PROTEOMIC CHARACTERISATION OF AN IMMUNODOMINANT 60-kDA RO/SSA AUTOANTIBODY IN PRIMARY SJÖGEN'S SYNDROME

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Summary

The Ro (SSA)/La (SSB) ribonucleoprotein complex is a frequent target of long-lived humoral autoimmunity in primary Sjögren's syndrome (SS), systemic lupus erythematosus and the neonatal lupus syndrome. High-titer IgG autoantibodies to Ro60 are a serological hallmark of primary SS and have been proposed as a key event in the initiation of human systemic autoimmunity. Whilst most research on Ro60 has been focused on mapping of Ro60 epitopes, little is known about the molecular characteristics of the anti-Ro60 autoantibodies themselves. Evidence for the existence of forbidden (autoreactive) B cell clones and secreted clonotypic autoantibodies in human systemic autoimmune diseases is limited. This thesis exploits the recent finding of an immunodominant epitope present on Ro60 (termed Ro60peg) to characterise a Ro60-specific autoantibody proteome in patients with primary SS.

A proteomic approach based on high resolution Orbitrap mass spectrometry (MS) was utilised to determine the clonality, isotype, and variable-region sequences of human autoantibodies directed Ro60peg in 7 patients with primary SS. Anti-Ro60peg IgGs purified from polyclonal sera by epitope-specific chromatography were analysed by 2-dimensional gel electrophoresis followed by combined database and de novo mass spectrometric sequencing. Remarkably, anti-Ro60peg-specific autoantibody responses comprised an IgG1 kappa restricted monoclonal species that was shared (public) across unrelated patients and specified by a V_H3-23 heavy (H) chain paired with a V_K3-20 light (L) chain.

The public anti-Ro60peg clonotype was specified further by common mutations in the H chain and L chain complementarity determining regions.

Further studies using high resolution Orbitrap MS were performed to track the evolution of the V_H3-23/ V_K3-20 Ro60peg public clonotypic autoantibody in 4 patients with primary SS. Direct sequencing of variable-region molecular signatures of clonotypes purified from serial serum samples collected retrospectively over 10 years revealed sequential clonal replacement. Prospective longitudinal studies confirmed clonotypic loss and replacement at approximately 3 monthly intervals. Levels of secreted anti-Ro60 clonotypes fluctuated markedly over time, despite minimal changes in affinity.

In conclusion, the mass spectrometric sequencing of a secreted anti-Ro60 autoantibody proteome has revealed a class-switched clonotypic autoantibody that is common (public) to different patients and specified by a unique H and L chain pairing. The expression of this somatically mutated public B cell clonotype implies a common breach of B cell tolerance checkpoints in patients with primary SS followed by antigen-driven clonal selection. These findings provide evidence in humans for Burnet's "forbidden" clone hypothesis. The application of proteomic technology to track the evolution of the secreted anti-Rop60peg autoantibody proteome in serial serum samples demonstrates a dynamic process of repeated clonal turnover that masquerades as long-lived Ro60 humoral autoimmunity.

Declaration

‘I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text’.

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Signed:

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Other publications during PhD candidature

Arentz, G., Thurgood, L. A., Lindop, R., Chataway, T. K. and Gordon, T. P. (2012) 'Secreted human Ro52 autoantibody proteomes express a restricted set of public clonotypes', *Journal of Autoimmun*ity, 39(4), 466-70.

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Abstracts and Communications

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List of Abbreviations

2DGE ..... two-dimensional gel electrophoresis

aa ..... amino acid

AUC ..... area under the dose-response curve

C ..... constant

CDR ..... complementarity determining region

D ..... diversity

ELISA ..... enzyme-linked immunosorbent assay

EBV ..... Epstein-Bar virus

FR ..... framework region

FT ..... Fourier transform

GAD ..... glutamic acid decarboxylase

H ..... heavy chain

Ig ..... immunoglobulin

IgG ..... immunoglobulin G

IGV ..... immunoglobulin variable

IT ..... ion trap

J ..... joining

k ..... kappa

K_D ..... equilibrium dissociation constant

L ..... light chain

MBP ..... maltose binding protein

MS ..... mass spectrometry

OD ..... optical density

PBS.....phosphate saline buffer

RNP.....ribonucleoprotein

SLE..... systemic lupus erythematosus

SS .....Sjögren's syndrome

UV .....ultra violet

V ..... variable

VH ..... variable-heavy

VL ..... variable-light

vWFA.....von Willebrand factor A

Chapter 1: Introduction

1.1 Introduction

Primary Sjögren's syndrome (SS) is a chronic autoimmune condition causing secretory gland dysfunction (for recent reviews see Fox 2005; Peri *et al.,* 2012). This leads to dryness of the main mucosal surfaces such as the mouth, and eyes. Primary SS may be more serious, with morbidity from other manifestations including vasculitis, lymphopenia, and interstitial nephritis. Currently, diagnosis is difficult and there are no proven pharmacotherapies to prevent or delay primary SS. The development of disease-specific diagnostics and potential therapeutic approaches has been prevented due to the poor understanding of the aetiology and pathogenesis of primary SS. This is probably multifactorial, involving a combination of genetic, environmental, and immune regulatory aspects.

The Ro/La ribonucleoprotein complex (RNP) complex is the most frequently targeted autoantigen in primary SS and is regarded as a primordial autoantigen involved in the initiation of human systemic autoimmunity (Heinlen & Scofield, 1991). Anti-Ro60 autoantibodies can occur prior to the onset of clinical symptoms (Arbuckle *et al.*, 2003; Eriksson *et al.,* 2011); and may exert pathogenic effects by initiating tissue damage in congenital heart block (Garcia *et al.,* 1994; Buyon *et al.,* 2003; Karnabi *et al.,* 2011). However, it is unknown whether the development of primary SS is fundamentally associated with the presence of anti-Ro/La autoantibodies, and whether they are a cause of primary SS or a parallel anomaly. Identification and characterisation of sub-sets of

anti-Ro60 autoantibodies can provide a valuable entry point to study the pathogenesis of primary SS and may offer potential improved diagnostic markers.

This thesis is concerned with the proteomic characterisation of an immunodominant Ro60 autoantibody in patients with primary SS. Chapter 1 presents an overview of the literature with an emphasis on primary SS and the interrelationship with anti-Ro60 autoantibodies. Chapter 2 describes related methodology. Chapters 3 and 4 present the experimental findings of this thesis. The final chapter 5 summarises the findings of the thesis and comments on future developments.

1.2 Primary Sjögren's syndrome

1.2.1 Clinical features of primary Sjögren's syndrome

Primary SS is a chronic autoimmune disorder characterised by mononuclear cell infiltration of the exocrine glands, predominantly lacrimal and salivary glands. Clinically, the disease is manifested by two severe symptoms: dryness of the mouth (xerostomia), and the eyes (keratoconjunctivitis). These symptoms are frequently accompanied by systemic involvement, and extraglandular manifestations, such as arthritis, vasculitis, interstitial nephritis, and neuropathy. Table 1.1 outlines the principal clinical manifestations of this syndrome. Patients with primary SS also have an increased risk of B cell lymphomas (for recent reviews see Fox 2005; Peri *et al.,* 2012).

Primary SS has a population prevalence of approximately 0.5% and a large female preponderance (female to male ratio 9:1).

Table 1.1 Systemic manifestations of primary Sjögren's syndrome

Clinical Manifestation	Prevalence (%)	References
Arthritis	Up to 50%	Haga & Peen, 2007
Fatigue	50%	Kassan *et al.,* 2001; Segal *et al.,* 2008
Dermatologic manifestations	Up to 55%	Al-Hashimi *et al.,* 2001; Kittridge *et al.,* 2011
Neuropathy	Up to 31%	Delalande *et al.,* 2004; Sene *et al.,* 2011; Scofield *et al.,* 2012
Pulmonary involvement	23-30%	Palm *et al.,* 2013
Interstitial nephritis	5%	Tzioufas *et al.,* 2007
Vasculitis	11-30%	Tzioufas *et al.,* 2007
Lymphoma	5%	Ioannidis *et al.,* 2002
Thyroid dysfunction	Up to 44%	Perez *et al.,* 1995; D'Arbonneau *et al.,* 2003
Myalgias	Up to 44%	Lindvall *et al.,* 2002
Congenital heart block in mothers	2%	Press *et al.,* 1996

Furthermore, there are two age peaks for primary SS, the first during early adulthood, the twenties and thirties and the second after menopause in the mid-fifties (Bowman *et al.,* 2004; Fox 2005). There is no single disease-specific diagnostic criterion for SS. Diagnosis is guided by the presence of 4 of 6 revised international classification criteria, which must include either histopathological or autoantibody criteria (Vitali *et al.,* 2002). The diagnosis of primary SS may be problematic. Clinical symptoms are often vague and occur long after the onset of disease. Therefore, misdiagnosis of the condition is common and it is not unusual for years to pass before a confident diagnosis can be made. Moreover, diagnosis is often hampered by the heterogeneity of manifestations. Currently, there is no cure and therapies are limited to ameliorating the patient's symptoms. Artificial tear and saliva substitutes; steroidal and non-steroidal anti-inflammatory agents; disease modifying agents; and cytotoxic agents are recognised as possible management options in the very infirm, but are non-selective and limited by their side effects (Fox 2005; Peri *et al.,* 2012). As such, primary SS and its associated symptoms remain a significant clinical problem that requires the development of novel disease-specific diagnostic tools, together with effective preventative or curative interventions.

1.2.2 Aetiology and pathogenesis of primary Sjögren's syndrome

Despite decades of research the aetiology and pathogenesis of primary SS remain elusive. Susceptibility to the disease can be ascribed to an interplay between genetic factors, environmental triggers and stochastic events. The genetic predisposition in primary SS is not well defined. Several studies have demonstrated increased

concordance rates in monozygotic twins (Besana *et al.,* 19991; Scofield *et al.,* 1997; Bolstad *et al.,* 2000) and in familial aggregation studies (Reveille *et al.,* 1992; Becker *et al.,* 1998; Tanaka *et al.,* 2001). The best-documented genetic risk factors for the susceptibility to primary SS are the major histocompatibility complex haplotypes HLA-DR and HLA-DQ **(**Cruz-Tapias *et al.,* 2012). In a study, HLA-DQ2 and HLA-B8 were linked with Caucasian patients with primary SS. (Fye *et al.,* 1978; Chused *et al.,* 1977; Reveille *et al.,* 1991). The haplotype HLA-DR3-DQ2 has also been linked with primary SS in American Caucasians, Hungarians, French, and Colombian populations (Kang *et al.,* 1993; Anaya *et al.,* 2002; Kovacs *et al.,* 2006). However, these haplotypes are more strongly associated with specific autoantibody profiles rather than predisposition to the disease itself (Rischmueller *et al.,* 1998).

While genetic factors may be necessary for the development of primary SS, they are not sufficient as these haplotypes can occur in asymptomatic individuals (Johannesson et al., 2006). Environmental triggers may also required for the initiation of autoimmune disease in genetically predisposed individuals. Factors suspected to promote the development of primary SS include, physical agents, infection, hormonal factors, and autoantibodies (for reviews see Yamamoto, 2003). Viruses are considered potential candidates for triggering the immune response either by direct cell injury, activation of toll-like receptors or molecular mimicry (Kivity *et al.,* 2009). In particular, Epstein-Barr virus (EBV) is thought to play a role in the pathogenesis of this disorder (Fox *et al.,* 1986; Mariette *et al.,* 1991; Wen *et al.,* 1998). Recent work has shown that autoantibodies against Ro60 cross react with EBV nuclear antigen-1 (Kelly *et al.,* 2006). Other viruses that have been implicated in the pathogenesis of primary SS

include human cytomegalovirus (Hsieh *et al.,* 2011), Helicobacter pylori (Hasni *et al.,* 2011), human immunodeficiency virus (Itescu *et al.,* 1992), and hepatitis C (Haddad *et al.,* 1992). Hormonal factors have also been suggested to contribute to the aetiology of primary SS in both human populations (Hayashi *et al.,* 2004; Taiym *et al.,* 2004) and experimental models (Blank *et al.,* 1990). The strong female predominance observed in this disorder suggests sex hormone involvement, however the mechanism has not been elucidated.

A major serological hallmark of primary SS is the production of autoantibodies against the SSA/Ro (Ro60) and SSB/La (La) RNP. Anti-Ro/La antibodies are non-organ specific and are found in 50% to 90% of patients with primary SS depending on the method used for detection (Locht *et al.,* 2005; Franceschini & Cavazzona 2005; Wahren-Herlenius *et al.,* 1999). The presence of Ro/La autoantibodies is also associated with early disease onset, longer disease duration, and exaggerated clinical manifestations (Yamamoto 2003). However the precise role of these autoantibodies in the development of primary SS is yet to be settled. Studies have shown that maternal anti-Ro/La autoantibodies initiate and perpetuate inflammation, tissue damage and scaring in the atrioventricular node and endocardium of the foetal heart leading to congenital heart block, the most serious manifestation of the neonatal lupus syndrome (Horsfall *et al.,* 1991; Buyon *et al.,* 1992; Garcia *et al.,* 1994; Buyon *et al.,* 2003). In a recent study Toker *et al.,* (2004) documented that severity of sicca symptoms in patients with primary SS is associated with the presence of autoantibodies against Ro/La in serum or tear fluid. These findings raise the question of whether anti-Ro/La antibodies play an important role in the development of primary SS in genetically predisposed

individuals. Ro/La autoantibodies are therefore of particular interest to both basic investigators and clinical immunologists who wish to translate laboratory findings to the clinic and use these disease-specific biomarkers as entry points for studying the pathogenesis of human systemic autoimmune diseases. Adding to their importance as translational research tools, these autoantibodies are known to occur in serum years before clinical presentation of primary SS and can therefore be regarded as early markers of the intrinsic loss of B cell tolerance that appears to be causal for disease (Heinlen *et al.,* 2010; Eriksson *et al.,* 2011). Investigations into Ro/La autoantibodies as antecedents in the development of primary SS might improve the understanding of the pathogenesis of primary SS with the ultimate objective of developing novel strategies to retard its progression. Therefore, many investigators have explored the structure, origin, and precise targets of these autoantibodies. As a background for this thesis, the following section will review the structure and function of the Ro/La RNP complex.

1.3 The Ro/La ribonucleoprotein complex

The Ro/La RNP complex is a frequent target of humoral autoimmunity in primary SS and has been considered a primordial autoantigen involved in the initiation of human systemic autoimmunity (Heinlen *et al.,* 2010). The particle is composed of a 60-kDa polypeptide that is complexed with small non-coding cytoplasmic RNA's termed Y RNAs (Wolin & Steitz, 1984). The 48-kDa La protein is transiently associated with the Ro/La RNP complex through binding with Y RNAs and potentially Ro60 (Figure 1.1).

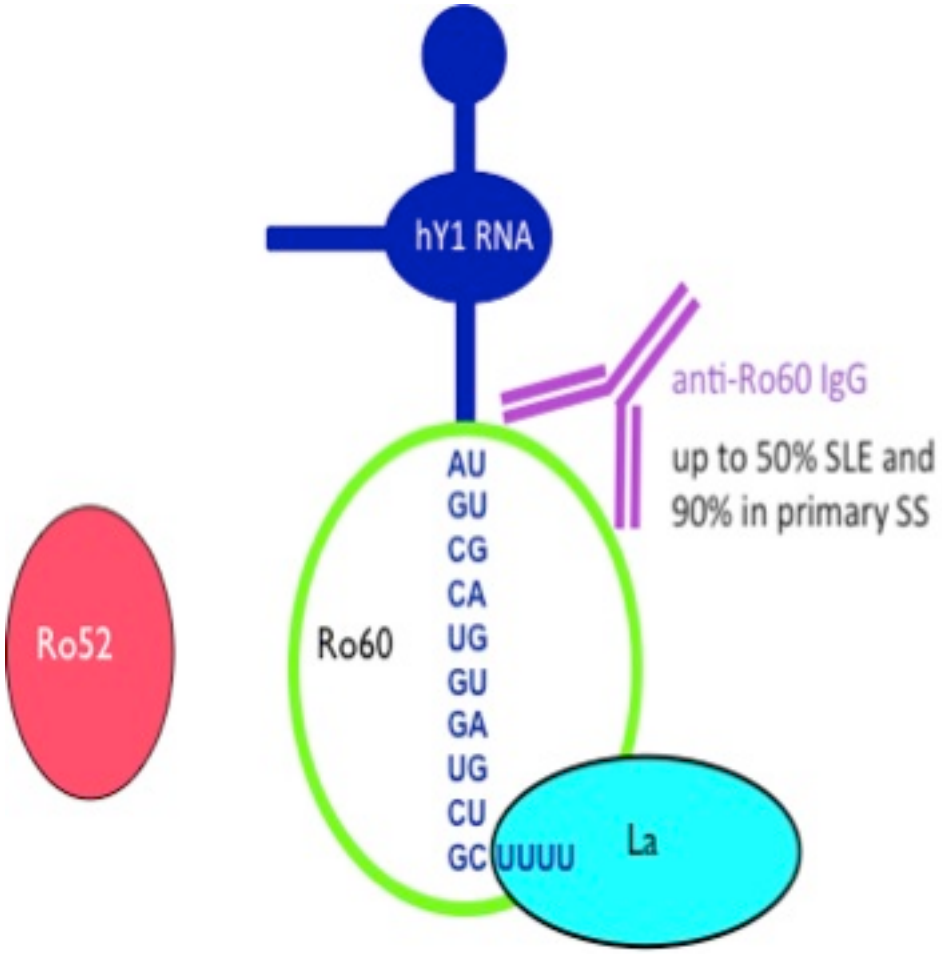


Figure 1.1. *The Ro/La ribonucleoprotein complex is composed of a 60-kDa Ro polypeptide complexed with small non-coding RNA termed hY RNA and a 48-kDa La protein. The complex is a frequent target of autoimmunity in primary* Sjögren's *syndrome (Adapted from van Venrooij et al., 1993).*

Most patients with anti-Ro60 antibodies produce autoantibodies to the structurally unrelated Ro52-kDa. Ro/SSA (Ro52) autoantigen, a member of the RING/B-box/coiled-coil tripartite motif protein family also termed TRIM21 (Schulte *et al.,* 2009). The localisation of the Ro/La complex is mainly cytoplasmic, although their protein components are also found in the nucleus (Harmon *et al.,*1984; Mamula *et al.,* 1989). Under certain circumstances (stress, UV radiation, or viral infection) components of the Ro/La RNP complex can also be found on the cell surface. The Ro/La complex is described briefly including notable structural and functional information.

1.3.1 Ro60 Structure

Ro60 is an evolutionary conserved and ubiquitously expressed RNA binding protein. This autoantigen is of relatively low abundance and therefore was not discovered prior to patient sera as a probe (Hendrick *et al.,* 1981). Studies have revealed that Ro60 is present in both the cytoplasm and nucleus (O'Brien & Wolin, 1994). Ro60 binds small cytoplasmic RNAs, RNA polymerase III transcripts, (called Y RNAs). In humans four Y RNAs have been identified (hY1, hY3, hY4 and hY5) of approximately 80 to 130 nucleotides (Wolin & Steitz, 1984). The Y RNAs are bound to the outer surface of Ro60 via the lower stem of the RNA formed by base pairing the 5' and 3' ends (Figure 1.2). Recently, the crystal structure of the *Xenopus laevis* Ro60 (78% identical to human Ro60) was solved (Stein *et al.,* 2005). This revealed that Ro60 consists of multiple alpha-helical (HEAT) repeats that form a toroid or 'doughnut-like' structure with a von Willebrand Factor A (vWFA) domain at the COOH-terminus (Figure 1.2).

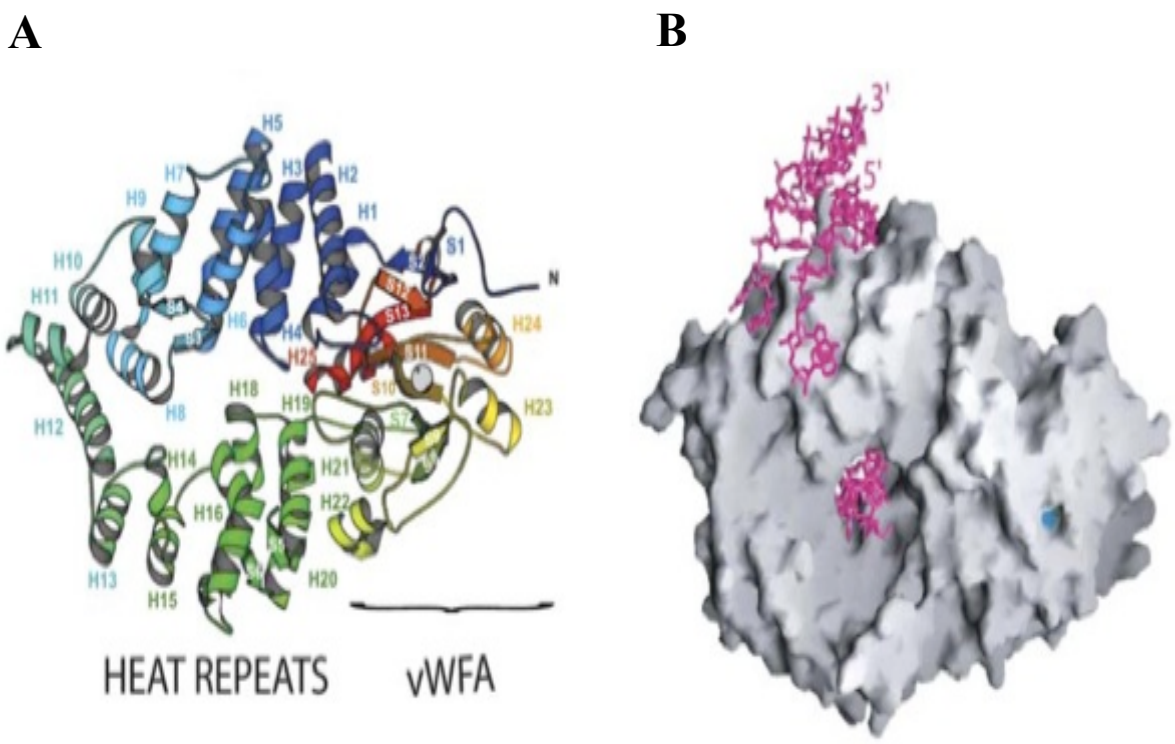


Figure 1.2. *The molecular structure of Xenopus Lavis Ro60.* ***A.*** *Ro60 is a toroid of alpha helical repeats with a von Wilebrand factor A (vWFA) domain at the COOH-terminus. Helices are labelled H1-H25 and the β strands are S1-S13. Blue shading indicates the NH_2-terminus; red shading distinguishes the COOH-terminus and the metal ion-dependent adhesion site (MIDAS) is shown as a grey circle.* ***B.*** *Crystal structure of the Ro60 polypeptide with bound RNA (pink). A fragment Y RNA is bound on the outer surface and a single stranded RNA is bound in the central cavity. The MIDAS motif is shown in blue. (Adapted from Stein et al., 2005).*

The vWFA domain is frequently found in extracellular matrix proteins and functions in cell adhesion and protein-protein interaction (Whittaker & Hynes, 2002). Within the vWFA domain is a metal ion-dependent adhesion site (MIDAS) motif, that functions as a cation-dependent ligand binding site (Dickeson & Santoro, 1998).

The 48 kDa La protein is physically linked with the Ro60 protein via the Y RNA; approximately half of patients with anti-Ro60 antibodies possess antibodies to La (Tan, 1989). The spreading of the immune response from Ro to La is thought to arise by intermolecular B-T cell help after initiation of T cell immunity to one component of the Ro/La RNP, most likely to be Ro60 (reviewed in McCluskey *et al.,* 1998). La associates with a range of RNA polymerase III transcribed RNA molecules including precursors of 7S RNA, 5S rRNA, tRNA, U6 RNA and Y RNAs as well as some virally encoded RNAs (van Wenrooij *et al.,* 1993). An oligouridine stretch at the 3' end of all RNA polymerase III transcribed RNAs constitutes the La binding site (Stefano, 1984). The physiological role of La is to serve as a termination factor for RNA polymerase III (Stefano, 1984) and to stabilise single stranded DNA during DNA metabolism (Roy *et al.,* 2009).

Autoantibodies to a structurally unrelated protein, Ro52 are invariably part of the anti-Ro/La linked sets but can also occur as an isolated species (Schulte *et al.,* 2009). Structural analysis of Ro52 reveals a zinc finger motif termed the RING finger at the NH_2-terminus and a Cys/His domain termed the B box. Other notable features of Ro52 include a predicted alpha-helical domain adjacent to the B box and leucine zipper motif.

The physiological role of Ro52 protein is not well understood; however, the protein had been reported to act as an E3 ligase in the ubiquitination process (Espinosa *et al.,* 2006). Determination of the Ro60 structure has made it possible to define the epitopes against which anti-Ro60 autoantibodies are directed.

1.3.2 Ro60 function

Although Ro60 was first described over 20 years ago the cellular function has remained enigmatic. Recent structural analysis has provided further insight into the role of the Ro60 autoantigen. O' Brien & Wolin (1994) documented that the inner hole of Ro60 forms a complex with 5S RNAs in *Xenopus laevis* oocytes. Interestingly, the inner hole of Ro60 is wide enough to accommodate single stranded RNA but not double stranded RNA (Stein *et al.,* 2005). Ro60 was also found to bind misfolded variant U2 snRNAs in mouse embryonic cells (Chen *et al.,* 2003). In another study Belisova *et al.,* (2005) showed that Ro60 can promote correct folding of a misfolded RNA substrate *in vivo*. These findings suggest that Ro60 may function in the quality control pathway for ribosome biogenesis and has led to the proposal that Ro60 may assist in destabilising RNA helicies and promoting correct folding. Further insight into the function of Ro60 comes from characterisation of an ortholog in *Dienococcus radiodurans* that confers resistance to ultraviolet (UV) light in this bacterium (Chen *et al.,* 2000). Moreover, murine embryonic cells lacking Ro60 exhibit a lower survival rate following UV radiation (Chen *et al.,* 2003) and mice lacking Ro60 show a 2-fold enhancement of apoptotic keratinocytes upon UV exposure (Xue *et al.,* 2003). These observations suggest that Ro60 may also play a key role in cell survival mechanisms following cell

stress.

1.3.3 The autoimmune response to Ro60

Over the past decade, most research has focused on defining the fine specificity of autoantibodies to Ro60 by identifying antigenic determinants (B cell epitopes and more recently apotopes) (Scofield *et al.,* 1999; Wahren-Herlenius *et al.,* 1999, Reed *et al.,* 2008). It has long been recognised that autoantibodies against Ro60 are mainly directed against conformational epitopes (Boire *et al.,* 1991; Gordon *et al.,* 2004). The conformational-dependence of Ro60 epitopes has hampered attempts for finer mapping using peptides. Nevertheless, several investigators have identified Ro60 epitopes using overlapping synthetic peptides and recombinant proteins tested by ELISA or in Western blotting (Figure 1.3). Major epitopes have been documented within the central third of the protein and minor epitopes at the carboxyly- and amino-terminal (for reviews see Scofield et al., 1999; Wahren-Herlenius *et al.,* 1999). Wharen *et al.,* (1992) documented a major antigenic determinant located in the middle of Ro60 (amino acid (aa) residues 181-320) in 86% of sera, and a further two antigenic domains located in the amino- and carboxyl-terminal (aa 1-134 and 397-525, respectively) in approximately 20% of sera. These observations correlate with other studies demonstrating a major antigenic region in the middle of Ro60 proteins (aa 155-326) (McCauliffe *et al*., 1994; Sattia *et al.,* 1994; Routsias *et al*., 1996). In addition, McClain *et al.,* (2005) mapped the initial (prior to clinical disease onset) epitope of the Ro60 autoantigen to aa 169-180 in patients with systemic lupus erythematosus (SLE). Over time these patients developed responses to multiple epitopes throughout the Ro60 protein, consistent with epitope spreading.

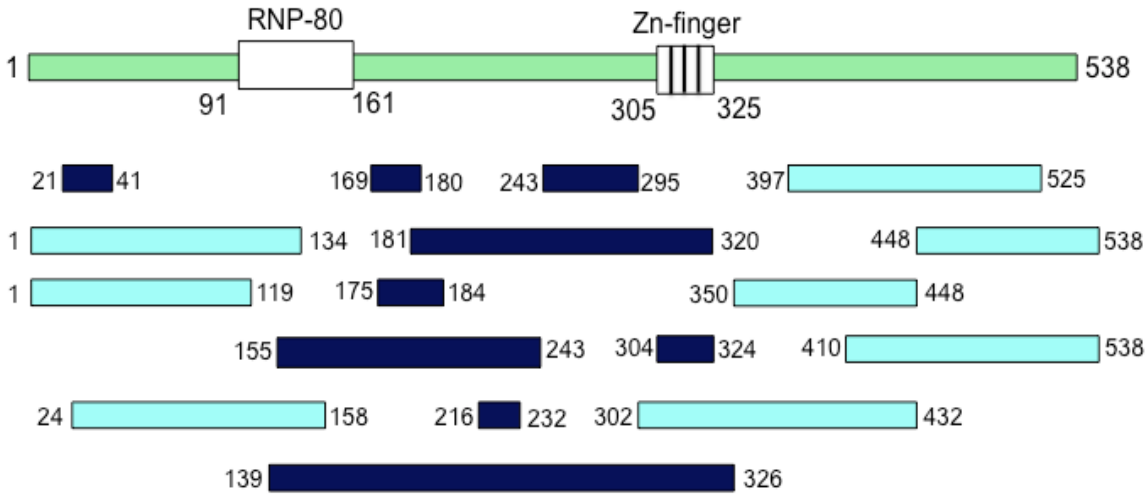


Figure 1.3. *Schematic representation of the structural features and epitopes of Ro60. The RNA recognition motif (RNP-80) contains two highly conserved sequence elements: RNP1 and RNP2. The Zn-finger consists of cysteine and histidine residues. Major epitopes are shown in dark blue and minor epitopes are shown in pale blue. Immunodominant epitopes tend to be confide to the central region of the protein. Numbers refer to amino acids (aa) (Adapted from Wahren-Herlenius et al., 1999).*

Similarly, Scofield & Harley (1991) identified numerous epitopes spaning the entire length of Ro60, revealing that the autoantibody response is heterogeneous. Several other groups have also described peptide epitopes that are recognised by subsets of patient anti-Ro sera, further supporting the concept of disease-specific pathways (Routsias *et al.,* 1998; Scofield *et al.,* 2001; Tzioufas *et al.,* 2002).

In a more recent study Reed *et al.,* (2008) developed a new approach to B cell mapping that identifies apotopes (defined as an epitope expressed on the surface of apoptotic cells) expressed on native Ro60. Interestingly, an immunodominant apotope was identified within the Ro60 aa 82-244 region that is highly specific for a subset of patients with SLE with anti-Ro60 without anti-La but not detected patients with primary SS. In a subsequent study, Reed *et al.,* (2010) identified two distinct B cell determinants contained within Ro 60 aa 193–236 that stratify anti–Ro 60 responses in primary SS and SLE (section 1.3.4).

Although considerable effort has gone into mapping the B cell epitopes recognised by anti-Ro/La autoantibodies, such studies fail to explain why these autoantigens are selected as targets of the immune system. Investigating the molecular characteristics of the autoantibodies themselves may, in addition to mapping epitopes and apotopes, shed light on the nature of the immune response that leads to autoimmunity. The recently reported immunodominant epitope of Ro60 (aa 193-236) can be viewed as a model to directly characterise autoantibodies in patients with primary SS (section 1.3.4).

1.3.4 An immunodominant B cell determinant of Ro60 protein that is exposed as an intracellular epitope or surface-exposed apotope.

Our laboratory showed recently that a major antigenic target of the Ro60 response in primary SS and SLE patients is directed against a domain encoded by aa 193 to 236 (Reed *et al.,* 2010). This region forms a helix-loop-helix structure (or a 'peg' like structure) located at the apical tip of the toroid structure of the Ro60 molecule (Figure 1.4), and has been termed Ro60peg. Ro60peg can form either an intracellular epitope or a surface-exposed apotope, depending on the subcellular location of the autoantigen. This dichotomy of determinant expression translates to distinct human anti-Ro60 autoantibody subsets. Reactivity with the intracellular Ro60peg epitope is highly specific for anti-Ro/La responses in both primary SS and SLE. The association of anti-Ro60peg epitope reactivity in patients with linked anti-Ro/La antibodies indicates that reactivity with this determinant is a biomarker for intermolecular spreading. On the other hand, autoantibodies directed against the Ro60peg apotope are prevalent in a subset of patients with SLE with isolated anti-Ro responses. Finally, the Ro60peg apotope is absent in patients with primary SS, irrespective of the presence of anti-La. In summary, these differential binding characteristics and distinctive subcellular locations of the Ro60peg domain signify the presence of two mutually exclusive B cell determinants that stratify anti-Ro60 responses in primary SS and SLE. First, an intracellular epitope that is exposed upon binding of Ro60peg protein to the solid phase, and second, a Ro60peg apotope that is expressed on the surface of early apoptotic cells. Experiments in this thesis will focus on the proteomic characterisation and sequencing of human autoantibodies directed against theRo60peg intracellular epitope.

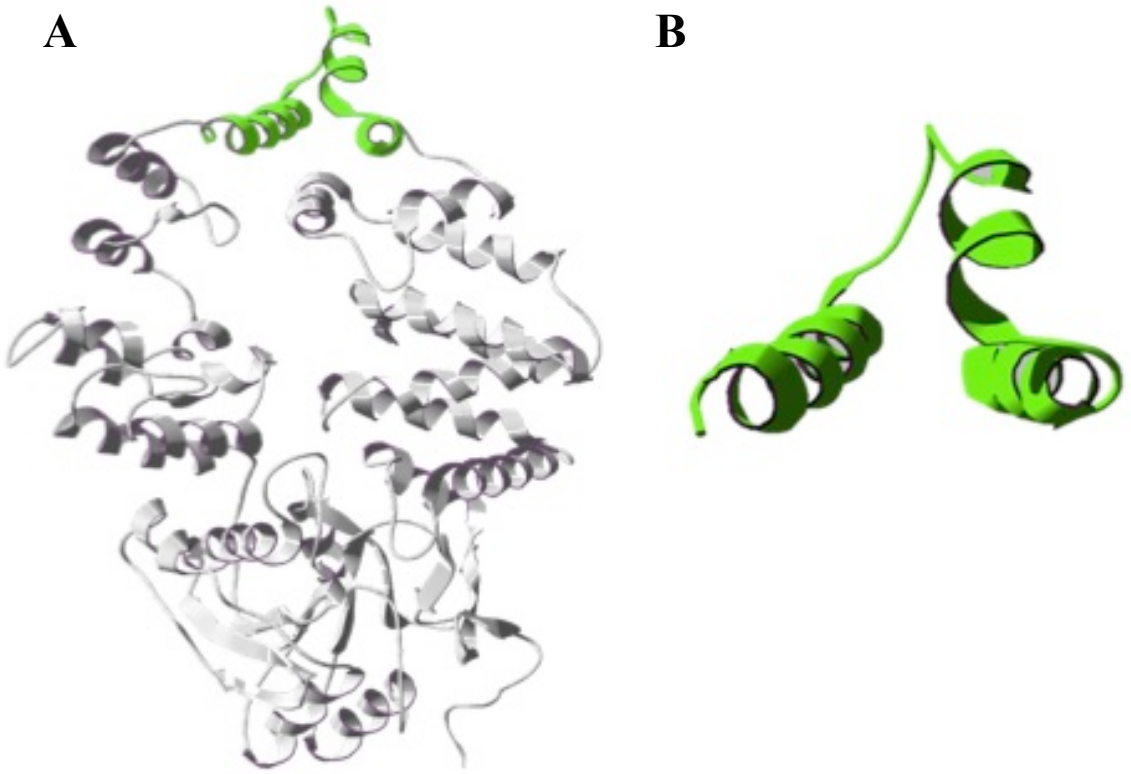


Figure 1.4. *The predicted structure of human amino acids (aa) 193-236.* ***A.*** *The Ro60 ribbon structure with aa 193-236 highlighted in green. Ro60 aa 193-236 forms an helix-loop-helix structure located at the apical tip of the toroid structure of human Ro60, termed Ro60peg.* ***B****. Representation of isolated Ro60peg shown in the same orientation as the intact structure (Adapted from Reed et al., 2010).*

1.4 Historical aspects of Burnet's clonal selection theory and the "forbidden" clone hypothesis

Fifty years ago the concept of autoimmunity was still very much in its infancy. In 1960, Sir McFarlane Burnet was awarded the Nobel Prize for Medicine and Physiology for his work on the immunological recognition of self. However, Burnet's most important contribution to immunology was the clonal selection theory, which in brief proposed that the antibody repertoire is predetermined before exposure to antigen; that the repertoire is expressed in a clonal fashion on the surface of immunocytes (one cell one antibody); and that single cells (each expressing a unique antibody on the surface) are selected by antigen for clonal proliferation (Figuure 1.5A)

Burnet wrote (Burnet 1957, page 647):

"The capacity to produce a given antibody is a genetically determined quality of certain clones of mesenchymal cells, the function of the antigen being to stimulate cells of these clones to proliferation and antibody production".

The "forbidden" clone hypothesis, a corollary of the clonal selection theory, proposes that autoimmune disease develops as a result of the emergence of "forbidden" (autoreactive in the modern parlance) clones that should have been deleted via normal immune tolerance. These "forbidden" clones are hypothesised to arise through a combination of inherited and somatic mutations that enable a self-reactive clone to bypass sequential tolerance checkpoints. The autoreactive clones proliferate on exposure to autoantigen, differentiate to plasma cells, and secrete a specific autoantibody. These autoantibodies target tissue to produce end-organ damage by

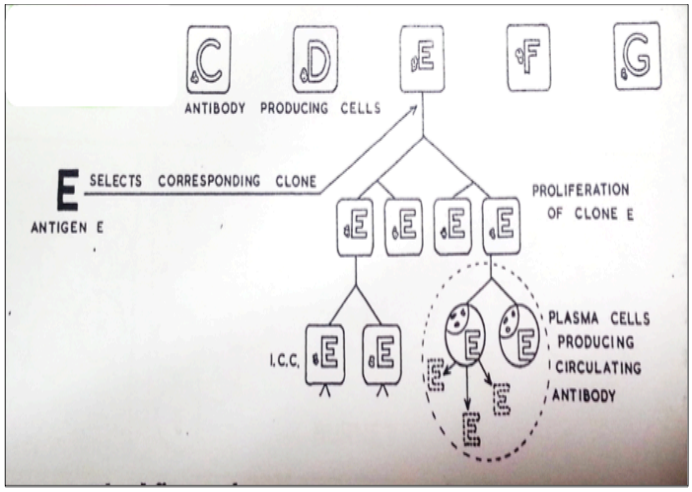
peripheral self-antigen recognition (Mackay & Burnet, 1963; Burnet, 1972) (Figure 1.5 B).

Burnet wrote, in relation to the development of a forbidden clone (Mackay & Burnet, 1963, page 39):

"In health, immunological competent cells carrying self-reactive patterns are deleted by homeostatic mechanisms. Failure of deletion of such cells, possibly associate with weak homeostasis, allows the development of a self-reactive or forbidden clone of cells, potentially capable of causing autoimmune disease. The origin of the forbidden clone is indicated as being due to mutation but is should be emphasised that more precise knowledge in the future may show that the forbidden pattern represents a persistence of one of the original embryonic cell reactivities or the release of a repression rather than a mutation".

To this day Burnet's clonal selection theory remains the foundation of our understanding of the adaptive immune system. However, more than 50 years after Burnets proposed "forbidden" clone hypothesis, there is limited evidence for "forbidden clones" specific for authentic autoantigens in human systemic autoimmune disease. Over that time, there have been major advances in our understanding of the structure of immunoglobulins (Igs), generation of antibody diversity, B cell development pathways, and phenotypic and functional diversity of B lymphocytes.

A



B

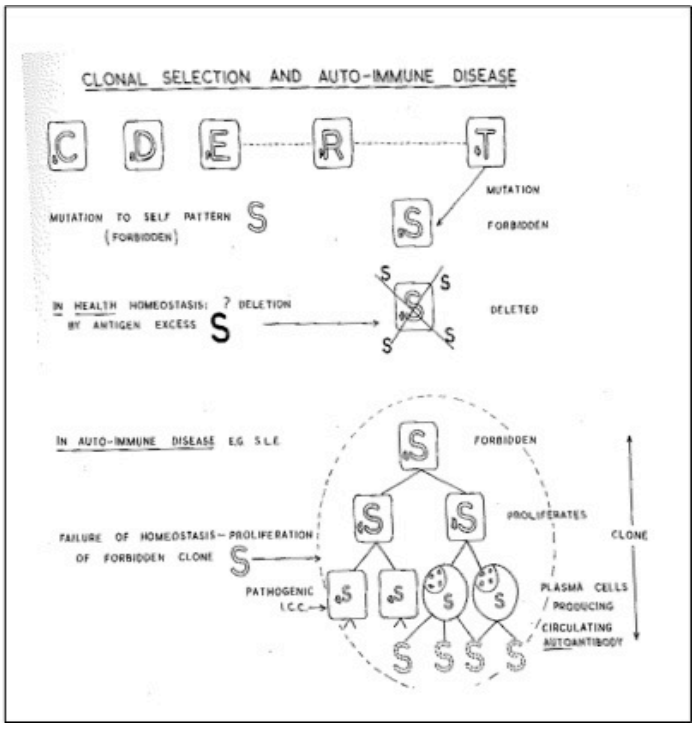


Figure 1.5. *Clonal selection and autoimmune disease.* ***A.*** *The clonal selection theory: the antigen E stimulates the proliferation of a clone of cells with a predetermined pattern established at or near birth to produce the corresponding antibody* ***B.*** *Forbidden clone hypothesis: the forbidden clone arises from a single cell from the B cell repertoire due to mutation. In health, immunologically competent cells carrying self reactive patterns are deleted by homeostatic mechanisms. In autoimmune disease, failure to delete such cells allows the development of a self-reactive or "forbidden clone" which may be capable of causing autoimmune disease (Adapted from Mackay and Burnet, 1963).*

1.5 B cell development

B cell development has been extensively studied in mice (for review see Hardy & Hayakawa, 2001; LeBien & Tedder, 2008). B cells arise from hematopoietic stem cell precursors in the foetal liver before birth and in the bone marrow afterward. Early B cell development is characterised by intermediate stages of differentiation (pro-B cell and pre-B cell) before becoming immature B cells (Figure 1.6). During this differentiation, rearrangement at the Ig locus results in the generation of heavy (H) and surrogate light (L) chain (composed of the invariant λ5 and VpreB polypeptides) pairs on the cell surface, which culminate in the expression of a mature BCR (comprised of rearranged H- and L- chain genes) capable of binding antigen. Cells then migrate to secondary lymphoid organs as transitional type 1 cells and differentiate into transitional type 2 that give rise to follicular B cells (or marginal-zone B cells). When naïve B cells traffic through secondary lymphoid tissue and encounter antigen, they can differentiate into short-lived antibody secreting plasmablasts or long-lived memory or plasma cells depending on the type, strength, and timing of signals they receive within the lymphoid microenvironment (Elgueta *et al.,* 2010). Of note, multiple cell surface-associated proteins and the expression of key transcription factors are required for each stage of B cell development and differentiation (Fuxa *et al.,* 2007). Additionally, it must be acknowledged that T lymphocytes play an important role in antibody production and cell-mediated immune responses. A series of checkpoints normally controls B cell selection, both centrally in the bone marrow (central tolerance) and in peripheral lymphoid tissues (peripheral tolerance), whereby self-reactive B cells are purged (for review see Meffre and Wardemann, 2008).

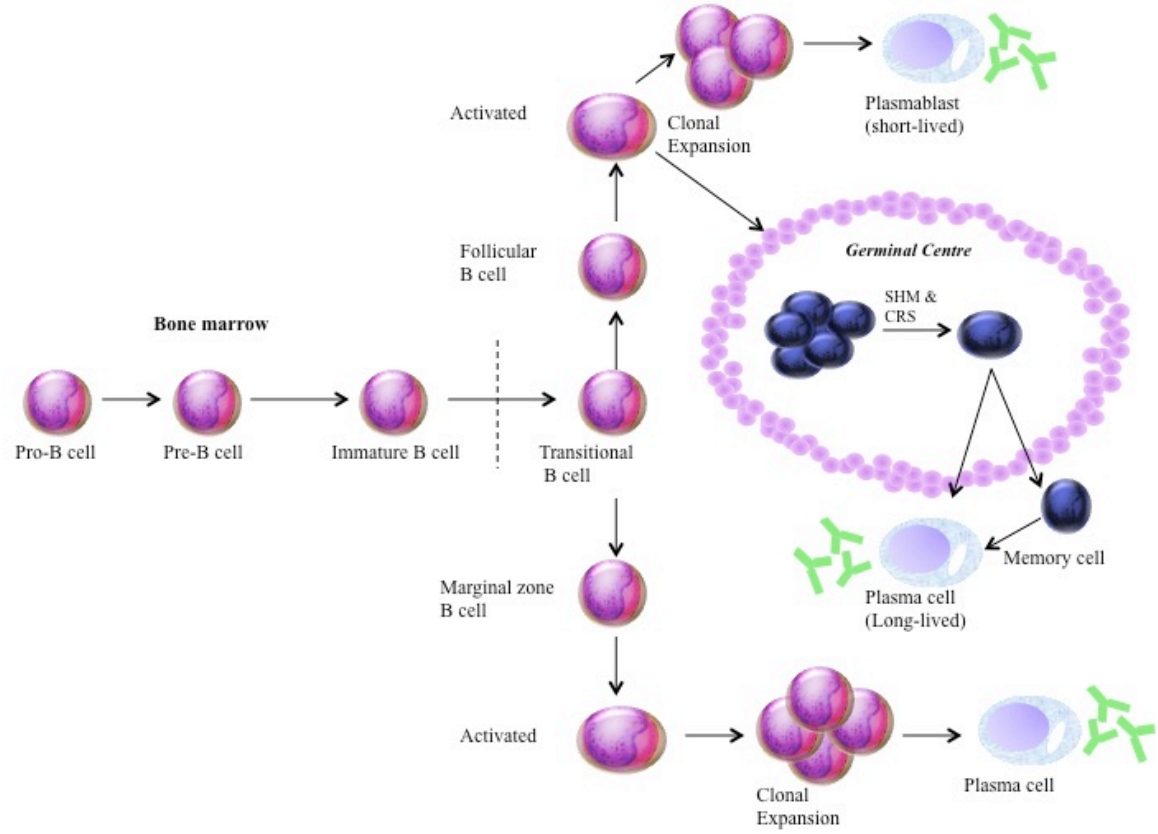


Figure 1.6. *Schematic representation of B cell development and differentiation. The figure shows the broad overview of B cell development stages. B cell development takes place in the bone marrow, thereafter the immature cells migrate to the periphery where maturation is complete. Following encounter with antigen, naïve B cells can differentiate into short-lived plasmablasts. Alternatively B cells can seed a GC, where affinity maturation, and differentiation into long-lived memory and plasma cells occur. SHM indicates somatic hypermutation; CSR, class switch recombination (Adapted from LeBien & Tedder, 2008).*

This process begins in the bone marrow at the immature stage of development where self-reactive B cells are clonally deleted (apoptosis) or undergo receptor editing (IgL chain secondary recombination). Despite the efficient removal of large numbers of autoreactive B cells in the bone marrow, some self-reactive clones escape central tolerance and migrate to the periphery. The second checkpoint occurs in the periphery, where strongly self-reactive B cells are eliminated via deletion or anergy (non-responsiveness to antigen) (Wadermann *et al.,* 2003; Wadermann *et al.,* 2007).

A small number of self-reactive B cells escape and compete poorly with normal B cells for microenvironment niches and survival factors (such as cytokines and BAFF) (Mackay & Tangye, 2004). Wardemann and colleagues (2003) found that as many as 75% of early immature B cells in healthy subjects were self-reactive and/or polyreactive. The frequency was reduced to approximately 20% after the second, peripheral checkpoint. Acting together, these tolerance checkpoints ensure autoreactive B cells are removed and preclude autoimmunity.

1.6 Generation of antibody diversity

The antibody molecule consists of an identical pair of H- and L- chains, each of which contains a variable (V) domain for antigen recognition and a constant domain for effector functions. The VH chain domain is created by assembly of V, diversity (D), and joining (J) segments, while L chains are assembled from V and J segments only. Within the V-region there are three hypervariable regions (known as complementarity determining regions; CDRs) embedded into four framework regions (FR) (Figure 1.7

A). The CDRs are the most diverse regions on the antibody molecule and form the antigen binding-site (Tonegawa, 1983; Alzari *et al.,* 1988; Kabat *et al.,* 1991). The CDR3 is the most variable portion of the Ig molecule and is in direct contact with the antigen. The enormous diversity of the antibody repertoire is produced in early B cell development through gene rearrangement, whereby V, D, and J gene segments are recombined (The mechanisms of Ig gene rearrangement have been reviewed in detail elsewhere, Tonegawa, 1983; Alt *et al.,* 1992; Schatz *et al.,* 1992) (Figure 1.7 B). Rearrangement of one D gene to one J_H gene is followed by the addition of one of the numerous V_H to the fused D-J_H segments. Recombination-activating genes, RAG1 and RAG2, mediate the recombination process (Schatz *et al.,* 1989; Oettinger *et al.,* 1990). The combination of different V(D)J segments creates considerable diversity, which is further enhanced by the random pairing of different H- and L- chains (Brezinschek *et al.,* 1998). Theoretically, this diversity yields approximately 10^{15} different antibodies. Further diversity is introduced by exonuclease activity and the incorporation of P and N nucleotides at the joining sites by terminal deoxyribonucleotidyl transferase. Finally, antigen binding induces somatic hypermutation, thereby introducing another layer of diversity in the antibody repertoire. These various mechanisms contribute to the generation of a highly diversified array of IgV gene products. However, loss of B cell tolerance and defects in diversity are believed to result in the emergence of "forbidden" clones or autoreactive B cells that are capable of causing autoimmune diseases.

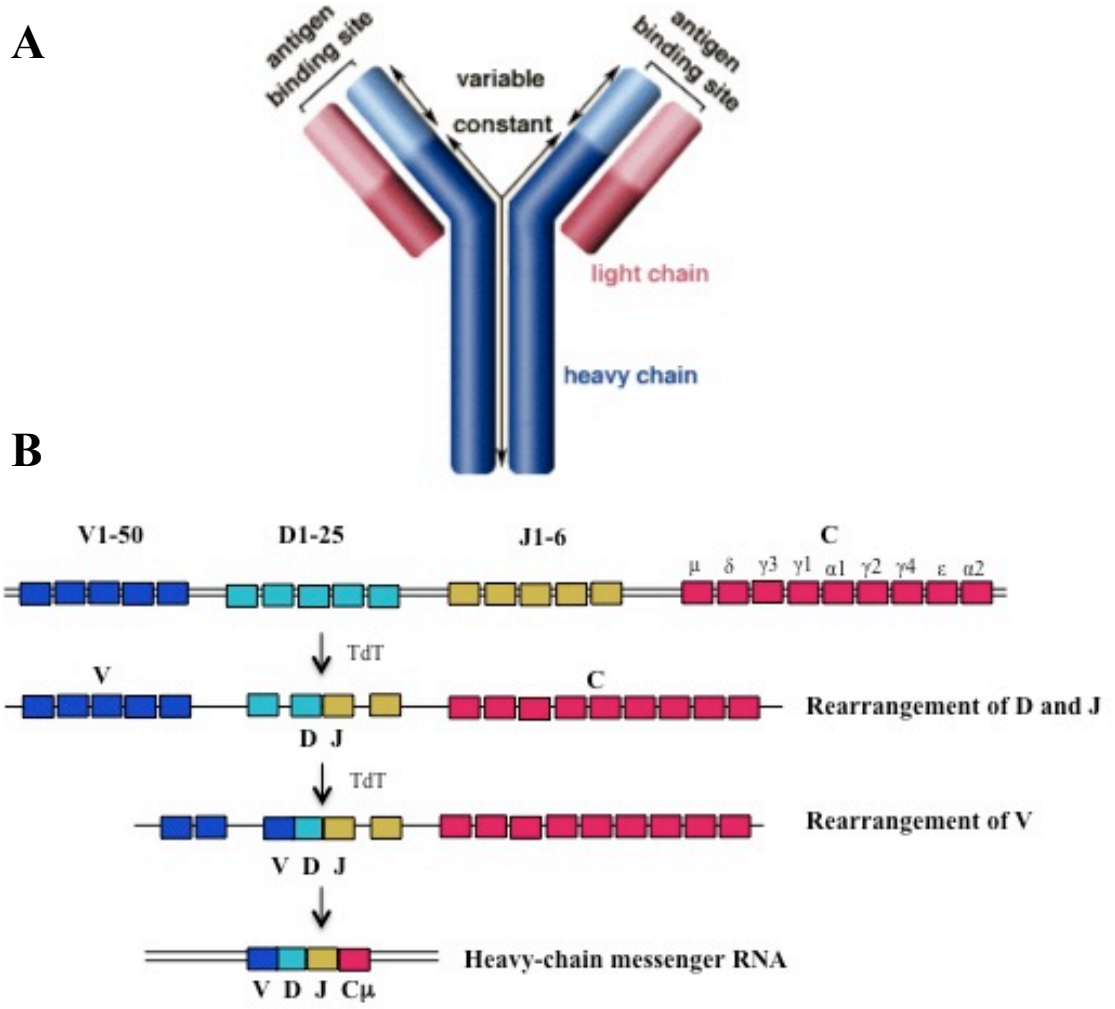


Figure 1.7. *Generation of diversity in the Ig repertoire.* ***A.*** *Generalised structure of an antibody. The B cell receptor comprises a pair of heavy (H) (blue) and light (L) (pink) chains. Each chain consists of a constant (C) (dark blue and pink) and a variable (V) (pale blue and pink) regions. The V-regions each contain three hypervariable complementarity determining regions (CDRs).* ***B.*** *The V(D)J rearrangement process. The events involved in gene rearrangement for the H chain are shown. The diversity (D) and joining (J) gene segments are recombined first. This is followed by recombination of the V segment to the DJ segment. The complete VDJ segment is then transcribed into mRNA, which is translated into μH chains (Adapted from Delves & Roitt, 2000; Martensson et al., 2010).*

1.7 Models of anti-Ro60 autoantibody production

The underlying cause of anti-Ro/La autoantibody production remains unclear (for review see Dziarski, 1988). One model hypothesises that these autoantibodies are the result of polyclonal B cell activation, but this appears unlikely given the fine specificity of anti-Ro60 humoral responses and recruitment of common sets of immunodominant epitopes in unrelated patients.

The model favoured by most researchers proposes that Ro60 autoantigen-driven B cell activation causes sustained B cell activation and specific autoantibody production. Evidence supporting this model include (1) the presence of high titer anti-Ro60 autoantibodies (mg/ml concentrations) suggesting preferential selection (Derksen & Meilof, 1992; Heinlen *et al.,* 2010); (2) spreading of the response to other epitopes on Ro60 (intramolecular spreading) and the physically linked La molecule (intermolecular spreading) (McCluskey *et al.,* 1998; Heinlen *et al.,* 2010); (3) of different patterns of anti-Ro/La autoantibody production, an apparent increase in autoantibody affinities over time (Kurien & Scofield, 2009); and (4) association with particular MHC class II alleles (Rischmueller *et al,* 1998).

1.8 Early studies on the clonality of anti-Ro60 autoantibodies

While Burnet's forbidden clone hypothesis posits that autoantibodies directed against Ro60 epitopes should be clonally distinguishable, little is known about the clonality of Ro60 (or La) autoantibodies, particularly at a molecular level (i.e. their V-region gene usage). Early studies on humoral responses to the linked La autoantigen using two-

dimensional gel electrophoresis (2DGE) revealed oligoclonality of kappa (k) L-chains for both NH2- and COOH-terminal epitopes (Bini *et al.,* 1990), while isoelectric focusing and affinity immunoblotting of serial serum samples from an anti-Ro60-positive patient revealed an oligoclonal response to Ro60 autoantigen that increased in complexity over time (Kurien *et al.,* 2009). This interesting finding on a single patient has not been confirmed by studies on other patients with anti-Ro60 humoral autoimmunity, in particular whether common oligoclonal banding patterns occurs in unrelated patients.

1.9 Immunoglobulin variable gene usage of autoantibodies

Whether the production of pathogenic autoantibodies arise from intrinsic genetic abnormalities in the generation of the Ig V repertoire remains a matter of controversy. Although antibody diversity is almost limitless in potential and the probability of finding identical V(D)J gene segments is extraordinarily low, recent work demonstrates that certain V(D)J combinations are favoured in the normal repertoire (Boyd *et al.,* 2010). Studies have shown that V_H3-23*01 is the most commonly expressed VH gene in the peripheral repertoire (Brezinschek *et al.,* 1995). Given the biased gene usage in the normal population, studies have explored the V(D)J rearrangement in response to specific infections, malignancies, and autoimmune disease (Deulofeut & Robinson, 1997; Owens *et al.,* 2003; Hadzidimitriou *et al.,* 2006; Binley *et al.,* 2008; Bahler *et al.,* 2009; Roghatgi et al., 2009). These studies have revealed production of antibodies with restricted H- or L- chain V(D)J combinations in unrelated patients. One example is the vaccine for *H. influenza* type B, where antibodies perferentially use V_H3-23, J_H4 or J_H6,

and CDR-3 (Gly-Tyr-Gly-Phe/Met-Asp) gene rearrangement (Liu & Lucas, 2005). Another study found that antibodies against 23F polysaccharide of *Streptococcus pneumonia* frequently use IG_KV2-24 or IG_K3-11 for the L chain and IG_H3-30 for the H-chain (Thomson *et al.,* 2011). Furthermore, an analysis of splenic germinal centre B cells of a SLE patient demonstrated biased expression of V_H5 and J_H4 family genes (Fraser *et al.,* 2003). These observations suggest that public or restricted usage of V genes occurs in the autoantibody repertoire. However, the number of patients whose Ig V genes have been analysed in detail is too small to establish any correlations between gene utilisation and disease-specific autoantibodies. Whether preferential pairing of VH and VL genes occurs in pathogenic autoantibodies associated with systemic autoimmune diseases such as primary SS remains unclear. This thesis explores a novel proteomic method of analysing the clonality and Ig V usage of humoral autoimmunity, directed against the immunodominant Ro60peg epitope.

1.10 Current methods to analyse antibody repertories

Several technologies have been developed for characterising autoantibodies at a molecular level, but are limited by their inability to represent the overall secreted autoantibody repertoire.

1.10.1 Hybridoma method

Kohler and Milstein (1975) originally developed a method to produce monoclonal antibodies in hybridomas, that is, cells resulting from the fusion of B cells with myeloma cells. This technique has traditionally been exploited to develop novel therapeutic monoclonal antibodies (for review see Sullivan *et al.,* 2011). Early

development efforts used rodent systems to generate hybridomas. More recently, human monoclonal antibodies have evolved from hybridoma B cell lines derived from transgenic mice bearing human Ig genes (Lonberg *et al.,* 1994; Bruggemann *et al.,* 1996; Lonberg *et al.,* 2005). However, transgenic mouse systems are not necessarily equivalent to humans and cannot precisely imitate the human immune response. In humans however, this method suffers from low transformation frequencies, and yields monoclonal antibodies mostly IgM class, whereas the autoantibodies of patients with autoimmune diseases are generally of the IgG class (Kozbor, 1986). As a result, this method is generally unsuitable for the direct isolation and molecular characterisation of antibodies from the native human repertoire.

1.10.2 Repertoire cloning by phage display

Phage display was developed as an alternative to traditional hybridoma technology to isolate diverse antibodies. This technology is based on the presentation of peptides or protein fragments on the surface of bacteriophages. It involves constructing combinatorial libraries of separate V-region H- and L- chain V domain genes of the antibody that are expressed on the phage surface (for review see Kretzschmar & von Ruden 2002). This technique is a high-throughput mode for generating human antibodies from diverse libraries, which allows comprehensive protein expression. Furthermore, this method has evolved as a powerful technology in drug discovery and thus in identifying human therapeutic antibodies. Phage display libraries have been extensively advocated in literature (Bruggeman *et al*., 1996; Johns *et al*., 2000; Marget *et al*., 2000; Goletz *et al*., 2002). Previously, Suzuki *et al*., (1997) used phage display

technology to isolate Ro60 Fab clones from salivary gland lymphocytes of patients with SS. Moreover, in a recent study two anti-Ro52 monoclonal antibodies were derived by combinatorial cloning (Salomonsson *et al*., 2004). A major concern with this technique is that the random reassortments of H- and L- chains do not recapitulate their native rearrangements. Furthermore, this method does not allow distinction between disease-relevant and irrelevant autoantibodies (Forman *et al*., 2007).

1.10.3 Single-cell sorting techniques

An alternate technique that allows isolation of human antibody repertoires with preservation of the H- and L- chain pairing is single-cell sorting, which harbours the potential to isolate functional antibodies against conformational epitopes from human blood. The Nussenzweig group originally described this approach to explore human B cell selection and tolerance (Wadermann *et al.,* 2003; Wadermann & Nussenzweig, 2007). Over the last decade many studies have employed this technique to evaluate basic B cell concepts in health and autoimmunity (Battye *et al.,* 2000; Wramment *et al.,* 2008; Scheid *et al.,* 2009). A recent analysis of recombinant antibodies cloned from single circulating B cells from one patient with SLE showed a high frequency of anti-Ro/La memory B cells, none of which were clonally related (Mietzner *et al.,* 2008). In another study single-cell techniques have also obtained preliminary data on IgV gene usage of anti-Ro52 and anti-La (Elagib *et al.,* 1999; Mietzner *et al.,* 2008). However, genomic studies of single cells have been unable to provide a comprehensive snapshot of the B cell receptor repertoire of systemic autoantibodies. Moreover, this approach involves a selection bias; and cannot be used conveniently in large unrelated patient

populations, in whom it is a challenge to determine whether immune responses against Ro60 recruit shared (public) self-reactive B cell clonotypes.

1.11 Autoantibody clonality analysis and sequencing by mass spectrometric based proteomics: a new approach for examining specific autoantibody repertories

An alternative strategy to single cell techniques is to apply proteomic technologies to analyse clonality and V gene usage of serum-derived autoantibodies. Such an approach would have the advantage of providing the actual protein sequence of an authentic autoantibody, rather than giving a predicted translated sequence. Direct sequencing of autoantibodies has been difficult to implement in the past because of the marked diversity and polyclonality of established humoral responses and the lack of a reference database. Until recently, the only proteomic method available for *de novo* sequencing analysis of unknown proteins has been Edman degradation (Pharm *et al.,* 2003). The advantage of Edman degradation is that sequencing occurs in a linear fashion, beginning at the N-terminus. However, Edman degradation is characterised by short peptide reads, requires proteolytic digestion, peptide fractionation, and peptide-by-peptide sequencing, therefore rendering it a low-throughput and time-consuming approach (Edman, 1967). Advances in mass spectrometric sequencing now make it feasible to sequence antibodies using high-resolution Orbitrap mass spectrometry (MS). Recent studies have used MS to identify monoclonal antigen-specific antibody sequences from immunised animals (Banderira *et al.,* 2008; De Costa *et al.,* 2010; Van Duijun *et al.,* 2010; Cheung *et al.,* 2012). The proteomic approach offers distinct advantages over conventional methods in terms of analysing the autoantibody repertoire. Orbitrap MS in particular has

high mass accuracy, resolution, and sensitivity; effectively controls for sequencing errors; can be applied easily to multiple samples in a relatively short time; achieves close to full-length sequencing in a single run; and requires smaller quantities (< 50 ng) than Edman degradation. Overall, the proteomic approach potentially provides a direct method for determining the clonality, and VH and VL gene expression of a purified autoantibody, as well as mapping any V-region somatic mutations. This thesis utilises a mass spectrometric approach for the first time to characterise the autoantibody repertoire specific for a Ro60 determinant (Ro60peg) in patients with primary SS.

1.12 Specific Aims and Hypothesis

As described above, a major hallmark of primary SS is the production of autoantibodies against Ro60. However, little is known about the molecular characteristics of the anti-Ro60 autoantibodies themselves or their relationship with the development of primary SS. Evidence for the existence of "forbidden" B cell clones specific for Ro60 or their secreted counterparts (clonotypic autoantibodies) is limited (section 1.4). Precise identification of these putative clonotypic autoantibodies from polyclonal serum has not been feasible because of the complexity of mature anti-Ro60 autoantibody patterns in patient sera. This thesis exploits the structurally stable Ro60peg epitope (expressed as a recombinant protein) to select (affinity purify) a specific anti-Ro60 autoantibody population from complex serum samples and applies advanced proteomic techniques to characterise its clonality, V gene usage, and pattern of somatic mutations. Together these constitute the V-region molecular signature of the autoantibody.

The specific aims and hypotheses of this thesis are as follows:

Aim 1. To purify anti-Ro60peg autoantibodies from the sera of patients with primary Sjögren's syndrome by epitope selection and determine their clonality by 2DGE (Chapter 3).

Hypothesis 1. Humoral responses to the Ro60peg epitope are monoclonal and reflect the secreted autoantibody product of a "forbidden" B cell clone.

Aim 2. To determine the V-region molecular signature of the clonotypic anti-Ro60peg autoantibody by de novo mass spectrometric sequencing (Chapter 3).

Hypothesis 2. The anti-Ro60peg clonotypic response utilises restricted VH- and VL-chain gene segments that are public (common) to all patients, consistent with an antigen-driven response.

Aim 3. To determine the IgV-region molecular signatures of Ro60peg clonotypic autoantibodies in serial serum samples from patients with primary SS, and analyse the clonotypic variation over time (Chapter 4).

Hypothesis 3. The Ro60peg-specific clonotype undergoes a dynamic process of clonotypic turnover, characterised by a periodic succession that masquerades as long-lived anti-Ro60 humoral autoimmunity.

1.13 Significance of work described in this thesis

This thesis describes a novel proteomic approach to characterise the clonality and V-region molecular signatures of systemic autoantibodies present in human serum samples, and tests Burnet's "forbidden" clone theory in humans in the context of an authentic disease-associated autoantigen (Ro60). This approach provides insight into key questions concerning the production of autoantibodies in primary SS: autoantibody clonality; public versus private gene usage; dynamics of antibody-secreting clones; and generation of long-lived humoral autoimmunity. The novel proteomic approach used for anti-Ro60peg autoantibodies can also be applied to other human autoantigens in which the B cell determinants have been characterised biochemically, such as Sm/RNP and ribosomal P in SLE; citrullinated peptides in rheumatoid arthritis; or glutamic acid decarboxylase (GAD) in type I diabetes. Identification of autoantibody-specific V-region signatures may be used to develop both autoantibody-specific diagnostics and B cell targeted therapeutics. With regard to the former, surrogate V-region peptides and targeted MS can be used as a next generation molecular diagnostic for direct detection of autoantibodies in patient samples, as reported in a related publication Arentz *et al.,* (2012).

Chapters 3 and 4 are based directly on peer-reviewed publications arising from the work in this thesis. Accordingly, a copy of the relevant publication is inserted at the end of each chapter. An editorial based on the work in chapter 4 has been included at the end of the chapter.

Chapter 2: Materials and Methods

2.1 Proteins

2.1.1 Preparation of recombinant maltose binding protein fusion constructs

Soluble overlapping fragments of Ro60 encompassing aa 82-244, and 82-539 expressed as maltose-binding protein (MBP) were prepared from pMAL-x2X vectors (provided by Dr. Ken Kaufman, Oklahoma Medical Research Foundation, Oklahoma City, OK, USA).

MBP fusion constructs were prepared according to manufactures recommendations (New England BioLabs, Ipswich, MA, USA). Laboratory glycerol stocks of subcloned *Escherichia coli* (M15) containing MBP fusion constructs (15 % glycerol stored below -80 ^{o}C) were recovered in rich media (10 g/L tryptone, 5 g yeast extract, 5 g NaCl, 2 g glucose) and 0.1 mg/ml ampicillin at 37 ^{o}C with constant shaking until the optical density (OD) at 600 nm was approximately 0.5. Isopropyl-β-thiogalactopyranoside (Promega, Mountain View, CA, USA) was added to a final concentration of 0.3 mM and incubated at 37 ^{o}C for 2 hrs to induce vector expression. Cells were harvested by centrifugation (4,000 x g, 20 min); the pellet was resuspended in column buffer (20 mM Tris, 200 mM NaCl, 1mM EDTA, pH 7.4) to a final volume that was 5% of the initial culture and stored at -20 overnight. Cells were thawed in an ice-water bath; sonicated in short pulses, 3 x 1 min intervals, (Sonicator W-375 Heat System-Ultrasonics, Planview, USA). Cell debris was removed by ultra-centrifugation (9,000 x g, 30 min, 4 ^{o}C). MBP recombinant fusion proteins were affinity-purified from the supernatant by maltose-

affinity chromatography, dialysed against 1 L of phosphate buffered saline (PBS) overnight at 4 ^{o}C and quantitated by a Bradford assay (2.1.3).

2.1.2 Protein concentration determination by Bradford assay

Bradford reagent (Bradford, 1976) was prepared from 10% (w/v) Coomassie Blue in 20% (v/v) ethanol, and 10% (v/v) 85% orthophosphoric acid. Protein samples of unknown concentrations were diluted 1:10, 1:50, 1:100, 1:200, 1:500 in PBS; 0.1 ml of each sample was added to 0.9 ml Bradford reagent. The OD was measured at 595 nm (UV-160A UV-visible recording spectrophotometer, Shimadzu Corporation, Kyoto, Japan) and converted to a protein concentration based on a standard curve constructed from 0.1 ml bovine serum albumin (Sigma-Aldrich, St Louis, MO, USA) solutions at defined concentrations (0, 5, 10, 15, 20 μg/ml) mixed with Bradford reagent.

2.1.3 Verification of recombinant maltose binding protein fusion construct expression

SDS-PAGE analysis was carried out to verify the expression of recombinant MBP fusion protein. An equal volume of reducing sample buffer (1 M Tris pH 6.8, 20% glycerol, 2% SDS, 30% RO water, 0.001% Bromophenol blue) was added to 4 μg MBP recombinant fusion protein and heated at 100 ^{o}C for 5 min. SDS-PAGE was performed using a Mini-Protean 3 Cell apparatus (BioRad, Hercules, CA, USA). A 10% (w/v) stacking and resolving gel was used (Laemmil, 1970). Electrophoresis was run at a constant current 40 mA for 45 min. After, the gel was stained with Coomassie Blue for 1 hr, followed by destaining (25% methanol, 7% acetic acid, 68% RO water) overnight.

2.1.4 Additional protein reagents

Native bovine Ro60 was provided by Dr. Neil Cook, (Arotec Diagnostics, Wellington, New Zealand). All other Ro60 peptides were obtained from Mimtopes (Clayton, Victoria, Australia).

2.2 Patients and Controls

2.2.1 Patient and control sera

Sera were collected from patients with primary SS who fulfilled at least four of the six American-European consensus classification criteria (Vitali *et al.,* 2002). Anti-Ro52/Ro60/La specificities were characterised by counterimmuno-electrophoresis, line immunoassay (EUROLine, Euroimmun, Lübeck, Germany), and enzyme linked immunosorbent assay (ELISA) (RELISA™, Immunoconcepts, Sacramento, USA). Patients were considered monospecific if their sera only reacted with one autoantigen (e.g. monospecific for Ro60 was characterised as positive for Ro60, but negative against La/Ro52). None of the patients were being treated with steroids or other immunosuppressives or had evidence of a malignant B cell disorder. Control sera were obtained from healthy donors. The study was approved by the Clinical Ethics Committee of the Flinders Medical Centre. The investigation conforms with institutional guidelines; written informed consent was obtained prior the study.

2.2.2 Purification of immunoglobulin G by Protein A

Total immunoglobulin (IgG) was purified from patient and control sera by protein A-Sepharose CL-4B (Pharmacia, Uppsala, Sweden) chromatography. Serum was loaded

onto protein A columns and washed with 50 column volumes of PBS (pH 7.4). Bound IgG was eluted with 0.1 M glycine, 0.5 M NaCl (pH 2.3) and neutralized with 1 M Tris (pH 8.0). Eluted IgG was dialysed against 1 L of PBS overnight at 4 ^{o}C, concentrated with an Amicon Ultra Centrifugal Filter (Millipore, Billerica, MA, USA) and quantified by nephelometry (ICS Analyser II; Bechman Instruments, Brea, CA, USA).

2.2.3 Isolation of anti-Ro60 IgG by epitope-specific affinity chromotography

Approximately 7 mg of recombinant fusion protein (2.1.1) was dialysed against coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) overnight at 4 ^{o}C. Two milligrams of cyanogen bromide-activated Sephrose 4B beads (GE healthcare, Uppsala, Sweden) were resuspended in 1 mM HCl for 30 min at room temperature with agitation, and washed with 0.5 L coupling buffer. Dialysed recombinant protein was then bound to beads overnight at 4 ^{o}C. Unbound protein was removed by centrifugation (100 x g, 5 min) and several washes with 0.1 L coupling buffer. To block the remaining active sites, 0.2 M glycine in coupling buffer was added to the beads for 2 hrs at room temperature. Beads were then washed with 3 cycles of alternating pH consisting of a wash with an acetate buffer (0.1 M $NaO_2C_2H_3$, 0.5 M NaCl, pH 4) and then coupling buffer. The prepared beads were poured into a 2.5 x 10 cm column and equilibrated with PBS for affinity purification.

Specific autoantibodies present in patient sera were bound to the appropriate recombinant fusion protein columns, washed with 50 column volumes of PBS and eluted with 0.1 M glycine, pH 2.3. Eluted autoantibodies were neutralised in 1 M Tris, pH 8.0 and dialysed against 1 L PBS at 4 ^{o}C and concentrated with an Amicon Ultra

Centrifugal Filter (Millipore, Billerica, MA, USA). The concentration and specificity of the anti-Ro60 affinity purified IgG was quantified and confirmed by ELISA as detailed in 2.3.1 and 2.3.2 respectively.

2.3 Enzyme Linked Immunosorbent Assay (ELISA)

2.3.1 Quantification ELISA

The concentration of affinity purified human IgG was determined by ELISA (Tran *et al.,* 2002). Maxisorb 96 well ELISA plates (Nunc, Roskilde, Denmark) were coated with affinity purified anti-human IgG (H&L) [goat] (Rockland, Immunochemicals Gilbertsville, PA, USA), diluted 1/500 in 0.03 M carbonate buffer (pH 9.6) for 1 hr at 37 ^{o}C. Non-specific sites were blocked with 3% non-fat milk in PBS for 1 hr at 37 ^{o}C, and washed with 0.05% Tween-20 in PBS. 0.1 ml human IgG of known concentration, serially diluted 1/3 and affinity purified human IgG of unknown concentration, serially diluted 1/10 were applied to the wells for 1 hr at 37 ^{o}C. After repeated washing bound IgG was detected with an anti-human IgG alkaline phosphatase-conjugated antibody (Sigma, St Louis, MO, USA), followed by p-nitrophenyl phosphate substrate (Sigma, St Louis, MO, USA) with 1 mg/ml 90 mM diethanolamine, pH 9.6. All samples were tested in duplicate. OD values were measured at 405 nm and results were expressed as the mean of duplicates. Human IgG of known concentrations were used as a standard for calculating the concentration of unknown values.

2.3.2 Specificity ELISA

Maxisorb 96 well ELISA plates (Nunc, Roskilde, Denmark) were coated with saturating concentrations (5 μg/ml) of soluble recombinant proteins for 1 hr at 37 ^{o}C in 0.03 M carbonate buffer (pH 9.6). Non-specific sites were blocked with 3% non-fat dried milk in PBS for 1 hr at 37 ^{o}C, washed with 0.05% Tween-20 in PBS. Starting serum (diluted 1:500), flow-through fraction (normalised to the starting serum), and bound/eluted fraction (2.5 μg/ml) were added to the plate for 1 hr at 37 ^{o}C. After further washing, wells were incubated with an alkaline phosphatase-conjugated rabbit anti-human IgG for 1 hr at 37 ^{o}C (Sigma, St Louis, MO, USA). Bound antibody was detected by the addition of substrate (disodium p-nitrophenyl phosphate, 1 mg/ml in 90 mM diethanolamine, pH 9.6). All samples were tested in duplicate, the OD was read at 405 nm and results were expressed as the mean of duplicates. Values reported were corrected for background binding and results were expressed as the difference between the protein of interest coated ELISA plates and the OD values on a control fusion protein (MBP or GST) tested in parallel.

2.3.3 Antibody avidity determination

Relative autoantibody binding affinities of serial serum samples were compared by potassium thiocynate (KSCN) elution method as previously described (Pullen *et al.,* 1986; Gordon *et al.,* 1991). Sera diluted 1/500 was bound to fusion protein coated plates, as described in section 2.3.2 for 1 hr at 37 ^{o}C. Following incubation, the plates were washed and PBS containing either 0.5, 1.0, 2.0 or 3.0 M were added to each well for 15 min at 37 ^{o}C. After further washing wells were incubated with an alkaline

phosphatase-conjugated rabbit anti-human IgG (Sigma, St Louis, MO, USA) and developed (section 2.3.2). Results were expressed as a percentage of absorbance readings in the absence of thiocynate. The affinity index representing the molar concentration of thiocynate at 50% binding was estimated.

2.3.4 Measurement of autoantibody levels

Plates (Maxisorb, Nunc) were coated with recombinant MBP-Ro60peg at 5µg/ml in 0.03 M carbonate buffer (pH 9.6). Non-specific sites were blocked with 3% skim milk in PBS. After washing 3 times with PBS containing 0.05% Tween-20, plates were incubated with sera serially diluted (twofold dilutions starting at 1:100) in 1% skim milk in PBS. Bound IgG was detected with goat anti-human IgG alkaline phosphatase-conjugated antibody followed by p-nitrophenyl phosphate substrate. All samples were tested in duplicate, the OD was read at 405 nm, and autoantibody levels were expressed as the area under the dose-response curve (AUC) (Segwick et al 1983).

2.3.5 Isotype analysis

Afinity purified IgG subclass was determined as described in section 2.3.2. Briefly, after affinity purified IgGs were added to the plate and washed, wells were incubated either with polyclonal rabbit anti-human kappa L chains (Dako, Glostrup, Denmark), polyclonal rabbit anti-human lambda L chains (Dako, Glostrup, Denmark) or mouse monoclonal to human (H chain specific) IgG1, IgG2, IgG3 and IgG4 (Abcam, Cambridge, MA, USA).

2.4 Western blot

2.4.1 Immunoblots of IgG samples

Immunoblot analysis was carried out to confirm the fine specificity of affinity purified IgG. Recombinant proteins (2 μg) were separated by SDS-PAGE (Laemmli, 1970) in 10% gels under reducing conditions (section 2.1.3). Proteins were transferred to a nitrocellulose membrane using a Mini Trans-Blot Electrophoretic Transfer Cell (BioRad, Hercules, CA, USA). Nitrocellulose was blocked with 5% non-fat milk in Tris-Buffered Saline Tween-20 for 2 hr at room temperature and washed three times for 5 min. Nitrocellulose was probed with either affinity purified IgG, normal protein A IgG (negative control) or mouse monoclonal anti-MBP (New England BioLabs, Ipswich, MA, USA) (positive control) at 2.5 μg/ml in 1% non-fat milk for 1 hr at room temperature. After washing, nitrocellulose was probed with horseradish peroxidase-conjugated anti-human IgG antibody (Abcam, Cambridge, MA, USA) or goat anti-mouse IgG (BioRad, Hercules, CA, USA) for 1 hr at 37 ^{o}C. The washing procedure was repeated and the reactivity with MBP-Ro60 fusion proteins was detected by SuperSignal® West Pico chemiluminescent substrate (Pierce) with a LAS-4000 luminescent image analyser (Life Science, Fuji Film, Tokyo, Japan). Prestained Precision Plus protein standards (BioRad, Hercules, CA, USA) were used to estimate molecular mass.

2.5 Immunofluorescence

HEp2000^{TM} slides with HEp-2 cells overexpressing the human Ro60 protein (Immunoconcepts) were incubated with anti-Ro60peg IgG (2.5 μg/ml) for 60 min at

room temperature, washed, incubated with FITC-conjugated anti-human IgG, and viewed with an Olympus BX51 florescence microscope.

2.6 Measurement of autoantibody binding affinities

Anti-Ro60peg binding affinities (equilibrium dissociation constants, K_D) were analysed using surface plasmon resonance technology using a Biacore X100 (GE Healthcare). Anti-human IgG were immobilised on a research grade CM5 sensor chip through primary amine groups according to the manufacturers recommendations. Surfaces were first conditioned by multiple 30 sec injections of regeneration buffer. Anti-Ro60peg IgGs were then captured at a flow rate of 10µl/min to a level of 79 to 89 resonance units (RU). Recombinant MBP-Ro60peg was then injected at concentrations ranging from 6.3nM to 1600nM in multiple cycles for 9 min at a flow rate of 10µl/min. After each cycle the surface was regenerated with regeneration buffer to remove anti-Ro60peg IgGs and MBP-Ro60peg. At equilibrium binding the responses were plotted against the concentration of MBP-Ro60peg. A steady state binding model was used to determine the K_D.

2.7 Proteomics

2.7.1 Two-dimensional gel electrophoresis (2DGE)

Affinity purified IgG or whole IgG were prepared for 2DGE using a ReadyPrep™ 2-D Cleanup Kit (BioRad, Hercules, CA, USA), according to manufactures recommendations. After, IgG was dissolved in rehydration solution (7 M Urea, 2 M Thiourea, 4% CHAPS, 30 mM Tris, 1% Pharmalytes 3- 7, 65 mM DTT, and 0.002%

Bromophenol blue) and applied to 24 cm IPG strips covering a nonlinear pH 3-7 gradient (GE Healthcare, Uppsala, Sweden). Strips were rehydrated using an IPGphor (GE Healthcare, Uppsala, Sweden) at 21 ^{o}C using a voltage of 50 V for 10 hrs. Following, strips were focussed for 80, 000 V hrs using a focussing protocol (step and hold 500 V 30 min, step and hold 1,000 V 30 min, gradient 10, 000 V 30 min, step and hold 10,000 V 60,000 V hrs, gradient 1,000 V 30 min, step and hold 1,000 V hold).

Focussed IPG strips were reduced with equilibration solution (6 M urea, 75 mM Tris-HCL, pH 8.8, 2% (w/v) SDS, 29.3% (v/v) glycerol) containing 1% dithiothreitol (GE Healthcare, Uppsala, Sweden) for 15 min, followed by alkylation in equilibration solution containing 4% idoacetamide for 15 min.

Second-dimension separations used 8% to 19% gradient polyacrylamide gels (40% acrylamide bis, 1.5 M Tris HCl, pH 8.8, water, 10% SDS, 10% APS, 0.005% TMED). SDS-PAGE was conducted at 350 V, 400 mA, 100 W using an Ettan DALT 6 gel castor. After electrophoresis, gels were fixed with 40% (v/v) methanol and 10% (v/v) acetic acid for 2 hrs and stained with Sypro Ruby overnight (BioRad, Hercules, CA, USA). Following staining, the gels were rinsed in water three times for 10 min. Imagining was performed using an Typhoon 9400 Variable Mode Imager (GE Healthcare, Uppsala, Sweden) at 200 μ. Gel spots were excised manually using a modified pipette tip and stored at -20 ^{o}C.

2.7.2 Preparation of samples for mass spectrometry

For in-solution digestions, two or more affinity purified IgGs from each serum sample were precipitated using a 2D Clean Up Kit (BioRad) according to manufacturer's

instructions and resuspended in 20 µl of 100mM ammonium bicarbonate, pH 8.0. IgG was reduced by the addition of DTT to 5 mM with incubation at 100 ºC for 5 min and alkylated by the addition of iodoacetamide to 20 mM. Samples were digested with 100 ng of Trypsin Gold (Promega) followed by incubation for 16 h at 37 ºC. For in gel digestions, gel plugs were excised from gels, washed 5 times with 100 mM ammonium bicarbonate and shrunk with acetonitrile. The gel plugs were dried in a 37 ºC oven and 10 µl of Trypsin Gold (10 ng/ µl, Promega) was added followed by incubation on ice for 15 minutes. Excess Trypsin Gold was removed and 10 µl of 100mM ammonium bicarbonate was added. Samples were incubated for 16 h at 37 ºC. Digest solutions were removed from each sample and placed in MS vials. Gel plugs were shrunk with 2 µl of acetonitrile and the extracted solution was added to the appropriate digestion vials.

2.7.3 Mass spectrometry

Peptides were analysed using a LTQ Thermo Orbitrap XL linear ion trap (IT) mass spectrometer fitted with a nanospray source (Thermo Electron Corporation). Digested peptides were applied to 206 a 100 mm i.d. 2 cm PepMap100 NanoViper trap column (Dionex) and separated on a 100 mm i.d. 150 mm C18 5 mm 100 Å column (Nikkyo Technos), using a Dionex Ultimate 3000 HPLC (Dionex) and eluted with a 55 min gradient from 2% acetonitrile to 45% aceto-nitrile containing 0.1% formic acid at a flow rate of 200 nl/min, followed by a step to 77% acetonitrile for 12 min. The mass spectrometer was operated in positive ion mode with one Fourier transform (FT) MS scan of mass/charge (m/z) 300-2000 at 60,000 resolution followed by collision induced dissociation fragmentation and FT or IT MS product ion scans of the six most intense

ions. The instrument method contained a dynamic exclusion of 25 s with an exclusion list of 500, 10 ppm low and high mass width relative to the reference mass and collision energy of 35%. Only multiply charged ions were selected for MS/MS

2.7.4 Protein sequence data analysis

Database searches were carried out with Proteome Discoverer version 1.2 (Thermo Electron Corporation) using the Sequest algorithm against a combined database of IMGT sequences (http://www.imgt.org), containing human H and L chains and the Uniprot 2010-06 database. The database search parameters were: a maximum of 2 missed trypsin cleavages, cross-correlation scores (Xcorr) of matches were greater than 1.5, 2.0, 2.25, 2.5 and 2.75 for charge states 1, 2, 3, 4 and 5 peptide ions respectively; peptide probability was high and 2 or more unique peptides were sequenced for each protein. The mass tolerance for peptide identification of precursor ions was 10 ppm and 0.01 Da for product ions. Searches were carried out with the following variable modifications; the oxidation of methionine, phosphorylation of serine, threonine and tyrosine, and carboxymethylation of cysteine.

2.7.5 *De novo* analysis

De novo sequencing was performed on raw data files with Peaks Studio version 5.3 (Bioinformatics Solutions Inc.). Sequences in Peaks were searched against the combined IMGT/ Uniprot 2010-06 database. Data refinement was carried out on the raw data files with the following parameters: scans were merged with a retention time of 30 secs, a precursor m/z error tolerance of $\leq$10 ppm and a minimum charge state of 2, maximum of 4, scan quality value >65, with data processing (peak centroiding, charge

deconvolution deisotope). *De novo* sequencing of the input spectrum was performed using the Peaks algorithm with a precursor mass 10 ppm and product ion error tolerances of 0.02 Da (in-solution digests of the affinity purified IgG) or 0.8 Da (gel digests), digestion enzyme as trypsin, and the variable modifications of the oxidation of methionine, phosphorylation of serine, threonine and tyrosine, deamidation of asparagine and glutamine, and the carboxymethylation of cysteine. The peaks module was used to perform germline assignments against a combined database of human IGMT sequences (http://www.igmt.org) and the Uniprot 2010-06 database under the previously described parameters. A V gene family was assigned when more than two unique germline peptides were identified. The SPIDER module was used to identify mutations or variations from the germline IMGT/Uniprot sequences using a homology match query type under the previously described paramaters. Spectra was analysed in the inChorus search tool which combined the Peaks Protein ID, X!Tandem and OMSSA search tools with the following parameters: a parent and fragment ion error tolerance of 10 ppm and 0.02 Da respectively, up to 3 missed trypsin cleavages and the previously described variable modifications. All peptide matches from both the Peaks and Spider search modules were manually checked to assure quality before inclusion in the final data analysis.

Chapter 3: Molecular Signature of a Public Clonotypic Autoantibody

A "Forbidden" clone in Systemic Autoimmunity

3.1 Introduction

Burnett's Forbidden Clone Hypothesis, a corollary of Clonal Selection Theory, posits that the emergence of "forbidden" (autoreactive) clones of lymphocytes is causal for autoimmune disease. However, 50 years later there is little evidence for the existence of forbidden-B cell clones or their secreted clonotypic autoantibodies in human systemic autoimmunity. Ro60 is a major target of humoral autoimmunity in primary SS and SLE and has been considered a primordial autoantigen involved in the initiation of human systemic autoimmunity (chapter 1.2.2). Pathogenic maternal anti–Ro60 auto- antibodies bind cognate antigen on apoptotic cells in the foetal heart and can initiate tissue damage in congenital heart block (Clancy et al., 2006). While considerable effort has been focused on Ro60 epitope recognition, and more recently on the mapping of Ro60 apotopes (Scofield et al., 1999; Wahren-Herlenius et al., 1999; Reed *et al.,* 2008), little is known about the molecular characteristics of the anti-Ro60 autoantibodies themselves. Analysis of the B cell receptor repertoire against a key human autoantigen such as Ro60 will lead to a better understanding of how B cell tolerance is perturbed in systemic autoimmunity, and may lead to novel therapeutic strategies aimed at removing autoreactive B cell clones. A recent analysis of recombinant antibodies cloned from single circulating B cells from one patient with SLE showed a high frequency of anti-

Ro/La memory B cells, none of which were clonally related (Mietzner *et al.,* 2008). However, single cell techniques are not easily applicable to the study of larger unrelated patient populations, in whom a challenge is to determine whether immune responses against Ro60 recruit shared (public) self-reactive B cell clonotypes. Furthermore, animal models are of limited value in understanding B cell repertoire selection in Ro60 autoimmunity because spontaneous anti-Ro60 autoantibodies similar to those in humans are not produced in standard mouse models of lupus.

A direct way of analysing the clonality and *IGV* gene usage of humoral anti-Ro60 autoimmunity is to study the proteome of the circulating autoantibodies. However, the molecular characterisation of clonotypic autoantibody populations in whole serum is hampered by the marked diversity and polyclonality of established humoral anti-Ro60 responses, which have generally undergone marked intra- and inter-molecular spreading to the linked Ro52 and La/SSB proteins by the onset of symptoms (Heinlen *et al.,* 2010; McCluskey *et al.,* 1998). Thus by the time of clinical presentation, individual clonotypes are likely to be obscured by polyclonal anti-Ro/La autoantibody reactivity. This complexity in patient serum samples has been highlighted recently by isolelectric focusing and affinity immunoblotting of serial serum samples from a single anti-Ro60-positive patient (Kurien *et al.,* 2009).

It was reasoned that an initial positive selection of complex anti-Ro/La polyclonal sera on a structurally defined Ro60 epitope might narrow the clonotypic diversity of established anti-Ro60 responses sufficiently for direct sequencing of purified autoantibodies. In this study, Ro60peg, an immunodominant epitope present on an

apical peg-like structure of the Ro60 protein was selected (chapter 1.3.4). High titres of autoantibodies directed against this domain, encompassing Ro60 aa 193 to 236, have been linked recently with a subset of patients with primary SS who have diversified Ro/La responses (Reed *et al.,* 2010). Proteomic analysis of anti-Ro60peg IgGs purified from the sera of these patients revealed a long-lived, class-switched clonotypic autoantibody that is common (public) to all patients and specified by a unique H and L chain pairing with shared mutations in the CDRs.

3.2 Results

3.2.1 Isolation of monospecific anti-Ro60peg IgGs from anti-Ro/La sera

This study has led to development of a proteomic approach to analyse self-reactive clonotypes concealed in polyclonal sera that can be applied to multiple patient serum samples. To first select for clonotypic autoantibodies reactive with the Ro60peg B cell epitope, anti-Ro/La-positive sera from 7 patients with primary SS (clinical and demographic characteristics are summarised in table 3.1) was passed over a MBP-Ro60 aa 82-244 affinity column that expresses immunoreactive Ro60peg. The larger Ro60 fragment was used in the column after failing to express the Ro60peg epitope on immobilised MBP-Ro60 aa 193-236. The specificity of eluted IgGs for Ro60peg was confirmed by analysing starting, flow-through and eluted column fractions on Ro52/Ro60/La ELISAs (Figure 3.1 A) and Ro60 subfragment ELISAs (Figure 3.1 B). Immunoblots of overlapping Ro60 subfragments confirmed that reactivity was restricted to the Ro60peg determinant (Figure 3.2).

Table 3.1 *Characteristics of patients with primary SS**

Patient	Gender	Age *(yr)*	Symptoms *(yr)*	Autoantibodies: [a]				Total IgG[b] *(mg/ml)*	RF[b] *(IU/ml)*
				Ro60peg	Ro60	Ro52	La		
pSS1	F	53	21	1.7	2.4	2	0.6	21.5	203
pSS2	F	70	24	2.2	1.4	1.1	0.3	25.2	<40
pSS3	F	78	29	1.6	1.9	1.9	2.1	50.41	1110
pSS4	F	40	12	1.6	1.5	1.3	0.4	10.7	82
pSS5	F	52	13	2.2	1.3	1.7	1.7	29.5	154
pSS6	F	31	14	1.9	1.6	1.4	1.9	68	638
pSS7	F	55	0.5	1.9	2.2	2.9	1.7	52.5	2280

*pSS = primary Sjögren's syndrome; RF= rheumatoid factor.

[a] Autoantibodies were measured by enzyme-linked immunosorbent assay, with optical density (OD) < 0.2.

[b] Total IgG and § Rheumatoid factor measured by nephelometry.

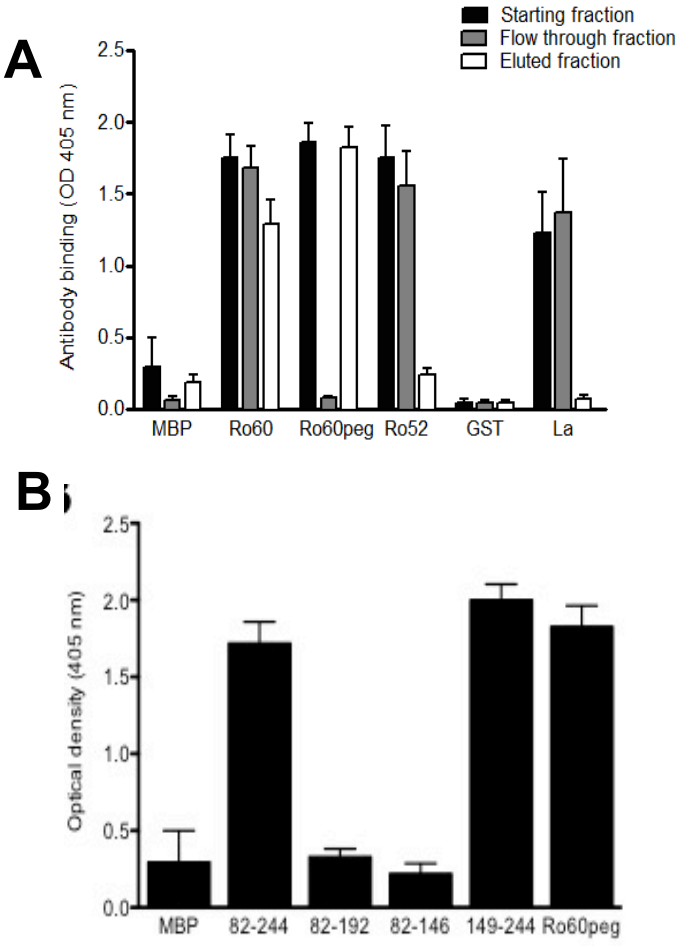


Figure 3.1. *Selection of monospecific anti-Ro60peg IgGs from anti-Ro/La primary SS sera. Epitope-specific affinity chromatography was used to purify anti-Ro60peg IgGs from sera with anti-Ro52/Ro60/La autoantibodies.* ***A.*** *Specificity of affinity-purified IgG was first tested by enzyme-linked immunosorbent assay (ELISA) using native Ro60, maltose binding protein (MBP) expressing Ro52, glutathione S-transferase (GST) expressing La, and MBP and GST controls. Starting, flow through, and eluted fractions were compared. Values are the mean ± SEM of duplicate ODs (n = 7 patients with primary SS patients).* ***B.*** *The fine specificity of the anti-Ro60 IgG was characterised by ELISA using MBP expressing various regions of Ro60. The subfragments 82-244 and 149-244 encompass the Ro60peg epitope. Data is from 7 primary SS patients and depicted as the mean ± SEM.*

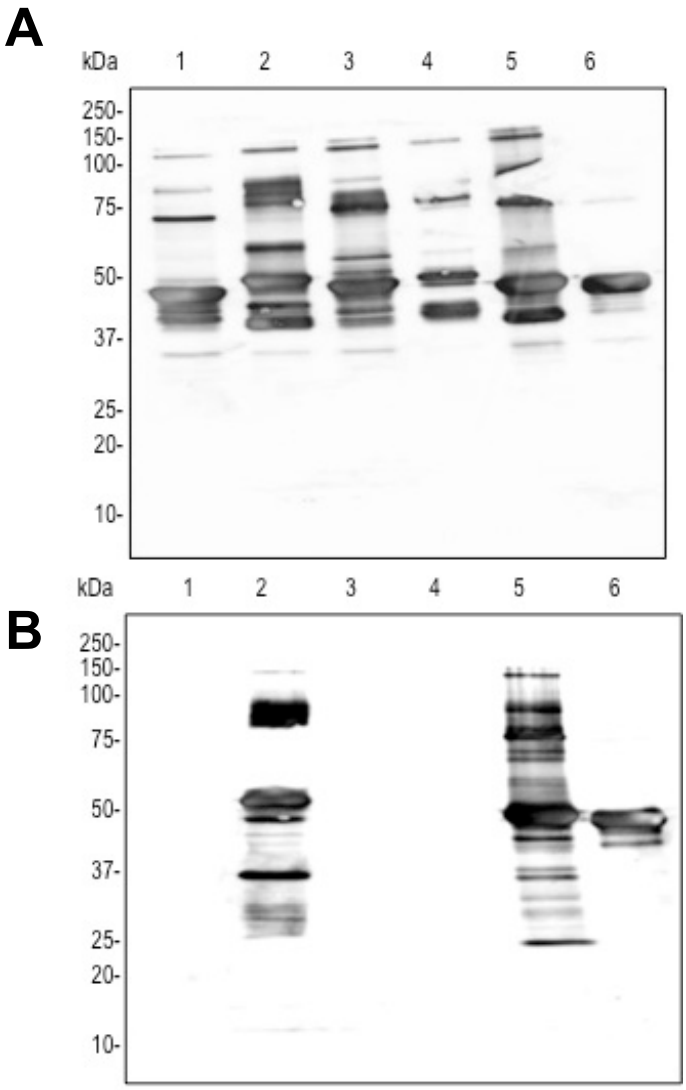


Figure 3.2 *Fine specificity of anti-Ro60peg IgG confirmed on Immunoblots of overlapping MBP-Ro60 fragments.* ***A.*** *Positive control* ***B.*** *Representative immunoblot of recombinant MBP-Ro60 fragments probed with anti-Ro60peg IgG from a patient with primary SS. Columns 1-6 correspond in serial order to the sub-fragments in Figure 1B. Subfragments amino acid (aa) 82-244 (coulumn 2) and 149-244 (column 5) express the Ro60peg epitope.*

The purified autoantibodies also bound human Ro60 expressed in HEp-2 Ro60 transfectants, verifying that the Ro60peg determinant is expressed by intracellular Ro60 protein (Figure 3.3). In control experiments, IgGs were not detectable after passing normal human sera (n=4) or sera from primary SS patients with anti-Ro/La who were negative for anti-Ro60peg (n=2) over the MBP-Ro60 aa 82-244 column; or after passing anti-Ro60peg-positive sera (n=2) over a sham MBP column.

3.2.2 Anti-Ro60peg IgGs are clonally restricted

The electrophoretic properties and clonality of anti-Ro60peg monospecific IgGs purified from patients with primary SS were then assessed using high-resolution 2D-GE. Resolution of Ro60peg-selected H and L chains revealed a markedly restricted pattern when compared with polyclonal IgG, with a dominant L chain species migrating at the same pI of 6.8 in all patients, consistent with a shared clonotypic autoantibody. The corresponding Ro60peg-selected H chains resolved into a number of equally spaced gel spots consistent with charge microheterogeneity of H chains (Figure 3.4). These charge variants are generally observed on isoelectric focusing of monoclonal antibodies and known to result from post-translational modifications that involve both H and L chains (Layer *et al.,* 1999; Liu *et al.,* 2008).

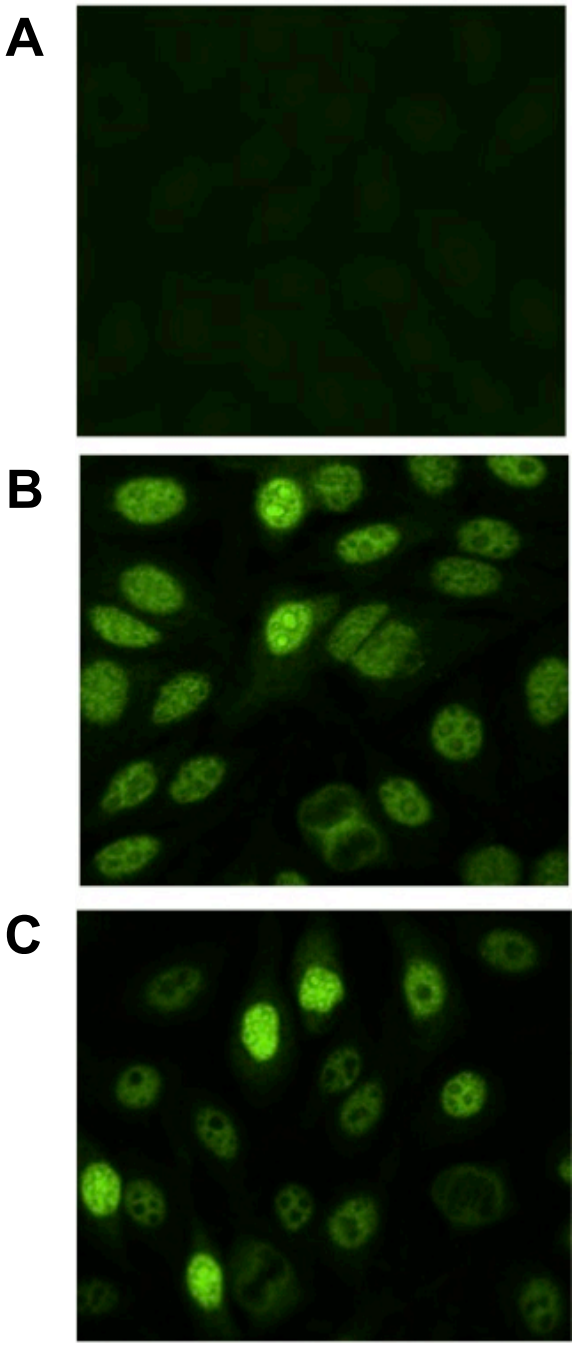


Figure 3.3. *Indirect immunofluorescence of HEp-2 Ro60 transfectants. The brightly stained cells are over-expressing human Ro60* ***A.*** *Negative control.* ***B.*** *Transfected HEp-2 cells (positive control) stained with IgG (10 μg/ml) containing Ro60 autoantibodies.* ***C.*** *Hep2 Ro60 transfectants probed with anti-Ro60peg IgG (2.5 μg/ml) from a representative patient with primary SS.*

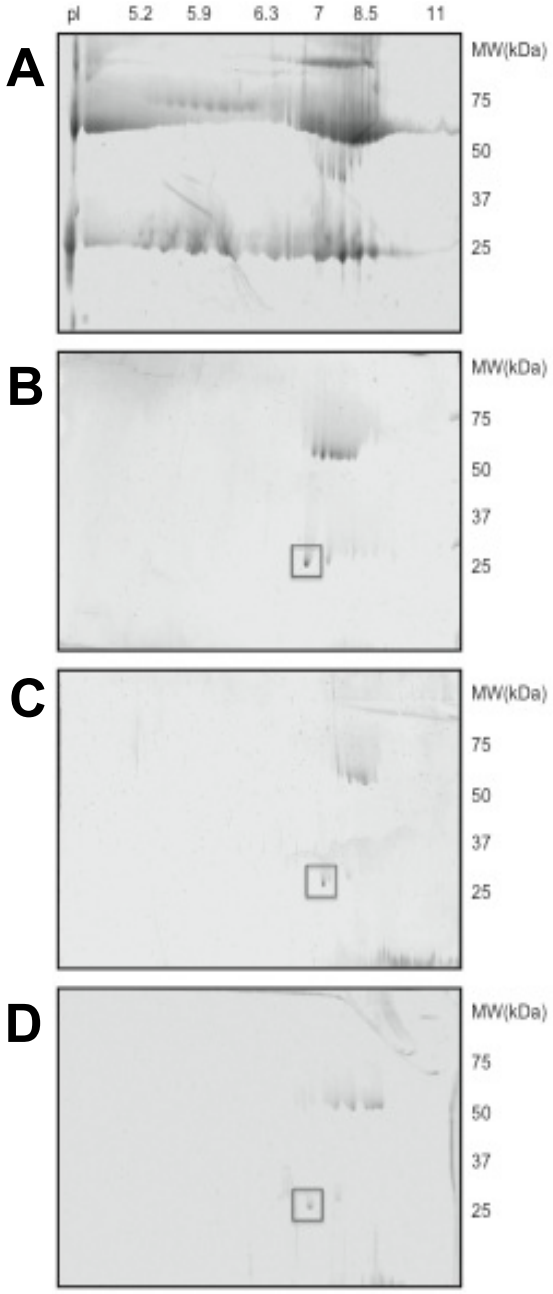


Figure 3.4. *Purified anti-Ro60peg autoantibodies are clonally restricted. Anti-Ro60peg IgGs were separated on high-resolution 24 cm pH 3-11 8-15% gradient gels.* ***A.*** *Whole IgG displays a diverse antibody repertoire (representative of 1 primary SS patient).* ***B-D.*** *2D-GE of affinity purified anti-Ro60peg IgG populations revealed a common clonotypic L chain (boxed) migrating at pI 6.8 and closely spaced H chain isoforms (representative of 3 primary SS patients).*

3.2.3 Ro60peg-reactive clonotypic IgGs are public and specified by a unique immunoglobulin heavy and light chain pairing signature

High-resolution Orbitrap mass spectrometric sequencing was then performed on gel-plugs and in-solution digests to confirm clonality and characterise at a protein level the C region, *IgV* gene usage and V-region mutational status of anti-Ro60peg IgGs from the 7 primary SS patients. Sequencing of C regions confirmed a kappa-restricted monotypic population of the IgG1 isotype. No lambda L chain sequence was detected. An early study (Reed *et al.,* 2010) reported that Ro60peg autoantibody populations were IgG1 kappa and lambda, which conflicts with the definitive kappa restriction identified by mass spectrometric sequencing of the purified anti-Ro60peg IgG in this thesis. The anti-lambda conjugate used in the 2010 study was subsequently found to be cross-reactive with kappa L chains. This issue was readdressed using new specific anti-kappa and anti-lambda conjugates on the Ro60peg ELISA that confirmed clonotypic restriction to IgG1 kappa (data not shown), now concordant with MS data.

Combined database-driven and *de novo* aa sequencing of the V-regions of H and L chains was then performed for the first time on a purified human autoantibody. These techniques achieved near full-length V-region sequence in a single run of less than 50 ng of affinity-purified human IgG in the multiple patient samples. In agreement with recent reports of *de novo* Ig sequencing, reliable H-CDR3 sequence was not obtained because of the highly random nature and variable sequence quality through this region (De Costa *et al.,* 2010; VanDuijn *et al.,* 2010). Accordingly, analysis of Ro60peg-specific Ig V-region sequences revealed a single V_K*3-20*-encoded L chain in each

patient that was further specified by arginine substitutions in the H-CDR3 at either position 91 (3 primary SS) or 92 (4 primary SS) of the CDR3s (Figure 3.5 A) (Spectra and MS data are shown in appendix 3). Faint adjacent L chain 2D-GE gel spots near the dominant pI 6.8 species were excised as gel plugs, sequenced and shown to be *V_K3-20*-encoded, in keeping with L chain isoforms of monoclonal antibodies commonly observed on 2D-GE (Layer *et al.,* 1999; Harrison *et al.,* 1992). Similarly, mass spectrometric sequencing of individual Ro60peg-specific IgGs revealed a single *V_H3-23*-encoded H chain in all patients (Spectra and MS data are shown in appendix 3), thereby confirming, by direct protein sequencing, that the clonotypic autoantibody identified on 2D-GE is a monoclonal autoantibody with a unique H/L pairing signature. H chains from different patients contained shared mutations within the H-CDR1 with either aspartic acid substitutions at positions 30 or 31 (4 primary SS) or an asparagine substitution at position 30 (2 primary SS) (Figure 3.5 B). The sequence at position 31 of subject 7 was unattainable due to trypsin cleavage at the adjacent aa site. Apparent random mutations were noted in the L-CDR1 and H-CDR2 of individual patients. As with L chains, sequencing of closely spaced H chain gel plugs confirmed *V_H3-23*-encoded H chain isoforms that are assumed to arise from post-translational modifications such as N-glycosylation and/or deamidation. Since flow-trough fractions were depleted of anti-Ro60peg binding activity (Figure 3.1 A), it is apparent that all of the anti-Ro60peg reactivity is accounted by the V_H3-23/V_K3-20 clonotype.

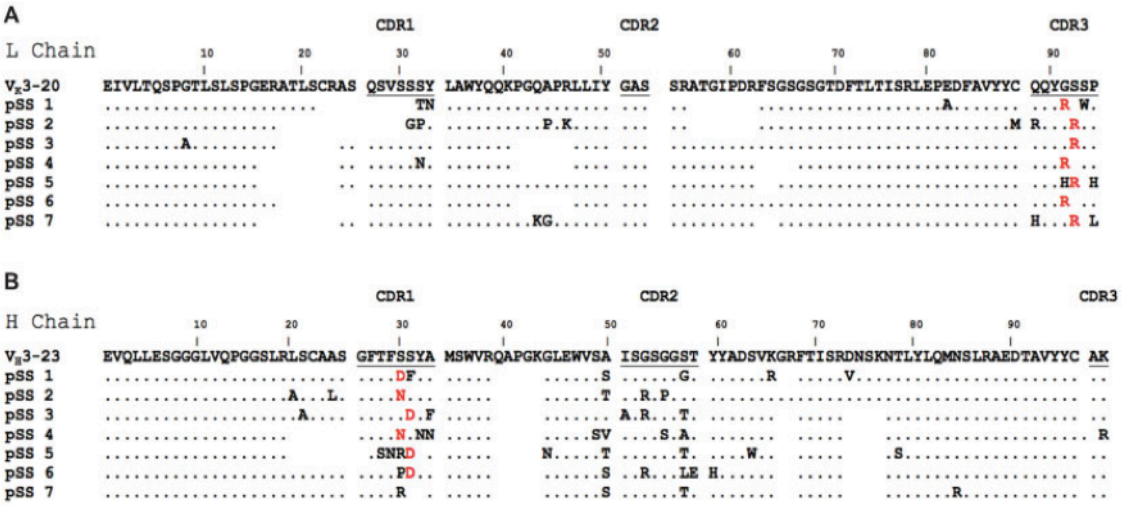


Figure 3.5. *Direct sequencing of anti-Ro60peg IgGs reveals a public clonotype with a distinctive molecular signature. De novo sequencing was performed on Ro60peg-purified IgGs from 7 primary SS patients (pSS), using nano-liquid chromatography Orbitrap mass spectrometry.* ***A.*** *Light-chain (L chain) V-region sequences are aligned with V_k3-20 germline. A common arginine (R) mutation was noted in the L chain third complementarity-determining region (CDR3) with a S-to- R substitution at position 92 (in 4 patients with primary SS) or a G-to-R substitution at the adjacent position 91 (in 3 patients with primary SS).* ***B.*** *Heavy-chain (H chain) V-region sequences are aligned with germline V_H 3-23. Shared mutational patterns were observed in the H-CDR1 with S-to-D substitutions at positions 30 or 31 (in 4 patients with primary SS) or a S-to N substitution in position 30 (in 2 primary SS). Dots indicate homology with germline sequence derived from the IMGT database. Germline CDRs are underlined. Amino acids divergent from the germline are indicated in text with recurring mutations highlighted in red. Spaces indicate areas of incomplete sequence.*

3.2.4 The anti-Ro60peg clonotype is a stable phenotype

Studies on serum samples collected retrospectively from four of the primary SS patients indicate that the anti-Ro60peg immune response is long-lived with high-titre humoral responses persisting over many years (Figure 3.6 A). Furthermore, relative autoantibody affinities as assessed by KSCN elution of bound anti-Ro60peg IgGs remain remarkably constant over years, consistent with a stable phenotype (Figure 3.6 B). We have also detected high titre anti-Ro60peg autoantibodies expressing the V_H3-23/V_K3-20 clonotype in an asymptomatic patient with anti-Ro/La as well as in the asymptomatic mother of a baby with congenital heart block, raising the possibility that this self-reactive B cell clonotype emerges early in disease or precedes clinical disease.

3.3 Discussion

A key finding of this study is that a subset of unrelated primary SS patients, defined by an immune response against an identical determinant, express a public Ro60peg-reactive B cell clonotype specified by a H chain derived from the *V_H3-23* gene segment paired with a L chain derived from the *V_K3-20* gene segment. An implied corollary is that primary SS patients who express this B cell clonotype share a common pathway of autoimmunity to the Ro60peg determinant. To date this specific H/L chain pairing has not been reported previously for human autoantibodies. *V_H3-23* is commonly employed by human autoantibodies and over-represented in the normal B-cell repertoire (Dorner *et al.,* 2005), and is also used preferentially in some MALT lymphomas (Adam *et al.,* 2008; Sakuma *et al.,* 2007).

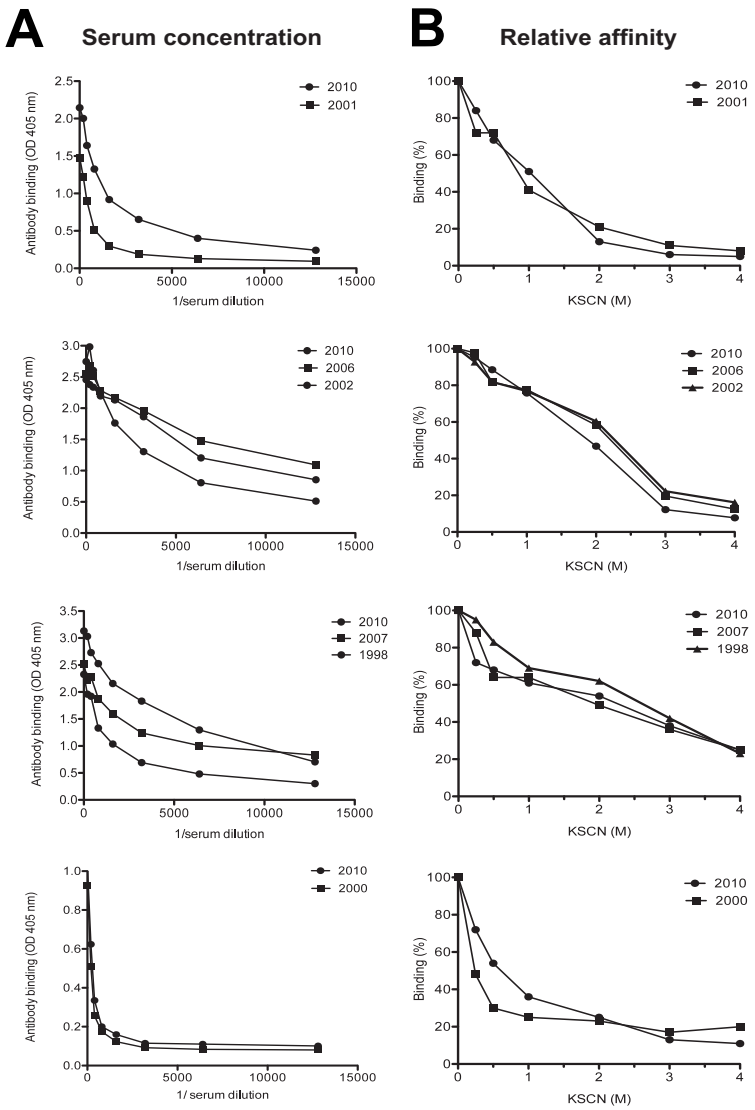


Figure 3.6. The Ro60peg-reactive clonotypic autoantibody is a long-lived stable phenotype ***A***. *Anti-Ro60peg concentrations were compared in serial serum samples from 4 patients with primary Sjögren's syndrome (SS) by testing 2-fold dilutions of sera in a maltose binding protein (MBP)-Ro60peg enzyme linked immunosorbent assay (ELISA).* ***B***. *Relative affinities of anti-Ro60peg IgG binding were compared in the same sera, using KSCN elution profiles. Sera were bound to MBP-Ro60peg coated on ELISA plates and incubated with varying concentrations of KSCN. Values are the mean from duplicate samples. OD = optical density.*

V_K3-20 on the other hand is expressed in B-cell clones of type II mixed cryoglobulinaemia, an autoimmune disorder that shares a risk for lymphoma in primary SS (De Re *et al.,* 2006).

The presence of common mutations in the CDRs of both the H and L chains in these patients, notably arginine and aspartic acid mutations, supports the notion that Ro60peg-reactive clonotypic B cells are positively selected during an active immune response characterised by antigen-driven somatic mutation. However, the possibility that some of the recurring mutations may originate from underlying genetic variation cannot be fully excluded. Further studies including molecular modelling will be useful in determining the structural role of these recurring mutations in specific binding to the Ro60peg domain. Intriguingly, L chain substitution experiments of a human anti-double-stranded DNA monoclonal antibody have revealed the potential importance of pairing a *V_H3-23*-encoded H chain with a L chain containing arginine residues in the CDR3 regions in Ro60 binding, although the Ro60 epitope specificity was not determined (Haley *et al.,* 2004).

The mechanisms that lead to a breakdown in immune tolerance to the helix-loop-helix structure of the Ro60peg domain and secretion of the long-lived public clonotypic autoantibody in these patients have yet to be determined. Some contributing factors may include the immunogenetic background of the host (Rischmueller *et al.,* 1998); increased availability of Ro60 self-antigen resulting from delayed apoptotic cell clearance; elevated levels of the pro-survival cytokine BAFF in patients with primary SS (Groom *et al.,* 2002; Mariette *et al.,* 2003); and T cell help from IL-21-producing T

follicular helper cells which promotes exit from germinal centres and differentiation to long-lived memory B cells and plasma cells (Simpson *et al.,* 2010; DiPlacido *et al.,* 2010).

Perturbation in the B cell repertoire with a biased V_H3-23/V_K3-20 chain pairing in this primary SS subset cannot be fully rule out; however, Ig chain pairing appears random and unrestricted in the normally expressed repertoire and is comparable in healthy subjects and SLE donors (Brezinschek *et al.,* 1998; DeWildt *et al.,* 1999; DeWildt *et al.,* 2000). Accordingly, a model of Ro60 antigen selection and clonal expansion of Ro60peg-specific B cell clonal precursors formed by the stochastic pairing of a heavy chain expressing IGV_H3-23 and light chain expressing IGV_K3-20 is favoured (Figure 3.7). These precursors presumably bypass early B cell tolerance checkpoints in the bone marrow and periphery to be subjected to positive selection by B cell receptor engagement with Ro/La ribonucleoprotein complexes released from dying cells in germinal centres of secondary lymphoid organs, or ectopic lymphoid tissue (Figure 3.7).

One possible mechanism of evasion of pre-germinal centre checkpoints may be so-called "clonal ignorance" due to unavailability of Ro60 ligand in the periphery (Shlonchik *et al.,* 2008). Such tolerance through indifference has been demonstrated for the intracellular La autoantigen in a double La/anti-La transgenic mouse model (Aplin *et al.,* 2003). Follicular dendritic cells may be involved in clonal survival by capturing apoptotic remnants and presenting Ro60 autoantigen to B cells (Vinuesa *et al.,* 2009).

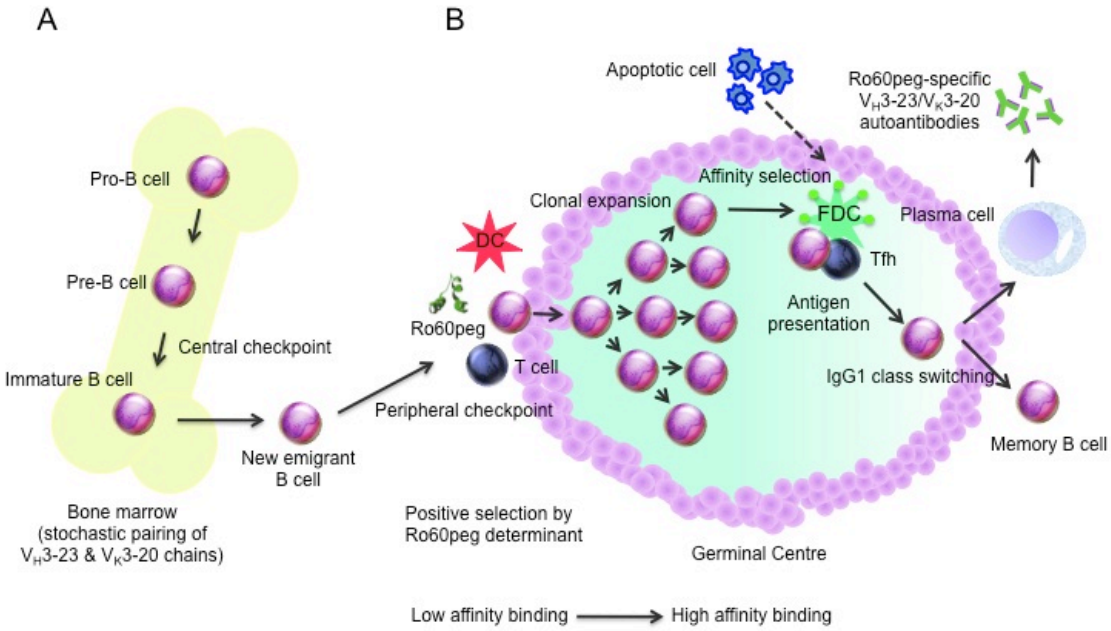


Figure 3.7. *A simplified model of Ro60peg humoral autoimmunity in primary Sjögren's syndrome (SS)* ***A.*** *Ro60peg clonal precursors are formed in the bone marrow by stochastic pairing of a heavy chain expressing V_H3-23 and a light chain expressing V_K3-20. These precursors bypass central and peripheral B cell tolerance checkpoints and are subjected to positive selection in germinal centres of secondary lymphoid organs or ectopic lymphoid tissue. B cell tolerance checkpoints 1 and 2 are defined by Meffre & Wardemann (2008).* ***B.*** *Mature naïve B cells migrate to the boundary between the B cell follicles and T cell zone in search of T cell help. After T cell dependent antigen-mediated activation naïve B cells migrate into B cell follicles, undergo clonal expansion and activate somatic hypermutation (SHM) on their Ig V-regions. B cells then move from the dark zone to the light zone where they are selected for high affinity of their B cell receptor for cognate antigen (Ro60peg) derived from apoptotic cells. The surface immunoglobulin undergoes class switching and Ro60peg-specific B cells differentiate into either memory B cells or plasma cells that secrete Ro60peg-specific V_H3-23/V_K3-20 clonotypic autoantibody. FDC indicates follicular dendritic cell; Tfh, T follicular helper cell; SHM, somatic hypermutation.*

The finding of a mutated public clonotypic autoantibody directed against a member of the clinically relevant extractable nuclear antigen family implicates Ro60 protein as a self-antigen capable of stimulating B-cell clonal proliferation. As such, it represents a rare example of a "forbidden" autoreactive B cell clone in human disease that reacts with a determinant on a bona fide systemic autoantigen. The H/L chain pairing signature offers a unique opportunity to track the development and fate of a self-reactive clonotype in human subjects. As such, a future aim will be to use labelled antigen to isolate B cells that produce the clonotypic autoantibody from peripheral blood and/or tissues and determine their B cell subset distribution.

This methodology has been used recently to delineate B cell tolerance checkpoints bypassed by a subset of DNA-reactive autoantibodies in patients with systemic lupus erythematosus (Jacobi *et al.,* 2009). The proteomic approach reported herein represents the first combined database and *de novo* sequencing of a genuine serum-derived human autoantibody, and may be applicable to other diseases with complex autoantibody systems. The methodology can be applied to multiple patient samples, achieves close to full-length V-region sequence in a single run, and requires far smaller quantities of IgG (<50ng) than Edman degradation. As noted in a recent proteomic study, shared Ig sequence motifs combining germline sequence and somatic mutations may provide unique biomarkers of specific autoantibodies in human disease (VanDuijn *et al.,* 2010).

Finally, if the clonotypic anti-Ro60 population is determined to be a pathogenic species in this subset of patients with primary SS, such as by driving autoantibody spreading or producing type I interferons, or is found to be a precursor clone of a lymphoma, then

selective silencing of the autoreactive B cell clonotype by an anti-idiotypic or antigen-specific approach remains a consideration.

The next chapter of this thesis will address a key question in human systemic autoimmune disease, namely the genesis of life-long humoral autoimmunity. The proteomic-based approach developed herein will be used to track the evolution of the public Ro60peg-specific clonotypic autoantibody in serial serum samples.

Chapter 4: Long-term Humoral Autoimmunity is Maintained by Rapid Clonal Turnover

4.1 Introduction

The immune mechanisms that sustain high-titer anti-Ro60 autoantibodies over a patient's lifetime are poorly understood. Humoral responses to RNA binding proteins such as Ro60 are thought to remain relatively stable over time, as opposed to anti-dsDNA responses that show marked variation in titer in many patients (Pisetsky, 2012). However, an early study with frequent measurements of anti-Ro60 autoantibody levels in a patient with systemic lupus erythematosus over 80 months revealed unexpected fluctuations in autoantibody levels unrelated to disease activity, immunosuppressive treatment, and anti-dsDNA antibody levels, suggesting variable production of anti-Ro60 autoantibodies (Derksen & Meilof, 1992). It is not known whether these findings are generalisable to other anti-Ro60-positive patients, or whether these fluctuations are indicative of more significant changes in the autoantibody response that may be missed by standard solid-phase immunoassay.

Long-term humoral autoimmunity is thought to be generated by short-lived plasma cells arising from the continuous activation of autoreactive B cells, and/or by long-lived plasma cells that survive for years in survival niches in the bone marrow (Hoyer et al., 2004; Radbruch *et al.,* 2006) (Figure 4.1). However, these models of autoantibody production have yet to be tested against a bona fide human autoantigen such as Ro60.

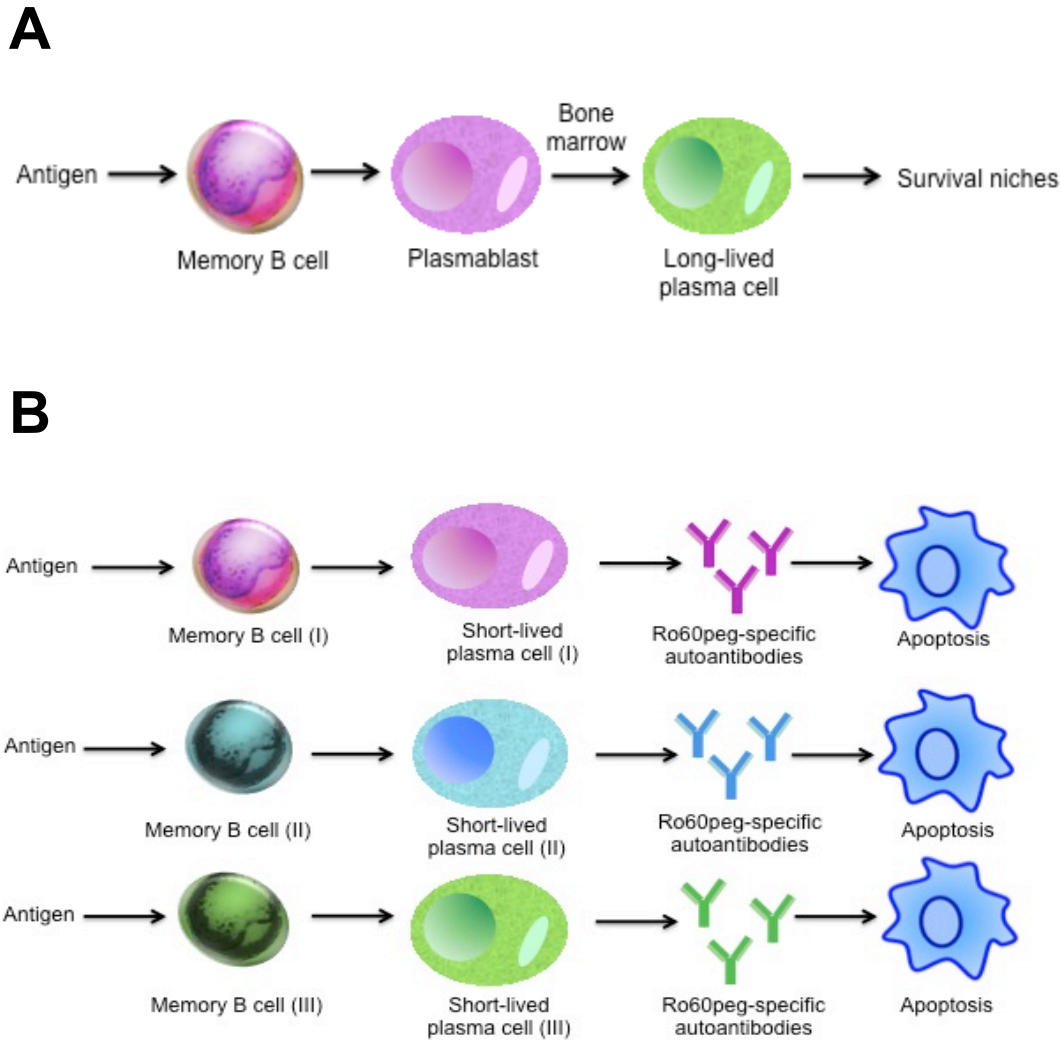


Figure 4.1**. Mechanisms for long-term humoral autoimmunity.* ***A. *Persistence of anti-Ro60 autoantibodies could be generated by long-lived plasma cells that reside in the survival niches and last for years.* ***B.*** *An alternative model is the clonal succession model in which there is continual turnover of short-lived plasma cells that masquerades as long-lived humoral autoimmunity. In this scenario short-lived plasma cell clones undergo clonal exhaustion or deletion to be replaced with a new clone derived from a pool of memory B cells.*

The fluctuation of the humoral anti-Ro60 response has not been previously studied at the level of the secreted autoantibody repertoire because of technical limitations in sequencing low abundant autoantibodies in complex sera with multiple anti-Ro/La specificities.

In the previous chapter high resolution Orbitrap mass spectrometric autoantibody sequencing was used to identify a clonotypic signature and unique V_H3-23/V_K3-20 chain pairing that is public (shared) across unrelated patients. This clonotype, specific for Ro60peg, appears to be phenotypically stable on conventional immunoassay (Lindop *et al.,* 2011). In this chapter, the secreted anti-Ro60 autoantibody proteome was directly sequenced in serial samples from patients with the prototypic human systemic autoimmune disease of primary SS, and analysed the clonotypic variation over time. This study demonstrates a dynamic process of repeated turnover in Ro60 humoral autoimmunity, characterised by a periodic succession of Ro60-reactive clonotypes masquerading as long-lived Ro60 humoral autoimmunity.

4.2 Results

4.2.1 Isolation of monospecific anti-Ro60peg IgG from polyclonal serum

In chapter 3 an apparently long-lived public Ro60peg-reactive clonotypic autoantibody specified by a unique V_H3-23/V_K3-20 chain pairing was identified (Lindop *et al.,* 2011). The present study investigates whether this clonotype is stable over long periods or is replaced periodically. These mutually exclusive possibilities were examined by aa sequencing of anti-Ro60peg clonotypic autoantibodies purified from serial serum samples of 4 patients with primary SS. Ro60peg-sepcific IgGs were initially purified by

passing sera over an affinity column that expresses immunoreactive Ro60peg. The eluted IgGs were confirmed as monospecific for Ro60peg by analysing starting, flowthrough, and eluted column fractions on Ro52/Ro60/La ELISA's (Figure 4.2). Absence of Ro60peg binding activity in flowthrough fractions indicated that the entire anti-Ro60peg response is accounted for by the V_H3-23/V_K3-20 clonotype.

4.2.2 Anti-Ro60peg autoantibodies undergo sequential clonal replacement

Next, a retrospective longitudinal analysis of Ro60peg-specific IgGs purified from serial serum samples of up to 10 years duration in patient's pSS1, pSS2 and pSS3 was carried out (clinical and demographic characteristics are summarised in Table 4.1). De novo MS sequencing of purified Ro60peg IgGs was performed to obtain V-region molecular signatures. Near full-length sequences confirmed expression of a single anti-Ro60peg clonotype at each time point, defined by a V_H3-23 heavy chain paired with a V_K3-20 light chain, with conserved aa substitutions as demonstrated previously in Chapter 3 (Lindop *et al.,* 2011). Further de novo sequencing revealed that the V_H3-23 and V_K3-20 regions rearrange with J_H5 and J_K2, respectively, further confirming clonality.

V-region sequences revealed a process of periodic Ro60peg-specific clonotypic turnover, simulating long-lived humoral autoimmunity (Figure 4.3) (spectra and MS data are shown in appendix 4). In patient pSS1, the anti-Ro60peg clonotype remained unchanged over 3 months between Feb 2002 and April 2002; by 7 months later a new clonotype had arisen (Nov 2002). Uniquely mutated clonotypes with the same H and L chain gene pairing then appeared in 2005 and 2006 (Figure 4.3 A). Clonotypic turnover was also observed in patient pSS2 over periods of at least 5 months (Figure. 4.3 B).

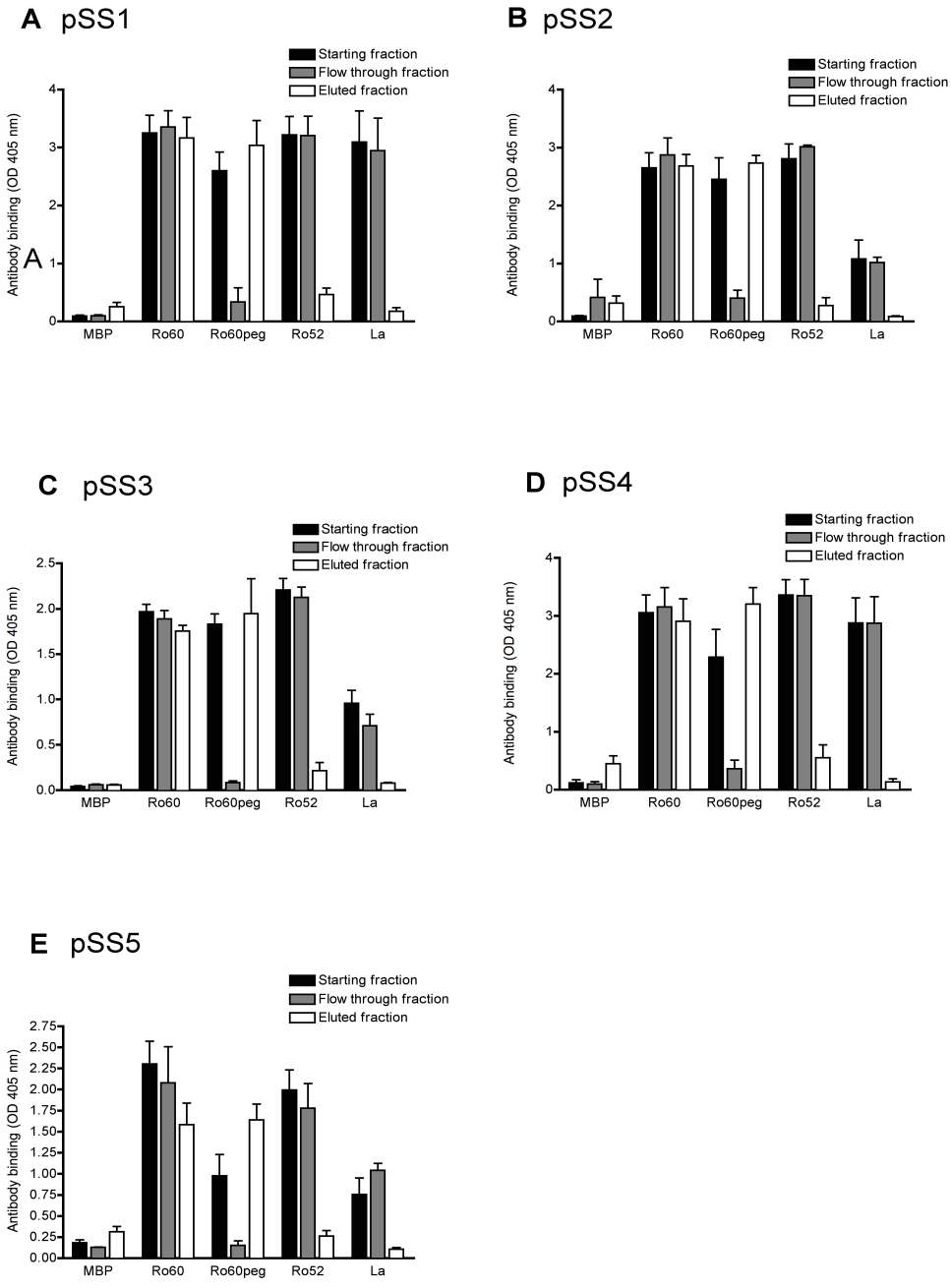


Figure 4.2. *Affinity-purified anti-Ro60peg IgGs are monospecific for Ro60peg determinant. Ro60peg-specific IgGs were affinity purified from sera of patients pSS1-4 purification confirmed by enzyme-linked immunosorbent assay (ELISA) on multiple runs (pSS1 n=7, pSS2 n=5, pSS3 n=5, pSS4 n=6, pSS5 n=4). (A-E) Specificity of anti-Ro60peg IgGs from patients pSS1-5 demonstrating isolation of Ro60peg-specific IgGs, without significant contamination with Ro52- or La-specific IgGs. Optical density (OD) expressed as mean ± SEM.*

Table 4.1 *Characteristics of patients with primary SS**

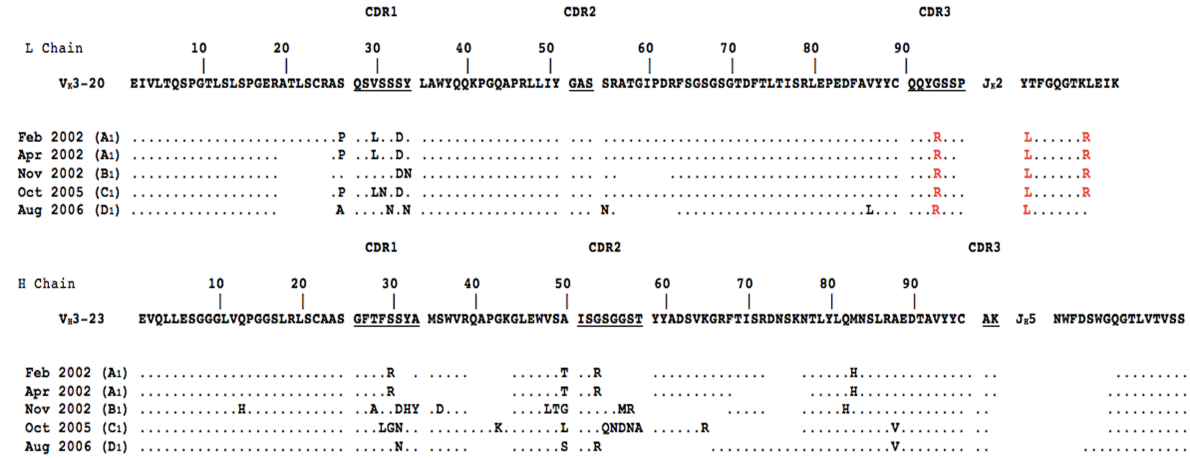
Patient	Gender	Age *(yr)*	Symptoms *(yr)*	Autoantibodies: [a]				Total IgG[b] *(mg/ml)*	RF[b] *(IU/ml)*
				Ro60peg	Ro60	Ro52	La		
pSS1	F	70	24	2.2	1.4	1.1	0.3	25.2	<40
pSS2	F	31	14	1.9	1.6	1.4	1.9	68	638
pSS3	F	42	12	1.6	1.5	1.3	0.4	10.7	82
pSS4	F	41	13	0.9	1.8	1.8	1.8	50.1	<40
pSS5	F	55	0.5	1.9	2.2	2.9	1.7	52.5	2280

*pSS = primary Sjögren's syndrome; RF= rheumatoid factor.

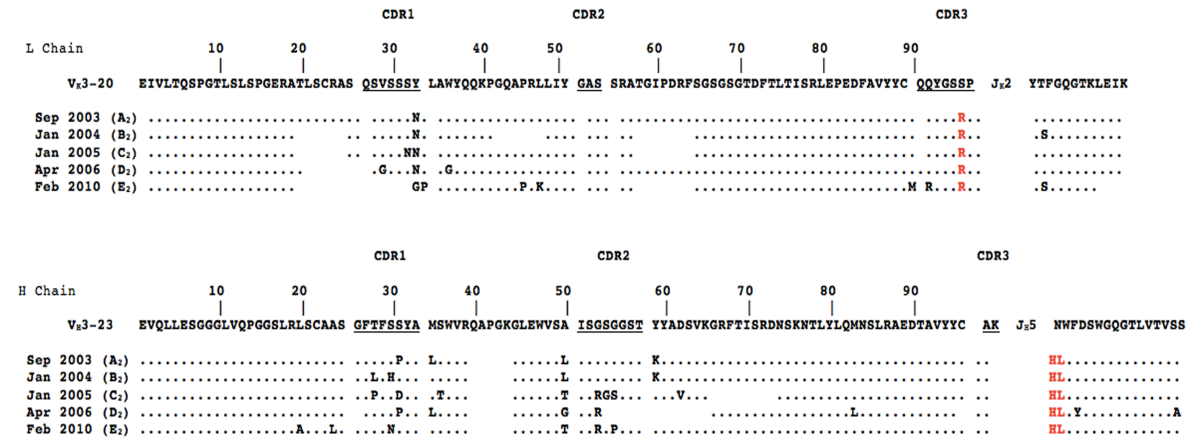
[a] Autoantibodies were measured by enzyme-linked immunosorbent assay, with optical density (OD) < 0.2.

[b] Total IgG and § Rheumatoid factor measured by nephelometry.

A pSS1



B pSS2



C pSS3

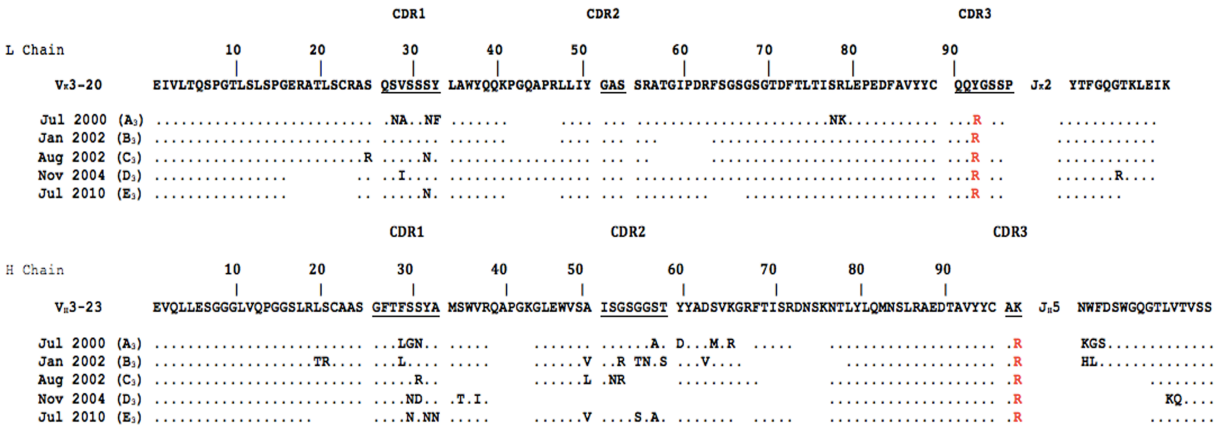


Figure 4.3. *Retrospective longitudinal analysis of Ro60peg-specific clonotypic autoantibodies from 3 patients. Serial samples were analysed by Orbitrap mass spectrometry and de novo V-region sequencing. H- and L-chain sequences are aligned with germline VK3–20/JK2 and V_H3–23/JH5, in which CDRs are underlined.* ***A.*** *In pSS1, clonotype A1 is unchanged over 3 months (Feb 2002 to April 2002), but a replacement clonotype has emerged by November 2002 (B1; 7 month interval). Similarly, clonotypic replacement occurred by October 2005 (C1) and August 2006 (D1).* ***B.*** *In pSS2, clonotype A2 (September 2003) is replaced by January 2004 (B2; 5 month interval). Replacement clonotypes are observed in January 2005 (C2), April 2006 (D2), and February 2010 (E2). Dots represent homology with germline sequence. Amino acids divergent from germline are indicated as text with common mutations in red. Spaces indicate incomplete sequence.* ***C.*** *In pSS3, clonotype B3 (Jan 2002 to Aug 2002) is replaced by a new clonal variant after 5 months. New clonal variants appeared at each subsequent time point in Nov 2004 and Jul 2010.*

Sequential clonotypes had unique V-region mutational signatures, indicating that every clone arises de novo and has not evolved from its predecessor by sequential addition of new mutations. A similar pattern of clonotypic turnonver at 6 months or longer was observed in serial samples taken from pSS3. New clonal variants with a distinct mutational signature appeared at 6 months (Aug 2002) and subsequent time points (Nov 2004, Jul 2010) (Figure 4.3 C), consistent with the clonal turnover model.

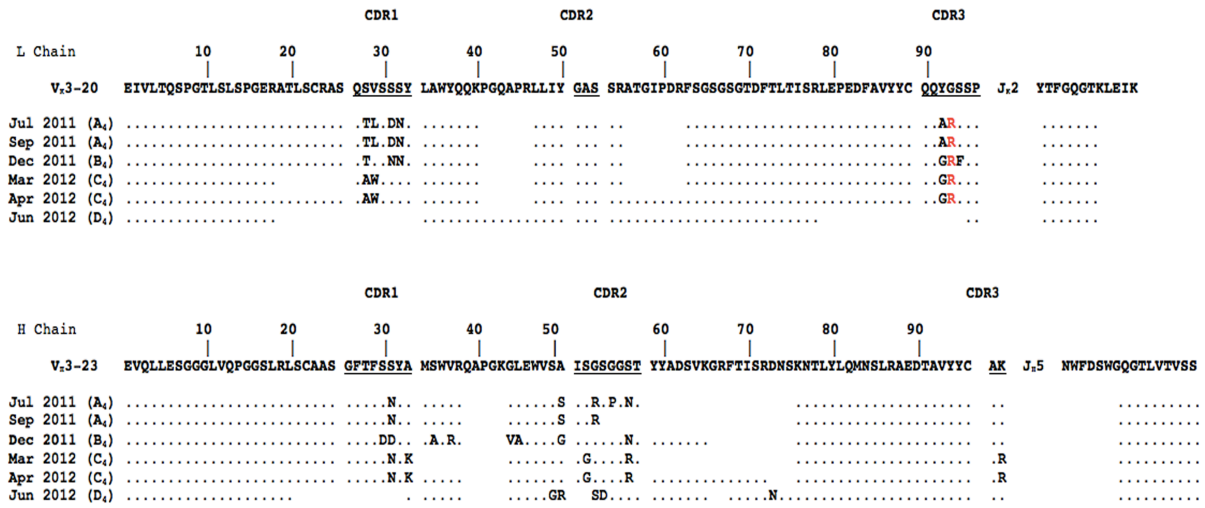
4.2.3 Clonotypic replacement occurs at regular intervals

Retrospectively, clonotypes appeared to undergo repeated replacement every 3-7 months. To better define the kinetics of the clonotypic autoantibody turnover, anti-Ro60peg clonotypes were prospectively tracked in two additional patients (pSS4 and pSS5) (Spectra and MS data are shown in appendix 4). In patient pSS4, the clonotype changed three times, at approximately three-monthly intervals (Figure 4.4 A). In pSS5, consecutive samples over 3 months indicated a stable clonotype over this period (Figure 4.4 B). These data indicate clonal turnover with a period of no less than 3 months.

4.2.4 Anti-Ro60peg levels fluctuate over time without change in affinity

Anti-Ro60peg levels and affinities from samples collected retrospectively and prospectively over months to years were then measured to study the kinetics of autoantibody production. Autoantibody levels in all patients fluctuated markedly over time (the mean coefficient of variation was 12%), despite minimal change in affinity, consistent with an affinity ceiling in the face of sequential clonotypic turnover (Figure 4.5).

A pSS4



B pSS5

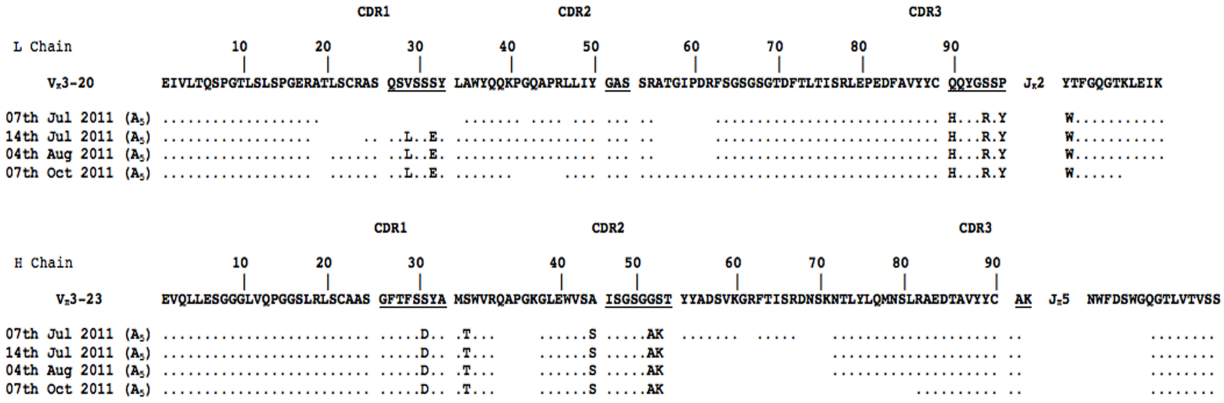


***Figure 4.4**. Prospective longitudinal analysis of Ro60peg-specific clonotypic autoantibodies from 2 patients. Serial samples were analysed and are presented as in Figure 4.4. **A.** In pSS4, clonotype A3 is unchanged over 3 months (July 2011 to September 2011, but clonotypic replacement occurs by December 2011 (B3; 6 month interval), and again by March 2012 (C3; 3 month interval). This clonotype remains for at least 6 weeks (March to April 2012), and is then replaced by June 2012 (D3; 3 month interval). **B.** In pSS5, serial samples over 12 weeks demonstrate an unchanged clonotype.*

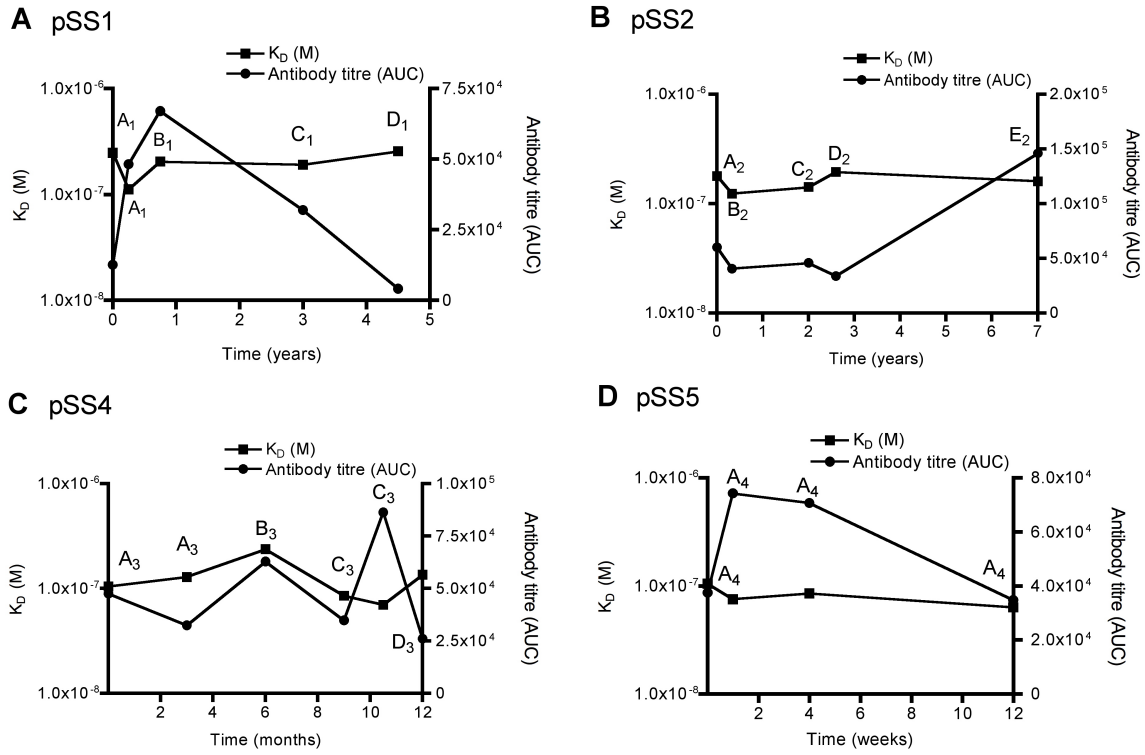


Figure 4.5. *Comparison of Ro60peg-specific clonotypic autoantibody levels and affinities over time in patients pSS1–5. Autoantibody levels determined by area under curve (AUC) of Ro60peg ELISA, and binding affinities (equilibrium dissociation constants, KD) by Biacore X100. Clonotypes denoted by Ax, Bx, Cx, Dx, and Ex. In pSS1 and pSS2, autoantibody levels fluctuate markedly over years. Antibody levels and affinities for pSS3 were not measured due to limited sample availability. In pSS4, levels rise and fall a number of times in 12 months. In pSS5, autoantibody level rises over 1 week, and falls over 11 weeks. There is minimal change in affinity in all patients over time.*

The more detailed kinetic study of a single clonotype in pSS5 revealed a rapid rise in level over 1 week, followed by a steady decline over the following 11 weeks (Figure 4.5 D), consistent with a brief period of monoclonal anti-Ro60peg production, followed by gradual autoantibody clearance.

4.3 Discussion

This study addresses the mechanism of long-term anti-Ro60 autoantibody production in patients with primary SS by direct sequencing of a secreted public clonotype. Autoantibodies directed against RNA-protein complexes, including anti-Ro60, are generally resistant to immunosuppressive and anti-CD20 agents and persist for the life of the patient, leading to the suggestion that these autoantibodies are produced by long-lived plasma cells (Isaksen *et al.,* 2008; Liu *et al.,* 2011).

Here, it was shown that humoral responses against an immunodominant determinant on Ro60 are maintained over years by a periodic turnover of short-lived V_H3-23/V_K3-20 clonotypic variants, each of which is specified by a unique V-region mutational signature. Conventional immunoassays such as ELISA that detect only antibody binding, fail to identify this perpetual yet subtle clonal turnover, whereas sequential clonotypes are revealed here for the first time by using mass spectrometric sequencing of the secreted autoantibody proteome. These findings seem incompatible with a model of long-lived autoreactive plasma cells that secrete stable Ro60-specific clonotypes, but rather infer cycles of clonal expansion and exhaustion over days of short-lived plasma cells or plasmablasts (Figure 4.6).

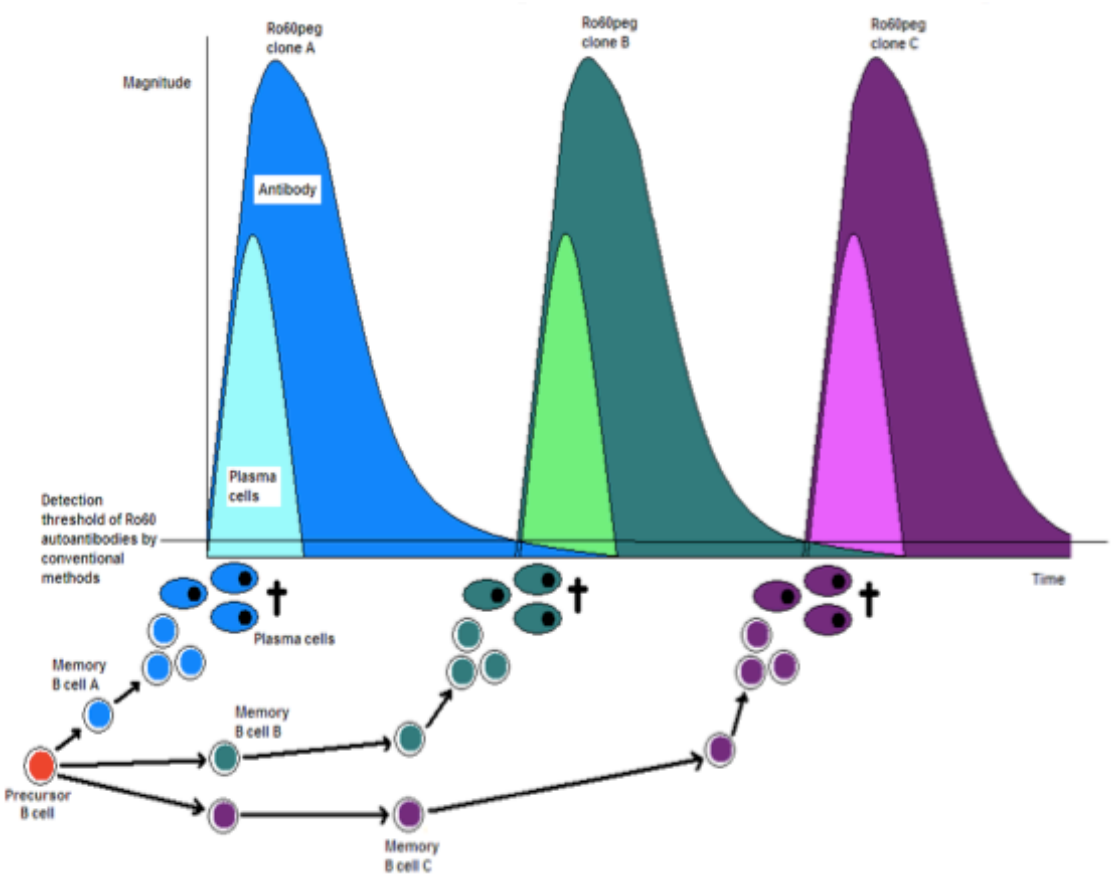


Figure 4.6. *Clonal succession model of long-lived humoral Ro60 autoimmunity. Primary antigen challenge leads to the generation of a pool of Ro60peg-specific memory B cells that reside in inflamed salivary glands. Rechallenge by Ro60peg antigen induces unrelenting cycles of B cell clonal expansion, production of Ro60-specific short-lived plasma cells or plasmablats and clonal depletion. Each uniquely mutated Ro60 clonotype replaces the one before it, and the kinetics of clonal replacement approximates the half-life of IgG. The repeated turnover of Ro60 clonotypic autoantibodies masquerades as long-lived humoral autoimmunity and results in life-long production of anti-Ro60 autoantibodies in patients with primary Sjögren's syndrome (SS).*

This study suggests that there are two potential pathways by which waves of short-lived clonotypic autoantibodies might be produced, both of which would depend on continuous stimulation by self antigen. First, short-lived plasmablasts may be generated by Toll-like receptor-driven activation of autoreactive B cells in extracellular foci, as shown experimentally for rheumatoid factor. T cells can have a facultative role in these reactions leading to isotype switch and somatic hypermutation of IgV regions (Sweet *et al.,* 2011). Alternatively, new clonotypes might arise from expansion of short-lived plasma cell clones generated by repeated recall responses on a pool of Ro60-specific memory B cells that reside in inflamed salivary glands. Successive clonotypes do not appear to result from affinity maturation, since autoantibody affinities change little over years of disease.

The selection pressures driving this dynamic process of clonal succession therefore currently remain unknown. The Ro60 determinants responsible for the ongoing auto-immunogenic stimulation are thought to derive from disordered apoptosis, either as membrane-bound Ro60 on apoptotic bodies or as part of intact Ro/La RNP complexes released after secondary necrosis (Reed *et al.,* 2010). The unrelenting cycles of B-cell clonal expansion, production of Ro60-specific plasmablasts and clonal depletion, inferred from de novo sequencing of serial autoantibody proteomes, may contribute to autoimmune pathogenesis via multiple mechanisms, including the formation of anti-Ro/La IgG-immune complexes; presentation of Ro60 and/or somatically mutated IgV determinants on MHC class II with T-cell activation and epitope spreading; production of cytokines and type I interferons; and salivary gland inflammation (Chan et al., 1999; Lindop et al., 2012; Salinas *et al.,* 2013; Yao *et al.,* 2013).

An intriguing finding of this longitudinal study, in which the de novo mass spectrometric sequencing method can detect a second clonal variant to within a sensitivity of detection of 0.05% of the dominant clonotype (Arentz *et al.,* 2012), was the presence at each serum sample point of a single anti-Ro60peg clonotypic variant, as specified by its unique IgV-region mutational signature. Thus in patient pSS4 clonotypes were completely replaced every three months, implying enhanced clearance of clonotypic IgGs (half-life approximately 9 days) compared to normal serum IgG (half-life approximately 21 days). Overall, the proteomic data are consistent with secretion of repeated short-lived waves of Ro60peg-specific monoclonal IgGs that are cleared rapidly from tissues and circulation following binding to cognate Ro60 protein exposed on apoptotic and necrotic debris. The disappearance of serum clonotypes is consistent with efficient removal or suppression of their putative feeder B-cell clones; such feedback autoregulation may conceivably come from the secreted clonotypic autoantibodies themselves in the form of IgG immune complexes that co-ligate inhibitory Fcγ receptors on Ro60-specific B-cells (Crowley et al., 2009; Dorner *et al.,* 2011).

It will be important to determine whether our findings for the Ro60peg clonotype extend to other epitopes on Ro60 and/or to other extractable nuclear antigen targets of life-long humoral autoimmunity. We have recently reported that secreted human Ro52 autoantibody proteomes express a restricted set of public clonotypes (Arentz *et al.,* 2012). Since anti-Ro52/Ro60 responses are tightly linked, it is likely that Ro52-specific clonotypes undergo a similar pattern of clonal turnover. The continual changes in V-region signatures observed in serial studies may limit the diagnostic utility of single V-

region peptide sequences in detecting serum clonotypic species by targeted MSF. Moreover, patient-specific idiotypic vaccines based on a single clonotype are unlikely to be effective in the face of ongoing somatic mutation. Accordingly, efforts to reduce circulating pathogenic anti-Ro autoantibodies might better be directed towards reducing ongoing exposure of the immune system to immunogenic Ro determinants, perhaps by modulating apoptotic cell turnover in patients with systemic autoimmunity. An alternative way of breaking the cycles of autoantibody production might involve blocking specific B cell access to exposed Ro60 protein by using synthetic peptides designed to bind and mask immunodominant epitopes.

Chapter 5: Concluding Remarks

5.1 Summary and Conclusions

This thesis describes a novel proteomic approach to characterise the clonality and V-region molecular signatures of human autoantibodies based on de novo sequencing using Orbitrap MS. This method has a key advantage over traditional hybridoma and recombinant antibody single-cell approaches in that it represents the global autoantibody repertoire against structurally defined determinants; and allows near full-length protein sequencing of epitope-specific populations of autoantibodies purified from complex whole serum.

The MS approach used herein lead to the discovery of a public (shared) clonotypic autoantibody directed against a major Ro60 antigenic determinant (Ro60peg) in unrelated patients with primary SS. Remarkably, the clonotype was specified by a characteristic V_H3-23 H chain paired with a V_K3-20 L chain and common mutations in both the H and L CDR regions. The utilisation of a highly restricted B cell clonotypic repertoire that is shared among outbred individuals implies a common pathway for the initiation of autoimmunity to the Ro60peg determinant, and challenges the general view that these responses are highly diversified, heterogeneous, and polyclonal. A model proposed is that in patients with primary SS intrinsic defects in early B cell tolerance checkpoints may allow the emergence of low affinity germline-encoded clonotypes that preferentially use V_H3-23/V_K3-20 chain pairings (Meffre and Wardemann, 2008). These may be exposed in secondary lymphoid organs or ectopic lymphoid tissues to Ro/La RNP complexes released from apoptotic and/or necrotic remnants and undergo clonal

selection, expansion, and affinity maturation in dysregulated germinal centres (Vinuesa *et al.,* 2009) (Figure 3.9). The presence of shared aa replacement mutations in the CDR regions in both the H and L chains, notably arginine and aspartic acid mutations supports the role of clonal selection in the generation of Ro60peg-sepcific clonotypes. Furthermore, the finding of a public clonotypic autoantibody directed against Ro60peg represents the final product of autoreactive B cell and plasma cell clones. As such it provides the strongest evidence to date for Burnet's "forbidden" clone hypothesis in an authentic human autoimmune disease.

The immunodominant Ro60peg determinant was studied further by serial autoantibdody sequencing to determine the mechanisms of long-term anti-Ro60 autoantibody production in patients with primary SS. Remarkably, analysis of serial V-region molecular signatures revealed a dynamic process of clonal turnover, characterised by clonotype loss and replacement at approximately 3 monthly intervals. Surprisingly, earlier clonotypes never reappear in the periphery, and a unique molecular signature specifies each new clone. Moreover, levels of secreted anti-Ro60peg clonotypes fluctuated over time despite minimal changes in clonal affinity. These findings challenge the widespread belief that long-lived plasma cells are the key drivers of persistent autoimmunity in patients with primary SS and provide an alternative model for autoantibody production. A model is presented in figure 4.6 in which there is a continual turnover of Ro60peg clonotypes that masquerades as long-lived humoral autoimmunity. In this scenario there is ongoing clonal expansion and exhaustion over days of short-lived autoreactive plasmas cells, or plasmablasts, generated by repeated recall responses on a pool of Ro60peg memory B cells that reside in inflamed salivary

glands, as opposed to a single event generation of long-lived plasma cells (Odendahl *et al.,* 2000).

These findings have important diagnostic and therapeutic implications. The discovery of a single Ro60peg clonotypic autoantibody that is specified by a unique H and L chain pairing and common arginine mutation, in the face of ongoing clonal turnover, could be used as a disease-specific biomarker for targeted diagnostics. This may be particularly useful for early disease diagnosis where a patient with anti-Ro60 autoantibodies may have overlapping symptoms with other diseases, such as SLE, and diagnosis is unclear. Moreover, direct intervention with a small peptide mimic to bind and mask the Ro60peg immunodominant epitope may be a therapeutic approach to retard circulating pathogenic autoantibodies.

5.2 Future Directions

The discovery of a public clonoytypic autoantibody directed against an immunodominant epitope on Ro60, descried in chapter 3, taken together with recent findings for the linked anti-Ro52 (Arentz *et al.,* 2012) and anti-La (Thurgood *et al.,* in press) responses suggest that humoral responses against protein-RNA complexes maybe mediated by a remarkably restricted set of autoreactive public B cell clonotypes. Whether circulating autoantibody proteomes associated with other systemic autoimmune diseases, such as Sm/RNP and ribosomal-P in SLE; citrullinated peptides in rheumatoid arthritis; or β2-glycoprotein in anti-phospholipid antibody syndrome, are driven by restricted sets of public clonotypes needs to be determined.

The continual changes in V-region signatures observed in serial studies of Ro60peg autoantibodies imply a relentless turnover of short-lived clonotypic variants, masquerading as long-lived Ro60 humoral autoimmunity. Further studies are required to determine whether the clonotypic turnover model for the Ro60peg clonotype applies to other epitopes on Ro60 and/or to other extractable nuclear antigen targets of long-lived humoral autoimmunity. Our laboratory has recently found that Ro52 autoantibodies express a highly restricted B cell clonotype repertoire that is public among unrelated patients with systemic autoimmunity (Arentz *et al.,* 2012) (see appendix 2). Since anti-Ro52/Ro60 responses are thought to be associated, Ro52-specific clonotypes may undergo a similar pattern of clonal turnover. This may provide insight into the immune mechanisms that sustain high titer autoantibody production over a patients lifetime.

This thesis has identified a public (common) V-region molecular signature in the L chain CDR3 of the anti-Ro60peg autoantibody among serial samples of unrelated patients with primary SS. The discovery of this unique mutated V-region peptide from the Ro60peg autoantibody proteome (see below) could be translated to a novel biomarker in a targeted MS based diagnostic platforms. This method has the potential to track autoantibody variants and protein modifications that are not detectable by current ELISA and immunoblot methods. The application of targeted peptide MS for clinical diagnosis has been applied in the candidate's laboratory to identify Ro52-specific V-region peptide motifs (Arentz *et al.,* 2012) (refer to appendix 2). As yet, the application has not been applied to other human autoantibodies such as anti-Ro60/La. The tryptic peptide fragment in question, spanning the FW region 3 and CDR3 of the anti-Ro60peg

clonotype (LEPEDFAVYYCQQYG**R** or LEPEDFAVYYCQQY**R**) contains a unique arginine substitution at aa 91 or 92 that is not present in the IMTG or Uniprot databases; this mutated tryptic fragment may be an ideal Ro60peg surrogate peptide for targeted MS detection of anti-Ro60 autoantibodies in complex serum samples. Ultimately, the performance of a targeted MS method for detection of anti-Ro60peg autoantibodies will need to be compared with solid-phase antibody detection methods in the routine clinical setting

Appendices

Appendix 3: Spectra with fragment ion and mass error tables of V_H3-23 and V_K3-20 gene family peptides detected in affinity purified anti-Ro60peg IgG from 7 patients with primary SS (pSS 1-7) (refer to chapter 3). To view refer to the CD attached.

Appendix 4: Spectra with fragment ion and mass error tables of V_H3-23/J_H5 and V_K3-20/J_K2 gene family peptides detected in retrospective and prospective anti-Ro60peg IgG samples from 5 patients with primary SS (pSS 1-5) (refer to chapter 4). To view refer to the USB attache

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