



Long-term Ro60 humoral autoimmunity in primary Sjögren's syndrome is maintained by rapid clonal turnover

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Abstract Long-term humoral autoimmunity to RNA–protein autoantigens is considered a hallmark of systemic autoimmune diseases. We use high resolution Orbitrap mass spectrometric autoantibody sequencing to track the evolution of a Ro60-specific public clonotypic autoantibody in 4 patients with primary Sjögren's syndrome. This clonotype is specified by a V_H3–23/V_K3–20 heavy and light chain pairing. Despite apparent stability by conventional immunoassay, analysis of V-region molecular signatures of clonotypes purified from serum samples collected retrospectively over 7 years revealed sequential clonal replacement. Prospective longitudinal studies confirmed clonotype loss and replacement at approximately three-monthly intervals. Levels of secreted anti-Ro60 clonotypes fluctuated markedly over time, despite minimal changes in clonal affinity. Our novel findings indicate a relentless turnover of short-lived clonotypic variants, masquerading as long-lived Ro60 humoral autoimmunity.

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1. Introduction

Long-term humoral autoimmunity to RNA–protein autoantigens is considered a hallmark of systemic autoimmune diseases. This is true of autoantibodies against the 60 kDa Ro/SSA (Ro60) ribonucleoprotein, the most common member of the extractable nuclear antigen (ENA) family and frequent target of humoral autoimmunity in systemic lupus erythematosus, primary Sjögren's syndrome (SS) and the neonatal lupus syndrome [1,2]. Ro60 may be a primordial autoantigen involved in

Abbreviations: 60 kDa Ro/SS, Ro60; AUC, area under the dose–response curve; ELISA, enzyme-linked immunosorbent assay; ENA, extractable nuclear antigen; FT, Fourier transform; RU, resonance units; SS, Sjögren's syndrome.

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the initiation of human systemic autoimmunity [3], as anti-Ro60 autoantibodies can be present in serum years before clinical autoimmunity, when they may become pathogenic under conditions of increased antigenic exposure. For example, tissue damage in congenital heart block may be triggered by binding of maternal anti-Ro60 IgGs to cognate antigen on the surface of apoptotic fetal cardiocytes during a critical window of cardiac remodeling [4–6].

Despite their clinical importance, the immune mechanisms that sustain high-titer anti-Ro60 autoantibodies over a patient's lifetime are poorly understood. Humoral responses to RNA binding proteins such as Ro60 are thought to remain relatively stable over time, as opposed to anti-dsDNA responses that show marked variation in titer in many patients [7]. However, an early study with frequent measurements of anti-Ro60 autoantibody levels in a patient with systemic lupus erythematosus over 80 months revealed unexpected fluctuations in autoantibody levels unrelated to disease activity, immunosuppressive treatment, and anti-dsDNA antibody levels, suggesting variable production of anti-Ro60 autoantibodies [8]. It is not known whether these findings are generalizable to other anti-Ro60-positive patients, or whether these fluctuations are indicative of more significant changes in the autoantibody response that may be missed by standard solid-phase immunoassay.

Long-term humoral autoimmunity can be mediated by short-lived plasma cells arising from the continuous activation of autoreactive B cells, and/or by long-lived plasma cells that survive for years in survival niches in the bone marrow [9,10]. However, these models of autoantibody production have yet to be tested against a bona fide human autoantigen such as Ro60. The fluctuation of the humoral anti-Ro60 response has not been previously studied at the level of the secreted autoantibody repertoire because of technical limitations in sequencing low abundant autoantibodies in complex sera with multiple anti-Ro/La specificities. We have recently applied high resolution Orbitrap mass spectrometric autoantibody sequencing to identify a clonotypic signature and unique V_H3-23/V_K3-20 chain pairing that is public (shared) across unrelated patients. This clonotype, specific for an immunodominant epitope termed Ro60peg, appears to be phenotypically stable on conventional immunoassay [11]. In the present study, we directly sequenced the secreted anti-Ro60peg autoantibody proteome in serial samples from patients with the prototypic human systemic autoimmune disease of primary SS, and analyzed the clonotypic variation over time. We demonstrate a dynamic process of repeated turnover in Ro60 humoral autoimmunity, characterized by a periodic succession of Ro60peg-reactive clonotypes masquerading as long-lived Ro60 humoral autoimmunity.

2. Materials and methods

2.1. Patients

Retrospective and prospective serial serum samples were collected over weeks to years from 4 patients with primary SS and anti-Ro/La autoantibodies who expressed the V_H3-23/V_K3-20 Ro60peg clonotypic autoantibody [11]. Demographic characteristics and serologic findings of the patients are

shown in Table 1. Patients fulfilled at least 4 of the 6 American–European Consensus Group classification criteria [12]. None of the patients was treated with corticosteroids or other immunosuppressive agents at the time of serum collection. The study was approved by the Southern Adelaide Human Research Ethics Committee.

2.2. Preparation and specificity analysis of affinity purified anti-Ro60peg IgGs

Anti-Ro60peg IgG was purified by epitope-specific affinity chromatography. Sera were passed over a CNBr-activated Sepharose 4B column (Pharmacia) coupled with a soluble recombinant MBP-Ro60 aa 82–244 fragment that expresses the Ro60peg epitope. Bound IgG was eluted with 0.1 M glycine, 0.5 M NaCl (pH 2.3), and neutralized in 1 M Tris (pH 8.0). Eluted IgG was dialyzed against PBS (pH 7.4), and concentrated using an Amicon concentrator. The activity and specificity of affinity-purified IgG were determined by comparing the starting serum, flow-through fractions, and bound/eluted fractions for reactivity against MBP-Ro60peg, native Ro60, full length Ro52, full-length glutathione S-transferase La, and an MBP control, as previously described [11].

2.3. Measurement of anti-Ro60peg autoantibody levels

Anti-Ro60peg levels were determined by antibody titre ELISA. Plates (Maxisorb, Nunc) were coated with recombinant MBP-Ro60peg at 5 µg/ml in 0.03 M carbonate buffer (pH 9.6). Non-specific sites were blocked with 3% skim milk in PBS. After washing 3 times with PBS containing 0.05% Tween-20, plates were incubated with sera serially diluted (twofold dilutions starting at 1:100) in 1% skim milk in PBS. Bound IgG was detected with goat anti-human IgG alkaline phosphatase-conjugated antibody followed by p-nitrophenyl phosphate substrate. All samples were tested in duplicate, the optical density was read at 405 nm, and Ro60peg-specific IgG levels were expressed as the area under the dose–response curve (AUC) [13].

2.4. Measurement of anti-Ro60peg autoantibody binding affinities

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

Table 1 Characteristics of patients with primary SS^a.

Patient	Age/sex	Symptoms (year)	Autoantibodies ^b :				Total IgG ^c (mg/ml)	RF ^d (IU/ml)
			Ro60peg	Ro60	Ro52	La		
pSS1	70/F	24	2.2	1.4	1.1	0.3	25.2	<40
pSS2	31/F	14	1.9	1.6	1.4	1.9	68	638
pSS3	41/F	13	0.9	1.8	1.8	1.8	50.1	<40
pSS4	55/F	0.5	1.9	2.2	2.9	1.7	52.5	2280

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

^b Ro60peg, Ro60, Ro52, and La autoantibodies measured by ELISA (OD values) with a normal cut-off of 0.2.

^c Total IgG measured by nephelometry.

^d Rheumatoid factor measured by nephelometry.

MBP-Ro60peg. A steady state binding model was used to determine the K_D .

2.5. Mass spectrometry and protein sequence analysis

Affinity purified anti-Ro60peg IgGs were precipitated using a 2D-Clean Up Kit (BioRad) and resuspended in 20 μ l of 100 mM ammonium bicarbonate (pH 8.0). IgG was reduced by the addition of DTT (5 mM) and incubation at 100 °C for 5 min and alkylated by the addition of iodoacetamide to 20 mM. Samples were digested with 1 μ l of Trypsin Gold at 100 ng/ μ l (Promega) overnight at 37 °C.

Peptides were analyzed using a LTQ Thermo Orbitrap XL linear ion trap mass spectrometer fitted with a nanospray source (Thermo Electron Corporation), as previously described [14]. Digested peptides were applied to a PepMap100 NanoViper trap column (Dionex) and separated on a C18 5 μ m 100 Å column (Nikkoy Technos), using a Dionex Ultimate 3000 HPLC (Dionex). The samples were eluted with a 55 min 2% to 45% acetonitrile containing 0.1% formic acid gradient at 200 nl/min flow rate. The mass spectrometer was operated in positive ion mode with one Fourier transform (FT) MS scan of mass/charge (m/z) 300–2000 at 60,000 resolution followed by collision induced dissociation fragmentation and FT product ion scans. Dynamic exclusion was used, with an exclusion list of 500, 10 ppm low and high mass widths relative to the reference mass and collision energy of 35%. Only multiple charged ions were selected for MS/MS.

The mass spectra were analyzed using the Peaks Studio v5.3 software (Bioinformatics Solutions Inc.), as previously described [14]. Briefly, de novo sequencing of the input spectrum was performed using the Peaks algorithm with a precursor ion error tolerance of 10 ppm and product ion error tolerance of 0.02 Da, digestion enzyme as trypsin, and the variable modifications of oxidation of methionine, carbamidomethylation of cysteine, and deamidation of asparagine and glutamine. The input spectrum was searched against a combined database of IMGT sequences (<http://www.imgt.org>), containing human H and L chains and the UniProt 2010-06 database. The SPIDER module was used to identify mutations or variations from the germline IMGT/UniProt sequences using a homology match query type. All peptide matches from both the Peaks and Spider search modules were manually checked to assure quality before inclusion in the final data analysis.

3. Results

3.1. Isolation of monospecific anti-Ro60peg IgG from polyclonal serum

We have previously identified an apparently long-lived public Ro60peg-reactive clonotypic autoantibody specified by a unique V_H3-23/V_K3-20 chain pairing [11]. In the present study we investigated whether this clonotype is stable over long periods or is replaced periodically. Anti-Ro60peg clonotypic autoantibodies purified from serial serum samples of 4 patients with primary SS were confirmed as monospecific for Ro60peg by ELISA (Fig. 1). Absence of Ro60peg binding activity in flowthrough fractions indicated that the entire anti-Ro60peg response is accounted for by the V_H3-23/V_K3-20 clonotype.

3.2. Anti-Ro60peg autoantibodies undergo sequential clonal replacement

Next, we carried out a retrospective longitudinal analysis of Ro60peg-specific IgGs purified from serial serum samples of up to a 7 year duration in patient's pSS1 and pSS2. De novo MS sequencing of purified Ro60peg IgGs was performed to obtain V-region molecular signatures. Near full-length sequences confirmed expression of a single anti-Ro60peg clonotype at each time point, defined by a V_H3-23/J_H5 heavy chain paired with a V_K3-20/J_K2 light chain, with conserved amino acid substitutions as demonstrated previously [11].

V-region sequences revealed a process of periodic Ro60peg-specific clonotypic turnover, simulating long-lived humoral autoimmunity (Fig. 2). In patient pSS1, the anti-Ro60peg clonotype remained unchanged over 3 months between Feb 2002 and April 2002; by 7 months later a new clonotype had arisen (Nov 2002). Uniquely mutated clonotypes with the same heavy and light chain gene pairing then appeared in 2005 and 2006 (Fig. 2A). Clonotypic turnover was also observed in patient pSS2 over periods of at least 5 months (Fig. 2B). Sequential clonotypes had unique V-region mutational signatures, indicating that every clone arises de novo and has not evolved from its predecessor by sequential addition of new mutations.

3.3. Clonotypic replacement occurs at regular intervals

Retrospectively, clonotypes appeared to undergo repeated replacement every 3–7 months. To better define the kinetics

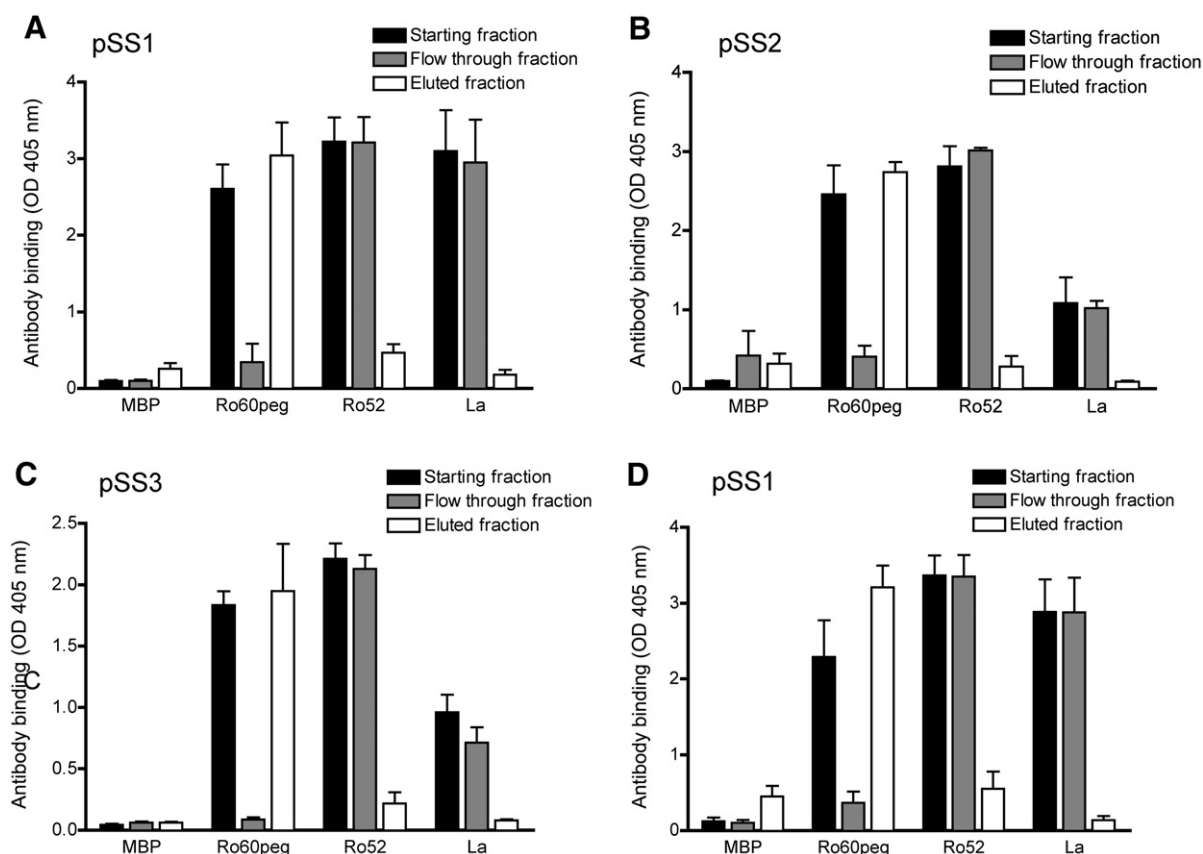


Figure 1 Affinity-purified anti-Ro60peg IgGs are monospecific for Ro60peg determinant. Ro60peg-specific IgGs were affinity purified from sera of patients pSS1–4 purification confirmed by ELISA on multiple runs (pSS1 $n = 5$, pSS2 $n = 5$, pSS3 $n = 6$, pSS4, $n = 4$). (A–D) Specificity of anti-Ro60peg IgGs from patients pSS1–4 demonstrating isolation of Ro60peg-specific IgGs, without significant contamination with Ro52- or La-specific IgGs. Optical density (OD) expressed as mean $\pm$ SEM.

of the clonotypic autoantibody turnover, we prospectively tracked anti-Ro60peg clonotypes in two additional patients (pSS3 and pSS4). In patient pSS3, the clonotype changed three times, at approximately three-monthly intervals (Fig. 3A). In pSS4, consecutive samples over 3 months indicated a stable clonotype over this period (Fig. 3B). These data indicate clonal turnover with a period of no less than 3 months. Clinical examinations of patients by a single clinician at each blood sample point revealed no overt change in their sicca symptoms, although objective tests of salivary gland function were not performed.

3.4. Anti-Ro60peg levels fluctuate over time without change in affinity

We then measured anti-Ro60peg levels and affinities from samples collected retrospectively and prospectively over months to years to study the kinetics of autoantibody production. Autoantibody levels in all patients fluctuated markedly over time, despite minimal change in affinity, consistent with an affinity ceiling in the face of sequential clonotypic turnover (Fig. 4). The more detailed kinetic study of a single clonotype in pSS4 revealed a rapid rise in level over 1 week, followed by a steady decline over the following 11 weeks (Fig. 4D), consistent with a brief period of

monoclonal anti-Ro60peg production, followed by gradual autoantibody clearance.

4. Discussion

This study addresses the mechanism of long-term anti-Ro60 autoantibody production in patients with primary SS by direct sequencing of a secreted public clonotype. Autoantibodies directed against RNA–protein complexes, including anti-Ro60, are generally resistant to immunosuppressive and anti-CD20 agents and persist for the life of the patient, leading to the suggestion that these autoantibodies are produced by long-lived plasma cells [15,16]. Here, we show that humoral responses against an immunodominant determinant on Ro60 are maintained over years by a periodic turnover of short-lived V_H3-23/V_K3-20 clonotypic variants, each of which is specified by a unique V-region mutational signature. Conventional immunoassays such as ELISA that detect only antibody binding, fail to identify this perpetual yet subtle clonal turnover, whereas sequential clonotypes are revealed here for the first time by using mass spectrometric sequencing of the secreted autoantibody proteome. These findings seem incompatible with a model of long-lived autoreactive plasma cells that secrete stable Ro60-specific clonotypes, but rather infer cycles of clonal

A pSS1

	CDR1	CDR2	CDR3	
L Chain	10	20	30	40
V _κ 3-20	EIVLTQSPGTL	SLSPGERATL	SCRAS	QSVSSSY
Feb 2002 (A ₁)	P	..L..D.	R..	L.....R
Apr 2002 (A ₁)	P	..L..D.	R..	L.....R
Nov 2002 (B ₁)	P	..DN	R..	L.....R
Oct 2005 (C ₁)	P	..LN.D	R..	L.....R
Aug 2006 (D ₁)	A	..N.N	L..	L.....R

	CDR1	CDR2	CDR3	
H Chain	10	20	30	40
V _α 3-23	EVQLLESGGGLV	QPGGSLRLS	CAAS	GFTFSSYA
Feb 2002 (A ₁)	R	T	R	H..
Apr 2002 (A ₁)	R	T	R	H..
Nov 2002 (B ₁)	H	A..DHY	D..	LTG
Oct 2005 (C ₁)	LGN	K	L	QNDNA
Aug 2006 (D ₁)	N	S	R	V

B pSS2

	CDR1	CDR2	CDR3	
L Chain	10	20	30	40
V _κ 3-20	EIVLTQSPGTL	SLSPGERATL	SCRAS	QSVSSSY
Sep 2003 (A ₂)	N	R	R	S
Jan 2004 (B ₂)	N	R	R	S
Jan 2005 (C ₂)	NN	R	R	S
Apr 2006 (D ₂)	G..N	G	R	S
Feb 2010 (E ₂)	GP	P.K	M	R

	CDR1	CDR2	CDR3	
H Chain	10	20	30	40
V _α 3-23	EVQLLESGGGLV	QPGGSLRLS	CAAS	GFTFSSYA
Sep 2003 (A ₂)	P	L	K	HL
Jan 2004 (B ₂)	L.H	K	R	HL
Jan 2005 (C ₂)	P.D	T	RGS	HL
Apr 2006 (D ₂)	P	G	R	HL
Feb 2010 (E ₂)	A..L	N	T	R.P

Figure 2 Retrospective longitudinal analysis of Ro60peg-specific clonotypic autoantibodies from 2 patients. Serial samples were analyzed by Orbitrap mass spectrometry and de novo V-region sequencing. H- and L-chain sequences are aligned with germline V_κ3-20/J_κ2 and V_α3-23/J_α5, in which CDRs are underlined. (A) In pSS1, clonotype A₁ is unchanged over 3 months (Feb 2002 to April 2002), but a replacement clonotype has emerged by November 2002 (B₁; 7 month interval). Similarly, clonotypic replacement occurred by October 2005 (C₁) and August 2006 (D₁). (B) In pSS2, clonotype A₂ (September 2003) is replaced by January 2004 (B₂; 5 month interval). Replacement clonotypes are observed in January 2005 (C₂), April 2006 (D₂), and February 2010 (E₂). Dots represent homology with germline sequence. Amino acids divergent from germline are indicated as text with common mutations in red. Spaces indicate incomplete sequence.

expansion and exhaustion over days of short-lived plasma cells or plasmablasts.

We propose two potential pathways by which waves of short-lived clonotypic autoantibodies might be produced, both of which would depend on continuous stimulation by self antigen. First, short-lived plasmablasts may be generated by Toll-like receptor-driven activation of autoreactive B cells in extracellular foci, as shown experimentally for rheumatoid factor. T cells can have a facultative role in these reactions leading to isotype switch and somatic hypermutation of IgV regions [17]. Alternatively, new clonotypes might arise from expansion of short-lived plasma cell clones generated by repeated recall responses on a pool of Ro60-specific memory B cells that reside in inflamed salivary glands. Successive clonotypes do not appear to result from affinity maturation, since autoantibody affinities change little over years of disease. The selection pressures driving this dynamic process of clonal succession therefore

currently remain unknown. The Ro60 determinants responsible for the ongoing auto-immunogenic stimulation are thought to derive from disordered apoptosis, either as membrane-bound Ro60 on apoptotic bodies or as part of intact Ro/La ribonucleoprotein complexes released after secondary necrosis [18]. The unrelenting cycles of B-cell clonal expansion, production of Ro60-specific plasmablasts and clonal depletion, inferred from de novo sequencing of serial autoantibody proteomes, may contribute to autoimmune pathogenesis via multiple mechanisms, including the formation of anti-Ro/La IgG-immune complexes; presentation of Ro60 and/or somatically mutated IgV determinants on MHC class II with T-cell activation and epitope spreading; production of cytokines and type I interferons; and salivary gland inflammation [19–22].

An intriguing finding of this longitudinal study, in which the de novo mass spectrometric sequencing method can detect a second clonal variant to within a sensitivity of

A pSS3

	CDR1	CDR2	CDR3	
L Chain	10 20 30 40 50 60 70 80 90			
V ₃ -20	EIVLTQSPOTLSLSPGERATLSCRAS	QSVSSSY LANVQQKPGQAPRLIIY GAS	SRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVIYC QQYGSSP	J ₁ 2 YTFGQGTKEIK
Jul 2011 (A ₃)				
Sep 2011 (A ₃)				
Dec 2011 (B ₃)				
Mar 2012 (C ₃)				
Apr 2012 (C ₃)				
Jun 2012 (D ₃)				

	CDR1	CDR2	CDR3	
H Chain	10 20 30 40 50 60 70 80 90			
V ₃ -23	EVQLLESGGGLVQPGGSLRLSCAAS	GFTFSSYA MSWVRQAPGKLEWVSA	ISGSGGST YYADSVKGRFTISRDNKNTLYLQMNSLAEDTAVIYC AK	J ₁ 5 NWFDSSWGQGTLTIVSS
Jul 2011 (A ₃)				
Sep 2011 (A ₃)				
Dec 2011 (B ₃)				
Mar 2012 (C ₃)				
Apr 2012 (C ₃)				
Jun 2012 (D ₃)				

B pSS4

	CDR1	CDR2	CDR3	
L Chain	10 20 30 40 50 60 70 80 90			
V ₃ -20	EIVLTQSPOTLSLSPGERATLSCRAS	QSVSSSY LANVQQKPGQAPRLIIY GAS	SRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVIYC QQYGSSP	J ₁ 2 YTFGQGTKEIK
07th Jul 2011 (A ₄)				
14th Jul 2011 (A ₄)				
04th Aug 2011 (A ₄)				
07th Oct 2011 (A ₄)				

	CDR1	CDR2	CDR3	
H Chain	10 20 30 40 50 60 70 80 90			
V ₃ -23	EVQLLESGGGLVQPGGSLRLSCAAS	GFTFSSYA MSWVRQAPGKLEWVSA	ISGSGGST YYADSVKGRFTISRDNKNTLYLQMNSLAEDTAVIYC AK	J ₁ 5 NWFDSSWGQGTLTIVSS
07th Jul 2011 (A ₄)				
14th Jul 2011 (A ₄)				
04th Aug 2011 (A ₄)				
07th Oct 2011 (A ₄)				

Figure 3 Prospective longitudinal analysis of Ro60peg-specific clonotypic autoantibodies from 2 patients. Serial samples were analyzed and are presented as in Fig. 1. (A) In pSS3, clonotype A₃ is unchanged over 3 months (July 2011 to September 2011, but clonotypic replacement occurs by December 2011 (B₃; 6 month interval), and again by March 2012 (C₃; 3 month interval). This clonotype remains for at least 6 weeks (March to April 2012), and is then replaced by June 2012 (D₃; 3 month interval). (B) In pSS4, serial samples over 12 weeks demonstrate an unchanged clonotype.

detection of 0.05% of the dominant clonotype [14], was the presence at each serum sample point of a single anti-Ro60peg clonotypic variant, as specified by its unique IgV-region mutational signature. Thus in patient pSS3 clonotypes were completely replaced every three months, implying enhanced clearance of clonotypic IgGs (half-life approximately 9 days) compared to normal serum IgG (half-life approximately 21 days). Overall, the proteomic data are consistent with secretion of repeated short-lived waves of Ro60peg-specific monoclonal IgGs that are cleared rapidly from tissues and circulation following binding to cognate Ro60 protein exposed on apoptotic and necrotic debris. The disappearance of serum clonotypes is consistent with efficient removal or suppression of their putative feeder B-cell clones; such feedback auto-regulation may conceivably come from the secreted clonotypic autoantibodies themselves in the form of IgG immune complexes that co-ligate inhibitory Fcγ receptors on Ro60-specific B-cells [23,24].

It will be important to determine whether our findings for the Ro60peg clonotype extend to other epitopes on Ro60 and/

or to other ENA targets of life-long humoral autoimmunity. We have recently reported that secreted human Ro52 autoantibody proteomes express a restricted set of public clonotypes [14]. Since anti-Ro52/Ro60 responses are tightly linked, it is likely that Ro52-specific clonotypes undergo a similar pattern of clonal turnover. The continual changes in V-region signatures observed in serial studies may limit the diagnostic utility of single V-region peptide sequences in detecting serum clonotypic species by targeted mass spectrometry. Moreover, patient-specific idiotypic vaccines based on a single clonotype are unlikely to be effective in the face of ongoing somatic mutation. Accordingly, efforts to reduce circulating pathogenic anti-Ro autoantibodies might better be directed towards reducing ongoing exposure of the immune system to immunogenic Ro determinants, perhaps by modulating apoptotic cell turnover in patients with systemic autoimmunity. An alternative way of breaking the cycles of autoantibody production might involve blocking specific B cell access to exposed Ro60 protein by using synthetic peptides designed to bind and mask immunodominant epitopes.

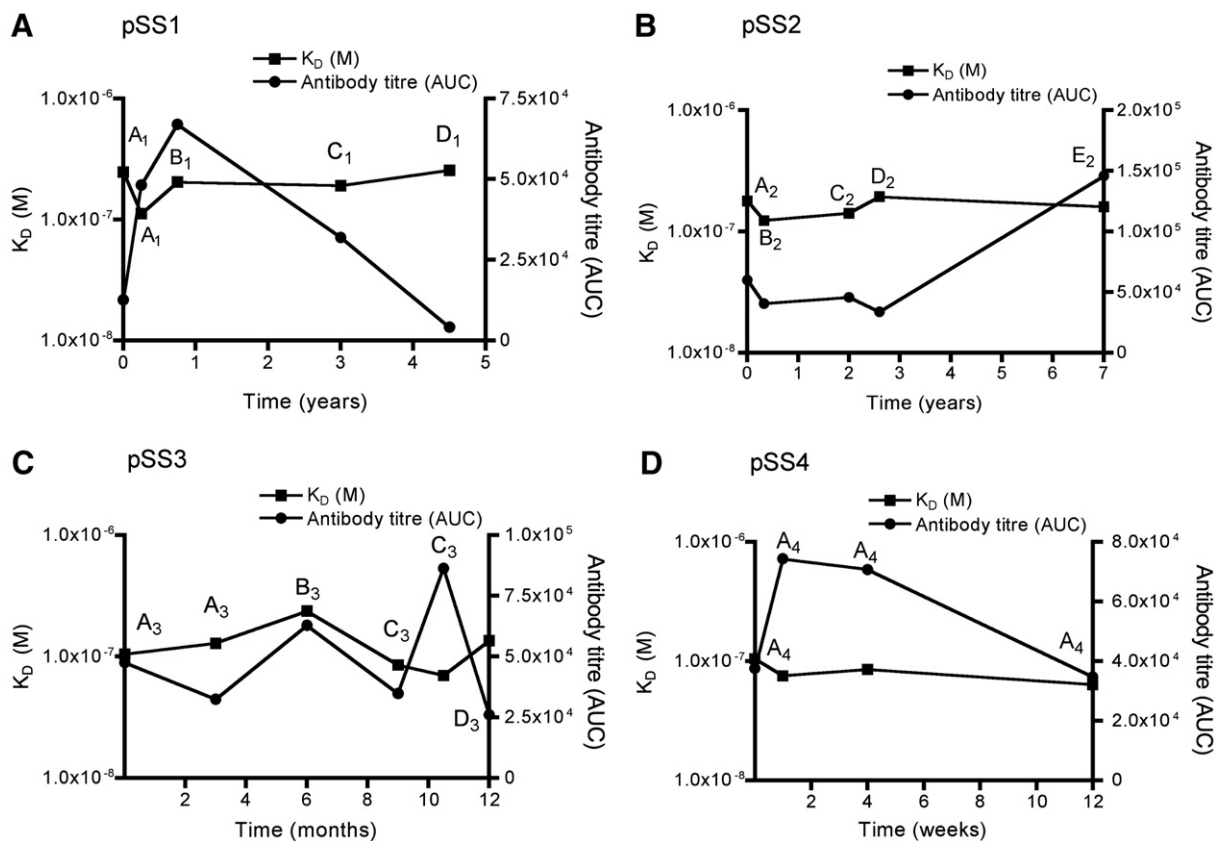


Figure 4 Comparison of Ro60peg-specific clonotypic autoantibody levels and affinities over time in patients pSS1–4. Autoantibody levels determined by area under curve (AUC) of Ro60peg ELISA, and binding affinities (equilibrium dissociation constants, K_D) by Biacore X100. Clonotypes denoted by A_x , B_x , C_x , D_x , and E_x . In pSS1 and pSS2, autoantibody levels fluctuate markedly over years. In pSS3, levels rise and fall a number of times in 12 months. In pSS4, autoantibody level rises over 1 week, and falls over 11 weeks. There is minimal change in affinity in all patients over time.

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