sjögren's syndrome: a prospective cohort study. *Arthritis Rheum* 50(4)*:*1262-1269.

Theander E, Henriksson G, Ljungberg O, Mandl T, Manthorpe R, Jacobsson LT (2005). Lymphoma and other malignancies in primary Sjögren's syndrome. A cohort study on cancer incidence and lymphoma predictors. *Ann Rheum Dis*, e-pub ahead of print.

Triantafyllopoulou A, Moutsopoulos HM (2005). Autoimmunity and coxsackievirus infection in primary Sjögren's syndrome. *Ann N Y Acad Sci* 1050:389-396.

Uccelli A, Aloisi F, Pistoia V (2005). Unveiling the enigma of the CNS as a B-cell fostering environment. *Trends Immunol* 26(5)*:*254-529.

van Blokland SC, Versnel MA (2002). Pathogenesis of Sjögren's syndrome: characteristics of different mouse models for autoimmune exocrinopathy. *Clin Immunol* 103(2)*:*111-124.

Vincent A (2002). Unravelling the pathogenesis of myasthenia gravis. *Nat Rev Immunol* 2(10)*:*797-804.

Vitali C, Bombardieri S, Moutsopoulos HM, Balestrieri G, Bencivelli W, Bernstein RM, Bjerrum KB, Braga S, Coll J, de Vita S, et al. (1993). Preliminary criteria for the classification of Sjögren's syndrome. Results of a prospective concerted action supported by the European Community. *Arthritis Rheum* 36(3)*:*340-347.

Vitali C, Bombardieri S, Jonsson R, Moutsopoulos HM, Alexander EL, Carsons SE, Daniels TE, Fox PC, Fox RI, Kassan SS, Pillemer SR, Talal N, Weisman MH (2002). Classification criteria for Sjögren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. *Ann Rheum Dis* 61(6)*:*554-558.

Vora KA, Wang LC, Rao SP, Liu ZY, Majeau GR, Cutler AH, Hochman PS, Scott ML, Kalled SL (2003). Cutting edge: germinal centers formed in the absence of B cell-activating factor belonging to the TNF family exhibit impaired maturation and function. *J Immunol* 171(2)*:*547-551.

Voulgarelis M, Dafni UG, Isenberg DA, Moutsopoulos HM (1999). Malignant lymphoma in primary Sjögren's syndrome: a multicenter, retrospective, clinical study by the European Concerted Action on Sjögren's Syndrome. *Arthritis Rheum* 42(8)*:*1765-1772.

Voulgarelis M, Giannouli S, Anagnostou D, Tzioufas AG (2004). Combined therapy with rituximab plus cyclophosphamide/doxorubicin/vincristine/prednisone (CHOP) for Sjögren's syndrome-associated B-cell aggressive non-Hodgkin's lymphomas. *Rheumatology (Oxford)* 43(8)*:*1050-1053.

Vyberg M, Torlakovic E, Seidal T, Risberg B, Helin H, Nielsen S (2005). Nordic immunohistochemical quality control. *Croat Med J* 46(3)*:*368-371.

Wahren M, Ringertz NR, Pettersson I (1994a). IgM and IgG subclass distribution of human anti-Ro/SSA 60 kDa autoantibodies. *Scand J Immunol* 39(2)*:*179-83.

Wahren M, Skarstein K, Blange I, Pettersson I, Jonsson R (1994b). MRL/lpr mice produce anti-Ro 52,000 MW antibodies: detection, analysis of specificity and site of production. *Immunology* 83(1)*:*9-15.

Waterman SA, Gordon TP, Rischmueller M (2000). Inhibitory effects of muscarinic receptor autoantibodies on parasympathetic neurotransmission in Sjögren's syndrome. *Arthritis Rheum* 43(7)*:*1647-1654.

Wen S, Shimizu N, Yoshiyama H, Mizugaki Y, Shinozaki F, Takada K (1996). Association of Epstein-Barr virus (EBV) with Sjögren's syndrome: differential EBV expression between epithelial cells and lymphocytes in salivary glands. *Am J Pathol* 149(5)*:*1511-1517.

Weyand CM, Goronzy JJ (2003). Ectopic germinal center formation in rheumatoid synovitis. *Ann N Y Acad Sci* 987:140-149.

Wynn TA (2005). T(H)-17: a giant step from T(H)1 and T(H)2. *Nat Immunol* 6(11)*:*1069-1070.

Xanthou G, Polihronis M, Tzioufas AG, Paikos S, Sideras P, Moutsopoulos HM (2001). "Lymphoid" chemokine messenger RNA expression by epithelial cells in the chronic inflammatory lesion of the salivary glands of Sjögren's syndrome patients: possible participation in lymphoid structure formation. *Arthritis Rheum* 44(2)*:*408-418.