The Autonomy of Different Aspects of Sjögren's Syndrome and their Treatment in an Experimental Model

The use of comprehensive biomarker analyses to characterize the disease and the effect of heat-shock proteins in treatment intervention

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Dedicated to my mother and my beloved Sibel

There is a theory which states that if ever anyone discovers exactly what the universe is for and why it is here, it will instantly disappear and be replaced by something even more bizarre and inexplicable.

There is another theory which states that this has already happened.

Don't panic!

Douglas Adams - The Restaurant at the End of the Universe

Research environment

The research leading up to the thesis here presented was initiated in 2005 and was conducted in the framework of the Bergen Research School in Inflammation (BRSI) at the Broegelmann Research Laboratory, The Gade Institute, University of Bergen, Norway, under the leadership of Professor Roland Jonsson.

Collaborators were affiliated with: the Section of Oral Pathology and Forensic Odontology, The Gade Institute, University of Bergen, Norway; the Section for Physiology, the Department of Biomedicine, University of Bergen, Norway; the Section of Pathology, The Gade Institute, University of Bergen, Norway; the Department of Pathology, Haukeland University Hospital, Bergen, Norway; the Department of Pathology, Immunology and Laboratory Medicine, University of Florida, Gainesville, United States; the Department of Rheumatology and Department of Otolaryngology, Head and Neck Surgery, Haukeland University Hospital, Bergen, Norway.

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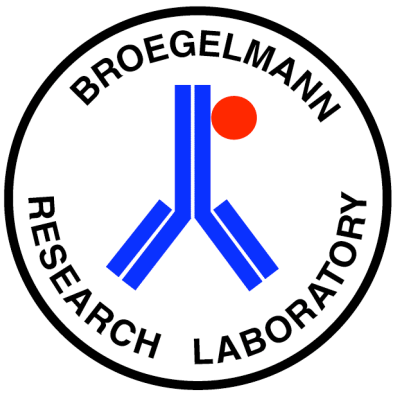


Summary

Sjögren's syndrome (SS) is a systemic rheumatic disease, in which the salivary and lacrimal glands are the principal targets of a pathological autoimmune reaction. Clinically, SS is manifested by keratoconjunctivitis sicca (dry eyes) and xerostomia (dry mouth). Histopathologically, the disease is characterized by persistent focal mononuclear cell infiltration in the salivary glands. Traditionally, loss of secretory capacity, degree of lymphoid infiltration and production of specific autoantibodies were anticipated to correlate with each other and indicate disease state and disease severity. The correctness of this assumption was, however, difficult to prove.

The aim of the first study was to clarify the chronology of the SS disease course and to describe possible interrelationships between sialoadenitis and hyposalivation. We could confirm that the SS in non-obese diabetic (NOD) mice is characterized by at least two distinct phases. In our study, inflammation of the salivary glands preceded the onset of hyposalivation with a considerable amount of time. Interestingly, the onset of hyposalivation was not associated with a significantly higher degree of inflammation. Significant alterations in cytokines in serum and saliva paralleled the transition from the pre-clinical to the overt disease state.

The purpose of the second study was to acquire knowledge on 87 immunologically relevant proteins measured in serum and 75 proteins analyzed in saliva. Thirty-eight biomarkers analyzed in serum and 34 proteins in saliva from NOD mice were significantly altered when compared to Balb/c mice. Eighteen biomarkers in serum and 3 chemokines in saliva had the potential to predict individual strain-membership with 80-100% accuracy. Computation of a correlation network led to the conclusion that processes related to the adaptive immune system promote SS with a strong implication of T-helper (T_H)2 related proteins in hyposalivation. In addition, the SS-related disease manifestations appeared to be associated with different immunological processes and did not correlate with each other. Cluster of differentiation (CD)40, CD40L, interleukin-18, granulocyte chemotactic protein-2 (GCP-2) and anti-muscarinic M3 receptor immunoglobulin G_3, may, however, interrelate the different aspects of SS. The study further established saliva as an attractive biofluid for biomarker discovery in SS and provides a basis for the comparison and selection of potential drug targets and diagnostic markers.

The third study was conducted to investigate a potential immunomodulatory effect of heat-shock protein 60kDa (Hsp60) and Hsp60-derived peptide aa437-460 (aa437-460) immunization on spontaneous experimental SS. Administration of Hsp60 and aa437-460 significantly reduced SS-related histopathology compared to the untreated NOD controls. In addition, 50% of Hsp60 and 33% of aa437-460 injected mice retained normal exocrine function. Both treatments induced similar changes in the biomarker profiles, which indicated decreased inflammatory chemotaxis and strengthened anti-inflammatory regulatory pathways. Successful prevention of hyposalivation was accompanied by quantitative alterations in 36 biomarkers, of which 19 inflammatory mediators declined to levels comparable to the measurements obtained in Balb/c mice. Molecules involved in inflammatory chemotaxis, neovascularization and regulatory pathways coined the differences displayed by the biomarker profiles. In addition, specific biomarker signatures had the capability to accurately predict the received treatment, treatment efficacy and impaired exocrine function.

Common to all three studies were the appearing independence of the different aspects of SS and the valuable information we could extract from biomarker analyses especially in saliva.

Table of contents

Abbreviations

AECG	American-European Consensus Group
Apo A1	apolipoprotein A1
AQP	aquaporin
ArKO	aromatase-knockout
BAFF	B-cell activating factor
Bax	BCL-2 associated X protein
BCL-2	B-cell lymphoma-2
BCR	B-cell receptor
CAII	carbonic anhydrase II
CC	C-C motif
CCP	cyclic citrullinated peptide
CD	cluster of differentiation
CDR	complementarity-determining region
CFA	complete Freund's adjuvant
CTLA-4	cytotoxic T-lymphocyte-associated protein-4
CX3C	C-X3-C motif
CXC	C-X-C motif
DA	discriminant analysis
DC	dendritic cell
E2f1	E2F transcription factor 1
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbant assay
FDC	follicular DC
FGF	fibroblast growth factor
Fmoc SPPS	9H-fluoren-9-ylmethoxycarbonyl based solid-phase peptide synthesis
Foxp3	forkhead box P3
FS	focus score
GC	germinal center
GCP-2	granulocyte chemotactic protein-2
GM-CSF	granulocyte macrophage-colony stimulating factor
GRO	melanoma growth stimulatory-activity protein
HCV	hepatitis C virus
HPLC	high-performance liquid chromatography
H&E	haematoxylin and eosin
Hsp60	heat-shock protein 60kDa
HSPs	heat-shock proteins
ICA69	islet cell autoantigen 69kDa
Id3	inhibitor of DNA binding 3
IDDM	insulin-dependent diabetes mellitus
IFA	incomplete Freund's adjuvant
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IL-1RA	IL-1 receptor antagonist
IP-10	inducible protein-10
IS	insulitis score
IVIG	intravenous immunoglobulin

JAK	Janus family of cytoplasmatic tyrosine kinases
Klk-13	kallikrein-13
LDL	low-density lipoprotein
LFA-1	lymphocyte function-associated antigen-1
LIF	leukemia inhibitory factor
LPS	lipopolysaccharide
M3R	muscarinic M3 receptor
MAP	multi-analyte profile
MCMV	murine cytomegalovirus
MCP	monocyte chemoattractant protein
M-CSF	macrophage-colony stimulating factor
MDC	macrophage-derived chemokine
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
MMP	matrix metalloproteinase
MPO	myeloperoxidase
MZ	marginal zone
NK	natural killer
NOD	non-obese diabetic
OSM	oncostatin M
PANTHER	protein analysis through evolutionary relationships
PAS	periodic acid-Schiff
PCA	principal component analysis
PI3K Ia	phosphoinositide 3-kinase class IA
PNAd	peripheral node addressin
pSS	primary Sjögren's syndrome
r	Pearson's correlation coefficient
R*	canonical correlation
RA	rheumatoid arthritis
RANTES	regulation upon activation, normal T-cell expressed and secreted
RF	rheumatoid factor
RI	ratio index
SCID	severe combined immunodeficiency
SLE	systemic lupus erythematosus
SS	Sjögren's syndrome
sSS	secondary Sjögren's syndrome
STAT	signal transduction and activators of transcription
TCR	T-cell receptor
TGF	transforming growth factor
T_H	T-helper
TIMP	tissue inhibitor of metalloproteinase
TLR	Toll-like receptor
TNF	tumor necrosis factor
TPO	thrombopoietin
T_{reg}	regulatory T-cell
TUNEL	TdT-mediated dUTP-biotin nick end labeling
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
vWF	von Willebrand factor
XC	C-motif

List of papers

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

Paper I

Malin V. Jonsson, **Nicolas Delaleu**, Karl A. Brokstad, Ellen Berggreen, Kathrine Skarstein (2006)
Impaired salivary gland function in NOD mice: association with changes in cytokine profile but not with histopathologic changes in the salivary gland
Arthritis & Rheumatism 54: 2300-2305

Paper II

Nicolas Delaleu, Heike Immervoll, Janet Cornelius, Roland Jonsson (2008)
Biomarker profiles in serum and saliva of experimental Sjögren's syndrome: associations with specific autoimmune manifestations
Arthritis Research & Therapy 10:R22

Paper III

Nicolas Delaleu, Ana C. Madureira, Heike Immervoll, Roland Jonsson (2008)
Inhibition of experimental Sjögren's syndrome through immunization with heat-shock protein 60kDa and its peptide aa437-460
Arthritis & Rheumatism, in press

Related publications

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

2. **Delaleu N**, Jonsson R, Koller MM. (2005)
Sjögren's syndrome
Eur J Oral Sci 113: 101-113

3. 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: 168-176

4. Jonsson MV, **Delaleu N**, Jonsson R. (2007)
Animal models of Sjögren's syndrome
Clin Rev Allergy Immunol 32: 215-224

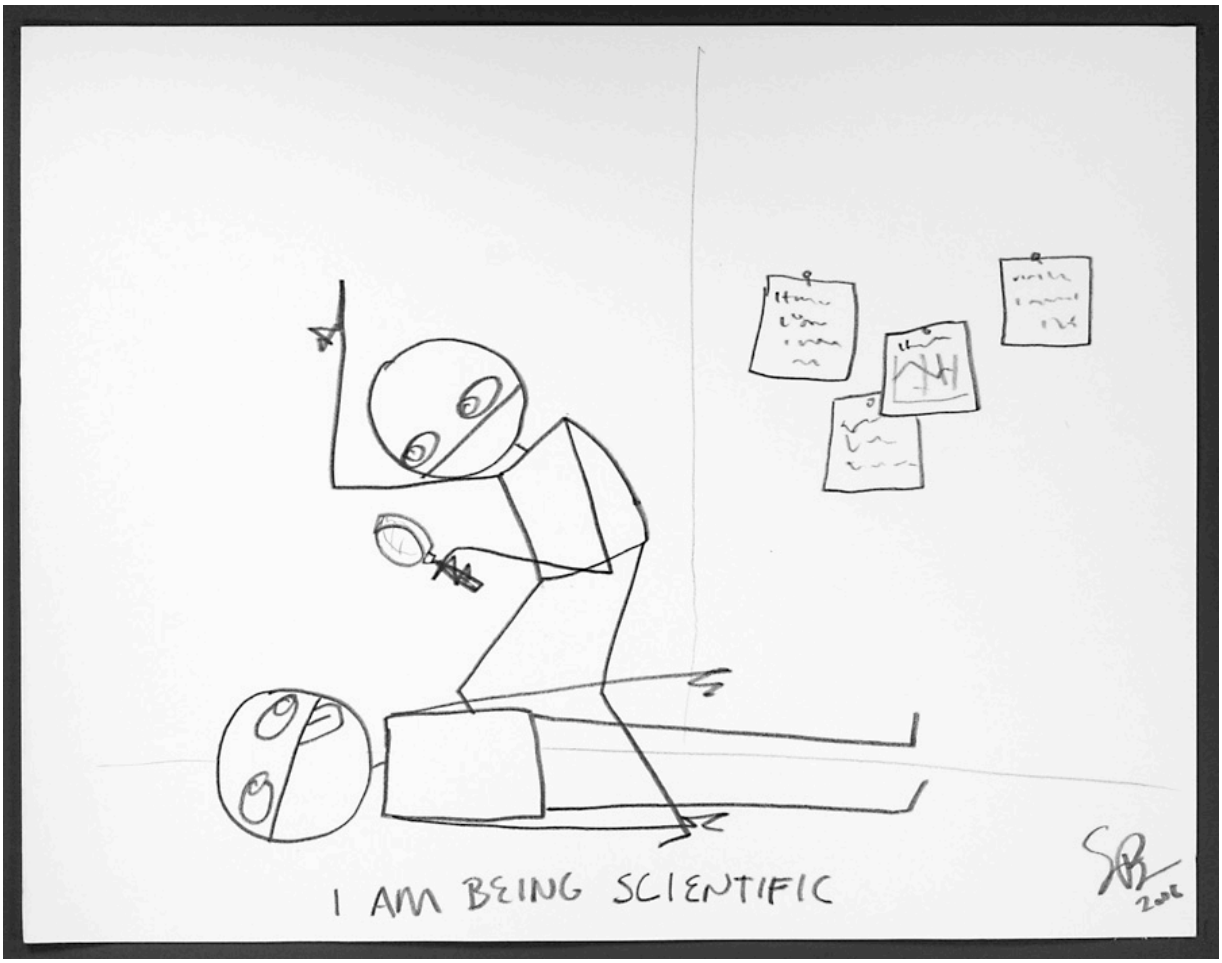
5. **Delaleu N**, Jonsson MV, Appel S, Jonsson R.
New concepts in the pathogenesis of Sjögren's syndrome
Rheum Dis Clin N Am, in press

6. Berggreen E, Nyløkken K, **Delaleu N**, Hajdaragic-Ibricevic H, Jonsson MV.
Impaired vascular responses to parasympathetic nerve stimulation and deficient muscarine receptor activation in submandibular glands of NOD mice
In manuscript

Background

On science

In the broadest sense, science refers to any systematic knowledge or practice. Applying a more restrictive definition, science refers to a system of acquiring knowledge applying the scientific method. The scientific method is based on the collection of data through observation and experimentation, and the formulation and testing of hypotheses. The organized body of knowledge gained through such research is as well referred to as science. Scientists shall never claim absolute knowledge of nature or the behavior of the subject of study, as a scientific theory is empirical and always open to falsification.



I am being scientific; illustration by Sam Brown (www.explodingdog.com)

The two major principles applied in science are the principle of reductionism and the principle of holism. Until today the scientific method has primarily tended towards reductionism what led to enormous scientific advances since the age of enlightenment. In many ways

reductionism also liberates us from the annoyance of complexity. Descartes argued in 1637 that the world is like a machine, its pieces like clockwork mechanisms. The machine could be understood by taking its pieces apart, studying them, and by putting them back together one would grasp the larger context [1]. Reductionism in science is based on the philosophical position that a complex system is nothing but the sum of its parts. Metaphorically, one reduces complexity to causal principles. Complex systems can therefore be accounted for with a hierarchy of organizations, of which each higher level can be described by the levels lower in the hierarchy [2]. While it is widely accepted that mathematics underlie most aspects of physics and physics provide the basis for chemistry and so forth, at levels of organization with higher amounts of complexity, formed from assemblies of large numbers of interacting components, such statements may become controversial [3].

Aristotle concisely summarized holism by stating, *"The whole is more than the sum of its parts"* what implies that complex systems are inherently irreducible and a holistic approach is needed to understand them. Promoted by the revolution in information technology and the recent developments of powerful bioanalytical platforms, system-based approaches have emerged within the field of bioscience [4, 5]. System biology promotes integration rather than reduction, driven by the ultimate goal to display a complete and accurate model of a biological system at a given time. Intuitively and intellectually, one may conclude that both, hypothesis-driven component focused research, and discovery-oriented systematic approaches are needed to properly account for a specific system state. The challenges in the years to come will also consist of finding ways to bridge between these two principles of research [4, 5].

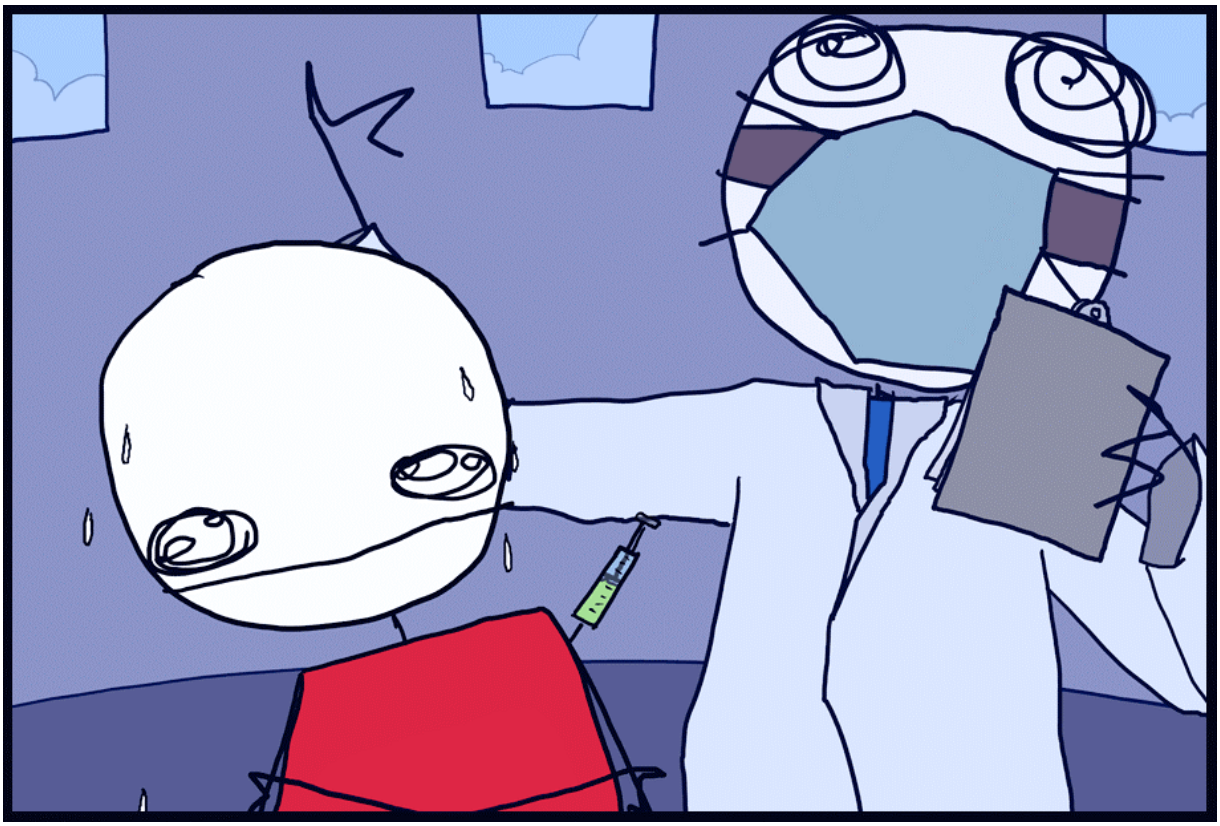
On immunity

"The story of immunity is the story of the self. The immune system, like the brain, creates, records and protects our individuality" Irun R. Cohen [6].

The immune system greatly defines us as a species and an individual of this species by defining what is allowed to reside within us. It protects our body against pathogens, rejects foreign tissue and eliminates tumor cells [7]. In addition, the immune system is involved in body maintenance and paves the path for reconstruction, which follows non-lethal injury [7].

Immunology is a relatively new science, having its origins in Edward Jenner's discovery made in 1796. He described that cowpox had the capacity to induce protection against human smallpox [8]. The causal relationship between a specific pathogen and a specific infectious

disease, however, was only defined at the end of the 19th century. The triumph of vaccination in fighting infectious diseases led to emergence of the research discipline termed immunology [7].



You are not immune; Illustration by Sam Brown (www.explodingdog.com)

Immunology covers the study of all aspects of the immune system in all organisms and in states of health and disease. Malfunctions of the immune system include hypersensitivities, immune deficiency, allograft rejection and autoimmune diseases [7]. The latter are addressed in further detail in the following chapters.

It should be recognized, that every organism has an immune system capable of protecting it from specific forms of harm. Nevertheless, the different species maintain immune systems of different complexity, often involving layered defense mechanisms with varying specificities. Most simply, surface barriers such as physical barriers *e.g.* skin, chemical barriers *e.g.* alterations in the pH and biological barriers *e.g.* commensal flora competing with potential pathogens for resources, prevent pathogens from invading the body [7].

Innate immunity

The next layer of defense, termed the innate immune system, exists in nearly all forms of life and elicits an immediate immune response in case surface barriers have been breached. The symptoms of inflammation that are redness, heat, swelling, pain and loss of function indicate the activation of the innate immune system. These clinical manifestations reflect vasodilation of local blood vessels in favor of the recruitment of immune cells to sites of injury. In addition, the complement cascade is activated, representing the mobilization of the humoral component of the innate immune response. Elevation of the body temperature is mainly caused by endogenous pyrogens such as tumor necrosis factor (TNF)-α, interleukin (IL)-1 and IL-6, which in their turn initiate the acute-phase response approximately 90 minutes after the onset of a systemic inflammatory reaction [7]. In many cases, the invading pathogens can be recognized and eliminated through preformed nonspecific effectors. Subsequently, processes mediating tissue repair replace the inflammatory reaction.

In other cases, although the innate immune system lacks fine specificity, certain cells such as macrophages, neutrophils and dendritic cells (DCs) recognize common microbe-associated molecular patterns through receptors located on their cell membranes or in their intracellular compartments [7]. In mammals, Toll-like receptor (TLR)4 binds lipopolysaccharide (LPS), an important component of the outer membrane of Gram-negative bacteria [9]. TLR engagement triggers the production of chemokines and cytokines and the expression of co-stimulatory molecules. The expression of the latter is crucial to initiate an adaptive immune response through a process referred to as antigen presentation [7]. In addition to the cell types already mentioned, mast cells, eosinophils and basophils belong to the cell types categorized as innate leukocytes [7]. At the interface of the innate and adaptive immune system, minor subsets of innate-like lymphocytes (B-1 cells, γ:δ T-cells and natural killer [NK] T-cells) have been described [7]. They only express a limited diversity of receptors and are mainly confined to specific locations in the body. Furthermore, they are not required to undergo clonal expansion before responding efficiently to the antigens they recognize. However, their exact role in host defense and immunological disorders is still a matter of speculation. Common to all processes belonging to the innate immune system is the lack of adaptation subsequently to a pathogen encounter and their incapability of generating a noteworthy immunological memory [7].

Adaptive immunity

The adaptive immune system is thought to have arisen in the first jawed vertebrates. It is composed of highly specialized cells and processes, which become activated when pathogens evade the innate immune system. Compared to the innate immune response, the adaptive immune system provides the individual with the ability to selectively recognize and remember specific pathogens and to mount a more potent immune response each time the pathogen is reencountered (immunological memory) [7]. T-cells and B-cells are the two cell types involved in specific antigen recognition and capable of generating immunological memory. Both subsets use different, but structurally similar molecules, which specifically bind a certain antigen. The antigen-recognition molecules of T-cells are membrane bound and referred to as T-cell receptors (TCRs). Antigen-recognition molecules on B-cells are membrane bound immunoglobulins (Ig's) and are referred to as B-cell receptors (BCRs) [7]. Ig's can also be secreted by highly differentiated B-cell subsets (plasma cells, plasma blasts). Secreted Ig's, also known as antibodies, form the humoral component of the adaptive immune response. The large repertoire of the TCRs and the BCRs is based on the mechanism of V(D)J recombination (an irreversible genetic recombination of antigen receptor gene segments). BCRs are furthermore modified by mutations, known as somatic hypermutations, of the variable regions within the Ig. These two mechanisms allow a small number of genes to generate a vast number of different antigen receptors, which has theoretically been estimated at 10^{18} for T-cells and $5x10^{13}$ for B-cells [7]. Because the gene rearrangements lead to irreversible changes in the DNA, all of the progeny of one specific T- or B-cell will inherit genes encoding the same receptor specificities.

The activation of T-cells is dependent upon recognition of considered "non-self" protein fragments (peptides) presented within a protein structure, termed the major histocompatibility complex (MHC) [10]. MHC class I molecules present peptides derived from proteins synthesized in the cytosol (*e.g.* viruses). MHC class II molecules stably bind peptides from proteins degraded in the endocytic vesicles (*e.g.* phagocytized bacteria). MHC class I and MHC class II are differentially recognized by the co-receptor molecules cluster of differentiation (CD)8 and CD4, respectively [7]. At the same time the expression of CD4 and CD8 categorizes T-cells into two major subsets, the $CD8^+$ and $CD4^+$ T-cells. $CD8^+$ T-cells are mainly cytotoxic T-cells specialized to release preformed cytotoxins *e.g.* perforin and granzymes. Tightly focused at the site of contact between the two cells, cytotoxins either perforate the membrane or induce apoptosis of the target cells [7]. Whereas MHC class

I/peptide complexes can be expressed on nearly all cells, MHC class II molecules are almost exclusively expressed on professional antigen presenting cells. $CD4^+$ T-cells recognize the MHC class II/peptide complexes on antigen presenting cells and are specialized to activate other immune effector cells, *e.g.* B-cells and macrophages, to act against the antigen they are presenting [7].

B-cells, through their BCR, have the unique capability of recognizing their cognate antigen in its native form. Alike other professional antigen presenting cells, B-cells present protein antigens in form of peptide/MHC class II complexes to $CD4^+$ T-cells. Upon ligation, T-cells secrete cytokines, providing B-cells with so-called T-cell help. These cytokines trigger B-cell proliferation, isotype switching to IgG, IgA, and IgE, and promote differentiation towards plasma cells and memory B-cells. Many B-cells are dependent on the co-stimulatory signals provided by T-cells. However, other antigens, such as repeating carbohydrate epitopes, are considered to be T-cell independent, meaning they deliver all necessary signals to the B-cells to become activated. B-cells recognizing T-cell independent antigens require extensive BCR cross-linking to mount an immune response, which is characterized by only few or no somatic mutations, limitations in Ig class switching and weak memory. Indeed, T-cell independent immune reactions comprise many characteristics initially associated with the innate immune system [7].

The goal of every immune response must be the survival of an infection by either eliminating or confining the specific pathogen in a manner so it cannot inflict harm on the host any longer. At the same time, damages on the surrounding tissue have to be limited. The immune system also needs to ensure the restoration of tissue homeostasis, whenever possible, without causing secondary effects such as fibrosis. These multiple tasks require the generation of fine-tuned and controlled immune responses.

Mediators of the immune system

Cytokines

The term cytokine is used for a diverse group of soluble proteins and peptides that act as humoral regulators at nano- to picomolar concentrations [7]. Under normal and pathological conditions they modulate the functional activities of individual cells and tissues. Their mechanisms of action may be classified as autocrine (acts on the cytokine secreting cell itself), juxtacrine (involving specific cell-to-cell contacts), paracrine (effect is restricted to the immediate vicinity of the cytokine secreting cell) and endocrine (diffuses to distant regions of the body) [7]. Because cytokines are characterized by redundancy, pleiotropism, synergism, and antagonism and can be secreted by a wide range of cell-types, the initial concept of "one producer cell - one cytokine - one target cell" has been falsified for practically every cytokine investigated. Generalizing the functions of cytokines is also impossible because beside their central role in immune system, not all their functions are restricted to immune reactions. They are, furthermore, involved in several developmental processes such as embryogenesis and hematopoiesis [7].

Nonetheless, classification according to structural homology has been able to subdivide cytokines in several, sometimes rather large families: 1) the hematopoietins, *e.g.* many ILs and growth factors such as the granulocyte macrophage-colony stimulating factor (GM-CSF), 2) the interferons (IFNs) and 3) the TNF superfamily *e.g.* TNF-α, CD40L, FasL and B-cell activating factor (BAFF) [11], 4) the more recently discovered IL-17 family *e.g.* IL-17 [12] and 5) the chemokines [13]. Accordingly, their specific receptors can be grouped similarly: 1) The class I cytokine receptors recognizing the hematopoietins, 2) the class II cytokine receptors binding IFN-α, -β, -γ and IL-10 superfamily and so forth [7]. However, cytokines, which are sharing structural similarities, may, but often do not, exert similar biological functions. Nevertheless, hematopoietins and IFNs, all signal through similar pathways, involving members of the Janus family of cytoplasmatic tyrosine kinases (JAK) and signal transduction and activators of transcription (STAT). Because the JAK and STAT protein families are comprised of different members, they can be individually activated to achieve very specific effects [7].

Based on the cytokines secreted, T-helper (T_H) cells are currently subdivided into three distinct functional effector subsets, termed T_H1, T_H2 and T_H17 [12, 14, 15] (Figure 1). They differentiate from not yet committed T_H0 cells in response to exposure to specific cytokines.

Such stimuli are followed by the induction of independent patterns of gene transcription. T_H1 cells tend to be involved in host defense against intracellular pathogens and thought to perpetuate autoimmune responses [12, 14]. T_H1 cell differentiation from T_H0 cells is dependent on IFN-γ and IL-12. In their turn, T_H1 cells produce IL-2 and IFN-γ. T_H2 cells on the other hand tend to engage in immune responses, which depend on a substantial humoral component to eliminate the pathogen [12, 14]. Key molecules of a T_H2 dominated immune response are IL-4, IL-5 and IL-13. T_H1 and T_H2 cells counteract each other, mainly because their respective cytokines can exert an inhibitory effect on the opposite T_H cell subset [12, 14].

Hence, an optimal scenario, before the emergence of T_H17 and regulatory T-cells (T_{reg}) cell subsets, seemed to consist of a well balanced T_H1 and T_H2 response, tailored in accordance with the encountered immune challenge [14]. Indeed, considering the limited knowledge about cytokines at the time the T_H1/T_H2 model was developed [14] and despite its flaws, the T_H1/T_H2 paradigm showed remarkable durability and still conserved some of its validity [16].

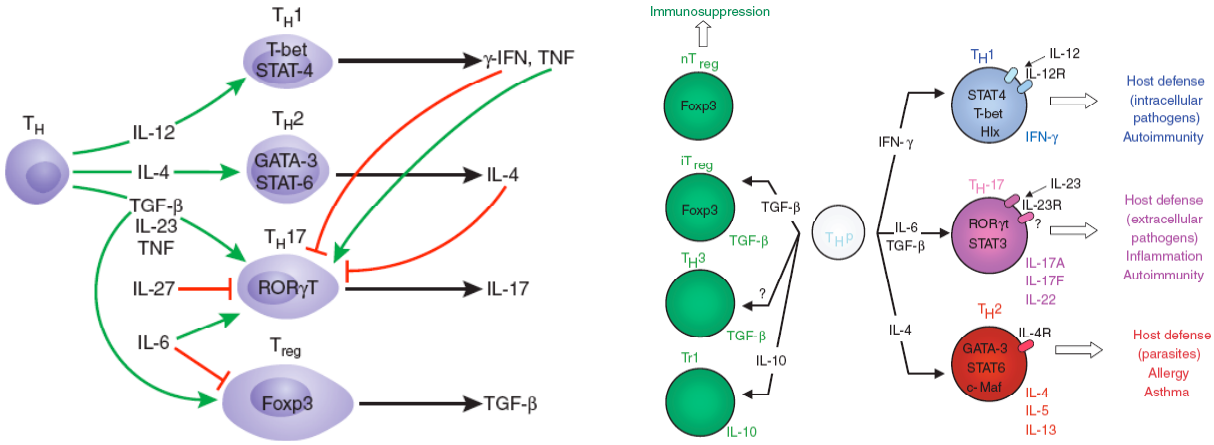


Figure 1) *T-helper cell differentiation and regulation. Green arrows and black indicate induction, while red lines indicate inhibition. Transcription factors for particular lineages are placed in the nucleus. Differentiation of $CD4^+$ T-cell lineages: Peripheral naive $CD4^+$ T-cell precursor cells (T_Hp) can differentiate into three subsets of effector T-cells (T_H1, T_H2 and T_H17) and several subsets of T_{regs}, including induced T_{reg} cells (iT_{reg}), Tr1 cells and T_H3 cells. Naturally occurring T_{reg} cells (nT_{reg}) are generated from $CD4^+$ thymic T-cell precursors. The differentiation of these subsets is governed by selective cytokines and transcription factors, and each subset accomplishes specialized functions. Figures fully borrowed from L. Steinman [12] and E. Betelli et al. [17].*

More recently the T_H1/T_H2 paradigm has been challenged by the emergence of the T_H17 subset producing IL-17 (IL-17A), IL-17F and IL-22 [12, 15]. A major role of IL-17 has now been described in various models of immune mediated tissue injury related with host defense against microorganisms and organ-specific autoimmunity [15, 17]. IL-6, transforming growth

factor (TGF)-β and IL-23 induce the differentiation, survival and expansion of T_H17 cells, whereas the major cytokines produced in a T_H1 and T_H2 context, IFN-γ and IL-4, respectively, antagonize T_H17 related immune responses [18]. Furthermore, reciprocal interactions involving T_H17 cells have been reported when studying the differentiation of a class of T_{regs} termed forkhead box P3 $(Foxp3)^+$ T_{regs}. This process involves IL-6 and TGF-β (Figure 1). TGF-β, initially thought of as anti-inflammatory, was proposed to function as a critical regulator of both tissue-damaging T_H17 cells when collaborating with IL-6, and as an inducer of anti-inflammatory T_{regs} when acting in absence of IL-6 [17] (Figure 1). The mechanism by which T_{regs} limit effector responses *in vivo*, however, remains poorly understood. Crucial however, seems the secretion of anti-inflammatory cytokines, such as TGF-β and IL-10 by T_{regs} [19, 20]. In addition, T_{regs} may reduce the ability of DCs to subsequently activate effector T-cells [21].

It is certain, that the current concept of how cytokines and T-cell subsets achieve an appropriate balance between protection from "non-self", while sparing the "self" from self inflicted damage, will continue to increase in terms of size and complexity. Even though T-cell populations have received most attention over the recent years, it is important to remember that virtually all populations of cells, especially in close proximity of the inflammation, may contribute in one or another way to the outcome of the immune reaction.

Chemokines

Chemokines are a family of 8-10kDa small, secreted proteins, mainly involved in leukocyte chemoattraction during the initiation and orientation of the innate and adaptive immune response. Beside their role in inflammation, chemokines are of importance in regulating cell trafficking in states of tissue development and tissue homeostasis [7, 13]. This large family of related molecules is classified according to structural properties related to the number and position of conserved cysteine residues. C-C motif (CC) and C-X-C motif (CXC) chemokines are the two major, and C motif (XC) and C-X3-C motif (CX_3C) the two minor chemokine subfamilies [22] (Figure 2). Accordingly, the different subfamilies bind to specific receptors, all belonging to the G-protein-coupled receptor superfamily. Most chemokines can bind to several receptors [13], named according to the subfamily of chemokines they recognize *e.g.* CCR for CC chemokines, CXCR for CXC chemokines an so fourth [22]. Indeed, redundancy in chemokine-ligand/chemokine-receptor interactions seems evident (Figure 2). However, these interaction charts are mainly based on results obtained *in vitro* and it is probable that

this apparent redundancy is artificial in nature and absent *in vivo*. Further knowledge regarding subtle regulatory mechanisms, such as contribution of chemokine receptors to lymphocyte extravasation and serial usage of different chemokine receptors, might help to break this seeming redundancy [13]. Although the main task of cytokines is to orchestrate leukocyte recruitment, they also exhibit other cytokine-like activities including regulation of angiogenesis [23], fibrosis [24] and apoptosis [25].

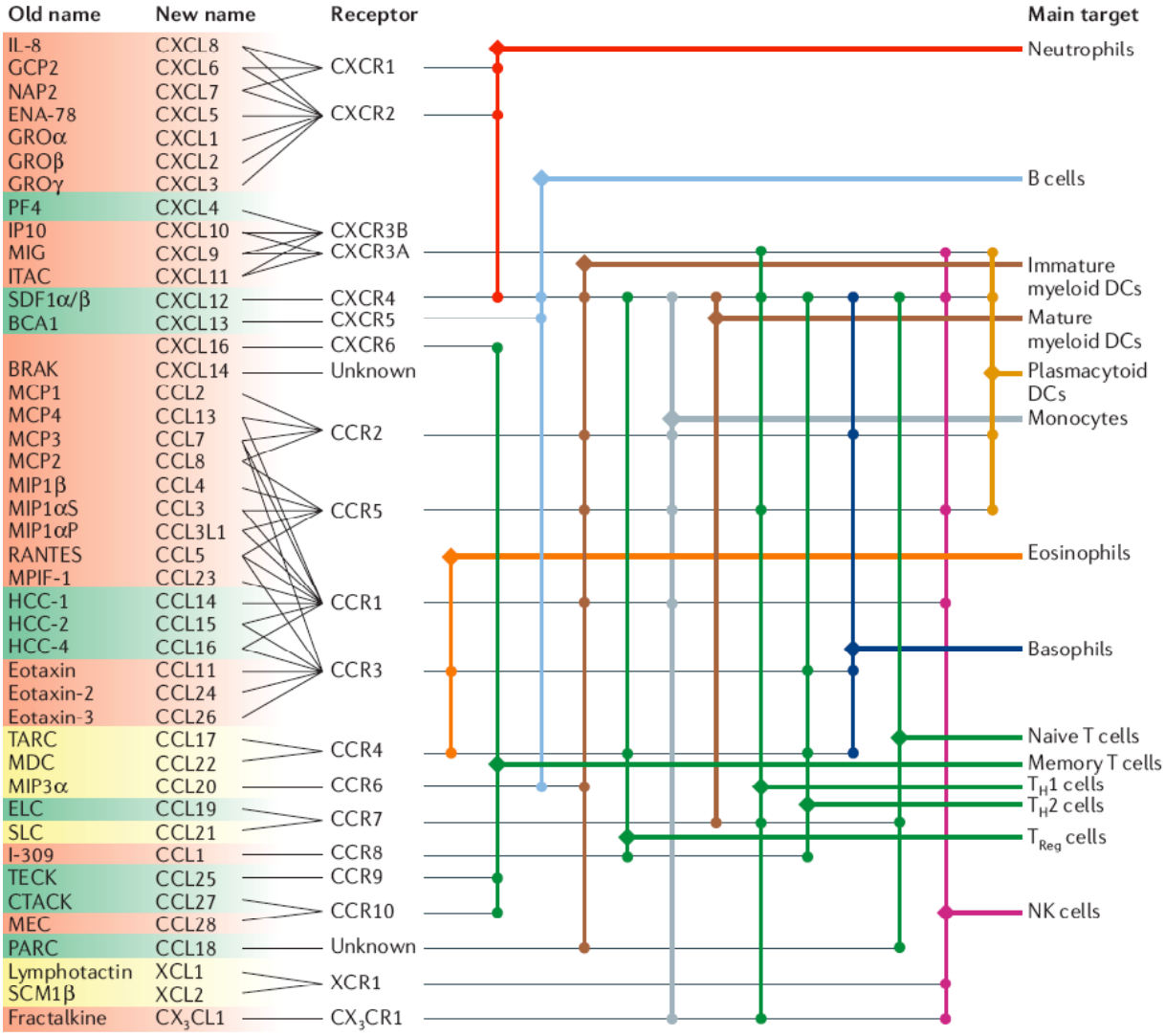


Figure 2) *Human chemokines, encoded by 43 genes, are classified into four families on the basis of structural differences. Chemokines can also be classified as pro-inflammatory (red), homeostatic (green) and those with mixed function (yellow). Chemokines bind to a subfamily of seven-transmembrane G-protein-coupled receptors, which at present include 18 receptors that are classified as CCR, CXCR, CX3CR and XCR on the basis of the class of chemokines they are able to bind. Their main expression pattern on leukocytes is listed in the right column. To show the connection between receptors and cell types a notation inspired by electronic circuit representation has been used. To connect receptors to target cells, follow a horizontal line and turn on a vertical one at each node; the rhombs (diamond shapes) aggregate vertical lines to the cell type. Figure fully borrowed from A. Mantovani et al. [13].*

Growth factors

The term growth factor refers to proteins capable of stimulating cellular proliferation and cellular differentiation. Growth factors play a crucial role in a variety of cellular processes. For example, the vascular endothelial growth factor (VEGF) family consists of important signaling proteins involved in both vasculogenesis (the *de novo* formation of the embryonic circulatory system) and angiogenesis (the growth of blood vessels from pre-existing vasculature) [26]. The term growth factor became interchangeable with the term cytokine, when it became apparent that lymphocytes were also using some signaling proteins originally identified in hematopoietic context [27]. However, the term growth factor implies a positive effect on cell division, whereas cytokine is a neutral term with respect to whether a molecule does or does not affect cell proliferation.

Peptide hormones

It is known that hormones can suppress or activate the immune system depending on the specific context [28]. In opposition to cytokines, which can be produced by a wide variety of cells, specialized cells, mainly residing within endocrine glands, such as the pancreas, form peptide hormones. However, also non-endocrine glands, such as adipose tissue, produce peptide hormones *e.g.* leptin [29]. In addition, it should be taken into account that mononuclear cells express receptors specific for neurotransmitters, another class of signaling molecules mainly produced by highly differentiated cells [30]. Unfortunately however, the crosstalk between the nervous and the immune system is poorly explored [31].

On autoimmunity, immune tolerance and autoimmune diseases

Autoimmunity refers to the capacity of somatically generated antigen receptors to recognize self-molecules. Through the process of recognition, self-molecules can become self-antigens or autoantigens [6, 7]. The classical view on the relationship between autoimmunity and autoimmune diseases is based on the assumption that autoimmunity cannot be physiological. Hence, a healthy immune system must be free from autoimmunity. Several clues are used by the immune system to distinguish self-ligands from non-self ligands, *e.g.* the encounter with the ligand while the lymphocyte is still immature and the exposure to high and constant concentration of the ligand in absence of co-stimulatory signals. The latter is usually dependent on the activation state of the innate immune system [7]. This view defines autoimmunity in general as a random accident, which has no purpose and is always forbidden [32]. Autoimmune diseases, in such a case, must either be caused by a failure in eliminating an autoimmune clone during its development, through the mutation of a mature clone, which accidentally resulted in the recognition of a self-molecule, or be initiated by mechanisms of molecular mimicry. The absence of an autoimmune disease would therefore be marked by the absence of autoreactive clones. Such clones, because they evidently arise through random generation of TCRs and BCRs must all be deleted. Following this train of thought, the only specific cure for an autoimmune disease is the elimination of autoreactive cells. F.M. Burnet and P.B. Medawar were awarded the 1960 Nobel Prize for the discovery of acquired immune tolerance also known as the clonal deletion theory [32].

Despite the plausibility of the clonal deletion theory, it became evident that the concept could neither always fit the state of a healthy immune system nor explain certain basic phenomena from autoimmune diseases [6, 7]. Nevertheless, autoimmune diseases are the result of a loss in immunological tolerance, which is the ability to appropriately respond to self-molecules. The exact conception of the nature, origin and maintenance of the immunological tolerance is still elusive, but several theories have been proposed, complementing, and in certain aspects opposing the clonal deletion theory *e.g.* the clonal anergy theory [33, 34] and the idiotype-anti-idiotype network theory [35]. Furthermore, hypotheses involving specific regulatory cell populations with the function to limit or downregulate pathogenic autoimmune reactions are under intense investigation [19, 36].

Today, one may imagine the existence of two types of autoimmunity: On one hand natural or physiological autoimmunity and on the other hand pathological autoimmunity. The latter is

manifested as a disease caused by an immune reaction specifically directed against self-antigens (autoimmune disease) [6, 37].

Indeed, autoimmunity might be an intrinsic and essential part of the healthy immune system, vital to its development and function especially in the context of immunological tolerance. According to I.R. Cohen, physiological autoimmunity should be viewed as an organized entity of a few key self-molecules [38]. The entity was termed the "immunological homunculus" analogous to the concept of the neurological homunculus, which refers to a functional map of the body in the brain [6]. Applying the same concept, the immunological self-determinants forming the immunological homunculus would define the borders between the self and the non-self. Indeed, the immune system consists of a relatively high number of autoreactive T- and B-cells, which recognize a relatively small number of immune-, maintenance- and tissue molecules [39-41]. Accepting the principle of natural autoimmunity has far reaching consequences as it implies that our immune system possesses a dynamic picture of the self. Such a picture would be drawn by the components involved in the maintenance of physiological autoimmunity [38]. Autoimmunity would no longer only be an aberration, but specific self-molecules would have the central role in shaping the immune response in accordance with the system's state. Thereby they are subjected to strict anti-autoimmune regulation [42]. These self-determinants and their corresponding counterparts of the immune system would be crucial in preventing the conversion of autoimmunity into an autoimmune disease. Hence, autoimmune diseases would arise from a divergence between the actual tissue state and the immune system's perception of the situation and be marked by insufficient anti-autoimmune regulation against key self-molecules. Treatment of autoimmune diseases should be designed to reinitiate healthy immune regulation, which in its turn would be followed by reinstatement of physiological autoimmunity [36]. Administration of self-antigens taking into account dose, dose schedule, anatomical site and context can prevent or in some cases induce remission of autoimmune diseases in murine models [43-45]. Similar results can be achieved by antigen unspecific stimulation of regulatory mechanisms [46].

The number of medically defined autoimmune diseases is relatively small and the different diseases mostly present distinct clinical and immunological signatures, which involve specific sets of self-antigens. Nevertheless, the lack of an knowledge regarding causative mechanisms renders disease classification difficult [47]. Nonetheless, autoimmune diseases can be clustered into organ-specific autoimmune diseases *e.g.* insulin-dependent diabetes mellitus (IDDM), primarily affecting the insulin producing pancreatic islets, and into systemic

autoimmune diseases, which can affect many and very diverse tissues of the body. The latter category includes diseases such as scleroderma, rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and Sjögren's syndrome (SS). It is important to notice that in several medical conditions described above, not only inappropriate destruction, but also inappropriate healing may contribute significantly to the pathogenesis [16]. Both processes are closely connected to the immune system and the state of inflammation. It is evident that the most difficult task of the immune system is the establishment of effective host-defense against foreign pathogens as well as against cancer cells while sparing healthy tissue and insuring proper instauration of tissue homeostasis.

Heat-shock protein 60kDa and the regulation of inflammation

Classical anti-inflammatory drugs which can be grouped into corticosteroids, cytotoxic drugs and fungal and bacterial derivatives are very broad in their actions [7] and opportunistic infection are common complications of immunosuppressive therapies. Recent treatment strategies specifically induce the blockade of several pro-inflammatory cytokine pathways, such as TNF-α [48] and IL-1 [49]. However, none of these strategies reverse the pro-inflammatory process and still harbor the risk of opportunistic infections [50, 51]. Indeed, it is obvious that patients suffering from autoimmune diseases would greatly benefit from restoration of immunological tolerance. Among other agents, heat-shock protein 60kDa (Hsp60) [52] and anti-CD3 [19] are representatives of a new category of biological agents endowed with the unique capacity to promote immunological tolerance in absence of long-termed and generalized immunosuppression.

Heat-shock proteins (HSPs) are ubiquitously expressed molecular chaperones, which have crucial housekeeping functions in both prokaryotic and eukaryotic cells by ensuring proper folding of proteins [53]. The expression of HSPs is markedly upregulated under conditions of cellular stress, such as an infection, inflammation and exposure of the cell to toxins. HSPs were also proven to be highly immunogenic [53]. Analyses of the T-cell response against *Mycobacterium tuberculosis* demonstrated that 10-20% of the response was directed against *M. tuberculosis* Hsp60 [54]. Exceptionally conserved throughout evolution, molecular mimicry between HSPs from microbial and HSPs form mammalian origin was suspected to underlie several autoimmune and inflammatory conditions such as RA [55] and atherosclerosis [56]. Hsp60 was also identified to be the crucial component in Bacille Calmette-Guérin mediated inhibition of IDDM in NOD mice [57]. Subsequently, a peptide vaccine (DiaPep277, aa437-460), based on eukaryotic Hsp60, has been developed for the treatment of IDDM and is currently tested in phase II clinical trials [58]. In contrast, HSPs remain a poorly investigated field in SS [59]. However, molecular mimicry does not seem to be the only origin of Hsp60 autoimmunity. Interestingly, despite multiple checkpoints, which could ensure central tolerance to self-Hsp60, healthy individuals maintain T- and B-cells recognizing exclusively self-Hsp60 [41]. In addition, abundant thymic overexpression of Hsp60 could not abolish T-cell responses to Hsp60 [60].

It is most probable that T-cells recognizing self-Hsp60 receive yet undefined survival signals during negative selection in the thymus. As postulated by I.R. Cohen, Hsp60 belongs to a set

of conserved self-antigens at the core of physiological autoimmunity, which he termed the immunological homunculus [6]. After entering the periphery, the lack of clonal exclusion in the thymus must be compensated by mechanisms of peripheral tolerance. Several lines of evidence indicate that under certain conditions $CD4^+$ T-cells specific for Hsp60 self-epitopes critically regulate inflammation [52].

Therapeutic and preventive effects, irrespective of the specific autoimmune condition studied, have been attributed to the capacity of Hsp60 to trigger anti-inflammatory and regulatory mechanisms. Importantly, it seems that the protective effects of Hsp60 do not require an antigenic relationship between Hsp60 and the disease causing antigen [52]. Disease severities of RA and more recently of juvenile idiopathic arthritis have been related to the extent of Hsp60 induced propagation and activation of T_{regs} [61, 62]. Hsp60 and the Hsp60 derived peptide aa437-460, via TLR2 signaling, altered inflammatory chemotaxis and downregulated T-cell migration *in vitro* [63, 64]. T_{regs}, identified to be innately responsive to Hsp60, are supposedly rendered more effective in downregulating CD4 and CD8 effector responses after Hsp60 and aa437-460 engagement [20]. In addition, Hsp60 may regulate T_H1/T_H2 transcription factors and cytokine expression [65].

Research efforts also expanded the focus from Hsp60 specific T-cells to antigen presenting cells, including macrophages and DCs. Antigen presenting cells may thereby directly interact with endogenous Hsp60 through identified receptors such as CD14, CD40, CD36, CD91, lectin-type oxidized low-density lipoprotein (LDL) receptor, TLR2 and TLR4, and other yet unidentified cell specific surface receptors [66]. If proven correct, self-HSPs would represent an interface between the innate and the adaptive immune system. However, the proclaimed induction of pro-inflammatory cytokine secretion, mediated by self-HSPs, has been suspected to originate from LPS contamination of the HSP preparations [67]. Nevertheless, subsequent studies were able to identify specific effects of mammalian HSPs, which could not be assigned to microbial HSPs. These effects seemed to be regulatory and anti-inflammatory in nature [52]. In summary, compared to microbial Hsp60, its mammalian counterpart may, instead of promoting a pro-inflammatory response, pave the path for a regulatory response through the adaptive arm of the immune system.

Biomarker discovery

A biomarker is a substance or measurement that is indicative for a certain state of a living organism [68, 69]. Identification of specific biomarkers of autoimmunity and tolerance represents a major goal of clinical immunology [70]. At first, biomarkers should provide the physician with information about the patient's individual disease risk or benefit his diagnosis. Biomarkers may further predict the disease course or point the physician towards the use of a specific treatment. Repeated measuring of adequate biomarkers may further allow to follow-up on treatments responses.

A validated biomarker, possessing one or several properties described above, reduces the often not graspable complexity behind the process it indicates, to a rather simple measurement. For, example, a high concentration of LDL combines complexities related to factors such as genetic background, metabolism, inflammation and state of blood vessels walls, to an indicator of atherosclerosis [71]. Compared to defined infectious diseases, where the presence of a defined causative agent facilitates such enterprise, the diagnosis and classification of autoimmune diseases remains largely based on clinical examination combined with traditional laboratory tests [47].

Due to accessibility and the lack of extraction procedures, biological fluids are the targets of choice for the detection of biomarkers [68, 69]. The rationale, however, is strongly dependent on the assumption that the tissue of interest is in close contact with the biological fluid. In such a case alterations in the tissue-state would be prone to be reflected in the spectrum and amount of proteins liberated into the biofluid [68]. The recent development of sophisticated, large-scale and high-throughput genomic and proteomic platforms created unprecedented possibilities to identify novel biomarker signatures for autoimmune diseases [5, 68, 69]. The ultimate goals of such studies are to improve diagnosis, to assess individual risk, to predict and characterize the response to treatment and the identification of possible targets for therapeutic treatment intervention. However, there is an obvious gap between theory and reality, which is clearly illustrated when comparing the enormous amount of data acquired through genomic and proteomic methods focusing on body fluids and the rare emergence of new, clinically applicable biomarkers [68]. Technologies applied to biomarker discovery are either, at least theoretically, unbiased platforms *e.g.* mass spectrometry or biased technologies, such as bead-based multiplex immunoassays, antigen array and antibody array platforms [70]. The inherent bias in the latter category is due to the use of existing capture

agents such as purified proteins, peptides or antibodies. The choice of technology needs to be dictated by the hypothesis that is being tested and the availability of specific reagents [70]. Which technology platform most accurately mirrors the true situation is often difficult to estimate, highlighting the importance of appropriate and uniform guidelines for quality assurance and quality control. One important task in the future consists in the creation of bioinformatics for the analysis of disparate data sets such as transcript profiles, protein profiles, and cell-surface phenotypes [70, 72].

Sjögren's syndrome

Overview

SS is a chronic autoimmune disease mainly affecting the exocrine glands. Nearly all patients complain of a persistent feeling of dry mouth (xerostomia) and dry eyes (keratoconjunctivitis sicca) [73, 74]. These symptoms can be confirmed by objective tests, which show significant functional impairment of the salivary and lacrimal glands. Histological evaluation of these glands show large and persistent infiltrates of mononuclear cells, so-called foci. These consist of mainly T-cells and fewer B-cells. Beside lymphoid infiltration, acinar epithelial cell atrophy and progressing fibrosis can be observed.

Affecting approximately 0.3-0.6% of the total population, SS is considered a very common rheumatic disease. With a ratio of 9:1 SS exhibits one of the highest female to male ratios of all autoimmune diseases. SS may extend from an autoimmune exocrinopathy to the manifestation of diverse extraglandular symptoms, such as involvement of the musculoskeletal, pulmonary, gastrointestinal, hepatobiliary, hematologic, vascular, dermatologic, renal and nervous system. Furthermore, the risk of developing non-Hodgkin's lymphoma is thought to be increased 16 fold compared to the general population [75].

SS may occur alone, then defined as primary SS (pSS), or in association with another defined autoimmune disease, *e.g.* SLE, RA or scleroderma, and is then defined as secondary SS (sSS).

As true for most autoimmune diseases, the etiology of SS is at present unknown. However, environmental factors set against an appropriate genetic background may be capable of triggering SS.

Traditionally, the loss of secretory capacity has been thought to be the direct consequence of glandular tissue being destroyed or replaced by infiltrating cells. The correctness of this assumption was, however, challenged by frequently observed discrepancies between the extent of destroyed glandular tissue and the remaining salivary secretion capacity. Processes disturbing the physiological cascade of saliva production and secretion have been proposed as a possible explanation for this phenomenon [76].

History, epidemiology, diagnosis and patient classification

In 1933 Henrik Sjögren, a Swedish ophthalmologist, presented his doctoral dissertation entitled "Zur Kenntnis der Keratoconjunctivitis sicca" [77]. He reported detailed clinical and

histological findings in women presenting xerostomia and keratoconjunctivitis sicca. Together with RA, SS is one of the most frequent autoimmune diseases and occurs in large parts of the world. The disease can appear at any age with a significant peak incidence between 40-50 years of age.

As there is no single test for the diagnosis of SS, different classification criteria have been proposed. Depending on the applied classification criteria, the prevalence of SS reaches from 0.5% of the adult female population, when applying the Californian Fox Criteria [78], to 1-3% of the total population, when using the European Community Criteria [79] or the American-European Consensus Group criteria (AECG) [47]. However, most studies act on the assumption of a prevalence of 0.3-0.6% of the total population. All three methods of classification listed above are based on the following disease manifestations: 1) subjective feeling of dry mouth and dry eyes, 2) objective ocular and oral signs of dryness, 3) defined focal lymphoid infiltrates within the salivary glands and 4) the presence of specific autoantibodies.

Clinical manifestations of Sjögren's syndrome

Beside general discomfort, dry mouth is accompanied by problems in swallowing, speaking and alterations in taste, and the lack of tears by photosensitivity and fluctuating vision [80]. Indeed, deficiencies in saliva quantity and quality have a negative impact on dental and oral health. Dental caries, mucositis, oral candidiasis and swelling of the salivary glands are the most frequent oral signs of SS and edentulous patients are common [81]. Increased levels of acidogenic and aciduric microorganisms were found in most patients with SS [82]. Consequently, prevention of caries and oral infections is strongly indicated and drugs with anti-cholinergic side effects, *e.g.* anti-depressants and anti-histamines, should be avoided. Beside sicca symptoms, pain and especially fatigue contribute to the significant decrease in quality of life and a worsened psychological status in SS patients compared to the general population [83].

Treatment of patients with Sjögren's syndrome

Treatments applied today provide merely marginal symptomatic relief [84] and patients with SS have to face a life with the disease. Keeping the mouth moistened, using water followed by a saline mixture, is the simplest solution to treat xerostomia. Extensive use, however, may remove the small amounts of mucous saliva, resulting in the dilution of protective agents present in saliva *e.g.* mucins. Dry eyes can be treated with protective measures, ocular wetting

agents and occasionally with local application of cyclosporine [85] and IFN-α [86]. To stimulate salivary flow, oral administration of muscarinic receptor agonists (pilocarpine hydrochloride or cevimeline) [87] were shown to significantly improve local and systemic symptoms of SS.

Drugs acting as muscarinic agonists have become accepted for the treatment of SS [88, 89]. However, the effectiveness of these depends upon intact receptor structures and parenchyma. Another potential treatment involves the administration of intravenous immunoglobulin (IVIG) [90]. Antiidiotypic antibodies, being part of IVIG, remove the inhibitory activity of anti-muscarinic M3 receptor (M3R), an antibody potentially involved in the pathogenesis of SS, *in vitro* [91].

Hydroxychloroquine [92] and nandrolone [93] also improved systemic symptoms of SS, whereas the usage of cyclosporine was rather ineffective [85]. Depletion of mature B-cells using anti-CD20 antibodies, first tested in small patient groups, which developed lymphoma [94, 95] or manifested severe systemic symptoms [96], also ameliorated several symptoms in a more representative SS cohort [97]. The use of TNF-α antagonists for the treatment of SS was not sustained since promising results could not be confirmed [98, 99].

Unfortunately, all therapies applied today are inadequate to cure SS and regrettably, the number of clinical studies addressing SS is significantly lower than for most other, as common, autoimmune diseases.

Etiology of Sjögren's syndrome

A possible scenario for the emergence of SS comprises a viral infection of the target organs [100-102]. Subsequently, the immune attack, initially serving the purpose of host-defense, may, through molecular mimicry, be converted into a pathological immune reaction, which directly targets self-molecules.

Another concept follows the perception that autoimmune diseases arise from a divergence between the actual tissue state and the immune system's perception of the situation [38]. Autoimmune diseases, in such a case, would be marked by insufficient anti-autoimmune regulation against key self-molecules.

As for other autoimmune diseases, a genetic predisposition for SS seems to exist [103]. Analyses of HLA-DR and HLA-DQ gene segments in patients with SS, revealed an increased prevalence of haplotypes B, Drw52 and DR3 [104]. However, better correlations were found

between HLA-DR haplotypes and the presence of anti-Ro and anti-La than with disease severity or disease development [104]. Recently, a variant of the minor histocompatibility antigen HA-1 was associated with reduced risk of pSS [105]. In contrast, no association with polymorphisms in Fas and FasL [106], IL-10, TNF-α, IL-1 receptor antagonist (IL-1RA) [107], cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) [108], TNF-α R2 [109] and CCR7 [110] was observed.

The infectious agents which have received most attention in the field of SS are Epstein-Barr virus [100], human T-cell leukemia virus-1 [101] and hepatitis C virus (HCV) [102]. Association of SS with all these candidates remained, however, rather weak. Sialoadenitis, now considered as extrahepatic manifestation of chronic HCV infection, received some attention in the newly proposed AECG, in which HCV infection is listed as an illegibility criterion in clinical studies investigating SS [47]. A more recent study identified a Coxsackie virus as a potential agent involved in the induction or maintenance of SS in a Greek population [111]. However, the results could not be validated in a French patient cohort [112]. Konttinen *et al.* speculated that due to their anatomical localization and their short excretory ducts, the small exocrine glands should be considered as a *locus minoris resistentiae*, where apoptotic acinar cells may be unconventionally displayed to the immune system [113]. Interestingly, autoantigens associated with SS (Ro52, Ro60 and La48) are nuclear proteins, which are redistributed and exposed on the cellular surface during apoptosis [114].

Microarray-based investigations of the salivary gland tissue transcriptome in SS patients [115, 116] and in congenic mouse models of SS [117, 118] showed an activated type I and type II IFN system. Such activation, potentially originating from a viral infection, may be perpetuated by RNA-containing immune complexes, in their turn activating plasmacytoid DCs to prolong IFN-α production at the tissue level [119]. The finding that U1 snRNA and hY1RNA have the capacity to induce IFN-α further argues for the existence of such a vicious circle [120].

Female predominance and the late onset of SS directed the attention towards sex hormones and their role in the etiology of SS. In general, androgenic hormones have been considered to protect from autoimmunity and it has been proposed that women with SS are androgen deficient [121]. Whereas neither estrogen receptor-α nor estrogen receptor-β deficient mice develop SS, another model of estrogen deficiency, the aromatase-knockout (ArKO) mouse develops a lymphoproliferative autoimmune disease resembling SS [122]. Microchimerism of

fetal cells may also play a role in generating an autoimmune potential in women who have been pregnant [123].

Experiments in non-obese diabetic (NOD) mice, which exhibited severe combined immunodeficiency (SCID) [124] and inhibitor of DNA binding 3 (Id3) deficient mice [125] indicate a possible relationship between defective organ development and the initiation of pathological immune reaction targeting the salivary glands. Interestingly, in the latter model, depletion of B-cells ameliorated the SS-related symptoms [126].

The salivary glands in Sjögren's syndrome

A scheme of possible events taking place in the salivary glands of SS patients is proposed in (Figure 3). Primarily for diagnostic purposes, a salivary gland biopsy is evaluated for the presence and frequency of focal cellular aggregates, defined as clusters of at least 50 mononuclear cells [127].

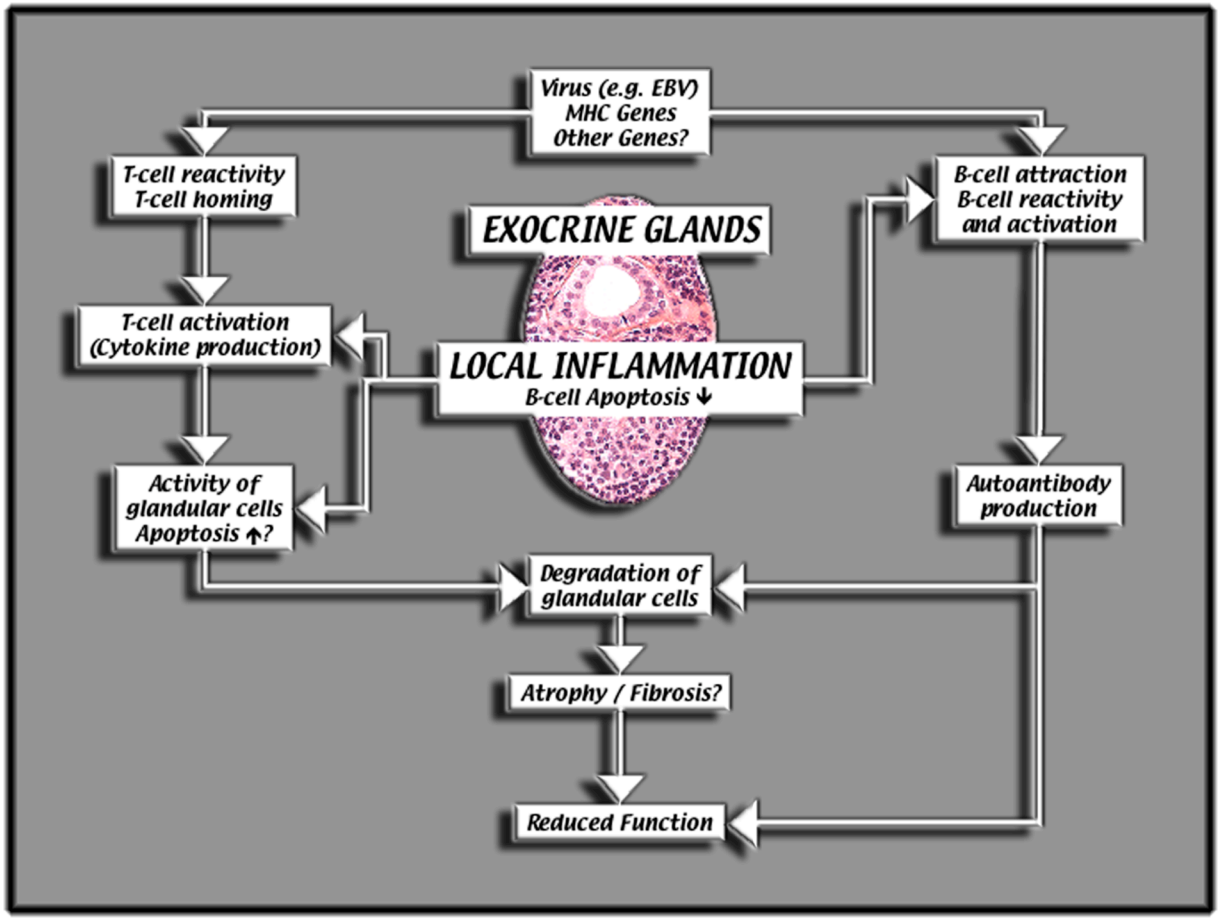


Figure 3) *Proposed scheme of events related to the initiation and pathogenesis of SS. Figure adapted from N. Delaleu et al. [84].*

The infiltrates consist of T-cells, B-cells, DCs [119, 128] and macrophages [129]. Similar infiltrates were also found in other organs *e.g.* the lacrimal glands, the lungs, the thyroid gland or the liver obtained from patients with SS [74]. Analyses of gene expression profiles in salivary gland tissue from SS patients confirmed the presence of many aspects of chronic inflammation [115, 116]. Recent studies reported that approximately one in four SS patients exhibit ectopic germinal center (GC)-like structures in the minor salivary glands, a feature which might be associated with a more severe disease [130]. To what extent GC-like structures in the salivary glands overtake processes normally assigned to GC in the secondary lymphoid tissue remains to be proven. Furthermore, GC-like structures have been associated with the occurrence of lymphoproliferative disorders in SS [131]. However, similar signs of ectopic lymphoid tissue formation and lymphoid neogenesis also occur in synovia from RA patients [132], in thyroid glands of Hashimoto's thyroiditis [133] and in gut mucosa in relation with infectious agents *e.g. Helicobacter pylori* [134].

Endothelial cells

Lymphocyte migration is partly mediated by vascular cell adhesion molecule (VCAM)-1 and peripheral node addressin (PNAd) expressed on vascular endothelium (Figure 4). Inhibition of these two molecules or their ligands (α4-integrin, L-selectin and lymphocyte function-associated antigen-1 [LFA-1]) showed a nearly complete block of lymphocyte migration into the lacrimal glands in NOD mice [135]. In contrast, mucosal addressin cell adhesion molecule-1, E-selectin or P-selectin did not seem to contribute to monocyte migration into lacrimal glands in NOD mice [135].

Epithelial cells

The glandular epithelium in SS seems to be activated [136, 137], resulting in local production of several pro-inflammatory cytokines [138]. In addition, the presence of chemokines in epithelial cells has also been reported [139, 140]. Furthermore, epithelial cells of SS patients also express TLRs, which argue for a role of these cells in the disease [141, 142]. Nevertheless, whether epithelial cell activation is an initiating event or a secondary phenomenon requires further investigation. Epithelial cells also express Fas, FasL and BCL-2 associated X protein (Bax), suggesting apoptosis as a mechanism of acinar cell destruction [143, 144]. However, despite aberrant expression of pro-apoptotic molecules, apoptosis might be a rare event in acinar and ductal cells of patients with SS [145]. Increased matrix metalloproteinase (MMP)-2, MMP-3 and MMP-9 activity was associated with changes in the

structural organization in the basal lamina and the apical surface of acini observed in patients with SS [146, 147].

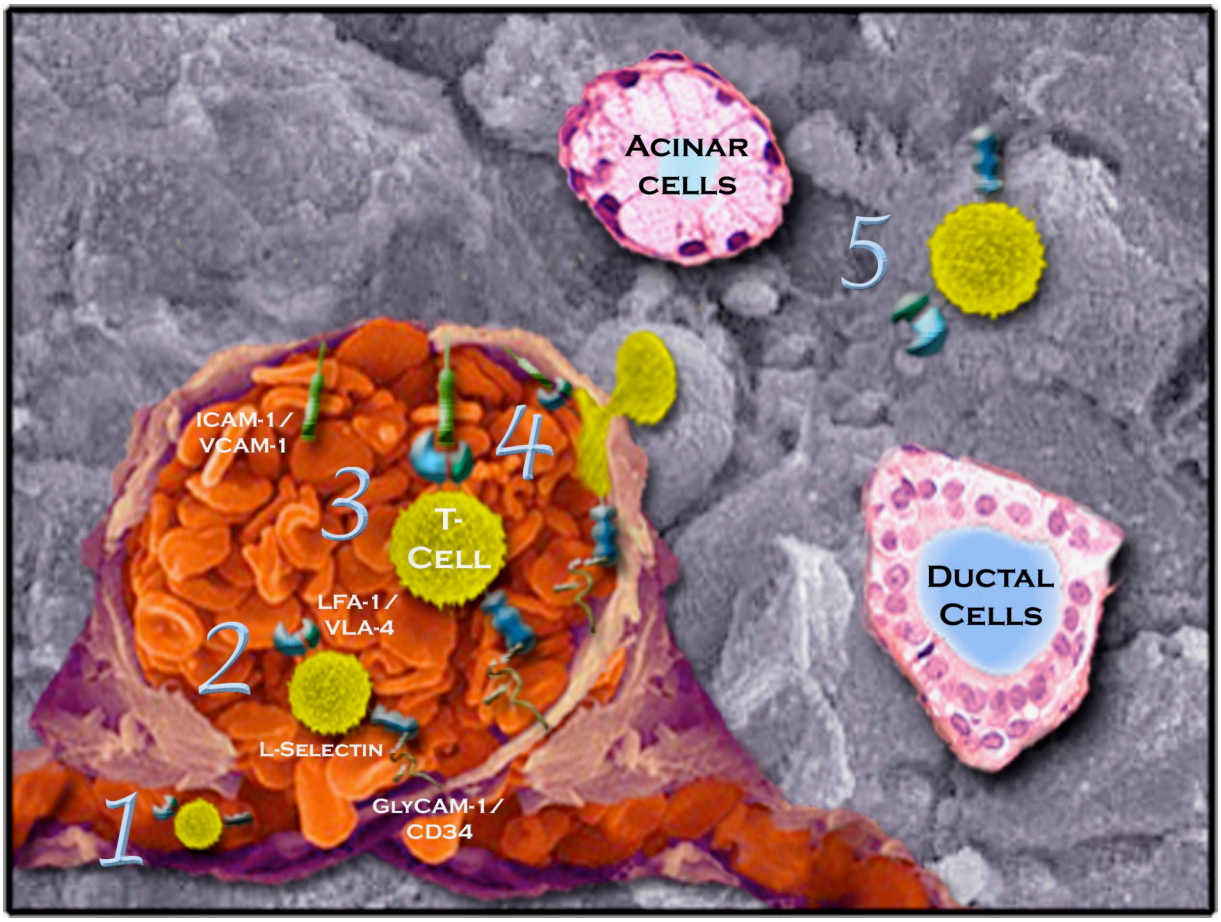


Figure 4) *An important feature in the regulation of lymphocyte recirculation is the ability of lymphocytes to recognize and bind to the surface of endothelial cells before migrating through the vessel into surrounding tissue. (1) Circulating lymphocytes enter high endothelial venules. (2) Initial transient tethering and rolling: most lymphocyte adhesion molecules such as L-selectin are found on the tips of the lymphocytes microvilli where they can easily contact the endothelium by binding glycosylation dependent cell adhesion molecule (GlyCAM)-1 or CD34. (3) Appropriate activating factors: chemokines such as CXCL13 encountered in the local environment render possible a first lymphocyte activation step which facilitates firm adhesion. Activated integrins LFA-1 and VLA-4 on lymphocytes interact with intercellular adhesion molecules (ICAMs) and VCAMs. (4) Lymphocyte diapedesis: migration through endothelium, from circulation to tissue, a process probably also directed by chemokines. (5) Migrated lymphocytes contribute to the local immune response. Figure adapted from N. Delaleu et al. [84].*

T-cells

Mononuclear cell infiltrates consist of up to 80% T-cells, representing a $CD4^+$ to $CD8^+$ T-cell ratio of more than two. Most $CD4^+$ T-cells are of a primed memory phenotype ($CD45R0^+$) and over 50% of all T-cells express CD40/CD40L [148]. In context with T-cell activation, HLA-DR and IL-2R were found on a large fraction of T-cells [136, 149]. Analyses of

complementarity-determining regions (CDR)3 showed some conserved amino acid motifs, suggesting a relatively limited number of recognized antigens [150]. High proportions of T-cells express Fas and/or members of the B-cell lymphoma 2 (BCL-2) family [151]. However, it was also reported that neither Fas/FasL induced apoptosis nor apoptosis implicating the Bax/Bcl-2 pathway occur more often among infiltrating mononuclear cells in SS compared to control individuals [145, 152]. Analyses of circulating T_{reg} population showed inconsistent results [153, 154], whereas in numbers they may be underrepresented in the salivary glands [154].

Cytokines and chemokines in Sjögren's syndrome

As crucial modulators of the immune system, cytokines represent potential drug targets. The therapeutic impact of agents neutralizing the pro-inflammatory cytokine TNF-α in patients with RA is indeed remarkable [48] and proves the coherence of this rationale. Cytokine profiles have been studied in blood and salivary gland tissues from patients with SS [138, 155, 156] and in mouse models for SS [157]. However, because cytokines are characterized by redundancy, pleiotropism, synergism, and antagonism, the interpretation of cytokine-related data is often difficult. Applying the by now expanded concept of T_H1/T_H2 cytokines and autoimmunity, it has been postulated that T_H2 cytokines may be predominant in an early phase of SS, while T_H1 cytokines would be associated with a later stage of the disease [158]. In opposition stands the proposed principle, that the decrease in salivary flow, which is thought to follow the emergence of glandular inflammation [159], might be associated with T_H2 cytokines [160-162]. In NOD mice the development of sialoadenitis and hyposalivation seems to critically depend on IFN-γ [163], whereas transition between the pre-clinical and the overt disease state, manifested by the onset of hyposalivation, has been associated with IL-4 [160, 161] and the STAT6 pathway [162]. The revision of the T_H1/T_H2 paradigm was caused by the emergence of a T-helper cell subset termed T_H17, producing IL-17 (IL-17A), IL-17F and IL-22 [15]. A major role of IL-17 has been described in various models of immune mediated tissue injury and autoimmune diseases, *e.g.* RA [15]. Interestingly, a recent study, also reported an activated T_H17/IL-23 system in patients with SS [164].

Chemokines are small secreted proteins implicated in leukocyte chemoattraction, angiogenesis, fibrosis and malignancy [13]. Despite their uncontested potential as candidates for therapeutic intervention therapies, few studies examined chemokines in SS [155]. The chemokines CXCL13, CXCL12 and CCL21 were expressed in acinar and ductal epithelium

cells from patients with pSS and sSS [165]. However, in NOD mice injection of anti-CXCL12 antibodies was effective in preventing diabetes and insulitis, but did not affect the SS-like disease [166].

Generation of the patient's individual cytokine and chemokine profile, using a bead-based multiplex immunoassay, revealed biomarker signatures in serum potentially indicating the presence or absence of GC-like structures in the salivary glands [155]. Among 25 biomarkers, discriminant function analysis identified BAFF, CCL11 and IFN-γ levels to discriminate best between patients with and without GC-like structure formation [155].

B-cells

Phenotypical analyses of B-cells from patients with SS revealed decreased numbers of circulating $CD27^+$ memory B-cells [167, 168], which selectively overexpressed CXCR3 and CXCR4 [169]. In contrast, the fraction of $CD27^+$ memory B-cells within the glands was enlarged [168]. Extensive analyses of mature B-cell subsets (Bm1-Bm5) in blood further showed altered proportions of most mature B-cell subsets in pSS compared to healthy donors and patients with RA [170].

The percentage of B-cells expressing mutated V(H) genes was significantly higher in B-cells isolated from parotid glands compared to B-cells in circulation [171]. Furthermore, V(L) gene analysis of B-cells isolated from the glands revealed biased usage of V(L) chain genes [172]. However, if these alterations result from disturbed B-cell maturation and abnormal selection processes or if the biased repertoire reflects a normal antigen-driven local immune response is unclear. Polyclonal B-cell activation may develop into an oligo- or monoclonal B-cell expansion during disease progression. Such expansion may provide a basis for the initiation of a malignant lymphoproliferative disease [173, 174].

B-cell activating factor

BAFF, a relatively new member of the TNF superfamily, is regulated by IFN-γ and has received considerable attention in the field of SS after it was reported that mice transgenic for BAFF develop a secondary pathology reminiscent of SS [175]. Closer investigation of the BAFF system in the field of B-cell biology demonstrated the need of an obligate survival signal for both, maturing and fully differentiated B-cells [11]. Taken together, BAFF is suggested to lower the threshold required for B-cell survival what may allow autoreactive B-cells to escape from apoptosis and to exhibit their autoimmune potential [11].

In patients with SS, elevated levels of circulating BAFF were reported to correlate with increased autoantibody titers [176]. Increased BAFF expression was furthermore observed within salivary glands of patients with SS [177], together with a significantly lower rate of apoptosis among the BAFF expressing cells [178]. BAFF levels were also increased after the discontinuation of an anti-CD20 therapy what may promote the reemergence of autoreactive B-cells [179]. Counteracting the BAFF triggered repopulation of the periphery with pathogenic autoreactive B-cells may therefore further improve the success of B-cell depletion in SS [97].

Autoantibodies in Sjögren's syndrome

Patients with pSS often present increased levels of polyclonal IgG in the serum [74]. Augmented production of IgM and IgG compared to IgA was further detected within labial salivary glands from patients with SS [180]. Autoantibodies specific for Ro (SSA) and La (SSB) are associated with SS and of major importance in SS diagnosis and patient classification [47]. 60-80% of the patients present anti-Ro and 40-60% anti-La antibodies in the serum [74]. Studies have shown that reciprocal spreading to the Ro52, Ro60 and La polypeptides occurs following immunization with a single component [181, 182]. Intermolecular epitope spreading suggests little tolerance to Ro and La in the B-cell and the T-cell compartment [183, 184]. Even though the role of Ro and La in the pathogenesis of SS is still elusive, recent research efforts reanimated the discussion, if Ro52 might directly contribute to the induction of autoimmune T- and B-cells in SS [185]. In addition, immunization of Balb/c mice with specific Ro peptides recapitulated the serological pattern, pathological findings and salivary gland dysfunction of SS [186].

Rheumatoid factor (RF) is produced in approximately 60% of the patients [187], whereas anti-cyclic citrullinated peptide (CCP) antibodies are rarely found in patients with SS [188]. Other antibodies found in patients with SS are anti-α-fodrin [189, 190] and anti-phospholipid antibodies [191]. The latter antibody could not be associated with any of the typical clinical manifestations of SS [192] and anti-α-fodrin autoantibodies are a controversial issue, since the original findings were difficult to verify [193].

Despite sizable research especially on anti-Ro and anti-La, the role of autoantibodies in the pathogenesis of SS remains ambiguous. Additional research efforts are necessary to reveal, if autoantibodies may be more than just bystanders of a T-cell mediated autoimmune disease as it has been proposed initially. Research initiatives, which identified autoantibodies inhibiting

neuronal innervation of acinar cells in the process of saliva secretion, are considered a major advance and these studies assigned for the first time a specific pathogenic role to an autoantibody in SS [194, 195].

Mechanisms mediating salivary gland dysfunction

The original model to explain impaired salivary secretion in SS suggested the loss of glandular epithelial cells, secondary to apoptosis and T-cell mediated cell death, as the main reason for hyposalivation [196]. However, the frequently observed lack of correlation between the amount of destroyed glandular tissue and the often disproportional decrease in salivary flow [197] is difficult to fit into this model. In addition, glandular tissue from SS patients retains a certain functionality when tested *in vitro* [198]. Subsequent studies investigated immune-mediated mechanisms, which could cause chronic inhibition of the secretory function prior to glandular destruction and atrophy (Figure 5). A model of SS pathogenesis comprising mechanisms of glandular dysfunction [76] is further supported by the observation that certain mouse strains retain full secretory function despite severe glandular inflammation [199].

Acinar cell innervation and humoral immunity

The secretion of water and electrolytes from acinar cells is directly induced by acetylcholine and substance P released by the innervating parasympathic nerve ends [200]. In the salivary glands M1R and M3R are the predominant acetylcholine receptor subtypes, whereas M3 is predominant in the lacrimal glands [201]. Moreover, experiments in knockout mice revealed the crucial role of the M3R in the process of saliva secretion [202].

NOD Igμ^{null} mice lack functional B-cells and despite the focal appearance of T-cells in the salivary glands NOD Igμ^{null} mice failed to develop hyposalivation [194]. Reversible induction of hyposalivation through serum transfer from old NOD mice and IgG fractions from SS patients in these mice [194] further supports the notion of a contribution of serum components to the pathogenesis of SS. Functional analyses demonstrated that IgG fractions from patients with pSS reduced the carbachol-evoked increase in $Ca2^{+}$ in murine and human acinar cells [195]. Due to the laborious method, however, both tests described above may be inadequate to screen large patient cohorts. The use of M3R tranfectants in combination with flow cytometry [203] or the application of newly developed enzyme-linked immunosorbant assays (ELISAs)

[204], may represent an alternative to further investigate the role and diagnostic value of anti-M3R antibodies in SS.

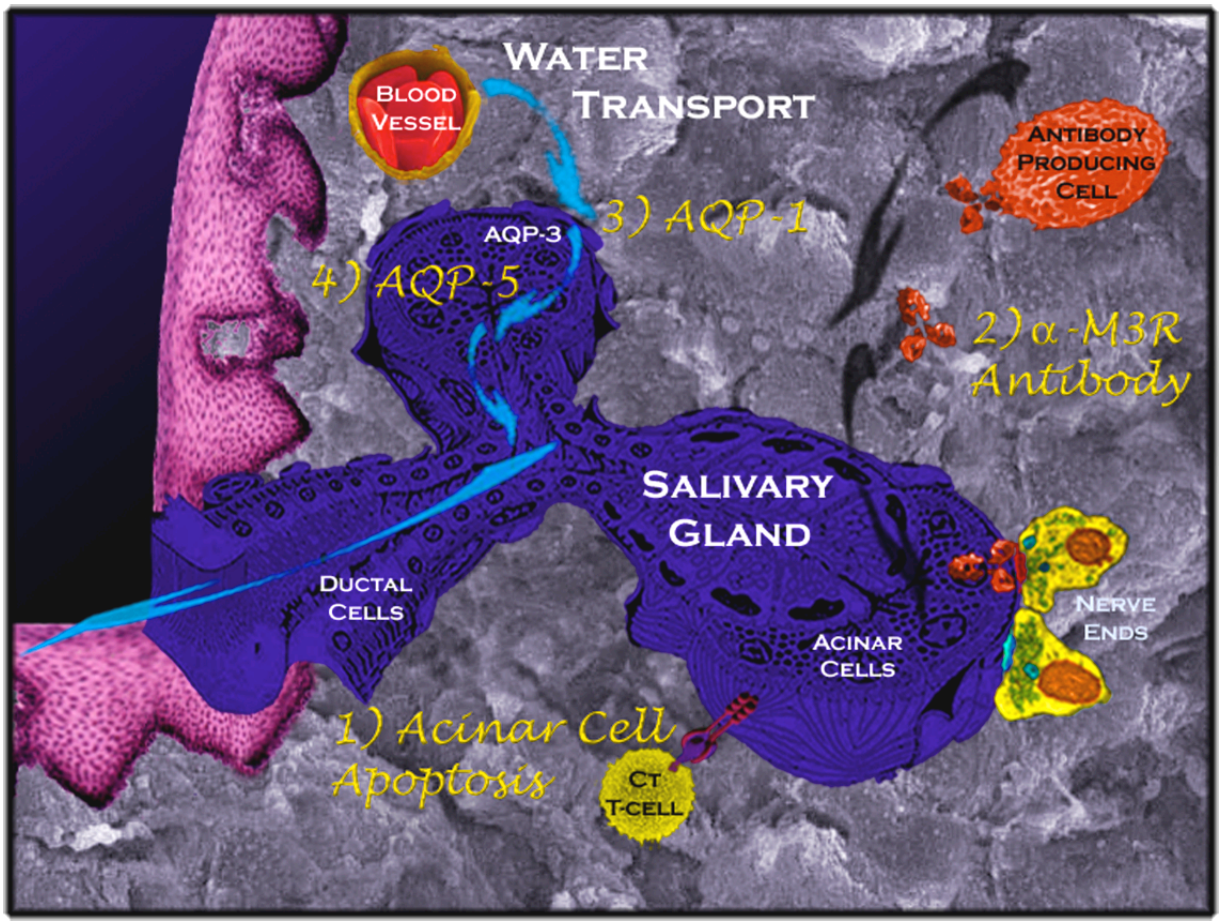


Figure 5) *Proposed mechanisms mediating glandular destruction and dysfunction in SS. (1) Altered apoptosis of glandular epithelial cells. (2) Antibodies targeting the M3R may directly be related to impairment of the salivary glands by inhibiting neuronal innervation of acinar cells. (3) Aquaporins (AQPs) are suggested to play a major role in water transport through cell membranes. AQP-1 expression was decreased in myoepithelial cell from patients with SS. (4) AQP-5 expression may be decreased in acinar cells from patients with SS. Figure adapted from N. Delaleu et al. [73].*

Acetylcholine ligation to M3R has also been shown to protect cells from apoptosis *in vitro* [205]. Inhibition of anti-apoptotic effects mediated trough M3R signaling could potentially link functional quiescence with cellular destruction. In addition, it should be taken into account that mononuclear cells express muscarinic receptors, secrete acetylcholine and encode the synthesizing choline acetyltransferase and the degrading acetylcholine esterase [30].

Aquaporins

Aquaporins (AQP) are transmembrane proteins, which form channels in the cell membrane and increase the water permeability of the lipid bi-layer by up to 100 fold [206]. Different isoforms exist and show specific cellular and subcellular distributions in salivary and lacrimal glands. AQP-5 is located in the apical membrane of acinar cells, AQP-3 is present within the basal membrane of acinar cells and AQP-1 is expressed on myoepithelial cells [207]. Monoclonal anti-M3R antibodies had the potential to prevent translocation of AQPs to the plasma membrane [208]. Since then, AQPs have been suspected to contribute to the loss of exocrine gland secretory function in SS. Mice deficient of AQP-5 exhibit an approximate decrease of 65% in salivary secretion capacity compared to wild-type mice [209]. However, analysis of AQP-5 distribution within the glandular tissue from SS patients showed controversial results [210, 211]. AQP-1 expression was decreased by 38% in salivary glands of patients with SS suggesting a possible insufficient AQP-1 dependent water flow across myoepithelial cells [212].

Inflammatory mediators

Cytokines such as IL-1β may disturb the process of saliva secretion by inhibiting the release of acetylcholine from cholinergic nerves [213, 214]. Evidence against interferences of such kind was, however, presented recently. The study showed no relation between concanavalin A-induced cytokine production and acetylcholine evoked Ca^{2+} mobilization [215].

Model organisms in Sjögren's syndrome research

A model organism is a species, which can be studied extensively to explain a biological phenomenon observed in another species. One thereby anticipates that discoveries made in the model organism can provide information about the organism of primary interest. Model organisms are chosen based for several traits such as genetic background, possibilities for experimental and genetic manipulation, size, generation time, accessibility and potential economical benefit. In immunological research, the classical model vertebrate, the mouse, is the most widely studied.

The use of murine models in SS research allows to follow the progression of the disease in a controlled environment [199]. Genetically altered strains enable the close investigation of a specific protein, a certain cell type or a certain mechanism and how it may contribute to the aspects of the disease. Furthermore, studying potential treatments regarding their capacity to modulate SS may lead to better therapies in the future.

Ideally, a model for SS should present a high disease penetrance rate and exhibit clinical, histopathological and immunological features of the human disease.

Spontaneous models of Sjögren's syndrome

Mouse strains which naturally develop an autoimmune condition resembling SS were first described in 1968 [216]. Subsequently, numerous studies confirmed the existence of focal inflammation within the lacrimal and salivary glands from strains traditionally studied for SLE. These include NZB, NZB/NZW [216] and the MRL mouse with its substrains [217]. Later a SS-like disease has also been described in NOD mice [218], NFS/*sld* mice [219] and IQI/Jic mice [220]. The fact that these models develop SS spontaneously mimics the complex situation found in patients. However, such complexity is challenging and may often hinder the formulation of clear-cut answers regarding the contribution of a single process. Nevertheless, the overall functioning principles cannot easily be predicted by studying the properties of its isolated components because they may strongly rely on each other or arise from the interaction of numerous constituents.

NZB and NZB/NZW F1 mice

In NZB/NZW F1 mice, glandular inflammation is more pronounced in females compared to males, whereas this phenomenon is generally less apparent in NZB mice [216, 221]. These

reports established the NZB/NZW F1 mouse as a model of progressive focal sialoadenitis [222, 223], in which, however, glandular function and the presence of SS related autoantibodies have not yet been explored.

MRL/+ and MRL/*lpr* mice

In 1982 MRL mice were first reported to present periductal lymphoid infiltrates in the salivary glands [224]. Subsequently, a system to quantify the inflammation in the salivary glands was introduced [225]. Like in humans, aberrant antigen presentation by glandular ductal epithelium in close proximity to lymphoid infiltrates is thought to perpetuate activation of $CD4^+$ T-cells. MRL/+ and MRL/*lpr* mice differ with respect to a mutation involving the Fas gene [226]. However, negative selection in thymus does not seem to be impaired [227] and despite a defective genetic background, MRL/*lpr* mice express a detectable amount of apoptosis-related Fas protein on lymphoid cells [228]. Nevertheless, defective apoptosis related to the /*lpr* mutation [228] resulted in increased susceptibility and severity of the disease, most likely through acceleration of the disease course.

Immunohistochemical analyses suggested local activation of T-cells [225, 229] and their importance was further confirmed in T-cell transfer experiments [230]. Inflammatory lesions in autoimmune MRL/*lpr* mice are sites of local IFN-γ production [229] and B-cells produced IgA and IgM RF in salivary glands [231].

A differentiated, age-related pattern of cytokines has been suggested to influence the development and progression of the autoimmune sialoadenitis in MRL/*lpr* mice. IL-1β and TNF gene transcripts were detected in the salivary glands before the onset of sialoadenitis, whereas IL-6 mRNA expression was detected at the time of onset [232]. Furthermore, perpetuation of autoimmune sialoadenitis in MRL/*lpr* mice was described in conjunction with low expression of TGF-β and IL-10 [233].

Nevertheless, despite female predominance and the rare occurrence of anti-Ro, the clinical hallmark of SS, hyposalivation, is absent in this model.

NFS/*sld* mice

The NFS/*sld* mouse provides a model for pSS, in which aberrant immune responses against α-fodrin are facilitated [219]. A defect in salivary gland development leads to the cleavage of the structural protein fodrin by caspase. Subsequent to neonatal thymectomy, 3d-Tx NFS/*sld*

mice develop T-cell dominated infiltrates in the salivary and lacrimal glands. Sera from patients with SS were suggested to contain antibodies specific for fodrin 125kDa [189], however, the specificity of these autoantibodies for SS was not as high as originally thought [193]. In addition, secondary to the SS-like disease, 3d-Tx NFS/*sld* mice developed severe autoimmune lesions involving arthritis and interstitial pneumonia, as they aged [234].

IQI/Jic mice

Moreover, IQI/Jic mice have been suggested as an animal model for SS [220]. Focal infiltration of lymphocytes with parenchymal destruction was noted in the lacrimal and salivary glands. Sialoadenitis progressed over time and seemed more prominent in females than in males. IQI/Jic mice also developed inflammatory lesions in multiple organs, such as the lungs, pancreas and kidney [235]. Kallikrein-13 (Klk-13) has recently been suggested to play a role as an autoantigen in the disease development in IQI/Jic mice [236].

Non-obese diabetic mice

The NOD strain descends from a cataract-prone strain of outbred Jcl:ICR mice and is considered the best characterized model for SS and IDDM. Although some genetic loci related to diabetes (idd-loci) may contribute to the inflammatory changes in the exocrine glands, it seems that diabetes and SS develop independently from each other [237]. Diabetes in NOD mice is restricted to the expression of MHC I-Ag7, whereas NOD.B10.H2b mice, which are resistant against diabetes, still exhibit sialoadenitis and hyposalivation [238]. Nevertheless, these mice still presented perivascular inflammatory infiltrates and in some cases insulitis [239]. In addition, the extent and cellular composition of the glandular inflammation still remains to be explored.

NOD mice in which the MHC H2g7 haplotype was exchanged for an H2q or H2p haplotype were also investigated [240]. In summary, the exchange of the H2 haplotype did not affect the frequency of sialoadenitis, although disease severity varied among the strains. In contrast, the development of insulitis was almost completely inhibited [240]. Interestingly, introduction of the H2q haplotype directed the autoimmune response towards production of lupus-types of autoantibodies and a higher incidence of kidney involvement. The latter phenomenon was also reported in relation with immunomodulatory treatments, capable of preventing diabetes in NOD mice [241].

Nonetheless, the parental NOD strain remains the best characterized model for SS and represents a complex model involving genetics, sensitivity to exogenous factors and defects in central and peripheral tolerance [242]. The extensive general and diabetes related knowledge about the immune system of the NOD mouse might benefit SS-related research in the future. Intrinsic alteration in immune tolerance may further contribute to the reported susceptibility of the strain to develop autoimmune thyroiditis [243], SLE [241], myasthenia gravis [244] and autoimmune encephalomyelitis [242] as a result of specific manipulations.

Focal lymphoid infiltration in the submandibular salivary glands can be observed from approximately 8 weeks of age onwards and they are similar in structure and cellular composition as the infiltrates in humans [199]. In NOD mice, alike in humans, chronological order of glandular inflammation, glandular dysfunction and glandular destruction, and the relation between histopathological manifestations and hyposalivation, still remain matters of speculation.

In diabetes, several lines of evidence indicate that progression from early insulitis to overt diabetes is promoted by the loss of immunoregulatory cells, such as T_{regs} and invariant NK T-cells within the islets [43]. Unfortunately, little is known about the role of such cells in the progression of SS. However, NOD mice deficient for E2F transcription factor 1 (E2f1), a regulator of T-cell proliferation, differentiation, and apoptosis, have a profound decrease in $CD4^{+}CD25^{+}$ T_{regs} and seem highly predisposed to IDDM and SS [245].

Another possible link between SS and IDDM in NOD mice may involve common autoantigens. Disruption of the islet cell autoantigen 69kDa (ICA69) gene in NOD mice, a self-antigen associated with diabetes, which is expressed in the pancreas, but also in the exocrine glands, greatly reduced SS-related histopathology and glandular hypofunction [246]. T- and B-cell autoreactivity to ICA69 was detected in patients with pSS. In addition, immunotherapy, using a high-affinity peptide targeting ICA69-specific T-cells, reduced overt SS in wild-type NOD mice. However, a study based on a larger cohort of patients failed to confirm a role or true frequency of ICA69 autoimmunity in human SS [247].

In contrast to diabetes, experiments in NOD $Ig\mu^{null}$ mice revealed no crucial role for B-cells in the initiation phase of SS. Instead, the lack of functional B-cells circumvents the onset of hyposalivation in these mice [194]. Similar to SS in humans, AQP-5 may contribute to the reduced saliva secretion observed in NOD mice [248].

Protection from IDDM in NOD mice has been associated with a shift from a T_H1 to a T_H2 cytokine expression profile in β-cell specific autoreactive T-cells, which were observed as a result of appropriate treatment or genetic modification [43]. Subsequent studies indicated, however, that compartmentalization into disease promoting T_H1 and protective T_H2 cytokines may represent an oversimplification, which cannot always be applied to the pathogenesis of IDDM [249].

Cytokines in the exocrine glands in NOD mice have been analyzed [250, 251] and local expression of IL-10 and IL-12 mRNA during the disease was reported. In addition, the presence of IL-1β, IL-2, IFN-γ and TNF-α was reported and IL-10 mRNA expression was associated with detectable expression of IL-2 and IFN-γ [250, 251]. The roles of T_H1 and T_H2 cytokines in the pathogenesis of SS were assessed in NOD IL-$4^{-/-}$[161], NOD.B10-H2b $IL4^{-/-}$ [160], NOD.B10-H2(b).C $STAT6^{-/-}$ [162], NOD IFN-$\gamma^{-/-}$ and NOD IFN-γ $R^{-/-}$ [163] mice. IL-$4^{-/-}$ alike $STAT6^{-/-}$ deficient NOD mice retained salivary secretion rates similar to Balb/c mice and failed to produce M3R antibodies of the IgG_1 isotype, despite the development of sialoadenitis [160, 162]. NOD IFN-$\gamma^{-/-}$ and NOD IFN-γ $R^{-/-}$ mice did not develop inflammation in the salivary glands and retained normal salivary secretion capacity, while the inflammation within the lacrimal glands persisted [163]. Interestingly, the strains defective for IFN-γ or IFN-γ R did no longer exhibit the alterations in glandular organogenesis, which were reported in the parental strain [163].

Congenic mice, in which NOD specific genetic loci were introduced on either a B6 [237] or a B10 [117] background, were generated to investigate the importance of specific gene regions in SS. Both strains were extensively analyzed regarding changes in gene expression [117, 118]. Especially the C57BL/6.NOD-Aec1Aec2 strain may represent a valuable mouse model for SS [118, 164, 237, 252]. Nevertheless, the background strain C57BL/6 develops spontaneous organ-specific autoimmune lesions in the salivary glands and multiple other organs and produces a wide variety of autoantibodies [253]. These phenomena render it more difficult to draw conclusions regarding disease causing and disease promoting gene segments. Similar accounts for B10 mice, which, independently from genetic manipulation, spontaneously develop sialoadenitis [117]. Unfortunately this latter model has not yet been assessed for salivary gland hypofunction [117].

Transgenic and knock-out models of Sjögren's syndrome

Cytokines

By promoting growth and effectiveness of T_{regs}, IL-2 plays a crucial role in the maintenance of immunological self-tolerance. Inhibition of circulating IL-2 led to the aggravation of diverse autoimmune manifestations in NOD mice [243]. IL-2 and IL-2Rα deficient C57BL/6 mice develop sialoadenitis, hyposalivation and histopathological manifestations related to other autoimmune diseases [254]. In conclusion, the impact of IL-2 indicates that in conditions with decreased regulatory cell populations the salivary glands are prone to exhibit autoimmune manifestations.

C57BL/6 mice transgenic for IL-10 exhibit progressive histopathology and hyposalivation evocative of SS [255]. However, IL-10 gene transfer into NOD mice partially suppressed the appearance of SS-like features [256], what indicates a dual role of IL-10 in SS.

Two research groups developed mice transgenic for BAFF, of which one has been assessed for SS pathogenesis. Both strains showed severe autoimmune manifestations resembling SLE including circulating immune complexes, anti-DNA antibodies, and immunoglobulin deposition in the kidneys [257, 258]. The strain investigated for SS presented lymphoid infiltrates in the salivary glands together with an approximate 70% decrease in salivary secretion capacity [175]. Major proportions of the infiltrating cells were classified as B-cells displaying a marginal zone (MZ)-like phenotype, which appeared to be crucial for the development of the SS-like disease in this strain [259]. Smaller populations were classified as B-1 cells [175]. Disruption of the TNF-α gene in BAFF transgenic mice revealed a crucial role of the anti-tumor activity of TNF-α in this model [260].

A resent study investigated the role of IL-14α, in the pathogenesis of SS [261]. IL-14α overexpression in C57BL/6 mice was accompanied by increased numbers of B-1 cells, followed by sialoadenitis and the development of $CD5^+$ B-cell lymphoma. Unfortunately, salivary gland function was not evaluated and SS related antibodies were not found. In addition to SS-like features, IL-14α transgenic mice exhibit immune-complex mediated nephritis [261].

TGF-ß1 is a multifunctional molecule, which has regulatory effects on many developmental, physiological and immunological processes. Animals homozygous for the mutated TGF-ß1 allele present a syndrome marked by mixed inflammatory cell responses and tissue necrosis

leading to organ failure and death [262]. The syndrome also includes inflammation of the exocrine glands, in about 50% of the animals. The inflammation could be prevented by systemic injections of synthetic fibronectin peptides and subsequent reversal of the acinar and ductal alterations suggest that salivary gland development is not impaired in the absence of TGF-β1 [263].

Id3$^{-/-}$ knock-out mice

Id proteins are inhibitors of basic helix-loop-helix transcription factors and act as positive and negative regulators of proliferation and differentiation of immune and non-immune cells, both in embryonic and developed tissue [264]. The immune system of Id3$^{-/-}$ mice is characterized by alteration in humoral immune reactions, MZ B-cell development, B-cell precursor survival and both MHC I and MHC II restricted positive and negative selection [265]. Tissues such as salivary glands, which undergo epithelial-mesenchymal interactions, seem to have a tendency to express Id genes. Id3 is, furthermore, implicated in angiogenesis in normal and tumor tissue [264, 266]. In the Id3$^{-/-}$ model, T-cell dominated focal inflammation developed between 6 and 12 months, together with production of anti-Ro and anti-La. These mice, however, showed severe exocrine gland dysfunction at 6 to 18 weeks already, long before the appearance of focal mononuclear cell infiltration in the exocrine glands [125]. Nevertheless, the notion that a single gene disruption triggers distinct pathological changes, almost exclusively limited to the salivary and lacrimal glands, encourages the investigation of possible interrelationships between organ development and the initiation of autoimmune diseases. Interestingly, depletion of B-cells ameliorated the disease symptoms of SS in these mice [126].

Aromatase-deficient mice

Estrogen is a crucial factor regulating gender differences of the immune system. The reported effects of estrogen are, however, contradictory [267, 268]. Whereas neither estrogen receptor-α nor estrogen receptor-β deficient mice develop SS, another model for estrogen deficiency, ArKO mice develop a lymphoproliferative autoimmune disease resembling SS in parallel with B-cell infiltration in the kidneys and enlargement of the spleen [122].

R1Δ/R2n mice

Mice with a T-cell specific loss of phosphoinositide 3-kinase class IA (PI3K Ia) develop inflammation resembling SS in addition to lymphoid infiltration in the lungs, liver and intestines [269]. Whereas secretory function was not addressed in detail, the authors reported

decreased T_{regs} in the periphery and increased anti-Ro and anti-La antibodies as a result of the genetic modification.

Models of experimentally induced Sjögren's syndrome

Carbonic anhydrase

Patients with autoimmune diseases including patients with SS may produce autoantibodies against carbonic anhydrase II (CAII) [270]. Indeed, experimental sialoadenitis was induced through CAII immunization in PL/J mice [271]. PL/J mice with different H-2 haplotypes, bearing H-2s and H-2u, were also susceptible to CAII-induced sialoadenitis [271]. However, further studies are required to be able to estimate the resemblance of this model with SS in humans.

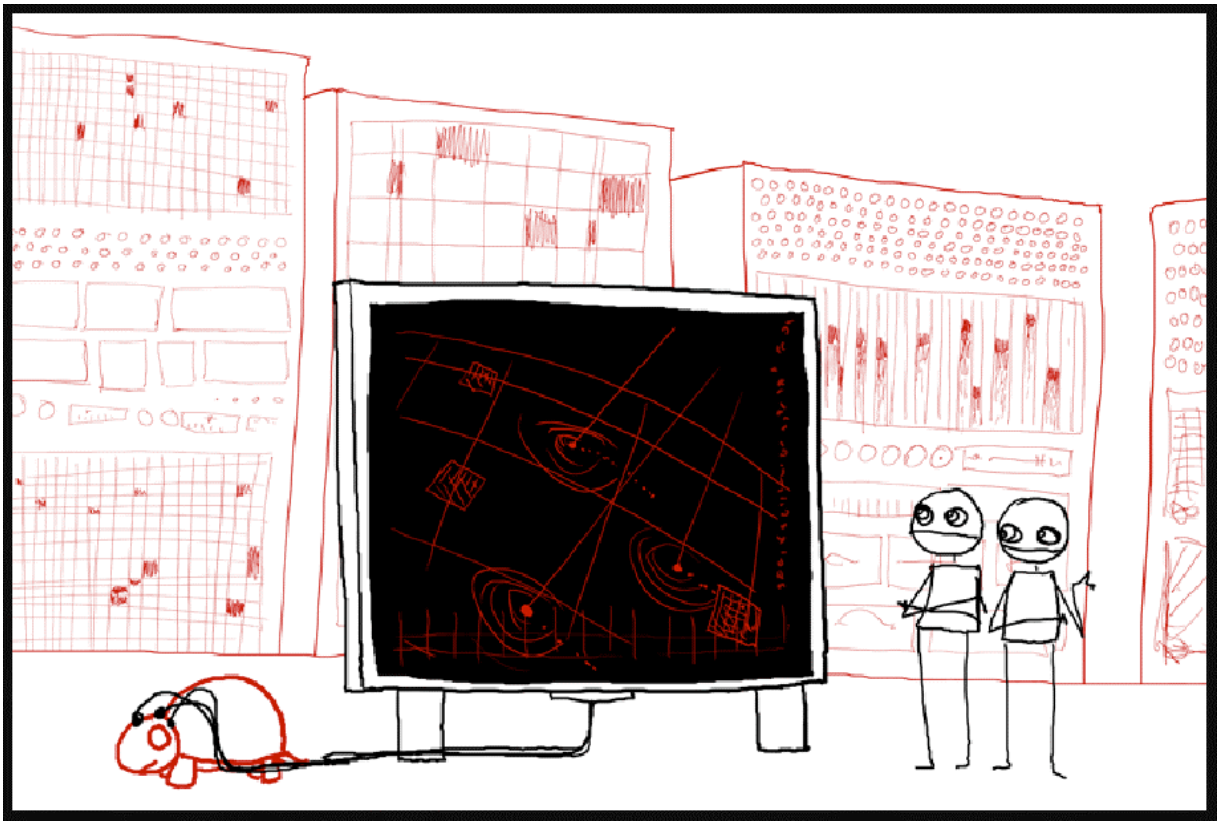
Ro peptide

Repeated intraperitoneal injection of Ro peptides emulsified in complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA) recapitulated SS in Balb/c mice [186]. These mice showed decreased salivary gland function, SS-like histopathology and production of anti-Ro and anti-La at 38 weeks of age [186]. Oral feeding of Ro or Ro peptide could, in addition, partly prevent the induction of the SS-like disease through the immunization protocol described above [45]. These findings reanimate the discussion, whether autoreactivity towards Ro, due to molecular mimicry or aberrant expression, may play a role in the etiology of SS.

Murine cytomegalovirus

To elaborate a possible viral etiology of SS, four different murine strains (B6, Fas-deficient B6-lpr/lpr, B6-tnfr1$^{-/-}$, and B6-tnfr1$^{-/-}$-lpr/lpr) were infected intraperitoneally with murine cytomegalovirus (MCMV) [272]. Beside sialoadenitis, B6-lpr/lpr mice, developed anti-Ro and anti-La antibodies. Apoptotic cells were detected during the acute, but not during the chronic phase of the inflammation. Both Fas- and TNF RI-mediated apoptosis seemed to contribute to the clearance of MCMV infected cells in salivary glands. Indeed, such defects may lead to a post-infectious, chronic inflammation resembling the histopathology of SS. In a subsequent study, FasL mutant B6-gld/gld mice were infected with MCMV to evaluate the therapeutic value of FasL expression by local gene transfer using recombinant adenoviral vectors [273]. Despite high levels of FasL expression after injection of recombinant vectors,

less than 5% of ductal and acinar cells were TdT-mediated dUTP-biotin nick end labeling (TUNEL) positive. Nevertheless, a significant reduction in the number of inflammatory foci and tissue destruction in salivary glands was observed as a result of local FasL gene transfer [273].



None of this makes sense; Illustration by Sam Brown (www.explodingdog.com)

Aim of the studies

Paper I

The aim of the study was to characterize the chronology of the disease course of SS in NOD mice and to study possible interrelationships between salivary gland inflammation and hyposalivation. In addition, ten cytokines were analyzed in saliva and serum to identify alterations in cytokine patters associated with a specific disease stage.

Paper II

The aim of the study was to expand the knowledge regarding 87 analytes in serum and 75 proteins in saliva. Based on direct comparison with Balb/c mice we intended to identify differentially expressed proteins and investigate their potential to discriminate between the disease model and the control strain. This pool of data should also allow computation of a correlation network, representing associations of biomarkers with relevant clinical features of SS in non-diabetic NOD mice, both systemically and locally.

Paper III

The aim of the study was to investigate a potential immunomodulatory effect of Hsp60 and aa437-460 on spontaneous experimental SS. Comprehensive biomarker profiles were generated to identify biomarker signatures related to the treatment, treatment efficacy and exocrine function.

Material and Methods

Mice (Paper I-III)

Animals studied in *Paper I*, female NOD and Balb/c mice, were purchased from Taconic (Bomholtgård, Denmark). The animals were maintained under standard animal-housing conditions at the Section for Physiology, Department of Biomedicine, University of Bergen, Norway. In *Paper II & III*, female NOD/LtJ (stock #001976) and Balb/cJ (stock #000651) mice (The Jackson Laboratory, Bar Harbor, ME, USA) were housed in sterilized individually ventilated cages at the same animal facility alluded to above. Mice were fed with autoclaved RM1 pellets, (Special Diet Service, Witham, UK). All handling procedures were carried out in a laminar flow hood. All studies have been approved by the Committee for research on animals/Forsøksdyrutvalget, Project #79-04/BBB and Project #12-05/BBB, respectively.

Immunization (Paper II & III)

In *Paper II*, at 7 weeks of age, 22 NOD mice and 20 Balb/c mice were injected subcutaneously with 25 μl IFA emulsified in PBS. In *Paper III*, additional 32 female NOD mice (16/group) were immunized subcutaneously with 50 μl of one of the following agents emulsified 1:1 in IFA: 50 μg low-endotoxin (< 0.05 endotoxin units/μg) human recombinant Hsp60 (#ESP-540G; Stressgen, Ann Arbor, MI, USA) [274] or 100 μg of aa437-460 (VLGGGVALLRVIPALDSLTPANED) synthesized using 9*H*-fluoren-9-ylmethoxycarbonyl based solid-phase peptide synthesis (Fmoc SPPS) at Invitrogen (Carlsbad, CA, USA). The peptide was purified using high-performance liquid chromatography (HPLC) to ensure > 95% purity.

Assessment of diabetes (Paper I-III)

In *Paper I*, serum glucose levels were measured the day of euthanasia using Reflotron Plus Glucose test kit (Roche Diagnostics, Laval, Quebec, Canada). In *Paper II & III*, from 10 weeks onwards NOD mice were screened weekly for diabetes. Two repetitive measurements of glucosuria (Figure 6) (> 50 mg/dl; Keto-Diabur-Test strips, Roche, Mannheim; Germany) were considered to represent onset of diabetes and confirmed by measurement of blood glucose levels (> 300 mg/dl; Ascensia microfill, Bayer healthcare, Mishawaka, IN, USA). At week 20 and 21, all mice were screened for hyperglycemia.

Figure 6) *Urine collection for the screening for glucosuria in mice. The procedure is carried out in a laminar flow hood.*

Measuring salivary secretion capacity (Paper I-III)

Mice were fasted for a minimum of 2 hours, but given water *ad libitum* and anesthetized with an intramuscular injection of ketamine/medetomidine (0.01 ml/g bodyweight). In *Paper I*, after stimulation of secretion by pilocarpine (#P6503; Sigma, St. Louis, MO, USA) in saline (0.5 μg/g bodyweight) administered via the femoral artery, saliva was collected with capillary tubes for 10 minutes, and the volume was determined. The samples were stored at -80°C until analyzed. In *Paper II & III*, salivary secretion was induced by intraperitoneal injection of 0.5 μg pilocarpine per gram bodyweight (#P6503; Sigma) and collected during 10 minutes (Figure 7). Pre-weighted tubes were weighted again after collection to determine the amount of saliva (1 μg = 1 μl). Protease inhibitor cocktail (#P8340; Sigma) was added at a concentration of 1:500 and samples were kept at -80°C until analysis.

Figure 7) *Saliva collection and measurement of the salivary flow rate in anesthetized mice subsequent to the intraperitoneal injection of pilocarpine.*

Blood sampling and organ collection (Paper I-III)

Blood was collected from the saphenous vein from non-anesthetized mice and by heart puncture on the day of euthanasia. The blood was allowed to clot and centrifuged at 800 *g* to obtain serum. In *Paper I*, submandibular and sublingual salivary glands were surgically removed, snap-frozen in isopentane by liquid nitrogen and stored at -80°C. In *Paper II & III*, organs were fixed in 4% formalin before embedding in paraffin, sectioning and staining with haematoxylin and eosin (H&E). Sections obtained from the kidneys were also stained using the periodic acid-Schiff (PAS) staining technique.

Histopathology and quantification of inflammation (Paper I-III)

Histopathological evaluations in *Paper I* are based on frozen tissue and in *Paper II & III* on paraffin embedded tissue. H&E staining was carried out to determine the degree of inflammation. In *Paper I*, submandibular and sublingual salivary glands were snap-frozen in isopentane by liquid nitrogen, and stored at -80°C. Five μm thick sections were cut using a cryostat (Leica Instruments, Nussloch, Germany) and placed onto SuperFrost Plus glass slides (Menzel, Braunschweig, Germany). Salivary gland sections were evaluated and analyzed

using a Leica DMLB light microscope connected to a ColorView III-camera and AnalySIS software (Soft Imaging System GmbH, Munster, Germany), to determine the focus score (FS; number of foci of 50 or more mononuclear cells/mm^2 of glandular tissue), and the ratio index (RI; area of inflammation/area of glandular tissue). In *Paper II & III*, FS and RI were determined as followed: three independent H&E stained sections of each salivary gland were qualitatively evaluated; the section displaying the highest degree of inflammation was recorded as a multiple image composite, displaying the whole surface of the gland (80x magnification); total glandular area and the individual size of each focus were morphometrically analyzed (Lucia, Laboratory Imaging, Hostivař, Praha). To determine the insulitis score (IS), at least five H&E stained tissue sections of the pancreas were analyzed in a blindly fashion. On average, 32 islets *(Paper I)* and 46 islets *(Paper II)* per mouse were evaluated in a blinded manner and scored as described by E.H. Leiter [275]. Sections of the kidneys, thyroid gland, thymus, heart, lungs, liver, stomach, small and large intestine, appendix and the skin were also stained with H&E.

Immunohistochemistry (Paper I)

Immunostaining was performed by the AvidinBiotinComplex using the following antibodies: for T-cells (CD4), rat $IgG_{2b,k}$, clone GK1.5 (R&D Systems, Abingdon, UK); for B-cells (B220), rat IgG_{2b}, clone RA3-6B2 (R&D Systems); for proliferating cells (Ki-67) rat IgG_{2a}, clone TEC-3; and for follicular DCs (FDC), rat IgG_{2c}, clone FDC-M1 (BD Biosciences, San Jose, CA, USA). Briefly, following fixation in cold acetone, endogenous peroxidase (Blocking kit) and biotin were blocked (Avidin D and Biotin blocking solution, Vector Laboratories, Burlingame, CA, USA). Non-specific binding was inhibited by normal rabbit serum. Diaminobenzidine was used as chromogen. Unless otherwise specified, all reagents were purchased from DAKO (Glostrup, Denmark).

Multiple analyte profiles (Paper I-III)

In *Paper I*, serum and saliva samples obtained from the mice were analyzed using a bead-based multiplex sandwich immunofluorescence assay (#LMC0001; BioSource, Nivelles, Belgium), as recommended by the manufacturer, measured on a Luminex 100 system (Luminex Corporation, Austin, TX) and analyzed using StarStation software (Applied Cytometry Systems, Dinnington, Sheffield, UK).

In *Paper II & III*, a similar bead-based multiplex assay (Figure 8) was applied to generate multi-analyte profiles (MAPs) from serum and saliva from the 12 non-diabetic NOD and 12 Balb/c mice comprising 82 analytes for serum and 75 for saliva. MAPs were generated for every individual non-diabetic NOD mouse assessed for SS (n = 36) and 12 Balb/c, which were randomly selected from the original cohort of 20 PBS/IFA injected Balb/c mice. Results were obtained using a multiplex sandwich immunofluorescence assay based on color-coded and antibody coated beads, carried out at Rules Based Medicine Inc. (Austin, TX, USA). Using an automated system for liquid handling, each sample was introduced into the capture microsphere multiplex of the MAP. After incubation, multiplexed cocktails of biotinylated, reporter antibodies were added, developed with a streptavidin-phycoerythrin solution and analyzed using a Luminex 100 instrument (Luminex Corporation). For each multiplex, 8-point calibrators and 3-level controls were included. Antibodies used in the MAP to recognize and quantify the specific autoantibodies were directed against all Ig isotypes.

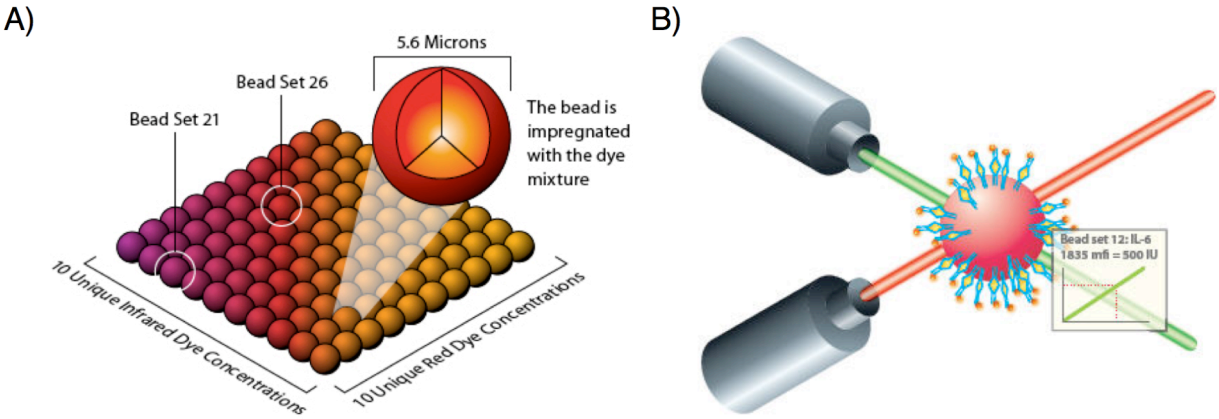


Figure 8) *Bead-based multiplex immunoassay A) The combination of two unique dye intensities allows the labeling of up to 100 sets of color-coded microspheres each possessing a unique spectral signature. Each color-class of beads is subsequently labeled with capture antibodies. Subsequent to a classical sandwich immuno-assay procedure, a Luminex analyzer is used to measure the quantity of each analyte included in the assay. Fluidics based on the principle of flow cytometry cause the stream of suspended microspheres to line up in a single file prior to passing the detection chamber. One laser classifies the microparticle and determines the analyte that is being detected and the second laser determines the magnitude of the streptavidin-phycoerythrin-derived signal, which is in direct proportion to the amount of bound analyte. Figure adapted from www.rndsystems.com.*

Quantification of anti-M3R antibodies (Paper II & III)

Levels of anti-M3R autoantibodies were measured as described previously [160]. In brief, aliquots of 2 x 10^5 Chinese hamster ovary cells, transfected with pcDNA5/FRT/V5-His MsM3R-Flp-In cells, were incubated for 1.5 hours at 4°C with 10 µl of serum before

incubation with one of the following FITC-conjugated goat anti-mouse detection antibodies purchased from Southern Biotech (Birmingham, AL, USA) diluted 1:50: isotype control, goat IgG (#0110-02); IgG H+L (#1031-02); IgG_1 F(ab')2, (#1072-02); IgG_{2b} F(ab')2 (#1092-02); IgG_{2c} F(ab')2 (#1079-02) and IgG_3 F(ab')2, (#1102-02). The cells were analyzed using a FACSCalibur flow cytometer using Cell Quest software (BD Biosciences) and FlowJo (Tree star Inc., Ashland, OR, USA).

Statistical analyses

Paper I: Data were analyzed using one-way analysis of variance followed by the Bonferroni posttest for selected groups (modified unpaired Student's 2-tailed *t*-test for multiple group comparisons). To normalize skewed distributions and improve the homogeneity of variance, cytokine data were log-transformed prior to all statistical analyses. To determine the linear relationship between 2 variables, values were compared using Pearson's correlation test (2-tailed). Correlation analyses were restricted to NOD mice and data sets in which a scientific reason for a causal connection was given. *P* values less than 0.05 were considered significant. Statistical analyses were performed using GraphPad Prism 4.0 software (GraphPad Software Inc., San Diego, CA, USA).

Paper II: Means were compared using independent Student's *t*-test (2-tailed). Bivariate linear associations, used to generate the correlation matrices, were computed using 2-tailed Pearson correlation (r). Strain-membership prediction was assessed by discriminant analyses (DA) and subsequent cross-validated (leave-one-out) group prediction. The quality of the DA function is expressed by its canonical correlation (R*).

Principal component analyses (PCA) were computed from MAPs obtained from NOD mice with the purpose to uncover the latent structure within protein families. Protein family membership was defined based on the Protein ANalysis THrough Evolutionary Relationships (PANTHER) classification system (http://www.pantherdb.org). PCA seeks a linear combination of variables so that the maximum variance is extracted from the variables. It then removes this variance and seeks a second linear combination and so forth. Loadings above 0.6 were considered as defining parts of the component. For proper model specification, variables being either differently expressed between the two strains ($P < 0.05$) and/or significantly correlated ($r > 0.6$; $P < 0.05$) with one of the disease parameters were included. As a rotation method Varimax was chosen. The number of components was determined by the Kaiser criterion (Eigenvalue > 1.0). An explanatory criterion (> 80%) was applied, in addition, for

growth factors and cytokines in serum. Variables being the defining parts of a component were also combined for DA and entered simultaneously. In serum no data was missing and in saliva missing values were excluded pair-wise from all analyses except PCA and DA. All analyses were computed using SPSS 13 (SPSS Inc., Chicago, IL, USA).

Paper III: Means were compared to the specific reference group using 1-way Anova and Dunnett's post-test (2-tail), accounting for multiple group comparison. Due to comparison of all pairs, salivary flow means were analyzed using Bonferroni's posttest. Prior to original and cross-validated (leave-one-out) group prediction the discriminant potential of the respective variables was computed using DA. Variables were entered simultaneously or in a forward stepwise manner, using Wiki's lamda as the inclusion/exclusion criteria. Relative risk and incidences were compared using Fisher's exact test (2-tail). *P* values less than 0.05 were considered significant. Statistical analyses were computed in SPSS 13 (SPSS Inc.) and Prism 4.0 (GraphPad Software Inc.).

Summary of the results

Paper I

NOD mice retained stable salivary secretion during the period of time in which the major histopathological changes in the salivary glands took place. The significant decrease in salivary secretion capacity occurring between 17 weeks and 24 weeks of age, was, in contrast, not accompanied by significant changes in the degree of salivary gland inflammation. T- and B-cells marked the chronic inflammatory cell infiltrates within the salivary glands and in one third of the mice early signs of ectopic lymphoid organization were observed. Significant quantitative changes in IL-2, IL-5, and GM-CSF in serum and in IL-4 and TNF-α in saliva occurred within the time period characterized by the onset of hyposalivation. Correlation analyses revealed a negative association between salivary secretion and the levels of IL-4, IFN-γ and TNF-α in saliva obtained from NOD mice, while correlation between salivary flow rates and parameters of glandular inflammation were consistently weak.

Paper II

A total of 162 analytes, for which often only scant or no information in SS has been published, was analyzed in serum and saliva from NOD and Balb/c mice. The application of multiplex technology significantly diminished the sample volume required and enabled the investigation of the analytes' connectivity through correlation networks. At 21 weeks of age all NOD mice presented glandular inflammation and impaired salivary secretion. The latter, when applied as a discriminant function, classified 100% of the mice according to their strain membership, confirming the onset of overt SS in all NOD mice. In addition, subsets of NOD mice presented histopathological changes in the kidneys and the lungs reminiscent of SS-related extraglandular disease manifestations.

Thirty-eight biomarkers in serum and 34 in saliva obtained from NOD mice were significantly different from Balb/c mice, which served as reference strain. Eighteen biomarkers in serum and 3 chemokines measured in saliva had the capacity to predict strain membership with 80-100% accuracy. The extraction of principal components reduced the size of the correlation matrix considerably, from 9'604 to 1'994 coefficients. Correlation networks revealed a complete lack of original variables or components correlating with salivary flow and either FS or RI. This lack was also obvious when analyzing inter-component correlations. In contrast,

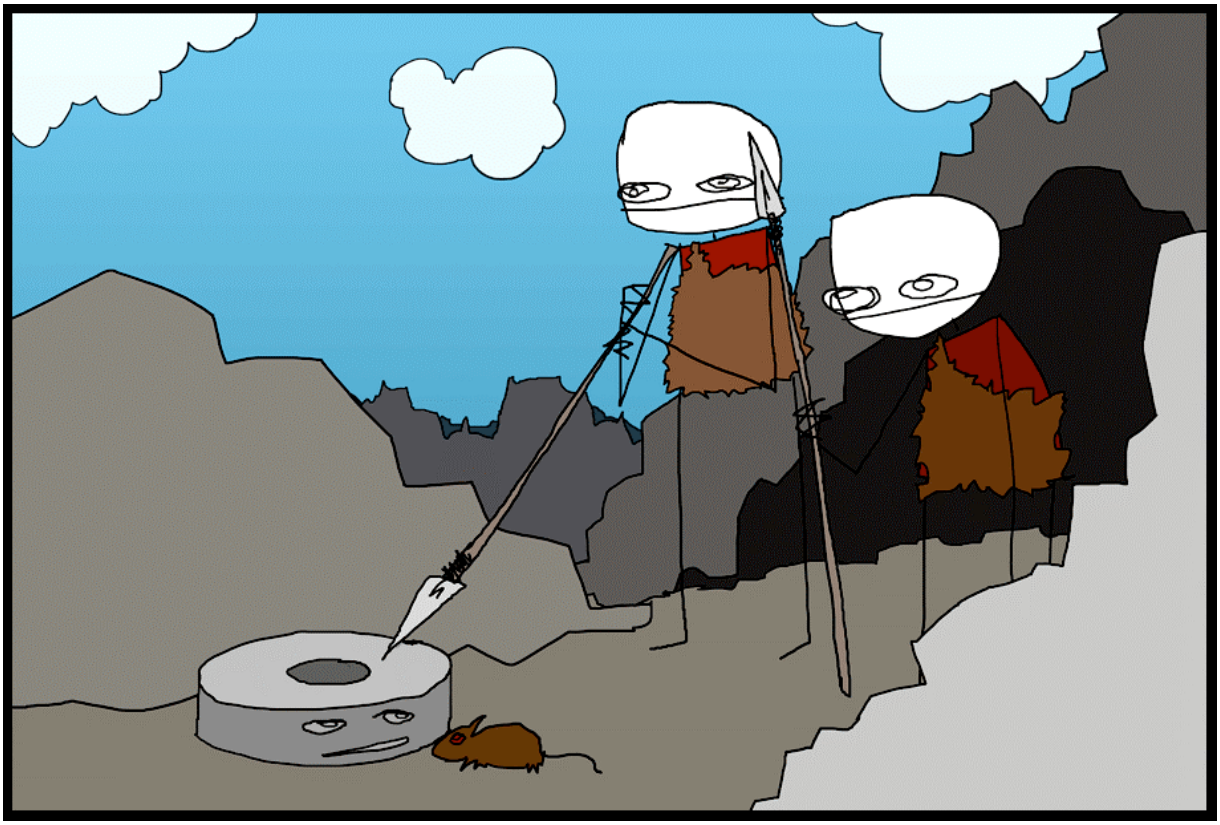
there were some components associated with either salivary flow or glandular inflammation having positive or negative interrelationships with components related to autoantibody levels. Among components associated with salivary flow, an antagonistic interplay between cytokine Sa-C-3 (associated with decreased salivary flow) and all three components extracted from serum (positively associated with salivary flow) became apparent. Components associated with FS and/or RI correlated strongly with each other, indicating a process in which cytokine Sa-C-2 and CXCL Sa-C-2 opposed acute-phase reactant Sa-C-1 and secreted IgA. In addition, cytokine Se-C-2 and coagulation factor Sa-C-2 played a dual role by combining the positively associated IL-1α and von Willebrand factor (vWF) with the negatively associated CD40L and fibrinogen, respectively.

Paper III

Immunizing NOD mice with Hsp60 and aa437-460 at 7 weeks of age significantly reduced SS-related histopathology compared to PBS/IFA injected NOD controls. At 21 weeks of age, salivary secretion in PBS/IFA injected NOD controls was decreased by 42% compared to Balb/c and DA confirmed the onset of hyposalivation in all PBS/IFA injected NOD controls. Mean salivary flow was higher in Hsp60 immunized mice and similar in the aa437-460 injected group. Nevertheless, we observed an obvious dichotomy regarding salivary secretion capacity in both treated groups. Evidentially, they could be separated by choosing the highest salivary flow rate measured in PBS/IFA injected NOD controls as a threshold. Notably, salivary flow rates of mice immunized with Hsp60 or aa437-460 with salivary flow rates above the threshold did no longer present impaired salivary secretion compared to Balb/c mice.

Importantly, except salivary secretion capacity, treated mice with retained salivary flow (n = 6 Hsp60 and n = 4 aa437-460 treated) and treated mice with decreased salivary flow (n = 6 Hsp60 and n = 8 aa437-460 treated) were very similar with respect to FS, RI and IS. In addition, we did not identify significant alterations in the frequency of extraglandular disease manifestations in response to the two treatments investigated. Both treatments induced similar changes in biomarker profiles. Notably, circulating inducible protein-10 (IP-10) and eotaxin decreased significantly as a consequence of the treatment. In contrast, anti-M3R IgG_1, IL-10 and leptin discriminated most accurately between the different treatment groups and, in addition, the PBS/IFN injected NOD controls (hitrate: 86.1% original and cross-validated).

Successful prevention of hyposalivation (treated mice with retained salivary flow) was accompanied by quantitative alterations in 36 biomarkers when compared to treated mice with decreased salivary flow. Nineteen inflammatory mediators, measured in the treated mice with retained salivary flow, thereby declined to levels comparable to Balb/c. Low salivary VEGF-A was the best individual predictor of successful treatment, whereas high VEGF-A indicated the onset of hyposalivation despite Hsp60 or aa437-460 administration (hitrate 87.5% original and cross-validated). Expanding the model by including circulating apolipoprotein A1 (Apo A1) and secreted macrophage-colony stimulating factor (M-CSF) further improved prediction accuracy (hitrate: 91.7% original, 95.8% cross-validated). DA were also computed to identify sets of biomarkers with the highest potential to discriminate between normal and decreased salivary secretion capacity, irrespective treatment-group membership and strain. As most accurate single predictor granulocyte chemotactic protein-2 (GCP-2) in saliva was identified (hitrate 81.3%, original and cross-validated). A forward stepwise model combining salivary GCP-2 with circulating IL-1α and salivary myeloperoxidase (MPO), myoglobin and macrophage inflammatory protein (MIP)-3β resulted in 93.8% accurately classified cases, original and cross-validated.



It might be useful one day; Illustration by Sam Brown (www.explodingdog.com)

General discussion

The clinical symptoms of SS are thought to develop late in the disease course; thus, in most patients, SS is most likely diagnosed at an advanced stage [74]. Investigation of the SS disease course in humans is ethically difficult due to the lack of a non-invasive method for the quantification of salivary gland inflammation. Consistent with the results reported by other investigators [159], our results described in *Paper I* suggest that hyposalivation in NOD mice follow the emergence of focal lymphoid infiltration with a certain delay in time. Conventionally, the extent of impairment in exocrine function has been anticipated to be the direct outcome of the degree of glandular inflammation replacing and destroying parenchyma. Nevertheless, despite the soundness of the hypothesis, the frequently observed lack of correlation between the amount of destroyed glandular tissue and the disproportional decrease in salivary flow [197] was difficult to fit into this model. Consequently, processes disturbing the physiological cascade of saliva production and secretion have been proposed as a possible explanation for this phenomenon [76]. A model of SS pathogenesis acknowledging the existence of other pathogenic mechanisms than the sole destruction of glandular epithelium is further supported by several murine models of SS. MRL/*lpr* mice retain a stable salivary flow rate over time despite pronounced inflammation in the salivary glands and several other features of human SS [199]. The absence of overt SS in presence of sialoadenitis was, furthermore, described in NOD IL-$4^{-/-}$ [161], NOD.B10-H2b IL-$4^{-/-}$ [160] and NOD.B10-H2(b).C STAT$6^{-/-}$ mice [162].

One conclusion, common to all three studies combined in this thesis, is the apparent autonomy of the major SS-related disease manifestations. Results summarized in *Paper I* indicate that the development of hyposalivation is independent from a significant quantitative increase in glandular inflammation. In *Paper II* we describe the complete lack of original variables and extracted components correlating with salivary flow and either FS or RI. This lack was also evident when analyzing inter-component correlations. In *Paper III*, the effective protection from hyposalivation was associated almost exclusively with changes reflected by the MAP. At the same time we found neither protection nor manifestation of hyposalivation to be related to the quantitative degree of glandular inflammation.

The findings in all three studies also support the notion that alterations within the salivary gland tissue are prone to be reflected in the spectrum and amount of proteins liberated into saliva. The identification of a set of biomarkers in human saliva, similar to the 3 chemokines

we described in *Paper II*, predicting the presence or absence of SS with high accuracy, or as described in *Paper III*, specific biomarker signatures capable of predicting treatment efficacy and capable to distinguish normal from impaired salivary secretion, would represent a major advance in the field. Due to the non-invasive collection method and the lack of extraction procedures, saliva based approaches are more applicable in clinical practice for prognostic, diagnostic and follow-up purposes than methods requiring salivary gland biopsies [47, 68, 115]. Importantly, as described in *Paper II*, we could exclude a general concentration effect on biomarker quantities measured in saliva in relation with lower fluid secretion. This conclusion was based on the observation that in a non-inflammatory situation, represented by Balb/c mice, the average correlation coefficient of all analytes and analytes included in the modeling process did not show any sign of a negative association with salivary flow. Saliva, because it is directly collected from the site of inflammation, is most probably superior to measurement in serum in reflecting the immunological situation in the salivary glands [276]. The findings regarding extraglandular autoimmune manifestations in *Paper II* and *Paper III* also support this hypothesis.

However, the presence of SS-unrelated histopathology and other subclinical autoimmune disease features remain a common problem of several murine models of SS [117, 199]. Such divergence from an "ideal" SS-specific situation may lead to false conclusions drawn from measurements in serum. Due to the complexity of this biofluid, one may also conclude that biomarker profiles solely relying on serum or plasma require considerable validation efforts to prove their specificity and sensitivity for a specific autoimmune disease such as SS [5, 68]. Nevertheless, analyzing the SS patient's individual cytokine and chemokine profile in serum, using a bead-based multiplex immunoassay similar to the technology applied in *Paper I-III*, revealed that biomarker signatures in serum might indeed be able to indicate specific histopathological situations in the salivary gland. In this study, among 25 biomarkers, BAFF, CCL11 and IFN-γ levels discriminated best between patients with and without GC-like structures [155].

The formation of GC-like follicles in non-lymphoid organs has been suggested to indicate a more severe disease and through histopathological and immunohistochemical screening such structures were found in approximately every forth patient with SS [130]. Interestingly, in *Paper I*, the early signs of lymphoid organization in the salivary glands, defined by distinct areas of T-cells and B-cells, proliferating cells and FDCs, were associated with higher FS.

However, to what extent such structures overtake functional tasks normally assigned to secondary lymphoid organs remains to be proven.

In *Paper I* we describe significant alterations in circulating IL-2, IL-5, and GM-CSF and secreted IL-4 and TNF-α occurring during the same period of time as the onset of hyposalivation. In contrast, no significant increase in glandular inflammation was observed within this specific period. By promoting growth and suppressor functions of T_{regs}, IL-2 is crucial in the maintenance of immunological self-tolerance. Neutralization of circulating IL-2 led to aggravation of diverse autoimmune manifestations in NOD mice [243] and deletion of IL-2 and IL-2Rα in C57BL/6J mice caused the emergence of sialoadenitis, hyposalivation and SS-unrelated histopathological manifestations [254]. Even though the role of regulatory processes in the progression and perpetuation of SS is still elusive, the results published in *Paper III* indicate that lower salivary gland inflammation and especially the prevention of hyposalivation depends on the efficacy of Hsp60 and aa437-460 to strengthen regulatory processes.

Although recent studies indicated that absence of IL-5 could induce a shift toward adaptive immune responses [277, 278], the decrease in circulating IL-5 reported in *Paper I* is difficult to interpret. Murine IL-5 acts on B-cells and contributes to the maintenance and differentiation of B-1a cells [279]. In atherosclerosis, natural autoantibodies secreted by B-1a cells recognizing LDL may exert a protective effect by preventing the implication of the adaptive immune system against this particular antigen [277, 280]. The significant negative correlation of secreted IL-5 with salivary flow reported in *Paper II* and of IL-4 with salivary secretion rates in *Paper I* further corroborates with the notion of T_H2 cytokines and B-cells in the pathogenesis of SS [160, 161]. Most importantly, depletion of the patient's B-cells, using anti-CD20 antibodies, was shown to ameliorate several symptoms of the disease [97]. TNF-α was upregulated in saliva during the overt stage *(Paper I)* and significantly decreased in NOD mice protected from hyposalivation *(Paper III)*. The therapeutic impact of agents neutralizing TNF-α in patients with RA is indeed remarkable [48], however, the use of TNF-α antagonists in SS was discontinued since initial promising results could not be confirmed [99].

In *Paper II* we present data on a considerable number of immunologically relevant proteins, for which often only little or no information in SS has been published. The possibility of multiplexing analytes significantly diminished the required sample volume and enabled investigation of the analytes' connectivity through correlation networks [5]. Compared to a

component-focused experimental study design, such a study cannot be as conclusive in defining the role of a single protein. It rather represents a novel way of analyzing the implication of multiple molecules in a specific condition, also by utilizing the explanatory power of interrelationships, which define a specific system state [4]. Most system states in fact, arise from and strongly rely on the interactions of their numerous constituents [5].

Analyses of protein levels instead of mRNA expression data could rule out certain factors of uncertainty such as mRNA stability and correlation between mRNA levels and the corresponding protein levels. A recent study combining global mRNA expression analyses with 2-D gel electrophoresis coupled with mass spectrometry to analyze pooled saliva obtained from SS patients for the purpose of biomarker identification reported poor correlations between levels of transcripts and protein quantities [281]. The same study identified 42 proteins, significantly altered when comparing pooled saliva from SS patients with healthy controls [281]. Technologies and techniques in the field of quantitative mass spectrometry are evolving at a fast pace, now allowing first identifications of peptides, which could be of interest for preliminary validation steps [69]. Nonetheless, identification of immunological regulators, operating at nano- to picomolar concentrations using mass spectrometry, still represents a great challenge, as abundant proteins may mask the immunological modulators in the biofluid of interest [69]. The gold standard for such quantitative measurements is still the application of immunoassays as a result of their unmatched sensitivity provided by antibodies [68].

Stable-isotope protein tagging or subtractive proteomics may, however, harbor the potential to significantly improve the number of immune system related proteins identified by mass spectrometry [69]. Nevertheless, the two approaches, antibody-based biomarker identification and mass spectrometry-based global proteome profiling, may well complement one another in delineating SS-specific disease signatures. Both technologies, however, need further technological advances to obtain a more complete coverage of crucial immune mediators and of the whole proteome, which consists of at least 100’000 constituents [69, 70]. Certain molecules of proven importance in the pathogenesis of SS, such as BAFF [175] and IFNs [119], were not part of our MAP. Storage of data in a structured format represents one option of including results from future studies in existing databases or correlation networks and would enable exciting new possibilities for automated and integrative biological research [5, 70]. Standardizing reagents, assays, data storage, and normalization techniques are mandatory to achieve such possibilities in the future [70].

In line with the results described in *Paper I* the data acquired in *Paper II* also argues against close interrelationships between the different SS-related disease manifestations. Only RI and anti-La/SSB correlated directly with each other and the segregation between proteins being either associated with hyposalivation or glandular inflammation was absolute. The chemokines, which were associated with higher salivary flow (MIP-1α and monocyte chemoattractant protein [MCP]-5) are considered T_H1 related chemokines and are negatively regulated by STAT6 [282]. In contrast, the STAT6 dependent T_H2 associated chemokines [282, 283] eotaxin and macrophage-derived chemokine (MDC) were associated with decreased salivary flow. Normalization of secreted MDC quantities to levels comparable with Balb/c mice was, in addition, observed in relation with the treatment-induced protection from hyposalivation described in *Paper III*. Both, eotaxin and MDC are produced by T_H2-promoting DC cell types upon engagement of CD40/CD40L [284] and these two molecules were associated with low salivary secretion capacity in the correlation network as well. Interestingly, CD40 and CD40L are expressed on salivary gland epithelial cells and infiltrating lymphocytes in biopsies obtained from SS patients [285].

The observed correlation between CD40/CD40L and anti-M3R IgG_3 levels may be associated with the primary role of CD40 and CD40L which is related to B-cell survival, B-cell proliferation, Ab production and Ab isotype switching [286]. In *Paper II*, all antibody isotypes against M3R were upregulated, with, perhaps most importantly, the positive associations between M3R IgG_3 and secretory CD40, CD40L, IL-18, GCP-2 and MMP-9. IgG_2 subclasses and IgG_3 are generally considered to be significantly more potent in mediating pathogenic effects than IgG_1 [287]. In contrast to other isotypes, the effect of IgG_3 is FcR independent and strictly related to complement activation, a component of the immune system recently related to SS pathology [252]. In addition, IgG_3 can form complexes through self-association and generate cryoglobulins [287], a feature in SS [74]. However, previous studies concluded IgG_1 to be the crucial isotype in anti-M3R antibody mediated hyposalivation due to its absence in STAT6 [162] and IL4 [160] deficient NOD related strains. In contrast, in *Paper III*, the induction of anti-M3R IgG_1 was strongly related to immunization with Hsp60. However, in *Paper III*, the alterations in anti-M3R IgG_1 levels were not related to the manifestation or protection from hyposalivation.

Beside B-cell fate, CD40/CD40L ligation plays a central role in converting a tolerogenic antigen presentation into a pathogenic immune activation [288]. In conditions with chronic inflammation, CD40 and other inflammatory mediators can exert adverse effects on tissue

renewal and repair processes [289]. Indeed, we found specific growth factors *e.g.* epidermal growth factor (EGF) to be negatively correlated with CD40/CD40L and secreted autoantibodies.

Furthermore, negatively correlated with CD40/CD40L was circulating IL-10, whose anti-inflammatory properties may apply to our model, since in *Paper II* increased circulating IL-10 did correlate with higher salivary flow and lower autoantibody concentrations in saliva. This observation is in line with the reported benefit of IL-10 gene transfer, which could suppress the emergence of certain SS-related manifestations in NOD mice [256]. However, IL-10 in saliva was associated with glandular inflammation, and the findings reported in *Paper III* did not mirror the conception of increased IL-10 secretion by T_{regs} in favor of immunosuppression [20]. These findings reinforce the results obtained in IL-10 transgenic mice, which developed progressive histopathology and hyposalivation, evocative of SS [255].

With the exception of vWF, increased concentrations of proteins in saliva related to acute tissue injury were all associated with a lower degree of glandular inflammation. These events may be followed later on by inflammatory cell invasion into the glandular tissue and/or may mirror an imbalance between processes restoring homeostasis and factors promoting chronic inflammation. Higher levels of IL-1 family members correlated with both worsening of hyposalivation and increased glandular inflammation. GCP-2, inducible through IL-1β [290], was, alike IL-1β, associated with glandular inflammation. The levels of all these pro-inflammatory proteins (IL-1α, IL-1β and GCP-2) declined in treated mice with retained salivary flow to levels comparable with Balb/c mice *(Paper III)*. Leukemia inhibitory factor (LIF), also inducible through IL-1β [291], which was included in a component together with IL-1β and IL-10, has been shown to have parallels with IL-1, TNF-α and IL-6 related to the perpetuation of inflammation in RA [291]. Alike anti-TNF-α the inhibition of IL-1 pathway using recombinant IL-1RA has become an accepted treatment strategy in RA [292]. Unfortunately, clinical or experimental studies using IL-1RA in SS are still lacking.

Among the biomarkers analyzed in saliva, GCP-2, IP-10 and regulation upon activation, normal T-cell expressed and secreted (RANTES), revealed the highest potential to distinguish between NOD and Balb/c. In addition, in *Paper III*, GCP-2, individually or combined with 4 other analytes, accurately predict impaired salivary secretion capacity irrespective of treatment group-membership and strain. For clarification, GCP-2 is expressed at neutrophil

and macrophage dominated inflammatory sites, IP-10 is related to T_H1 immune responses, and RANTES is involved in T_H1 and T_H2 immune responses [293].

GCP-2 and IP-10 have opposite roles in angiogenesis [23], an aspect which, despite the recognized importance of neovascularization in promoting the influx of inflammatory cells [294], has not yet been addressed in SS. Proteins, such as GCP-2, VEGF, EGF, IL-1, VCAM-1 and MMP-9, which were significantly increased in NOD mice compared to Balb/c *(Paper II)*, are involved in angiogenesis [294]. In addition, also angiostatic molecules, such as IP-10, tissue inhibitor of metalloproteinase (TIMP)-1 and endostatin-1 [294] were also altered *(Paper II)*. In *Paper III*, low VEGF-A, a key molecule mediating vascularization [26], was the primary predictor indicating the success or failure in preventing hyposalivation followed by low M-CSF, which is alike thrombopoietin (TPO) and oncostatin M (OSM), an inducer of VEGF-A [295]. Other molecules implicated in the process of neovascularization, which were decreased in saliva in concordance with prevention of hyposalivation in treated mice are, MMP-9, fibroblast growth factor (FGF)-9 [294], as well as all CXCR2 ligands measured (GCP-2, melanoma growth stimulatory-activity protein [GRO] and MIP-2) [13, 23]. The importance of neovascularization is greatly recognized in RA and has been explored as a target for therapeutic intervention [296]. Anti-VEGF therapies are also important in the treatment of certain cancers [297] and in age-related macular degeneration [298]. Anti-VEGF therapies can either involve monoclonal antibodies or orally available small molecules that inhibit the tyrosine kinases stimulated by VEGF [297]. Unfortunately, the issue of pathogenic neovascularization has not yet been addressed as a pathogenic mechanism in SS.

The study presented in *Paper III* describes the first preventive antigen-specific intervention, successfully ameliorating spontaneous experimental SS. Despite the antigenic relationships between Hsp60 and antigens specifically related to SS are unknown, we provide further evidence that immune responses to Hsp60 are relevant in the control of pathogenic autoimmunity [52]. In line with the notion that Hsp60 and aa437-460 treatment can alter chemotaxis of T-cells *in vitro* [63, 64], decreased inflammation was associated with lower serum levels of chemoattractants for T_H1 and T_H2 cells [13] even 14 weeks after immunization. In addition, MCP-1 and MCP-3, to which monocytes and plasmacytoid DCs are most responsive [13], were also reduced in serum as a result of the treatment.

Disease severity of RA and juvenile idiopathic arthritis have been related to the extent of Hsp60 induced propagation and activation of T_{regs} [61, 62]. Suggested key molecule involved

in this process is IL-10, which may be secreted upon Hsp60 signaling through TLR2 [20]. As described above, our findings, however, do not support a general anti-inflammatory role of IL-10 in SS. In contrast, increased salivary IL-2 and the observed modulation of leptin as an effect of Hsp60 and aa437-460 immunization support the notion of strengthened regulatory processes as a result of the treatments. Compared to IL-2, the adipokine leptin exerts a reciprocal effect on effector and T_{reg} populations [29]. Besides T_{regs}, IL-17 cells raised considerable attention in the field of autoimmune diseases [12, 15] and a major role of IL-17 has now been described in various models of immune mediated tissue injury related with host defense against microorganisms and organ-specific autoimmunity [15, 17]. Even though results in *Paper II* showed an increase of IL-17 in saliva from NOD mice, the treatment interventions described in *Paper III* were not accompanied by detectable alterations in IL-17, neither in saliva nor in serum. Recent results suggest that the T_H17/IL-23 system is activated in SS patients and C57BL/6.NOD-Aec1Aec2 mice during the overt disease state. Functional associations between IL-17/IL-23 expression and specific clinical manifestations have, however, not yet been identified [164].

In *Paper III*, prevention of hyposalivation was achieved in a substantial number of mice at least for the duration of the experiment. Nevertheless, future studies need to elucidate if NOD mice can permanently be protected from developing hyposalivation and if such treatments may also be able to induce remission of SS. Refinement of treatment regimes, as it has been shown recently for diabetes using tandem repeated aa437-460 [299], may further increase the percentage of mice protected from hyposalivation.

As in *Paper II*, the MAPs generated a comprehensive, but not all-embracing overview of the serum and salivary proteome in *Paper III*. The purpose of the analyses in this study was also to characterize the different disease phenotypes, which resulted from the treatments. The biomarker signature associated with successful prevention of hyposalivation in NOD mice was primarily coined by the decrease and normalization of multiple chemokine levels and proteins related to angiogenesis. Such modulation was potentially promoted by the simultaneous decrease in TNF-α and IL-7, both key antagonist of T_{reg} induced immunoregulation [300]. Based on these variables, forward stepwise DA identified a model in which the combination of VEGF-A and M-CSF in saliva and Apo A1 levels in serum predicted the prevention of hyposalivation with 96% accuracy.

Downregulation of CD40, IL-10 and IL-11 [301] may further suggest T_H2 associated responses to be modulated by the treatment. An effect of Hsp60 on B-cells through innate signaling pathways has been reported [302] and may also contribute to the modulation of autoimmune diseases with a confirmed or potential B-cell aspect. Nevertheless, in *Paper III* the levels of most autoantibodies remained unaffected. Another encouraging aspect of our study regards the capacity of GCP-2 which individually or combined with 4 other analytes, accurately predict impaired salivary flow.

As alluded to already, we did not find significant associations between extraglandular disease manifestations and the biomarker profiles. Similarly, we did not identify significant alterations in extraglandular disease manifestations as a result of the two treatments investigated in *Paper III*. Nevertheless, it would be of interest to investigate in further detail the involvement of kidneys and lungs in both experimental and human SS [303-305]. The development of malignancy in one mouse remains difficult to interpret, as lymphoma develop rather frequently in old NOD mice [306]. There are conflicting data about whether there is an increased risk for lymphoma and solid malignancies with anti-TNF therapies for RA [292]. Nevertheless, anti-inflammatory treatment need to be critically reviewed regarding alterations induced, which could create immune privileges for tumor cells and microbial pathogens or host-defense related immunity.

Conclusions

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

- The development of major focal inflammation in the salivary glands precedes the onset of hyposalivation with a considerable amount of time.
- The onset of hyposalivation, which marks the onset of overt SS, can occur without a significant increase in salivary gland inflammation.
- Changes in cytokine patterns in serum and saliva coincide with the transition from pre-clinical to overt disease.
- Processes related to the adaptive immune system seem to promote SS with a strong association between T_H2 related proteins and the severity of hyposalivation.
- The different disease manifestations of SS appear to be greatly autonomous from each other and they are associated with different immunological processes.
- CD40, CD40L, IL-18, GCP-2 and anti-M3R IgG_3 may represent a pivotal point, which intersects the different aspects of SS pathology.
- Saliva represents an attractive biofluid for biomarker discovery in SS.
- Immunization with Hsp60 led to inhibition of SS in NOD mice.
- The Hsp60-derived peptide aa437-460, currently tested in phase II clinical trials for the treatment of IDDM, mimicked in general lines the beneficial effects of Hsp60.
- Successful prevention of hyposalivation was related to a significant decrease in inflammatory mediators related to pathological neovascularization, inflammatory chemotaxis and cell activation.
- Comprehensive analyses revealed specific biomarker signatures capable to predict the treatment received, treatment efficacy and impaired exocrine function.
- MAP analyses provide a basis for the comparison and selection of potential drug targets and diagnostic markers and allow the characterization of treatment responses.

Future perspectives

Although several hypotheses have been proposed over the last years, the etiology and several aspects regarding the pathogenesis of SS are still elusive. Unfortunately, all therapies applied today are inadequate to cure SS and regrettably, the number of clinical studies addressing SS is significantly lower compared to most other, as common, autoimmune diseases.

In all three studies here presented, we found direct evidence that the immunological processes mediating the different SS-related disease manifestations are considerably independent from each other. Stratification of the disease course in patients with SS and the close investigation of the interrelationships between the different aspects of disease are important goals for the future. Given the limits of the current disease assessment, the development of non-invasive precise methods for the quantification of salivary gland inflammation would greatly facilitate such studies. In mice specific biomarker signatures had, on an individual scale, the potential to discriminate "sick" from "healthy", successful prevention of hyposalivation from ineffective treatment of hyposalivation and intact salivary secretion capacity from impaired salivary secretion. It is unclear if these successful pilot studies conducted in an experimental model will translate into similar findings in an outbred human population. However, the methodology regarding MAP, data analyses and last but not least the results here presented will hopefully encourage researchers to conduct similar studies in samples collected from SS patients.

Promoted by the revolution in information technology, advanced analytical platforms, such as multiplex immunoassays, mass spectrometry and mRNA microarrays, take an important place in today's biomedical research. As reflected in this work and research efforts undertaken by other groups, such methodologies have to some extent been applied in SS. We believe that discovery-driven studies such as the one presented in *Paper II*, can widen the horizon and inspire new hypothesis-focused research. Bridging between these two approaches represents a great challenge for the years to come, which may, however, be rewarded with a more integrated perspective on immunology and autoimmune diseases.

The enigma of the initiating event leading to the accumulations of mononuclear cells in the exocrine glands characteristic for SS has not yet been solved and the hunt for "the causing agent" over the last decades has yet left most questions unanswered. The paradigm of insufficient immune regulation against key self-molecules being at the origin of the

conversion from physiological autoimmunity into an autoimmune disease led to the emergence of new concepts for the treatment of autoimmune diseases. As indicated by our study investigating the immunomodulatory potential of Hsp60 and aa437-460, one may assume that these and similar biologics, which strengthen regulatory mechanisms, may lead to valuable alternatives for the treatment of SS. Such agents are often extensively studied and successfully applied in other autoimmune conditions than SS and appear to not induce long-term immunosuppression. More frequent interdisciplinary collaborations may indeed take us all one crucial step towards a deeper understanding of SS and more appropriate measures for the treatment of SS.

No one lets me know what I think; Illustration by Sam Brown (www.explodingdog.com)

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Cyborg Fb 7c, Sirevåg, Stavanger, Norway. Photographer: Ronny Sjøthun

Bergen, 13th march 2008

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