B cell specificity and pattern in primary Sjögren's syndrome

- Studies in humans and a murine model

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Unforgettable

As I gaze outside my window
On this sunny winter's day
My mind embarks on a journey
As my thoughts drift away

I recall the many joyous memories
Of moments that we all have shared
Alas, of life's unpredictable nature
One cannot be spared

Our calamities and laughter
Your guidance and warm embrace
Are forever cherished
As the smiles you brought across my face

So in spite the distance
On this you can rely
We parted on "till we meet again!"
And not "goodbye…"

By Lara A. Aqrawi

Dedicated to all the inspirational people that have crossed my path
Thank you for being you.

SCIENTIFIC ENVIRONMENT

This doctoral work was conducted over the course of 4 years (2010 to 2014), and performed at the Broegelmann Research Laboratory, Department of Clinical Science, University of Bergen, within the frame of the Bergen Research School of Inflammation, and Gade Laboratorium for Pathology, Department of Clinical Medicine at the University of Bergen. Supervision and guidance were provided by Senior Scientist Karl A. Brokstad and Professor Kathrine Skarstein. Moreover, the collaborative study on Ro52 expression pattern was performed during a four-month exchange period at the Experimental Rheumatology Unit, Department of Medicine, Karolinska Institute, in Stockholm, Sweden under the supervision of Professor Marie Wahren-Herlenius.

SUMMARY

Sjögren's syndrome (SS) is a chronic autoimmune disease characterised by focal inflammation of exocrine glands, particularly salivary and lacrimal glands. Here, mononuclear cells, including B cells, infiltrate the glands, leading to dysfunction and later destruction of the glandular tissue. It thereby results in the common symptoms of dry eyes (keratoconjunctivitis sicca) and dry mouth (xerostomia). Another distinctive feature of this disease is the systemic production of autoantibodies such as Ro/SSA and La/SSB. This autoantibody production results from the activation of B cells into antibody secreting short- and long-lived plasma cells. Hence, although the etiology of SS remains unclear, B cells do play an important part in the pathogenesis of this disease.

In this doctoral work we address the concept of B cell specificity and pattern in primary SS (pSS), where we consider both the general and the autoantigen-specific B cell pattern in the peripheral blood and the salivary glands of patients with pSS. Additionally, we also account for the expression pattern of the Ro52 autoantigen in the salivary glands of pSS patients with regard to level of inflammation. Furthermore, in order to compare the plasma cell pattern before the onset of disease in relation to advanced disease and characterise the plasma cell compartment in the parotid and submandibular salivary glands and in the bone marrow, we explore a congenic NOD mouse strain, namely NOD.B10.H2b.

Our general findings disclose a low number of autoantigen-specific memory B cells that are observed alongside high levels of plasma cells both in the peripheral blood and the salivary glands of patients with pSS. Moreover, we also demonstrate a correlation between the ductal epithelial expression of Ro52 and the level of inflammation in the salivary glands of pSS patients. By the application of the NOD.B10.H2b model, we observe an accumulation of long-lived plasma cells in the parotid and submandibular salivary glands of mouse that coincides with our observations in lower labial salivary glands of the pSS patients.

LIST OF PUBLICATIONS

The doctoral thesis is based on the following publications, which will be referred to in the text by use of their Roman numerals (I-V)

I. **Aqrawi, L.A.**, Skarstein, K., Bredholt, G., Brun, J.G. & Brokstad, K.A. Autoantigen-specific memory B cells in primary Sjogren's syndrome. *Scand J Immunol* 75, 61-68 (2012)

II. **Aqrawi, L.A.**, Brokstad, K.A., Jakobsen, K., Jonsson, R. & Skarstein, K. Low number of memory B cells in the salivary glands of patients with primary Sjogren's syndrome. *Autoimmunity* 45, 547-555 (2012)

III. **Aqrawi, L.A.**, Skarstein, K., Oijordsbakken, G. & Brokstad, K.A. Ro52- and Ro60-specific B cell pattern in the salivary glands of patients with primary Sjogren's syndrome. *Clin Exp Immunol* 172, 228-237 (2013)

IV. **Aqrawi, L.A.**, Kvarnström M., Brokstad, K.A., Jonsson, R., Skarstein K. & Wahren-Herlenius, M. Ductal epithelial expression of Ro52 correlates with inflammation in salivary glands of patients with primary Sjögren's syndrome. *In manuscript*

V. Szyszko, E.A., **Aqrawi, L.A.**, Jonsson, R., Brokstad, K.A. & Skarstein, K. Non-proliferating plasma cells detected in the salivary gland and bone marrow of NOD.B10.H2b mice, a model for primary Sjögren's syndrome. *Submitted*

Appendix: **Aqrawi, L.A.**, Ro52- og Ro60-spesifikke celler og Sjögrens syndrom. *Best practice, Reumatologi* Nr. 17, 22-25 (2013)

ABBREVIATIONS

ABC	Avidin Biotin Complex
AECC	American-European Consensus Criteria
AID	Activation induced cytidine deaminase
ANA	Antinuclear antibodies
APRIL	A proliferation inducing ligand
BAFF	B cell activating factor
Bcl-6	B cell lymphoma 6 protein
Blimp-1	B lymphocyte induced maturation protein 1
BrdU	Bromodeoxyuridine
CTLA-4	Cytotoxic T lymphocyte antigen 4
DAB	Diaminobenzidine
DAMPs	Danger-associated molecular pattern
DC	Dendritic cell
FS	Focus score
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot
ER	Endoplasmic reticulum
GC	Germinal centre
H&E	Haematoxylin and eosin
HEV	High endothelial venules
HRP	Horseradish peroxidase
IFN	Interferon
IRF	Interferon regulatory factor
LPR	Liquid permanent red
MHC	Major histocompatibility complex
MRL	Murphy Roth's Large
NK	Natural killer

NG	Normal gland
NOD	Non-obese diabetic
PAMPs	Pathogen-associated molecular patterns
Pax-5	Paired box protein 5
PBMC	Peripheral blood mononuclear cell
PNAd	Peripheral node addressin
PVP	Polyvinylpyrrolidone
pSS	Primary Sjögren's syndrome
RA	Rheumatoid Arthritis
RF	Rheumatoid factor
RING	Really interesting new gene
SS	Sjögren's syndrome
sSS	Secondary Sjögren's syndrome
TMBH	Tetramethylbenzidine
TLR	Toll-like receptor
TGF	Transforming growth factor
TNF	Tumour necrosis factor

TABLE OF CONTENTS

1 INTRODUCTION

1. 1 THE IMMUNE SYSTEM

The immune system is often regarded as an entity consisting of an organised network of different layers that work together and interact in order to protect the host. These different components communicate with the external environment and with each other in order to initiate an immune response against potentially harmful pathogens such as viruses, bacteria, fungi, parasites and toxins[1]. This highly advanced system attempts to protect the host from constituents that are recognised as foreign whilst preventing reactions to self and to other non-harmful external elements[2]. Primarily, the immune system has been divided into the innate and the adaptive, which differ with regard to reaction times and the ability to generate memory[3] (Figure 1).

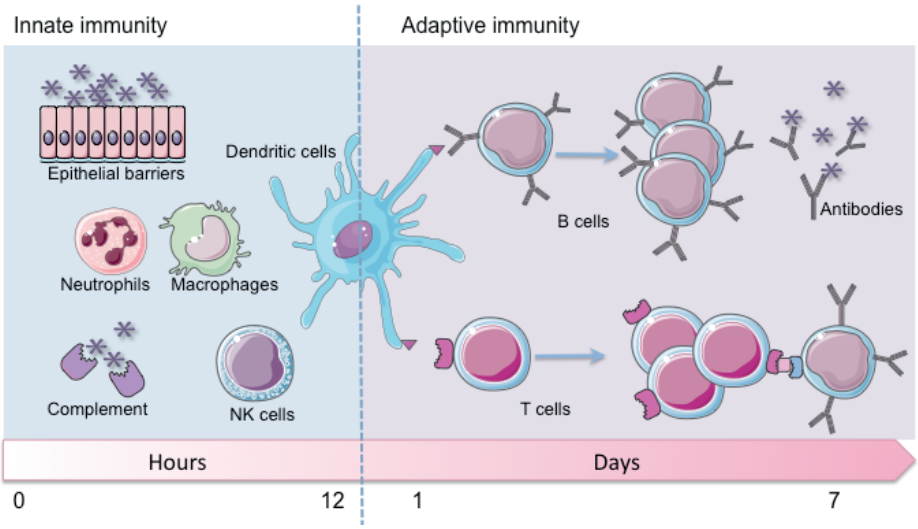

Figure 1. Cellular components of innate and adaptive immunity. Innate immunity has low specificity and affinity, but reacts within hours of an infection by the activation of phagocytic cells (macrophages and neutrophils) and natural killer (NK) cells through the recognition of common conserved bacterial and viral components. It therefore represents the first line of defence against microbes. The adaptive immune response develops later upon the activation of T and B lymphocytes by the dendritic cells through antigen presentation on MHC. This process requires more time yet results in a specific immune response, where the dendritic cells act as mediators between the innate and the adaptive immune systems. Moreover, the generation of T and B cell memory during adaptive immunity can aid in mounting a rapid immune response upon recurrent infection. Figure was produced using Servier Medical Art and inspired by Abbas *et al*[3].

1.1.1 Innate immunity

The innate immune system is considered the host's first line of defence against pathogens, thus facilitating a rapid immune response within minutes to hours of infection. Components of innate immunity include (i) epithelial barriers between the environment and the host, comprising of skin and mucosal surfaces of the gastrointestinal and respiratory tract; (ii) phagocytes including monocytes, macrophages and neutrophils and other immune cells such as dendritic cells, NK cells, basophils and eosinophils; and (iii) members of the complement system, anti-microbial peptides and cytokines[3].

All the aforementioned constituents of innate immunity recognise pathogens through their pattern-recognition receptors that are generated in the germ-line. These receptors therefore have limited diversity where identical receptors are expressed on all cells of the same lineage (nonclonal). This is why the innate immune system is often referred to as "non-specific" and/or naïve. Such pattern recognition receptors include Toll-like receptors (TLR)[4,5], N-formyl-methionyl receptors, mannose receptors and scavenger receptors. These receptors recognise and bind microbial patterns on the surfaces of microbes, also known as pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs), which are essential for the survival of the microbes. To optimise protection of the host, the 11 TLRs discovered thus far are partially localised inside the cell, particularly inside the endosome where they recognise and bind phagocytosed bacterial nucleic acids (single- and double-stranded RNA and DNA), while some TLRs are also localised on cell surfaces[6].

Phagocytes of innate immunity i.e. monocytes, macrophages and neutrophils are responsible for identifying, ingesting and destroying bacteria, where the phagocytosed microbes are killed by lysosomal enzymes, reactive oxygen species and nitrogen species[7,8]. Also, when encountering pathogens these phagocytic cells secrete pro-inflammatory cytokines (e.g. TNF-α, IFN-α/β, IL-1, IL-6, IL-12) that

lead to the stimulation and maturation of dendritic cells[9]. Unlike other phagocytes, dendritic cells are considered the most effective of antigen-presenting cells, since they are capable of antigen presentation on both major histocompatibility complex (MHC) class I and MHC class II molecules, leading to activation of $CD8^+$ and $CD4^+$ T cells, respectively[10]. This results in subsequent activation of the adaptive immune system. Meanwhile, other cellular components of the innate immune system such as eosinophils, basophils and mast cells are known to eliminate parasites. Moreover, NK cells are involved in the elimination or "killing" of tumour cells and virally infected cells where cells that do not express MHC class I molecules on their surface are identified by the NK cells and accordingly eliminated[11,12].

1.1.2 Adaptive immunity

When a pathogen manages to penetrate the innate immune system of the host, the adaptive immune system becomes activated. Although this adaptive response takes a longer time frame in order to reach its full-scale potential (days to weeks), it remains the most specialised out of the two systems with increased specificity to the encountered pathogen alongside ability to generate B and T cell memory that assists in mounting a rapid immune response upon recurrent infection with the previously encountered pathogens[2].

The most important cells of the adaptive immune system are the B and T lymphocytes, where the B cells originate mainly in the bone marrow while T cell development starts in the thymus, hence the acronyms "B" and "T". Another explanation for the "B" acronym is the fact that these B cells were first discovered in chicken in the organ bursa fabricius, where haematopoiesis takes place in this organism. Haematopoiesis is the process where the different blood cells develop from haematopoietic stem cells (from the Ancient Greek "blood" and "to make"). Mammals generally do not seem to display an equivalent organ. Instead, the bone

marrow has been shown to be the site of both haematopoiesis and B cell development. After development in the bone marrow and the thymus these "naïve" B and T lymphocytes migrate to secondary lymphoid organs (lymph nodes and spleen), where they become activated. There are two main lineages of T cells, namely $CD8^+$ cytotoxic T cells and $CD4^+$ helper and regulatory T cells. The $CD4^+$ T cells recognise antigens presented on MHC class II molecules by antigen presenting cells, including dendritic cells, which causes them to become activated. Thereafter, the $CD4^+$ T cells differentiate into different subclasses, including Th1 and Th2 helper T cells, both of which produce pro-inflammatory cytokines. The Th1 subset produces interleukin-2 (IL-2) and interferon-γ (IFN-γ) and in turn triggers macrophages to phagocytose antigens, while the Th2 subset secrets IL-4, IL-5, IL-13 and IL-25 and results in B cell activation and antibody production[3]. Unlike the $CD4^+$ T cells, the $CD8^+$ cytotoxic T cells recognise antigens presented by pathogens on MHC Class I molecules, and in turn trigger apoptotic mechanisms in the infected cells[13,14]. Additionally, the regulatory T cells (Tregs) are regarded as suppressors, since they are involved in maintaining tolerance to self-antigens and produce anti-inflammatory cytokines such as IL-10, IL-35 and TGF-β[15,16].

In order to activate naïve T cells into effector T cells three signals are required[17] (i) presence of peptide on the MHC molecule; (ii) expression of co-stimulatory molecule B7 (CD80/CD86) on the antigen presenting cell that is recognised by CD28 on the T cell; and (iii) secretion of stimulatory cytokines by the antigen presenting cell (e.g. IL-12). However, the B7 molecule on the antigen presenting cell is not only recognised by CD28 on the T cell, it can also bind cytotoxic T lymphocyte antigen 4 (CTLA-4) that belongs to the T cell inhibitory receptors, and could thus result in inhibition of T cell responses[18]. This is why the outcome of antigen presentation to the T cell depends on maintaining a balance between these stimulatory and inhibitory molecules. Once activated these T cells differentiate into effector T cells ($CD4^+$, $CD8^+$, Tregs) and memory T cells that enter circulation and may migrate further to peripheral tissues[3].

Although B cells drive the humoral part of adaptive immunity, they are the producers of antibodies, and are also capable of antigen presentation. Here the B cell receptor captures the soluble antigen, and then the internalised antigen is processed and presented on the MHC class II molecule to the $CD4^+$ T cell. This is followed by binding of the $CD4^+$ T cell to the MHC class II molecule, which in turn triggers co-stimulatory signals mediated by CD40 ligand and effector B cell cytokines (e.g. IL-2, IL-4, IL-6, IL-12, TNF-α, IFN-γ) that result in B cell activation[19-21]. Moreover, although TLR activation of cells is considered a part of innate immunity, the activation of B cells may also occur via binding of their TLRs to antigenic ligands. An example of this is binding of B cell TLR9 (in the endosome) to internalised CpG-containing bacterial DNA[22]. Regardless of the means of activation, once activated the B cells differentiate into antigen-specific memory B cells, in addition to short- and long-lived antibody secreting plasma cells, all of which are the focus of this doctoral thesis work and will therefore be discussed in more detail in the following sections.

1.2 B CELL DEVELOPMENT

Before birth B cell development takes place in the fetal liver where they develop into B1 B cells. Meanwhile, during fetal development pluripotent stem cells migrate from the liver to the bone marrow as the bones develop. These newly produced B cells in the bone marrow then develop into a new lineage of B cells, namely the B2 B cells. After developing in the bone marrow these B2 B cells then migrate to the periphery where they differentiate further upon activation in secondary lymphoid organs. The first step of B cell development in the bone marrow occurs when the B cell initially develops into a pro B cell that expresses surface marker CD19. This is followed by a process known as VDJ-rearrangement that allows the pro B cell to rearrange its variable (V) diversity (D) joining (J) immunoglobulin (Ig) gene segments to form a pre B cell receptor that consists of a heavy chain and a light chain[23]. This results in both combination diversity followed by joining diversity where the recombination of V, D and J gene segments allows enormous diversity in the specificity of the B cell receptor. Once the formation of the heavy chain of the B cell receptor is successful it will be followed by production of the light chain. Combined, the heavy chain and the light chain then form membrane bound IgM, as expression of surface marker CD20 now becomes upregulated. At this stage the B cell is termed “immature”.

However, before the immature B cell can exit the bone marrow and migrate to the periphery it must first undergo several checkpoints to ensure that this newly developed B cell is not self-reactive, a process referred to as central tolerance[24-26] (explored further in segment 1.3.1). During this stage of development surface marker IgD is also upregulated. The mature B cell co-expressing IgM and IgD is now ready to leave the bone marrow, migrate to secondary lymphoid organs and meet its antigens in order to differentiate further into memory B cells and antibody producing plasma cells. The maturation of the B2 subset of B cells consists of five developmental stages, referred to as Bm1, Bm2, Bm3, Bm4 and Bm5. This stage of B cell development involving bone marrow maturation is

often referred to as the Bm1 stage. A schematic illustration that provides an overview of B cell development is presented in Figure 2.

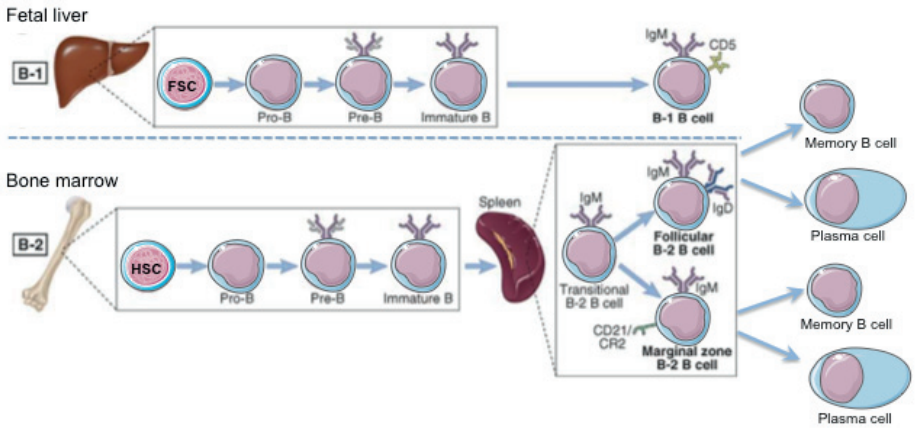

Figure 2. The different stages of B cell maturation. The B1 subset of B cells originates in the fetal liver. The development of the B2 subset of B cells originates in the bone marrow, followed by migration and activation in secondary lymphoid organs where the mature B cells differentiate further into memory B cells and plasma cells. Figure was produced using Servier Medical Art and adapted from Abbas *et al*[3].

1.2.1 Secondary lymphoid organs and germinal centre formation

Secondary lymphoid organs include the spleen and lymph nodes, consisting of follicles that contain B cell zones and T cell zones where B cells and T cells reside and differentiate, and in turn form structures known as germinal centres. Once in the peripheral lymphoid organ the B cell can meet its antigen and migrate to the T cell zone. Here the Bm1 transitional B cell is activated upon binding of CD40 on the B cell to CD40 Ligand on the $CD4^+$ Th2 cell[27]. The activated B cell then migrates to the germinal centre (Bm2 stage of development) where it proliferates further and the differentiating B cells form the dark zone of the germinal centre (Bm3 stage of development). Moreover, changes in the *Ig* locus of the B cell also

occur at this stage. These changes involve both somatic hypermutations in the locus and isotype class switch recombination, two processes that are regulated by the enzyme activation induced cytidine deaminase (AID)[28]. During somatic hypermutations changes are induced in the variable region of the B cell receptor aiming to enhance the binding affinity of the B cell receptor to the antigen. This is followed by migration of the B cell to the light zone of the germinal centre and antigen presentation by Fc receptor of the follicular dendritic cells to the BCR of the B cell (Bm4 stage of development)[29]. This is a checkpoint where high-affinity recognition of antigen by the B cell receptor leads to selection of that particular B cell and its survival[30]. Meanwhile, B cells with low affinity on their B cell receptor that fail to bind strongly to the antigen presented by the follicular dendritic cell undergo apoptosis and are eliminated[31].

The non-variable region of the heavy chain, on the other hand, can undergo isotype class switching. This is an irreversible process of DNA recombination where the B cells switch isotype to produce antibodies with heavy chains of different classes, including IgG, IgA and IgE. The cytokine environment provided by the $CD4^+$ T cells is the main determinant for the isotype induced. More precisely, IL-4 stimulates IgG_4 and IgE production, IL-10 induces IgG_{1-3} and IgA, while IL-21 promotes IgE[32-36]. In general, viruses and bacteria lead to IgG class switching, protozoa induce IgE production, while IgA is produced for protection interceded over mucosal membranes. In addition, a number of stimulatory factors have also been shown to induce class switching, these include CD40 Ligand, B cell activating factor (BAFF), a proliferation inducing ligand (APRIL), TLR3, 4, 8 and 9[37-44].

After surviving the germinal centre process the B cell then goes on to develop into either a memory B cell or a plasma cell (Bm5 stage of development). An illustration of germinal centre reactions and $CD4^+$ T cell dependent B cell activation is shown in Figure 3.

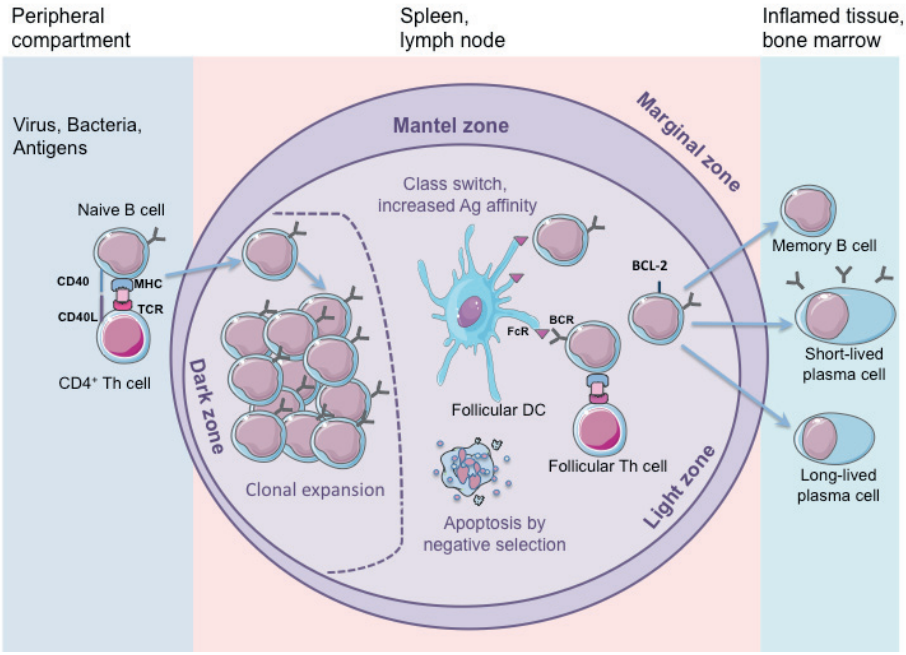

Figure 3. Germinal centre reactions and T cell dependent activation of B cells. At the edge of a primary follicle in secondary lymphoid tissue the B cell is activated by a helper T cell, migrates into the germinal centre and proliferates, forming the dark zone. This is followed by somatic hypermutation in Ig variable genes of the proliferating B cells and their migration to the light zone where they encounter follicular dendritic cells that present the B cells with antigen. B cells with the highest affinity on their Ig receptor are selected to survive, undergo isotype class switch recombination in their Ig non-variable chain, and differentiate further to memory B cells or antibody secreting plasma cells. Figure was produced using Servier Medical Art, adapted from Dr. Lilian Vasaitis, and inspired by Abbas *et al*[3].

1.2.2 Genetics of memory B cell and plasma cell development

B cells that successfully complete the germinal centre reaction develop either into a memory B cell or an antibody secreting plasma cell. The main role of a memory B cell is to rapidly proliferate and differentiate into a plasma cell upon re-encounter and re-stimulation with its specific antigen. Hence, the primary infection in the host is “remembered” and a swift second immune response can be

mounted when needed. These memory B cells have undergone somatic hypermutations upon initial encounter with the antigen, and are in most cases also class switched. During a second encounter with the same antigen these antigen-specific memory B cells will undergo affinity maturation through further somatic hypermutations, where some will also develop into plasma cells[3]. Memory B cells are known to survive in the host for long periods of time (even as long as the host)[45]. Although the bone marrow is known to be the homing site of memory B cells[46], these cells are also found in the periphery and can recirculate between secondary lymphoid organs of the host via the blood stream while scouting for their specific antigens[47].

Apart from memory, mature B cells can also develop into plasma blasts that still express surface-bound antigen receptors. These later give rise to professional antibody producing plasma cells that can secrete up to several thousands of antibodies per second! The differentiation of mature B cells into plasma cells is the result of changes in gene expression of certain regulatory factors in these cells[48]. More precisely, an upregulation of the transcription factor B lymphocyte-induced maturation protein 1 (Blimp-1) takes place[49]. Blimp-1 is normally under strong suppression by plasma cell inhibitors, including B cell lymphoma 6 (Bcl-6) protein and Paired box protein 5 (Pax-5)[50]. This repression is eliminated when NF-κB activation leads to the induction of interferon regulatory factor (IRF) 4 and the subsequent downregulation of the suppressive plasma cell inhibitors[51]. The upregulation of Blimp-1 that results from this process transforms the B cell from a non-secretory plasma blast to a highly specialised plasma cell with a well-developed endoplasmic reticulum (ER)[52]. More concisely, Blimp-1 promotes the production of X-box binding protein 1 (Xbp-1), which allows the expansion of the ER thus improving the cell's tolerance for large-scale protein synthesis and antibody secretion[53,54].

Fully developed plasma cells either exist as short-lived where they remain in circulation for weeks, or long-lived plasma cells that home to secondary lymphoid

organs, inflamed tissue, and more importantly the bone marrow where they can survive for decades given the right microenvironment and the presence of survival niches[55-58]. Plasma cell survival is further dependent on the migration capability of these cells to the survival niches, where bone marrow stromal cells have been shown to interact with receptors on the surface of the plasma cells and provide all the necessary signals required for plasma cell survival[59,60]. This long-lived subset of plasma cells is unique in its ability to produce antibodies despite antigen stimulation[61]. Moreover, due to their long life span, long-lived plasma cells in a way also contribute to the immunological memory of humoral immunity[62].

1.2.3 Characterisation of B cell subsets and their surface markers

B cells are usually identified by their expression of surface marker **CD19**[63]. This is also known as the general B cell marker on the surface of B cells from the pro B cell stage until the activation stage where it is expressed on both short- and long-lived plasma cells. Another common B cell marker is **CD20**, also expressed early in B cell development when the B cell reaches the "immature" stage before it exits the bone marrow[64,65]. However, although CD20 is present on memory B cells, it is downregulated once the B cell becomes activated and is therefore absent from the surface of plasma blasts, short- and long-lived plasma cells. Nonetheless, both CD19 and CD20 are components of the B cell receptor. Also, **CD24** is normally expressed on almost all B cells, promoting antigen dependent proliferation, yet prevents activation into plasma cells[3]. The different B cell subsets are otherwise identified by the expression of distinct additional B cell surface markers that will be explored further in this section.

B1 B cells

B1 B cells develop in the fetal liver of the host before birth. They are characterised by expression of surface marker **CD5**, and are believed to be self-renewing and long-lived. They give rise to so-called natural antibodies, which

participate in the host's first line of defence. These antibodies are T cell independent and of the **IgM** class. They therefore do not undergo class switching or affinity maturation, but instead recognise general pathogenic surface molecules[3].

Transitional B cells

During the development of B2 B cells in the bone marrow the expression of BAFF-receptor is initiated. After leaving the bone marrow, the survival of these immature B cells in the periphery depends on signals generated by BAFF (ligand of BAFF-receptor), thereby allowing the immature B cells to develop into first transitional-1 B cells and then further into transitional-2 B cells. This subset of B cells is characterised by expression of surface markers **IgM**, **IgD** and **BAFF-receptor**[66]. Overexpression of BAFF-receptor results in the differentiation of these transitional-2 B cells into marginal zone and follicular B cells. It has been shown, however, that overexpression of BAFF-receptor ligand (BAFF) could lead to the generation of self-reactive transitional B cells and consequently lead to the breakdown of tolerance[67].

Marginal zone B cells

Marginal zone B cells are present in the marginal zone of the spleen, hence their name, and are characterised by expression of surface marker **CD21** (also known as complement receptor 2 or CR2). These cells are able to communicate directly with antigen presenting cells, including macrophages and dendritic cells, and in turn are capable of giving rise to T cell *independent* responses via engagement of TLR9[68]. Similar to B1 B cells, marginal zone B cells have limited diversity and produce the **IgM** class of antibodies. This is why marginal zone B cells play a casting role in primary responses to antigens where they rapidly can proliferate into IgM secreting short-lived plasma cells and induce cytokine production[3].

Follicular B cells

Most mature naïve B cells are follicular B cells that have not yet met their antigen. They undergo VDJ-rearrangement and co-express membrane bound **IgM** and **IgD** on their surface. This co-expression grants these mature B cells the possibility to recirculate and reside in peripheral secondary lymphoid organs. They require antigen stimulation to become activated and therefore require collaboration with dendritic cells and $CD4^+$ helper T cells in the periphery[3].

Germinal centre B cells

After the B cell meets its antigen in secondary lymphoid tissue, it may migrate to the T cell zone where the B cells form a germinal centre with the T cells. Here both somatic hypermutations in the locus and isotype class switch recombination take place, as explained in the previous section[28]. After surviving the germinal centre reaction the B cell then goes on to develop into either a memory B cell or an antibody secreting short- or long-lived plasma cell (Figure 3).

Memory B cells

Memory B cells have the ability to rapidly proliferate and differentiate into antibody secreting plasma cells upon re-stimulation with their specific antigen in a T cell independent manner. They are characterised by expression of surface marker **CD27**[69]. Moreover, memory B cells that have yet to undergo class switching also express IgM and IgD, while both these surface markers are absent on class switched memory B cells[70,71].

Plasma blasts

Plasma blasts are considered the pre-stage of plasma cells. They are distinguished by expression of surface marker **CD38** and **CD27**, in addition to **IgD**[65]. This subset of B cells is known for being highly proliferative and migratory, two features that are diminished once they develop into plasma cells. A gradual upregulation of Blimp-1 occurs in these plasma blasts as they gradually mature into antibody secreting plasma cells[49,53,54].

Short-lived plasma cells

The short-lived subset of plasma cells usually ascends early in an immune response or develops from marginal zone B cells. Much like plasma blasts, short-lived plasma cells are also characterised by expression of surface markers **CD38** and **CD27**, yet lack IgD surface expression[65,72]. In addition to this, **CD138** (also referred to as Syndecan-1) is regarded the general plasma cell marker and is present on both the short- and long-lived subset of plasma cells[73].

Long-lived plasma cells

The long-lived subset of plasma cells is usually derived from the germinal centre reaction, where these class switched antibody secreting cells then migrate to the bone marrow and are able to continue producing antibodies for years, even after the antigen is eliminated, given the right microenvironment[55-57]. Similar to the short-lived subset, long-lived plasma cells are also distinguished by their expression of surface marker **CD138** and **CD27**, while lack IgD[72,73]. However, CD38 is absent on this long-lived subset of antibody secreting cells.

B regulatory cells

Much like the subset of T cells that have regulatory properties and can moderate immune responses, there exists a subset of regulatory B cells that have been characterised by **CD25** expression and production of the anti-inflammatory cytokine IL-10[74]. This IL-10 producing subset of B cells is of particular importance in autoimmune models[75,76].

An overview of the B cell subsets that have been covered in this doctoral thesis, and their consequent surface markers during the different stages of development are illustrated in Figure 4.

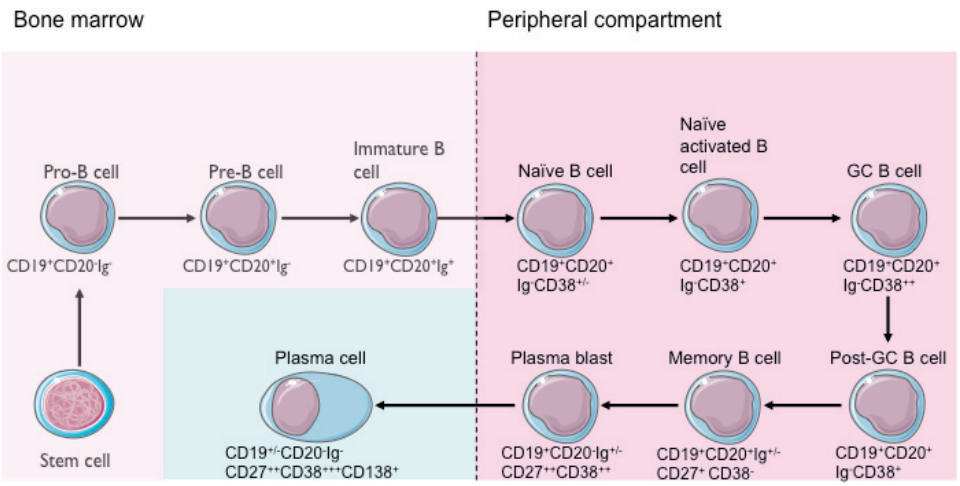

Figure 4. B cell differentiation and surface marker expression. The general B cell marker CD19 is present on all subsets of B cells from the pro B cell stage, while CD20 is expressed from the "immature" B cell stage of development up until memory B cell formation and is absent on antibody secreting plasma cells. CD27 is present on memory B cells, plasma blasts and plasma cells. CD38 is expressed when reaching the plasma blast stage of development and is also present on the short-lived subset of plasma cells, yet becomes downregulated when these develop into long-lived plasma cells. The general plasma cell marker is CD138, present on both short- and long-lived antibody secreting plasma cells, and is therefore absent on plasma blasts. Figure was produced using Servier Medical Art and adapted from Edwards and Cambridge, Nature Reviews Immunology 2006[77].

1.3 SELF-TOLERANCE AND AUTOIMMUNITY

1.3.1 Immunological tolerance

An important feature of B and T cell development is the eradication of self-reacting cells that could pose a potential threat in the host[78]. This concept is known as self-tolerance, and comprises of both *central* and *peripheral* tolerance[3,79,80]. In the case of B cells, central tolerance takes place in the bone marrow, where immature B cells that recognise self-antigens change their specificity by undergoing receptor editing and thereby express a new Ig light chain[81,82]. If in turn receptor editing fails to eliminate autoreactivity, the immature B cells may become deleted. As for T cells, central tolerance takes place in the thymus, where immature T cells that recognise self-antigens are either deleted or develop into T regulatory cells (a process that requires IL-2 and the transcription factor FoxP3)[80,83].

Peripheral tolerance comprises of mechanisms like anergy, deletion or immune suppression. Anergy is often defined as a state of "unresponsiveness" that does not involve cell death. Here the cell is either not stimulated at all, or undergoes incomplete stimulation. Anergic T cells are the results of recognition of self-antigens (presented by antigen presenting cells on MHC class-I molecules), while the second co-stimulatory signal (usually provided by binding of B7 on the antigen presenting cell to CD28 on the T cell) is blocked by the engagement of B7 with T cell inhibitory receptors (e.g. CTLA-4)[84]. B cells may also undergo a state of anergy when they recognise self-antigens in peripheral tissue in the absence of $CD4^+$ T helper cells. Autoreactive B and T cells can also be eliminated in the periphery either by overexpression of pro-apoptotic proteins or by activation-induced cell death. The latter involves both Fas and Fas ligand, where B cells undergo apoptosis upon binding of Fas (receptor on the B cell) to Fas ligand (on the follicular T cell), while activated T cells of the same cohort are eliminated by co-expression and binding of Fas and Fas ligand[85]. Another form of peripheral tolerance involves immune suppression via $CD4^+CD25^+$ T regulatory cells[86] or

$CD25^+$ B regulatory cells, and the secretion of anti-inflammatory cytokines by these cells, including IL-10[87,88].

1.3.2 Autoimmunity and autoimmune diseases

Despite the immune system's efficient ability to eliminate autoreactive B and T cells through central and peripheral tolerance, it sometimes fails to distinguish self from non-self, a concept known as autoimmunity[89]. This is a state where the host's immune system initiates an immune response against its own cells, tissues and proteins. These autoimmune reactions are antigen-specific and involve the presence of self-reactive lymphocytes[90]. Most individuals have some form of autoreactive lymphocytes without any apparent clinical signs. However, autoimmune processes can lead to the development of autoimmune diseases in some instances, affecting approximately 5% of the world's population[91].

Autoimmune diseases are either regarded as organ-specific, as in the case of type I diabetes and myasthenia gravis, or systemic, as for instance rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and Sjögren's syndrome (SS)[92,93]. Interestingly, women are more prone to most autoimmune diseases, especially in the case of SLE and SS, where 90% of those affected are female[94,95]. It has been suggested that sex hormones and gene-dosage effects of X chromosome genes might be involved[96-99]. Additional genes associated with SS include *IRF5*, *STAT4* and other genes that are involved in both innate and adaptive immune responses[100-104]. Moreover, recent studies from Swedish and Norwegian registries exposed several single nucleotide polymorphisms (SNPs) in the lymphotoxin α, lymphotoxin β, TNF (LTA/LTB/TNFα) locus that lead to amino acid changes associated with primary SS (pSS)[105].

1.4 SJÖGREN'S SYNDROME

SS is a systemic rheumatic autoimmune disease affecting the exocrine glandular function, where the salivary and lacrimal glands are the primary sites of inflammation[106]. Here, progressive focal mononuclear cell infiltration is seen in the glandular epithelium, leading to glandular dysfunction. Other sites of inflammation might also include glands in the skin, gastro-intestinal and genital tracts, as well as in the lungs and kidneys[107,108]. SS patients often suffer from the common symptoms of dry mouth and dry eyes (xerostomia and keratoconjuctivitis sicca), in addition to general symptoms of systemic inflammation such as fatigue, muscle and joint aches, low-grade fever and clinical depression.

SS can either exist on its own i.e. pSS, or as secondary (sSS) in combination with other rheumatic autoimmune diseases, most commonly RA and SLE[109,110]. Moreover, approximately one third of pSS patients develop extra-glandular manifestations, including different skin manifestations, arthritis, leucopenia and vasculitis. When classified according to the American-European Consensus Criteria (AECC)[111] the prevalence of pSS in the general population is very low. It mostly affects post-menopausal women, with a female to male ratio of 9:1 and a peak incidence at 40-50 years of age. Much like other autoimmune diseases the cause of SS remains unknown. However, it has been suggested that genetic predisposition, hormonal factors and viral agents, such as Epstein-Barr virus and retroviruses, could be potential triggers in disease development[112,113].

1.4.1 Circulating B cells in Sjögren's syndrome

Many features of pSS underline the important role of B cells in disease pathogenesis. For instance, patients with pSS produce high levels of circulating autoantibodies that target the self-antigens SSA (Ro52 and Ro60) and SSB (La48)[114,115]. This is observed in approximately 70% of the patients, and it has

recently been shown that autoantibodies could be detected in the patients long before symptom onset (as early as 18 years)[116]. Other autoantibodies include rheumatoid factor (RF), anti-nuclear antibodies (ANA), and anti-muscarinic acetylcholine M3 receptor antibodies[117-120]. In addition to this, B cell hyperactivity in pSS may also result in hypergammaglobulinaemia, with increased levels of IgG in patient sera[121]. Interestingly, patients with pSS show a characteristic alteration in their circulating peripheral B cell subsets, where decreased frequencies of $CD27^+$ memory B cells are observed in combination with increased levels of naïve B cells and plasma cells[122-124]. Moreover, an increase in the $CD5^+$ B cell population has also been described[125]. Nonetheless, whether B cell activation is a primary cause or a secondary effect in SS remains unclear[126].

1.4.2 Salivary gland involvement and disease pathogenesis

Focal chronic inflammation within the salivary gland of pSS patients is usually the result of infiltration and accumulation of mononuclear cells such as B cells, T cells, short- and long-lived plasma cells, macrophages and dendritic cells[127,128] (Figure 5).

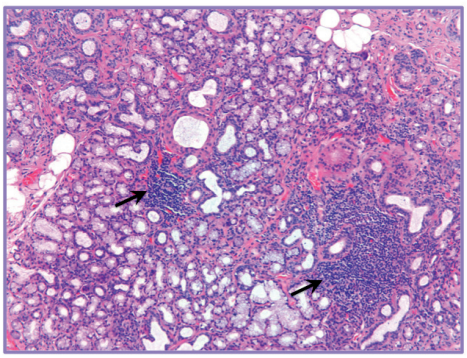

Figure 5. Lower labial minor salivary gland of a pSS patient. A haematoxylin and eosin (H&E) stained section of the minor salivary gland showing focal mononuclear cell infiltrates of >50 cells/mm^2 indicated with arrow. This patient has a focus score value of 1.

These infiltrating cells are in some cases able to organise themselves into B and T cell areas (zones) where the infiltrating B cells in the salivary gland tissue constitute approximately 20% of the total mononuclear cell infiltration[129,130]. This could in turn result in the formation of tertiary lymphoid structures, referred to as ectopic germinal centre-like structures, at the site of inflammation[131-134]. Structurally, these ectopic germinal centres in the salivary gland appear similar to the conventional germinal centres observed in secondary lymphoid organs, but whether there is a functional similarity between the two still requires further studies[135]. Similar ectopic structures have also been detected in other autoimmune diseases, such as RA, where germinal centre-like reactions in synovial tissue lead to the production of RF secreting plasma cells[136,137]. In pSS, however, local autoantibody producing plasma cells have been detected in the inflamed tissue[138,139].

Another important consequence of B cell accumulation in the target tissues of pSS patients is the development of non-Hodgkin's lymphoma in some instances[128,140,141]. This depends on whether the polyclonal B cell activation develops into an oligoclonal or a monoclonal B cell expansion, where the B cell proliferation is derived from either a few or a single clone(s) during disease progression, respectively. This could in turn lead to lymphoid malignancy, primarily low-grade marginal zone lymphomas[142]. In fact, the estimated prevalence of malignant lymphomas in pSS was considered around 6 to 15 times higher in pSS patients than in the general population, with a possible relation to the formation of the aforementioned ectopic germinal centre-like structures at the site of inflammation[142,143].

Aside from mononuclear cell infiltrates, epithelial cells in the salivary gland also play a vital role in disease pathogenesis, since they are capable of expressing factors needed in B cell migration, activation, maintenance and survival[144-146]. Consequently, several pro-inflammatory cytokines are usually overexpressed in pSS, such as IL-1β, TNF-α and IL-6[147,148]. Moreover, in relation to B cell

development, the novel TNF family members BAFF and APRIL, and their receptors (BAFF-receptor, BCMA and TACI) have been thoroughly explored in order to determine their possible role in the pathogenesis of pSS[39,149]. It has been shown that elevated levels of circulating BAFF in SS patients correlated with autoantibody production in these individuals[67,150]. Also, BAFF expressing cells have been detected in the salivary glands of SS patients and associated with attenuated apoptosis, where excess BAFF "rescues" the autoreactive B cells from peripheral deletion and inhibits B cell receptor-mediated apoptosis[67,151,152]. This in turn results in the accumulation of self-reactive B cells and allows them to enter the "forbidden" follicular and marginal zone niches, where they are prone to becoming activated.

1.4.3 The Ro52 autoantigen

Ro52, also known as TRIM21, was first recognised in rheumatology as an autoantigen in pSS and SLE, where autoantibody production against Ro/SSA has been shown since the late 1960s[153,154]. It was later reported that the autoantigen Ro/SSA appears to consist of two distinct proteins with a size of approximately 52 and 60 kDa, respectively[155]. Hence, the proteins were denoted Ro52 and Ro60. Although the term Ro52 is commonly used in autoimmunity when discussing autoantibodies, in the context of cellular functions the official name of the gene and the protein is TRIM21, as it belongs to the TRIpartite Motif (TRIM) protein family[156].

TRIM proteins are involved in innate and anti-viral immune responses, in addition to targeting certain molecules that are involved in cell proliferation, survival and apoptosis. They are RING-dependent E3 ubiquitin ligases that have been shown to act in the process of ubiquitination[157]. This is a post-translational modification, a process that labels protein for degradation, trafficking and activation[158-160], which in turn allows eukaryotic cells to control important

biological processes[161]. Structurally, TRIM proteins contain a Really Interesting New Gene (RING) and a B-box motif at its N-terminal, followed by a coiled-coil domain and a B30.2 (PRYSPRY) variable region in the C-terminal end[162]. Here, the N-terminal of the protein contains its active site, while the C-terminal mediates specificity (Figure 6).

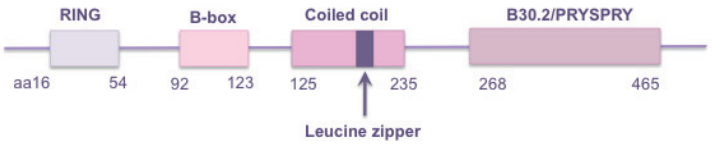

Figure 6. The structural domains of the Ro52/TRIM21 protein. The N-terminal of the protein contains its enzymatically active regions also known as the RING and B-box domains, followed by the coiled-coil. At the C-terminal the B30.2 (PRYSPRY) region is situated. Figure was adapted from Oke V *et al*[162].

Ro52 is an intracellular protein located predominantly in the cytoplasm and has been shown to have a regulatory role in inflammation as it has been found to ubiquitinate interferon regulatory factors (IRF) 3, 5, 7 and 8. This ubiquitination modifies the transcriptional activity of the abovementioned IRFs, which results in increased pro-inflammatory cytokine production including IL-12/IL-23p40, TNF, IL-6 and type I IFN[158,159,163-169]. Interestingly, SS patients seem to have autoantibody specificities against different epitopes of the Ro52 protein, including its RING, B-box and coiled-coil domains[170,171].

1.4.4 Classification criteria and diagnosis

The diagnosis of SS patients involves the application of a set of classification criteria that were initially developed for research cohorts and are now used in clinical practice. Since these classification criteria were established for research purposes they tend to favour symptoms of established disease, making it harder to diagnose a patient with a recent development of the disease. Currently the AECC are most widely used, and both pSS patients and subjects with sicca complaints (non-pSS controls) included in this doctoral work were classified accordingly[111]. Here pSS is defined by (A) the presence of four out of *six inclusion items* that includes both subjective and objective elements (ocular symptoms, oral symptoms, ocular signs, histopathology, salivary gland involvement and detection of circulating autoantibodies against the Ro/SSA and/or La/SSB antigens), or by (B) the presence of three out of *four objective items* (ocular signs, histopathology, salivary gland involvement and detection of circulating autoantibodies). An overview of the AECC is presented in Table 1.

Table 1: Revised AECC for Sjögren's syndrome (2002)

I. Ocular symptoms – a positive response to at least one of the following questions:

1. Have you had daily persistent troublesome dry eyes for more than 3 months?
2. Do you have a recurrent sensation of sand or gravel in the eyes?
3. Do you use tear substitutes more than 3 times a day?

II. Oral symptoms – a positive response to at least one of the following questions:

1. Have you had a daily feeling of dry mouth for more than 3 months?
2. Have you had recurrently or persistently swollen salivary glands as an adult?
3. Do you frequently drink liquids to aid in swallowing dry food?

III. Ocular signs – objective evidence of ocular involvement defined by testing positive for either:

1. Schirmer's I test, performed without anaesthesia (≤5 mm in 15 minutes)
2. Rose bengal score or other ocular dry score (≥4 according to van Biijsterveld's scoring system)

IV. Histopathology – in minor salivary glands (obtained from normal-appearing mucosa):

Focal lymphocytic sialoadentitis evaluated by an expert histopathologist with a focus score ≥1. This is defined as the number of lymphocytic foci (which are adjacent to normal-appearing mucous acini and contain more than 50 lymphocytes) per 4 mm^2 of glandular tissue.

V. Salivary gland involvement – objective evidence of salivary gland involvement by testing positive for at least one of the following:

1. Unstimulated whole salivary flow (≤1.5 ml in 15 minutes)
2. Parotid sialography showing the presence of diffuse sialectasias (punctuate, cavitary or destructive pattern), without evidence of obstruction in the major ducts
3. Salivary scintigraphy showing delayed uptake, reduced concentration and/or delayed excretion of taste

VI. Autoantibodies – presence in the serum of the following antibodies:

1. Antibodies to Ro/SSA or La/SSB antigens, or both

Adapted from Vitali et al[111]

Although the AECC have a high specificity (92.5%)[111], this set of criteria also includes subjective symptoms as part of the classification[172]. However, the Sjögren's International Collaborative Clinical Alliance (SICCA) research group proposed a new set of classification criteria in 2012 that was approved by the American College of Rheumatology (ACR). A comparison of the AECC and ACR criteria was recently performed on a well-characterised sicca cohort showing how both sets of criteria yield concordant results in the majority of cases[173]. Interestingly, the SICCA group criteria are based only on objective measures, where the concept of ocular staining score was introduced[174]. An overview of the ACR approved classification criteria is presented in Table 2.

Table 2: ACR approved classification criteria for Sjögren's syndrome (2012)

The classification of Sjögren's syndrome, which applies to individuals with signs/symptoms that may be suggestive of SS, will be met in patients who have at least two of the following three objective features:

I. Autoantibodies

Positive serum anti-SSA (Ro) and/or anti-SSB (La) or (positive rheumatoid factor and ANA ≥1:320)

II. Histopathology

Labial salivary gland biopsy exhibiting focal lymphocytic sialadenitis with a focus score ≥1 per 4 mm^2

III. Ocular staining

Keratoconjunctivitis sicca with ocular staining score ≥3
(assuming that the individual is not currently using daily eye drops for glaucoma, and has not had corneal surgery or cosmetic eyelid surgery in the last 5 years)

Adapted from Shiboski et al.[174]

In addition to the aforementioned sets of criteria, new diagnostic tools are being considered that may influence the sensitivity of diagnosis. An example of this is ultrasonography of the salivary gland, where one is able to explore some aspects

of the inflammation pattern in the parotid and submandibular glands[175]. This may be a helpful and practical tool for diagnosis and follow up in the future.

1.4.5 Treatment and B cell therapy

Thus far, the treatment of SS has been more focused on relieving the common symptoms of dry eyes and dry mouth, in addition to constraining the extraglandular manifestations when present[176-178]. Hence, various saliva and tear substitutes are often administered to patients for their sicca symptoms, and in some instances the salivary flow might be re-stimulated with the muscarinic receptor agonist pilocarpine[179]. In spite of its efficient improvement of dryness this drug also possesses unpleasant side effects such as increased perspiration and frequent urination. Hydroxychloroquin is commonly used for musculoskeletal manifestations whereas corticosteroids and immunosuppressive drugs are restricted to cases with severe extraglandular manifestations such as interstitial lung disease or interstitial nephritis[177,178].

Targeted therapeutic advances focus on the administration of monoclonal antibodies that target B cell subsets or B cell activating factors. This is due to the central role of B cells in the pathogenesis of SS. For instance, small studies with rituximab, a monoclonal antibody that binds the B cell surface antigen CD20, have shown a reduction in fatigue and an increase in salivary flow in the patients[180,181]. Other ongoing therapeutic attempts include the targeting of B cell surface markers CD22 and CD138, as well as inhibition of B-Lymphocyte stimulator (BLyS)/BAFF[182-184]. Inhibition of TNF-α, which has good efficacy in the majority of patients with RA, has not shown to be effective in pSS[178].

1.5 MOUSE MODELS FOR SJÖGREN'S SYNDROME

Various animal models have been developed for SS throughout the years, which elicit both spontaneous and experimentally induced disease development. The use of such animal models makes it possible to study different stages of disease development in a controlled environment, in addition to testing different therapeutic approaches[185]. Mouse strains that naturally develop an immune condition resembling SS were first described in the late 1960s[186]. Ideally, a model for SS should display the common symptoms of ocular and oral dryness, in addition to chronic inflammation in the lachrymal and salivary glands, and systemic immunological features that resemble the human form of the disease, such as antinuclear antibodies, autoantibodies and hypergammaglobulinemia[187].

1.5.1 The NOD and the NOD.B10.H2b mouse

Some of the best characterised animal models for studying SS include the Murphy Roth's Large (MRL/lpr) and the Non-obese Diabetic (NOD) mouse[77,188-192]. To date, the NOD mouse is one of the most used and thus best described out of these models[193]. However, the NOD model has also been applied to study insulin dependent diabetes mellitus[194]. Nonetheless, due to the loss of secretory function alongside lymphocytic infiltration in its exocrine glands this model has also been widely used for studying SS like disease development[195].

As the NOD mouse also develops diabetes, this model seems more appropriate for studying the secondary rather than primary form of SS. To overcome this predicament another congenic NOD strain has been developed, namely the NOD.B10.H2b mouse[156]. Here the NOD MHC I-A^{g7} locus has been replaced with the non-diabetogenic MHC I-A^{b} locus of C57BL/10 mice. Hence, due to the

lack of the diabetogenic locus this novel NOD.B10.H2b strain does not develop diabetes[73,196]. Moreover, the NOD.B10.H2b mouse also exhibits all the immunopathological manifestations of the human form of pSS such as loss of secretory function, histological features with lymphocytic infiltration of exocrine glands (lacrimal and salivary glands), the presence of hypergammaglobulinaemia and the production of antinuclear autoantibodies[162,197,198]. However, in contrast to human pSS, in NOD.B10.H2b mice anti-Ro/SSA and anti-La/SSB autoantibodies have not been detected, and there seems to be an equal distribution of disease development amongst both males and females[73].

Taking all the aforementioned features into account, the NOD.B10.H2b mouse represents a promising model for the study of pSS, and was therefore used in this doctoral work to characterise the plasma cell compartment in the salivary glands and bone marrow in order to gain better insight into disease pathogenesis.

2 AIMS

The overall objective of this study was to explore B cell specificity and pattern in pSS, by analysing the B cell pattern and autoantigen expression both systemically and in the salivary glands of pSS patients. Furthermore, a novel experimental model for pSS was used to investigate the B cell pattern with regard to disease progression both in the salivary glands and in the bone marrow.

The specific aims were:

I. Characterise the autoantigen-specific memory B cell and plasma cell pattern in peripheral blood from pSS patients, with regard to disease progression

II. Explore the general memory B cell and plasma cell pattern in the lower labial salivary glands of pSS patients

III. Characterise the Ro52- and Ro60-specific B cell pattern in the lower labial salivary glands of patients with pSS

IV. Investigate the expression of the SS target autoantigen Ro52 in lower labial salivary glands of patients with pSS, with regard to level of inflammation

V. Characterise the plasma cell pattern in the salivary glands and bone marrow of NOD.B10.H2b mice, and compare the plasma cell pattern before and after onset of disease

3 MATERIALS AND METHODS

3.1 PATIENTS AND CONTROL SUBJECTS

3.1.1 Peripheral blood from patients with pSS and healthy controls (Paper I)

In paper I we used heparinised whole blood from 23 pSS patients and 20 healthy control subjects (Table 3). The patients were recruited from the Department of Rheumatology, Haukeland University Hospital, Bergen, Norway. These consecutive patients were diagnosed according to the AECC[111], as explained more elaborately in the introduction. Informed consent was attained from all participants, and the Committee of Ethics at the University of Bergen approved the study (#2009686).

By applying an already established memory B cell ELISPOT assay[199] to an autoimmune disease such as SS, we examined the Ro/SSA and La/SSB specific memory B cell pattern in the peripheral blood of our pSS patients. Then, by assessing the total amount of Ro/SSA and La/SSB antibody secreting cells in the peripheral blood of these same individuals, as performed previously[115], we tried to establish a more complete picture of the B cell repertoire that is specific for Ro/SSA and La/SSB in these pSS subjects. Medical records and clinical data were obtained from patients' charts at the Department of Rheumatology, Haukeland University Hospital. This provided information collected during routine laboratory assessments such as rheumatoid factor (RF) detection, antinuclear antibodies (ANA), anti-Ro/SSA and anti-La/SSB. A summary of the patients' clinical characteristics is shown in Table 3.

Table 3: Clinical characteristics of patients included in paper I

Patient ID	Age (years)	Gender	ANA	SSA	SSB	RF titer	IgG (g/L)	IgA (g/L)	IgM (g/L)	Focus* score	Schirmer's¶ test	Salivary¶¶ flow
137	53	F	+	+	-	-	15.4	1.25	0.64	0	nt	nt
138	58	F	-	-	-	+	7.62	3.78	0.47	4	nt	nt
139	48	F	+	+	-	-	12.5	2.89	0.37	nt	+	6.7
141	64	F	+	+	+	+	21.4	4.38	3.1	3	+	1.0
144	69	F	+	+	-	-	6.58	1.08	0.85	2	-	12.0
146	79	F	+	+	+	+	11.6	2.37	0.73	-	-	nt
147	77	F	+	-	-	-	14.4	6.59	0.28	1	+	-
148	65	F	+	+	+	+	13.3	2.28	1.07	0	nt	nt
149	71	F	+	+	-	+	9.08	1.85	1.18	4	nt	0.2
150	60	F	+	-	-	+	9.76	3.13	1.08	4	-	0.0
151	62	F	+	+	-	-	11.5	4.04	0.86	2	-	2.0
152	60	F	+	+	-	-	7.89	2.21	1.00	0	+	1.4
153	50	F	+	+	+	-	14.7	2.32	0.61	2	-	1.0
155	68	F	+	+	+	+	17.6	2.76	2.37	nt	-	0.7
156	27	F	+	+	-	+	14.4	2.17	0.85	nt	-	nt
158	30	F	+	+	+	-	39.4	2.39	1.16	0	+	nt
159	67	F	+	+	+	-	27.2	3.25	1.05	-	+	1.0
160	42	F	+	-	-	+	11.1	1.82	1.00	2	-	0.5
161	68	M	+	+	+	-	14.3	3.29	1.41	nt	+	1.1
162	69	F	+	+	+	+	14.0	2.72	1.06	-	+	nt
163	65	F	+	+	-	-	15.7	2.17	0.63	1	-	0.0
165	51	F	-	-	-	-	8.49	1.94	0.59	1	+	0.6
166	67	F	+	+	-	-	8.11	2.15	1.47	nt	+	2.4

*Values are the number of focal infiltrates/4 mm^2 area containing >50 mononuclear cells. ¶Measure for tear production; normal tear production (negative test) ≥10mm/5minutes. ¶¶Values are in ml/15minutes (unstimulated flow); normal flow >1.5ml/15 minutes. ANA: antinuclear antibodies, RF: rheumatoid factor, NT: not tested.

3.1.2 Salivary gland tissue from pSS patients and controls (Paper II, III and IV)

In paper II and III we used lower labial minor salivary gland biopsies attained from 10 patients that were diagnosed with pSS, 9 of which fulfilled the AECC[111] for pSS. The biopsies were performed between the years 1992 and 2009 at the Department of Otolaryngology/Head and Neck Surgery at Haukeland University Hospital, Bergen, Norway. Haematoxylin and eosin (H&E) sections were evaluated by an oral pathologist in order to determine their focus score. This is defined as the number of mononuclear cell infiltrates (foci) with >50 mononuclear

cells per 4mm^2 of salivary gland tissue[200]. Since focus scoring is a semi-quantitative method, where focus score values may differ depending on how deep in the gland the sections were taken, the focus score values were re-evaluated for all 10 pSS patients. The re-assessed focus score range was found to be from 1 to 3. Individuals that were evaluated for pSS, but did not fulfil the AECC criteria and also displayed normal gland (NG) morphology with no focal inflammation served as non-pSS tissue controls with a focus score value of zero. All studied subjects gave their informed consent, and the Committee of Ethics at the University of Bergen approved the studies (#2009686).

In paper II the pSS patients were divided in four groups according to the degree of inflammation in their salivary glands; focus score 0, 1, 2 and 3, where the "focus score 0" group showed apparent non-inflamed salivary gland tissue and therefore served as a negative control. The general memory B cell pattern was examined in these salivary gland biopsies via double immunohistochemical staining, using markers CD20 and CD27. Additionally, CD138 was used to distinguish the plasma cells. Moreover, peripheral blood attained in the year 2010 from these 10 patients has also been examined in our previous study[201] (paper I) where the Ro/SSA- and La/SSB-specific memory B cell pattern and autoantibody production was assessed.

In paper III we examined the Ro52- and Ro60-specific B cell pattern in the salivary glands of the 10 pSS patients from our previous studies[201,202] (paper I and II). Expression of B cell markers CD19, CD5, CD20 and CD27 were studied alongside the Ro52 and Ro60 antigens using double immunohistochemical staining. The Ro52- and Ro60-specific cells were also quantified in these glands. An overview of the clinical characteristics of the pSS patients included in paper II and III is presented in Table 4.

Table 4: Clinical characteristics of patients included in paper II and III

Patient no.	Focus* score	BCZ¶ in SG	Ro52+¶ cells in SG	Ro60+¶ cells in SG	Ro52¶¶ secreting cells in PB	Ro60¶¶ secreting cells in PB	%Ro52⌘ of total IgG+ MBC	%Ro60⌘ of total IgG+ MBC	MBC¶ in SG
138	2	4	149	141	7	1	7	2	4
141	3	6	46	143	9	5	11	0	17
144	0	0	23	104	1	4	5	3	0
147	1	5	69	18	35	22	0	1	5
149	1	2	17	46	0	0	1	0	6
152	0	0	31	89	7	5	1	1	0
158	3	6	3	17	66	29	6	1	6
160	0	0	69	54	6	6	1	1	0
163	2	5	138	56	7	9	1	0	11
165	1	9	222	137	3	3	0	1	12

*Re-evaluated focus scores where values are the number of focal infiltrates/4mm^2 area containing >50 mononuclear cells. ¶Values are presented per 10mm^2 of salivary gland (SG) tissue. ¶¶Anti-Ro52 and anti-Ro60 secreting B cells measured by direct EISPOT previously and presented as spot forming cells/100000 PBMCs. ⌘Autoantigen-specific memory B cells measured by ELISPOT previously and presented as a percentage of IgG+ memory B cells. BCZ: B cell zone, SG: salivary gland, PB: peripheral blood, MBC: memory B cell.

In paper IV we used lower labial minor salivary gland biopsies from 28 pSS patients fulfilling the AECC[111]. These individuals were diagnosed at the Department of Medicine, Karolinska University Hospital, Stockholm, Sweden and the Department of Otolaryngology/Head and Neck Surgery at Haukeland University Hospital, Bergen, Norway between the years 1992 and 2013. Salivary gland biopsies from 20 subjects evaluated for SS at the same period and departments, but not fulfilling the criteria served as non-pSS tissue controls. This collaborative Swedish/Norwegian study included the 10 pSS patients used previously[201-203] (papers I, II and III) as part of the Norwegian cohort. The expression of Ro52 protein was assessed by single immunohistochemical staining of frozen and paraffin-embedded tissue sections with anti-human Ro52 (7.8C7) monoclonal antibody[167]. Furthermore, secreted Ro52 protein was measured in saliva and serum samples from these same individuals through a capture-ELISA assay. A schematic overview of the distribution of patient material for papers I, II, III and IV is illustrated in Figure 7.

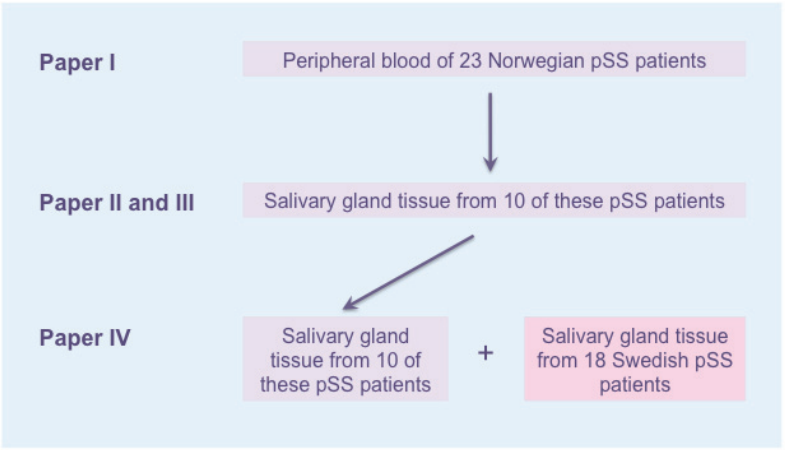

Figure 7. Analysed patient material. Peripheral blood from 23 pSS patients was included in paper I. Out of these 23 patients, salivary gland biopsies from 10 pSS patients were used in paper II, III and IV. Salivary gland biopsies from 18 Swedish pSS patients were also part of the study group in paper IV, as this was a collaborative study performed at the Experimental Rheumatology Unit, Karolinska University Hospital, Stockholm, Sweden.

3.2 MICE

In paper V we used female NOD.B10.H2b mice that were purchased from Jackson laboratories. This congenic mouse model exhibits all the immunopathological manifestations of the parental NOD mouse strain. However, due to the replacement of the NOD MHC I-A^{g7} locus with the non-diabetogenic MHC I-A^{b} of the C57BL/10 mouse, the NOD.B10.H2b model does not develop the complications of autoimmune diabetes and insulitis[156,198]. Nonetheless, in consistence with human pSS patients, the NOD.B10.H2b mouse develops the common clinical symptoms of secretory impairment, in addition to histological features such as lymphocytic infiltration of exocrine glands, particularly lacrimal and salivary glands.

3.2.1 Experimental layout of murine study

We wished to perform a histopathological characterisation of the target organs in the NOD.B10.H2b pSS diseased mouse in order to delineate the time sequence of plasma cell accumulation in both the salivary glands and bone marrow. Female NOD.B10.H2b mice and BALB/c mice were thus divided into 6 different age groups, those being 8, 11, 17, 24, 32 and 40 weeks. The distinction of short- and long-lived plasma cells was made feasible via the incorporation of bromodeoxyuridine (BrdU) in the mice through their drinking water. Since it has previously been reported that $BrdU^+$ plasma cells stabilise after 10 days of BrdU feeding[204], each age group of mice was given 1mg/ml of BrdU in their drinking water 11 days before sacrifice (Figure 8). Post sacrifice, both the salivary glands and the lymph nodes were fixed in formalin, while the humerus bone was first fixed in formalin and then placed in decalcification medium consisting of ethylenediaminetetraacetate (EDTA) and polyvinylpyrrolidone (PVP)[91]. The bone and tissue collected was further embedded in paraffin and used for immunohistopathological analysis of tissue sections using BrdU and CD138 monoclonal antibodies in order to gain better insight in disease pathogenesis and therapy (paper V).

Figure 8. Experimental layout of the murine study. Mice were treated with BrdU in drinking water for 11 days *before sacrifice*. They were then sacrificed at 11, 17, 24, 32 and 40 weeks of age. Figure was inspired by Dr. Ewa A. Szyszko.

3.3 METHODS

3.3.1 Direct and memory B cell ELISPOT (Paper I)

Enzyme linked immunospot assay (ELISPOT) is a well-established method for the detection and quantification of antibody producing cells in peripheral blood[205]. In our study we wished to assess the presence of anti-Ro/SSA and anti-La/SSB secreting cells in the peripheral blood of pSS patients, as demonstrated in previous studies [115,206,207]. In short, 96-well filter plates were coated with recombinant Ro 60-kD, Ro 52-kD and La 48-kD antigen (10 μg/ml), in addition to purified goat anti-human heavy chain-specific IgG (4 μg/ml) that was included as a positive control. The plates were then blocked, washed and incubated with the PBMCs isolated from the peripheral blood of pSS patients and healthy controls. Finally, the plates were developed by addition of Tetramethylbenzidine (TMBH) to each well and the enzyme activity was visualised as blue spots, where each spot represents one antibody secreting cell specific for Ro52, Ro60, La48 or IgG, respectively. These antigen-specific spots were counted using the ELISPOT plate reader.

Tracking of human antigen-specific *memory* B cells has also been made feasible through a generalised and sensitive memory B cell ELISPOT assay[199]. It has been used previously to quantify the number of memory B cells present in peripheral blood in response to different vaccines, hence evaluating vaccine efficiency in the recipients[208-212]. In our study this memory B cell ELISPOT assay was adapted to examine the Ro/SSA and La/SSB *specific* memory B cell pattern in the peripheral blood of pSS patients. Hence, by assessing these autoantigen-specific *memory* cells in addition to the total amounts of Ro/SSA and La/SSB antibody secreting cells in the same subjects, we tried to establish a more complete picture of the B cell repertoire specific for Ro/SSA and La/SSB in pSS patients. This memory B cell ELISPOT assay utilizes a 6-day polyclonal stimulation of PBMCs with a mitogen-mix, consisting of pokeweed mitogen[213] (1:100000), a CpG oligonucleotide[214,215] (3 μg/ml), and pansorbin[216,217]

(1:10000). Eight wells were cultured per individual, four of which were stimulated with the optimised mix of polyclonal mitogens, while the other four were non-stimulated negative control wells. The supernatant from both stimulated and non-stimulated wells was collected post this 6-day incubation for further analysis via ELISA, as described below. This was followed by an antigen-specific direct ELISPOT for the detection of memory B cells that have differentiated into antibody secreting cells in vitro as described above. An illustration of the experiment outline for memory B cell ELISPOT is illustrated in figure 9.

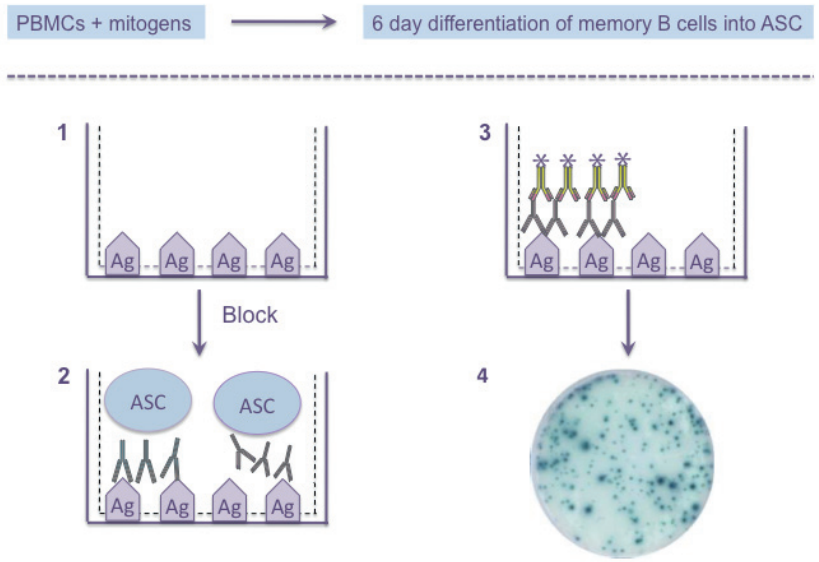

Figure 9. Experimental outline of the memory B cell ELISPOT. PBMCs were stimulated with a mitogen-mix consisting of pokeweed mitogen, a CpG oligonucleotide and pansorbin. This was followed by a 6-day incubation where the memory B cells could differentiate into antibody secreting cells (ASC). Then an antigen-specific direct ELISPOT was performed for the detection of memory B cells that have differentiated into ASC in vitro. Figure was produced using Servier Medical Art and inspired by Dr. Geir Bredholt.

3.3.2 Indirect and capture ELISA (Paper I and IV)

Enzyme linked immunosorbent assay (ELISA) is a colorimetric technique used to measure the presence of antibody (indirect ELISA) or antigen (capture ELISA) in a sample, most commonly in serum or saliva. In paper I indirect ELISA was employed where the autoantibody levels were measured in the plasma of the patients, in addition to the supernatant of cultured cells from all subjects, both cells stimulated with pokeweed mitogen and those non-stimulated (memory B cell ELISPOT assay). The ELISA plates were coated with recombinant Ro 60-kD, Ro 52-kD and La 48-kD antigens (10 μg/ml) and polyvalent goat anti-human IgG (2 μg/ml)[206]. Non-specific binding sites were blocked, after which the plates were incubated with blood plasma and supernatant, thereby allowing the antibodies to bind to the coated antigen, while human IgG was used as a standard. The unbound antibodies were then washed off in PBST and peroxidase-conjugated goat anti-human IgG was used as a detection antibody. The plates were developed by the addition of substrate solution that consisted of OPD diluted in ddH_2O and H_2O_2. The reaction was stopped with H_2SO_4 thus yielding a yellow colour. Finally, the absorbance was measured at 490 nm where absorbance values 1 s.d. above mean values obtained from our 20 healthy negative control subjects were considered positive. A schematic illustration of an indirect ELISA setup is illustrated below (Figure 10A).

In order to measure secreted Ro52 protein in saliva and serum samples (paper IV) a capture ELISA was established where ELISA plates were coated with Ro52 mouse monoclonal 7.1F2 antibody[167] (1 μg/well). The plates were then blocked, followed by incubation with patient sera or saliva in order to allow the secreted Ro52 protein in the samples to bind to the coating antibody. Purified full-length recombinant Ro52 protein expressed from the pMAL-vector was used as a positive control (1μg/well), while wild-type vector encoded maltose-binding protein was used as a negative control (1 μg/well). After washing the plates,

biotinylated Ro52 mouse monoclonal 7.8C7 antibody[167] was added, permitting the biotinylated antibody to bind to the secreted Ro52 protein in the sample already bound to the coating antibody. ALP-conjugated streptavidin was used for detection of bound antibodies and the absorbance was measured at 405 nm. A schematic illustration of a capture ELISA setup is illustrated below (Figure 10B).

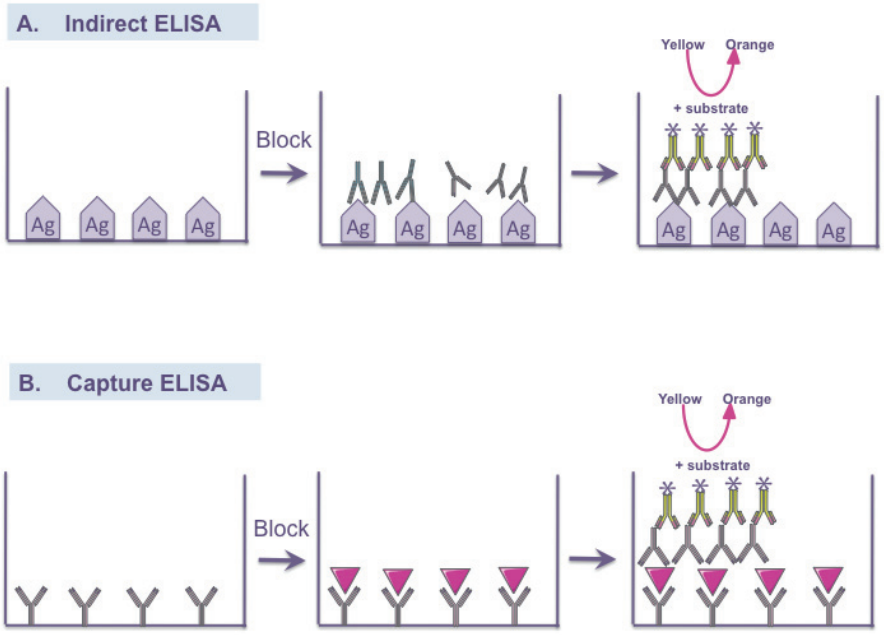

Figure 10. Indirect and capture ELISA. (A) Indirect ELISA was applied to measure the autoantibody levels in the plasma of patients, in addition to the supernatant of cultured cells from all subjects (memory B cell ELISPOT assay). **(B)** Capture ELISA was employed to measure secreted Ro52 protein in saliva and serum samples (paper IV). Figure was produced using Servier Medical Art and inspired by Oddgeir Selaas.

3.3.3 Immunohistochemistry (Paper II, III, IV and V)

Immunohistochemistry is a method used to detect antigens or antibodies in cells of tissue sections by utilizing the principle of antibodies binding antigens (or vice

versa) in biological tissue. Immunohistochemical staining (single- and double-staining) was employed in this thesis on human (paper II, III and IV) and murine (paper V) salivary gland tissue and bone marrow (paper V) sections, in addition to immunofluorescence (paper V). Both methods were based on firstly performing heat-induced epitope retrieval using the recommended buffer solutions. Then endogenous enzyme activity was blocked with 0.3% peroxidase (Dako), and blocking for avidin and biotin using Blocking Kit (Vector Laboratories). This was followed by incubation with monoclonal antibodies against the antigens of interest, except in the case of paper III where we first used Ro52- and Ro60-specific *antigens* (Arotec Diagnostics Limited, Wellington, New Zealand) to target the autoantibody-secreting plasma cells in the salivary glands of pSS patients, prior to incubation with the relevant primary antibodies. Then the appropriate secondary antibodies were used, and finally detection with a chromogen such as diaminobenzidine (DAB) or liquid permanent red (LRP). An overview of the primary antibodies used in all 4 studies is presented in table 5.

Table 5: Primary antibodies used in immunohistochemical experiments (paper II, III, IV and V)

Name		Clone	Supplier	Paper
Mouse monoclonal anti-human	CD27	137B4	Nordic BioSite	II, III
Mouse monoclonal anti-human	CD20	L26	Dako	II, III
Mouse monoclonal anti-human	CD138	MI15	Dako	II, III
Mouse monoclonal anti-human	CD19	LE-CD19	Dako	II, III
Mouse monoclonal anti-human	CD5	4C7	Dako	III
Mouse monoclonal anti-human	Ro52		Progen	III
Mouse monoclonal anti-human	Ro60		Progen	III
Mouse monoclonal anti-human	Ro52 (7.8C7)		Self-generated	IV
Rat anti-mouse	CD138	281-2	BD Biosciences	V
Mouse anti-BrdU			BD Biosciences	V
Rat anti-PNAd		MECA-79	BD Biosciences	V
Rabbit anti-mouse	CD27	EPR8569	Abcam	V

The immunohistochemical staining was performed using Envision technique for all human studies, while the Avidin Biotin Complex (ABC) method was applied additionally in the mouse study (paper V). The ABC method is based on using secondary antibodies that are conjugated to biotin. This biotinylated secondary antibody then functions as a link between tissue-bound primary antibodies and the avidin-biotin-peroxidase complex used for detection. The modern Envision staining method, on the other hand, utilises a horseradish peroxidase (HRP) polymer dextran backbone with detector secondary mouse Ig and rabbit Ig antibodies intact[218]. This results in an amplification of the detector signal, where biotinylated secondary antibodies are not needed. A schematic illustration of how the ABC and Envision staining systems work is shown in figure 11A and 11B, respectively.

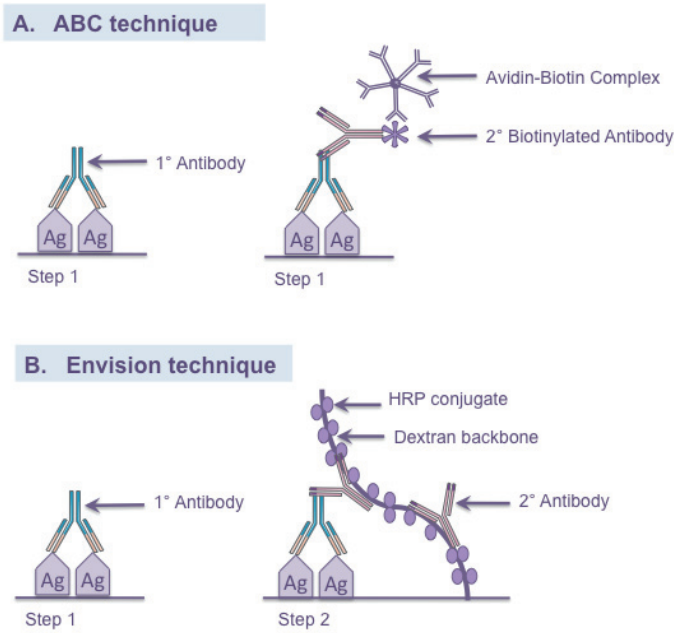

Figure 11. The ABC and Envision immunohistochemical staining systems. In the human studies the Envision staining system was utilised. The ABC method was applied additionally in the mouse study (paper V). Figure was produced using Servier Medical Art and inspired by Dako's Immunohisotchemical Staining Methods booklet (Fifth Edition).

3.3.4 Evaluation of staining

The minor salivary gland sections were evaluated post immunohistochemical staining by three investigators with the use of a light microscope (Leica, DMLB, Leica Microsystems Wetzlar, Wetzlar, Germany). A critical assessment of the microenvironment in the salivary gland tissue has been a great focus of this thesis. For this reason, we characterised both the mononuclear cells in focal infiltrates and also those located interstitially i.e. in close proximity to the acinar or ductal epithelium. Cells were considered positive when 50% or more of the cell membrane, cytoplasm or nucleus were positively stained. However, the intensity of cellular staining was not emphasised, except with regard to the ductal epithelial staining of Ro52 (paper IV), where we analysed the degree of ductal epithelial staining in relation to level of inflammation in the salivary gland tissue. With regard to our double-staining experiments (paper II, III, V), cells were considered in contact when more than 10% of the cell membrane of each cell was in tact with the other.

Furthermore, morphometry was also applied (paper IV). Here we quantitated the area of the salivary gland sections, and the mononuclear cell infiltrates stained with Ro52 in each gland (both frozen and paraffin-embedded tissue sections). Hence, we could then calculate the ratio index for each salivary gland section i.e. the inflammatory area of focal infiltrates in the section divided by the total glandular tissue area.

3.3.5 Statistical analysis

Statistical significance between two groups was evaluated by the Student t-test and presented as mean. Differences were considered significant when $p \leq 0.05$. In addition, the Spearman non-parametric correlation test was used to examine the association between the different parameters. All statistical analyses were performed using GraphPad Prism for Mac.

3.3.6 Methodological considerations

Verification of the memory B cell ELISPOT assay

In paper I the reliability of the adapted method for the stimulation of memory B cells was verified by the use of a memory B cell isolation kit (Miltenyi Biotech, Bergisch Gladbach, Germany), where memory B cells were isolated from PBMCs of pSS patients through depletion of unwanted non-B cells and subsequent positive selection with CD27Microbeads. The purity of the different eluates was then assessed by flow cytometry. This was followed by culturing of the different cell populations isolated with and without memory B cells, and subsequent stimulation with pokeweed mitogen and CpG, as described previously. As anticipated, lymphocyte blasting and clustering were only observed in the stimulated culture when the memory B cell population was present.

Paraffin-embedded versus frozen tissue sections

With regard to the immunohistochemical experiments (paper II, III, IV and V), we used formalin fixed paraffin-embedded minor salivary gland tissue sections, which could result in loss of immunoreactivity by antigens in the tissue. This is why we tested our primary antibodies on frozen tissue sections to address this matter. Moreover, in our collaborative study, the Swedish cohort consisted mostly of frozen tissue sections (paper IV). Also, in order to obtain staining on paraffin-embedded material, efficient heat-induced epitope retrieval was a crucial element for optimal staining, as staining is highly dependent on the fixation of the tissue. This is why we selected the appropriate epitope-retrieval solutions recommended by the different antibody producers for each staining experiment. Furthermore, we used the secondary antibody without primary antibody as a control for possible cross-reactivity with the tissue. As anticipated, these controls were negative. In addition, tissue sections from human tonsils were used as positive tissue controls for staining of lymphoid cells.

Focus scoring of salivary gland tissue

Although focus scoring of H&E stained sections is the standardised approach for assessing the degree of inflammation in the salivary gland biopsies when evaluating a patient for SS, this method has its limitations. Given that focus scoring is a semi-quantitative method, focus score values may differ depending on how deep in the salivary gland biopsy the sections were taken. This is why it is fundamental to make sure that one has sectioned the glands deep enough when performing H&E staining, to be certain that they are truly negative for mononuclear cell infiltration, as "false-negative" instances may otherwise occur. In order to avoid discrepancies and make focus score values more relatable to the different stainings in our studies, new H&E staining was performed on paraffin-embedded sections and the focus score values were re-evaluated.

Detection methods- Envision versus ABC

We preferred using the Envision technique in our studies in favour of the traditional ABC method[218-220], except in the case of paper V where both methods were applied. This is because the Envision based staining system utilizes a polymer backbone conjugated with both secondary antibodies and enzyme, which amplifies the signal, making it more sensitive than the ABC method. In turn, one is able to use higher dilutions of the primary antibody. Moreover, unlike the ABC method, the Envision system does not contain biotin, which is an advantage when working with salivary gland biopsies, as endogenous biotin may give rise to undesired background staining. Also, as the secondary antibody and enzyme (HRP or AP) are applied simultaneously, the protocol is less time consuming. However, the Envision system is unfortunately limited to antibodies generated in either mouse or rabbit.

4 SUMMARY OF RESULTS AND GENERAL DISCUSSION

4.1 CIRCULATING AUTOANTIGEN-SPECIFIC MEMORY B CELLS IN pSS

Over the years it has become more evident that B cells do indeed play a central role in the pathogenesis of SS[121,221,222]. More precisely, the continuous activation of B cells into antibody secreting plasma cells observed in SS patients contributes to the high levels of autoantibodies detected in those subjects[129]. Strikingly, this abnormal B cell differentiation goes hand in hand with a depressed percentage of circulating memory B cells detected in these individuals[122]. But what about the autoantigen-specific memory B cell pattern in SS patients? By adapting a memory B cell ELISPOT assay[199] that is usually used in vaccine studies to assess vaccine efficiency in generating memory B cells in the recipients[208-212], we were able to examine the Ro/SSA and La/SSB *specific* circulating memory B cell pattern in pSS patients. In combination with the direct ELISPOT assay[115] we were able to attain a more complete picture of the autoantibody-specific B cell pattern in the peripheral blood of pSS patients.

Our results show that when comparing the average numbers of Ro/SSA and La/SSB specific spot forming cells in both the direct and memory B cell ELISPOT assays, we observed a significant decrease in IgG^+ Ro and La specific memory B cell levels in comparison with the total IgG^+ antigen-specific B cell population. This might indicate that a greater portion of these memory B cells have been activated and have differentiated into antibody secreting plasma cells in these individuals. It has been shown earlier that there appears to be a skew in B cell differentiation in pSS leading to a decline in total circulating memory B cells, and a subsequent increase in levels of short- and long-lived plasma cells[122,223]. The phenotypic diversity of these plasma cells in the peripheral blood of pSS subjects has also been characterised previously by our group[223]. This was demonstrated via flow cytometry to quantify the different B cell subsets in the

peripheral blood of pSS patients. However, by utilizing the aforementioned technique of the memory B cell ELISPOT we were able to quantify the Ro/SSA and La/SSB *specific* memory B cells, in addition to the autoantigen-specific plasma cells (via direct ELISPOT). We henceforth were able to demonstrate that this decrease also applies for the autoantigen-specific memory B cells in the peripheral blood of pSS patients. Moreover, these lower Ro/SSA and La/SSB specific memory B cell levels and elevated levels of autoantigen-specific plasma cells may indicate that greater portions of the autoantigen-specific B cells are in an activated stage in the patients, which in turn results in high serum titres of autoantibodies[115,224,225].

Having established that there are low numbers of circulating autoantigen-specific memory B cells in the peripheral blood of pSS patients, we postulated the memory B cell pattern in the salivary glands, one of the major target organs in SS. Keeping in mind that the bone marrow has been shown to be the major homing site for B cell memory[46,226-228], could it also be that these memory B cells are migrating to the salivary glands in SS patients, and are therefore present in scarce numbers in the peripheral blood? It has previously been reported that these reduced levels of memory B cells in the periphery coincided with their accumulation in the salivary glands of pSS patients[229], a concept that we wished to examine further in our patient cohort (papers II and III).

4.2 GENERAL MEMORY B CELL PATTERN IN SALIVARY GLANDS OF pSS PATIENTS (PAPER II)

Focal inflammatory infiltrates are often observed in the salivary glands of SS patients. These consist of mononuclear cells such as B cells, plasma cells, T cells, macrophages and dendritic cells[127,128,230,231]. Nonetheless, B cells have been found to make up 20% of the infiltrating cell population in exocrine glands[207], where the majority of these comprises of short- and long-lived plasma cells in an activated stage[58]. Interestingly, memory B cells have also been shown to

accumulate in the salivary glands of SS patients[229,232], where this retention coincided with diminished levels of memory B cells in the peripheral blood of the subjects. This was shown by the immunoflourescent staining of parotid tissue attained from one SS patient that was also suffering from lymphoma, a diagnosis that could have a profound effect on B cell pattern in this individual. We have also previously observed a significant reduction of Ro/SSA and La/SSB specific memory B cells in the peripheral blood of our subjects[201], an observation consistent with what had been reported earlier on the general memory B cell pattern in peripheral blood of pSS patients[122,123,229]. This might have been due to the differentiation and activation of memory B cells into antibody-secreting plasma cells, but it could also be the result of retention of these cells in the target organs. For these reasons it was of particular interest to explore the implications diminished levels of circulating memory B cells had on the number of memory B cells infiltrating the salivary glands.

By applying double immunohistochemical staining with CD27[69,124] and CD20[65,71,233] on paraffin-embedded salivary gland biopsies we managed to identify the $CD27^{+}/CD20^{+}$ memory B cells and the $CD20^{+}$ B cell zones. Furthermore, single-staining with CD138[58,223,234] that was carried out on serial sections from the same patients in order to distinguish the $CD27^{++}/CD20^{-}/CD138^{-}$ plasma blasts[235], from the $CD27^{++}/CD20^{-}/CD138^{+}$ plasma cells[69,236-238]. Also, by using immunohistochemistry we were able to gain insight into the morphology and distribution of these cells within the gland, which would not have otherwise been possible with the use of immunofluorescence.

Contrary to what had been reported previously[229], we found the total number of $CD27^{+}/CD20^{+}$ memory B cells infiltrating the salivary gland to be very low and situated within the B cell zones of all the patients tested. Meanwhile, a high number of $CD27^{+}/CD138^{-}$ plasma blasts and $CD27^{++}/CD138^{+}$ short- and long-lived plasma cells were detected in these same subjects[58,202]. This was in consistency with what had been shown earlier by our group concerning

expression of $CD138^+$ plasma cells in the salivary glands of pSS patients and identification of markers necessary for plasma cell survival[58]. Consequently, lower levels of Ro/SSA and La/SSB specific memory B cells in the peripheral blood of our pSS patients resulted nonetheless in a low number of memory B cells in their salivary gland tissue. Regardless, high numbers of plasma cells and plasma blasts were still detected in both the peripheral blood and salivary glands of these subjects, implying an activation of these memory B cells into short- and long-lived plasma cells both in circulation and at the site of inflammation in the gland. This might explain why present therapeutic approaches in SS that target and eliminate $CD20^+$ cells result in a reduced number of B cells in the patients, while serum levels of autoantibodies remain unaltered, since the $CD20^-$ autoantibody producing plasma cells, which can be the long-lived subset, remain unaffected by treatment[239-241]. This is why attempts to target and eliminate $CD138^+$ short- and long-lived plasma cells have already been initiated in another more severe autoimmune disease, namely systemic lupus erythematosus[242]. However, although the effects of the anti-CD20 antibody Rituximab® are usually short-term and SS patients may experience relapse, it remains the most successful antibody so far developed for therapeutic purposes of this disease[243]. It has also proven to be effective and well tolerated by those SS patients that were additionally diagnosed with non-Hodgkin's lymphoma[180,244-247].

Given that our pSS patient cohort displayed a reduced number of Ro/SSA and La/SSB specific memory B cells in their peripheral blood that coincided with a reduced number of total memory B cells in their salivary glands, we were also curious about the autoantigen-specific B cell pattern in the salivary glands of these individuals and wished to investigate this further (paper III).

4.3 AUTOANTIGEN-SPECIFIC B CELL PATTERN IN SALIVARY GLANDS OF pSS PATIENTS (PAPER III)

Anti-Ro/SSA and anti-La/SSB autoantibody-producing cells have previously been identified in the salivary glands of SS patients[132,138,248,249], where the infiltrating B cells lead to the formation of ectopic germinal centre-like structures (GC) in approximately 25% of the cases, also known as the on-site dome of B cell differentiation in the inflamed tissue[133,250,251]. Haven characterised the autoantigen-specific memory B cell and plasma cell pattern in the peripheral blood (paper I)[201] of our pSS patients, in addition to the general memory B cell and plasma cell pattern in their salivary glands[202] (paper II), we were inspired to further explore the Ro/SSA-specific B cell pattern in the salivary glands of these same individuals.

Although Ro/SSA-specific cells have previously been detected in the salivary glands of SS patients through the ABC immunohistochemical staining method and the use of biotinylated Ro52 and Ro60 antigens[139,207,252], we applied the Envision double-staining system to our study cohort[218]. By using non-biotinylated Ro52 or Ro60 antigens along with CD19[65,223], CD5[253], CD20[65,71,233] and CD27[69,236], respectively, we managed to identify the Ro/SSA-specific cells in the salivary gland, and also account for the different subtypes of Ro52- and Ro60-specific B cells present. The application of immunohistochemistry on paraffin-embedded tissue additionally gave us insight into the distribution of the identified cells and salivary gland morphology.

As predicted, our results showed both Ro52- and Ro60-specific cells to be $CD19^{+}$, confirming that they are indeed B cells. Also, no Ro52- and Ro60-specific cells were found to be positive for CD5, indicating that these Ro/SSA-specific cells are of the B-2 subset of B cells. This is known as the B cell subset originating in the bone marrow, followed by the migration of the cells to

secondary lymphoid organs where they differentiate further into memory B cells and antibody producing plasma cells[253]. Furthermore, these Ro/SSA-specific cells were shown to be CD20 negative, and therefore not belonging to the memory B cell compartment. However, the Ro52- and Ro60-specific cells identified were nonetheless CD27 positive, suggesting that they could either be plasma blasts, short- or long-lived plasma cells. This is in tune with our aforementioned findings where low levels of *total* memory B cells in the salivary glands (paper II), and of autoantigen-specific memory B cells in the peripheral blood (paper I) of these same individuals have been observed alongside elevated levels of short- and long-lived plasma cells[201,202]. As memory B cells have been found to migrate to the bone marrow [226,254,255], the salivary glands might not be the major homing site of memory B cells in the pathogenesis of pSS. Still, it has also been established that long-lived plasma cells also tend to migrate to the bone marrow[56,58,183,234,256]. Therefore, another possible explanation for this lack of Ro52- and Ro60-specific memory B cells and elevated levels of short- and long-lived plasma cells in the salivary glands of our study group could be the result of activation of these Ro/SSA-specific memory B cells into CD20-negative plasma cells at the site of inflammation. This would in turn make these Ro/SSA-specific cells resistant to the CD20-directed therapeutic approach in SS.

Interestingly, these Ro/SSA-specific cells were detected in the salivary glands of all subjects, including the controls. Moreover, local production of SSA-specific autoantibodies in the salivary glands has previously been found to coincide with high levels of circulating autoantibodies in sera of SS patients[139], while our findings suggest that the presence of SSA-specific cells could possibly also be independent of the systemic serum levels, since only 40% of our patient group were positive for Ro/SSA-specific serum antibodies. Also, no correlation was found between the number of Ro/SSA-specific infiltrating cells and increase in focus score of our study population. However, as these Ro52- and Ro60-specific cells were located sporadically within the salivary glands and also interstitially, the degree of Ro/SSA-specific cells could in turn be independent of focus score.

Hence, as these Ro/SSA specific cells were detected in the salivary glands of all pSS patients and controls, regardless of serological positivity or focus score in the individuals, one could suggest this immunohistochemical staining for autoantigen-specific cells in salivary gland tissue as an additional predictor for SS development. However, due to the limited number of subjects included in our studies, this hypothesis needs to be tested further on a greater cohort for additional verification.

After accounting for the autoantigen-specific B cell pattern in one of the major target organs in SS, one cannot help but wonder about the expression pattern of the antigen itself. This postulation gave rise to our next collaborative study, where we explored the expression pattern of the Ro52 autoantigen in the salivary glands of pSS patients from both Norwegian and Swedish registries (paper IV).

4.4 RO52 AUTOANTIGEN EXPRESSION IN SALIVARY GLANDS OF pSS PATIENTS (PAPER IV)

To date the Ro52-specific cells have previously been explored in the salivary glands of pSS patients[138,139,203,207], while little is known about the Ro52 protein expression[248,257]. Haven already accounted for the B cell pattern of Ro52-specific antibody producing cells in the salivary glands of pSS patients, we now aimed to investigate the expression of the SS autoantigen target Ro52. We performed immunohistochemical staining on both frozen and paraffin-embedded tissue sections of lower labial salivary gland biopsies from Swedish and Norwegian pSS patients and controls. A previously generated and validated anti-human Ro52 monoclonal antibody that targets the coiled-coil region of the Ro52 protein (7.8C7)[167] was used in the staining experiments, and the Ro52 expression pattern was assessed thereafter.

Interestingly, while the Ro52 antibody producing cells were detected outside the focal infiltrates in our previous study[203] (paper III), the Ro52 autoantigen, on the other hand, was upregulated in the focal infiltrates themselves. Moreover, unlike the expression pattern of the Ro52 antibody producing cells, the Ro52 autoantigen was also observed in the ductal epithelium of the salivary gland tissue in both the patients and the controls. However, pSS patients with more focal mononuclear cell infiltration and a consequent upregulation of Ro52 in their infiltrates also exhibited a significantly higher Ro52 expression in their ductal epithelium compared to the non-pSS controls.

We then wished to investigate whether the Ro52 staining of ductal epithelium correlated to the degree of inflammation in the gland. Given that our cohort comprised of patients from two countries, where each health institution had their own routines for biopsy collection and focus scoring of the salivary glands, we wanted to re-evaluate the sections stained with Ro52 and re-assess the degree of inflammation in the glands. Since focus scoring is a semi-quantitative method that only accounts for focal infiltrates that comprise of >50 mononuclear cells/4mm^2 of tissue, we decided to apply morphometry and score the sections by calculating the ratio-index in each gland. The ratio-index is defined as the total inflammatory area of focal infiltrates in the section divided by the total glandular tissue area[55, 267]. This thus provided more information on inflammation severity and pattern for each patient in addition to the focus score. Interestingly, we found that the degree of ductal epithelium expression of Ro52 correlated to the level of inflammation in the subjects. Given that Ro52 is a ubiquitously expressed protein[158,170] known to be upregulated by several pro-inflammatory stimuli[160,161,258], together our findings further demonstrate how Ro52 expression is induced by inflammation. A similar pattern was evident in a previous study where the staining of skin lesions of SLE patients and healthy individuals showed that the expression of Ro52 coincided with the level of inflammation, and in turn was overexpressed in the patients[259].

Having detected high ductal epithelial expression of Ro52 in the salivary gland, we wished to investigate whether Ro52 protein could be secreted to saliva or serum, as is the case for other inflammation-related molecules such as HMGB1[260]. This is why a capture ELISA was established in which saliva and serum samples from both our patient cohort and non-pSS sicca controls were analysed. To our surprise, very little to no secreted Ro52 protein could be detected in saliva and serum samples of both pSS patients and non-pSS controls, suggesting that Ro52 protein is not secreted as part of the inflammatory process.

The increased chronic expression of Ro52 in an autoimmune setting may lead to peripheral breakage of tolerance especially in the salivary gland. Nonetheless, the development of autoantibodies against the Ro52 antigen is not merely dependent on high Ro52 protein expression levels, as the genetic background of the individual might also play a role in determining whether Ro52-specific autoantibodies do indeed arise during inflammation[166,171,261]. Furthermore, since overexpression of Ro52 has been associated with decreased cell proliferation and apoptosis induction[158], this could in turn explain how excessive mononuclear cell infiltration in the salivary glands eventually leads to tissue degeneration and impairment in saliva production, consequently resulting in the common symptom of dry mouth in the subjects.

4.5 PLASMA CELLS IN SALIVARY GLANDS AND BONE MARROW OF NOD.B10.H2b MICE (PAPER V)

We wished to further characterise the plasma cell compartment in the parotid and submandibular salivary glands, in addition to studying the plasma cell pattern in the bone marrow, since it was shown to be the homing site for the long-lived subset of plasma cells[61,240,262,263]. To address this notion, we used a congenic NOD strain, namely the NOD.B10.H2b murine model[156]. This strain exhibits all the immunopathological manifestations for SS present in the parental NOD[188]

mouse strain without the complication of developing diabetes. Moreover, by using a murine model we would also be able to compare the plasma cell pattern before the onset of disease in relation to advanced diseases progression[204], and in turn gain better insight in disease pathogenesis and present therapeutic approaches in SS.

After exposing the mice to BrdU, a synthetic nucleoside commonly used in the detection of proliferating cells[264], double immunohistochemical staining of both paraffin-embedded and frozen tissue sections from 8- and 40-week-old diseased mice were performed using BrdU and CD138 monoclonal antibodies. We identified both short- and long-lived plasma cells residing in the salivary glands and bone marrow of the autoimmune NOD.B10.H2b mouse, where the short-lived subset were $CD138^+/BrdU^+$, while the long-lived subset were $CD138^+$ but negative for BrdU. Interestingly, there was an accumulation of long-lived plasma cells in the salivary glands as the disease progresses, strengthening the idea that this particular long-lived subset of plasma cells is a major contributor to chronic humoral autoimmunity[183,204,265].

In the bone marrow, however, a rather similar staining pattern was observed for both the 8-week-old and 40-week-old NOD.B10.H2b mice, where some of the $CD138^+$ cells also expressed BrdU, while single-positive cells were also observed for each CD138 and BrdU, respectively. These single positive cells expressing CD138 alone illustrate the possible presence of long-lived plasma cells in both age groups. Meanwhile, the detection of long-lived plasma cells residing mainly in the bone marrow have previously been shown to produce autoantibodies without antigen stimulation, in addition to being able to withstand effects of today's therapeutic advances [61,240,262].

Furthermore, we also observed megakaryocytes in close proximity to the plasma cells in the bone marrow of these autoimmune mice, illustrating a possible presence of survival niches that propagate essential plasma cell survival

signals[266]. Actually, long-lived plasma cells have been shown to survive in the tissue with the support of such specific survival signals[193]. We verified this notion in our model by single immunohistochemical staining of the salivary glands and lymph nodes for PNad. These $PNad^+$ structures are known as high endothelial venules that are involved in the migration of lymphocytes to secondary lymphoid organs, and have also been associated to lymphoid neogenesis in autoimmune diseases (including SS) in accompanying mouse models[77,187,194-196]. Consequently, the detection of megakaryocytes in the bone marrow of our studied mouse strain is an additional implication of this.

Henceforth, our findings on the accumulation of long-lived plasma cells in the parotid and submandibular salivary glands of the NOD.B10.H2b mouse coincides with our observations in lower labial salivary glands of the pSS patients (paper II and III). Moreover, the detection of megakaryocytes in close proximity to the plasma cells in the bone marrow of these mice shows a possible presence of survival niches that provide essential plasma cell survival signals. However, a further investigation of the bone marrow microenvironment is needed to confirm this notion.

5 CONCLUSIONS

The main conclusions deduced from the studies enclosed in this doctoral thesis include the following:

- Decrease in Ro/SSA and La/SSB *specific* memory B cell levels in the peripheral blood of pSS patients
- High numbers of Ro/SSA and La/SSB specific autoantibody producing plasma cells in the peripheral blood of pSS patients
- Low number of total memory B cells in the salivary glands of pSS patients
- High number of plasma blasts, short- and long-lived plasma cells in the salivary glands of pSS patients
- Ro/SSA-specific cells identified in the salivary glands of pSS patients are B cells that belong to the B-2 subset.
- No Ro/SSA-specific memory B cells and elevated numbers of plasma blasts, short- and long-lived plasma cells are detected in the salivary glands of pSS patients
- Activation of memory B cells into plasma cells at the site of inflammation
- Upregulation of Ro52 in ductal epithelium might be a triggering factor for disease progression
- Detection of short- and long-lived plasma cells in the salivary glands and bone marrow of NOD.B10.H2b mice
- Accumulation of a possible long-lived subset of plasma cells in the salivary glands of NOD.B10.H2b mice as the disease progresses

6 FUTURE PERSPECTIVES

It has become more evident over the years that B cells do play a central role in the progression and pathogenesis of SS. In the present studies we have focused on addressing B cell specificity in pSS through a detailed characterisation of the general and autoantigen-specific B cell pattern, both in circulation and in the target organ of pSS patients. Interestingly, we detected Ro/SSA specific cells in all subjects, both in the pSS and control group, regardless of serological positivity or focus score values. Hence, immunohistochemical staining for autoantigen-specific cells in salivary gland tissue could be proposed as an additional predictor for SS development. However, due to the limited number of subjects included in our studies, this observation needs to be verified and our hypothesis should therefore be tested further on a greater cohort consisting of salivary gland biopsies from pSS patients and also control subjects that do not fulfil the AECC and have normal gland morphology with no signs of focal inflammation. Perhaps tonsils could also be used as an additional control for subjects that do not suffer from the common SS symptoms of dry eyes and dry mouth.

When examining the short- and long-lived plasma cell pattern in the bone marrow of the NOD.B10.H2b model, the detection of megakaryocytes in close proximity to plasma cells suggests the presence of plasma cell survival niches. However, a further investigation of the bone marrow microenvironment would aid in exploring this concept further. Also, further studies could be conducted on a pSS murine model in order to gain a better understanding of memory B cell homing during the pathogenesis of SS. This could include a more detailed examination of the bone marrow microenvironment, in addition to memory B cell pattern in both the salivary glands and lymph nodes of these mice.

Moreover, a further characterisation of Ro52 expression pattern is necessary with regard to B and T cell pattern in the minor salivary glands of pSS patients. This can be performed through immunohistochemistry and a series of double-staining experiments with the use of general B and T cell markers such as CD20 and CD3.

One of today's therapeutic challenges is targeting autoreactive plasma cells in autoimmunity. This is partially due to the lack of markers that distinguish the different plasma cell subsets. In addition to the immunohistochemical characterisation performed in this study, perhaps a further examination of the fundamental properties of short- and long-lived plasma cells could be conducted. This could be approached by firstly isolating these cells from tissue, followed by, for instance, B cell specific microarrays. One would thereby be able to identify possible new markers to distinguish the long-lived subset of plasma cells, and in turn target these cells in therapy and develop new treatments for SS.

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— *The best is yet to come!*
Frank Sinatra

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