Chronic lymphocytic leukemia often arises by a multiclonal selection process

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Received: August 23, 2024.
Accepted: February 12, 2025.
Early view: February 20, 2025.

https://doi.org/10.3324/haematol.2024.286380

Abstract

Although chronic lymphocytic leukemia (CLL) is diagnosed by identifying a circulating B-cell clone that exceeds 5x10^3/μL, additional distinct clones (ADC) have been identified in various studies. Notably, the numbers of ADC documented in these studies has increased as the various technologies evolved. To better define the frequency and the characteristics of ADC in CLL, we used a next-generation sequencing platform that affords high sequencing depth along with steps that limit over-counting to analyze IGHV-IGHD-IGHJ gene rearrangements in circulating CD5$^+$ B cells from 57 patients. Notably, all patients had at least one ADC, in addition to the clinically relevant clone. In 46 patients for whom lymphocyte count data were available, 44 had at least one ADC above the threshold of 1 B cell/μL and, remarkably, the average number of ADC was 12 per patient. Notably, in two patients, the predominant ADC qualified clinically as a separate CLL clone and in the remaining cases as low/high-count monoclonal B-cell lymphocytosis clones. Moreover, in 11 patients studied longitudinally, predominant ADC were persistent and often increased in number. ADC in patients with CLL exhibited 4-fold more stereotyped IGHV-IGHD-IGH rearrangements than found in CD5$^+$ B cells from healthy individuals, and IGHV use, somatic mutations, and Ig isotype distribution were similar between predominant ADC and clinically relevant clones. Thus, finding multiple expanded clones within the CD5$^+$ B cells is the rule in patients with CLL, indicating that leukemogenesis is a multiclonal process that likely involves competition among B cells with special B-cell receptor features.

Introduction

Chronic lymphocytic leukemia (CLL) is defined as a monoclonal expansion of CD5$^+$ B lymphocytes that exceed a defined threshold.[1-4] However, the monoclonal definition can be violated by identifying another B-cell clonal expansion expressing a different IGHV-IGHD-IGHJ (IGHV-D-J) rearrangement. This has been observed at different frequencies in various studies, i.e., 2%,[5] 5%,[6] 13%[7] and 24%.[8] The presence of additional rearrangements has also been reported for monoclonal B-cell lymphocytosis (MBL), generally considered the pre-leukemic precursor of CLL.[9]

Knowledge accumulated over the past 20+ years indicates that the structure of the B-cell receptor (BCR) on CLL clones contributes strongly to the development and clinical course of the disease. For example, the mutation status of the expressed IGHV gene divides leukemic clones into two subsets, IGHV-mutated and IGHV-unmutated,[10,11] with unmutated CLL following a more virulent clinical course than mutated CLL.[12,13] Another prominent BCR structural feature of CLL is the non-random association of IGHV, IGHD, IGHJ genes that lead to the presence of remarkably similar VH CDR3 amino acid sequences by different clones,[14-17] referred to as “stereotyped BCR”. Moreover, patients whose clones express stereotyped BCR can exhibit similar characteristic genomic alterations, and experience comparable clinical

courses and outcomes.[18] Furthermore, CLL BCR can signal autonomously, i.e., without engaging antigens extrinsic to the membrane IG, because of BCR homo-dimerization.[19] Finally, specificity studies have indicated that many BCR display self- and poly-reactivity.[4] These collective findings and the significant clinical benefit from the use of small molecule inhibitors of BCR signaling[20] have led to the conclusion that BCR structure and function are intimately involved in the development of the disease.

In principle, CLL originates from a mature B-cell clone that progressively accumulates a series of transforming mutations, associated with ongoing BCR stimulation. This process could start at an earlier stage of hematopoiesis or B-cell maturation, even prior to the occurrence of IGHV-(D)-J gene rearrangement,[21-23] due to a somatic event or an inherited propensity, the latter implied by the strong familial association of CLL.[24] In this situation, many B cells, each with a distinct IGHV-D-J gene rearrangement, would have the opportunity to enter the leukemogenic process, with a series of events eventually favoring the emergence of a dominant, clinically relevant clone (CRC), with other clonal expansions being fingerprints of yet unachieved, and possibly ongoing, transformation.

In this study, we examined the IGHV-D-J gene repertoire of the entire CD5$^+$ B-cell population in 57 patients with CLL, employing a robust next-generation sequencing protocol that we developed.[25] Our findings show that the CD5$^+$ B cells from CLL patients contain additional clones (ADC) distinct from the CRC, and the BCR of the predominant ADC share features with the BCR repertoire in CLL. Thus, our data indicate that the development of CLL is initiated as a multiclonal process with the dominant clones being selected for structural features of the BCR in CRC. Our findings also support the notion that leukemogeneisis can begin at earlier stages of B-cell maturation.

Methods

Samples

The study was approved by the Institutional Review Board of Northwell Health in accordance with the Declaration of Helsinki. Written, informed consent was obtained before blood collection from patients with CLL. Peripheral blood mononuclear cells (PBMC) from CLL patients were separated by density gradient centrifugation (Ficoll, GE Healthcare), cryopreserved (in 10% dimethylsulfoxide, 45% fetal bovine serum and 45% RPMI medium), and stored in liquid nitrogen until used. IGH repertoire data derived from the PBMC of nine aged-matched healthy donors[26] were used as normal controls. A summary of the control data is provided in O*nline Supplementary Table S1*.

Cell sorting

Patients' PBMC were incubated with the following anti-human antibodies: FITC anti-CD19 (BD Biosciences) and PerCPcy5.5 anti-CD5 (BioLegend). Non-B cells were excluded with eFluor-450 anti-CD3 and anti-CD16, and dead cells by Sytox Blue staining (ThermoFisher). Up to 25,000 B cells from each donor were sorted directly into a single 200 μL polymerase chain reaction (PCR) tube containing 100 μL Dynabeads Oligo(dT) (ThermoFisher) in lysis buffer and stored at -80°C.

Library preparation and sequencing

Library preparation and subsequent sequencing were performed as described elsewhere.[25] Briefly, mRNA was isolated from B-cell lysates in 96-well plates using Dynabeads Oligo(dT) (ThermoFisher) according to the manufacturer's protocol. All the bead-immobilized mRNA was reverse transcribed and second-strand synthesis was performed in solid phase using IGHV leader-specific primers; in this step, 13 to 16 random nucleotides (unique molecular identifiers [UMI]) and partial Illumina adaptor sequences were incorporated. This allowed exponential PCR amplification using universal primers minimizing IGHV gene-specific bias. Additionally, the UMI, which tagged individual mRNA molecules, enabled building consensus sequences, allowing advanced error correction as well mitigating PCR amplification bias. Collectively, these improved the accuracy of quantitative analyses. The PCR product was indexed with Nextera XT kit (Illumina). The library was sequenced with MiSeq Illumina (v3 2 x 300 kit Illumina MS-102-3003) after loading at 12 pM with 10% PhiX. The list of the primers and the detailed protocol are provided in Vergani *et al.*[25] Raw data are deposited at SRA (BioProject ID PRJNA673787 and PRJNA807871).

Bioinformatic analysis

Raw reads were processed using a custom workflow built with pRESTO (REpertoire Sequencing TOolkit).[27] The IGHV-D-J sequences obtained were submitted to IMGT/HighV-QUEST[28] and analyzed using ChangeO,[29] and custom R scripts. Clonal assignment was performed with ChangeO with a distance threshold of 0.07.

Chronic lymphocytic leukemia stereotype assignment

CLL stereotyped sequences in the ADC were identified by submitting IGHV-D-J sequences to ARResT/AssignSubsets.[30] Sequences assigned to CLL stereotyped subsets with a confidence of "average" or higher were used in the analyses as was done for the control data in Bagnara *et al.*[31]

Results

Experimental design

PBMC were collected from 57 untreated patients with CLL. For 11 of these 57 patients, cells were available at two additional timepoints. CD19$^+$CD5$^+$ cells, purified from the PBMC of each patient or from nine aged-matched healthy donors,[31] were used to determine the full length IGHV-D-J sequence

as described previously.[25] The library preparation involved the use of UMI and universal primers for the exponential phase of the PCR amplification, minimizing possible IGHV gene-specific bias and the effect of PCR amplification for quantitative analysis (see Methods). Each biological sample was aliquoted, and the library prepared in two to eight replicates except for sample CLL1752 (O*nline Supplementary Table S2*). Only in-frame and productive IGHV-D-J rearrangements were selected for analysis. We included in the analysis only unique sequences considered of high quality, observed in ≥3 different UMI and derived from the consensus of ≥5 reads. Each unique IGHV-D-J sequence was assigned to a clone, defined as a collection of sequences carrying the same IGHV-D-J rearrangement (see Methods) including subclonal variants possibly derived by somatic hypermutation. The size of each clone was estimated by the count of mRNA transcripts, inferred from the UMI count. An average of >2,500,000 reads and >400,000 UMI per CLL patient sample was obtained after raw read processing and quality filter. A summary of the IGHV-D-J repertoire data is provided in O*nline Supplementary Table S2*.

Presence and frequency of clones distinct from the clinically relevant clone

Two groups of IGHV-D-J sequences were identified in each CLL patient. The first group, invariably the most abundant in every instance, corresponded to the sequence of the CRC, identified at diagnosis using Sanger methodology; this included subclonal variants differing in various somatic mutations.[26] The other group consisted of a collection of ADC bearing IGHV-D-J rearrangements that were different from those of the CRC and its descendants. Here, we have focused on the latter group.

Notably, ADC were detected in every CLL patient at least at one timepoint (Figure 1A), with an average frequency of 4.8% (range, 0.002-73%) relative to the total number of IGHV-D-J transcripts identified (thus including the CRC). The ADC group includes all clones regardless of their level of expansion. Additionally, in each patient the predominant ADC ($pADC_1$) was identified (Figure 1B); among the patients, the $pADC_1$ were found at an average frequency of 5.5% (range, 0.012-46%) (see Methods).

For 46 patients, white blood cell counts, lymphocyte counts, and flow cytometry data ($CD5^+CD19^+$ phenotype) were available, allowing us to calculate the absolute numbers of cells in the three most dominant ADC ($pADC_1$, $pADC_2$, and $pADC_3$) by multiplying the frequency of each individual clone by the count of $CD5^+CD19^+$ cells (cell/μL) in the patient's blood. We used a cut-off for clonal expansion of >1 B cell/μL, a threshold chosen because it represents the median value of B-cell expansion observed in MBL.[32] The $CD5^+$ B cells from the nine healthy individuals used as controls did not exceed this level (Figure 1C), whereas 96% (44 out of 46) of the patients exhibited at least one expanded ADC above 1 B cell/μL (Figure 1D), with an average of 12 expanded ADC per patient (range, 1-120 ADC). Overall, we observed a correlation between the levels of ADC expansion and their consistent presence in the replicates, although this was not always true in the samples with lower sequencing depths.

Comparison of the size of the additional distinct clones with those of chronic lymphocytosis leukemia and monoclonal B-cell lymphocytosis

The diagnosis of CLL, of high-count MBL, and of low-count MBL requires >5,000, 500-5,000, and ~1-499 monoclonal B cells/μL,[32-34] respectively. In two of the 44 patients with expanded ADC, the size of $pADC_1$ qualified as a second CLL clone by these criteria (5%), as high-count MBL in eight patients (18%), and as low-count MBL in 34 patients (75%) (Figure 1C). Extending the analysis to $pADC_2$ and $pADC_3$ and considering the total number of different timepoints used for the analysis (see below) indicated that 80% (37/46) of the patients had at least three MBL-like expansions at some point during the intervals studied.

Variations of the pattern of the predominant additional distinct clones at different timepoints

Since samples taken at two different timepoints were available for 11 patients, we next determined whether $pADC_1$ frequency changed over time. In ~82% (9/11) of patients, the $pADC_1$ remained the dominant clone over time (Figure 2A, black lines), increasing in absolute count in most cases (7/9, 88%). In 22% (2/9) of these patients, the $pADC_1$ decreased with time with one $pADC_1$ falling below the diagnostic level for CLL (Figure 2A, dashed black line). Notably, in two of the 11 patients investigated (18%, dashed red lines), the $pADC_1$ was replaced by a different clone at the second timepoint. Moreover, in three patients (27%), the $pADC_1$ was and remained the only ADC (Figure 2B, overlapping blue dashed line at or near 100%), so in these cases there was a single ADC. Notably, for these patients, the $pADC_1$ had the highest cell/μL count (Figure 2, green circle). Also, in two of these patients, the absolute count of the $pADC_1$ decreased over time (Figure 2A, dashed black line). In six of the 11 (54%) patients, the $pADC_1$ remained the major clone, although it increased in relative size (Figure 2B, black solid line). However, in the remaining two patients, 18%, (Figure 2B, red dashed line), the $pADC_1$ was surpassed by a different clone at the second timepoint.

The degree of expansion of the predominant additional distinct clone is independent of clinically relevant clone size

To determine whether there was a numerical relationship between the CRC and the $pADC_1$, we correlated the cell counts of the CRC with the cell counts of the $pADC_1$ (Figure 3). Notably, Pearson's correlations failed to demonstrate relationships between $pADC_1$ and CRC, indicating that the level of $pADC_1$ expansion did not directly relate to the size of the CRC (Figure 3).

Comparison between IGHV-D-J rearrangements of clinically relevant clones and additional distinct clones

To understand whether the IGHV-D-J rearrangements in ADC shared features with those found in CLL, we qualitatively compared the two groups for IGHV gene use and mutation status, IG isotype display, and the presence of CLL stereotyped IGHV-D-J rearrangements. To do so, when possible, we divided the ADC into two groups based on clonal frequency: a group with the three most represented pADC ($pADC_{1-3}$) observed in each patient and the remaining referred to as minor ADC (mADC).

IGHV gene use

In CLL, IGHV1-69 and IGHV4-34 are two of the most frequently used IGHV genes, with IGHV1-69 being expressed

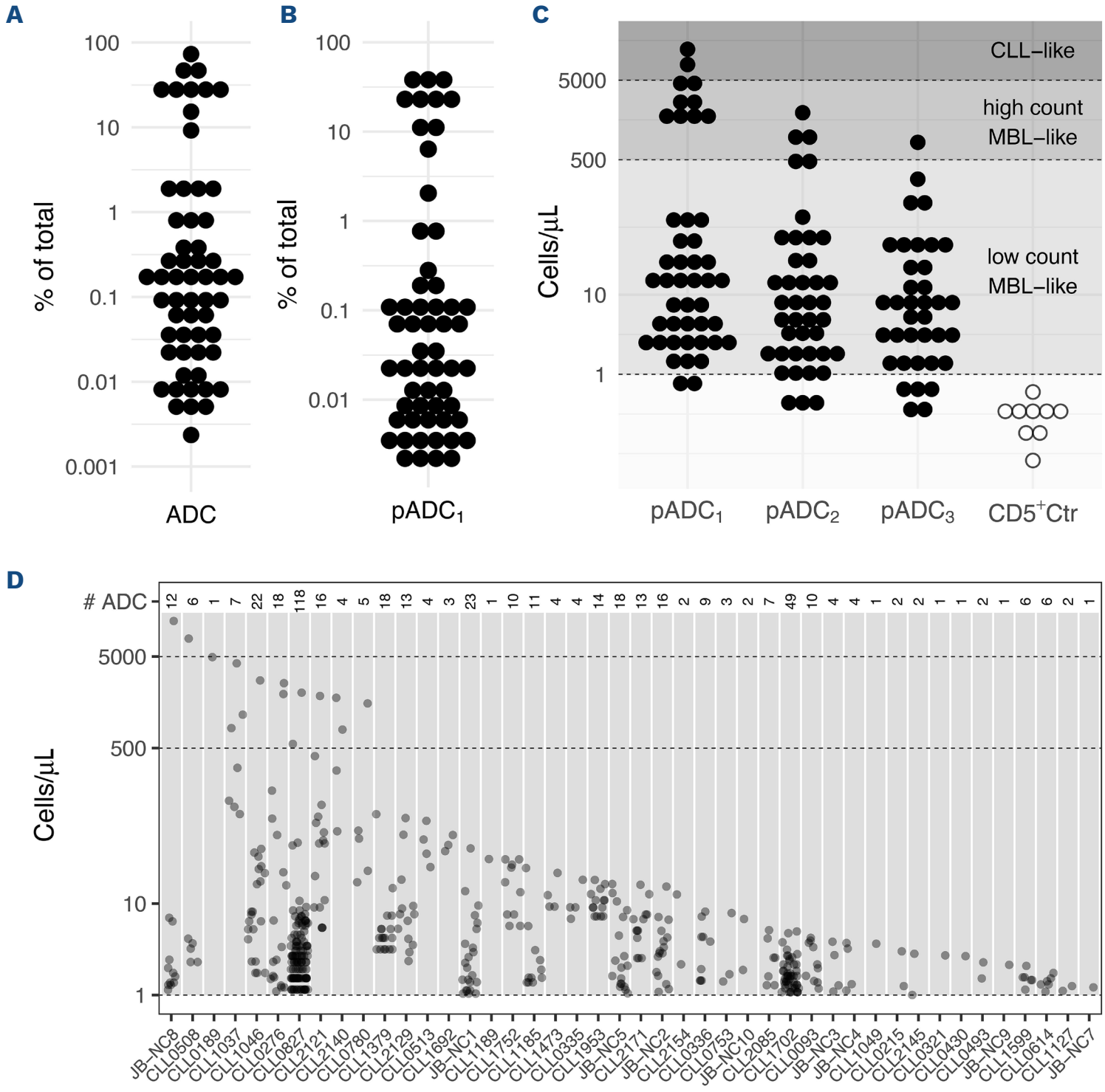

Figure 1. Quantitative analysis of additional distinct clones. (A) Cumulative frequency of additional distinct clones (ADC) relative to the total number of IGHV-D-J rearrangements identified in the circulating $CD5^+$ B cells of 57 patients with chronic lymphocytic leukemia (CLL). The values for each patient are expressed by a single dot. (B) Frequency of the predominant ADC ($pADC_1$) among the total number of IGHV-D-J rearrangements including the clinically relevant clone of 57 CLL patients. (C) Absolute cell counts (cells/µL) of the three most predominant additional clones ($pADC_1$, $pADC_2$, $pADC_3$) in the 46 CLL patients (black dots) for whom white blood cell counts were available. The plot is divided into four horizontal areas based on the interval defining CLL (>5,000 cells/µL), high-count monoclonal B-cell lymphocytosis (500-5,000 cells/µL), and low-count monoclonal B-cell lymphocytosis (1-499 cells/µL). Open dots indicate data obtained from $CD5^+$ B cells derived from normal donors. (D) Absolute cell counts (cells/µL) of every ADC having >1 cell/µL (44 patients). Data are presented in descending order of the $pADC_1$ expansion of each patient. The numbers on the top of the plot indicate the numbers of ADC >1 cell/µL in each sample. MBL: monoclonal B-cell lymphocytosis.

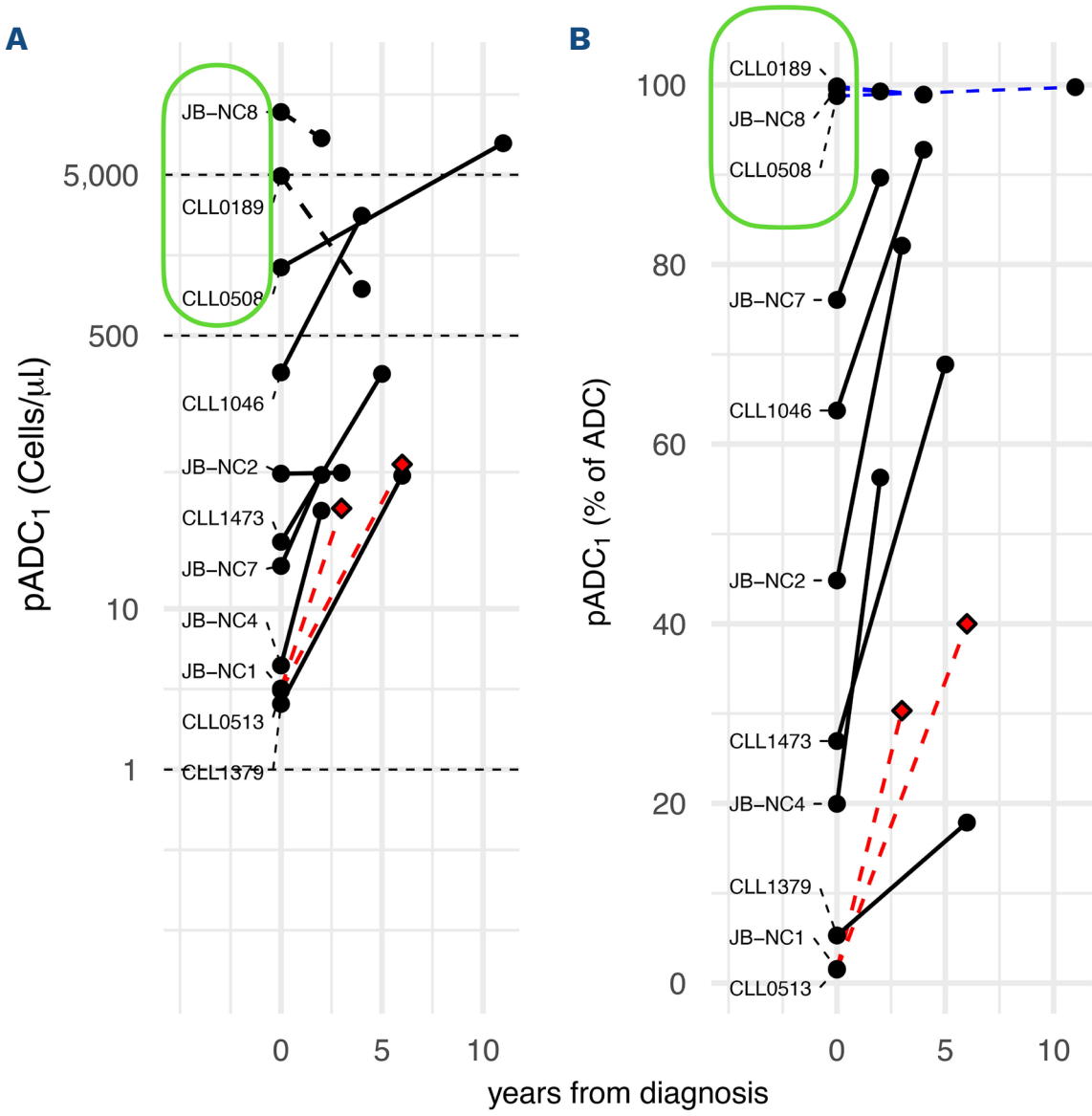

Figure 2. Longitudinal analysis of additional distinct clones. (A) Variation in the cell counts (cells/µL) of predominant additional distinct clones ($pADC_1$) in 11 chronic lymphocytic leukemia patients over time. The lines connect two samples from the same patient analyzed at different timepoints. Solid black lines indicate that the $pADC_1$ has the same IGHV-D-J rearrangement in the two samples. A dotted red line indicates that, at the second timepoint, the $pADC_1$ represented a distinct clone with a different IGHV-D-J rearrangement. (B) Percentage of $pADC_1$ relative to all ADC at different timepoints. Lines connect multiple samples from the same patients. Blue dotted lines indicate samples in which the fraction of $pADC_1$ remained substantially unchanged; black solid lines indicate samples in which the $pADC_1$ increased over time; red dotted lines mark samples in which the $pADC_1$ was a distinct clone, having an IGHV-D-J rearrangement different at the second timepoint from that seen at the first timepoint. The green circles highlight the same three patients in panel (A) and (B).

predominantly in unmutated CLL, and IGHV4-34 more often in mutated CLL.[11,35] First, we analyzed the frequencies and characteristics of these two genes detected in $CD5^+$ cells of normal donors and in ADC and then compared these to those found in a collection of 1,497 CLL cases from our laboratory's database. In contrast to other IGHV, mutated IGHV4-34 and unmutated IGHV1-69 were present at much lower frequencies in control $CD5^+$ B cells (Figure 4A, open dots and white bars) than in CLL, making them outliers in the gene frequency correlation. Likewise, the frequency of use of mutated IGHV4-34 and unmutated IGHV1-69 among mADC (Figure 4B, light blue square dots and bars) diverged considerably from that of CLL. In contrast, $pADC_{1\text{-}3}$ were closer to CLL clones in this regard. (Figure 4A, B, dark blue triangle dots and bars).

IGHV mutation status

The median IGHV mutation frequency in our internal database of CLL sequences was about 1.7%, while this frequency was only 0.7% in $pADC_{1\text{-}3}$ and virtually null in the mADC and $CD5^+$ normal B cells (Figure 4C). IGHV-unmutated and IGHV-mutated sequences, using the 2% mutation threshold, were equally distributed among CLL (52% and 48%) as well as in $pADC_{1\text{-}3}$ (55% and 45%) (Figure 4D). In contrast, mADC and $CD5^+$ B cells from healthy controls were unbalanced towards IGHV-unmutated rearrangements (72% and 28% for mADC, and 84% and 16% for $CD5^+$ controls) (Figure 4D). Thus, there were more IGHV-mutated sequences and sequences with higher numbers of

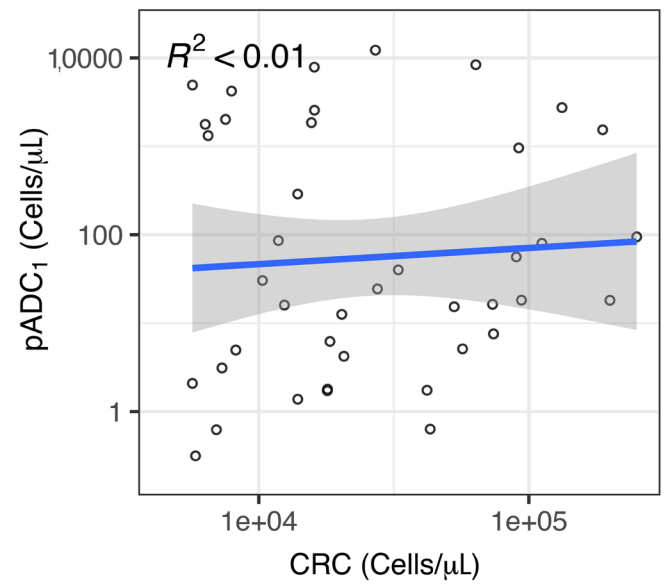

Figure 3. Correlations between clinically relevant clone and additional distinct clone cell counts. Correlations between the cell counts of clinically relevant clones (CRC) and predominant additional distinct clones ($pADC_1$) in 46 patients at all available timepoints Each dot represents a patient at one timepoint. The square of Pearson correlation (R^2 <0.01) indicates the absence of a correlation between CRC and $pADC_1$ expansion.

mutations in CLL and the $pADC_{1-3}$ compared to mADC and $CD5^+$ B cells from normal controls. Thus, the pADC were more like CLL using this parameter, and the mADC more like healthy $CD5^+$ B cells.

Although there was a statistically significant concordance in IGHV-mutation status in the CRC and the $pADC_1$ from the same patient ($P<0.007$) (Figure 4E), an association between IGHV, IGHD, IGHJ use and VH CDR3 sequence was not observed (*Online Supplementary Table S3*).

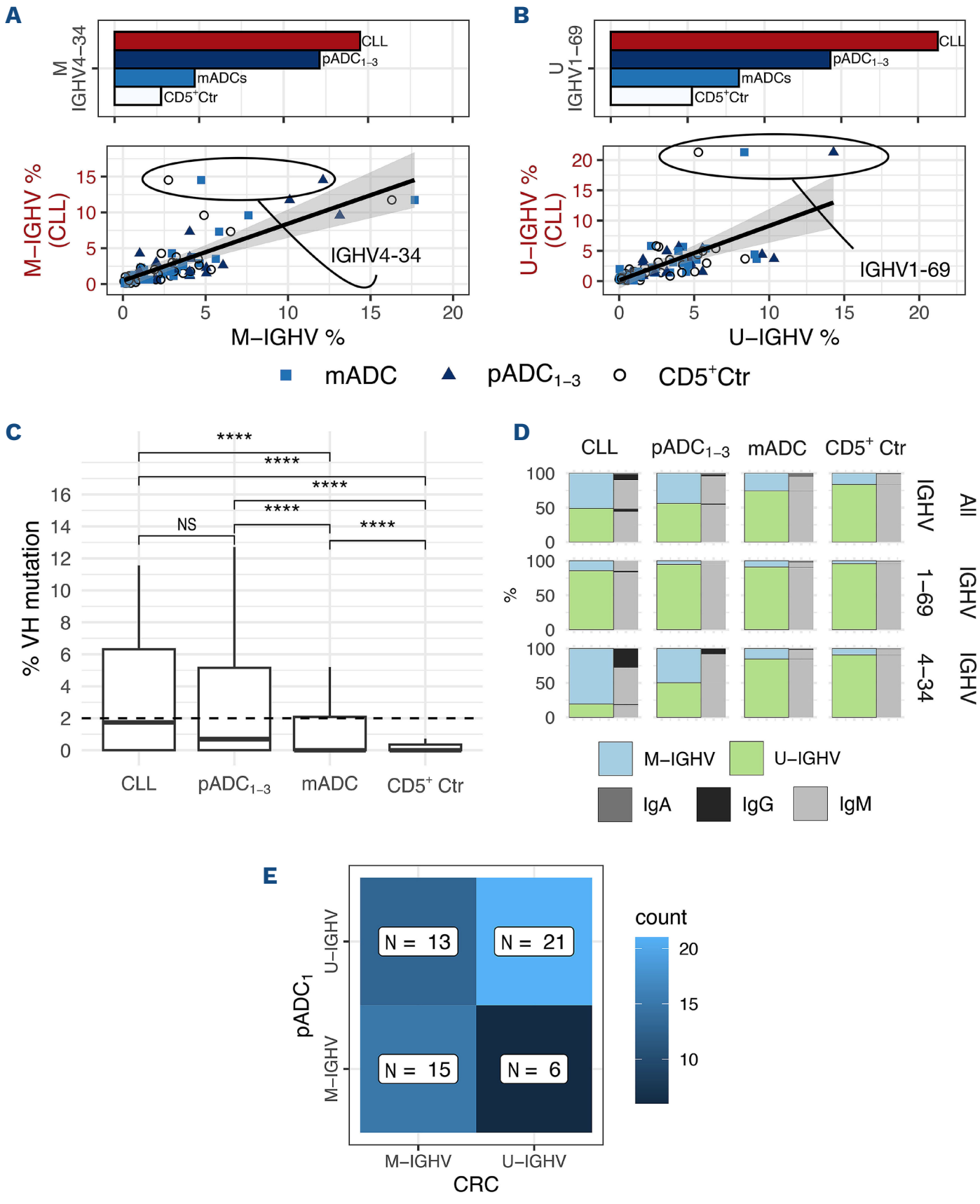

Figure 4. IGHV-D-J repertoire analyses of additional distinct clones. (A, B) Proportions of clones expressing mutated IGHV (A) and unmutated IGHV (B) genes. In the lower panels, the x-axes represent the percentages of the indicated populations: dark blue triangles for predominant additional distinct clones ($pADC_{1-3}$), light blue squares for minor additional distinct clones (mADC), and open circles for $CD5^+$ cells from control subjects (Ctr). The y-axes show the percentages of chronic lymphocytic leukemia (CLL) clones. The regression line illustrates the correlation between the CLL clones and the other populations. (C) IGHV mutation distribution in CLL, $pADC_{1-3}$, mADC, and $CD5^+$ B cells from healthy, age-matched controls (Ctr). In the boxplot, the thick horizontal lines show median values; the box describes the two quartiles and the whisker the 95th percentile. (D) Proportions of mutated and unmutated IGHV and IGH isotypes in IGHV4-34 and IGHV1-69 genes in the IGHV-D-J rearrangements from the different clones as indicated. (E) Association of IGHV mutation status between the clinically relevant clone and $pADC_1$ (Pearson χ^2 test, $P<0.007$). M-IGHV: mutated IGHV; U-IGHV: unmutated IGHV; CRC: clinically relevant clones.

IG constant region use

Notably, most rearrangements from all the ADC and CD5$^+$ normal B cells utilized the IgM isotype, like CLL cells. However, a substantial proportion of IGHV4-34 sequences were isotype class-switched to IgG in both CLL and pADC$_{1-3}$ (Figure 4D). Most IgA were detected in the mutated mADC, without an apparent enrichment for a specific IGHV gene.

Chronic lymphocytic leukemia stereotype-like sequences

Twenty-one of the total 5,846 ADC IGHV-D-J rearrangements (0.36%) were assigned to one of the major CLL stereotyped subsets (Figure 5). Notably, this frequency was four times higher than that in the repertoires from normal CD5$^+$ B cells (0.09%) (Figure 5A and Bagnara *et al.*[31]). Moreover, the stereotyped BCR frequency in the ADC from the same CLL cases was considerably less than that observed in the CRC (17%; 10/57) (Figure 5B). Notably, pADC were not further enriched in CLL stereotypes (*data not shown*), possibly because of the relatively small cohort of sequences available for analysis. Finally, two CLL patients had B cells with a stereotyped rearrangement in both CRC and the ADC.

Thus, the relative frequency of stereotyped rearrangements increases considerably when moving from a normal CD5$^+$ B-cell population to the ADC population to the CRC.

When counting both CRC and ADC, we identified the presence of more than one CLL stereotyped IGHV-D-J rearrangement in four patients (Figure 5B). For three (75%) of these patients, at least two IGHV-D-J rearrangements were assigned to the same stereotyped subset exclusively in the ADC or in the ADC and CRC.

Collectively, the data suggest a selection process favoring the expansion of B cells bearing BCR which have features shared by the BCR of the CRC in the same and other patients.

Discussion

In this study, we used next-generation sequencing to analyze the IGHV-D-J gene rearrangements of circulating CD5$^+$ B cells from patients with CLL. This revealed the presence of multiple expanded clones, including the CRC, in all patients, although the levels of expansion of these clones varied, indicating that multi-clonality is a common, possibly universal occurrence in CLL and not a feature of only a subset of patients.

Using a 1 B cell/μL cutoff to define an expanded B-cell clone, ~96% of the patients with CLL exhibited at least one ADC and 89% had more than one ADC (Figure 1C). Notably, in two cases, the size of the pADC met the criteria defining CLL and in the remaining cases achieved the definition for high- or low-count MBL (Figure 1D). These findings place a new perspective on the frequency of multiple expanded clones, independent of the CRC, in patients with CLL. In previous studies in which a second rearrangement was found,[5-8] the additional clone could have been the most predominant among a large series of different clonal expansions that were not detected because of the sensitivity of the technology available at the time. Consistent with this possibility, a retrospective next-generation sequencing analysis revealed the presence of a single, and sometimes of two distinct, expanded IGHV-D-J rearrangements in a few individuals several years prior to the onset of high count MBL or CLL.[36]

Our experimental approach minimized the likelihood of over-counting or counting inappropriate variants such as non-productive rearrangements. The latter is relevant for the possibility of the occurrence of allelic inclusion. Although B cells can potentially produce two rearranged IGHV-D-J,

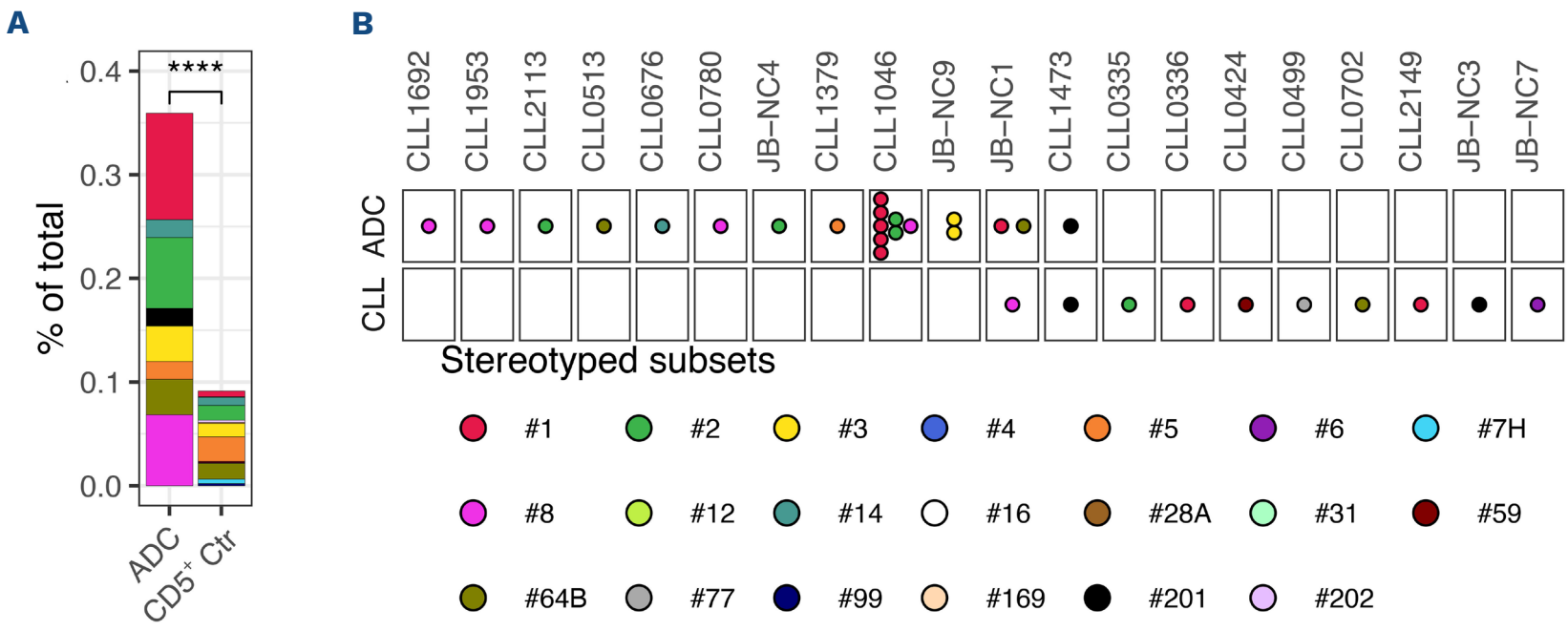

Figure 5. Analyses of the presence of stereotypy in additional distinct clones. (A) Frequency of chronic lymphocytic leukemia (CLL)-like stereotyped IGHV-D-J rearrangements detected in additional distinct clones (ADC) and CD5$^+$ normal B cells from healthy donors (Ctr); each color indicates a distinct stereotyped subset, as depicted in the graphic legend. (B) IGHV-D-J clones associated with a CLL stereotyped subset found in ADC and clinically relevant clones. Each dot represents a different IGHV-D-J rearrangement, and the colors indicate the stereotyped subset identified.

they normally express one. This phenomenon, called allelic exclusion, occurs because only one of the two allelic loci located on chromosome 14 is productively rearranged, while the other remains in the germline configuration, is partially rearranged (IGHD-IGHJ) or is rearranged non-productively. This last possibility raises the question of whether non-productive rearrangements originating from the CRC, as can occur in CLL,[37] could be erroneously interpreted as ADC in our analyses. However, since we excluded non-productive rearrangements, allelic inclusion is not a possibility. Moreover, the presence of numerous ADC per case (Figure 1D) cannot be explained based on the lack of allelic exclusion in the CRC, especially since allelic inclusion occurs in only 10% of CRC.[37] In addition, in our studies there was no correlation between the size of the CRC and the presence of $pADC_1$, and changes in $pADC_1$ size over time did not relate to those of CRC, both suggesting independence between the CRC, the $pADC_1$ and probably other ADC. However, it is possible that other normal/pre-leukemic B cells could lack allelic exclusion and produce two distinct IgH chains, and our bulk sequencing approach would not distinguish this. To do so, single-cell analyses are needed. Notably, our conclusions are consistent with the findings of other investigators[6,7] who used single-cell analyses or subclonal fractionation based on κ or λ chain expression.

The presence of multiple ADC might relate to clinical observations in patients with CLL, who develop Richter transformation to diffuse large B-cell lymphoma or more rarely Hodgkin disease.[38,39] In ~20% of these instances,[38,39] the IGHV-D-J rearrangement of the Richter transformation clone differed from that of the CLL CRC, suggesting that such lymphomas originated from a distinct ADC present in the patient's clonally expanded B-cell repertoire that was not recognized, most likely because of the sensitivity of the assays used.[40-43] Although these reports are each based on a limited number of cases, collectively they support this possibility.

The analysis of IGHV gene use, mutation status, and IG isotype expression indicated that pADC, more than mADC, exhibit characteristics typical for the repertoire of CLL clones (Figure 6A). This was highlighted when focusing on $pADC_{1-3}$ using the IGHV1-69 and IGHV4-34 genes. This showed a distribution of IGHV-mutated and IGHV-unmutated rearrangements, and an isotype use similar to that observed in the CLL repertoire, which differed substantially from that observed in mADC and in $CD5^+$ B cells from healthy individuals. Moreover, pADC utilizing IGHV4-34 genes were frequently switched to the IgG isotypes as in

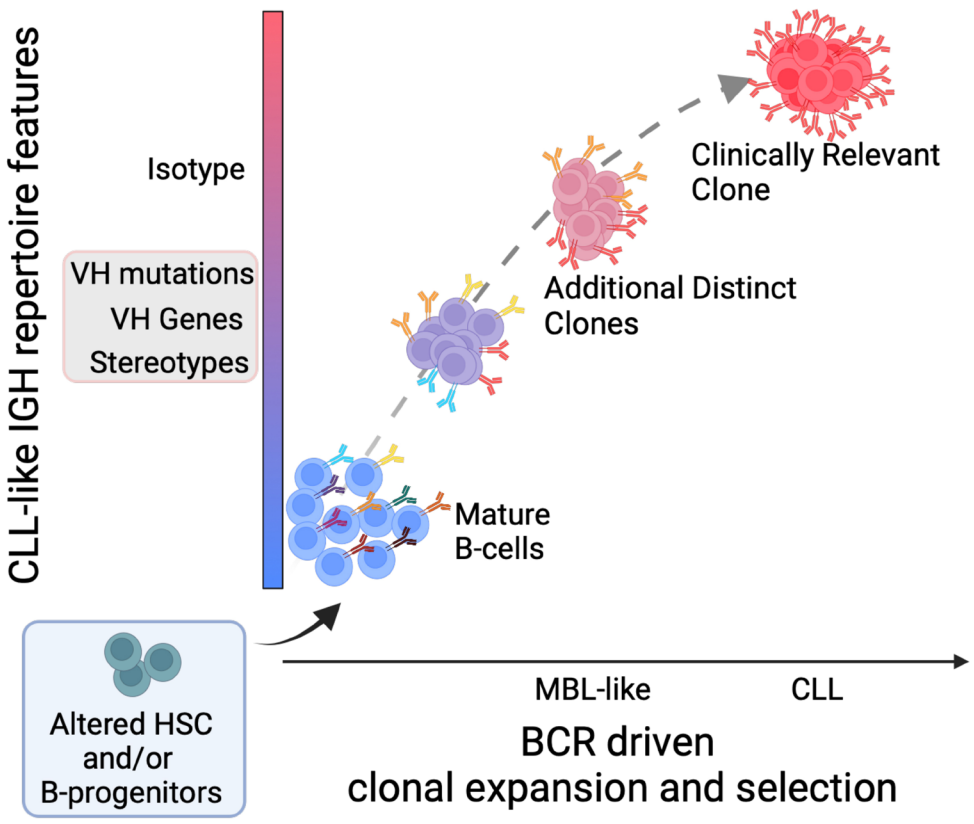

Figure 6. Proposed model of chronic lymphocytic leukemia ontogeny. (A) Hypothetical steps involved in shaping a patient's $CD5^+$ B-cell repertoire during chronic lymphocytic leukemia (CLL) ontogeny. The major repertoire features and level of clonal expansion are depicted on the left (gray background). The dark blue represents the IGHV-D-J repertoire characteristics of normal $CD5^+$ B cells; dark red represents CLL IGHV-D-J repertoire characteristics; intermediate colors represent the evolution to a CLL-like repertoire. (B) Hypothetical model of CLL ontogeny in which the initial lesion occurs early in B-cell maturation. HSC: hematopoietic stem cells; MBL: monocytosis B-cell lymphocytosis; BCR: B-cell receptor; $CD5^+$ Ctr: normal, control $CD5^+$ B cells; mADC: minor additional distinct clones; pADC: predominant additional distinct clones; CRC: clinically relevant clone.

CLL. Since IGHV4-34-bearing B cells are usually excluded from germinal centers in healthy subjects,[44] the presence of isotype-switched cells bearing somatically mutated IGHV4-34 is consistent with a defect in B-cell tolerance in people who develop CLL.[44,45] Finally, ADC showed biased expression of stereotyped rearrangements which was ~4 times greater than in $CD5^+$ normal B cells. We also found distinct IGHV-D-J rearrangements belonging to the same stereotyped subset in the same patient; indeed, in one case (CLL1046), five different clones assigned to subset 1 were identified. Additionally, IGHV-mutated rearrangements were more abundant in pADC and CRC than in mADC and normal $CD5^+$ B cells. Cells from mutated CLL are less prone to apoptosis when stimulated via BCR in a variety of experimental conditions *in vitro*, potentially mimicking what occurs *in vivo*.[46-50]

Finally, there was a significant concordance in IGHV mutation status between CRC and $pADC_1$ in individual cases, suggesting that the trajectories leading to their selection were similar, albeit differing for IGHV-mutated and unmutated cases. Notably, it has been reported that finding ADC that differ from CRC in IGHV mutation status might influence the patient's clinical course and outcome.[8] This was observed in subgroups of patients classified as having ADC by next-generation sequencing methodologies of lesser sequencing depths.

Collectively, these findings suggest a selection for specific BCR stereotypes resembling those of full-blown CLL or an inherited propensity to generate stereotypes. Additional studies will be needed to corroborate these possibilities, since our findings are limited to a low number of observations. The shared features also strongly suggest that ADC are part of the leukemogenic process, which appears to involved defined steps (Figure 6A): (i) an increase of stereotyped sequences in the mADC appears to be one of the earliest selection events; (ii) IGHV gene use, IGHV-mutation status, and IG isotype use attain a CLL-like fingerprint in the pADC, concomitant with clonal expansion. The mechanisms behind this IG repertoire modeling are not clear, although selection for BCR structure and signaling are feasible. Additionally, the possibility of molecular events involving pre-BCR rearrangements in early B-cell progenitors or hematopoietic stem cells, which may promote multiclonal B-cell expansions, needs to be considered.

Taken together, our findings strongly suggest that the genesis of CLL involves multiple clones which compete for survival/expansion, with the BCR playing a role in the process. This process could include selection for distinct abilities to respond to certain antigenic stimuli experienced by the competing B-cell clones. The ultimate complete transformation is likely facilitated by genetic alterations, either inherited or acquired early and/or during development, which promote clonality, as well as by microenvironmental inputs that contribute to the survival/expansion of the transforming B cells.

Disclosures

JRB has served as a consultant for Abbvie, Acerta/Astra-Zeneca, Alloplex Biotherapeutics, BeiGene, Genentech/Roche, Grifols Worldwide Operations, iOnctura, Kite, Loxo/Lilly, Merck, Numab Therapeutics, Pfizer and Pharmacyclics and has received research funding from BeiGene, Gilead, iOnctura, Loxo/Lilly, MEI Pharma and TG Therapeutics. KRR is author of a CLL chapter in UpToDate and has received royalties from it. SK is employed at Takeda Pharmaceuticals. JCB is a consultant for Jannsen, Abbvie, BeiGene and AstraZeneca.

Contributions

DB and NCh conceived the project and designed the experimental approach. DB, ANM, NCa, SV and MC performed experiments and analyzed data. JRB, SK, SF, KRR, JEK, JCB and SLA provided clinical samples. GF, FG and FF offered interpretations and conceptual insights. DB, ANM, MF and NCh wrote the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work was supported in part by philanthropic contributions to NCh and KRR from the Karches family, the Nash Family Foundation, the Muriel Fusfeld Foundation, and the Jean Walton Fund for Leukemia, Lymphoma, and Myeloma Research. FF was supported by the Italian Ministry of Health (Ricerca Corrente 2022-2024). ANM was supported by the European Union Horizon 2020 research and innovation program under Marie Sklodowska-Curie grant agreement n. 101023721 and Fondazione Umberto Veronesi grant agreement n. 5365.

Data-sharing statement

Raw data are deposited at SRA (BioProject ID PRJNA673787 and PRJNA807871). Processed and annotated data are available upon request to the corresponding author.

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