EXTENDED REPORT

Single cell cloning and recombinant monoclonal antibodies generation from RA synovial B cells reveal frequent targeting of citrullinated histones of NETs

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ABSTRACT

Objectives Rheumatoid arthritis (RA) is characterised by breach of self-tolerance towards citrullinated antigens with generation of anti-citrullinated peptide/proteins antibodies (ACPA). Currently, the nature and source of citrullinated antigens driving the humoral immune response within synovial ectopic lymphoid structures (ELS) is a crucial unknown aspect of RA pathogenesis. Here we characterised the autoreactive B-cell response of lesional B cells isolated from ELS+RA synovium.

Methods Single synovial tissue CD19+ cells were Fluorescence Activated Cell Sorting (FACS)-sorted and Valti/Vj Ig genes cloned to generate recombinant monoclonal antibodies (mAbs) from patients with ELS +/ACPA+ RA.

Results RA-mAbs immunoreactivity analysis provided the following key findings: (1) in a chIP-based array containing 300 autoantigens and in a ‘citrulline’ multiplex assay, a strong reactivity against citrullinated histones H2A/H2B (citrH2A/H2B) in ~40% of RA-mAbs, followed by cit-fibrinogen and cit-vimentin; (2) anti-citrH2A/H2B-reactive RA-mAbs (but not anti-citrH2A/H2B negative) selectively recognised neutrophil extracellular traps (NETs) from peripheral blood and/or RA joint neutrophils; (3) anti-citrH2A/citH2B and anti-NET immunobinding was dependent on affinity maturation and was completely abrogated following reversion of hypermutated Valti/Vj genes to germline sequences; (4) ELS+ (not ELS−) RA synovial tissues engrafted into Severe Combined Immunodeficiency (SCID) mice released human anti-citrH2A/citH2B and anti-NET antibodies in association with the intra-graft expression of CXCL13 and lymphotokxin (LT)-β, two major regulators of ELS.

Conclusion We provided novel evidence that B cells differentiated within synovial ELS in the RA joints frequent target deaminated proteins which could be generated during NETosis of RA synovial neutrophils including histones. Thus, NETs could represent a source of citrullinated antigens fuelling the ACPA autoimmune response within the RA synovium.

INTRODUCTION

Rheumatoid arthritis (RA) is characterised by breach of self-tolerance towards citrullinated proteins (anti-citrullinated peptide/proteins antibodies (ACPA)), which can occur years prior to clinical onset of RA at extra-articular sites. Several posttranslationally deaminated proteins have been indicated as a potential source of citrullinated antigens in the RA joints, but to date their cellular source and specific contribution to the lesional ACPA response is unknown.

Around 40%–50% of patients with RA display synovial ectopic lymphoid structures (ELS) characterised by B-cell follicles supporting a germinal centre (GC) response. Synovial ELS are self-sustained niches whereby autoreactive B cells undergo antigen-driven selection/differentiation with local antibody diversification through Ig genes somatic hypermutation (SHM) and class switching.

Citrullination, or arginine deimination, is catalysed by the enzyme peptidyl-arginine-deiminase (PAD). In the RA synovium, monocyte–macrophages are the main source of this enzyme. As a result, citrullination of fibrinogen, vimentin and α-enolase, among others, has been observed within the RA joints and associated with circulating ACPA. Accordingly, monoclonal antibodies generated from synovial fluid B cells frequently react against citrullinated antigens.

PAD-mediated deamination of core histones (H2A/H2B/H3/H4) has been described in neutrophils during the neutrophil extracellular traps (NETs) formation, or NETosis, a form of cell death which enhances the antimicrobial properties of activated neutrophils. Interestingly, RA synovial fluid neutrophils display an enhanced NETosis in the absence of microbial stimuli due to the RA proinflammatory milieu and RA sera react against citrullinated H4 from NETs.

At present, direct evidence that synovial B cells from ELS+RA recognise citrullinated proteins and the specific contribution of different citrullinated antigens in fuelling the lesional ACPA production is missing. To this aim, we investigated the immunoreactivity of recombinant monoclonal antibodies (mAbs) generated from single synovial B-cell clones obtained from patients with ELS+/ACPA+ RA.

MATERIALS AND METHODS

A full list of methods is reported in the online supplementary methods.

Patients

Three synovial tissues from total joint replacement were obtained after informed consent
Synovial mononuclear cell isolation and CD19+ cell FACS sorting
Mononuclear cells were isolated from fresh synovial tissue specimens as obtained above. Detailed method is reported in the online supplementary methods.

Generation of recombinant monoclonal antibodies
Single-cell real time-PCR reactions and IgV gene amplification were performed as described in refs. 21 and 22. Briefly, cDNA from CD3-CD19+B cells was amplified using reverse primers that bind the Cµ/Cγ or Cα constant region in three independent nested-PCR. The complete sequence of primers is reported in online supplementary table S1. Aliquots of Variable Heavy (VH)/ Vs/VL chains second PCR products were sequenced with the respective reverse primer and analysed by IgBlot. IgH complement determining region (CDR3) amino acids and length were determined as described.23 The V gene somatic mutations analysis was performed using IMmunoGeneTics/Variable (IMGT)/V)-QUeRy and STAndardization (QUEST) to characterise the silent versus non-silent mutation in each Framework Region (FR)/CDR region to determine the R:S ratio. The expression vector cloning strategy and the monoclonal antibody production were performed as described in ref. 21. Immunoglobulin Analysis Tool (IgAT) software was used to calculate the probability of antigen-driven selection within the Ig repertoire of the RA-rmAbs,25 as previously described.22

Multiplex autoantibody assay
The multiplex autoantibodies assay containing 20 citrullinated RA-associated antigens (see online supplementary table S2) was performed as previously published.5 Briefly, rmAbs were added at 10 μg/mL to custom Bio-Plex beads associated with RA putative autoantigens and incubated at room temperature (RT) for 1 h. After washing, PhycoErythrin (PE)-anti-human-IgG antibody was added to the beads and incubated at RT. The fluorescence of PE detected (Luminex200;Bio-Plex-Software V.5.0, Bio-Rad) reflects the amount of antibodies that bind to the beads.

ELISA assay for anti-citrullinated H2A and H2B
ELISA plates were coated with citrullinated or unmodified histones H2A or H2B at 10 μg/mL in Phosphate Buffered Saline (PBS). Samples at 10 μg/mL were transferred into the ELISA plate and incubated for 2 h (SCID serum diluted 1:10). Unbound antibodies were removed by washing before incubation for 1 h with horseradish peroxidase coupled goat-anti-human-IgG. Assays were developed using TetraMethylBenzidine (TMB) Substrate Reagent Set (Becton Dickinson Optical Enzyme ImmunoAssay (BDOptEIA)). Optical densities (ODs) were measured at 450 nm.

Stimulation of NETosis and immunofluorescence microscopy on NETs
Neutrophils were isolated from peripheral blood (PB) of healthy donors or synovial fluid of two patients with RA using discontinuous gradient centrifugation according to ref. 24 and seeded onto cell culture cover slides at 2×10⁵ cells/well. Cells were either fixed with 4% paraformaldehyde or before fixation activated with 100 nM Phorbol Myristate Acetate (PMA) for 4 h at 37°C. NETs were stained with RA-rmAbs or Sjögren’s syndrome (SS) control rmAbs diluted in PBS for 1 h (RT). After washing with Tris Buffered Saline (TBS), Alexa488-goat-anti-human-IgG (Invitrogen, 1:200) was added for 30 min (RT). 4’, 6-DiAmmidino-2-PhenylIndole (DAPI) (Invitrogen) was added to visualise the NETs. All sections were visualised using an Olympus BX60 microscope. All rmAbs have been tested at 10 μg/mL.

RA synovial tissue transplantation into SCID mice
RA SCID chimaeras were established as previously described.10 Sera and synovial grafts were harvested and analysed for auto-antibodies and gene expression profiling, respectively, as previously reported.10 Detailed method is reported in the online supplementary methods.

Statistical analysis
Differences in quantitative variables were analysed by the Mann–Whitney U test (two groups) and by the Kruskal–Wallis with Dunn’s post-test (multiple groups). χ² test with Yates’s correction or Fisher’s exact test when required were used to evaluate associations of qualitative variables in the different groups. All the statistical analyses were performed using GraphPad-Prism5.01. A p value <0.05 was considered statistically significant. For array reactivity, the Significance Analysis of Microarrays algorithm to normalise array median fluorescence intensity values was applied.

RESULTS
Ig gene analysis demonstrates intra-synovial antigen-driven B-cell maturation and clonal diversification in ELS +RA synovium
We sorted single CD19+B cells from synovial cell suspension obtained from three joint replacements of patients with ELS +/ACPA+RA (figure 1A, B). Sequence analysis of different VH/JH regions (n=139) and VH regions (Vκ=94;Vλ=81) demonstrated that the Vµ/Vλ gene repertoire of the synovial B cells was similar to PB CD5-IgM+B cells of healthy donors9 25 (figure 1E–G). We observed statistically different JH gene usage with JH2/JH3 and JH5 over-represented in the IgM and IgG synovial B cells, respectively, and JH4 less frequent in the IgG compartment (figure 1E). Generally, we observed a similar frequency in the distribution of IgM (33%), IgG (40%) and IgA (27%) isotypes across all sequences analysed (figure 1C, D). IgG and IgA synovial B-cell clones showed significantly higher number of SHM in their VH region compared with IgM, ~50% of which displayed germline sequences (see figure 2A and online supplementary figure S1A); additionally, SHM in Variable Light (Vλ) was higher in than λ chains (figure 2B). Switched B-cell clones also showed (1) high ratios of replacement (R) to silent (S) mutations in CDR1-2 compared with the FR1-3 regions (see figure 2C and online supplementary figure S1B), (2) a shorter CDR3 length compared with unswitched unmutated IgM +clones (figure 2D) and (3) a higher frequency of positively charged aa, which have been shown to be frequently used by autoreactive B cells6 (figure 2E). We estimated that between 24% and 30% of the sequences (probability 0.05 and 0.1, respectively) from the synovial B cells display evidence of antigen selection (see online supplementary figure S2). Full antibodies characteristics are provided in online supplementary table S4. Clonal relationship analysis of the VH gene sequences of RA...
synovial B cells showed evidence of intra-synovial diversification, as previously reported9 (figure 2f).

RA synovial rmAbs display frequent immunoreactivity towards citrullinated histones

To investigate the autoantigenic immunoreactivity of the synovial B-cell clones, we cloned matching VH+VL Ig genes from individual B cells into specific expression vectors and produced 66 in vitro whole rmAbs as complete IgG1 displaying identical VH+VL pairing and specificity of the parental B cells. Sufficient yield (>5 μg/mL) was obtained from 59 rmAbs (RA015/11=12; RA056/11=26; RA057/11=21), which were used for downstream analysis. First, we screened the RA-rmAbs in a synovial autoantigen microarray platform27 and in a multiplex RA-associated citrullinated antigen assay. Strikingly, RA-rmAbs (~40%) showed strong immunoreactivity towards citrullinated histones H2A (citH2A) and H2B by multiplex assay (figure 3A) with reactivity to histones H2A and H2B also frequently observed in the protein array heatmap (see online supplementary figure S3). Quantitative analysis confirmed that the strongest reactivity was directed against citH2A and citH2B followed by citrullinated vimentin and fibrinogen (figure 3B). Additionally, 5 rmAbs displayed binding to different citrullinated antigens, highlighting the existence of clones with multiple citrullinated reactivity. In particular, reactivity to citrullinated vimentin was demonstrated by ELISA in three RA-rmAbs (see online supplementary figure S4).

Overall, 41% (24/59) and 34% (20/59) of the RA-rmAbs were above the threshold of citH2A and citH2B reactivity, respectively (figure 3C). Such reactivity was confirmed to be disease-specific as it was not detectable in 30 control rmAbs from circulating naïve and memory B-cells of five patients with ANA+/ENA+ SS (see online supplementary figure S5). To confirm the importance of citrullination, we tested the RA-rmAbs towards the native versus citrullinated form of H2A and H2B by ELISA. A significant increase was detected in the binding to citH2A/H2B compared with native H2A/H2B histones in a large proportion of rmAbs from ELS+ACPA+RA synovial B cells but not in either

Figure 1  Histological characterisation of synovial ectopic lymphoid structures (ELS), single synovial CD19+ cell sorting and VH/VL Ig gene analysis.

(A) Representative immunohistological characterisation of synovial tissue samples from patients with rheumatoid arthritis (RA) used in this study (RA015/11, RA056/11 and RA057/11). To assess the presence of ELS, sequential paraffin tissue sections were stained for CD20 (B cells, left-panel), CD3 (T cells, central-panel) and CD138 (plasma cells, right-panel), respectively. (B) Isolation strategy of single CD19+ RA synovial B cells is shown. (C) The frequencies of VH, γ and α heavy chain among all CD19+ B cells for which VH sequences were obtained are shown. (D) Frequencies of VH, γ and α heavy chain among all CD19+ B cells for each synovial tissue are shown. (E) The VH and JH gene repertoire of single CD19+ synovial B cells for each individual chain type, μ (white), α (grey) and γ (black) is shown. (F) The Vκ and (G) Vλ gene repertoire are shown. The red bars indicate the VH+L gene repertoire of peripheral blood-naive B cells. The absolute number of sequences analysed is reported over each graph. Error bars in bar graph indicate SEM for individual patients. p Values compare data from RA synovial B cells with peripheral blood-naive B cells; *p<0.05; **p<0.001.
naïve or memory SS B cells or flu control rmAb (figure 3D, E). Interestingly, using as substrate synthetic citrullinated H2A peptides spanning the whole histone H2A length, we showed that different antibodies each recognised different citrullinated H2A epitopes (figure 3F), suggesting the occurrence of in situ ‘epitope spreading’. The immunoreactivity observed against citrullinated...
histones or multiple citrullinated antigens was not due to polyreactivity, a phenomenon frequently observed in rmAbs generated from naïve B cells as only 1/59 clones displayed polyreactivity against multiple structurally unrelated antigens (see online supplementary figure S6).

RA synovial rmAbs with cit-histones specificity bind neutrophils NETs

We next investigated the biological significance of the observed immunoreactivity towards citrullinated histones. First, we showed that the RA SF-neutrophils spontaneously undergo NETosis, as previously described (figure 4D, top). We assessed whether synovial RA-rmAbs reactive against citrullinated histones could specifically target NETs. A large proportion of RA-rmAbs displayed strong binding to NETs generated from either PB-neutrophils of healthy donors (figure 4A) or from RA SF-neutrophils (figure 4D) with 33%, 42% and 19% of the total synovial antibody response of patients RA015/11, RA056/11 and RA057/11, directed against NETs, respectively (figure 4E). Conversely, none of the control SS-rmAbs displayed anti-NET reactivity (figure 4B–D). Of relevance, the immunoreactivity of the RA-rmAbs was restricted to neutrophils undergoing NETosis with negligible or no binding to the nucleus of resting neutrophils.

Of relevance, the reactivity towards NETs was strongly associated with the level of immunobinding to citrullinated histones in the multiplex assay (see multiplex tiles in figure 4C) and in ELISA (figure 4F).

Synovial B cells anti-NET immunoreactivity is dependent on SHM and is lost after reversion to germline

A progressive increase in the mutational load within the VH Ig genes was associated with higher reactivity to citrullinated histones in all the isotypes obtained, with the strongest difference observed in IgG-switched B-cell clones (figure 5A). Therefore, to address the importance of affinity maturation and clonal
diversification via SHM in the anti-NET reactivity, we reverted selected highly mutated RA-rmAbs with strong NETs reactivity to the corresponding VH+VL Ig germline sequences by overlapping PCR. The germline RA-rmAbs invariably lost their reactivity towards NETs (Figure 5B). These data demonstrate that intra-synovial antigen-driven SHM is required for the immunoreactivity of RA synovial B-cell clones to NET-associated autoantigens.

ELS+RA synovia sustain anti-NET and anti-citrullinated histone antibodies in vivo in the Hu-RA/SCID chimeric mouse model

Finally, we investigated whether the anti-NET and anti-citH2A/H2B reactivity of the RA-rmAbs were reproduced in whole RA synovial tissue obtained from the same joints. For this purpose we used an in vivo chimeric human RA/SCID mouse transplantation model (Figure 6A). As we had previously shown, RA synovial ELS were self-maintained for several weeks in the absence of recirculating immune cells (Figure 6B) and released IgG-ACPA autoantibodies (measured as total anti-CCP-IgG, not shown). Mouse sera from mice transplanted with RA015/11 or RA056/11 synovial grafts contained autoreactive human anti-NET IgG (Figure 6C) and/or anti-citH2A/citH2B histones antibodies (Figure 6D). Additionally, in the synovial grafts of mice producing anti-citrullinated histones/NETs we observed significantly higher binding in anti-NET+ versus anti-NET− clones.
A common feature of RA is the formation of discrete clusters of infiltrating lymphomononuclear cells forming lymphoid aggregates or ELS. These structures resembling secondary lymphoid organs sustain a functional ectopic-GC response and support B-cell differentiation into high affinity autoantibody-producing cells. ELS formation is dependent on the activation of the LT-β/lymphoid chemokines pathway in ectopic sites and is driven by chronic antigenic stimulation. As such, ELS (also observed in non-autoimmune conditions such as chronic infections, allograft rejections and cancer) are unique in their ability to mount disease-specific and antigen-specific immune responses. Indeed, ectopic-GCs produce antibodies against citrullinated proteins in RA, ribonucleoproteins Ro/La in SS, thyroglobulin and thyroperoxidase in Hashimoto’s thyroiditis and acetylcholine receptor in myasthenia gravis.

Here, we exploited these unique features of ectopic-GC to unravel the nature and source of the citrullinated antigens driving adaptive immune responses in the RA joints generating full rmAbs from the joints of patients with ACPA+RA containing functional ectopic-GCs. As control, due to the absence of a direct comparator as ELS synovitis do not harbour B cells for cloning, we used rmAbs generated from circulating naïve and Ag-experienced memory B cells of patients with SS to exclude that any observed reactivity was due to non-specific autoimmunity, as circulating SS B cells are frequently autoreactive. We observed a high prevalence of IgA+ B cells in the RA joints in line with previous evidence that FDC-like synovial stromal cells directly induce IgA class-switch via a B cell Activating Factor of the TNF Family (BAFF) and APRoLiferation Inducing Ligand (APRIL)-dependent pathway. Importantly, we demonstrated that around 40% of RA synovial B cells from ectopic-GCs displayed reactivity against citrullinated antigens. Although, we observed frequent cross-citrullinated reactivity, using a multiplex ‘citrullinome’ assay, we showed that the strongest reactivity of ACPA-producing B cells was directed towards citrullinated histones, mainly H2A and H2B, followed by cit-vimentin and cit-brinogen. The data on histone reactivity were further confirmed (1) in vitro using several immunoenzymatic methods and cell-based NETs co-localisation experiments and (2) in vivo by engrafting ELS+ synovial tissues into SCID mice where we showed that the synovial production of anti-citH2A/citH2B and anti-NET autoantibodies is critically dependent on ELS formation and is sustained by high levels of CXCL13 and LT-β.

Figure 5 Immunoreactivity towards neutrophil extracellular traps (NETs) is dependent on somatic hypermutation. (A) Subanalysis of the anti-citH2A (top) and citH2B (bottom) histone reactivity in ELISA according to the number of somatic mutations in the VH regions of IgM (left), IgG (central) and IgA (right) clones demonstrates progressive increase in immunoreactivity according to the mutational load in all isotypes. (B) Reversal to germline (GL) sequences by overlapping PCR in representative individual anti-NET+ rheumatoid arthritis (RA) recombinant monoclonal antibody (rmAb) invariably abrogated the binding to NETs. The family usage, CDR3 sequence and the total number of somatic mutations in the FR and CDR regions of VH and VL Ig genes prior to reversal to GL sequences is shown beside each IF staining; *p<0.05; **p<0.01.
suggest that cit-histones are critically targeted by synovial-derived ACPA, further work dissecting the fine specificities of the rmAbs towards multiple citrullinated antigens is warranted, also considering that the binding of ACPA can be greatly influenced by immunodominant epitope density and citrullination protocols in different assays.

Histone citrullination is a critical step in the formation of NETs released from neutrophils in response to infectious or inflammatory stimuli to trap and kill pathogens. **19** Approximately 70% of all NETs proteins are histones in which arginine residues are deiminated to citrulline. Thus, NETosis allows the extracellular exposure of heavily citrullinated histones and, to a much lesser degree, also of other putative RA autoantigens such as citrullinated vimentin **19** which can become the target of the autoimmune ACPA response. Interestingly, the reactivity towards citrullinated vimentin, which was relatively low in our antibodies, was recently described in work using rmAbs derived from synovial fluid B cells, which however did not investigate the reactivity towards citrullinated histones and NETs. **16**

The RA-rmAbs reactive towards citH2A/H2B also strongly and specifically recognised NETs in cell-based immunoassays using either RA-synovial fluid or circulating activated neutrophils as substrate. These data are highly relevant to RA pathophysiology since recent observations showed that (1) RA-synovial fluid neutrophils undergo aberrant NETosis and release citrullinated antigens **19** and (2) patients with ACPA+RA sera recognise citrullinated histones from NETs. **2**

Importantly, the RA-rmAbs anti-NET immunoreactivity was entirely dependent on SHM in the IgVH genes since it was completely abrogated when the IgVH+VL genes were reverted into germline sequences. These data demonstrate that the anti-cit-histones/NET immunoreactivity is largely acquired within the synovial microenvironment on affinity maturation and intra-synovial diversification within ectopic-GC. Because these processes in B cells are largely dependent on cognate T-cell help, it is conceivable that Th cells with TCRs specific for processed NET peptides may be present in the RA synovium and engaged by antigen-presenting cells loading citrullinated NETs antigens. In this regard, recent evidence suggests that some NET
proteins (eg, LL37, HMGB1) promote the activation of professional antigen-presenting cells by facilitating antigen uptake, interaction with endosomal Toll Like Receptor (TLR) and the release of type-I Interferon (IFN) and other proinflammatory cytokines as in the case of plasmacytid DCs in SLE.\(^{42, 43}\) Similarly, in autoimmune vasculitis, NETs are highly immunogenic and mediate the transfer of cytoplasmic neutrophil antigens to myeloid dendritic cells, favoring the formation of anti-neutrophil cytoplasmic antibodies.\(^{44}\)

The clearance of NETs by macrophages is an immunologically silent process. However, in an inflammatory environment, the uptake of NETs can promote proinflammatory cytokines including interleukin (IL)-1β, IL-6, and TNF-α,\(^{45}\) which are critical in RA. Their processing followed by presentation of peptide antigens could elicit an adaptive immune response against citrullinated histones within GC in the RA joints, as also supported by long-standing evidence that synovial RA macrophages actively engulf neutrophil nuclear fragments.\(^{46}\) Although our previous work suggests that NETosis is an important source of citrullinated proteins in the joints, several alternative/complementary mechanisms also likely contribute to the generation of citrullinated antigens and ACPA in RA both within and outside the joints. Environmental factors such as bacterial infection in periodontal disease (ie, Porphyromonas gingivalis)\(^{47, 48}\) and smoking\(^{49}\) can lead to the formation of citrullinated antigens at mucosal sites which, in susceptible individuals, can lead to the production of ACPA prior to the clinical onset.\(^{4}\) Within the RA joints, the activation of PADs by macrophages in the RA synovium can bring to the citrullination of intracellular and extracellular proteins such as vimentin and fibrin.\(^{11-13}\) Based on our data, we propose that NETosis is an additional critical source of citrullinated antigens within the synovium, whereby normally sequestered citrullinated antigens within neutrophils, such as core histones, can be hypercitrullinated, exposed and be targeted by the immune system within the RA joints. Although a formal mechanistic demonstration is still necessary, we propose that the local release of citrullinated histones contribute to fuel the antigen-driven generation of highly mutated B-cell clones within synovial ELs resulting in the generation of high-affinity ACPA displaying anti-NET reactivity (see online supplementary figure S8).

A critical question which remains to be elucidated is whether anti-NET antibodies generated in RA ELS are pathogenic and contribute to chronic inflammation over and above ACPA generated in the peripheral compartment, as the pathogenic role of ACPA remains controversial.\(^{50, 51}\) Additionally, it is unclear whether anti-NETs are generated in patients with early RA which display a similar prevalence of ELS+ synovitis compared with established RA.\(^{52, 53}\) The combination of next-generation sequencing followed by single cell cloning and recombinant antibody production together with the availability of ultrasound-guided synovial biopsies could help in clarifying this aspect.\(^{52, 53}\)

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Supplementary Materials and Methods

Synovial mononuclear cell isolation and CD19+ cell FACS sorting

The synovial tissue was cut into small pieces and enzymatically digested with collagenase D (100 mg/ml) and DNase I (10 mg/ml) at 37 °C for 1 hour. 0.5 M EDTA was added to stop the reaction. The samples were then filtered through 40-µm cell strainer to remove undigested tissue. Cells viability was determined by Trypan blue exclusion test. For flow cytometry cells were stained with PerCP-Cy5.5-anti-human CD19 (clone-SJ25C1; BD Biosciences) and FITC-anti-human CD3 (clone-HIT3a; eBioscience). Flow cytometric analysis and sorting was performed with a FACSaria flow cytometer (Becton Dickinson). Single CD19+ cells were sorted directly into 96-well plates, as previously described [21].

Synovial tissue histological characterization of lymphocytic aggregates

Sequential paraffin-embedded 3µm sections of synovial tissue were stained for the markers CD3 (1:80 dilution; DAKO), CD20 (1:50 dilution; DAKO) and CD138 (1:50 dilution; DAKO) following routine H&E staining to classify the lymphocytic infiltration as aggregate or diffuse, as previously reported [10].

Synovial antigen microarray profiling

The synovial antigen microarray production, probing and scanning protocol has been previously described [27]. Briefly, arrays were probed with 10 µg/ml rmAbs for 1 hour at 4°C followed by washing and incubation with Cy3-conjugated goat anti-human secondary antibody. The arrays were scanned using a GenePix 4400A scanner and the net mean pixel intensities of each feature were determined using GenePix Pro 7.0 software. The net median pixel intensity of each feature above the background was used.
Arginine deimination of histone H2A and H2B

Histones H2A and H2B purified from bovine thymus tissue were incubated at 1 mg/ml with rabbit skeletal muscle PAD (7 U/mg) in 0.1 M Tris-HCl (pH 7.4), 10 mM CaCl₂, and 5 mM DTT for 2 h at 50°C. After incubation each histone was stored at -80°C in aliquots of 100 µl each.

ELISA for anti-citH2A peptides

ELISA plates were coated with citrullinated or peptides derived from H2A at 10 µg/ml in PBS and incubated over-night at 4°C. Samples at 10 µg/ml diluted in PBS, 0.5% Porcine gelatin, 0.05% Tween-20 were transferred into the ELISA plate and incubated for 2 hours at RT. Unbound antibodies were removed by washings before incubation for 2 hour RT with Alkaline-Phosphatase-coupled goat anti-human IgG. OD were measured at 405 nm.

ELISA for anti-mutated citrullinated vimentin (MCV)

Samples were tested for reactivity against mutated citrullinated vimentin (MCV) using commercially available ELISA kit (Orgentec anti-MCV). The ELISA test was performed according to the manufacturer’s instructions. Samples were added to the antigen coated plate and incubated 1 hour at RT at a concentration of 10 µg/ml. OD were measured at 450 nm.

Characterization of polyreactivity by ELISA

To test the reactivity against different allo- and auto-antigens, supernatants were tested for polyreactivity against double and single-stranded DNA (dsDNA and ssDNA), lipopolysaccharide (LPS) and insulin by ELISA as previously reported [21]. Antibodies that reacted against at least two structurally diverse self- and non-self-antigens were defined as polyreactive [21, 26]. Internal controls for polyreactivity were added on each plate consisting of the recombinant monoclonal antibodies mGO53 (negative), JB40 (low polyreactive), and ED38 (highly polyreactive) as previously reported [21].
Overlap-PCR to revert mutated IgH and IgL chain genes to germline sequence

Antibodies for reversion experiments are listed in Table S3 and included 5 synovial rmAbs with a strong reactivity in fluorescence microscopy on NETs. Mutated VH+VL regions were reverted into their germline (GL) counterpart sequence using a previously described overlap strategy [21]. This consisted of two (if J gene germline) or three (if J gene mutated) independent first PCR reactions followed by a nested overlapping PCR to join the amplicons generated with the first PCRs. As templates for the first reactions we used plasmids containing the rmAbs clone specific CDR3 regions and plasmids derived from naïve B-cells, containing the corresponding unmutated VH and VL genes (Fig.S7). PCRs were performed at 98 °C(1 min), 98 °C(20 sec), 56 °C(20 sec), 72 °C(20 sec), and 72 °C(2 min) for 32 cycles using the Q5-High-Fidelity DNA polymerase (NEB). Reverted IgH+IgL chain PCR products were sequenced to confirm the absence of mutations. GL antibodies were expressed and tested in fluorescence microscopy on NETs as described above. For the full list of primers used in the overlap PCRs see Table S3.

RA synovial tissue transplantation into SCID mice

Human arthroplasty synovium from the same 2 RA patients (RA015/11 and RA056/11) from which the monoclonal antibodies were generated was transplanted subcutaneously into Beige SCID-17 mice as previously described [10]. A total of 31 SCID mice were transplanted with synovial tissues from either patients. Four weeks post-transplantation animals were sacrificed and underwent terminal bleed. Serum was collected and stored at -20°C for subsequent analysis of human APCA, anti-NET and anti-citrullinated histone antibodies. Furthermore, at culling each synovial graft was harvested and divided into two parts; one part was paraffin embedded for later histological characterization and one part was stored in RNA-later at -80°C for quantitative real-time RT-PCR. All procedures were performed according to the Home Office regulations (PPL 70/7001).
Supplementary Figures

Figure S1: Single synovial CD19+ VH/VL Ig gene analysis for each synovial tissue.

(a) Frequencies of $\mu$, $\gamma$, and $\alpha$ heavy chain among all CD19+ B cells for each synovial tissue.

(b) Absolute numbers of somatic mutations in VH genes for IgM, IgG and IgA for each synovial tissue. (c) Frequency of replacement (R) and silent (S) mutation ratio in FR (white) and CDR (black) regions for IgM, IgG and IgA for each synovial tissue.
Figure S2: Interference of antigen selection in RA-rmAbs.

The graph shows the ratio of replacement mutations in CDR1 and CDR2 ($R_{CDR}$) to the total number of mutations in V region ($M_v$) plotted against $M_v$ for the RA-rmAbs. The dark and the light grey area indicate the 90% and 95% confidence limits for the probability of random mutations, respectively. A data point outside these areas represents a sequence that was antigen selected. The data were obtained using the Immunoglobulin Analysis Tool software [22].
Figure S3: Overall heatmap illustrating the binding of all RA synovial rmAbs in the autoantigen array.

RA-rmAbs antigen array reactivity. Heatmap tiles reflect the amount of IgG autoantibody binding reactivity based on the fluorescence intensity scale as indicated on the left bottom. Samples and antigens are shown in columns and rows, respectively. Blank and control flu antibody are reported on the left side of the heatmap.
Binding of the RA and control rmAbs (30 naïve and memory B-cell clones from SS patients) to mutated citrullinated vimentin (MCV) tested by ELISA. The dotted horizontal line represents the cut-off for positivity of the rmAbs which was determined as the mean+2SD of the reactivity of 30 SS control rmAbs (right panel).
Figure S5: Multiplex citrullinated antigen assay.
Luminex heatmap for control rmAbs derived from naïve and memory B-cells of SS patients. Heatmap tiles reflect the amount of IgG autoantibody binding reactivity based on the fluorescence intensity scale as indicated on the right top. Samples and synovial antigens are shown in columns and rows, respectively. ACPA negative and ACPA low, medium and high-reactive RA sera are included on the right hand side column. Blank and serum control beads are reported at the bottom of the heatmap.
Figure S6: Polyreactivity analysis of synovial rmAbs.
Synovial rmAbs were tested for reactivity with dsDNA (top left), ssDNA (top right), LPS (bottom left) and insulin (bottom right) by ELISA. Each graph shows the reactivity at a concentration of 1 μg/ml and it shows the result of two independent experiments. The cut-off OD (450 nm) at which antibodies were considered reactive is shown by the horizontal lines. Data points represent individual antibodies. Internal controls for polyreactivity are shown in each graph and include mGO53 (negative [26]), JB40 (low polyreactive [26]), and ED38 (highly polyreactive [26]).
Strategy to revert mutated Ig genes into their unmutated counterpart by (a) two (J gene germline) or (b) three (J gene mutated) separate PCRs for the V gene and the (D)J gene, followed by a final overlapping PCR to join the two PCR products.
Migration of neutrophils from the periphery followed by aberrant neutrophil NETosis during chronic inflammation within the RA compartment (synovial tissue and fluid, steps 1-2) provide a continuous source of externalised citrullinated antigens, such as citH2A, citH2B and citH4 histones that can be presented by antigen-presenting cells following engulfment of neutrophils nuclear fragments (step 3). Such process would sustain an antigen-driven autoimmune response towards citrullinated antigens within ELS in the RA joint (step 4) resulting in the production of high affinity ACPA displaying anti-NET reactivity (step 5) which may contribute to the perpetuation of chronic inflammation and autoimmunity.
Table S1. Complete list of primers used in this study

| 1° PCR HC | Forward 5’ to 3’ |
|-----------|-----------------|
| 5° L-VH 1 | ACAGGTGCCCACTCAGGAGTGCAG |
| 5° L-VH 3 | AAGGTGTCACGTGAGTGCAG |
| 5° L-VH 4/6 | CCCGATGAGTTCCTGAGGAGTGCAG |
| 5° L-VH 5 | CAAGGATCTGTCAGGAGTGCAG |

| Reverse 3’ to 5’ |
|-----------------|
| 3° Cµ CH1 ext | GGAAGAAGTTCCTGGCATGAGGC |
| 3° Cy CH1 ext | GGAAGAAGTTCGCCAGCCTGCAGTGC |
| 3° Cµ CH1 ext | TGGAAGAAGTTCCTGGCATGAGGC |

| 2° PCR HC | Forward 5’ to 3’ |
|-----------|-----------------|
| 5° Agel VH1 | CTGCCAAGGGTAGATCCTCTCGGAGTGCAG |
| 5° Agel VH1/5 | CTGCCAAGGTTGATACCTGCTGAGTGCAG |
| 5° Agel VH 1-18 | CTGCCAAGGGTAGATCCTCTCGGAGTGCAG |
| 5° Agel VH 1-24 | CTGCCAAGGGTAGATCCTCTCGGAGTGCAG |
| 5° Agel VH3 | CTGCCAAGGGTAGATCCTCTCGGAGTGCAG |
| 5° Agel VH3-23 | CTGCCAAGGGTAGATCCTCTCGGAGTGCAG |
| 5° Agel VH3-33 | CTGCCAAGGGTAGATCCTCTCGGAGTGCAG |
| 5° Agel VH 3-9 | CTGCCAAGGGTAGATCCTCTCGGAGTGCAG |
| 5° Agel VH4 | CTGCCAAGGGTAGATCCTCTCGGAGTGCAG |
| 5° Agel VH 4-34 | CTGCCAAGGGTAGATCCTCTCGGAGTGCAG |
| 5° Agel VH4-39 | CTGCCAAGGGTAGATCCTCTCGGAGTGCAG |
| 5° Agel VH 6-1 | CTGCCAAGGGTAGATCCTCTCGGAGTGCAG |

Reverse 3’ to 5’ |
|-----------------|
| 3° Cµ CH1 int | GGAAGAAGTTCCTGGCATGAGGC |
| 3° Cy IgG int | GTTCGGGAAGTAGATCCCTGAG |
| 3° Cµ CH1-2 int | GTTCCGTTGCTGCCAGGTCAGACT |

| 1° PCR λ LC | Forward 5’ to 3’ |
|-------------|-----------------|
| 5° L-1A    | GTAGAAGGGTCCTGCAGG |
| 5° L-1B    | GTAGAAGGGTCCTGCAGG |
| 5° L-1C    | GTAGAAGGGTCCTGCAGG |
| 5° L-1D    | GTAGAAGGGTCCTGCAGG |
| 5° L-1E    | GTAGAAGGGTCCTGCAGG |
| 5° L-1F    | GTAGAAGGGTCCTGCAGG |
| 5° L-1G    | GTAGAAGGGTCCTGCAGG |
| 5° L-1H    | GTAGAAGGGTCCTGCAGG |
| 5° L-1I    | GTAGAAGGGTCCTGCAGG |
| 5° L-1J    | GTAGAAGGGTCCTGCAGG |

Reverse 3’ to 5’ |
|-----------------|
| 3° Cγ3 | CACCACTGTCAGGCTTGGTGGCTTGG |

| 2° PCR 1 LC | Forward 5’ to 3’ |
|-------------|-----------------|
| 5° Agel VH1 | CTGCCAAGGGTAGATCCTCTCGGAGTGCAG |
| 5° Agel VH2 | CTGCCAAGGGTAGATCCTCTCGGAGTGCAG |
| 5° Agel VH3 | CTGCCAAGGGTAGATCCTCTCGGAGTGCAG |
| 5° Agel VH4 | CTGCCAAGGGTAGATCCTCTCGGAGTGCAG |
| 5° Agel VH5 | CTGCCAAGGGTAGATCCTCTCGGAGTGCAG |
| 5° Agel VH6 | CTGCCAAGGGTAGATCCTCTCGGAGTGCAG |

Reverse 3’ to 5’ |
|-----------------|
| 3° Xhol C3 | CTCTTTCTAGTACTGTGCTTGG |

| 1° PCR k LC | Forward 5’ to 3’ |
|-------------|-----------------|
| 5° L-2A | ATTAGAGGTCCTGTCAGG |
| 5° L-2B | ATTAGAGGTCCTGTCAGG |
| 5° L-2C | ATTAGAGGTCCTGTCAGG |
| 5° L-2D | ATTAGAGGTCCTGTCAGG |
| 5° L-2E | ATTAGAGGTCCTGTCAGG |
| 5° L-2F | ATTAGAGGTCCTGTCAGG |
| 5° L-2G | ATTAGAGGTCCTGTCAGG |
| 5° L-2H | ATTAGAGGTCCTGTCAGG |
| 5° L-2I | ATTAGAGGTCCTGTCAGG |
| 5° L-2J | ATTAGAGGTCCTGTCAGG |

Reverse 3’ to 5’ |
|-----------------|
| 3° Cκ494 | GTGTCAGCTTTGCTTCGTCAGT |

Specific k LC | Forward 5’ to 3’ |
|-------------|-----------------|
| 5° Agel Vκ1 | CTGCCAAGGGTAGATCCTCTCGGAGTGCAG |
| 5° Agel Vκ2 | CTGCCAAGGGTAGATCCTCTCGGAGTGCAG |
| 5° Agel Vκ3 | CTGCCAAGGGTAGATCCTCTCGGAGTGCAG |
| 5° Agel Vκ4 | CTGCCAAGGGTAGATCCTCTCGGAGTGCAG |
| 5° Agel Vκ5 | CTGCCAAGGGTAGATCCTCTCGGAGTGCAG |
| 5° Agel Vκ6 | CTGCCAAGGGTAGATCCTCTCGGAGTGCAG |
| 5° Agel Vκ7 | CTGCCAAGGGTAGATCCTCTCGGAGTGCAG |
| 5° Agel Vκ8 | CTGCCAAGGGTAGATCCTCTCGGAGTGCAG |
| 5° Agel Vκ9 | CTGCCAAGGGTAGATCCTCTCGGAGTGCAG |
| 5° Agel Vκ10 | CTGCCAAGGGTAGATCCTCTCGGAGTGCAG |
| 5° Agel Vκ11 | CTGCCAAGGGTAGATCCTCTCGGAGTGCAG |
| 5° Agel Vκ12 | CTGCCAAGGGTAGATCCTCTCGGAGTGCAG |
| 5° Agel Vκ13 | CTGCCAAGGGTAGATCCTCTCGGAGTGCAG |
| 5° Agel Vκ14 | CTGCCAAGGGTAGATCCTCTCGGAGTGCAG |
| 5° Agel Vκ15 | CTGCCAAGGGTAGATCCTCTCGGAGTGCAG |
| 5° Agel Vκ16 | CTGCCAAGGGTAGATCCTCTCGGAGTGCAG |
| 5° Agel Vκ17 | CTGCCAAGGGTAGATCCTCTCGGAGTGCAG |
| 5° Agel Vκ18 | CTGCCAAGGGTAGATCCTCTCGGAGTGCAG |

Reverse 3’ to 5’ |
|-----------------|
| 3° BsrWI Jc 1/4 | GCCACCGTAGCTTTGATYCTCACCTGAGTGCAG |
| 3° BsrWI Jc 2 | GCCACCGTAGCTTTGATYCTCACCTGAGTGCAG |
| 3° BsrWI Jc 3 | GCCACCGTAGCTTTGATYCTCACCTGAGTGCAG |
| 3° BsrWI Jc 5 | GCCACCGTAGCTTTGATYCTCACCTGAGTGCAG |
| 3° BsrWI Jc 6 | GCCACCGTAGCTTTGATYCTCACCTGAGTGCAG |
Table S2. List of citrullinated peptide and protein antigens in the Luminex assay

| Peptide antigens                          | Protein antigens         |
|-------------------------------------------|--------------------------|
| Fibrinogen A (211-230) cit cyclic         | Fibrinogen cit           |
| Fibrinogen B (246-267) cit                | Vimentin cit             |
| H2B/a (62-81) cit cyclic                  | Histone 2A cit           |
| Fibrinogen A (582-599) cit                | Histone 2B cit           |
| Clusterin (221-240) cit cyclic            | Apolipoprotein E cit     |
| Biglycan (247-266) cit cyclic             | CCP                      |
| H2A/a (1-20) cit cyclic                   |                          |
| Enolase 1A (5-21) cit                     |                          |
| Fibrinogen A (616-635) cit cyclic         |                          |
| Vimentin (58-77) cit cyclic               |                          |
| Filaggrin (48-65) cit cyclic              |                          |
| Fibrinogen A (556-575) cit cyclic         |                          |
| Clusterin (231-250) cit cyclic            |                          |
| Apolipoprotein E (277-296) cit cyclic     |                          |
Table S3. List of clones and primers used in the overlap PCR.

| CLONE       | PRIMER    | SEQUENCES (5'-3') * |
|-------------|-----------|---------------------|
| RA015-11_G58 | G58M38 VH Rev-F | TGAGGACACGGCTGTGTATTACTGT |
|             | G58M38 VH Rev-R | ACAGTAATAACACAGCCGTGTCCCTCA |
| RA015-11_K58 | K58 JH Rev-F   | ACTGGCCTCTAGTAGTACGTTGGCCGAAC |
|             | K58 JH Rev-R   | TTGGCCCAACGTACTCTGAGGCAGTT |
|             | K58 VH Rev-F   | TGCACTCTGAAGATTTTCGAGTTATTAAT |
|             | K58 VH Rev-R   | TAATACACGGAATATCTTTCAGACTGCA |
| RA015-11_M88 | M88 VH-Rev-F  | ACCGCCCAGCAGCCCGCTGTATTACTGT |
|             | M88 VH-Rev-R  | ACAGTAATAACAGCAGCCCGCTGTCCGCGGT |
| RA015-11_K88 | K88 VH-Rev-F  | ACCCTGAAGATATTACTGT |
|             | K88 VH-Rev-R  | ACGTAGTTACGTGCAAATATCTTTCAGGT |
| RA015-11_A91 | A91 JH-Rev-F  | ACACGGCTGTGTATTACTGTGAGGAC |
|             | A91 JH-Rev-R  | TCTCGCACAGTATACACAGCCGTGT |
| RA015-11_K91 | K91 VH-Rev-F  | AGCCTGAAGATATTTCAGACTATATTACTGT |
|             | K91 VH-Rev-R  | ACAGTAATATGTGCAATATCTTTCAGGCT |
| RA056-11_G29 | G29 JH Rev-F  | AGTGGCTTTTGACTACTGAGGAC |
|             | G29 JH Rev-R  | TGGCCCCAGTATACGAAAGCCACT |
|             | G29 VH Rev-F  | TGAGGACACGGCCGTGTATTAGACA |
|             | G29 VH Rev-R  | TGGTGTAATACGAGGCGGAGGCGG |
| RA056-11_KC29 | KC29 JH Rev-F | TGGGACCAAAGTGGATATCACAACGAA |
|             | KC29 JH Rev-R | TCTCGTATGACATCTGAGGAGGAGG |
|             | KC29 VH Rev-F | TGGTGTAATACGAGGCGGAGGCGG |
|             | KC29 VH Rev-R | CGTTGCAGCAGTAACTGACAGCAG |
| RA056-11G66  | 11G66-VHRevF | TGACCCCGCGACACAGCGGCTGTGTATTAT |
|             | 11G66-VHRevR | TATACACAGCCGCTGTCTGCAGGCGGAGG |
| RA056-11LC66 | 11LC66JH-F   | TACACTGTTGATTCCGCGAGGAGGACCA |
|             | 11LC66JH-R   | TGGTCCTTCGGCGAATACCACAGGTGTA |
|             | 11LC66-VHRevF| AGGCTAGGATGAGGCTGATATTACT |
|             | 11LC66-VHRevR| AATACACAGCTCTTCACCTCTACGCT |

* In red reverted base to GL
Table S4: Ig VH and VL gene repertoire analysis of CD19+ single B cells from 3 RA synovial tissues. Clones highlighted in grey have been expressed as recombinant monoclonal antibodies

| RA015/11 | VH | D | JH (| | CDR3(aa) | (+) | Length | Vκ/Vλ | Jκ/Jλ | (+) | CDR3 | (+) | Length |
|-----------|----|---|-----|-----|--------|-------|--------|------|------|-----|--------|-------|--------|
| 80        | 4-4| 3-10| 2-2| EVPTFYFDL | 0 | 9     | x | 1-39 | 2-0 | QQYSYPYFT | 0 | 9     |
| 94        | 1-3| 4-17| 3-4| GOEDGYGSNADF | 0 | 15    | x | 1-5  | 1-0 | QQYNSYWT | 0 | 9     |
| 58        | 3-64| 1-26| 5-1| EVGANRVPVPGP | 1 | 13    | x | 3-15 | 1-0 | QQYNYWPDST | 0 | 10    |
| 68        | 4-31| 6-25| 6-2| AISKADGYYMDV | 0 | 12    | x | 1-40 | 3-1 | QVQDSSLSGQV | 0 | 11    |
| 69        | 1-24| 5-6 | 6-1| ASSHYYYSMDV | 0 | 13    | x | 2-23 | 3-0 | CSSAGSAAV | 0 | 10    |
| 96        | 3-30| 1-1 | 5-3| DQKNENWPSYNWFD | 0 | 16    | x | 1-39 | 2-0 | QQYSYPYFT | 1 | 9     |
| 48        | 3-66| 3-3 | 4-3| EGDLWGGSGDY | 0 | 11    | | 1-44 | 2-0 | AAWDDELNQRV | 1 | 11    |
| 63        | 4-34| 3-1 | 5-2| AQREWVLPYPFD | 1 | 14    | x | 1-39 | 2-0 | QQYGYALYN | 0 | 9     |
| 74        | 4-59| 5-24| 6-1| GSKGGGYTYGMDV | 0 | 15    | x | 1-40 | 2-3 | QVQDSSLSGQV | 0 | 12    |
| 81        | 1-2| 4-17| 3-1| GAYGQPHI | 1 | 9     | | 3-21 | 1-3 | QWQDDSDRFD | 1 | 11    |
| 82        | 1-3| 2-2 | 4-3| DREDVVPTARILYGSYGSFD | 2 | 22   | x | 1-39 | 1-0 | QQYTYTPNT | 1 | 9     |
| 83        | 4-4| 2-21| 3-2| SPIGQATAFD | 0 | 12    | | 2-23 | 3-0 | CRMWWIGGWW | 1 | 10    |
| 91        | 3-21| 3-10| 4-1| WRAGVPSFYFD | 1 | 11    | | 1-33 | 3-0 | QQYAYNPTF | 0 | 8     |
| 95        | 1-38| 3-16| 4-4| GSSGYTD | 0 | 7     | | 4-1  | 2-0 | QQYQYPYFT | 1 | 9     |
| RA015/11 | VH | D | JH (| | CDR3(aa) | (+) | Length | Vκ/Vλ | Jκ/Jλ | (+) | CDR3 | (+) | Length |
| 11        | 1-3| 2-2 | 4-3| DRDVVPTARILYGSYGSFD | 2 | 22   | |       |     |       |     |       |     |       |
| 17        | 3-23| 3-1 | 4-3| SPITDFDDLYTFI | 0 | 15    | x | 3-20 | 1-0 | QQQYSYPYFT | 1 | 9     |
| 53        | 1-2| 3-10| 5-2| SGCPHYDFP | 1 | 10    | | 3-15 | 1-1 | QQYNYWPD | 0 | 9     |
| 62        | 3-48| 6-25| 4-2| EGNHSYDDY | 1 | 9     | | 3-11 | 3-0 | QQYNYWPDFT | 2 | 10    |
| 64        | 3-9| 3-22| 4-3| GSKGDTQYTY | 0 | 14    | x | 3-11 | 1-0 | QQYNYWPDFT | 1 | 9     |
| 66        | 4-4| 6-6 | 3-3| KOTYSTDYGDFD | 1 | 14    | x | 2-21 | 2-0 | QYQIEGSLYV | 1 | 11    |
| 72        | 1-69| 3-10| 5-2| ERLRDDLVPF | 1 | 11    | |       |     |       |     |       |     |       |
| 75        | 3-33| 3-16| 4-2| DVSGLPNTFD | 0 | 13    | |       |     |       |     |       |     |       |
| 80        | 1-9| 2-8 | 5-2| DSRQGGSAVF | 1 | 13    | |       |     |       |     |       |     |       |
| 86        | 3-23| 5-24| 4-2| GQDYNPSYFDY | 0 | 12    | |       |     |       |     |       |     |       |
| 88        | 3-11| 2-2 | 5-1| QPAWGTNWFD | 0 | 11    | |       |     |       |     |       |     |       |
| 91        | 3-11| 2-2 | 5-1| QPKWGTNWFD | 0 | 11    | |       |     |       |     |       |     |       |
| 95        | 3-11| 2-2 | 5-1| QPKWGTNWFD | 0 | 11    | |       |     |       |     |       |     |       |
| RA057/11 | VH | D | HJ | CDR3(aa) | (+) Length | (+) k | Ve/Vl | Je/Ub | (+) CDR3 | (+) Length |
|----------|----|---|----|----------|------------|------|-------|-------|----------|------------|
| 4-39     | 2-15 | 5 | 5  | 3-4      | 3-20 4  | 2-23 3  | 0     | QYYHHSPYT | 1 9        |
| 1-2      | 5 | 5  | 1  | 1-7      | 3-20 1  | 2-23 2  | 2     | CYAGILEV  | 0 9        |
| 4-34     | 6  | 6  | 1  | 2-6      | 3-20 4  | 2-23 2  | 2     | AWHDSILK  | 1 9        |
| 4-16     | 1-14 | 3 | 7  | 1-9      | 3-22 1  | 2-23 2  | 2     | CSAPAFISW | 0 9        |
| 3-21     | 1-17 | 1 | 6  | 4-17     | 3-20 1  | 2-23 2  | 2     | AWHDSILK  | 1 9        |
| 1-46     | 1-5  | 3 | 1  | 1-7      | 3-22 1  | 2-23 2  | 2     | CSAPAFISW | 0 9        |
| 4-38     | 3-20 | 3  | 6  | 1-7      | 3-22 1  | 2-23 2  | 2     | AWHDSILK  | 1 9        |
| 3-23     | 1-25 | 5 | 1  | 1-7      | 3-22 1  | 2-23 2  | 2     | AWHDSILK  | 1 9        |
| 3-22     | 1-25 | 1 | 7  | 1-7      | 3-22 1  | 2-23 2  | 2     | AWHDSILK  | 1 9        |
| 3-3      | 1-13 | 5 | 2  | 1-7      | 3-22 1  | 2-23 2  | 2     | AWHDSILK  | 1 9        |
| 4-35     | 1-13 | 1 | 1  | 1-7      | 3-22 1  | 2-23 2  | 2     | AWHDSILK  | 1 9        |
| 3-25     | 1-6  | 1 | 1  | 1-7      | 3-22 1  | 2-23 2  | 2     | AWHDSILK  | 1 9        |
| 2-8      | 2-7  | 7  | 1  | 1-7      | 3-22 1  | 2-23 2  | 2     | AWHDSILK  | 1 9        |
| 2-5      | 1-7  | 1 | 1  | 1-7      | 3-22 1  | 2-23 2  | 2     | AWHDSILK  | 1 9        |
| 2-4      | 1-7  | 1 | 1  | 1-7      | 3-22 1  | 2-23 2  | 2     | AWHDSILK  | 1 9        |
| 2-3      | 1-7  | 1 | 1  | 1-7      | 3-22 1  | 2-23 2  | 2     | AWHDSILK  | 1 9        |
| 2-2      | 1-7  | 1 | 1  | 1-7      | 3-22 1  | 2-23 2  | 2     | AWHDSILK  | 1 9        |
| 2-1      | 1-7  | 1 | 1  | 1-7      | 3-22 1  | 2-23 2  | 2     | AWHDSILK  | 1 9        |
| 1-7      | 1-7  | 1 | 1  | 1-7      | 3-22 1  | 2-23 2  | 2     | AWHDSILK  | 1 9        |