Clonal Characteristics of Circulating B Lymphocyte Repertoire in Primary Biliary Cholangitis

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Primary biliary cholangitis (PBC) is an autoimmune liver disease characterized by elevated serum anti-mitochondrial Ab and lymphocyte-mediated bile duct damage. This study was designed to reveal the clonal characteristics of B lymphocyte repertoire in patients with PBC to facilitate better understanding of its pathogenesis and better management of these patients. Using high-throughput sequencing of Ig genes, we analyzed the repertoire of circulating B lymphocytes in 43 patients with PBC, and 34 age- and gender-matched healthy controls. Compared with healthy controls, PBC patients showed 1) a gain of 14 new clones and a loss of 8 clones; 2) a significant clonal expansion and increased relative IgM abundance, which corresponded with the elevated serum IgM level; 3) a significant reduction of clonal diversity and somatic hypermutations in class-switched sequences, which suggested a general immunocompromised status; 4) the reduction of clonal diversity and enhancement of clonal expansion were more obvious at the cirrhotic stage; and 5) treatment with ursodeoxycholic acid could increase the clonal diversity and reduce clonal expansion of the IgM repertoire, with no obvious effect on the somatic hypermutation level. Our data suggest that PBC is a complex autoimmune disease process with evidence of B lymphocyte clonal gains and losses, Ag-dependent oligoclonal expansion, and a generally compromised immune reserve. This new insight into the pathogenesis of PBC opens up the prospect of studying disease-relevant B cells to better diagnose and treat this devastating disease. The Journal of Immunology, 2016, 197: 1609–1620.

Primary biliary cholangitis (PBC) is an autoimmune cholestatic liver disease. Histologically, PBC is characterized by dense portal lymphoplasmacytic infiltrates and ductal-centric granulomas with damage of biliary epithelial cells (BECs). The serological hallmark of PBC is the presence of high-titer anti-mitochondrial Abs (AMAs) that mainly target the E2 subunit of the pyruvate dehydrogenase complex (PDC-E2) (1). PDC-E2–specific Ab-secreting cells account for 23, ~10, and ~10% of total IgM, IgA, and IgG secreting cells in the circulation, respectively (2). No direct evidence so far has linked the destruction of BECs with AMAs, although several studies have provided clues in favor of the involvement of AMAs in the damage of BECs (3, 4). In addition to the elevated AMAs, high serum levels of IgM (1, 5), slightly increased IgG or IgA, and antinuclear Abs in up to 50% of PBC patients were seen (1, 6). Among the cells targeting bile ducts in portal areas, significant proportions were B lymphocytes and plasmacytes (7). Therefore, it is conceivable that Ag-driven oligoclonal expansion of B lymphocytes in the circulation exists, which could be responsible for the secretion of Abs of different isotypes and contribute to portal tract inflammation. Furthermore, disease-associated clonotypes of T lymphocytes have been uncovered recently in patients with PBC and primary sclerosis cholangitis (8). Further analysis of the clonal characteristics of B lymphocytes could gain

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Abbreviations used in this article: ALP, alkaline phosphatase; AMA, anti-mitochondrial Ab; BEC, biliary epithelial cell; CSR, class switch recombination; GC, germinal center; HEC, healthy control; HEC–lineage, lineage whose reads from all unique CDR3s account for >0.1% of the total reads represents a highly expanded and expressed clone; HEC–unique CDR3, unique CDR3 clone whose reads account for >0.1% of total reads stands for a highly expressed clone; HTS, high-throughput sequencing; IGH, Ig H chain; IGHL, J region of IGH; IGHV, V region of Ig H chain; IGL, Ig L chain; PBC, primary biliary cholangitis; PDC-E2, E2 subunit of the pyruvate dehydrogenase complex; SHM, somatic hypermutation; UDCA, ursodeoxycholic acid.
a complete picture of the immunological processes in PBC patients. Identifying the disease-specific B lymphocyte clones could also help us to achieve earlier specific diagnosis and design more targeted approaches for the effective treatment of the disease.

High-throughput sequencing (HTS) of the Ig repertoire is a platform that combines the multiplex PCR amplification of Ig genes, HTS of PCR products, and bioinformatics analysis of gene sequences. This platform has provided unprecedented insights into the understanding of immune repertoire development. HTS has now been widely applied in Ab discovery and in studies of infectious diseases and B lymphocyte–originated neoplasms (9). In patients with multiple sclerosis, HTS of the Ig repertoire has revealed evidence of clonal expansion and activation of B cells in cerebrospinal fluid (10). Expanded IgG4+ clones were observed in the affected tissue and blood of IgG4-associated cholangitis (11). Analysis of the autoreactive gut plasma cells in celiac disease and their corresponding memory compartment in the peripheral blood using HTS has demonstrated expanded, low somatic hypermutated and biased V region of Ig H chain (IGHV)–containing clones (12).

In this study, using HTS technology, we first attempted to identify the unique B cell clones in the PBMCs of PBC patients by comparing them with age- and gender-matched healthy controls (HCs). We then analyzed the features of the Ig repertoire, including IGHV and J region of IGH (IGHJ) gene segments usage, and clonal diversity and expansion in PBMCs of PBC patients. Our study has provided new insights into the clonal characteristics of B lymphocytes in PBC patients.

**Materials and Methods**

**Patients and samples**

After obtaining approval from the Institutional Ethics Review Board, we collected venous blood from 34 consenting healthy volunteers from FuXing Hospital, Capital Medical University, and 43 consenting PBC patients from Beijing YouAn Hospital, Capital Medical University, from September 2010 to August 2014.

The diagnosis of PBC was based on the established criteria (6). Patients with coexisting infectious disease, alcoholic liver disease, nonalcoholic steatohepatitis, PBC–autoimmune hepatitis overlap, other autoimmune disease, diabetes, recent inoculation, and immunosuppressive treatment and cancers were excluded from this study. Among the 43 PBC patients, 27 were noncirrhotic and 16 were cirrhotic. Among the 27 noncirrhotics, 10 were recently diagnosed before ursoodeoxycholic acid (UDCA) treatment, and 17 were previously diagnosed with PBC and had been treated with UDCA for >12 mo (13–15 mg/kg/d). Among the 16 cirrhotic PBC patients, 13 were UDCA treated and 3 UDCA untreated (Table I).

The selection criteria for healthy controls were 1) age and gender matched; 2) no apparent self-perceived discomfort and abnormality in the follow-up health checks; 3) no biological relationship with each other; 4) no medical history of autoimmune disorders, cancers, infectious diseases, liver diseases, allergy, and diabetes; and 5) no family history of autoimmune diseases.

**Sample collection and preparation**

From each 5-mL venous blood sample, 4–5 × 10^6 PBMCs were isolated by density gradient centrifugation using Ficoll-Paque plus (GE Healthcare, Pittsburgh, PA), deposited in RNAlater (Qiagen, Hilden, Germany), and stored at −80°C. RNA was extracted by using AllPrep DNA/RNA mini kit (Qiagen), whose quality was evaluated by the Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA).

**cDNA synthesis from IGH mRNA**

After DNase I treatment, reverse transcription (Invitrogen, Carlsbad, CA) was performed in a tube by mixing 200 ng of total RNA with 1 μl of reverse gene-specific primers (2.5 μM each) targeting the C region 1 of IGH of five isotopes. After denaturation for 5 min at 65°C, the mixture was quickly chilled on ice. After centrifugation with 4 μl of 5X first-strand buffer, 1 μl of DTT (0.1 M), 1 μl of RNaseOUT (40 U/μl), and 2 μl of dNTPmix (10 mM each), the system volume was adjusted to 19 μl by adding diethyl pyrocarbonate–treated water. After incubation at 25°C for 2 min, 1 μl SuperScript II reverse transcriptase was added and reverse transcription was performed at 42°C for 50 min before heat inactivation at 70°C for 15 min.

**PCR amplification of IGH**

Primers (12 targeting V and 5 targeting C gene segments), with a minimal and non–self-hybridizing primer set, were devised to acquire maximum coverage of a heterogeneous set of target sequences of V and C families, and amplification bias was evaluated and reduced to a minimum (13). Details of primers were filed as a Chinese patent (CN103184216A).

Multiplex PCR amplification of the framework region 3 → constant 1 region (including an intact CDR3 region) of IGH genes was implemented as described below: after 25 μl of 2X Qiagen multiplex PCR master mix, 5 μl of 5X Q solution, 1 μl of IGHV gene primer set pool, and 1 μl of IGHC gene primer set pool (0.2 μM each) were added into 18 μl of the reverse-transcribed products, a reaction system was formed, and the amplification process was initiated by using a multiplex PCR kit (Qiagen). Then PCR was performed at one cycle of 95°C for 15 min, 35 cycles of denaturation at 94°C for 30 s, and 30 cycles of both annealing at 60°C for 90 s and extension for 30 s at 72°C. After a final extension for 5 min at 72°C, the system was cooled down to 12°C.

**Purification of target fragment of multiplex PCR product**

The PCR products were run by agar gel electrophoresis. Target fragments of 200–300 bp were retrieved and purified by a QIAquick gel purification kit (Qiagen). DNA library preparation, cluster generation, template hybridization, isothermal amplification, linearization, blocking and

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**Table I. Clinical features of PBC patients included in the study**

| PBC (n = 43) |
|-------------|
| **PBC (Noncirrhotic) (n = 27)** |
| **PBC Untreated (n = 10)** | **PBC Treated (n = 17)** |
| Gender (F/M) | 10/0 | 14/3 |
| Age (y) | 56 (34–72) | 51 (30–73) |
| ALT | 87.6 (19–154) | 34.9 (17–172) |
| AST | 99.1 (26–229) | 34.9 (21–117) |
| Tbil | 20.35 (10.1–55.7) | 15.6 (8.1–63.5) |
| ALB | 36.45 (30–106) | 46.15 (13–98) |
| GGT | 559.45 (56–1213) | 76.15 (30–136) |
| ALP | 345.75 (82–882) | 30.15 (9.8–431) |
| IgG | 18.6 (12.8–22.5) | 18.3 (11–26) |
| IgA | 2.91 (2.01–5.26) | 2.76 (1.58–8.66) |
| IgM | 5.71 (1.37–9.65) | 4.75 (0.57–18.0) |
| AMA (+/−) | 170 | 160 |

ALB, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; F/M, female and male; GGT, γ-glutamyl transpeptidase; Tbil, total bilirubin.
denaturation, and hybridization of sequencing primers were performed as described elsewhere (14). Paired-end sequencing of samples was carried out with a read length of 151 bp using the Illumina HiSeq 2500 platform.

Analysis of illumina sequencing data

Raw sequencing data were processed and analyzed following the procedure established by Zhang et al. (13) by 1) filtering of raw reads to remove the undesired sequences, 2) merging of the remaining high-quality paired reads using COPE and FqMerger (BGI, Shenzhen, China) to obtain “contigs,” 3) aligning to reference IGH V(D)/J/C gene sequences (http://www.imgt.org/download/GENE-DB/) using the Basic Local Alignment Search Tool to determine the IGH V(D)/J/C gene segment usage of each contig, 4) spotting of the CDR3 region based on the combined IGH V(D)/J/C segments usage, 5) realigning the outer regions beyond CDR3 of contigs to ascertain the utmost matched IGH V(D)/J/C gene segments, and 6) refining the preliminary aligned contigs to acquire the optimal ones. Once the high-quality contigs were acquired, IgH CDR3 regions were identified within the sequencing reads according to the method proposed by the International ImMunoGeneTics collaboration (15). Some basic features and further analysis of contigs were implemented based on nucleic acid and amino acid level, respectively. Class-switched lineages were also constructed: IgM sequences and IgG sequences from the same patients were pooled together, and then lineages were constructed according to their definition. After construction, any lineages that contained both IgM and IgG sequences were deemed as class-switched lineages. A limitation of the HTS technique is that the original absolute number of Ig transcripts in a given sample could not be determined, and the actual number of B cells expressing each CDR3 cannot be known.

Statistical analysis

Because normal distribution could not be guaranteed, statistical comparisons (R software, version 3.0.3) were performed using nonparametric tests: Mann–Whitney test, or Kruskal–Wallis test followed by a Dunn post hoc analysis (when more than two groups were compared). The relationship between two variables was assessed by the Spearman rank correlation test. A two-tailed p value <0.05 was considered significant. All 34 HCs were used when comparing with PBC patients overall. To obtain matched sample sizes, 19 subjects were randomly selected from the 34 HCs when comparing with PBC patients overall. To obtain matched sample sizes, 19 subjects were randomly selected from the 34 HCs when comparing with PBC patients overall. To obtain matched sample sizes, 19 subjects were randomly selected from the 34 HCs when comparing with PBC patients overall.

Results

A total of 551 million raw reads representing 137 gigabase pairs from 77 samples were obtained after sequencing, with a final 257,823,005 contigs of BCR IGH CDR3 acquired. An average of 3,348,351 CDR3s and 55,785 unique CDR3s were generated per sample.

It is well known that the Ag specificity of a B cell clone is determined by the BCR, which is made up of IGH and IgL chain (IGL), but after cytolysis, IGH mRNA and IGL mRNA will not remain paired, so it is very hard to define a B cell clone by both IGH and IGL. In line with most investigators, B cell clones were defined by H chains (10–12, 16, 17). An amino acid unique IGH CDR3 sequence represents an actual existing B cell clone. Lineages represent biological clones that have undergone clonal expansion (16). Two unique CDR3 sequences that belong to the same V and J gene family, and whose sequences differ by no more than one amino acid, will be clustered into the same lineage.

Gains and losses of B lymphocyte clones in PBC patients

To look for the disease-associated clones, we first compared the degree of clonal overlap among PBC patients with that among HCs, and then searched the commonly shared clonotypes among PBC patients as well as among HCs.

Out of the overall 2,233,324 kinds of unique CDR3 clones among the 43 PBC patients, there were 40,565 kinds that were overlapping (each kind was present in at least 2 PBC patients). The ratio of these overlapping clonotypes to total unique CDR3 clones in PBC patients (1.816%, 40,565/2,233,324) was significantly higher than its counterpart among the 34 HCs (1.588%, 31,605/1,989,912) (p = 7.92 × 10^{-17}) (Table II).

From the perspective of clonal expression, the relative abundance (the ratio of the reads of the overlapping clones to the total reads from all PBC patients) of the 40,565 kinds of overlapping clones among the 43 PBC patients (34.53%, 44,203,350/128,010,057) was significantly higher than that among the 34 HCs (27.15%, 32,691,222/120,421,480) (p = 0.00000) (Table III).

We further searched for unique CDR3 clones commonly present in PBC patients and absent from HCs, as well as those present in HCs and absent from PBC patients. We identified 14 clones common among at least 6 of the 43 PBC patients and present in none of the 34 HCs, and 34 PBC patients in total had at least 1 of the 14 clones (Fig. 1A). The size of lineages (the number of unique CDR3s a lineage contained) to which each B-associated clonotype belonged in different PBC patients differed from one another (Fig. 1B). These shared clonotypes suggested a background of common Ag-driven positive selection in PBC patients. We also identified eight unique CDR3 clones common in at least 6 of the 34 HCs and present in none of the PBC patients, and 24 HCs altogether had at least one of the eight clones. A significant difference was not observed between the relative abundance of the 14 clones among PBC patients and that of the 8 clones among HCs (Supplemental Fig. 1A). Similarly, the difference in the relative abundance and the size of the lineages to which the clones belonged was not significant (Supplemental Fig. 1B, 1C). In PBC patients, the absence of these clonotypes commonly shared among HCs suggested a background of common negative selection.

V and J gene segment usage and the hydrophobicity and length of IGH CDR3 in PBC patients

IGHV (or IGHJ) segment usage in a sample is defined as the ratio of reads that contain the IGHV (or IGHJ) segment in question to the total reads (the relative frequency of the IGHV segment). Compared to HCs, preferential usage of IGHV5-51 (p = 0.03) reduced usage of IGHV1-18 (p = 0.04), and aberrant usage of 16 other IGHV segments whose relative frequency was <10% was

| Table II. Comparison of clonal overlap degree (unique CDR3 number rate) between PBC patients and HCs |

|            | Shared Unique CDR3 Number | Nonshared Unique CDR3 Number | Total Unique CDR3 Number | Shared Unique CDR3 Number Rate |
|------------|---------------------------|-----------------------------|--------------------------|-------------------------------|
| PBC        | 40,565                    | 2,192,759                   | 2,233,324                | 0.018164                      |
| HCs        | 31,605                    | 1,958,307                   | 2,029,912                | 0.015883                      |
| Total      | 72,170                    | 4,151,066                   | 4,223,236                | 0.017089                      |

Statistical analysis was performed by a Pearson χ² test with a Yates continuity correction: χ² = 325.7954, p = 7.92 × 10^{-17}. Shared unique CDR3 number represents the total unique CDR3 sequences at amino acid level that are shared by at least two samples of the 43 PBC patients, or the 34 HCs. Total unique CDR3 number represents the number of total unique CDR3 sequences of all the 43 PBC patients, or the 34 HCs. Nonshared unique CDR3 number represents the total number of unique CDR3 sequences that are not shared by any two or more samples of the 43 PBC patients, or 34 HCs. Shared unique CDR3 number rate is the ratio of shared unique CDR3s to the total unique CDR3s.
also observed in PBC patients (p < 0.05). Significant differences in the usage of IGHV1-46, IGHV2-26, IGHV2-70, IGHV3-13, IGHV3-15, and IGHV3-72 between cirrhotic and noncirrhotic PBC patients were discovered. IGHV2-5 usage in the UDCA-treated PBC patients was significantly reduced as compared with the untreated PBC patients (p = 0.03), but no significant impact of UDCA treatment on the usage of other IGHV segments was observed (Fig. 2).

We further compared the IGHV (or IGHJ) segments usage in all of the overlapping and nonoverlapping clones among PBC patients, and also those among HCs. Relative to the total nonoverlapping clones among PBC patients (or HCs), significantly different usage of most IGHV segments (>40 of all 48) was observed in all overlapping clones in both PBC patients and HCs. Usage of seven segments (IGHV1-2, IGHV1-46, IGHV1-69, IGHV2-5, IGHV3-33, IGHV3-53, and IGHV5-51) in all overlapping clones among PBC patients was significantly different as compared with HCs. There was no statistically biased usage of IGHJ segments in the overlapping clones among PBC patients as compared with that among HCs (Fig. 3A, 3B).

Table III. Comparison of clonal overlap degree (unique CDR3 reads rate) between PBC patients and health controls

|                  | Shared Unique CDR3 Reads | Nonshared Unique CDR3 Reads | Total Unique CDR3 Reads | Shared Unique CDR3 Reads Rate |
|------------------|--------------------------|----------------------------|-------------------------|-----------------------------|
| PBC              | 44,203,350               | 83,806,707                 | 128,010,057             | 0.34531                     |
| HCs              | 32,691,222               | 87,730,258                 | 120,421,480             | 0.27147                     |
| Total            | 76,894,572               | 171,536,965                | 248,431,537             | 0.30952                     |

Statistical analysis was performed by a Pearson $\chi^2$ test with a Yates continuity correction: $\chi^2 = 1582936$, p = 0.00E+00. Shared unique CDR3 reads represent the number of total unique CDR3 reads that are shared by at least two samples of the 43 PBC patients, or the 34 health controls. Total unique CDR3 reads represent the number of total unique CDR3 reads of the 43 PBC patients, or the 34 HCs. Nonshared unique CDR3 reads represent the total number of unique CDR3 reads that are not shared by any two or more samples of the 43 PBC patients, or 34 HCs. Shared unique CDR3 reads rate is the ratio of shared unique CDR3 reads to the total unique CDR3 reads.

FIGURE 1. Gains and losses of B lymphocyte clones in PBC patients. (A) The red boxes (small, medium, or large) represent the 14 commonly shared unique CDR3 sequences among PBC patients, but that are absent in 34 HCs. The blue boxes correspond to the eight unique CDR3s commonly present in HCs, but that are absent in PBC patients. In each row on the right, the number represents how many patients or HCs had a particular sequence; in each column, the number on top of the small red box or small blue box represents how many sequences a particular patient or HC had concurrently. (B) Diagram of seven lineage trees to which a representative unique CDR3 clone (ARDPSDY) that was present in seven PBC patients belonged. Each individual cluster of knots connected by lines represents a lineage. Each knot represents a unique CDR3 clone, and the size of the knot represents the total number of copies (reads). Knots of different colors denote different Ig isotypes, and knots inside a square share the exact same sequence among the seven PBC patients. Note that a lineage is comprised of clonally related unique CDR3s. Any two neighboring knots in a lineage connected by a line differ by a single amino acid. The more knots a lineage contains, the bigger the lineage size.
For the 14 commonly shared clones among PBC patients, significantly reduced usage of IGHV1-3, IGHV1-8, IGHV1-24, IGHV1-46, IGHV1-69, IGHV3-11, IGHV6-1, IGHV7-4-1, IGH3, IGHJ5, and IGHJ6 (p = 0.000) and significantly preferential usage of IGHV1-18, IGHV3-21, IGHV3-30, IGHV3-48, IGHV3-7, IGHV5-51, and IGHJ4 (p = 0.000) segments were observed when compared with the 8 commonly shared clones among HCs (Fig. 3C, 3D).

Compared to HCs, significant differences in both the length and hydrophobicity of CDR3s in PBC patients overall and its subgroups were not found (Supplemental Fig. 2). Relative to the total non-overlapping clones among PBC patients (or HCs), a significantly decreased length and hydrophobicity of CDR3 was observed in all overlapping clones in PBC patients and HCs (Supplemental Fig. 3A, 3B), suggesting that the overlapping clones were Ag experienced and highly expanded (18). For the 14 commonly shared clones among PBC patients, no significant difference in the length and hydrophobicity of CDR3 was observed when compared with the 8 clones among HCs (Supplemental Fig. 3C, 3D).

Potent antigenic stimulation often results in preferential usage of certain Ig gene segments (19, 20). The aberrant use of IGHV segments observed in PBC patients overall and in its subgroups, as well as in the overlapping clones among PBC patients, suggests that PBC patients have a distinct background of Ag exposure, which leads to an increase in clonal expansion and expression level and a reduction in the hydrophobicity and length of CDR3 (18).

A decreased efficiency in B lymphocyte class switching

We studied the composition of different Ig isotypes in PBMCs of PBC patients. Compared to HCs, the relative IgM abundance was significantly increased (except in UDCA-treated group), whereas the relative IgG abundance was significantly decreased in PBC patients overall and all its subgroups (Fig. 4). The clinical stage and UDCA treatment did not show significant effect on the dominance of IgM and the decreased IgG (Fig. 4C, 4D), which were consistent with the facts that there were no significant differences in serum IgM levels between noncirrhotic and cirrhotic PBC patients and between UDCA-treated and UDCA-untreated PBC patients (Supplemental Fig. 4).

Naive B cells, with IgM on their surface, are activated upon Ag stimulation, and after class switching the C region changes from IgM to other isotypes (IgG, IgA, and IgE). By pooling IgM and IgG sequences together, we were able to identify lineages that later underwent class switching (16). Significantly fewer class-switched lineages in PBC patients overall, UDCA-untreated patients, and cirrhotic PBC patients and an insignificant reduction in non-cirrhotic PBC patients (p = 0.064) than HCs were observed (Fig. 4E), suggesting a reduced class-switching efficiency, which could have contributed to the accumulated relative abundance of IgM and decreased abundance of IgG (16).

Reduction of average SMHs in class-switched isotypes

We investigated average mutations in the IGHV region generated by HTS from PBC patients and HCs. IgG and IgA average mutations were significantly fewer in PBC patients overall and subgroups with two exceptions, that is, the UDCA-treated group and IgG average mutation in the noncirrhotic group (p = 0.081) (Fig. 5). Clinical stage and UDCA treatment had no significant impact when noncirrhotic and cirrhotic, UDCA-untreated and UDCA-treated groups were compared, respectively (Fig. 5B, 5C). There was no significant difference of IgM average mutations between PBC patients and HCs, suggesting that the reduction of somatic mutations in PBC patients was limited to class-switched isotypes.

Reduced clonal diversity and enhanced clonal expansion and expression in PBC patients

The numbers of unique CDR3s and lineages were used as a measure to evaluate the clonal diversity of the B cell repertoire (16, 21). A unique CDR3 clone whose reads account for >0.1% of total reads stands for a “highly expressed clone” (HEC–unique CDR3) (22). A lineage whose reads from all unique CDR3s account for >0.1% of the total reads represents a “highly expanded and expressed clone” (HEC-lineage) (22). The ratio of
FIGURE 3. Usage of IGHV and IGHJ segments in all of the overlapping clones among PBC patients and those among HCs. (A) IGHV segments usage in all of the overlapping and nonoverlapping clones among PBC patients as well as those among HCs: aberrant usage of IGHV1-2, IGHV1-46, IGHV3-d, IGHV4-61, IGHV4-b, and IGHV5-a segments in the total nonoverlapping clones among PBC patients than those among HCs (p < 0.05) is shown; deviated usage of IGHV1-2, IGHV1-46, IGHV1-69, IGHV2-5, IGHV3-33, IGHV3-53, and IGHV5-51 segments in the all overlapping clones among PBC patients than those among HCs (p < 0.05) is also shown. (B) Significantly different usage of IGHJ1 and IGHJ6 segments in all of the overlapping clones among PBC patients, and significantly different usage of IGHJ3 and IGHJ6 in all of the overlapping clones among HCs, relative to both the nonoverlapping clones among PBC patients and those among HCs; no statistically biased usage of IGHJ segments in all of the overlapping clones among PBC patients as compared with those among HCs was found. (C) For the 14 commonly shared clones among PBC patients, reduced usage of IGHV1-3, IGHV1-8, IGHV1-24, IGHV1-46, IGHV1-69, IGHV3-11, IGHV6-1, and IGHV7-4-1 segments (p = 0.000) and preferential usage of IGHV1-18, IGHV2-21, IGHV3-30, IGHV3-48, IGHV3-7, and IGHV5-51 segments, compared with the eight commonly shared clones among HCs (p = 0.000), are shown. (D) Aberrant usage of IGHJ3, IGHJ4, IGHJ5, and IGHJ6 segments in the 14 clones among PBC patients, compared with the eight clones among HCs (p = 0.000). The p values were calculated by a Kruskal–Wallis test followed by a Dunn post hoc analysis (A and B), and by a Pearson χ² test with a Yates continuity correction (C and D).
HEC-lineage numbers to total lineage numbers was referred to as HEC-lineage number rate, and so was the HEC–unique CDR3 number rate, both of which were used to reflect the degree of clonal expansion and expression.

UDCA-untreated noncirrhotic PBC patients, who represent an early stage of the natural disease course, showed significantly decreased clonal diversity and increased clonal expansion and expression level, as measured by lineage numbers, unique CDR3 numbers, and HEC-lineage and HEC–unique CDR3 number rate of both IgM and IgG (Figs. 6E, 6F, 7E, 7F).

When UDCA-treated and untreated patients were compared, we found that UDCA treatment could restore the distorted IgM clonal diversity and increase clonal expansion and expression. This was evident in the CD4+ T cell population as well, where UDCA treatment led to a decrease in the percentage of CD4+ CD25+ FOXP3+ regulatory T cells, while increasing the percentage of CD4+ CD25- FOXP3- effector T cells.

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diversity, as well as clonal expansion and expression, by increasing the IgM-lineage numbers ($p = 0.06$) and unique CDR3 numbers ($p = 0.071$), and decreasing IgM HEC-lineage number rate ($p = 0.06$) and HEC–unique CDR3 number rate ($p = 0.013$) (Figs. 6E, 6F, 7E, 7F).

PBC patients overall and noncirrhotic patients demonstrated a significant reduction of IgG clonal diversity and increased IgG clonal expansion and expression compared with HCs, as measured by IgG lineage and unique CDR3 numbers (Fig. 6A–D), and IgG HEC-lineage and HEC–unique CDR3 number rate (Fig. 7A–D).

Relative to HCs, cirrhotic PBC patients showed a significant reduction of clonal diversity and elevation of clonal expansion and expression, as indicated by statistically lower IgA, IgG, and IgM lineage and unique CDR3 numbers, and significantly higher HEC-lineage and unique CDR3 number rate. Compared to the non-cirrhotic patients, a significant further reduction of clonal diversity and elevation of clonal expansion and expression were not observed in cirrhotic patients (Figs. 6C, 6D, 7C, 7D).

Taken together, these data suggested a compromised immune reserve potentiality and an enhanced humoral immune response existed in PBC patients, especially at the later clinical stage.
Enhanced clonal expansion and expression in patients with PBC. (A and B) Comparison of clonal expansion and expression degree of Ig isotypes between UDCA-untreated patients, UDCA-treated patients, and HCs. (C and D) Comparison of clonal expansion and expression degree of Ig isotypes between UDCA-untreated patients, UDCA-treated patients, and HCs.

Discussion
Using HTS technology and advanced bioinformatics analysis, we observed a higher degree of clonal overlap among PBC patients than HCs. Although these overlapping unique CDR3s only accounted for a small proportion of the total unique CDR3s (PBC, 1.8164%; HCs, 1.5883%), from the perspective of clonal expression, their corresponding relative abundances were much higher (PBC, 34.531%; HCs, 27.147%). These findings, in conjunction with the biased IGHV segment usage in all overlapping clones in PBC patients, suggest that an enhanced Ag-driven oligoclonal expansion occurs in PBC patients. We uncovered 14 commonly shared B cell clones only present in the periphery of PBC patients, and 8 commonly shared B cell clones only present in HCs. It is reasonable to speculate that some of the gained clones could be the culprit for the development of PBC and that some of the missing B lymphocyte clones could guard against the development of PBC. We have searched these highly repeated 14/8 unique CDR3 sequences in PubMed, and only three of them are present as part of three IGHV sequences with a length of ∼100 aa. We were unable to find any functional description of these three IGHV sequences. Ag-specific BCR sequences have been identified recently using public repertoire analysis (25). Because all PBC patients included here were AMA+, it will be important to study the repertoire of AMA-specific B cells to confirm whether these frequently repeated sequences among PBC patients target PDC-E2, which is the main Ag that binds AMAs. The function of B lymphocytes is regulated by T cells in T-dependent responses. Recently, Liaskou et al. (8) sequenced the TCR genes of 10 PBC patients and revealed 42 disease-associated T lymphocyte clones. Further characterization of these clones and extensive functional studies are required to pinpoint disease-specific B and T cell clones that could potentially be used as diagnostic markers and therapeutic targets.

Serum IgM and alkaline phosphatase levels closely associated with the distorted B cell repertoire
Circulating IgM-encoding memory B cells can rapidly expand upon Ag stimulation and differentiate into plasma cells in a T cell–independent manner, outside secondary lymphoid organs (23). Persistent Ag stimulation leads to highly expanded or expressed clones. We found that serum IgM level positively correlated with IgM HEC-lineage number rate and IgM HEC–unique CDR3 number rate (Fig. 8A, 8B), suggesting that higher serum IgM levels were at least partly a consequence of highly expanded and expressed circulating IgM-encoding memory or IgM-secreting plasmacyte clones.

Serum alkaline phosphatase (ALP), a marker of cholestasis, not only reflects disease severity (6), but also acts as a surrogate marker for the adverse outcomes of PBC patients (24). We observed that serum ALP level negatively correlated with the degree of clonal diversity in PBC patients (Fig. 8C–F), suggesting that the reduced clonal diversity in PBC patients indicates a disadvantageous clinical status.
B lymphocytes will experience clonal expansion in the presence of a cognate Ag, costimulatory signal, and Th cells. Somatic hypermutation of the V region will occur simultaneously, resulting in affinity maturation. As observed in this study, the highly expanded and expressed clones, which also resulted in the significantly biased usage of *IGHV* segments in PBC patients and its subgroups, could be due to the consequence of ongoing stimulation by endogenous bile duct epithelial Ags (1, 19, 20). The effective treatment of PBC patients who were refractory to UDCA with rituximab, which led to B cell depletion, through the reduction of AMA-secreting B cells, the titer of AMAs and serum IgM/G/A level, as well as serum ALP level, suggests that the highly expanded and expressed B lymphocytic clones have contributed to the abnormality of peripheral B cell immunity and may be involved in the pathogenesis of PBC patients (28). However, it is still unclear how and when the disease process was initiated. The reduced clonal diversity and increased clonal expansion of IgA, IgG, and IgM relative to HCs in cirrhotic PBC patients, as well as the significantly different *IGHV* segments usage from the noncirrhotic patients, could serve as potential markers for more severe and advanced clinical stages. The negative correlation between serum ALP and clonal diversity also suggested that the reduction in clonal diversity could also reflect disease severity and outcome (6, 24). The tendency of UDCA treatment to improve the distorted clonal diversity and expansion of IgM represents a new mechanism of UDCA treatment.

FIGURE 8. Correlation analysis between serum levels of IgM or ALP with variables of the Ig repertoire in PBC patients. (A and B) Serum level of IgM positively correlated with the HEC-lineage number rate and HEC–unique CDR3 number rate. (C–F) Serum level of ALP negatively correlated with the unique CDR3 number and lineage number of IgM and IgG. The *p* values were calculated by the Spearman rank correlation test.
A high level of serum IgM in most PBC patients is a unique phenomenon. Studies suggested it might be a consequence of hyperactive innate immune independent of T cells and mediated by IgM memory B cells either in the bloodstream or in the spleen (1, 5, 29, 30). Elevated serum IgM level has been used as a diagnostic marker for PBC, and the reduction of serum IgM level has been used as a marker of disease alleviation by clinicians (28, 31). It is conceivable, although there is a lack of direct evidence, to associate a higher serum IgM level with a more active inflammatory process in the liver. However, the exact role of IgM in the pathogenesis of PBC remains enigmatic. In this study, HTS of Ig genes using PBMCs revealed highly expanded and expressed IgM-expressing B lymphocytic clones in overall PBC patients and in its subgroups, resulting in significantly increased relative abundance of IgM. We also found that serum IgM level in PBC patients was positively correlated with the degree of IgM clonal expansion and expression, implying that the increased relative abundance of IgM was the result of an increased transcription level of expanded IgM-secreting circulating B lymphocyte clones, which could be an important source of the IgM secretion in PBC patients.

We also demonstrated a decreased IgG relative abundance in PBC patients, which might be due to the increased IgM relative abundance. This seems inconsistent with a higher proportion of overly expanded and expressed IgG-encoding clones and higher level of serum IgG. Indeed, we did not find any correlation between serum IgG level and any variables of the IgG repertoire, suggesting that the overexpanded IgG clones did not contribute significantly to IgG level. Other sources that overcome an increased IgG serum level might exist. In fact, B cell subsets such as IgM memory B cells, class-switched IgG/Iga memory B cells, and marginal zone B cells in secondary lymphoid organs all contribute to the serum Ab pool (23).

Previous research on patients with celiac disease has revealed limited SHMs in autoreactive plasma cells (12), which may be indicative of low-affinity Ag binding (32). In this study, limited SHMs in class-switched isotypes were also observed in PBC patients and their subgroups. SHMs occur in germinal centers (GCs) (33). Adaptive mutations and selections result in high-affinity Abs with higher activity (34). The limited Ab SHMs observed in PBC patients could be due to a flaw in the affinity maturation mechanism, or B cells might have experienced an extracellular evolution in GC reactions of short duration (33), or they could also be the result of a T cell–independent response. Class switch recombination (CSR) and somatic hypermutation are not necessarily interlinked processes, because there exist hypermutated IgM marginal zone B cells in the spleen and IgM memory B cells in the periphery, and CSR can occur outside of GCs without SHMs (19, 33). The limited SHMs in class-switched Ig isotypes suggested that CSR without apparent SHMs in IgG and IgA might also occur in PBC patients.

During the past decade, many genetic, environmental, and infectious factors that may contribute to the development of PBC have been studied (1, 6). However, there is limited knowledge regarding the precise role of B lymphocyte clonal changes in the pathogenesis of PBC. Our study demonstrates characteristics of the Ig repertoire in peripheral blood of PBC patients: gains and losses of certain B lymphocyte clones, aberrant properties of IgH CDR3, reduction of clonal diversity, enhanced clonal expansion and expression, fewer somatic hypermutations, and a decreased efficiency in B lymphocyte class switching. These abnormalities were more apparent at the advanced clinical stage, and UDCA treatment could partially restore some aspects of the deviated B cell repertoire, especially the IgM repertoire. This new insight into the pathogenesis of PBC could guide the development of a more balanced and targeted therapeutic approach.

Histologically, PBC is characterized by dense portal lymphocytic infiltration with a mixture of T cell, B cell, and plasmacyte components. Phenotyping the lymphocytic components could provide more direct evidence and further our understanding on the role of each component in the pathogenesis of PBC.

Disclosures
The authors have no financial conflicts of interest.

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