MOLECULAR SIGNATURES OF LUPUS-SPECIFIC AUTOANTIBODY PROTEOMES

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Summary

The structures of epitopes bound by autoantibodies against RNA-protein complexes have been well-defined over several decades, but little is known of the clonality, immunoglobulin (Ig) variable (V) gene usage and mutational status of the autoantibodies themselves at the level of the secreted (serum) proteome. The lupus-specific autoantibodies anti-Smith (anti-Sm) and anti-ribosomal P (anti-Rib-P) have been implicated in the pathogenesis of neurologic and renal complications in systemic lupus erythematosus (SLE), but these clinically important autoantibodies have yet to be characterized at a molecular level. The aim of this thesis is to determine the clonality and V-region usage of lupus-specific autoantibodies (anti-Sm and anti-Rib-P) with the hypothesis that secreted lupus autoantibody proteomes derive from restricted sets of public B cell clonotypes.

In this thesis, a novel proteomic workflow is presented based on affinity purification of specific Igs from serum, high-resolution two-dimensional gel electrophoresis (2-DE), and *de novo* and database-driven sequencing of V-region proteins by mass spectrometry (MS). Anti-Sm and anti-Rib-P Igs were purified from six anti-Sm- and six anti-Rib-P-positive sera, respectively by elution from ELISA plates coated with either native SmD or native Rib-P proteins. Furthermore, an 11- amino acid peptide (11-C peptide) representing the conserved COOH-terminal P epitope was used to specifically purify anti-Rib-P Igs directed to the COOH-epitope. SmD, Rib-P- and 11-C peptide-specific Igs were analyzed for heavy (H) and light (L) chain clonality and V-region expression using an electrophoretic and *de novo* and database-driven mass spectrometric

sequencing workflow. Purified anti-Rib-P and anti-SmD Igs were tested for cross-reactivity on ELISA and their proteome data sets analyzed for shared clonotypes.

Anti-SmD and anti-Rib-P autoantibody proteomes were IgG1 kappa (K) restricted and comprised each two public clonotypes defined by unique H/L chain pairings. Analysis of purified anti-SmD Igs revealed a major clonotypic population specified by IGHV1.69/IGKV2.28. Whilst, the major clonotypic population in Rib-P response was specified by IGHV1.3-JH4/IGK1.39-JK4 for the common COOH-terminal epitope. The second clonotype in both SmD and Rib-P was shared and sequenced as IGHV3.7-JH6/IGKV3.20-JK2, accounting for two-way immunoassay cross-reactivity between these lupus autoantibodies.

Sequence convergence of anti-SmD and anti-Rib-P proteomes suggests common molecular pathways of autoantibody production and identifies stereotyped clonal populations that are thought to play a pathogenic role in lupus. Shared clonotypic structures for anti-Rib-P and anti-Sm responses suggest a common B-cell clonal origin for subsets of these lupus-specific autoantibodies. The discovery of shared sets of specific V-region peptides can be exploited for diagnostic biomarkers in targeted MS platforms and for tracking and removal of pathogenic clones.

Declaration

I certify that this thesis does not incorporate without my acknowledgment any material previously submitted for a degree 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.

Mahmood Al Kindi

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signatures', Clin Exp Immunol, 184(1): 29-35 (Chapter 4). All of the experiments and the bioinformatic analyses in these articles and chapters were performed by myself with advice from the co-authors. The design and initial writing of the articles and chapters, and responses to the reviewers, were performed by myself under the guidance and editing of Professor Gordon.

Dedication

This work is dedicated to my home, Oman, a country of extraordinary uniqueness, and to Adelaide, my second home.

List of publications arising from this thesis

1. **Al Kindi, M.A.**, Chataway, T.K., Gilada, G.A., Jackson, M.W., Goldblatt, F.M., Walker, J.G., Colella, A.D., Gordon, T.P. (2015) 'Serum SmD autoantibody proteomes are clonally restricted and share variable-region peptides', *Journal of Autoimmunity,* 57, 77-81.

2. **Al Kindi, M.A.**, Colella, A.D., Beroukas, D., Chataway, T.K., Gordon, T.P. (2016) 'Lupus anti-ribosomal P autoantibody proteomes express convergent biclonal signatures', *Clinical and Experimental Immunology,* 184(1), 29-35.

3. **Al Kindi, M.A.**, Collela, A.D., Chataway, T.K., Jackson, M.W., Wang, J.W., Gordon, T.P. (2016) 'Secreted autoantibody repertoires in Sjögren's syndrome and systemic lupus erythematosus: A proteomic approach studied by proteomics', *Autoimmunity Review*s, 15(4), 405-10.

Other publications during PhD candidature

1. Wang, J.W., **Al Kindi, M.A.**, Colella, A.D, Dykes, L., Jackson, M.W., Chataway, T.K., Reed, J.H., Gordon, T.P. (2016) 'IgV peptide signatures of native Ro60 autoantibody proteomes in primary Sjögren's syndrome: molecular clues to Ro52/La diversification', *Clinical Immunology* (submitted).

2. Le, T.A., **Al Kindi, M.**, Tan, J.A., Smith, A., Heddle, R.J., Kette, F.E., Hissaria, P., Smith, W.B. (2016) 'The clinical spectrum of omega-5-gliadin allergy', *Internal Medicine Journal*, 46(6), 710-716.

List of abstracts and posters

1. **Al Kindi M**, Colella A, Jackson M, Chataway TK, Gordon TP. Autoantibodies to a major carboxyl-terminal epitope on ribosomal P proteins are clonally restricted and display a common heavy- and light-chain pairing signature: a forbidden clone in human lupus. Royal College of Pathologists of Australasia (RCPA) Annual Update, Melbourne, 2012.
2. **Al Kindi M**, Colella A, Jackson M, Chataway TK, Gordon TP. Lupus-specific autoantibodies comprise public (shared) clonotypes: a new paradigm in human systemic autoimmunity. St Georg Hospital Autoantibody Seminar, Sydney, 2013.
3. **Al Kindi M**, Colella A, Jackson M, Chataway TK, Gordon TP. Lupus-specific autoantibodies comprise public (shared) clonotypes: a new paradigm in human systemic autoimmunity. Annual Rheumatology Association (ARA) Scientific Meeting, Adelaide, 2013.

Other abstracts during PhD candidature

1. Wang J.J., **Al Kindi M.**, Chataway T., Colella A., Gordon T. Shared antibody heavy chain proteomic signatures in primary Sjögren's syndrome. Annual Rheumatology Association (ARA) Scientific Meeting, Adelaide, 2014.
2. Mendis T., Skärstrand H., Adamson P., **Al Kindi M.**, Jackson M., Gordon T. The use of MBP fusion proteins of ZnT8 to characterize autoantibody expression in T1D patients. Australia Society for Medical Research 54th National Scientific Conference, Adelaide, 2015.

3. Adamson P.J., **Al Kindi M.A.**, Colella A.D., Chataway T.K., Wang J.J., Gordon T.P. and Gordon D.L. Mass spectrometric sequencing of influenza haemagglutinin-specific antibody proteomes reveals shared clonotypes. Australasian Society for Infectious Diseases Conference, Tasmania, 2016.
4. Shields, D., Smith, A., Hissaria, P., **Al Kindi M**., Ferrante, A., Quach, A., Analysis of the NT5E (ecto-5'-nucleotidase) gene in Common Variable Immunodeficiency. International Congress of Immunology, Melbourne, 2016.

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2-DE……two-dimensional gel electrophoresis

aa……...amino acid

BSA ……...bovine serum albumin

CDR……complementarity determining region

D……..diversity

DH……..……...……..diversity-heavy

EBV……Epstein-Barr virus

ELISA……...….enzyme linked immunosorbent assay

FR……framework region

H-chain…….……heavy chain

HCDR3……...heavy chain complementarity determining region 3

HPLC……high performance liquid chromatography

Ig…….……..immunoglobulin

IgG……immunoglobulin G

IMGT…….……ImMunoGeneTics database

J……..joining

JH…….joining-heavy

JK …….joining-kappa

JL……...joining-light

K……..kappa

L-chain……..light chain

LKS…….liver kidney stomach

MRM……..…….multiple reaction monitoring

MS……..……mass spectrometry

NCBI……..The National Center for Biotechnology Information

NGS……..…….next generation sequencing

OD…….……...optical density

PBS……..……phosphate saline buffer

Q-TOF…….……...quadrupole-time of flight

RNP……..……ribonucleoprotein

SLE……..……systemic lupus erythematous

SS…….……Sjögren's syndrome

U-RNP……....uridine-rich ribonucleoprotein

UV…….ultra violet

V……...variable

VH……...variable-heavy

VL…… variable-light

Chapter 1: Literature review

1.1 Introduction

Autoantibodies play a key role as serum biomarkers in systemic autoimmune diseases and remain an important entry point for basic and clinical research into human autoimmunity (Mahler *et al*., 2014). These can be divided into two broad groups: "classical" autoantibodies directed against RNA-protein complexes such as Ro/La, Sm/RNP and ribosomal P that are present in high serum concentrations; have good disease specificity and are markers of future disease; and are generally detectable by standard solid-phase immunoassays (Tan *et al*., 1989; Heinlen *et al*., 2010); and "functional" autoantibodies against cell surface receptors and ion channels such as M3-muscarinic receptors in primary Sjögren's syndrome (SS) that are of low concentration; directly pathogenic following passive transfer; difficult to detect by standard immunochemical methods; and often require sensitive physiological assays for their detection (Dawson *et al*., 2005). Irrespective of their targets and biological effects, new approaches are required for the detection and molecular characterization of human autoantibodies in systemic autoimmunity.

Most studies of humoral autoimmunity to date have focused on the structural and biochemical characteristics of the target autoantigens and identification of their autoepitopes, with the hope of finding more clinically relevant biomarkers (Wolin *et al*., 2006; Hoffmann *et al*., 2010; Mahler *et al*., 2010). Related areas of research include disease specificity of apotopes (epitopes expressed on the surface of apoptotic cells) and human and murine studies on HLA class II phenotype and determinant spreading (McCluskey *et al*., 1998; Reed *et al*., 2008). Despite considerable progress in

identifying the amino acid (aa) sequences of linear and conformational epitopes in human systemic autoimmune diseases, such studies tell us little about the molecular characteristics of the autoantibodies themselves, in particular their clonality, immunoglobulin (Ig) variable (V) gene usage and mutational status. Analysis of serum autoantibody repertoires, if technically feasible, is likely to provide key insights into the emergence of autoreactive B cells in systemic autoimmunity, as well as leading to strategies aimed at removing these B-cell clones.

It is a salient reminder that although antibodies were discovered by Behring and Ehrlich at the end of the nineteenth century, little is known about the size and molecular composition of the actual secreted (serum) antibody repertoire in humans. These questions are beginning to be addressed by a combination of next generation sequencing (NGS) of B-cell repertoires and antibody proteomics (Sato *et al*., 2012; Cheung *et al*., 2012; Wine *et al*., 2013; Georgiou *et al*., 2014; Yadav *et al*., 2014 Lavinder *et al*., 2014; Wine *et al*., 2015; Lavinder *et al*., 2015) although progress remains slow. This thesis presents recent work on the proteomic analysis of serum autoantibodies in SLE based on *de novo* mass spectrometric sequencing of purified Igs from serum samples, and discusses how this new field of autoantibody research may lead to new diagnostic and therapeutic approaches in systemic autoimmune diseases.

1.2 Systemic lupus erythematosus (SLE)

Systemic lupus erythematosus (SLE) is a prototypic autoimmune rheumatic B-cell-mediated disease characterized by a variety of clinical manifestations and a high level of autoantibody production, accumulation of immune complexes and multiple organ

damage (Vyse *et al.*, 1996; Harley *et al*., 2012; Gottschalk et al., 2015). Its prevalence, incidence, and disease severity vary among ethnic groups. Increased prevalence has been reported among African-Americans, Asians, Hispanics, and Native Americans (Fernández *et al.*, 2007). It is mostly seen in females (9:1 prevalence) and has an early disease onset peaking in the child-bearing years (Gottschalk et al., 2015). Current treatment modalities include immunosuppressive and anti-inflammatory agents, which have severe off-target side effects and toxicities. Advances in B cell targeting are still ongoing aiming for novel and specific silencing therapeutics (Gottschalk et al., 2015).

1.2.1 Pathogenesis

Research on SLE is enormous and since 1946, around 56,000 papers have been published (Rekvig *et al*., 2014). More than 50 loci have been associated with SLE using genome wide association studies, involved mainly in clearance of nuclear debris, BCR signaling pathways, immune signal transduction pathways, TLR/type I interferon (IFN) pathways and immune complex processing (Guerra *et al*., 2012; Vaughn *et al*., 2012; Costa-Reis *et al*., 2013; Deng & Tsao, 2014; Niewold, 2015) (Figure 1.1). Environmental factors have also been attributed in the etiology of lupus (Powell *et al*., 1999). However, our understanding of the pathogenesis of autoimmunity in SLE and its various clinical manifestations is still incomplete and there are different theories and hypotheses that can explain the autoimmunity in SLE. The final outcome of immune dysregulation is the production of autoantibodies against nuclear complexes with variable presentation and profile among patients with SLE (Dörner *et al*., 2011). Deposition of immune complexes in various tissues and organs results in activation of local inflammatory pathways resulting in severe multisystem tissue damage. Activation

of type I IFNs by immune complexes containing RNA-protein autoantigens are also implicated in the pathogenesis of SLE and are relevant to the lupus-specific autoantibodies analyzed in this thesis (Savarese *et al*., 2006; Lövgren *et al*., 2006; Fairhurst *et al.*, 2006; Kariuki *et al*., 2015; Hagberg & Rönnblom, 2015).

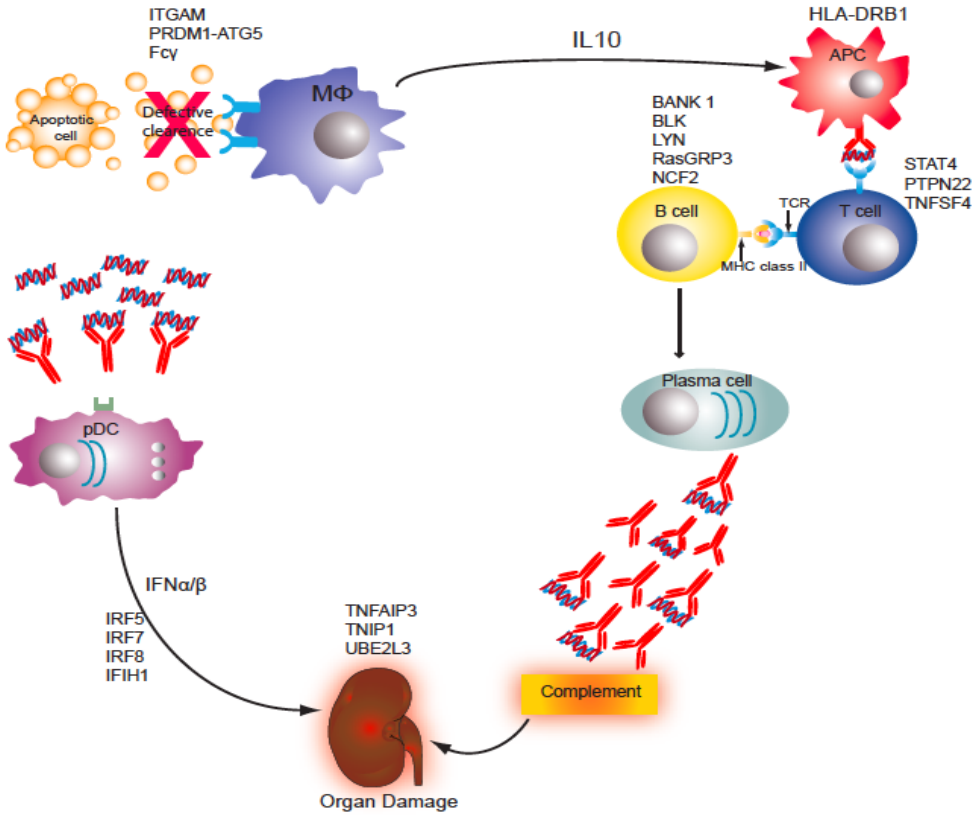

Figure 1.1. Simplified model of the immune dysregulation in systemic lupus erythematosus (SLE). Defective apoptotic clearance results in the deposition of immune complexes leading to the activation of B and T cells and secretion of autoantibodies. Hyperactive B cells produce autoantibodies that activate complements causing tissue and organ damage. Immune complex-activated plasmacytoid dendritic cells (pDCs) then release IFNα/β causing more tissue damage. At each pathway, the known associated genetic loci are indicated. (Adopted from Guerra *et al*., 2012)

1.2.2 Autoantibodies

Autoantibodies represent a major hallmark in the pathogenesis and diagnosis of SLE and are the key focus of this thesis. Although more than 100 autoantibodies have been detected in lupus patients (Sherer *el al*., 2004), two major autoantibody clusters have been identified Sm/RNP cluster and Ro/La cluster. These autoantibodies are directed against nucleic acid-protein complexes (Ching *et al*., 2012). Nucleic-acid components act as immunostimulatory ligands for toll-like receptor TLR-7 or TLR-9 augmenting the immune response in patients with SLE leading to the production of higher level of autoantibodies (Han *et al.* 2015). Another key lupus-specific autoantibody is anti-ribosomal P (anti-Rib-P) and has been associated with central nervous system manifestations and lupus nephritis (Elkon *et al.,* 1985; Pasoto *et al*., 2014).

1.2.2.1 Anti-Smith (anti-Sm) autoantibodies

Anti-Sm autoantibodies, discovered by Tan and Kunkel in 1966 as serum precipitins specific for patients with SLE, hold pride of place as founding members of the family of systemic autoantibodies targeting non-histone extractable nuclear antigens (Tan *et al*., 1966; Tsokos 2006). Anti-Sm responses are present in 10% to 30% of SLE patients and form part of the American College of Rheumatology criteria (ACR) for the classification of SLE (Tan *et al*., 1982; Migliorini *et al*., 2005). The pathogenic significance of these autoantibodies is underlined by their appearance shortly before the clinical onset of SLE and the reported clinical associations with lupus nephritis and neuropsychiatric disease (Arbuckle *et al*., 2003; Alba *et al*., 2003; Jurenacak *et al*., 2009). Sm proteins form the common core of U1, U2, U4 and U5 spliceosomal small nuclear ribonucleoprotein (sRNP) particles (Will & Lührmann, 2001). Sm autoantigens

comprise a ring of 7 snRNP common core conserved proteins B/B' (28,29 kDa), D1 (16 kDa), D2 (16.5kDa), D3 (18 kDa), E (12kDa), F (11 kDa) and G (9 kDa) with the snRNA passing through ring (Lemeier *et al*., 1990; Zieve *et al*., 2003) (Figure 1.2). The anti-Sm autoimmune response is directed mainly against B, D1, and D3 proteins and to a lesser extent D2 (Lehmeier *et al*., 1990; Brahms *et al.*, 1997). The E, F and G proteins are recognized less frequently and only in native conditions (Lehmeier *et al*., 1990; Brahms *et al.*, 1997). Anti-SmD is considered to have the highest specificity for SLE (Mahler *et al*., 2010). Monoclonal antibodies generated from MRL mouse were observed to recognize more than one snRNP core proteins and was the first evidence that Sm epitopes are shared between core proteins (Hirakata *et al*., 1993; Fury *et al*., 1999). Studies using overlapping peptides found that the dominant epitopes were found mainly in the C-terminal region of D proteins and are accessible in the native configuration (James *et al*., 1994; Riemekasten *et al*., Jaekel *et al*., 2001; McClain *et al*., 2002). These epitopes have significantly basic isoelectric points than non-antigenic regions (McClain *et al*., 2002) (Figure 1.2). Furthermore, post-translation modification and conversion of arginine in the C-terminal arginine-glycine (RG) dipeptide repeats to symmetrical dimethylarginine (sDMA) of B and D proteins is essential for specific binding of anti-Sm antibodies (Brahms *et al.*, 2000; Mahler *et al*., 2005). Immune complexes of anti-Sm and snRNP particles are thought to perpetuate systemic autoimmunity by inducing type I interferon in plasmacytoid dendritic cells via combined Fc receptor and TOLL-like receptor 7 engagement (Savarese *et al*., 2006; Lövgren *et al*., 2006).

Although the Sm protein-RNA antigenic complexes have been characterized in detail, the clonality and V-region composition of secreted (serum) human anti-Sm autoantibody proteomes have not been studied. An early study reported oligoclonality and restricted V- and J- region gene usage by anti-Sm hybridomas derived from MRL*/lpr* mice, raising the possibility of restricted anti-Sm clonality in human SLE (Bloom *et al*., 1993).

SmD C-terminal epitope (REAVA(GR)10GGPRR) has significant sequence homology with Epstein-Barr virus (EBV) nuclear antigen-1 (EBNA-1) (James *et al.*, 1994). The proline rich sequence (PPPGMRPP) is the predominant and early target in Sm B/B' immune response (Arbuckle *et al*., 1998). This proline rich sequence is similar to the (PPPGRRP) sequence in EBNA-1, and proline rich structures in U1-specific RNPs and retroviral proteins like HIV-1 p24gag (De Keyser *et al*., 1992; Arbuckle *et al*., 1998, Zieve & Khusial, 2003). This homology has been attributed to the cause-and-effect link between infections and autoimmunity (Arbuckle *et al*., 1998; Arbuckle *et al*., 1999). Immunization with the EBNA-1-derived proline rich peptide causes lupus-like autoimmunity (Arbuckle *et al*., 1998). Transgenic mice expressing EBNA-1 protein were found to produce anti-Sm and anti-dsDNA antibodies (Sundar *et al*., 2004).

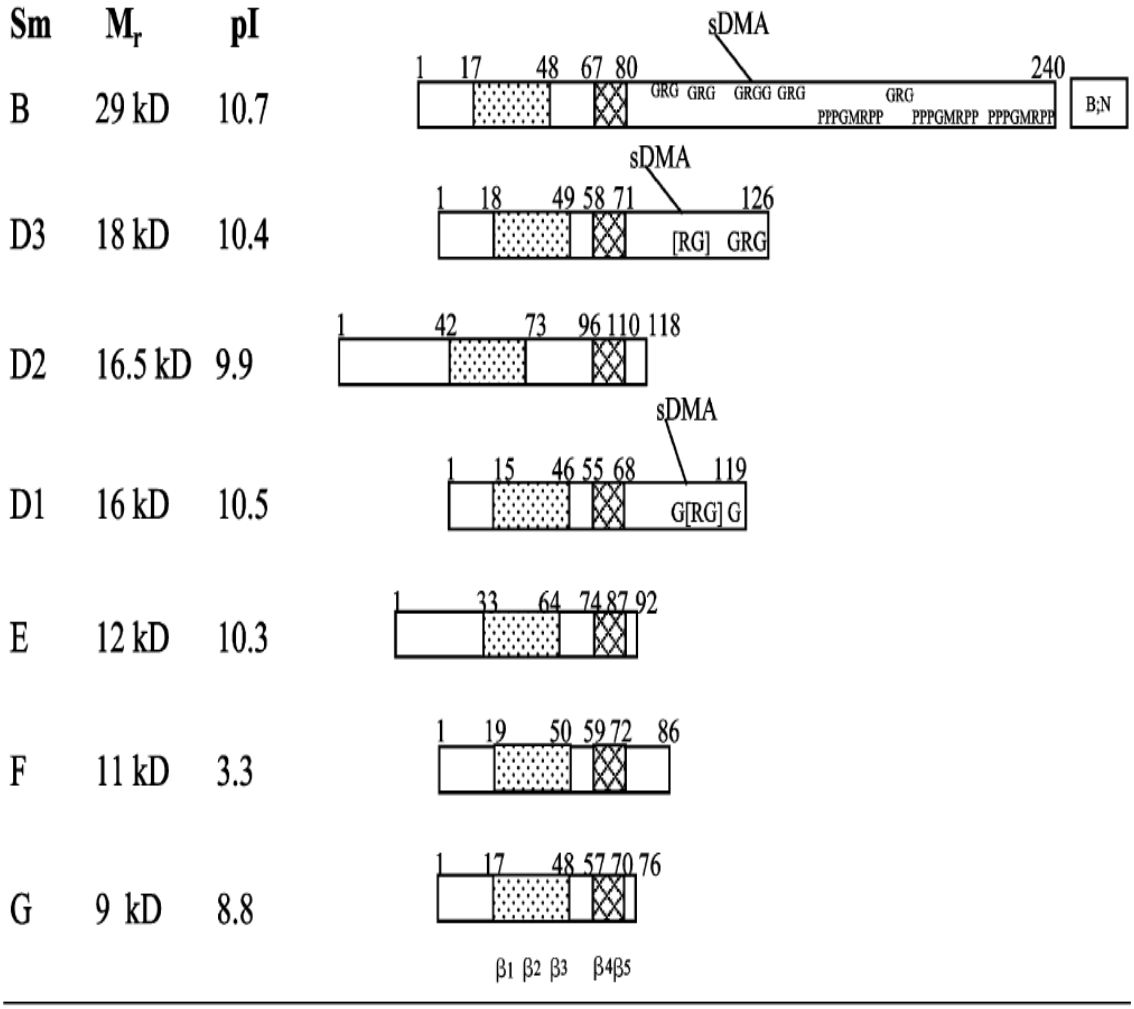

Figure 1.2. Sm core proteins. Sm complex is composed of at lease 7 proteins (B, D1, D2, D3, E, F, G). Symmetrical dimethylated arginine (sDMA) residues in the C-terminus are important for anti-Sm autoantibodies binding. (Adopted from Zieve & Khusial, 2003)

1.2.2.2 Anti-ribosomal P (anti-Rib-P) autoantibodies

Autoantibodies binding the P0, P1 and P2 ribosomal P (Rib-P) phosphoproteins are highly specific for SLE and have been reported in association with neurologic, renal and hepatic manifestations with some conflicting results (Elkon *et al.,* 1985; Pasoto *et al*., 2014). Autoantibodies to the three phosphoproteins are found in 10-40% of patients with SLE (Miyachi & Tan, 1979; Bonfa, & Elkon, 1986; Gerli & Caponi, 2005; Reichlin, 2006; Kessenbrok et al., 2007). Anti-Rib-P autoantibodies are detectable before SLE onset and bind mainly an immunodominant conserved COOH-terminal epitope (both linear and conformational determinants) common to all three P proteins in addition to other regions on the native complex (Elkon *et al*., 1986; Fabien *et al.*, 1999; Bruner *et al*., 2005; Heinlen *et al.*, 2010) Figure 1.3. Epitope-mapping studies performed by Mahler *et al*., 2003 found that the major epitope is precisely located within the last 11-C terminal amino acids SD(E/D)DMGFGLFD (11-C). Further studies of the C-terminal Rib-P epitope using nuclear magnetic resonance and SPOT epitope analysis and proposed that aa residues Phe^{111} and Phe^{114} displayed in the α-helical structure of the Rib-P core (GFGLFD) are important for the anti-Rib-P reactivity in SLE as compared to reactivity observed in Chagas' disease and HCV which is directed mainly to epitopes in the N-terminal part of the ribosomal proteins (Mahler *et al*., 2003; Kessenbrok *et al*., 2007). Bruner *et al*., 2005 identified other antigenic targets in ribosomal P0 protein including epitopes spanning amino acids 99-113 (RDMLLANKVPAAARA) and amino acids 139-151 (QALGITTKISRGT). Active and passive immunization studies in mice have underlined the clinical importance of anti-Rib-P by enhancing lupus nephritis and mediating depression, smell and memory deficits, potentially by cross-reacting with a neuronal antigen highly expressed in the

hippocampus termed the neuronal surface P antigen (NSPA) (Katzav *et al*., 2014; Ben-Ami *et al*., 2014; Bravo-Zehnder *et al*., 2015; Matus *et al*., 2007). Functional antibodies against the major 11-mer COOH epitope (11-C) bind NSPA and appear to mediate cognitive impairment by enhancing glutamatergic transmission in the hippocampus (Segovia-Miranda *et al*., 2015). Evidence of anti-Rib-P being able to penetrate living cells and alter protein synthesis leading to cell apoptosis and production of pro-inflammatory cytokines was reported (Koscec *et al*., 1997, Reichlin, 1998; Sun *et al*., 2001). Of note, anti-Rib-P were found in normal persons, but were masked by anti-idiotype antibodies (Stafford *et al.*, 1995). Disruption of this regulatory mechanism might be responsible for the autoimmunity in patients with anti-Rib-P autoantibodies.

While an early immunochemical study reported limited heterogeneity and mainly IgG1 kappa-restriction for COOH-terminal responses (Bonfa *et al*., 1987), nothing is known at the protein sequence level of the clonotypic structure and Ig V gene usage of these potentially pathogenic lupus autoantibodies, and whether they share V-region signatures with other systemic autoantibodies.

Mice and human studies have suggested a strong association between anti-Rib-P and anti-Sm autoantibodies (Elkon *et al*., 1989). Human anti-Rib-P antibodies purified from the native complex and anti-Rib-P antiserum raised in mice have been reported to cross-react with Smith (Sm) autoantigen suggesting an immunological link between these two lupus-specific autoantibodies, but the molecular basis of this cross-reactivity is unknown (Caponi *et al*., 1998; Ben-Ami *et al*., 2014).

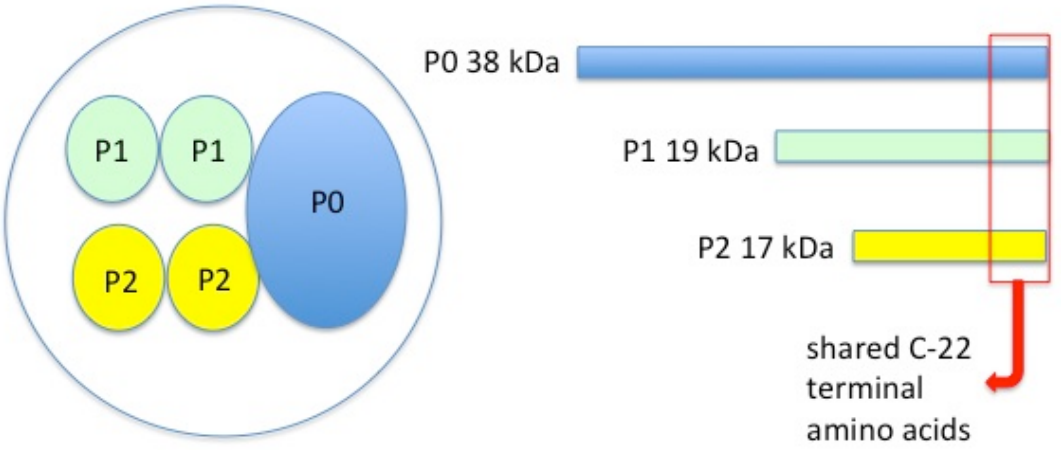

Figure 1.3. Ribosomal P phosphoproteins complex. Ribosomal P proteins are organized in a pentameric complex with one copy of P0 protein and two copies of P1 and P2 proteins. The three proteins share the C-terminal amino acids.

1.3 Proteomic workflows for molecular characterization of secreted (serum) autoantibodies

As discussed above, little is known about the clonality and V-region structures of systemic human autoantibodies. Early studies using two-dimensional gel electrophoresis (2-DE) suggested that anti-Ro60 and anti-La humoral responses were oligoclonal (Bini *et al*., 1990; Kurien *et al*., 2009), but aa sequencing of heavy (H) and light (L) V-regions of these putative clonotypic populations was not thought feasible because of the diversity of humoral responses and interference from normal serum immunoglobulins. These limitations were superseded by developing a proteomic approach for determining molecular signatures of serum autoantibodies, based on positive selection of polyclonal sera on defined antigenic determinants followed by high-resolution 2-DE and *de novo* and database-driven mass spectrometric sequencing.

The workflow begins with an autoantibody purification step from a human autoimmune serum followed by 2-DE and mass spectrometric sequencing and bioinformatics. Purified autoantibodies for sequencing were initially obtained by passing immune sera over affinity columns expressing recombinant antigen fragments or the complete autoantigen (Lindop *et al*., 2011; Arentz *et al*., 2012; Thurgood *et al*., 2013).

1.4 Analysis of secreted Ro/la autoantibody repertoires in primary Sjögren's syndrome (SS)

Applying the proteomic workflow to analyze patient serum samples has enabled us to determine B cell clonal diversification, VH/VL gene expression and somatic hypermutation profiles of Ro/La autoantibodies. Recent studies and findings of

convergent Ro/La-driven B cell responses and stereotyped autoantibody secretion represent rare examples of forbidden autoreactive B-cell clones (Lindop *et al*., 2011; Arentz *et al*., 2012; Thurgood *et al*., 2013).

Early studies utilizing combined high-resolution *de novo* mass spectrometric sequencing and 2-DE of serum anti-Ro60, anti-Ro-52 and anti-La autoantibodies have shown restricted and shared VH/VL regions among unrelated primary SS patients with Ro/La autoantibodies. The data is summarized in Table 1.1 and discussed below in sections 1.4.1 and 1.4.2.

Table 1.1

Systemic autoantibody proteomes in primary SS and SLE.

Autoantigen	Autoantibodies			
	V_H	V_K	J_H	J_K
Ro60 peg	V_H 3-23	V_K 3-20	J_H 5	J_K 2
Ro52	V_H 3-23	V_K 3-20	J_H 2	J_K 1
	V_H 3-7	V_K 3-20	J_H 6	J_K 5
LaA	V_H 3-43	V_K 3-20	J_H 6	J_K 2
	V_H 3-30	V_K 3-15	J_H 6	J_K 4

Abbreviations: SS, Sjögren's syndrome; SLE, systemic lupus erythematosus; V_H, heavy chain variable region; V_K, light chain (kappa) variable region; J_H, heavy chain joining region; J_K, light chain (kappa) joining region.

1.4.1 Anti-Ro60

Ro60 is considered to be a primordial autoantigen involved in the initiation of systemic autoimmunity, with anti-Ro60 occurring years before clinical autoimmunity and becoming pathogenic on binding Ro60 on the surface of apoptotic fetal cardiomyocytes in congenital heart block (Buyon *et al*., 2003; Reed *et al*., 2008; Heinlen *et al*., 2010). A subset of anti-Ro60 in patients with linked anti-Ro60/La responses react on solid phase immunoassay with an apical peg-like structure on Ro60 that is expressed on intracellular Ro/La-RNP complexes (Reed *et al*., 2010). In an initial 2-DE step to assess the clonality of affinity column-purified anti-Ro60 peg autoantibodies, an identical H- and L-chain monoclonal pattern was found in all patients. Mass spectrometric sequencing of individual gel spots revealed an IgG1 kappa-restricted monoclonal Ig specified by an IGHV3-23/IGKV3-20 chain pairing signature that is shared (public) among unrelated patients (Lindop *et al*., 2011). The V-regions of each chain were expressed both as germline-encoded and somatically mutated peptides, although it remains unclear as to whether shared aa replacements originate from positive selection or underlying genetic variation.

The discovery of this stereotypic monoclonal autoantibody directly against a bona fide systemic autoantigen argues against a random selection mechanism of autoantibody production. As pointed out in the recent editorial (Smith *et al*., 2013): "the fact that there is such an astonishing similarity between patients powerfully suggests that the entire pathway from original stimulus through the mechanism that generates autoimmunity must be virtually identical in its molecular details from patient to patient". Further studies are underway to sequence the entire serum Ro60 autoantibody

proteome of which anti-Ro60 peg is only a small component. It is likely that there will be significant differences in V-region molecular signatures and clonality among different diseases and among the Ro and Ro/La autoantibody subsets.

1.4.2 Anti-Ro52 and anti-La

Proteomic analysis of anti-Ro60 has been extended to include the linked Ro52 and La/SSB autoantigens in patients with primary SS. Interestingly, anti-Ro52 have a broader disease specificity to include systemic sclerosis, idiopathic inflammatory myopathies and primary biliary cirrhosis, and maternal anti-Ro52 are thought to be pathogenic in congenital heart block due to bifunctional inhibition of calcium channels (Salomonsson *et al*., 2005; Schulte-Pelkum *et al*., 2009; Dugar *et al*., 2010; Karnabi *et al*., 2010). Anti-Ro52 Igs purified on a full-length recombinant Ro52 protein showed a remarkable degree of clonal restriction on high-resolution 2-DE analysis, with a single IgG1 kappa species migrating at an isoelectric point of 7.5 being detected in all anti-Ro52-positive patients with primary SS and other systemic autoimmune diseases. Sequencing of gel plugs and solution digests of purified anti-Ro52 Igs revealed shared sets of public clonotypes with an IGHV3-7 or IGHV3-23 encoded H- chain paired with a common IGKV3-20 L- chain (Arentz *et al*., 2012). As reported for anti-Ro60 peg responses in primary SS, the Ro52-specific clonotypes were characterized by intraclonal diversification with shared and individual aa replacement mutations (Lindop *et al*., 2011; Arentz *et al*., 2012).

Autoantibody proteomes directed against the classical La autoantigen in primary SS were then sequenced and their signatures compared with linked anti-Ro60/Ro52

responses. In an effort to reduce sample size complexity, responses against a conserved NH2-terminal determinant termed LaA, which arise early in anti-La autoimmunity and are present in approximately 80% of mothers of children with congenital heart block were initially studied (McNeilage *et al*., 1992; Neufing *et al*., 2005). Using a combination of reduced and non-reduced 2-DE, it was possible to dissect two IgG1 kappa-restricted monoclonal species specified by an IGHV3-30 paired with an IGKV3-15 and an IGHV3-43/IGKV3-20 paired clonotype (Thurgood *et al*., 2013).

The Ro60/Ro52/La signatures were further delineated by partial or complete joining (J) sequences, indicating that while there is sharing of VH and VL gene families among these autoantibodies they do in fact arise from independent clonal precursors with distinct V-J combinations. The differences in mutation patterns of shared germline V gene segments among linked anti-Ro/La autoantibodies further support a model of independent antigen-driven clonal selection events.

The finding of shared convergent B-cell clonotypes is an excellent example of a forbidden autoreactive clone. Having identified public sets of clonotypic autoantibodies in anti-Ro/La humoral autoimmunity, the next step is to establish whether this principle applied to systemic autoantibodies in SLE. Accordingly, the aim of this thesis is to determine the clonality and V-region usage of lupus-specific autoantibodies (anti-Sm/RNP and anti-Rib-P) by isoelectric focusing and *de novo* mass spectrometric sequencing with the hypothesis that secreted lupus autoantibody proteomes derive from restricted sets of public B cell clonotypes.

1.5 B cell development

B cell came into attention after the description of antitoxins by Emil von Behring and Shibasaburo Kitasato in 1890 (von Behring & Kitasato, 1890). Animal models and clinical evaluation of patients with immunodeficiencies contributed to the discovery of B cells in the mid 1960s and early 1970s. Since then, B cell ontogenesis has been extensively studied both in animals and humans (for review check LeBien & Tedder, 2008; Pieper *et al*., 2013; Tobón *et al.,* 2013; Cooper, 2015). B cells and their secreted antibodies are the central elements of humoral immunity as part of the adaptive immune system. B cell development begins from hematopoietic precursor stem cells present in the bone marrow (BM) where they follow a program of differentiation including surface protein expression and Ig gene rearrangement forming BCR (Figure 1.5). V_H, D_H, and J_H combinatorial rearrangement of the H-chain locus together with the V_L-J_L of the L-chain locus generate a B-cell repertoire expressing antibodies which with the capability to recognize more than 5 x 10^{13} different antigens (Brack *et al*., 1978; Pieper *et al*., 2013). Immunoglobulin gene rearrangement occurs early in BM and defines the initial B cell development into three stages (pro-B cell, pre-B cell and immature B cell stages). Immature B cells then leave the BM and migrate into lymphoid organs where they develop and differentiate into naïve, follicular or marginal zone B cells. The germinal centre (GC) reaction is characterized by a dynamic process of clonal selection, somatic hypermutation (SHM), class switch recombination (CSR) and affinity maturation. Antigen selected B cells that leave the GC become memory B cells or plasmablasts that home to and reside in the BM and secret antibodies. Different transcription factors and cell surface proteins regulate the early stage of B-cell development (Nutt & Kee, 2007). B cell responses follow the two-signal paradigm with two signals (signal 1: BCR

ligation and signal 2: provided by other cells like T cells or molecules like TLRs) (Bretscher and Cohn, 1970). Antigen-specific interaction between B and T cells is essential in humoral immunity and cell-mediated immune responses (Lanzavecchia, 1985). Any alteration or defect occurring during theses critical steps can result in immunodeficiency of autoimmune diseases.

B cell tolerance is important in preventing the development of autoreactive B cells and the development of antibody responses to self-antigens. Both central and peripheral tolerance checkpoint mechanisms are implicated in B cell tolerance. In central tolerance, B-lymphocytes with high affinity are deleted in the BM or modified through a process of receptor editing. Anergic inactivation and apoptosis are other ways to control B cell development in the periphery (Yurasov *et al*., 2005; Meffre & Wardemann, 2008; von Boehmer & Melchers, 2010; Pillai *et al*., 2011; Eilat & Wabl, 2011). Older theories that attempted to explain the reactivity against self-antigens include "side-chain-theory & horror autotoxicus" by Paul Ehrlich and "Forbidden clone theory" by Frank Mcfarlabe Burnet (Ehrlich, 1900; Burnet, 1949). Autoreactive B cells escape the checkpoints and mature into either short-lived plasma cells or germinal centre-matured memory B cells and long-lived plasma cells depending on the nature of interaction of BCR with the antigen (Hoyer *et al*., 2004; William *et al*., 2005).

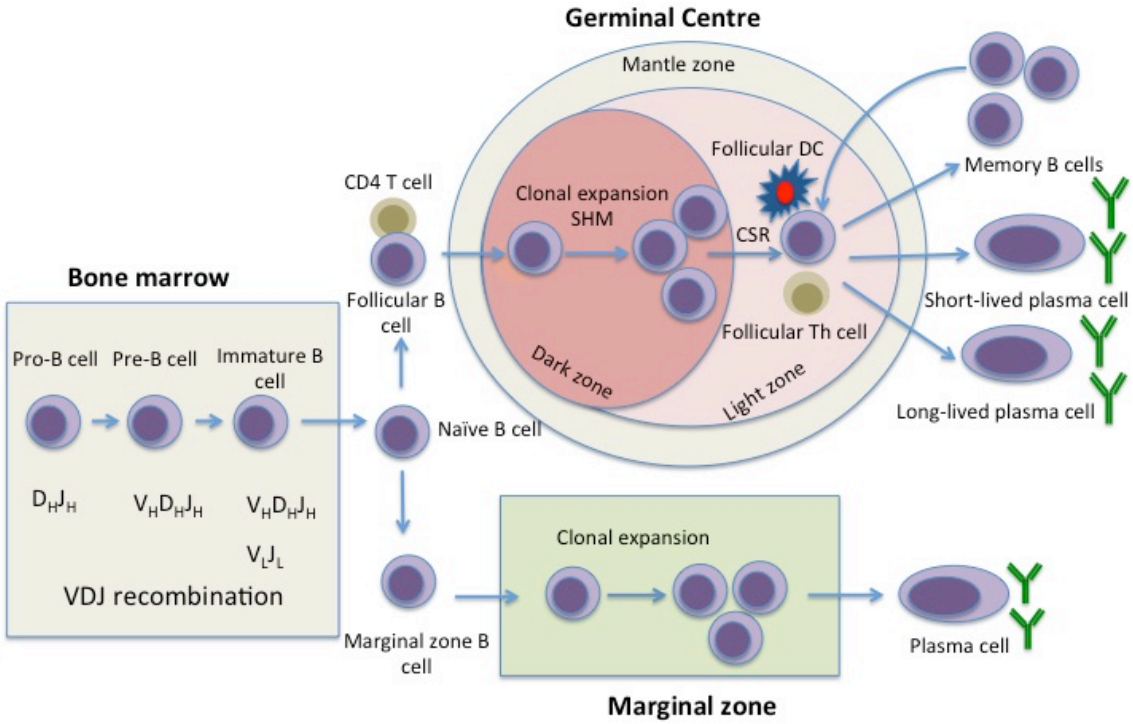

Figure 1.4. Schematic representation of B cell development leading to antibody secretion. B cell development starts in the bone marrow accompanied by VDJ gene recombination in the Ig H-chain gene followed by VJ recombination in the L-chain gene. The mature B cell (naïve B cell) can further develop in the germinal centre where affinity maturation, and differentiation into either plasmablasts or memory B cells occurs. Plasmablasts then home to and reside in the bone marrow where they become functional antibody secreting plasma cells. SHM indicates somatic hypermutation; CSR, class switch and recombination.

1.6 Antibody structure and sequence diversification

In the immune system, approximately 10^9 B lymphocyte pools are found in a mouse and nearly 10^{12} in a human adult (Fritz 2015). The RAG1 and RAG2 subunits are the generators of this diversity (Jankovic *et al*., 2004; Schatz & Spanopoulou, 2005). The antibody molecule is made up of an identical pair of H- and L-chains each of which consists of a (V) domain, (J) domain and diversity (D) domain in the H-chain only (Figure 1.5). V, D, and J genes are chosen from a number of genomic templates and joined together. Table 2 and 3 summarises the diverse numbers of V and J genes of the H- and L- chain families that can be selected to construct an antibody molecule as classified by ImMunoGeneTics (IMGT) database (http://www.imgt.org).

Immunoglobulins are composed of three hypervariable regions (known as complementary determining regions; CDRs) that determine the specific antibody binding with fours framework regions (FR) (Figure 1.5). When RAG genes are expressed, the joining of different VDJ domains results in variability so that it can bind to a diverse number of antigens. Antigen stimulation induces somatic hypermutation and affinity maturation which add another step of diversity after random pairing of H- and L- chain selection, V(D)J recombination and insertion of P and N nucleotides at the joining sites by terminal deoxynucleotidyl transferase (Brezinschek *et al*., 1998; Purugganan *et al*., 2001).

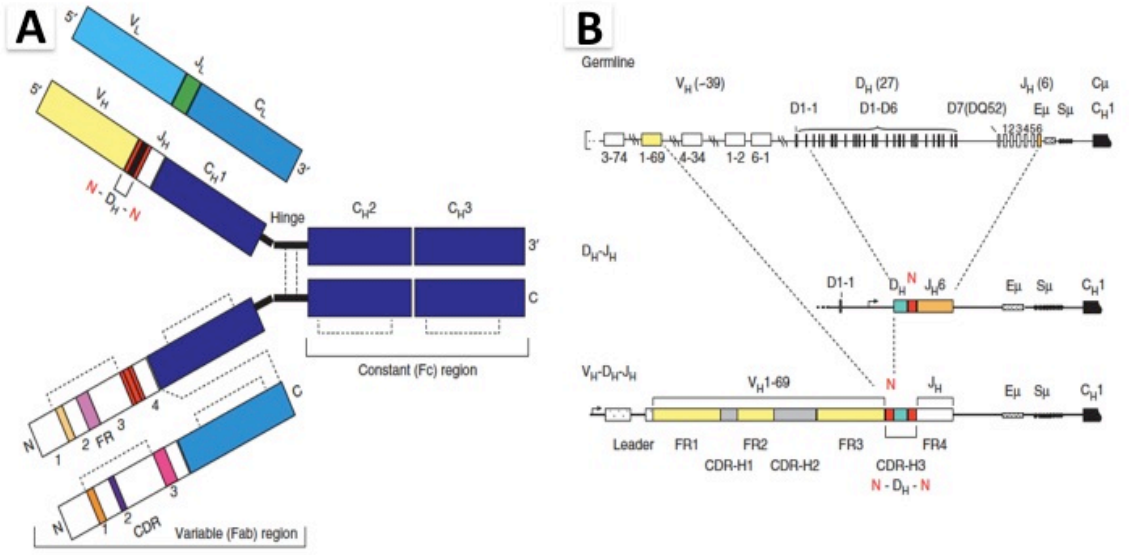

Figure 1.5. Structure of antibody molecule and sequence diversification mechanism. **A,** Schematic representation of antibody consisting of an identical pair of H- and L-chains each of which consists of a variable (V) domain, joining (J) domain and diversity (D) domain in the H-chain only. **B,** VDJ recombination early in B cell development that helps in creating Ig diversity and variable antigen binding capability. Adopted from Georgiou *et al*., 2014.

Table 1.2 Human heavy and kappa light chains genes as classified by the IMGT database.

Immunoglobulin locus	Number of genes
IGHV	58 functional genes (7 families) Up to 19 reported alleles per gene
IGHD	27 genes (7 families) 1-3 known alleles/gene
IGHJ	6 functional genes 1-4 known alleles/gene
IGKV	46 functional genes (6 families) 1-4 known alleles/gene
IGKJ	5 functional genes 1-4 known alleles/gene

Abbreviations: IGHV, immunoglobulin heavy variable; IGHD, immunoglobulin heavy diversity; IGHJ, immunoglobulin heavy joining; IGKV, immunoglobulin kappa variable; IGKJ, immunoglobulin kappa joining.

1.7 Clonality analysis and mass spectrometric sequencing of lupus-specific autoantibodies to characterize their molecular signatures

Despite advances in B cell biology, there is limited direct evidence for "forbidden clones" specific for authentic autoantigens in human systemic autoimmune disease. Models of autoimmunity including animal studies have limitations in contributing to our understanding of spontaneous human humoral autoimmunity. However, with the modern integration of proteomic tools like high-throughput mass spectrometric sequencing, we can now directly characterize the molecular signatures of human autoantibodies at their end-point in the serum antibody repertoire.

1.8 Specific aims and hypotheses

As described earlier, autoantibody production is the major hallmark of SLE. The lupus-specific autoantibodies anti-Sm and anti-Rib-P have been implicated in the pathogenesis of neurologic and renal complications in SLE, but these clinically important autoantibodies have yet to be characterized at a molecular level. This thesis exploits a novel ELISA plate-purification method to isolate antigen-specific autoantibodies (anti-Sm and anti-Rib-P) from complex serum samples using purified native autoantigens. This is followed by a combined 2-DE and high-resolution mass spectrometric sequencing technique to characterize the clonotypic structures and V-region signatures of these autoantibody proteomes.

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

Aim 1. To purify anti-SmD autoantibodies from sera of patients with systemic lupus erythematosus using purified native SmD autoantigen and determine their clonality and variable (V)- / Joining (J)- region usage by isoelectric focusing and *de novo* mass spectrometric sequencing.

Hypothesis. Secreted lupus anti-SmD proteomes derive from restricted sets of public B cell clonotypes and humoral responses to SmD are public among unrelated patients with SLE.

Aim 2. To purify anti-Rib-P autoantibodies from sera of patients with systemic lupus erythematosus using purified native Rib-P heterocomplex and 11-C peptide; and determine their clonality and variable (V)- / Joining (J)- region usage by isoelectric focusing and *de novo* mass spectrometric sequencing.

Hypothesis. Similar to anti-SmD proteomes, secreted lupus anti-Rib-P proteomes derive from restricted sets of public B cell clonotypes and humoral responses to Rib-P are public among unrelated patients with SLE.

Aim 3. To determine an immunological and molecular link between cross-reactive subsets of anti-Rib-P and anti-SmD autoantibodies based on shared public clonotypes.

Hypothesis. A subset of cross-reactive anti-Rib-P and anti-Sm autoantibodies arise from a common precursor B cell.

1.9 Clinical and diagnostic significance of work described in this thesis

This thesis describes a novel proteomic workflow based on affinity purification of specific Igs from serum, high-resolution 2-DE, and *de novo* and database-driven sequencing of V-region proteins by mass spectrometry (MS). The novel proteomic approach used herein can be applied to any other autoimmune diseases and infectious diseases. This thesis has pointed out the sharing of public V-peptide across unrelated patients with SLE. These findings could be translated to a novel biomarker in multiple reaction monitoring (MRM) diagnostic platforms and B cell targeted therapeutics. If the promise of MRM-based detection of specific human autoantibodies is fulfilled, development of multiplexed MS-based diagnostic platforms may supersede traditional solid phase immunoassays by detecting multiple autoantibodies at a molecular level in a single serum sample (Arentz *et al*., 2012).

Chapter 3 and 4 are adopted directly from peer-reviewed papers arising from the work in this thesis. Accordingly, a copy of the relevant paper is inserted at the end of each chapter.

Chapter 2: Materials and methods

2.1 Patients and controls

Sera were collected from six patients with SLE who were positive for anti-Sm autoantibodies and six patients with SLE who were positive anti-Rib-P autoantibodies by commercial line blot immunoassay (Euroline ANA profile 5, Euroimmun, Lubeck, Germany). Demographic characteristics and serologic findings in the patients are shown in Tables 1 and 2. Control sera were taken from eight healthy controls and three disease control patients who were age and sex matched with the patients. Patients fulfilled at least four of the eleven ACR criteria for SLE (Tan *et al*., 1982), including the presence of antibodies to double-stranded DNA by Farr assay (Trinity Biotech, Bray, County Wicklow, Ireland). The study was approved by the Clinical Ethics Committee of the Flinders Medical Centre.

2.2 Isolation of anti-SmD autoantibodies by plate purification

Anti-SmD IgGs were purified from ELISA plates (Maxi-Sorp; Nunc, Roskilde, Denmark) using a simple elution method. In brief, plates were coated with 1ug/ml of purified bovine native SmD protein (confirmed by mass spectrometric sequencing as SmD1, SmD2, SmD3) (Arotec Diagnostics, Wellington, New Zealand) diluted in 0.03M carbonate buffer, blocked with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS), incubated with serum from each subject diluted 1:50 in 1%BSA/PBS for one hour at 37 ^{o}C, washed four times with PBS, and bound Ig fraction eluted with 200ul of 0.1M glycine and 0.5M NaCl, pH 2.3. Eluted Ig fractions were neutralized in 1M Tris HCL, pH 8.0 and concentrated to 10ug/ml on an Amicon Ultra

Centrifugal Filters (Millipore, Billerica, MA, USA). The concentration and specificity of the anti-SmD affinity purified Ig was quantified and confirmed by EZQ and ELISA as detailed in section 2.4 and 2.5.1.

2.3 Isolation of anti-Rib-P autoantibodies by plate purification

Serum anti-Rib-P or 11-C peptide Igs were purified from ELISA plates (Maxi-Sorp; Nunc, Roskilde, Denmark after coating with 1ug/ml purified bovine native Rib-P heterocomplex consisting of the P0 (38 kDa), P1(19 kDa) and P2 (17 kDa) phosphoproteins (Arotec Diagnostics, Wellington, New Zealand) or 10ug/ml of the 11-C peptide (SDEDMGFGLFD) synthetized as a 4-branch MAP (Mimotopes, Notting Hill, VIC, Australia) diluted in 0.03M carbonate buffer, blocked with 1% BSA in PBS, incubated with serum from each subject diluted 1:50 in 1%BSA/PBS for one hour at 37 ^{o} C, washed four times with PBS, and bound Ig fraction eluted with 200ul of 0.1M glycine and 0.5M NaCl, pH 2.3. Eluted Ig fractions were neutralized in 1M Tris HCL, pH 8.0 and concentrated to 10ug/ml on an Amicon Ultra Centrifugal Filters (Millipore, Billerica, MA, USA). The concentration and specificity of the anti-Rib-P affinity purified Ig was quantified and confirmed by EZQ and ELISA as detailed in section 2.4 and 2.5.1.

2.4 Quantification of purified Ig by EZQ

EZQ protein quantification assay (Thermo Fisher Scientific, Waltham, MA, USA) was used to measure the concentration of plate purified Ig as the amount needed is only minimum (3ul). Simply, 1ul spot of samples was applied to the assay sheet in triplicates. After washing with methanol and drying, staining was done with EZQ

protein quantification fluorescent dye was added for 30min followed by destaining using EZQ destain solution. The final step was to read the fluorescence using Gel Doc EZ Imager (Bio-Rad, Hercules, CA, USA). Samples can be quantitated by comparison with a standard curve generated with serial dilution of BSA standard.

2.5 Enzyme linked immunosorbent assay (ELISA)

2.5.1 Specificity ELISA

ELISA was performed by initially coating the plate (Maxi-Sorp; Nunc, Roskilde, Denmark) with 1ug/ml of purified antigens (native SmD, Rib-P, recombinant Ro52 from Arotec Diagnostics, New Zealand; recombinant H1N1/2009 (HA-09) from Protein Sciences Corporation, Meriden, USA) or 10ug/ml of the 11-C peptide (SDEDMGFGLFD) synthetized as a 4-branch MAP (Mimotopes, Notting Hill, VIC, Australia) diluted in 0.03 carbonate buffer (pH 9.6) and kept at 4^{o} C overnight. Next day, plate was washed three times with PBS-T and non-specific sites were blocked with 250 ul of 1% BSA/PBS and incubated for 1 hr at 37^{o} C. Starting serum (diluted 1:100), unbound fraction (normalized to starting serum), and bound (eluted) fraction (2.5 ug/ml) were added to the plate and incubated at 37^{o} C for 1 hr. After further washing with PBS-T, secondary anti-human IgG alkaline phosphatase conjugate (Sigma, St Louis, MO, USA) diluted 1:1000 was added and incubated at 37^{o} C for 1 hr. The last step after washing with PBS-T was to detect bound antibody by addition of substrate (disodium p-nitrophenyl phosphate 1 mg/ml in 90 mM diethanolamine, pH 9.6). All samples were run in duplicates, the OD was read at 405nm and results were expressed as the mean of duplicates. Values were corrected for background binding.

2.5.2 Kappa and lambda distribution ELISA

Maxisorp 96 well ELISA plate (Nunc, Roskilde, Denmark) was coated with 1ug/ml of purified antigen diluted in 0.03 carbonate buffer (pH 9.6) and kept at 4^{o} C overnight. Next day, plate was washed three times with PBS-T and non-specific sites were blocked with 250 ul of 1% BSA/PBS and incubated for 1 hr at 37^{o} C. Then, distribution of bound anti-SmD Igs was determined by using specific rabbit anti-human kappa and anti-human lambda antisera (Dako, Glostrup, Denmark) detected by HRP-labeled donkey anti-rabbit antisera (Jackson ImmunoResearch, West Grove, PA, USA). All samples were run in duplicates, the OD was read at 450nm after adding a stop solution and results were expressed as the mean of duplicates.

2.6 Indirect immunofluorescence

2.6.1 Human epithelial type 2 (HEp-2)

HEp-2 cells (Immunoconcepts, Sacramento, CA, USA) were used to test for reactivity against human conformational epitopes. Briefly, slide was probed with purified Ig at 2.5 ug/ml and incubated at room temperature for one hour. After washing three times with PBS, secondary FITC-conjugated anti-human IgG was added and slide was incubated at room temperature for one hour. Slide was washed and read under Olympus BX51 fluorescence microscope.

2.6.2 Liver kidney stomach (LKS) block

MeDica slide (Encinitas, CA, USA) was used to check monospecificity of purified anti-Rib-P Ig. Slide was probed with 2.5 ug/ml of isolated Ig and incubated at room temperature for one hour. After washing step, secondary FITC-conjugated anti-human

IgG was added and slide was incubated at room temperature for one hour. Slide was washed and read under Olympus BX51 fluorescence microscope.

2.7 Countercurrent immunoelectrophoresis (CIEP)

In-house counterimmunoelectrophoresis was performed to confirm specificity of purified Igs using rabbit thymus extract (Bacto laboratories, Mount Pritchard, NSW, Australia) and anti-Sm and anti-RNP reference controls. Briefly, thymus extract was run as a line in one side of the agarose gel and the samples including controls, starting serum and purified Igs were run in wells on other side as wells. Electrophoresis was carried out at 100V for 1hr. Gel was then stained with Coomassie blue (Sigma, St Louis, MO, USA), destained and read to check for line of complete, or partial identity.

2.8 Commercial line immunoblot

Cross-reactivity against viral antigens (EBV, CMV, HSV-1, HSV-2, rubella) including EBNA-1 antigen was tested using EBV- profile 2 and TORCH-G line immunoblot kits (Euroimmun, Luebeck, Germany). Briefly, strips were incubated with either diluted serum (1:101) or 2.5 ug/ml of purified Igs at room temperature for 30 min. After washing, secondary alkaline-phosphatase-labeled anti-human antibodies was added and incubated fro 30 minutes at room temperature. After few washing steps, the bound antibodies are stained with a chromogen/substrate solution, which is capable of promoting a color reaction. An intense dark band at the line of the corresponding antigen appears if the serum sample contains specific cross-reactive antibodies.

2.9 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE analysis was carried out to confirm the purity of affinity isolated Igs. TGX-precast gel (Bio-Rad, Hercules, CA, USA) was used. Samples were initially reduced with 100 mM DTT and heated at 100^{o} C for 5 min. Then, alkylation was done by adding 200 mM of idoacetamide and kept for 30 min in the dark. An equal volume of SDS sample buffer (1 M Tris pH 6.8, 20% glycerol, 2% SDS, 30% RO water, 0.001% Bromophenol blue) was added and SDS-PAGE was performed using a Mini-Protein 3 cell apparatus (Bio-Rad, Hercules, CA, USA). Electrophoresis was run at a constant 300 V for 20 min. Imaging was performed using a stain0free Gel Doc EZ Imager (Bio-Rad, Hercules, CA, USA).

2.10 Western blot (WB)

Western blot was done to confirm specificity of serum anti-Sm against SmD protein. Affinity purified bovine Sm (2ug) was separated in SDS-PAGE under reducing condition (section 2.9). Proteins were transferred to a nitrocellulose membrane using a Mini Trans-blot Electrophoresis Transfer Cell (Bio-Rad, Hercules, CA, USA). Nitrocellulose membrane was blocked with 5% skim milk in Tris-Buffered Saline/ Tween-20 for 2hr at room temperature and washed three times each for 5 min. Membrane was then probed with starting sera diluted 1:250 in 1% skim milk overnight at 4^{o} C. After washing, secondary horseradish peroxidase-conjugated anti-human IgG antibody (Jackson ImmunoResearch, West Grove, PA, USA) diluted 1:5000 was added. The reactivity was detected with Bio-Rad-Clarity Western ECL substrate (Bio-Rad, Hercules, CA, USA) using a LAS-4000 Luminescent image analyzer (Life Science, Fuji Film, Tokyo, Japan).

2.11 Two-dimensional gel electrophoresis (2-DE)

2-DE was performed by initially exchanging the affinity purified IgG buffer with the 2-DE rehydration solution (7 M urea, 2 M Thiourea, 4% CHAPS, 30 mM Tris, 1% Pharmalytes, 65 mM DTT, and 0.002% Bromphenol blue) and applied to 13-cm, non-linear immobilized pH 3-11 IPG strips (GE Healthcare, Uppsala, Sweden). Strips were rehydrated at 20^{o} C using a voltage of 50 V for 10 hrs. Then, strips were focused for 83,000 V hrs using an isoelectric focusing protocol (step and hold 110 V 1 hr, step and hold 220 V 2hrs, step and hold 440 V 2hrs, gradient 9000 V 2hrs, step and hold 9000 V 65000 V hrs, gradient 220 V 1hr, step and hold 220 V 10 hrs).

After focusing, 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 and 0.002% Bromphenol blue for 15 min.

Second-dimension separation by molecular weight used 8-15% gradient Criterion stain free TGX gels (Bio-Rad, Hercules, CA, USA), at 300V, 400 mA, 100 W using a Criterion electrophoresis system (Bio-Rad, Hercules, CA, USA). After electrophoresis, Imaging was performed using a Gel Doc EZ Imager (Bio-Rad, Hercules, CA, USA). Gels were fixed with 40% (v/v) methanol and 10% (v/v) acetic acid for 4 hrs. Following fixation, gels were rinsed in water for 1 hr. Gel spots were excised manually using pipette tips under UV-box.

2.12 Preparation of samples for mass spectrometry

In-solution digests were performed on plate-purified Igs, while in-gel digests were performed on H- and L-chain spots excised from 2-DE gels using Trypsin Gold (Promega, Madison, WI, USA). For in-solution, affinity purified samples were buffered exchanged with 100 mM ammonium bicarbonate pH 8.0. with the final volume 20 ul. Ig was reduced by the addition of 5 mM DTT and incubated at 100^{o} C for 15 min and alkylated with 20 mM idoacetamide for 30 min in the dark. Trypsin Gold (Promega, Madison, WI, USA) was added in a ratio of 1:20 (enzyme/protein). Samples were digested at 37^{o} C overnight. For in-gel digestions, gel plugs were excised from gels, washed three times with 100 mM ammonium bicarbonate and shrunk with acetonitrile for 15 min. After pipetting out the excess acetonitrile, the gel plugs were dried at 37^{o} C. Then, the gel plugs were resuspended in Trypsin Gold/100 mM ammonium bicarbonate buffer in a ratio of 1:50. Samples were incubated on ice for 10 min and extra buffer was added to cover the gel plugs. Followed by incubation of samples for 16 hr at 37^{o} C. Digested solutions were removed from each sample and placed in MS vials ready for mass spectrometryic sequencing.

2.13 Mass spectrometry (MS)

Analysis of peptides was carried out using quadrupole-time of flight (Q-TOF) 5600+ mass spectrometer (AB Sciex, Framingham, MA, USA) coupled to an Eksigent nanoLC 400 high performance liquid chromatography (HPLC). Samples were applied to a C18 trap (Eksigent, Dublin, CA, USA) and eluted onto a 15 cm C18 column (Nikkyos Technos, Tokyo, Japan) using a 2-95% acetonitrile gradient over 33 min. The instrument was operated in high sensitivity positive ion mode; charge state of +2 to +5 ions selected; with one MS scan followed by 20 MS/MS scans exceeding 200 count per

second. TOF scans were acquired from m/z 350-1600 with an accumulation time of 0.15 second. Product-ion (PI) scans were acquired from m/z 100-1600 by applying dynamic accumulation. The PI scans selected a single precursor ion in Quadrupole 1 (Q1) mass filter cell that was transferred to Quadruple 2 (Q2) collision cell employing rolling collision energy. Fragment ions were then detected by TOF region. At least two technical and biological replicates were performed for each sample. Instrument was externally calibrated every 10 hours using trypsin-digested BSA MS standard (New England Biolabs, Ipswich, MA, USA).

2.14 Protein sequence data analysis

The MS/MS spectra were extracted from the .raw files into, mgf files using MS converter (ProteoWizard v3.0.5759). MS data was initially analyzed using Protein Pilot software (AB Sciex, Framingham, MA, USA). Re-calibrated MS data was then analyzed using Peaks Studio v7.5 software (Bioinformatics Solution Inc, Waterloo, ON, Canada) for variable Ig peptide sequence identification using the following parameters: digestion enzyme as trypsin; and the variable modification of oxidation of methionine, deamidation of asparagine and glutaimine, and the carboxymethylation of cysteine; 2 missed cleavages; precursor m/z tolerance of ≤10 ppm; product ion error tolerance of 0.01 Da; precursor charge state of +2 to +4; database search performed against a combined IMGT (http://www.imgt.org), NCBI, and the Uniprot 2015-08 databases. Two modules were used during analysis, Peaks and SPIDER. Peaks module was used to assign germline gene family. Gene family was assigned when two or more non-overlapping germline peptides were identified. The SPIDER module was used to identify any aa replacement mutation form the germline sequence using a homology

match query type under the previously described parameters. All peptides matched were manually checked for quality and ion coverage before inclusion in the final data analysis.

Chapter 3: Serum SmD autoantibody proteomes are clonally restricted and share variable-region peptides

3.1 Introduction

Anti-Smith (Sm) autoantibodies, discovered by Tan and Kunkel in 1966 as serum precipitins specific for patients with SLE, hold pride of place as founding members of the family of systemic autoantibodies targeting non-histone extractable nuclear antigens (Tan *et al*., 1966; Tsokos 2006). Anti-Sm responses are present in 10% to 30% of SLE patients and form part of the American College of Rheumatology criteria (ACR) for the classification of SLE (Tan *et al*., 1982; Migliorini *et al*., 2005). The pathogenic significance of these autoantibodies is underlined by their appearance shortly before the clinical onset of SLE and the reported clinical associations with lupus nephritis and neuropsychiatric disease (Arbuckle *et al*., 2003; Alba *et al*., 2003; Jurenacak *et al*., 2009). Sm autoantigens comprise a ring of 7 snRNP common core proteins (B, D1, D2, D3, E, F & G) with the snRNA passing through ring (Zieve *et al*., 2003). While SmB and SmD are the major targets of anti-Sm humoral autoimmunity, anti-SmD is considered to have the highest specificity for SLE (Mahler *et al*., 2010). Immune complexes of anti-Sm and snRNP particles are thought to perpetuate systemic autoimmunity by inducing type I interferon in plasmacytoid dendritic cells via combined Fc receptor and TOLL-like receptor 7 engagement (Savarese *et al*., 2006; Lövgren *et al*., 2006).

Although the Sm protein-RNA antigenic complexes have been characterized in detail, the clonality and V-region composition of secreted (serum) human anti-Sm

autoantibody proteomes have not been studied. Advances in mass spectrometric-based protein sequencing now allow H- and L-chain V-region peptide maps to be generated for the first time from microgram quantities of autoantibodies derived from complex patient sera (Lindop *et al*., 2012). Recently, we have combined 2-DE of anti-Ro52/Ro60/La autoantibodies with *de novo* and database-driven V-region protein sequencing to show that humoral responses against these protein-RNA complexes are mediated by public (shared) autoreactive B cell clonotypes (Lindop *et al*., 2011; Arentz *et al*., 2012; Thurgood *et al*., 2013). An early study reported oligoclonality and restricted V-region gene usage by anti-Sm hybridomas derived from MRL*/lpr* mice, raising the possibility of restricted anti-Sm clonality in human SLE (Bloom *et al*., 1993). In the present study, serum SmD autoantibody proteomes were characterized by high resolution mass spectrometric sequencing for the first time and find that the expression of these clinically important autoantibodies is dominated by unique sets of public clonotypes.

Sera were collected from six patients with SLE who were positive for anti-Sm autoantibodies by commercial line blot immunoassay (Euroline ANA profile 5, Euroimmun, Lubeck, Germany). Demographic characteristics and serologic findings in the patients are shown in Table 3.1

Table 3.1. Characteristics of patients with systemic lupus erythematosus (SLE)

Patient	Age/Sex	ANA (Pattern/ Titer)	Anti-dsDNA (IU/ml)	ENA	CIEP
SLE1	59/female	Speckled/2560	>100	Sm, RNP-A, Ro60, Rib-P	Sm, RNP, Ro60
SLE2	30/female	Speckled/2560	>100	Sm, RNP-A, RNP 70, Ro60,	Sm, RNP, Ro60
SLE3	46/female	Speckled/2560	>100	Sm, RNP-A, RNP 70	Sm, RNP
SLE4	45/female	Speckled/2560	80	Sm, RNP-A	Sm, RNP
SLE5	49/female	Speckled/2560	70	Sm, RNP-A, Ro-60	Sm, RNP, Ro60
SLE6	66/female	Speckled/2560	90	Sm	Negative

Antinuclear antibodies (ANA) were detected using HEp2 slides; anti-double stranded DNA (dsDNA) measured using Farr assay with a value ≥ 10 IU/ml considered positive; and extractable nuclear antigen (ENA) autoantibodies detected by line blot assay (Euroimmun, Germany) and counterimmunoelectrophoresis (CIEP) using rabbit thymus extract.

3.2 Results

3.2.1 Purification of anti-SmD IgGs from anti-Sm-positive sera

To purify anti-Sm autoantibodies for analysis of clonality by 2-DE and mass spectrometric sequencing, antibodies bound to native SmD-coated ELISA plates were eluted from the sera of six SLE subjects with mixed anti-Sm/RNP/Ro60 specificities. Western blot of sera blotted only SmD band (running at 16 kDa) as shown in (Figure 3.1A,B). Monospecificity of eluted IgGs for anti-SmD was confirmed by testing starting sera, unbound and eluted fractions on individual SmD/RNP-A/Ro60 ELISAs (Figure 3.2A). In addition, eluted IgGs gave a speckled nuclear immunofluorescence pattern with nucleolar and chromosomal sparing on HEp-2 cells (Figure 3.2B), and formed a precipitin line of identity to an anti-Sm reference serum and partial identity to an anti-RNP reference serum on CIEP, confirming reactivity with native Sm antigen (Figure 3.2C). Assessment of clonality of plate-purified anti-SmD IgGs by reduced 2-DE revealed two H-chain clusters of spots ranging from pI 6.4-7.6 (identified as IGHV3-7 on gel plug digests) and pI 8-9.2 (IGHV1-69) together with two distinct clusters of kappa L-chain spots ranging from pI 6.6-7.6 (IGKV2-28) and pI 8.2-9.2 (IGKV3-20) (Figure 3.3). Similar charge variants have been reported for clonotypic anti-Ro60 and anti-La autoantibodies and are presumed to arise from post-translational modifications (Lindop *et al*., 2011; Thurgood *et al*., 2013).

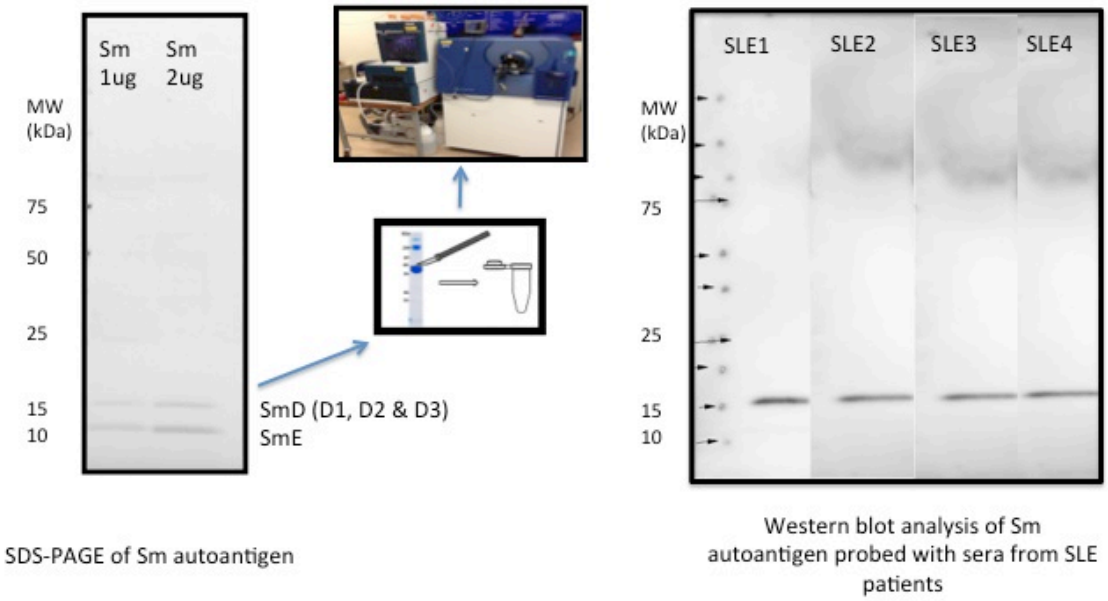

Figure 3.1. SDS-PAGE and Western blot analysis of Sm autoantigen probed with Sera from patients with SLE. **A.** SDS-PAGE of native bovine Sm autoantigen showing two bands running at 16 kDa (SmD1, SmD2, and SmD3) and 12 KD (SmE); the protein content of each band was confirmed by cutting the bands and sequencing by mass spectrometry (MS). **B.** Western blot analysis of Sm autoantigen probed with sera from SLE patients. The sera blotted specifically only SmD band.

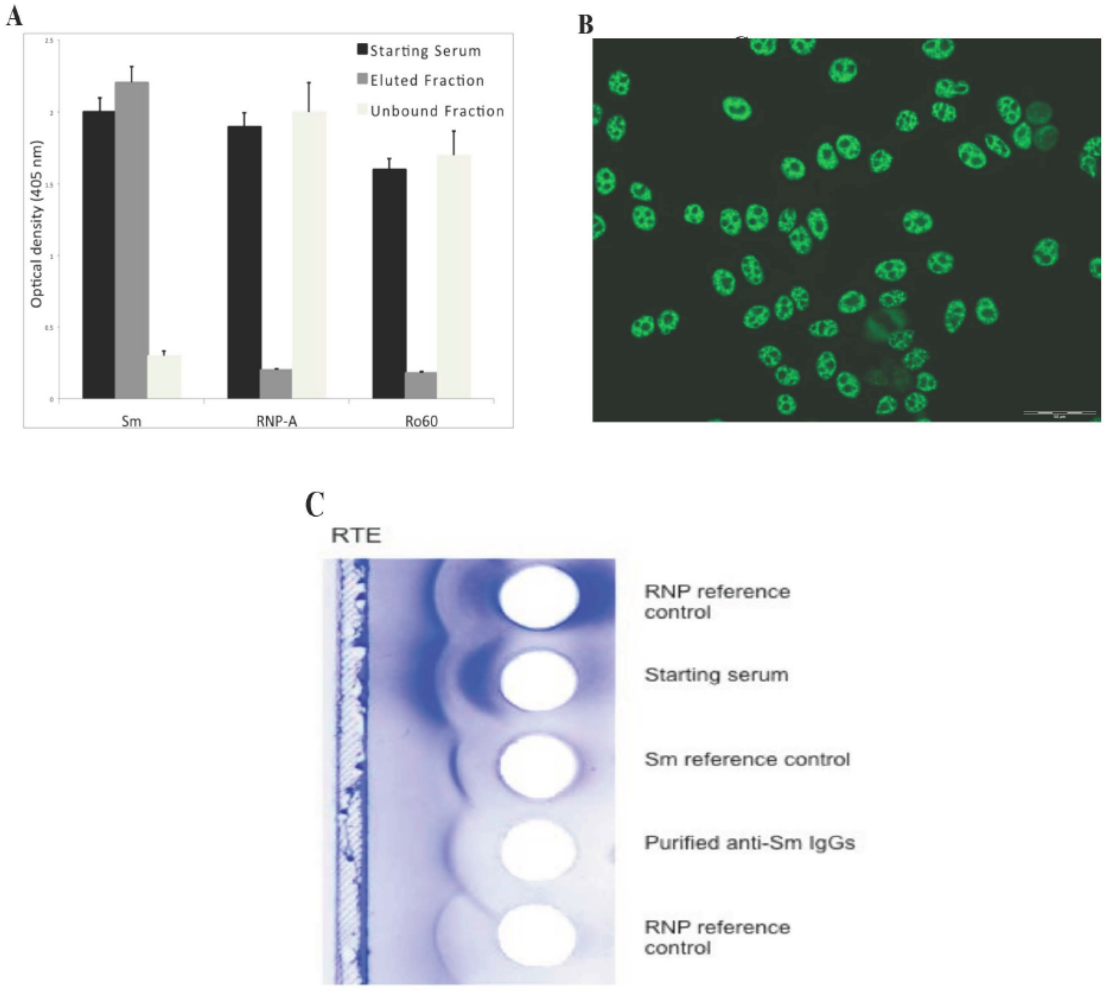

Figure 3.2. Specificity of anti-Sm IgGs purified from SmD-coated enzyme-linked immunosorbent assay (ELISA) plates from sera of six patients with systemic lupus erythematosus (SLE) containing mixed anti-Sm/RNP-A/Ro60 specificities. **A.** Purified anti-Sm IgGs tested by ELISA using native SmD, RNP-A and Ro60 proteins. Starting sera, bound and unbound fractions are compared. Bars show the mean ± SEM of duplicate optical density values. **B.** Indirect immunofluorescence of HEp-2 probed with anti-Sm IgG (2.5 ug/ml) from a representative patient with SLE. **C.** Counterimmunoelectrophoresis of purified anti-Sm IgGs (0.05ug/well) tested against rabbit thymus extract (RTE) with anti-Sm and anti-RNP reference controls.

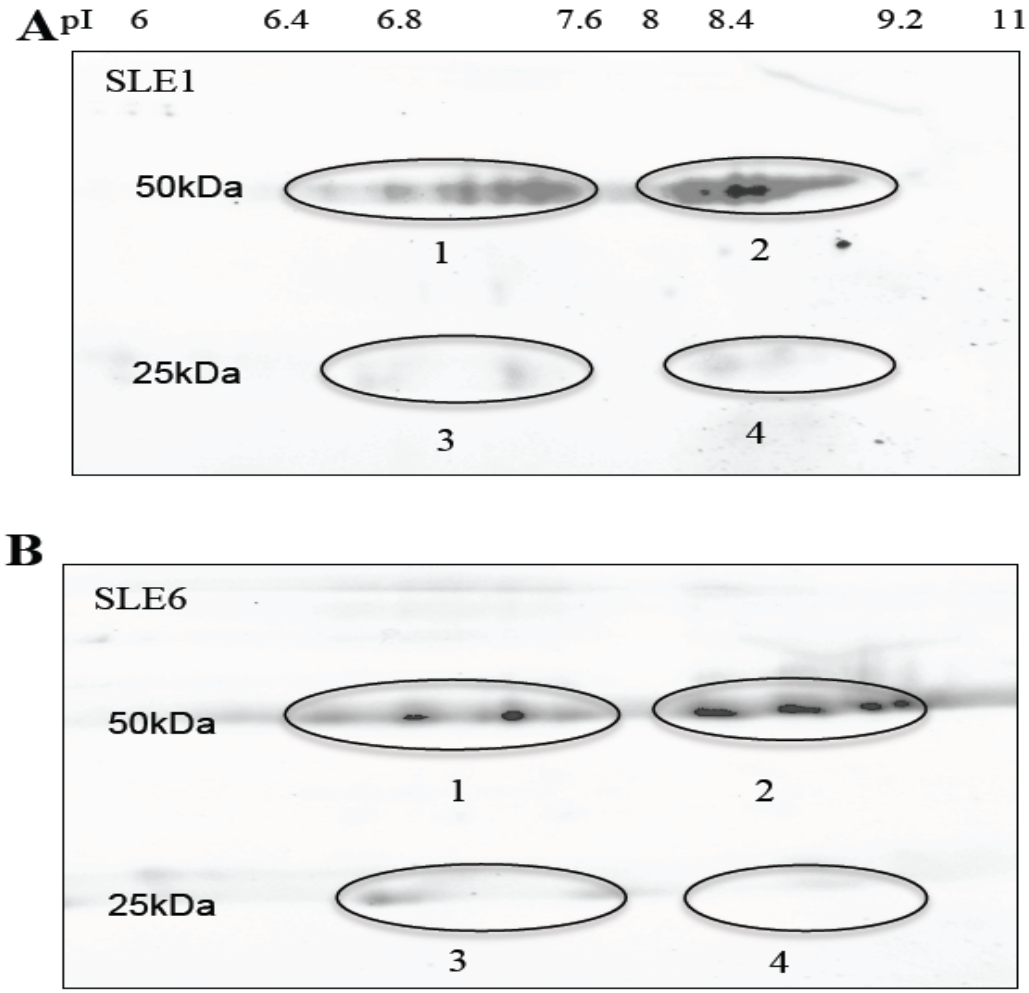

Figure 3.3. Clonal restriction of anti-SmD IgGs purified from the sera of patients SLE1 and SLE6. **A.** Reduced two-dimensional gel electrophoresis (2-DE) of SLE1 anti-Sm reveals 2 heavy chain (H-chain) clusters of spots ranging from pI 6.4-7.6 (gel plug 1, identified as IGHV3-7) and pI 8-9.2 (gel plug 2, identified as IGHV1-69) together with 2 clusters of light chain (L-chain) spots ranging from pI 6.6-7.6 (gel plug 3, identified as IGKV2-28) and pI 8.2-9.2 (gel plug 4, identified as IGKV3-20). **A.** The same pattern of H-and L-chain clusters with identical IGHV and IGKV gene families is observed for SLE6. H-chain spots have been overexposed in order to visualize L-chain spots.

3.2.2 SmD-reactive clonotypic IgGs are public and share common amino acid replacement mutations.

Mass spectrometric analysis was performed on solution trypsin digests of plate-purified anti-Sm IgGs from all six SLE patients. The lower limit of detection of H- and L-chain constant and V-region peptides by this method was determined as 50 pg/ml using serial tryptic digests of a mouse monoclonal antibody of known concentration as a surrogate IgG. This revealed the common expression of two IgG1 H-chain species, IGHV3-7 and IGHV1-69, and two kappa L-chains encoded by IGKV2-28 and IGKV3-20 respectively, confirming the plug digest data. No private clonotypes were expressed by individual patients. No lambda L-chain peptides were identified in plug or solution digests of plate-purified anti-SmD, showing kappa L-chain restriction at the level of L-chain protein sequencing. Kappa restriction was verified immunochemically by SmD ELISA (n=6 anti-SmD-positive SLE sera) probed with anti-kappa and anti-lambda antisera [mean (SD) anti-SmD kappa 0.60 (0.04) OD units; anti-SmD lambda 0.05 (0.01) OD units]. Sequencing of J-regions also revealed common usage of IGJH4 and IGJH6 while the two L-chain clonotypes were restricted to IGJK2 and IGJK4. As discussed previously, proteomic methodology cannot generally obtain full sequence through the H-chain D-region (Thurgood *et al.*, 2013). Detailed anti-Sm V-region tryptic peptide maps are shown in Supplementary figure 1 and reveal multiple clonotypic peptides with public (shared among patients) and private aa replacement mutations consistent with selection of intraclonal variants by self-antigen. The public mutations are summarized by a proteomic heat map and are present in both FR and CDRs of the H- and L-chains (Figure 3.4). The most common substitutions were a threonine to serine at position 14 of IGKV2-28 and a glutamine to lysine at position 6 of

IGKV3-20, both present in 5/6 anti-Sm-positive SLE patients. In control experiments, no IgG tryptic peptides were detected by MS of elutes from SmD-coated ELISA plates treated with normal human sera (n = 4) and SmD antibody-negative lupus sera with specificities for ribosomal P, RNP-A and Ro52/Ro60/La antibodies respectively (data not shown).

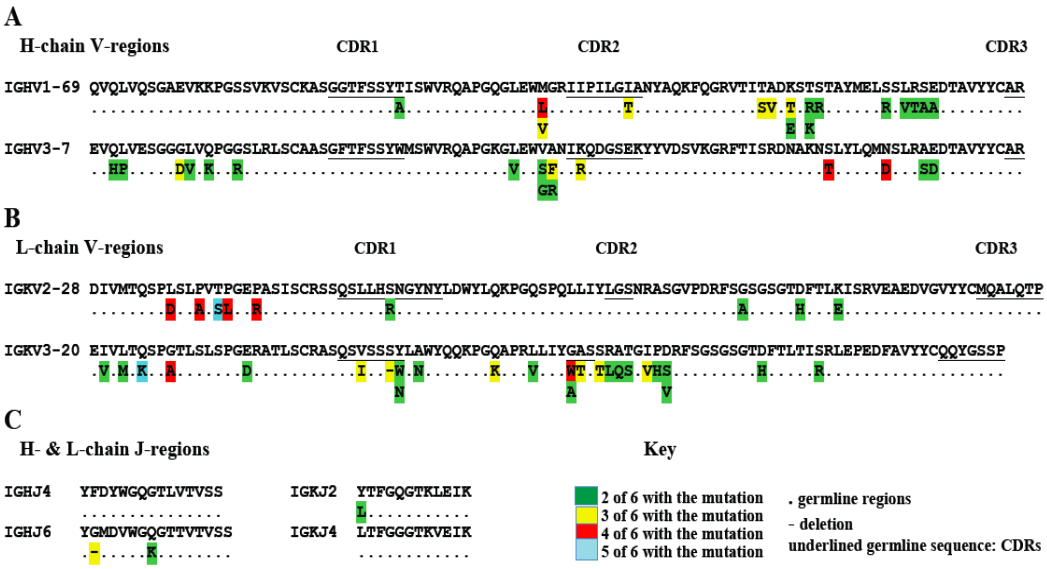

Figure 3.4. Variable (V)-region peptide heat map of compiled *de novo* sequencing data from six patients with systemic lupus erythematosus (SLE) showing public (shared among patients) mutations. **A.** Heavy chain (H-chain) V-region sequences align with germline IGHV1-69 and IGHV3-7. **B.** Light chain (L-chain) V-region sequences align with germline IGKV2-28 and IGKV-3-20. **C.** H-chain joining (J)-region with IGHJ4 and IGHJ6 germline sequence and L-chain J-regions aligned with IGKJ2 and IGKJ4. Common amino acid (aa) replacement mutations that diverge from the germline sequence are depicted in the text and color-coded according to the frequency of the mutation detected in the SLE patient cohort analyzed. Dots indicate aa matching to the germline sequence derived from the ImMunoGeneTics database. Germline complementary determining regions (CDRs) are underline.

3.3 Discussion

This study reveals that secreted anti-SmD autoantibody proteomes in unrelated patients with SLE are dominated by IgG1 kappa-restricted clonotypes specified by two distinct H- chains encoded by IGHV3-7 and IGHV1-69 gene segments and two L-chains derived from IGKV3-20 and IGKV2-28. While the finding of two common H- and L-chains is consistent with a biclonal anti-SmD autoantibody repertoire in lupus, the precise chain pairings are yet to be determined. Importantly, common usage of L- and H-chain V and J gene segments can now be extended from linked sets of anti-Ro60/Ro52/La responses to the SmD protein component of the Sm/RNP autoantibody cluster (Lindop *et al.*, 2011; Arentz *et al*., 2012; Thurgood *et al*., 2013). Taken together with the shared mutational signatures (Figure 3.4), these findings imply similar if not identical molecular pathways of anti-Sm autoantibody production in individual to individual. Although shared IGV usage has been reported for both infectious agents and autoantigens, this is generally H-chain restricted and has not been studied at the level of the secreted antibody proteome (Zhou *et al*., 2002; Krause *et al*., 2011; Breden *et al*., 2011; Bowers *et al*., 2014; Cho *et al*., 2014). While the relative importance of recombinatorial bias versus antigen-driven clonal selection events in shaping serum anti-SmD autoantibody proteomes remains unclear, the extensive V-region somatic hypermutation and intraclonal diversification observed at the level of SmD autoantibody proteomes are consistent with multiple rounds of Sm antigen-driven B-cell affinity maturation in germinal centres of secondary lymphoid organs.

The proteomic workflow developed herein utilized a simple ELISA plate-based method to purify anti-SmD autoantibodies from as little as one milliliter of complex SLE sera,

followed by combined electrophoretic analysis and *de novo* and database-driven mass spectrometric sequencing. The methodology requires as little as 20 micrograms of native or recombinant antigen for antibody isolation and sequencing of V-region clonotypic peptides; can be completed within a few days; and be used to determine V-region signatures for any serum autoantibody or humoral anti-viral response for which purified antigen or epitope is available. While these "front end" improvements have reduced autoantibody purification and sequencing times by days, sample size remains limited by the complexity of Ig bioinformatics analysis. The high-end- Q-TOF MS used herein performs higher mass accuracy protein sequencing and better protein coverage than Orbitrap MS used in previous studies, making it more suited to *de novo* peptide sequencing (Andrews *et al*., 2011). High-mass accuracy mass spectrometric L-chain constant region sequencing enables direct analysis of kappa/lambda restriction at the protein level, as opposed to standard immunochemical methods that may vary with antigen purity and specificity of anti-L-chain antibodies. Our finding of an absolute kappa restriction of purified anti-SmD IgGs as indicated by a unique kappa peptide profile by L-chain sequencing and kappa-specific anti-SmD solid phase immunoassay, differs from a 1980s study that used a different, antigen source and anti-L-chain (Eisenberg *et al*., 1985).

The discovery of shared anti-Sm autoantibody clonotypes will open new pathways of research for this prototypic systemic autoantigen, with unique V-region clonotypic peptides detected by mass spectrometric sequencing being potential novel biomarkers of anti-Sm autoantibody production. For example, mutated Sm-specific V-region clonotypic peptides might be used in targeted mass spectrometric platforms such as

MRM to quantitate specific V-region peptides in complex serum mixture, or to monitor expression of anti-Sm clonal turnover in patients undergoing treatment (Arentz *et al*., 2012; Lindop *et al*., 2013). A priority will be to sequence linked RNP/Sm autoantibody populations to test whether intermolecular sharing of autoreactive clonotypes holds true for the Sm/RNP autoantigen cluster. A long-term goal will be to correlate secreted autoantibody proteomes derived by MS with Sm-specific B cell and plasma cell receptor repertoires in blood and tissues.

In conclusion, a novel proteomic workflow was used to determine the molecular signatures of anti-SmD autoantibodies from unrelated patients with SLE. Remarkably, the response is restricted to the same H- and L-chain Ig families and shares V-region aa replacement mutations, a finding of pathognomonic and diagnostic significance for anti-Sm immunity in lupus.

The next chapter of this thesis will address the clonality and V-region signatures of another clinically important lupus-specific autoantibody directed against the Rib-P phosphoprotein complex. This will be characterized by 2-DE and high-resolution mass spectrometric sequencing and will include investigation at a molecular level the cross-reactivity of anti-Rib-P with Sm autoantigen.

Chapter 4: Lupus anti-ribosomal P autoantibody proteomes express convergent biclonal signatures

4.1 Introduction

Autoantibodies binding the P0, P1 and P2 ribosomal P phosphoproteins (Rib-P) are highly specific for SLE and have been reported in association with neurologic, renal and hepatic manifestations with some conflicting results (Pasoto *et al*., 2014). Anti-Rib-P autoantibodies are detectable before SLE onset and bind an immunodominant COOH-terminal epitope common to all three P proteins in addition to other regions on the native complex (Elkon *et al*., 1986; Bruner *et al*., 2005; Heinlen *et al.*, 2010). Active and passive immunization studies in mice have underlined the clinical importance of anti-Rib-P by enhancing lupus nephritis and mediating depression, smell and memory deficits, potentially by cross-reacting with a neuronal antigen highly expressed in the hippocampus termed the neuronal surface P antigen (NSPA) (Matus *et al*., 2007; Katzav *et al*., 2014; Ben-Ami *et al*., 2014; Bravo-Zehnder *et al*., 2015). Functional antibodies against the major 11-mer COOH epitope (11-C) bind NSPA and appear to mediate cognitive impairment by enhancing glutamatergic transmission in the hippocampus (Segovia-Miranda *et al*., 2015). While an early immunochemical study reported limited heterogeneity and mainly IgG1 kappa- restriction for COOH-terminal responses (Bonfa *et al*., 1987), nothing is known at the protein sequence level of the clonotypic structure and Ig V- gene usage of these potentially pathogenic lupus autoantibodies, and whether they share V-region signatures with other systemic autoantibodies.

Human anti-Rib-P antibodies purified from the native complex and anti-Rib-P antiserum raised in mice have been reported to cross-react with Smith (Sm) autoantigen suggesting an immunological link between these two lupus-specific autoantibodies, but the molecular basis of this cross-reactivity is unknown (Caponi *et al*., 1998; Ben-Ami *et al*., 2014). We have recently developed a novel proteomic workflow based on plate-purification of antigen-specific Igs, 2-DE and high-resolution mass spectrometric sequencing to identify two public immunodominant clonotypes that drive anti-SmD immunity, considered to have the highest specificity for SLE (Mahler *et al*., 2010; Al Kindi *et al*., 2015). In the present study, we employ direct antibody sequencing to compare clonotypic structures and V-region signatures of serum anti-Rib-P and anti-SmD autoantibody proteomes. The findings reveal a novel immunological link between subsets of anti-Rib-P and anti-SmD autoantibodies based on shared public clonotypes. Furthermore, an anti-Rib-P monoclonal population specific for the immunodominant COOH-terminal epitope was identified that may play a pathogenic role in neuropsychiatric lupus.

Serum samples positive for anti-Rib-P autoantibodies by commercial line blot immunoassay (Euroline ANA profile 5, Euroimmun, Lubeck, Germany) were retrieved from the clinical immunology serum repository at the Flinders Medical Centre from six female patients (median age 51 years; range 22-76 years) who met the American College of Rheumatology revised criteria for SLE (Tan *et al*., 1982). All patients were positive for antibodies to double-stranded DNA by Farr assay (Trinity Biotech, Bray, County Wicklow, Ireland). Demographic characteristics and serologic findings in the

patients are shown in Table 4.1. Control sera were obtained from four female healthy donors (median age 50 years; range 21-72 years).

Table 4.1. Characteristics of patients with Systemic lupus erythematosus (SLE)

Patient	Age/Sex	ANA (Pattern/titer)	Anti-dsDNA (IU/ml)	ENA
SLE1	62/female	Homogenous/2560	100	Rib-P
SLE2	54/female	Homogenous/2560	100	Rib-P, Ro60
SLE3	43/female	Homogenous/2560	100	Rib-P, Ro60
SLE4	48/female	Cytoplasmic/640	30	Rib-P
SLE5	76/female	Speckled/2560	40	Rib-P, Ro60
SLE6	22/female	Homogenous/2560	100	Sm/RNP, Rib-P, Ro60

Antinuclear antibodies (ANA) was detected using HEp2 slides, Anti-dsDNA was measured using Farr assay with value ≥ 10 IU/ml considered as positive and extractable nuclear antigen (ENA) autoantibodies were examined using Euroimmun euroline ANA profile 5.

4.2 Results:

4.2.1 The anti-Rib-P autoantibody population is biclonal and shares a cross-reactive clonotype with anti-SmD.

Monospecificity of eluted Igs for anti-Rib-P was confirmed by testing starting sera (anti-Rib-P/Ro60-positive), unbound and eluted fractions on individual Rib-P/Ro60 ELISAs (Figure 4.1A). Furthermore, eluted Igs gave the typical anti-Rib-P cytoplasmic and nucleolar immunofluorescence staining pattern on HEp-2 cells (Figure 4.1B). Indirect immunofluorescence of rodent LKS block probed with purified anti-Rib-P Igs stained chief cells (Figure 4.1C). Anti-Rib-P Igs purified from SLE patients with anti-Rib-P specifically bound Rib-P and SmD on ELISA but not Ro52 and HA-09 present in starting sera while conversely, purified anti-SmD Igs from anti-Sm-positive patients bound SmD and Rib-P, indicating weak two-way cross-reactivity between these autoantibody populations (Figure 4.1D). SDS-PAGE of the affinity purified Igs showed a single H- and L-chain bands, which were excised and sequenced by high-resolution MS (Figure 4.2).

The clonality of plate-purified anti-Rib-P Igs was then assessed by high-resolution 2-DE which revealed two H-chain clusters of spots shared between patients with pIs ranging from 6.0-7.6 (identified as IGHV3.7-JH6 on sequencing of gel plug digests) and 8.0-9.0 (IGHV1.3-JH4) linked with two shared clusters of kappa L-chain spots ranging from pI 5.8-7.4 (IGKV1.39-JK4) and pI 7.8-9.0 (IGKV3.20-JK2) (Figure 4.3A,B). Strikingly, the 11-C peptide-specific autoantibody population purified from patient SLE1 was monoclonal in terms of H- and L-chain usage and matched the same IGHV1.3-JH4/IGKV1.39-JK4 signature seen within the total anti-Rib-P population of

this patient (Figure 4.3A,C). This identical 11-C peptide-specific monoclonal pairing signature was detected in an additional three anti-Rib-P-positive patients SLE3, SLE5, SLE6 (data available on request), consistent with the presence of a dominant clonal population of anti-Rib-P directed against the common COOH- terminal epitope.

Analysis of purified anti-SmD Igs run in parallel by 2-DE revealed a clonotypic population specified by IGHV3.7-JH6/IGKV3.20-JK2 with the same electrophoretic mobility and clonal signature as the second anti-Rib-P clonotype (Figure 4.3 A,B,D). Significantly, 11-C peptide-purified Igs did not react with SmD protein by ELISA, nor did SmD-purified Igs bind the 11-C peptide (data not shown).

4.2.2 Rib-P-reactive clonotypic IgGs express convergent H-and L-chain signatures.

Mass spectrometric analysis was performed on solution trypsin digests of plate-purified whole anti-Rib-P Igs from the six SLE patients to confirm 2-DE assignment of clonotypes and obtain high-resolution V-region sequencing and mutational status. This confirmed the common expression of two IgG1 kappa-restricted clonotypic populations specified by IGHV3.7-JH6/IGKV3.20-JK2 and IGHV1.3-JH4/IGKV1.39-JK4 pairing signatures. No lambda L-chain tryptic peptides were detected in plug or solution digest of anti-Rib-P Ig, verifying kappa L-chain restriction at the level of direct protein sequencing. Complete anti-Rib-P V-region tryptic peptide maps are shown in Supplementary figure 2 and reveal multiple homologous clonotypic peptides with public (shared among patients) and private aa replacement mutations consistent with selection of intraclonal variants by persistent antigen stimulation. Public (common) mutations are present in both FR and CDRs of the H- and L-chains and are tabulated by

a proteomic heat map (Figure 4.4). In contrast, no IgG tryptic peptides were detected by MS in control experiments from Rib-P-coated ELISA plates treated with sera from healthy donors (n=4).

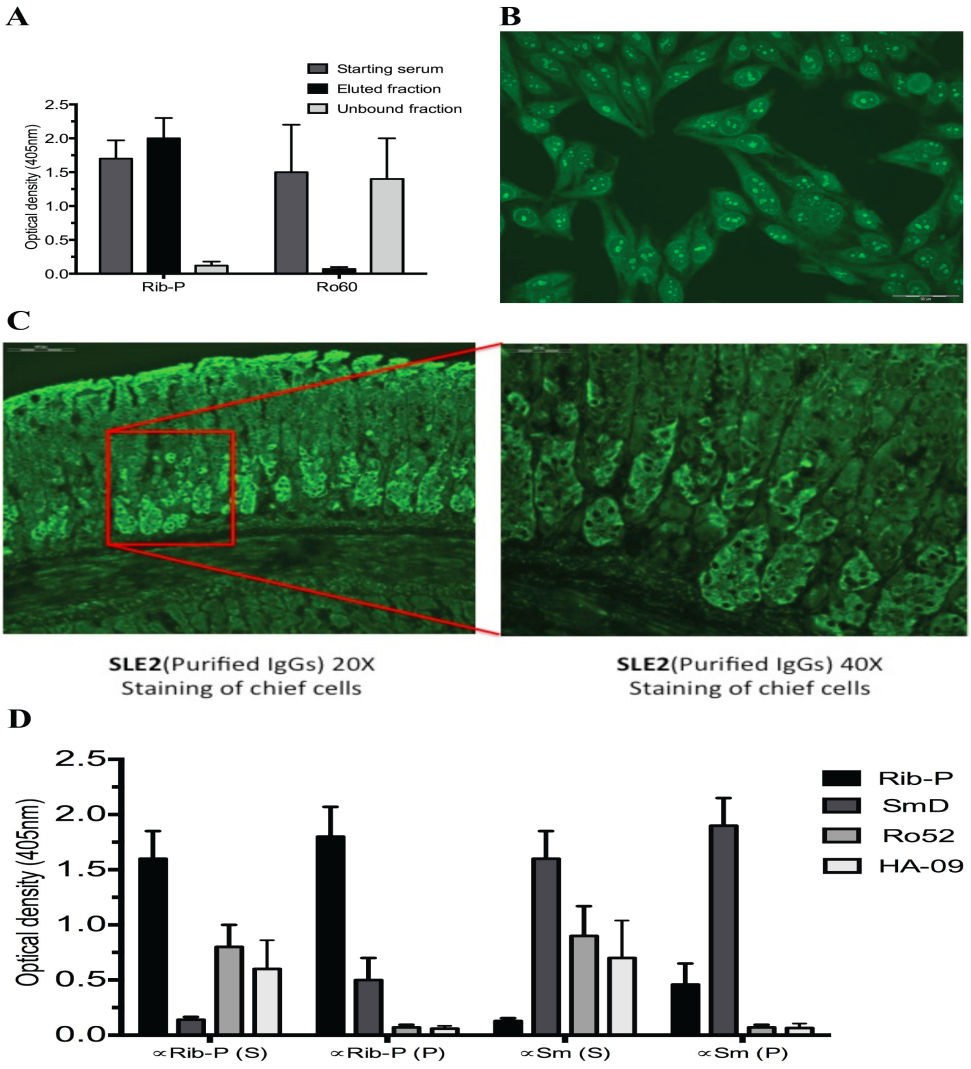

Figure 4.1. Specificity of anti-Rib-P IgGs purified from native Rib-P-coated ELISA plates from sera of six patients with SLE. **A.** Purified IgGs tested by ELISA using native Rib-P and Ro60 proteins. Starting, bound and unbound fractions are compared. Bars show the mean ± SD of duplicate optical density (OD) values. **B.** Indirect immunofluorescence of HEp-2 cells probed with anti-Rib-P IgGs (2.5 ug/ml). **C.** Indirect immunofluorescence of rodent LKS block probed with anti-Rib-P IgGs (2.5 ug/ml). **D.** Reciprocal reactivity between anti-Rib-P and anti-SmD IgGs against Rib-P and SmD, and Ro52 and HA-09 control proteins. S stands for starting serum and P for plate-purified IgGs. Bars show the mean ± SD of duplicate OD values.

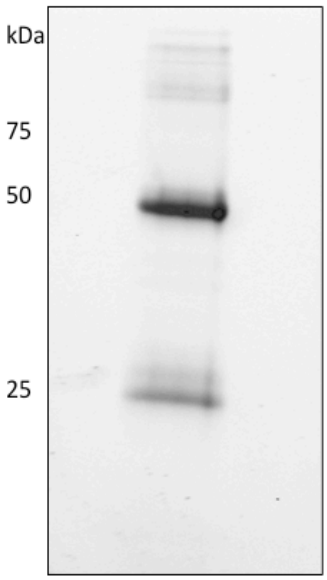

Figure 4.2. Representative SDS-PAGE of affinity purified Igs from patient with anti-Rib-P. A single H- and a single L-chain band were detected, which were excised and sequenced by high-resolution mass spectrometry (MS).

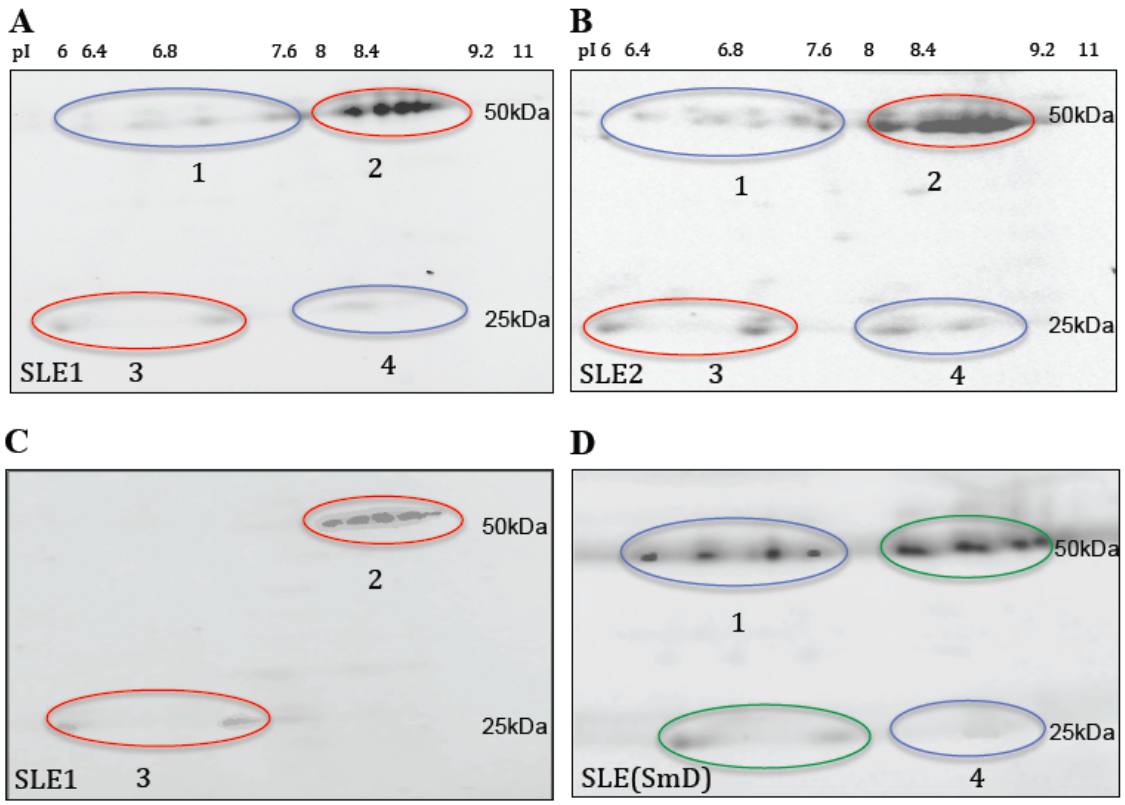

Figure 4.3. Clonal restriction of total anti-Rib-P IgGs and comparison with 11-C peptide- and SmD-specific IgGs. **A** and **B**. Representative two-dimensional gel electrophoresis (2-DE) of native Rib-P heterocomplex-purified IgGs from two SLE patients showing two H-chain sets of spots sequenced as IGHV3.7-JH6 (1) and IGHV1.3-JH4 (2) together with two sets of L-chain spots sequenced as IGKV1.39-JK4 (3) and IGK3.20-JK2 (4). **C.** 2-DE of 11-C peptide- purified IgGs from patient SLE1 reveals persistence of H-chain 2 and L-chain 3 clonotypic sets (circled in red). **D.** 2-DE of SmD-purified IgGs from an anti-Sm-positive SLE patient highlighting a shared IGHV3.7-JH6/IGKV3.20-JK2 clonotype with anti-Rib-P (circled in blue). The other anti-SmD H- and L- chain spots (circled in green) are anti-SmD-specific and sequenced as IGHV1.69/IGKV2.28.

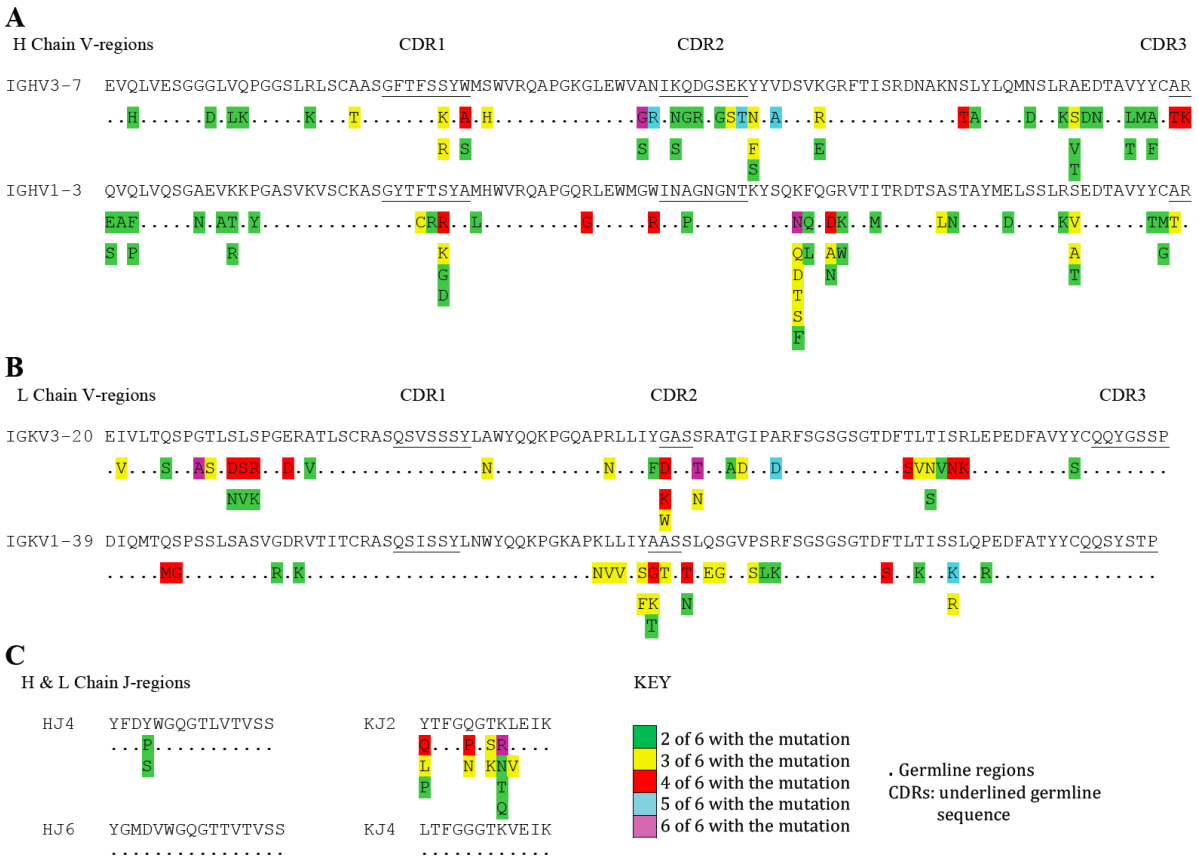

Figure 4.4. Variable (V)-region peptide heat map of compiled *de novo* sequencing data from six patients with SLE showing public (shared) amino acid (aa) replacement mutations. **A.** H-chain V-region sequences align with germline IGHV3-7 and IGHV1-3 (IMGT database). **B.** L-chain V-region sequences align with germline IGKV3-20 and IGKV1-39. **C.** H-chain J-region align with JH4 and JH6 germline sequence and L-chain J-regions align with JK2 and JK4 germline sequence. Shared aa replacement mutations divergent from the germline sequence are indicated in the text and color-coded according to the prevalence. Dots indicate aa matching to the germline sequence. Germline complementary determining regions (CDRs) are underlined.

4.3 Discussion

This study has utilized high-resolution electrophoretic and mass spectrometric techniques to discover two public clonotypic autoantibodies that dominate humoral Rib-P immunity in SLE. The proteomic data are consistent with humoral anti-Rib-P responses being driven by a dominant biclonal population, with one clone specific for the immunodominant COOH-terminal epitope and the second accounting for cross-reactivity with SmD protein. The expression of specific H/L chain pairings and sharing of V-J clonal signatures, combined with V-region mutations common to different patients, appear to be general properties of systemic autoantibodies and emphasize the importance of recombinatorial bias and antigen-driven clonal selection in shaping autoantibody repertories (Lindop *et al*., 2012, Al Kindi *et al*., 2015). Recent studies based on deep sequencing of antibody repertoires following vaccination and infection have revealed a similar convergence of H-chain responses in unrelated subjects, suggesting that natural selection against pathogens may guide autoantibody responses towards clonotypes readily available in the primary Ig repertoire (Boyd *et al*., 2015).

Human and rabbit antibodies purified on the 11-C peptide have recently been reported to impair memory in mice by binding NSPA on hippocampal neurons (Bravo-Zehnder *et al*., 2015), implicating the IGHV1.3-JH4/IGKV1.39-JK4 clonotype as a pathogenic species in neuropsychiatric lupus. Independent passive transfer studies have implicated anti-Rib-P in renal and neurological manifestations, although it is unclear whether the transferred Ig was monospecific for the COOH-terminal P epitope (Katzav *et al*., 2014; Ben-Ami *et al*., 2014). If the 11-C-peptide-specific clone proves to have pathogenic and functional properties in human disease, then removal or selective silencing of the clone may be a future therapeutic option in SLE. Early work suggests that this major autoreactive clonotype may bind to other cell

surface proteins sharing homology with the COOH- terminal P epitope on hepatocytes and T lymphocytes (Pasoto *et al*., 2014).

The IGHV3.7-JH6/IGKV3.20-JK2 clonotype, which binds an epitope outside the COOH-terminus yet to be mapped, is notable for its immunological cross-reactivity and shared germline H/L chain pairing signature with a recently identified anti-SmD clonotype (Al Kindi *et al*., 2015). These findings provide a novel molecular explanation for cross-reactivity between two lupus-specific autoantibodies based on shared clonotypic structures, suggesting a common clonal origin for at least a subset of these autoantibodies. We hypothesize that naïve germline-encoded IGHV3.7-JH6/IGKV3.20-JK2 B cells in the primary repertoire evade early B-cell checkpoints in SLE patients and escape to the periphery, where they can undergo either Rib-P- or Sm-driven clonal selection, expansion and affinity maturation in germinal centres (Figure 4.5). The secretion of mature autoantibodies with different maturation pathways and V-region mutation profiles would account for the weak cross-reactivity detected on immunoassay.

The stereotyped Ig gene rearrangements and conserved H/L-chain pairings in humoral anti-Rib-P responses recapitulate findings for anti-Ro/La and anti-Sm proteomes in primary (SS) and SLE and support clonotypic sharing of autoantibodies as a unifying feature of systemic autoimmune diseases (Chapter 3; Lindop *et al*., 2011; Arentz *et al*., 2012; Thurgood *et al*., 2013; Al Kindi et al., 2015). The marked clonal restriction and conserved V-region gene usage observed for both anti-Rib-P and anti-Sm Igs support a unifying mechanism of pathogenic autoantibody production in unrelated patients with SLE, based on highly similar if not identical B- cell activation pathways for the original stimulus through to the generation of

clones of autoantibody secreting cells. A corollary of the serum autoantibody proteome analysis is that

these lupus specific-autoantibodies are derived from a limited number of B-clonal precursors that have evaded early tolerance checkpoints and undergone antigen-driven clonal selection, expansion and affinity maturation in germinal centres. In a broader context, stereotyped B-cell receptors are becoming increasingly recognized in infections, B-cell cancers and autoimmune diseases, challenging the paradigm that Ig responses against the same antigen are randomly determined in different individuals (Henry *et al*., 2015). It remains unclear as to why identical determinants are selected on autoantigens among different patients (Scofield *et al*., 1999; Mahler *et al*., 2010; Hoffmann *et al*., 2010), and why their structural features appear to channel responses to a few shared clones. While stereotypy at the level of both autoantigen and cognate autoantibody points to a deterministic model of highly similar pathways of autoantibody production from patient to patient (Smith *et al*., 2013), the presence of intraclonal variants with individual somatic mutations and different antibody levels in individual patients is consistent with a secondary role for somatic selection in shaping the final autoantibody response. Both public and patient-specific V-region peptides have potential as diagnostic biomarkers using mass spectrometric MRM platforms, and may also be useful to track autoreactive B-cell clonal turnover.

Finally, this work reinforces the analytical power of direct mass spectrometric sequencing to determine molecular characteristics of serum autoantibody biomarkers, enabling identification of stereotyped responses at the level of secreted (serum) Ig proteomes. Prospective studies on SLE patients who have undergone formal neuropsychiatric assessment, together with new

experimental approaches in animal models will be required to unravel the diagnostic potential and pathogenic properties of these novel Rib-P immunoreactive clonotypes.

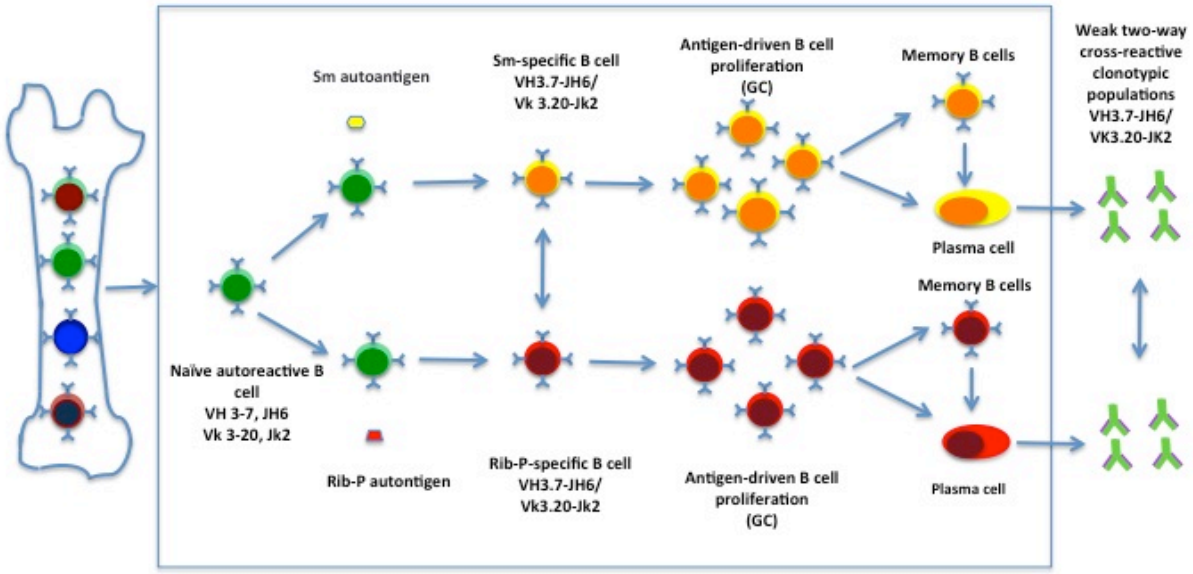

Figure 4.5 Model: A subset of anti-Rib-P and anti-Sm autoantibodies arises from a common precursor B cell. Naïve germline-encoded IGHV3.7-JH6/IGKV3.20-JK2 B cells in the primary repertoire evade early B-cell checkpoints in SLE patients and escape to the periphery, where they can undergo either Rib-P- or Sm-driven clonal selection, expansion and affinity maturation in germinal centres.

Chapter 5: Concluding remarks

5.1 Summary and Conclusion

This thesis presented a novel proteomic workflow based on affinity purification of specific Igs from serum, high-resolution 2-DE, and *de novo* and database-driven sequencing of V-region proteins by MS (Figure 5.1). MS-based antibody sequencing is a new field under development that can be performed in only a small number of laboratories. It has clear advantages over hybridoma and single-cell recombinant antibody cloning methods from B-cells and plasmablasts in being able to determine the complete secreted (serum) autoantibody proteome, the final product of humoral autoimmunity. An additional benefit of direct sequencing of serum autoantibodies is that H- and L- chain constant regions can be identified with complete accuracy at the level of aa sequence, as opposed to standard immunochemical methods that depend upon on the specificity of anti-L-chain antibodies. Early data on kappa/lambda restriction of autoantibodies, based on conventional immunochemistry, may need to be revised in light of this new technology. Other advantages include the ease of serum collection; stability of antibodies under storage; small volume requirements (a V-region peptide signature can be obtained on as little as one ml of sera); and applicability to archival sera.

Labor-intensive step of affinity purification has now been superseded by a simple ELISA plate-based elution method that dramatically reduces serum and autoantigen requirements and workflow times. Furthermore, the Orbitrap mass spectrometer used in earlier studies has been superseded in this thesis by a high-end high performance liquid chromatography quadrupole-time of flight (HPLC Q-TOF) mass spectrometer that performs higher MS/MS mass accuracy protein sequencing and improved protein coverage. While these improvements have

shortened purification and sequencing steps from weeks to days, and can be applied to any immune serum, sample size remains limited by the complexity of Ig bioinformatics analysis.

The novel MS workflow was used initially to characterize secreted autoantibody proteomes specific for the clinically important Sm autoantigen, in particular those directed against the SmD protein that have the highest specificity for SLE (Tan *et al*., 1966; Tan *et al*., 1982; Tsokos, 2006; Mahler *et al*., 2010; Al Kindi *et al*., 2015). The analysis revealed two public IgG1 kappa-restricted clonotypic species expressing IGHV3.7/IGKV3.20 and IGHV1.69/IGKV2.28 peptides with both common and individual V-region aa replacement mutations. The relative roles of V(D)J recombinatorial bias and antigen-driven clonal selection events in shaping this final autoantibody repertoire have yet to be unrevealed, but the combination of shared V-region gene usage and presence of random somatic hypermutations in these clonotypic Igs are consistent with both deterministic and stochastic effects.

Another classical lupus-specific autoantibody is directed against the P0, P1 and P2 ribosomal P phosphoproteins of the ribosomal P autoantigen (Pasoto *et al*., 2014). Anti-Rib-P autoantibodies bind an immunodominant COOH-terminal epitope common to all P proteins and other determinant (s) on the native complex (Elkon *et al*., 1986; Bruner *et al*., 2005). Combined 2-DE analyses and mass spectrometric sequencing on two populations of anti-Rib-P IgGs, one purified from the native ribosomal P complex and the other from the conserved COOH-terminal P epitope, revealed two major public clonotypic populations. One of these, defined as an IGHV3.7-JH6/IGKV3.20-JK2 clonotype, shared the same germline H- and L-chain pairing signature as a cross-reactive population of anti-SmD autoantibodies, although each clone expressed a different pattern of aa replacement mutations. This resolved a long-

standing issue between these lupus-specific autoantigens by identifying the molecular basis of their weak cross-reactivity, and suggests that a subset of autoantibodies have a common B-cell clonal origin (Caponi *et al*., 1998; Mahler *et al*., 2010; Al Kindi *et al*., 2016). The other clonotypic set was specific for COOH-terminal and was sequenced as IGH1.3-JH4/IGKV1.39-JK4.

These findings support a model of systemic autoimmunity in which humoral responses against protein-RNA complexes are mediated by public (shared) sets of autoreactive B-cell clonotypes with common molecular pathways of autoantibody production. Moreover, the discovery of shared V-region structures among linked autoantibody sets (Ro52/Ro60/La, Sm) supports models of intermolecular diversification based on major histocompatibility complex class II presentation of shared clonotypic V-region determinants by B cells specific to different proteins of an RNP complex to idiotypic-specific T helper cells; the so-called receptor presentation model of intermolecular autoantibody spreading (Zhang *et al*., 2001; Kalsi *et al*., 2002).

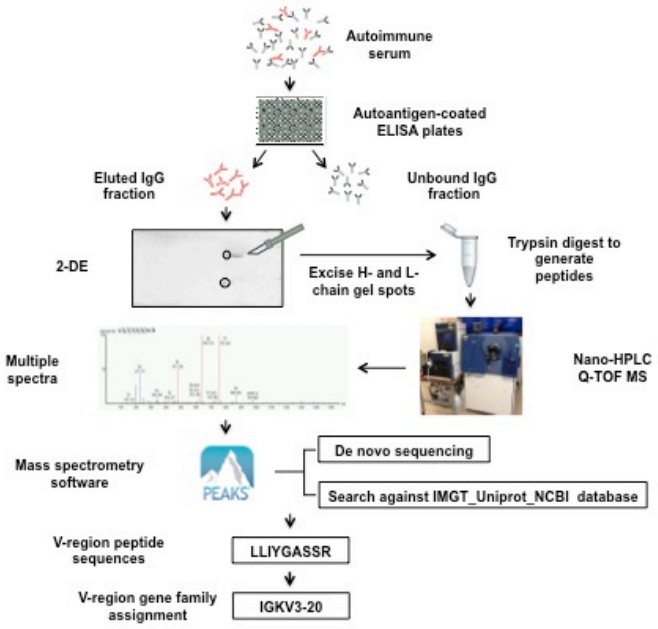

Figure 5.1. Proteomic workflow of secreted autoantibody repertoires. Serum autoantibodies are purified from autoantigen-coated ELISA plates by low pH elution. Clonality of eluted Ig is assessed by two-dimensional gel electrophoresis (2-DE). Heavy (H)- and light (L)- chain gel spots are digested with trypsin to generate peptides for Nano-HPLC Q-TOF MS. *De novo* sequencing is performed on raw MS spectra data using PEAKS Studio software. Variable (V)-region peptide sequences are derived by searching against a combined IMGT (http://www.imgt.org)_NCBI_Uniprot 2015-08 databases. V-region gene family is assigned from presence of unique peptide corresponding to the gene family.

5.2 Future Directions

De novo sequencing rarely obtains full sequence through the D-region of the H-chain and complete H-chain complementarity determining region 3 (HCDR3) sequence, compounded by the unavailability of HCDR3 sequences in databases. New developments in *de novo* sequencing of HCDR3 regions in conjunction with immunoproteogenomics approaches may overcome this limitation (Safonova *et al*., 2015). It has not yet been possible to sequence functional autoantibodies with this methodology because of their low serum concentration and lack of binding to antigen in the solid phase.

Future studies should be able to combine single-cell and proteomic methodologies to identify specific HCDR3 peptides from autoantigen-specific B-cells and plasma cells from blood or inflamed target tissues such as salivary glands biopsied from patients with primary SS (Mietzner *et al*., 2008; Maier-Moore *et al*., 2014). In the nearer term, the combination of mass spectra data with NGS sequencing of blood or tissue B-cell receptor repertoires, using the latter as patient-specific B-cell receptor transcriptome libraries for V-region peptide database searches, may allow robust HCDR3 identification and complete autoantibody reconstruction. Recent efforts to integrate NGS and MS to study serum and B-cell repertoires in human systemic autoimmunity have been promising, although much work needs to be done to improve bioinformatic analysis and *de novo* sequencing of Ig proteomes (Safonova *et al*., 2015; Halliley *et al*., 2015; Tipton *et al*., 2015).

Further studies will be directed at sequencing myositis- and scleroderma-specific autoantibodies, with the prediction that these autoantibody groups will also be dominated by public clonotypes with shared V-region peptide signatures (Walker *et al*., 2007; Limaye *et al*., 2009; Mehra *et al*., 2013; Betteridge *et al*., 2015). This will require careful selection of

autoreactive sera and purification of autoantibodies using purified native and recombinant autoantigens. Proteomic characterization of secreted antibody repertoires by MS need not be confined to human autoantibodies, and the approach described herein can potentially be used to identify shared serum V-region peptides in infectious diseases like in influenza, hepatitis and clostridium difficile among others, responses to vaccination, myeloma and immune deficiencies. Indeed, stereotyped B-cell receptors are now recognized in infections and B-cell cancers at the level of the transcriptome, further challenging the paradigm of random selection of antibody responses against the same antigen in different individuals (Krause *et al*., 2011; Parameswaran *et al*., 2013; Jackson *et al*., 2014; Boyd *et al*., 2015; Henry *et al*., 2015).

It is worth noting that conventional assays for autoantibody detection such as ELISA, while detecting binding of polyclonal antisera, are labor-intensive, require expensive purified antigens and are unable to identify disease-specific clonotypes. This thesis has pointed out the sharing of public V-peptide across unrelated patients with SLE. These findings could be translated to a novel biomarker in MRM diagnostic platforms. If the promise of MRM-based detection of specific human autoantibodies is fulfilled, development of multiplexed MS-based diagnostic platforms may supersede traditional solid phase immunoassays by detecting multiple autoantibodies at a molecular level in a single serum sample. MRM can also be applied to clone tracking, in which serial clonotypic Igs can be sequenced over time to check response to therapies. Finally, direct sequencing of serum autoantibody clonotypes in human disease may reveal idiotypic signatures that can be targeted by anti-idiotypic or antigen-specific approaches for clonal removal.

Appendices

Appendix 1: Supplementary figure 1 showing peptides of IGHV3.7, IGHV1.69, IGKV3.20 and IGKV2.28 gene families in affinity purified anti-SmD from six patients with SLE (refer to chapter 3) and CD attached.

Appendix 2: Supplementary figure 2 showing peptides of IGHV3.7, IGHV1.3, IGKV3.20 and IGKV1.39 gene families in affinity purified anti-Rib-P from six patients with SLE (refer to chapter 4) and CD attached.

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