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’.

Name: Rhianna Lindop

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).

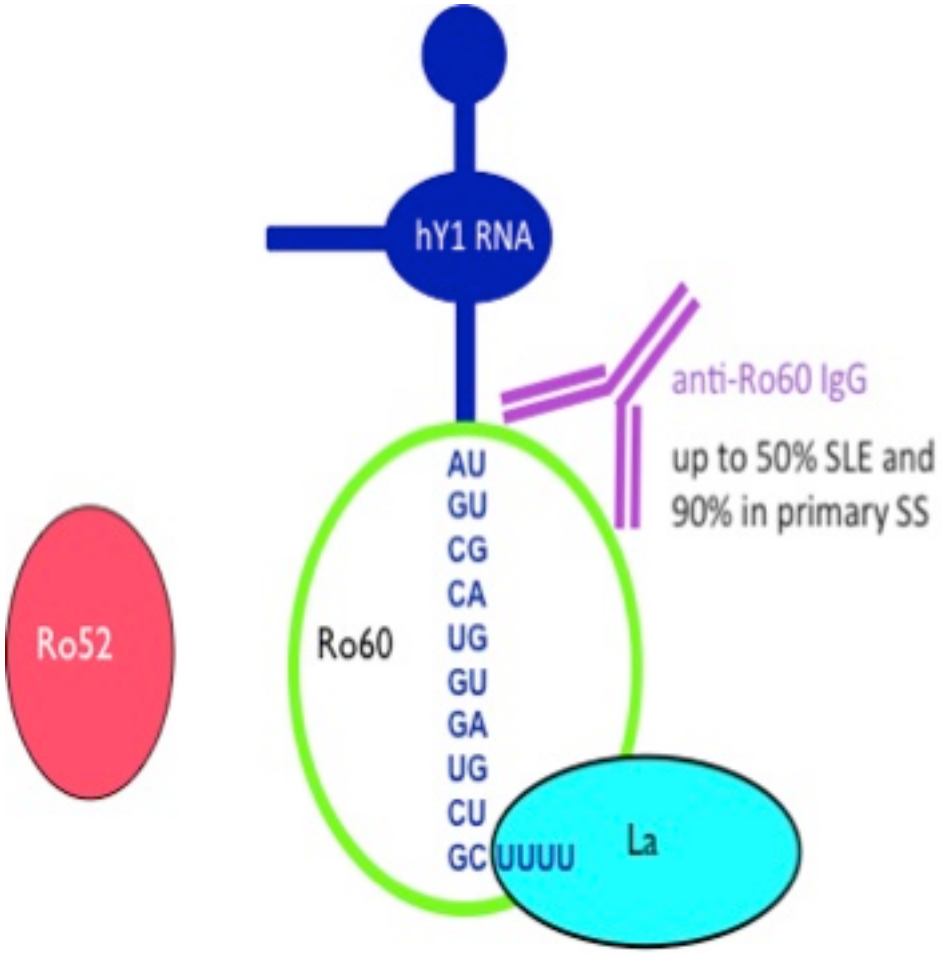


Figure 1.1. *The Ro/La ribonucleoprotein complex is composed of a 60-kDa Ro polypeptide complexed with small non-coding RNA termed hY RNA and a 48-kDa La protein. The complex is a frequent target of autoimmunity in primary* Sjögren's *syndrome (Adapted from van Venrooij et al., 1993).*

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).

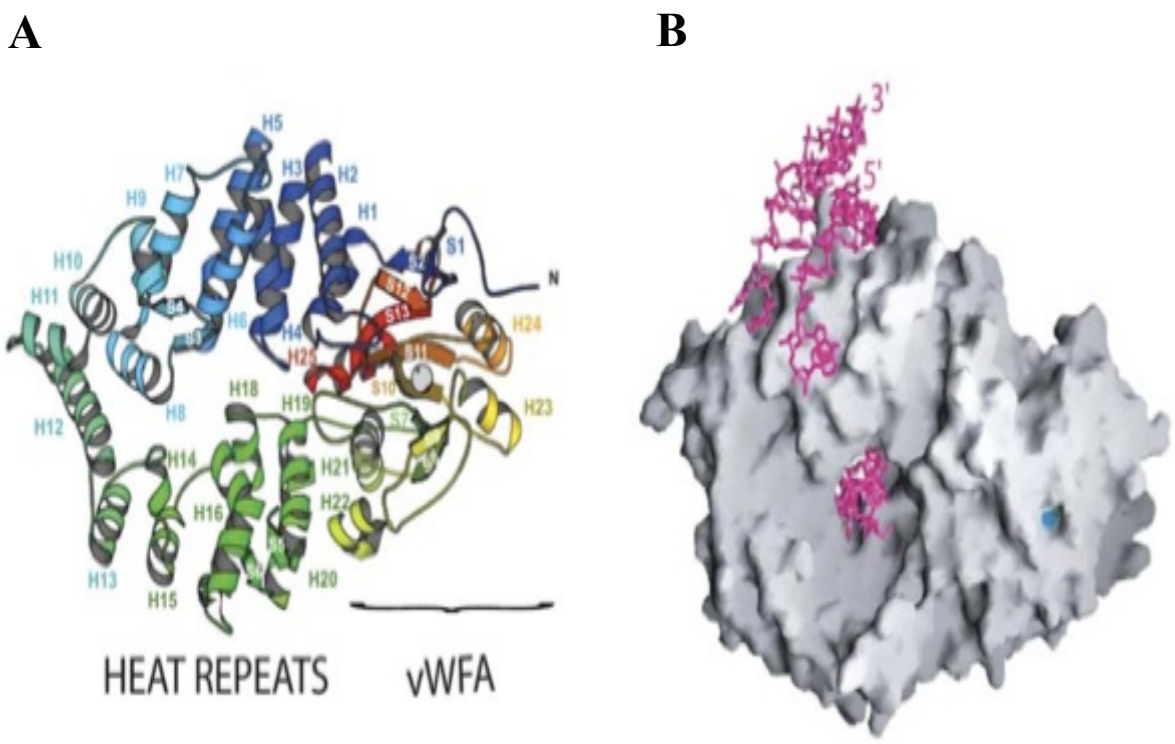


Figure 1.2. *The molecular structure of Xenopus Lavis Ro60.* ***A.*** *Ro60 is a toroid of alpha helical repeats with a von Wilebrand factor A (vWFA) domain at the COOH-terminus. Helices are labelled H1-H25 and the β strands are S1-S13. Blue shading indicates the NH_2-terminus; red shading distinguishes 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.

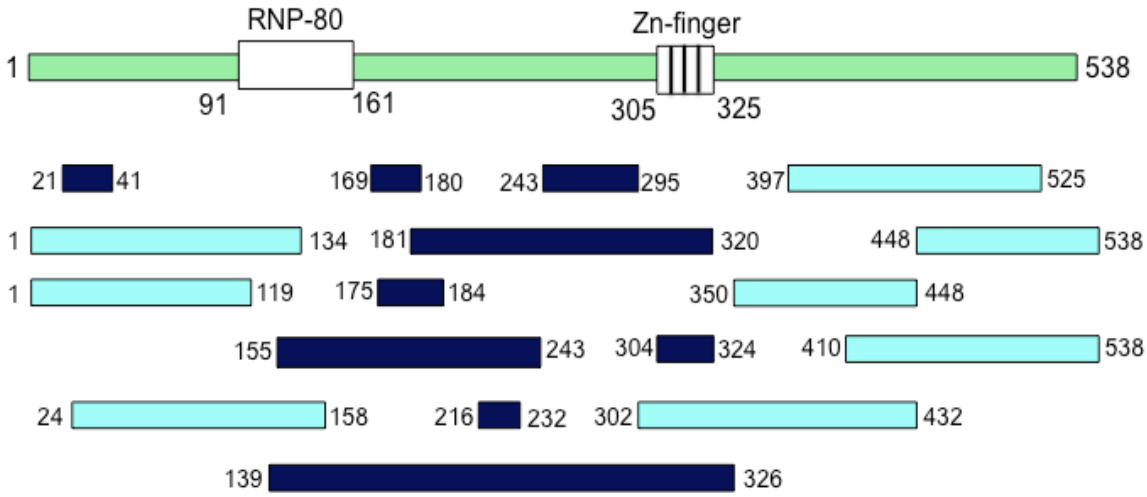


Figure 1.3. *Schematic representation of the structural features and epitopes of Ro60. The RNA recognition motif (RNP-80) contains two highly conserved sequence elements: RNP1 and RNP2. The Zn-finger consists of cysteine and histidine residues. Major epitopes are shown in dark blue and minor epitopes 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.

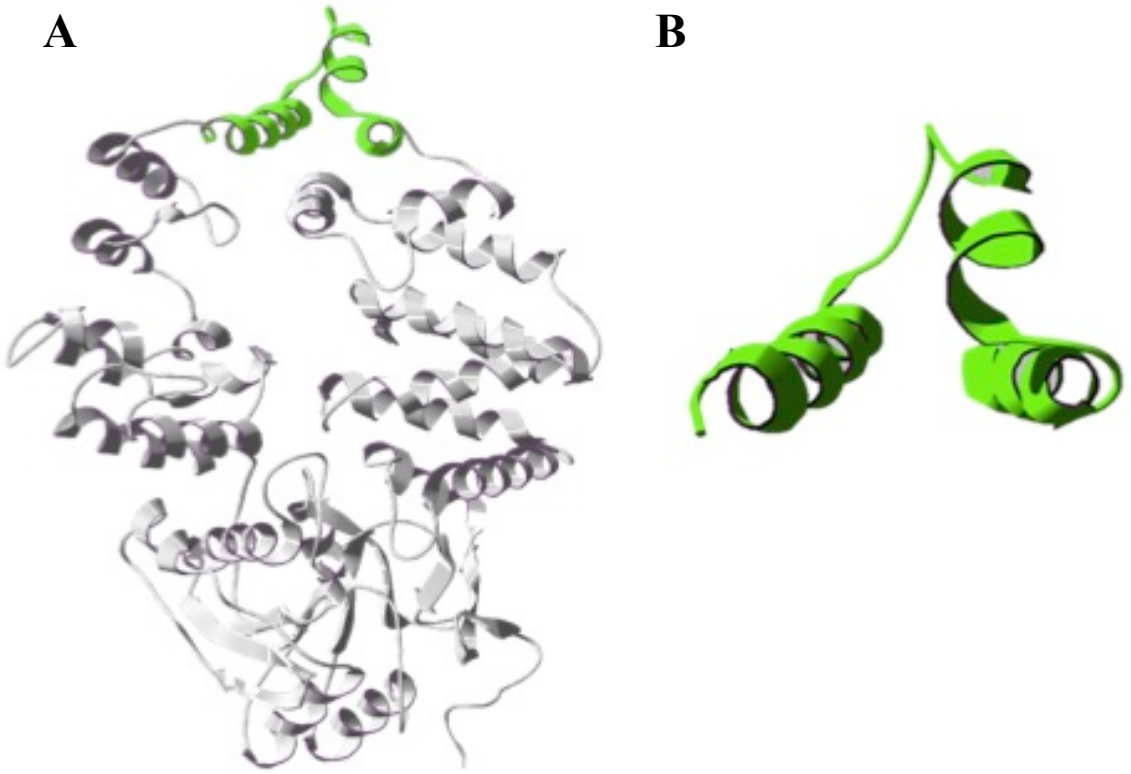


Figure 1.4. *The predicted structure of human amino acids (aa) 193-236.* ***A.*** *The Ro60 ribbon structure with aa 193-236 highlighted in green. Ro60 aa 193-236 forms an helix-loop-helix structure located at the apical tip of the toroid structure of human Ro60, termed Ro60peg.* ***B****. Representation 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

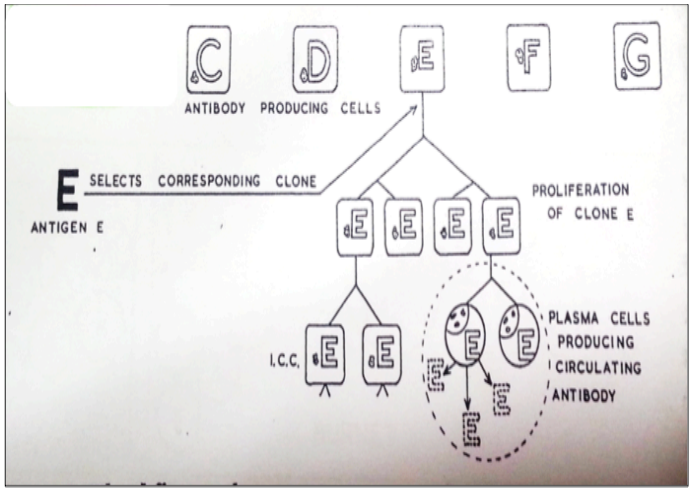
peripheral self-antigen recognition (Mackay & Burnet, 1963; Burnet, 1972) (Figure 1.5 B).

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

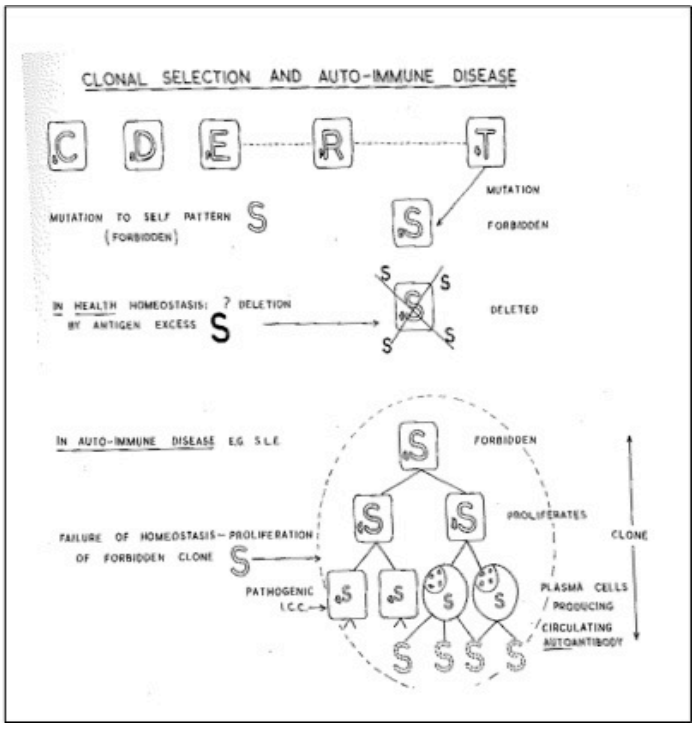


Figure 1.5. *Clonal selection and autoimmune disease.* ***A.*** *The clonal selection theory: the antigen E stimulates the proliferation of a clone of cells with a predetermined pattern established at or near birth to produce the corresponding antibody* ***B.*** *Forbidden clone hypothesis: the forbidden 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).

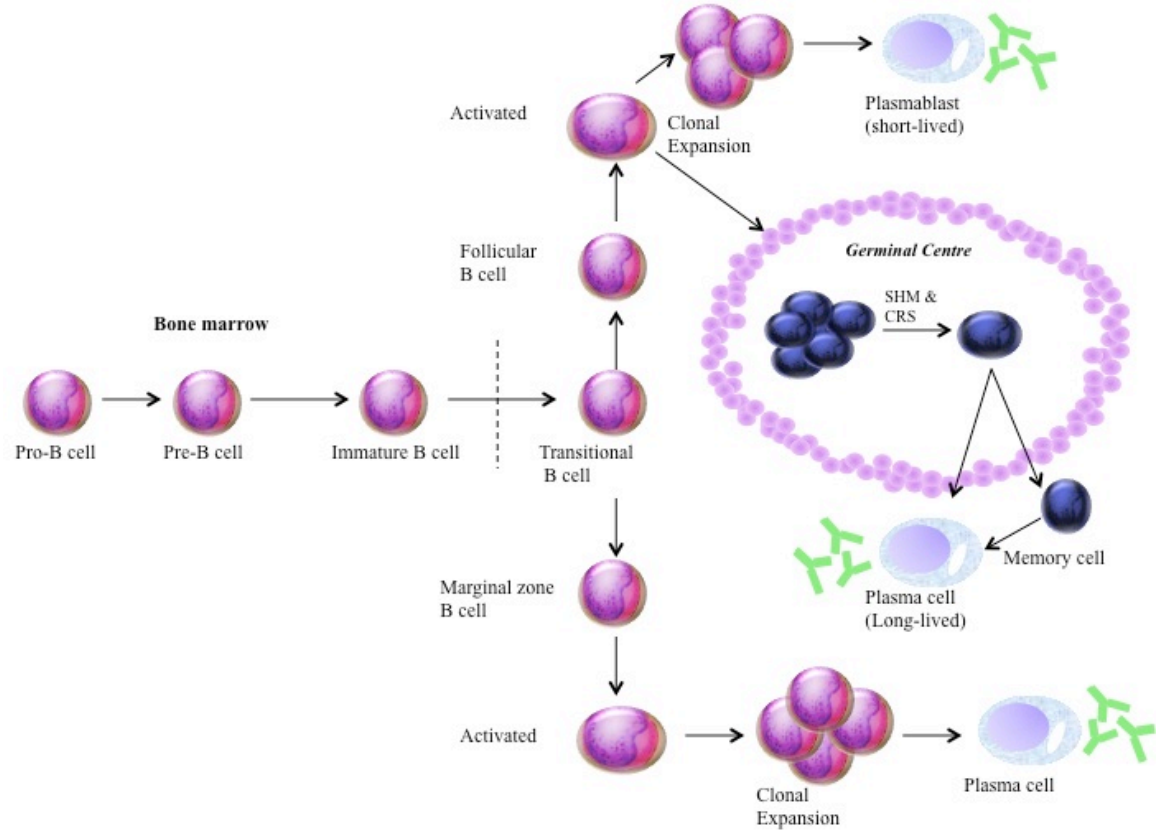


Figure 1.6. *Schematic representation of B cell development and differentiation. The figure shows the broad overview of B cell development stages. B cell development takes place in the bone marrow, thereafter the immature cells migrate to the periphery where maturation is complete. Following encounter 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.

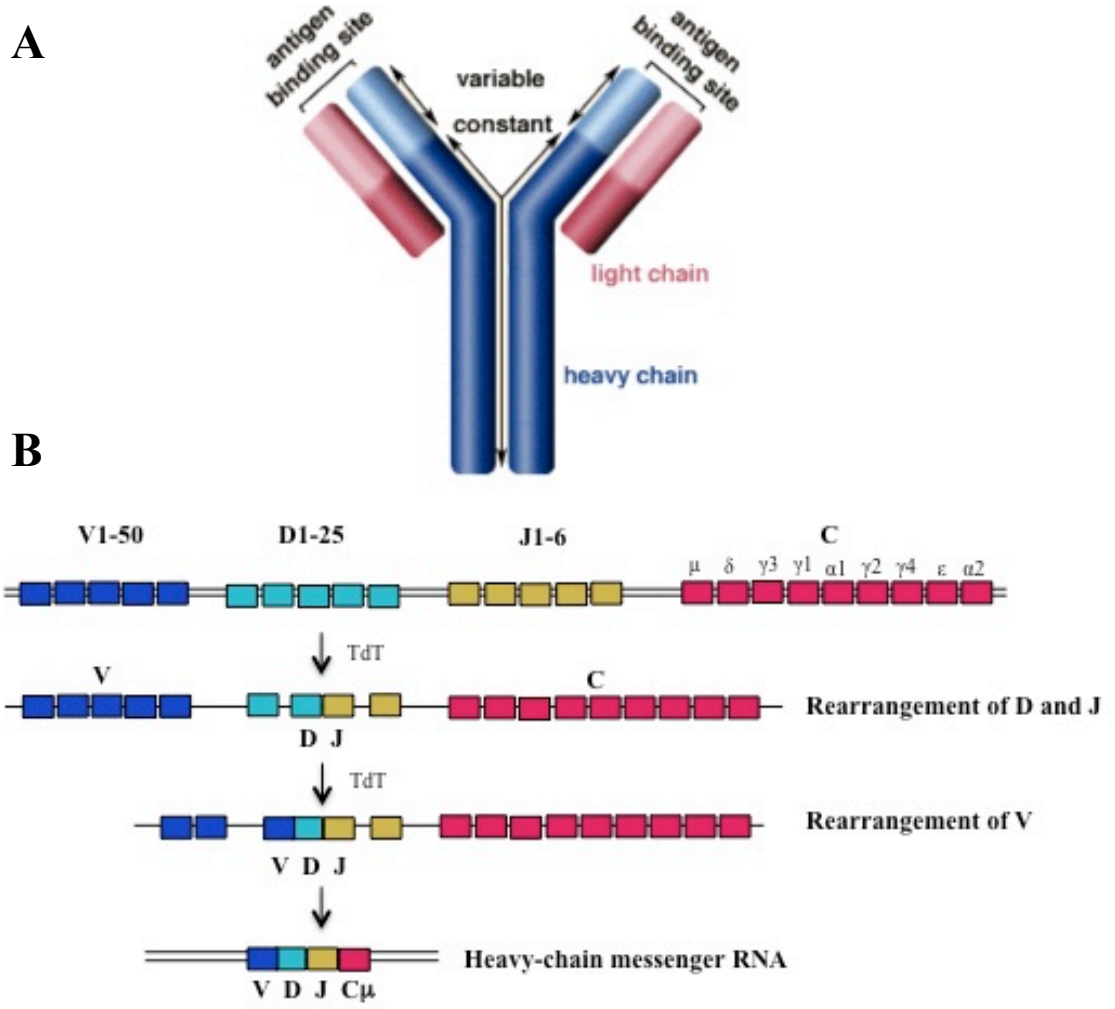


Figure 1.7. *Generation of diversity in the Ig repertoire.* ***A.*** *Generalised structure of an antibody. The B cell receptor comprises a pair of heavy (H) (blue) and light (L) (pink) chains. Each chain consists of a constant (C) (dark blue and pink) and a variable (V) (pale blue and pink) regions. 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.