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Autoreactive Monoclonal Antibodies from Patients with Primary Biliary Cholangitis Recognize Environmental Xenobiotics

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Abstract

A major problem in autoimmunity has been identification of the earliest events that lead to breach of tolerance. Although there have been major advances in dissecting effector pathways and the multi-lineage immune responses to mitochondrial self-antigens in primary biliary cholangitis (PBC), the critical links between environmental factors and tolerance remain elusive. We hypothesized that environmental xenobiotic modification of the E2 subunit of the pyruvate dehydrogenase (PDC-E2) inner lipoyl domain can lead to loss of tolerance to genetically susceptible hosts. Previously we demonstrated that serum anti-PDC-E2 autoantibodies cross-react with the chemical xenobiotics 2-octynoic acid (2-OA) and 6,8-bis (acetylthio) octanoic acid (SAC) and further that there is a high frequency of PDC-E2 specific peripheral plasmablasts. Herein we

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generated 104 recombinant mAbs based on paired heavy- and light-chain variable regions of individual plasmablasts derived from PBC patients. We identified 32 mAbs reactive with native PDC-E2, including 20 specific for PDC-E2 and 12 cross-reactive with both PDC-E2 and 2-OA and SAc. A lower frequency of replacement somatic hypermutations, indicating lower level of affinity maturation, was observed in the complementarity-determining regions (CDR) of the cross-reactive mAbs in comparison to mAbs exclusively recognizing PDC-E2 or those for irrelevant antigens. In particular, when the highly mutated heavy chain gene of a cross-reactive mAb was reverted to the germline sequence, the PDC-E2 reactivity was reduced dramatically whereas the xenobiotics reactivity was retained. Importantly, cross-reactive mAbs also recognized lipoic acid (LA), a mitochondrial fatty acid that is covalently bound to PDC-E2. Our data reflect that chemically modified LA or LA itself, via molecular mimicry, is the initial target that leads to the development of PBC.

Introduction

Primary biliary cholangitis (PBC) is characterized by the presence of anti-mitochondrial antibodies (AMAs), a serological hallmark, present over 95% of patients (1). Although extremely useful as a diagnostic marker, AMAs are not helpful for patient follow-up as their titer does not correlate with disease progression (2, 3). However AMAs can be detected long before the onset of symptoms. This unique feature suggests that data on the origin of AMA will help dissect the etiology of PBC (4–6). The major mitochondrial autoantigen in PBC is the E2 subunit of pyruvate dehydrogenase (PDC-E2) (7, 8). Other autoantigens recognized by AMAs are the E2 subunits of enzyme complexes belonging to the 2-oxo-acid dehydrogenase (2-OADC) family, including the branched-chain oxo-acid dehydrogenase (BCOADC), oxo-acid dehydrogenase (OGDC) complexes, the E3 binding protein (E3BP) and the E1 subunit of the pyruvate dehydrogenase complex (PDC-E1a) (7–10). All the epitopes of these autoantigens are localized within their respective lipoyl domains.

We have hypothesized that environmental xenobiotic modification of PDC-E2 inner lipoyl domain can lead to the loss of tolerance to PDC-E2 and have demonstrated the presence of anti-PDC-E2 autoantibodies that are cross-reactive to chemical xenobiotics including 2-octynoic acid (2-OA), a material commonly found in perfumes and cosmetics, and 6,8-bis (acetylthio) octanoic acid (SAc), a metabolite of acetaminophen that is structurally similar to lipoic acid (11). More recently, we demonstrated a high frequency of PDC-E2 specific plasmablasts in the periphery of PBC, suggesting ongoing activation of PDC-E2 specific B cells during the disease course of PBC (12). Herein we have investigated the origin and evolution of anti-PDC-E2 antibodies by analyzing the immunoglobulin (Ig) gene sequences of circulating plasmablasts and the reactivity of recombinant mAbs generated from Ig gene sequences.

Materials and methods

Study subjects and Blood samples

In this labor-intensive analysis, three AMA-positive PBC patients all stage I or II, diagnosed using established criteria, were enrolled. All participants provided written informed consent

and the study protocol was approved by the Institutional Review Board at the University of California, Davis prior to initiation of study. Blood samples were collected using acid-citrate-dextrose as the anticoagulant. B cells were isolated by negative selection with RosetteSep Human B cell Enrichment cocktail (Stemcell Technologies, Vancouver, BC, Canada); the purity was 70–80%.

Isolation of single plasmablasts by fluorescence activated cell sorting

Freshly isolated B cells were treated with Human Fc Receptor Binding Inhibitor (eBioscience, San Diego, CA) to block the Fc receptor prior to staining. Cells were then stained with FITC-conjugated anti-CD27, PE-conjugated anti-CD19, PE-Cy7-conjugated anti-CD20, BV421-conjugated anti-CD38 and APC-conjugated anti-CD3 (Biolegend, San Diego, CA). The LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit (Life Technologies, Carlsbad, CA) was used to determine the viability of cells. Single plasmablasts (CD3⁻CD19⁺CD20^{lo/-}CD27^{hi}CD38^{hi}) were sorted into 96-well plates with 4 µl of cell lysis buffer containing 10 mM DTT (Invitrogen, Carlsbad, CA) and RNase Inhibitor (Eppendorf) in each well (13). Cell sorting was performed using a BD Influx™ cell sorter.

Single cell RT-PCR and Ig gene amplification

cDNA was synthesized as previously described (13). Briefly, iScript cDNA Synthesis Kit (Bio-Rad) was used to synthesize cDNA in a total volume of 20µl/well in the original 96-well sorting plates. Variable region gene transcripts of IgH, Igκ, and Igλ were then amplified independently by nested PCR (13). The primer sequences for PCR amplification and cycling conditions have been described (13, 14).

Ig gene sequence analysis

Purified PCR products were sequenced at Quintara Biosciences. The resultant transcript sequences were analyzed by NCBI IgBLAST comparison with GenBank to determine the functional sequences and identify germline V(D)J gene segments with highest identity. The IgG, IgM or IgA isotype of each sorted plasmablast was determined by isotype gene-specific PCR during library preparation for sequencing.

Construction of Ig expression vectors

PCR products were digested with respective restriction enzymes to isolate the gene segments encoding the Ig heavy or light chain variable regions, which were then cloned into corresponding vectors containing a murine Ig signal peptide sequence (GenBank accession no. DQ407610) and a multiple cloning site upstream of the human Igγ1, Igκ or Igλ constant regions respectively, as described (14).

Production of recombinant mAbs

Ig expression vectors were transfected into Human embryonic kidney (HEK) 293 cells using the X-tremeGENE 9 DNA transfection Reagent (Roche, Mannheim, Germany) or JetPRIME transfection reagent (Polyplus transfection, NY, USA). Cells were seeded in 6 well-plates (2×10⁶ cells per well) or 75 cm² flasks (6×10⁶ cells per flask) in high Glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine serum (FBS),

then incubated overnight at 37°C with 5% CO₂ to allow attachment. Unattached cells were removed and culture medium replaced with serum-free DMEM. Transfection reagent was mixed with IgH or IgL (either Igκ or Igλ) expression vectors and incubated for 20 min at room temperature. The mixture was then added to cultured cells and incubated for 24 hours. The transfection culture medium was then changed to high Glucose DMEM supplemented with 5% FBS. The supernatant with secreted recombinant mAbs was harvested at days 6 and 12 post-transfection and the mAbs concentrated with Amicon Ultra-4 Centrifugal Filters or purified with HiTrap Protein G HP (GE Healthcare Life Sciences, NJ). The concentration of each mAb was measured with Human IgG ELISA quantitation sets (Bethyl Laboratories, Inc. TX).

Immunoblotting

Immunoblotting was performed with SDS-PAGE electrophoresis system. Briefly, 10 μg of each protein to be tested, including recombinant PDC-E2, BSA, rabbit serum albumin (RSA), 2OA-BSA, SAc-BSA, lipoic acid (LA)-BSA, and LA-RSA, was electrophoresed on 4–12% NuPAGE® Novex® Bis-Tris gels with NuPAGE MOPS SDS running buffer, then transferred to nitrocellulose membrane. Membranes were blocked in PBST containing 3% of skim milk for 2 hours at 4°C, then incubated with either concentrated or purified recombinant mAbs for 1 hours. A pool of plasma from PBC patients, at various dilutions (1:200,000 for PDC-E2 and 1:3,000 for 2OA-BSA or SAc-BSA), was used as a positive control. The membranes were then incubated with horseradish peroxidase-labeled anti-human IgG (H+L) (1:3,000) and developed with SuperSignal Chemiluminescent Substrates (ThermoFisher Scientific, Rockford, IL).

Statistical Analysis

Data were analyzed using the one-way analysis of variance (ANOVA). P value of 0.05 or less was considered statistically significant.

Results

Ig gene usage and isotype distribution of circulating plasmablasts in PBC patients

The Ig genes of 1,680 sorted single plasmablasts from 3 PBC subjects (S1, S2 and S3) were individually sequenced and analyzed for their antibody variable regions (Supplementary Figure 1). After excluding nonfunctional transcripts including those with out-of-frame sequences or stop codon in reading frame, a total of 922 variable regions of Ig heavy chain (IGHV) and 790 Ig kappa or lambda chain (IGKV or IGLV) transcripts were recovered, resulting in 568 VH:VL (variable heavy: light) pairs derived from the same cell. Out of these 568 pairs, IGHV3 family members, in particular IGHV3-23, IGHV3-30 and IGHV3-7, were the most frequent IGHV genes, following by IGHV4-39 and IGHV1-18 (Figure 1A). In Vκ gene usage, IGKV3-20 and IGKV3-15 were the most frequent IGKV3, followed by IGKV4-1 and IGKV1-39. In Vλ gene usage, IGLV2 family, in particular IGLV2-14, was the most represented gene followed by IGLV1 (Figure 1B). A total of 20 heavy and light chain gene combinations were utilized by three subjects (data not known), suggesting a diverse Ig repertoire of plasmablasts in PBC patients. Regarding the isotype distribution of the

circulating plasmablast repertoire, IgA isotype ($55.6 \pm 5.6\%$) predominated in all three subjects, followed by IgM ($20.6 \pm 8.5\%$) and IgG ($22.8 \pm 13.9\%$) (Figure 1C).

Identification of PDC-E2- and xenobiotics-reactive mAbs

We randomly selected 104 paired VH:VL cDNAs derived from single plasmablasts to construct human Ig γ 1, Ig κ or Ig λ expression vectors for expression of recombinant mAbs. The reactivity of each mAb against PDC-E2 and xenobiotics was examined by immunoblotting. Among these 104 single mAbs, 32 (30.8%) reacted to PDC-E2, including 20 reacted to PDC-E2 only and 12 cross-reacted with PDC-E2 and xenobiotics (Figure 2A). No mAbs that recognize xenobiotics but not PDC-E2 were identified. Indirect immunofluorescence staining of HEp-2 cells using a representative PDC-E2 reactive mAb showed a cytoplasmic staining pattern as expected for AMAs (Supplementary Figure 2). For the cross-reactive mAbs, we further confirm that their binding target was xenobiotics rather than the BSA conjugates, using unconjugated BSA or RSA and BSA conjugated with a biologically and structurally irrelevant molecule methacrylic acid (MA-BSA) as the testing antigens (Supplementary Figure 3). Finally we analyzed the original isotype distribution of the expressed plasmablast VH:VL pairs in each reactivity group. Among the total of 104 plasmablasts, 63 were IgA, 22 were IgM and 19 were IgG plasmablasts. Out of the 20 PDC-E2-specific mAbs, 15 (75%) were derived from IgA plasmablasts, which was higher than the IgA frequency in the PDC-E2/xenobiotics cross-reactive groups (7 of 12, 58.3%) and in the PDC-E2 negative group (41 of 72, 56.9%). In contrast, IgG appears to be underrepresented in the PDC-E2 specific and cross-reactive groups, as only one IgG mAb was identified in each of these groups. (Figure 2B). The frequency of IgM in PDC-E2 specific group (4 of 20, 20%) mAbs is similar to the frequency of IgM plasmablasts in the PDC-E2 negative group (14 of 72, 19.4%) (Figure 2B).

Somatic hypermutation load in CDR was significantly lower in cross-reactive antibodies

Introduction of somatic hypermutation (SHM) shapes the Ig repertoire diversity and increases the affinity of antibodies for their cognate antigens during antibody responses. We compared the SHM loads in the framework (FR) and CDR of IGHV (Figure 3A) and IGLV (Figure 3B) genes among PDC-E2/xenobiotics cross-reactive, PDC-E2-specific and PDC-E2 negative antibodies. The average number of all mutations in the entire variable region of heavy chain and light chain were significantly smaller in PDC-E2/xenobiotics cross-reactive antibodies as compared to PDC-E2 negative antibodies (Figure 3A). Importantly, a significantly lower rate of replacement mutation (R-mutation), was observed in the entire variable region of heavy chain and the CDR of both heavy and light chains used by PDC-E2/xenobiotics cross-reactive antibodies as compared to PDC-E2-specific or PDC-E2 negative antibodies, whereas such a difference was not observed for the silent mutations (S-mutation) (Figure 3A and 3B). Taken together, these results indicate that on average the mAbs recognizing PDC-E2 exclusively or those recognizing irrelevant antigens have adopted more amino acid (AA) changes from their germline sequences in comparison to the cross-reactive mAbs.

CDR1 sequence of the heavy chain in IGHV4-61:IGLV2-14 family is critical for PDC-E2 recognition

We explore the relationship between PDC-E2 reactivity and AA sequence of the mAbs by examining 6 evolutionarily closely related mAbs using the same IGHV4-61:IGLV2-14 pair (Figure 4A). The mAbs S1-3, S1-10, S1-11, S1-12 were PDC-E2-reactive, whereas S1-16 was PDC-E2-negative. Three of the PDC-E2 reactive mAbs (S1-3, S1-10 and S1-11) had a G→S replacement in CDR1, whereas the other two PDC-E2 reactive mAbs S1-12, S1-13 did not have the G→S replacement but had a nearby I→V replacement in CDR1, indicating the AA differences at the site of the two AA replacements (G→S and I→V) in CDR1 did not affect the reactivity to PDC-E2. The PDC-E2-negative mAb S1-16 had the same I→V replacement in CDR1 as the PDC-E2 positive mAbs S1-12 and S1-13, but an additional G→D replacement next to the I→V replacement. Based on 3D-structure modeling of these mAbs, this G→D replacement in the PDC-E2 negative S1-16 occurred on surface of heavy chain, resulting in alteration of surface structure in comparison to the other 5 mAbs (Figure 4B and data not shown). To evaluate the impact of AA variability in the light chain on the mAb reactivity to PDC-E2, we generated an artificial mAb S1-13HV: S1-16LV with the heavy chain of S1-3 (PDC-E2 reactive) and light chain of S1-16 (PDC-E2 negative) (Figure 4C). As shown in Figure 4D, this artificial mAb retained the reactivity to PDC-E2. These results suggested that within this group of mAbs using the IGHV4-61:IGLV2-14 pair, the conformational structure in CDR of heavy chain was critical for reactivity against the autoantigen PDC-E2.

Germline-encoded autoantibody reacts strongly to xenobiotics, but weakly to PDC-E2

Mutation analysis demonstrated that cross-reactive mAbs have less mutated CDRs in their heavy and light chains in comparison to the mAbs reactive to PDC-E2 exclusively (Figure 3). Two cross-reactive mAbs S1-8 and S1-9 were selected for further evaluation of the effects of SHM load on the antibody cross-reactivity. As shown in Figure 5A, these two mAbs used the same IGHV3-30 gene. S1-9 had a germline sequence-encoded heavy chain, whereas S1-8 had 5 replacement mutations in the heavy chain including one located in CDR3. In contrast, S1-8 utilized a germline sequence-encoded light chain Vκ4-1, whereas S1-9 utilized a Vλ1-44 gene with one mutation. As shown in Figure 5B, we swapped the light chains of S1-8 and S1-9 to generate two artificial mAbs S1-8' and S1-9', so that both the heavy chain and the light chain of S1-9' were encoded by germline sequences, whereas both the heavy chain and light chain of S1-8' were mutated. Next we compared the reactivity of these 2 native and 2 artificial mAbs to PDC-E2 and 2OA-BSA (Figure 5C). The artificial clone S1-9', with its heavily mutated heavy chain gene from the native S1-9 reverted to germline sequence, exhibited a sharply reduced reactivity to PDC-E2 while retaining its reactivity to 2OA-BSA (Figure 5C). In contrast, a comparably strong reactivity to 2OA-BSA was detected in the S1-9 and S1-8', which shared the lightly mutated Vλ1-44 gene. Taken together, these results suggest that while both heavy chain and light chain are important for antigen recognition, a heavily mutated IGHV3-30 heavy chain is associated with increased antibody reactivity to the autoantigen PDC-E2, whereas the germline encoded or lightly mutated light chains are associated with the cross-reactivity to the xenobiotics.

Cross-reactive mAbs recognize LA

Given the similarity between the structure of xenobiotic SAc and natural LA and the importance of LA in both enzymatic function and antigenicity of the PDC-E2 autoantigen, we examined the reactivity of cross-reactive mAbs to LA-conjugated RSA (LA-RSA) and unconjugated RSA. Four mAbs that were encoded by germline or lightly mutated (less than 3 total AA replacements) sequences were tested. All these mAbs recognized LA-RSA but not RSA (Figure 6), suggesting that LA is the dominant epitope of the cross-reactive mAbs.

Discussion

Epidemiological studies, clinical research and animal model studies have pointed to a role of environmental agents in triggering autoimmunity (15–17). However, the precise contribution of these agents to disease development is unknown. Our quantitative structure–activity relationship (QSAR) studies have demonstrated that AMA positive PBC sera react to a number of xenobiotic modified PDC-E2 structures, in particular those modified by SAc or 2OA (11, 18). To address this issue at the level of individual B cell clones, we have investigated the circulating plasmablasts, precursors of mature plasma cells, which enter circulation five to seven days after B cell activation before they migrate into inflamed tissues and bone marrow, the major site of antibody production (12, 19–21).

To date, very few studies have been conducted on immunological repertoire in PBC (22, 23). In the current study, we focused on the circulating plasmablasts, which are derived from recently activated naïve or memory B cells. We reasoned that analyses of the Ig gene sequence and Ab reactivity of B cells that were recently activated in PBC patients could reveal footsteps of B cell evolution at the initial stage of autoimmunity. We successfully generated 104 recombinant mAbs that were derived from single plasmablasts isolated from three patients with PBC. A total of 32 (30.8%) mAbs recognized PDC-E2. These included 20 (19.2%) mAbs that were PDC-E2-specific and 12 (11.5%) mAbs that were cross-reactive to PDC-E2 and xenobiotics 2OA- and SAc-BSA. Analyses of the Ig gene sequences demonstrated that the signature autoantibodies of PBC, AMAs, were encoded by a diversified Ig repertoire. Of note, the cross-reactive mAbs had fewer replacement mutations in their heavy chain and light chain genes in comparison to the mAbs that recognize PDC-E2 but not xenobiotics, suggesting that the high affinity autoreactive B cells were evolved from low affinity cross-reactive B cells through adoption of SHMs. Further evidence was provided by the artificial mAbs we generated, which demonstrated that reversion of highly mutated Ig gene to the germline sequence resulted in drastically reduced PDC-E2 reactivity with retained xenobiotics reactivity. In addition, we demonstrated that LA is the primary antigenic target of the cross-reactive mAbs. Taken together, our findings suggest that B cell autoimmunity in PBC is initiated with exposure to environmental xenobiotics, which activate xenobiotic-specific naïve B cells that cross-react with PDC-E2, and perpetuated with the mutation of Ig genes that increase the antibody affinity to the autoantigen. The affinity maturation of these antibodies results in highly reactive AMA, the serological hallmark of PBC.

Post-translational modifications, lead to generation of neoepitopes capable of eliciting both innate and adaptive immune responses, which may be the fundamental of autoantibody

recognition in autoimmune diseases (24). Upon continuous exposure to neo-antigen, B-cell epitope spreading expands the immune response from modified epitope to non-modified epitopes and eventually to other proteins complexed with the initial antigen during the course of an autoimmune response (17, 25). A limited number of proteins are known to be lipoylated in human, including PBC signature mitochondrial antigens PDC-E2 (26). 2-OA is widely used in detergents, perfumes and cosmetics, and SAc, a metabolite of acetaminophen that is structurally very similar to lipoic acid. 2-OA and SAc have the ability to modify the lipoyl domain of PDC-E2 resulting in the formation of neoantigens. SAc is a modified form of lipoic acid in which both sulfur atoms of the disulfide bond of the lipoyl ring are modified by acetyl groups, thereby maintaining PDC-E2 in a reduced state by preventing disulfide bond formation (18, 27–29). Our study demonstrates a subpopulation of anti-PDC-E2 autoantibodies, encoded with less mutated variable region transcripts, exhibits cross-reactivities to chemical xenobiotics including 2-OA and SAc. Of importance is the finding that, in fact, these cross-reactive mAbs also recognize lipoylated PDC-E2 and lipoic acid. These data strongly support the significance of epitope spreading and molecular mimicry as triggers in autoimmunity.

IgA anti-PDC-E2 antibodies have been detected not only in serum but also in bile, saliva and urine, and the presence of IgA anti-PDC-E2 in serum and in saliva has been associated with histological progression of PBC (30–32). Biliary epithelial cells have the capacity to export dimeric IgA to the bile through polymeric immunoglobulin receptor (pIgR)-mediated transcytosis (33, 34). Our data demonstrate that the majority of PDC-E2-specific and PDC-E2/xenobiotics cross-reactive plasmablasts (75% and 58%) are IgA. If IgA AMA binds to mitochondrial proteins during transcytosis through the biliary epithelium, mitochondrial function may be consequently disrupted, which further lead to epithelial apoptosis in PBC. Our finding of the predominant presence of IgA AMA+ plasmablasts shed light on IgA-mediated tissue damage in PBC. Moreover, our data reveal that one third of PDC-E2/xenobiotics cross-reactive are IgM plasmablasts. The presence of peripheral plasmablasts indicates recent activation of naïve B cell or memory B cells (20, 35). IgM plasmablasts may derive not only from newly activated naïve B cells (36, 37), but also from IgM memory B cells primed by PDC-E2 and xenobiotics. However we were unable to distinguish whether cross-reactive plasmablasts derived from naïve B cells or from IgM memory B cells because the specific markers for naïve-derived plasmablasts remain unclear. IgM+ plasmablasts bear less mutation loads, which might derive from newly activated naïve B cells. In support of our thesis, we note that a recent Ig repertoire analysis using two complementary approaches, including single cell-based next-generation sequencing and serum proteomics, revealed that the naïve B cell-derived circulating plasmablasts produce un-mutated or low mutated disease-specific autoantibodies in patients with systemic lupus erythematosus (SLE) (38). Taken together, our data obtained through single cell analyses of activated B cells in PBC, suggests that the autoreactive antibody response in PBC is initiated with exposure to environmental xenobiotics that mimic the native lipoic acid moiety on the autoantigen PDC-E2. Subsequent affinity maturation of the cross-reactive Ig genes results in a diversified high affinity antibody repertoire, which collectively forms the signature AMA in PBC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AMAs	anti-mitochondrial antibodies
PBC	primary biliary cholangitis
PDC-E2	E2 subunit of pyruvate dehydrogenase
2-OA	2-octynoic acid
SAc	6,8-bis (acetylthio) octanoic acid
Ig	immunoglobulin
PBMC	Peripheral blood mononuclear cells
PPAb	Plasmablast derived polyclonal Antibodies
mAbs	monoclonal antibodies
IGHV	Ig heavy-chain variable regions
IGLV	Ig light-chain variable regions
LA	lipoic acid

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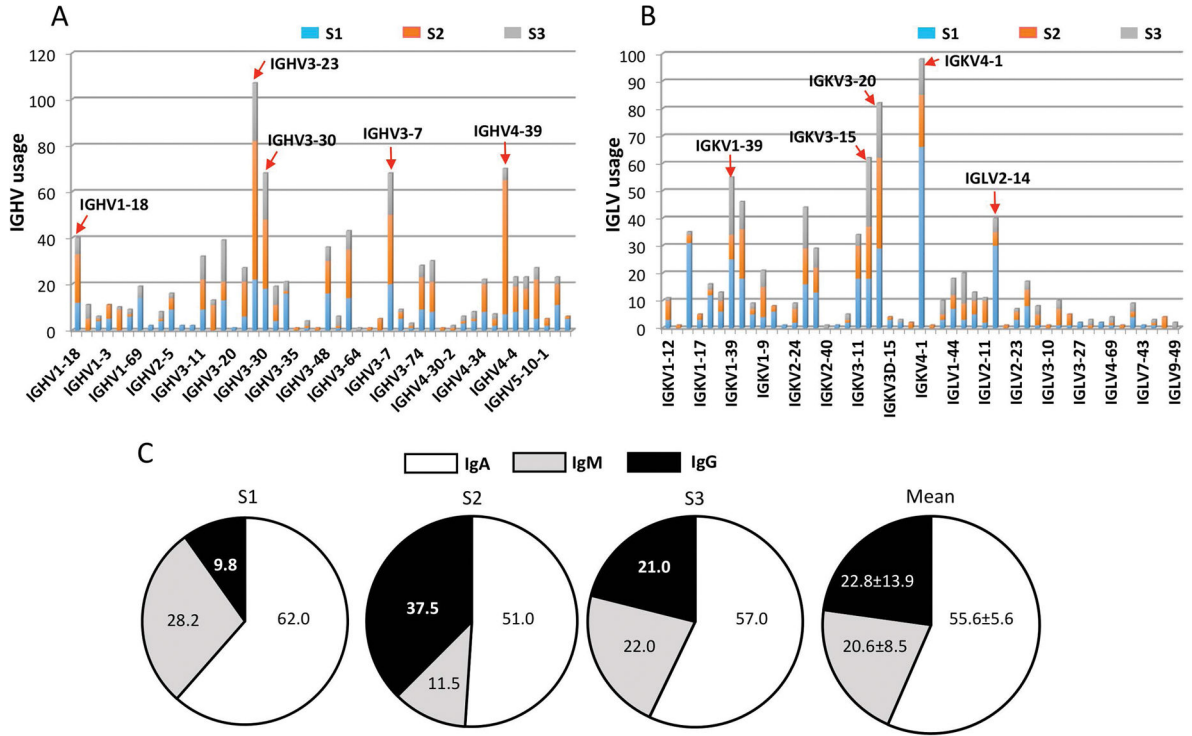


Figure 1. Single-cell analysis of antibody repertoire and isotype distribution. Antibody repertoire of plasmablasts A). 992 functional IGHV transcripts were identified, with IGHV3-23 transcripts being the most frequent. Red arrows indicate the frequently used IGHV families. B) 790 functional IGLV transcripts were identified, with the IGKV3-20 and IGKV4-1 transcripts being the most frequent. Red arrows indicate the frequently used IGLV families. C) Antibody isotype distribution. 568 functional and paired HV: LV antibody repertoire was analyzed. The variation in the frequency of IgA, IgM and IgG among the 3 PBC patients are presented in pie charts.

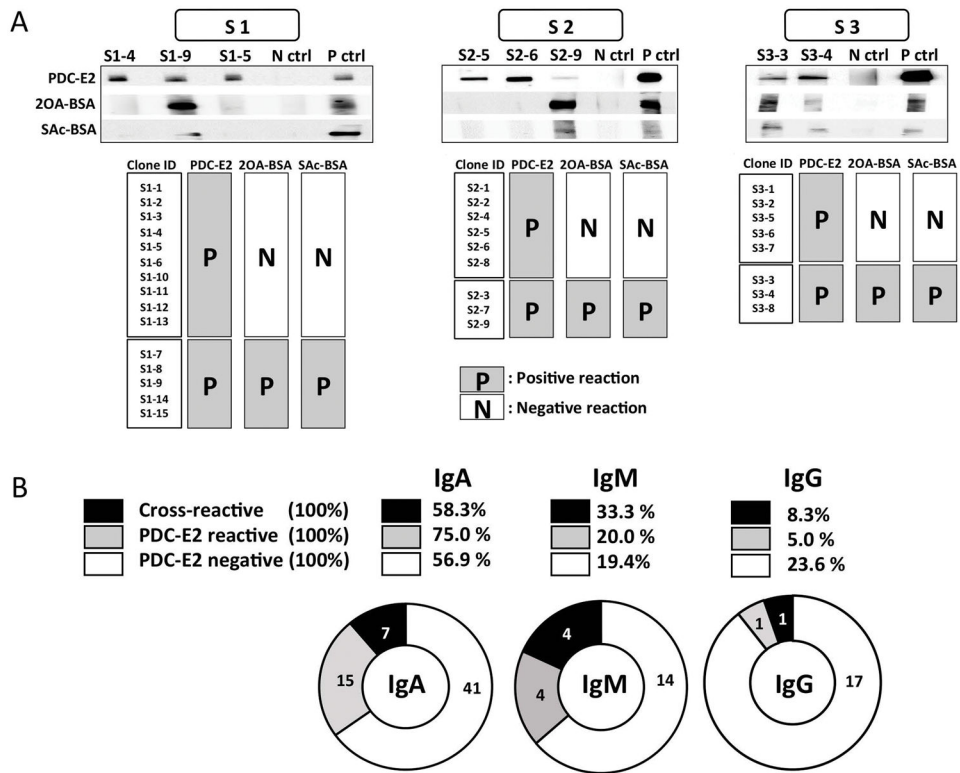


Figure 2. Antigen specificity and cross-reactivity of mAbs against PDC-E2, 2OA-BSA and SAc-BSA. A) Immunoblot assay was used to identify antigen specificity and cross-reactivity. mAbs cross-reacting with PDC-E2, 2OA-BSA and SAc-BSA were detected in all three subjects (S1, S2 and S3). Culture media only were served as negative controls (N ctrl); PBC patient sera diluted from 1:1,000 to 1:200,000 served as positive controls (P ctrl). B) The frequencies of PDC-E2 negative, PDC-E2 reactive and PDC-E2/xenobiotics cross-reactive clones. Antibody isotype distribution were examined from 104 randomly selected clones and screened for antigen specificity and cross-reactivity against PDC-E2, 2OA-BSA and SAc-BSA (IgA, n=63; IgM, n=22; IgG, n=19). The numbers in pie charts reflect the number of PDC-E2 negative, PDC-E2 reactive and PDC-E2/xenobiotics cross-reactive clones, respectively.

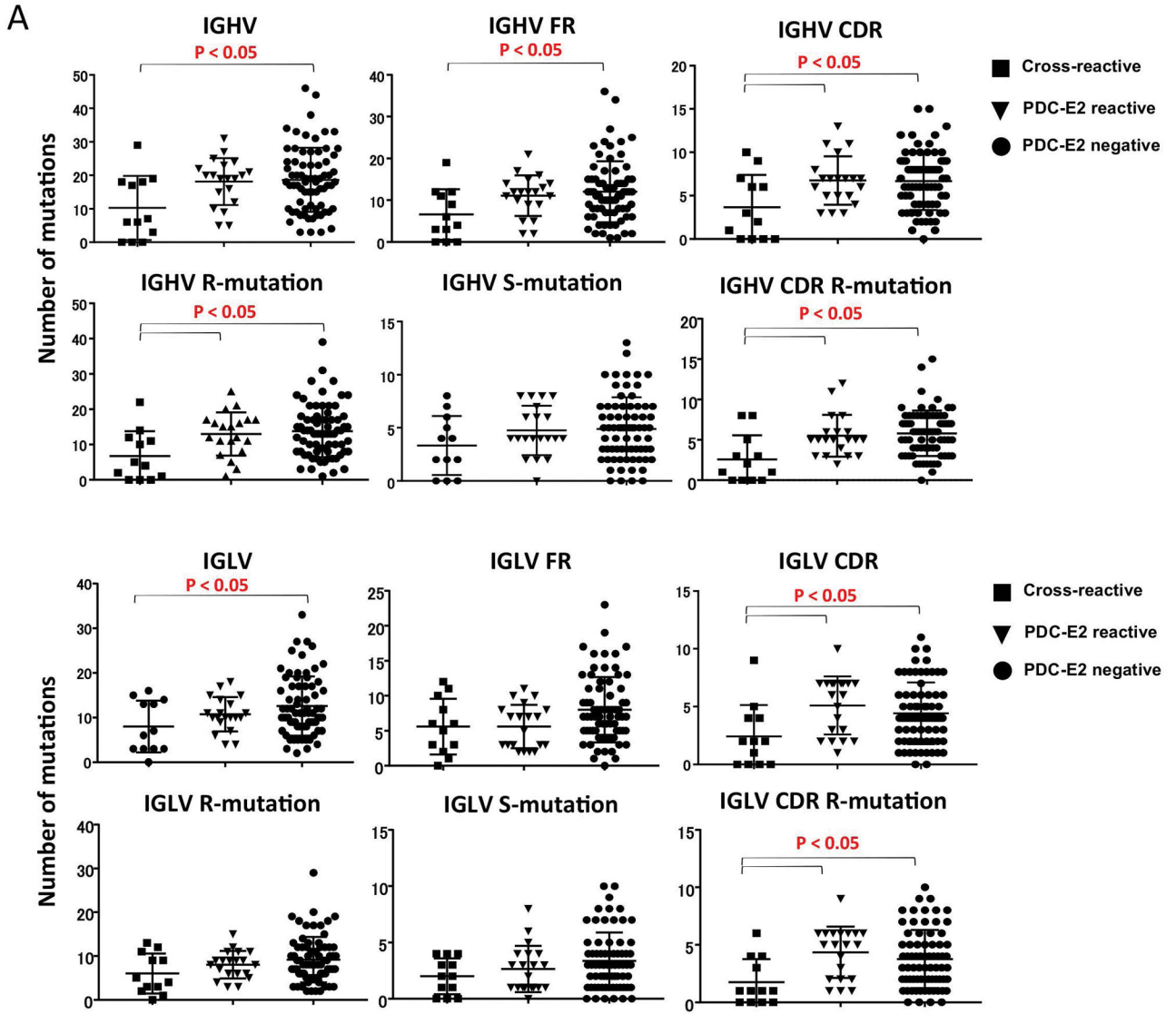


Figure 3. Comparison of number of mutations in IGHV regions and IGLV regions in PDC-E2/xenobiotic cross-reactive, PDC-E2 reactive and PDC-E2 negative mAbs
 A) Comparison of the number of mutations in IGHV, framework (FR) and CDR region of IGHV sequences (upper panels). Comparison of the number of replacement (R) and silent (S) mutations in IGHV and R-mutation in CDR region of IGHV-sequences (lower panels).
 B) Comparison of the number of mutations in IGLV, FR and CDR region of IGLV sequences (upper panels). Comparison of the number of R-and S-mutation in IGLV, and R-mutation in the CDR region of IGLV-sequences (lower panels).

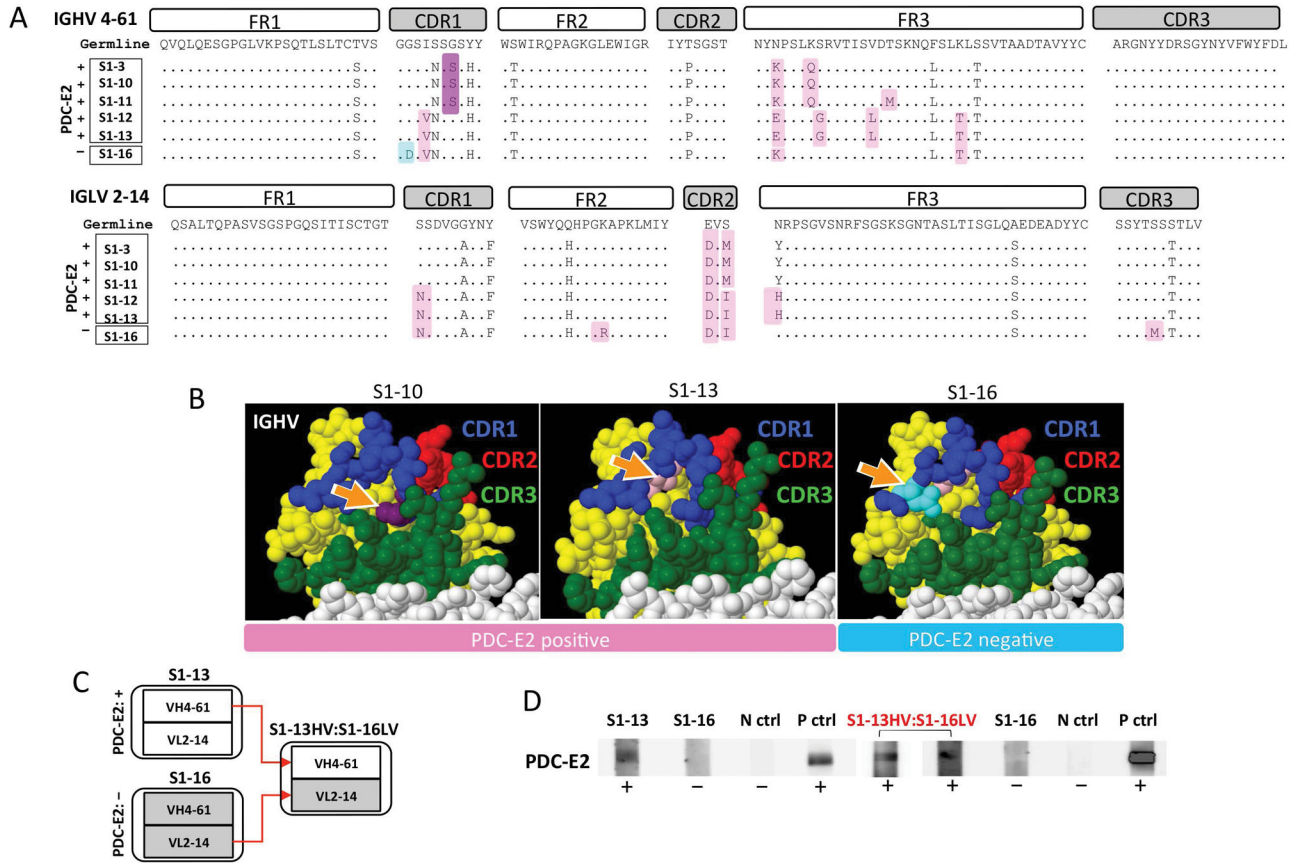


Figure 4. CDR1 amino acid (AA) sequence in IGHV 4-61:IGLV 2-14 family is critical for PDC-E2 recognition

A) Evolutionary AA replacements were analyzed in six evolutionarily closely related clones, which utilized IGHV4-61 and IGLV2-14. B) 3-D structures of PDC-E2 reactive (S1-3, S1-10, S1-11, S1-12 and S1-13) and a PDC-E2 negative clone (S1-16). Orange arrows indicate the sites of CDRs mutations. Note that 2AA replacements (G→S and I→V) in CDR1 did not affect the reactions to PDC-E2, whereas AA replacement (G→D) located on the surface in CDR1 region resulted in loss of PDC-E2 recognition. C) Illustration of artificial recombinant mAb construction. The IGHV segment was cloned from PDC-E2 positive antibody S1-13, and the IGLV segment was cloned from PDC-E2 negative S1-16. D) The AA replacements in IGHV were more critical for antigen reactivity than that in IGLV. Immunoblotting results demonstrated that PDC-E2 reactivity was comparable between the original clone S1-13 and designer clone “S1-13 IGHV: S1-16 IGLV” (S1-13 IGHV was constructed by pairing IGLV from the PDC-E2 negative clone S1-16).

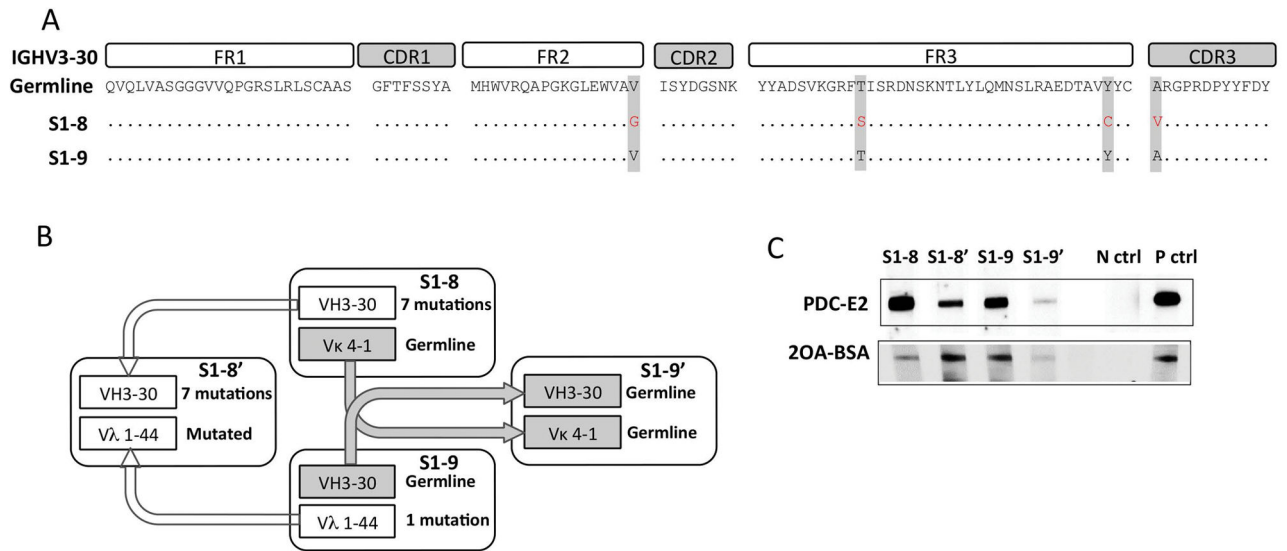


Figure 5. Germline-encoded clone strongly reacted to xenobiotics

A) AA sequences of cross-reactive clones S1-8 and S1-9 aligned with IGHV 3-30 germline sequence. B) Schematic representation of conversion of germline IGHV3-30-reverted antibody generation. C) A side-by-side comparison of antigen reactivities against PDC-E2 and 2OA-BSA using immunoblots. Equal concentrations of mAbs were used to probe PDC-E2 and 2OA-BSA.

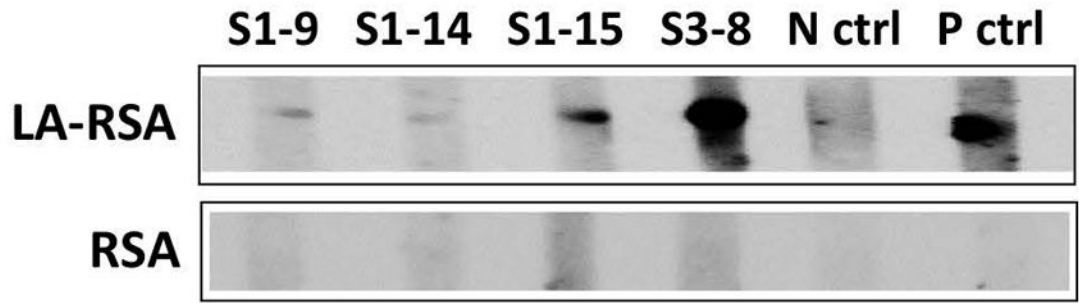


Figure 6. Cross-reactive mAbs recognize LA. Reactivity of PDC-E2/xenobiotic cross-reactive clones to lipoic acid (LA). Immunoblotting results demonstrate that PDC-E2/xenobiotic cross-reactive mAbs reacting to LA-RSA, but not RSA. N ctrl, negative control; P ctrl, positive control.