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Molecular and cytogenetic characterization of expanded B-cell clones from multiclonal versus monoclonal B-cell chronic lymphoproliferative disorders

Running head: Multiclonal B-CLPD: Molecular and cytogenetic features

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Abstract

Chronic antigen-stimulation has been recurrently involved in the earlier stages of monoclonal B-cell lymphocytosis, chronic lymphocytic leukemia and other B-cell chronic lymphoproliferative disorders. Among these individuals, expansion of ≥2 B-cell clones has been frequently reported; potentially, such coexisting clones have a greater probability of interaction with common immunological determinants. Here, we comparatively analyzed the B-cell receptor repertoire and the molecular profile, as well as the phenotypic, cytogenetic and hematological features of 228 chronic lymphocytic leukemia-like and non-chronic lymphocytic leukemia-like clones between multiclonal (n=85 clones from 41 cases) versus monoclonal (n=143 clones) monoclonal B-cell lymphocytosis, chronic lymphocytic leukemia and other B-cell chronic lymphoproliferative disorders. The B-cell receptor of B-cell clones from multiclonal cases showed a slightly higher degree of HCDR3 homology than B-cell clones from monoclonal cases, in association with unique hematological (e.g. lower B-lymphocyte counts) and cytogenetic (e.g. lower frequency of cytogenetically altered clones) features usually related to earlier stages of the disease. Moreover, a subgroup of coexisting B-cell clones from individual multiclonal cases which were found to be phylogenetically related, showed unique molecular and cytogenetic features: they more frequently shared IGHV3 gene usage, shorter HCDR3 sequences with a greater proportion of IGHV mutations and del(13q14.3), than other unrelated B-cell clones. These results would support the antigen-driven nature of such multiclonal B-cell expansions, with potential involvement of multiple antigens/epitopes.

Keywords: B-cell chronic lymphoproliferative disorders, monoclonal B-cell lymphocytosis, MBL, multiclonality, B-cell clones, BCR, cytogenetics, chronic lymphocytic leukemia, CLL.

Introduction

B-cell chronic lymphoproliferative disorders (B-CLPD) usually show a monoclonal expansion of a (single) mature-appearing aberrant B-cell clone.[1-4] However, patients diagnosed with composite lymphomas and other B-cell chronic lymphocytic leukemias – e.g. chronic lymphocytic leukemia (CLL) – have been reported in the literature for decades, particularly among immunocompromised subjects.[5-7] Although early reports considered this phenomenon as a rare event, it might have been underestimated due to the need for sophisticated multidisciplinary approaches encompassing combined histopathology, cytomorphology, immunophenotypic and cytogenetic techniques and/or molecular analyses of purified cell populations.[8] In fact, B-cell neoplasms consisting of two phenotypically distinct populations of clonally unrelated B-lymphocytes coexisting in the same patient (detected either simultaneously or at different time points during follow-up) have been reported in the literature,[9,10] with an estimated overall frequency among B-CLPD patients of around 5%.[11] Recently, it has also been shown that up to 20% of population-based non-CLL and CLL-like low count monoclonal B-cell lymphocytosis (MBL^{lo}) cases may also carry two different unrelated B-cell clones.[12-14] In addition, data from a small series suggest that the frequency of multiclonality could be particularly high among CLL-like MBL cases from CLL relatives, (4/6 cases analysed).[12] Altogether, these results support the existence of multiclonality in a significant proportion of both MBL cases and B-CLPD patients.

Multiclonal MBL and B-CLPD cases may consist of expansions of ≥2 B-cell clones potentially associated with chronic antigen-driven immune responses.[15-17] In fact, this is particularly frequent at the earlier MBL^{lo} stages, which would further support the potential reactive nature of MBL among individuals with normal lymphocyte counts, prior to the stepwise acquisition of genetic alterations and progression to clinical MBL (MBL^{hi}) and CLL.[13,14,18,19] If this hypothesis holds true, specific antigenic determinants could potentially be more frequently shared between the coexisting B-cell clones of multiclonal cases than between the expanded B-cells in different monoclonal MBL and B-CLPD patients, due to a greater probability of interaction with common immunological determinants. This might even be true when the

coexisting clones display clearly distinct immunophenotypic and cytogenetic, as well as clinical features.[20-22]

In order to test this hypothesis, in the present study we compared the B-cell receptor (BCR) repertoire and molecular profile, as well as the phenotypic, cytogenetic and hematological features of CLL-like and non-CLL-like clones (n=228) from multiclonal (n=41 cases) vs monoclonal cases (n=143, including both CLL and CLL-like MBL -n=128-, as well as B-CLPD patients other than CLL and non-CLL-like MBL subjects -n=15-).

Methods

Patients and samples. A total of 184 subjects with one (n=143 monoclonal cases) or ≥2 (n=41 multiclonal cases) CLL/non-CLL B-CLPD (n=140) and/or CLL-like/non-CLL-like MBL (n=88) B-cell clones -as defined by the World Health Organization criteria 2008- [23] were included. Binet staging[24] of CLL subjects was retrospectively collected.

From the 41 multiclonal cases, 2 (5%) corresponded to healthy individuals with CLL-like MBL^{lo}, 8 (19.5%) were CLL-like MBL^{hi} cases, 23 (56%) had CLL and 8 (19.5%) had B-CLPD other than CLL; 4 of these latter cases showed coexistence of either one or two CLL-like MBL B-cell population(s). In 3/41 multiclonal cases, three coexisting B-cell populations were detected. From the 143 monoclonal cases, 13 (9%) corresponded to healthy adults with CLL-like MBL^{lo}, 26 (18%) were CLL-like MBL^{hi}, 89 (62%) had CLL, 2 (1%) were non-CLL-like MBL^{lo}, 2 (1%) non-CLL-like MBL^{hi} cases and 11 (8%) had other B-CLPD. The precise criteria used for the classification of MBL^{lo} and MBL^{hi} is detailed in *Online Supplementary Methods*. The age/gender distribution for each diagnostic group, is detailed in Supplementary Table 1.

Peripheral blood samples were obtained from each subject after written informed consent was given, and the study was approved by the local ethics committees of the University Hospital of Salamanca and the Blood and Transplantation Center of Coimbra/Portuguese Institute of Blood and Transplantation, in accordance with the Helsinki Declaration of 1975, as revised in 2008.

Immunophenotypic analyses. Immunophenotypic studies to screen for the presence and full characterization of clonal B-cell populations were performed by high-sensitive multiparameter flow cytometry on erythrocyte-lysed peripheral blood samples, according to previously described procedures[18,25-29] which are also detailed in *Online Supplementary Methods*. All cases showed a clonal (imbalanced SmIgκ:SmIgλ ratio of >3:1 or <1:3) and/or an aberrant $CD5^+$ B-cell population.

Cytogenetic and molecular studies. Cytogenetic analyses were performed by multicolor interphase fluorescence *in situ* hybridization on slides containing FACS-purified and fixed

aberrant B-cells, as previously described in detail.[18,30] In parallel, analysis of the patterns of rearrangement of the immunoglobulin heavy chain variable region genes (IGHV) and immunoglobulin K (IGKV) and λ (IGLV) light chain genes was performed for each FACS-purified B-cell clone [18,31-32] (see the *Online Supplementary Methods* section for detailed descriptions). To investigate the level of phylogenetic relationship among IGHV aminoacid sequences, a sequence distance tree was built using the neighbor-joining method implemented in the freely available Molecular Evolutionary Genetic Analysis (MEGA) software.[33] Two different co-existing BCRs were considered as being phylogenetically related when their IGHV aminoacid sequences, going from framework region 1 to HCDR3 (both regions included) showed an identity ≥ 60%. This "identity" threshold was based on previously published concepts about the phylogeny of human IGHV genes based on their aminoacid sequences[33], and on the minimum identity percentage observed in colocalized sub-branches (presumably with the highest evolutionary relationship[33]) of the sequence distance tree built in this study (see the *Online Supplementary Methods* section for more detailed descriptions). HCDR3-alignments were carried out for each multiclonal case whose co-existing B-cell clones showed HCDR3 regions with identical or one aminoacid differing lengths using the bioinformatic tools available at the web services of the European Bioinformatics Institute (EMBL-EBI Cambridge, UK). Through the EMBL-EBI tools, the identical aminoacids or those with analogous side-chain polarity per paired intra-case HCDR3-alignment were highlighted (see the *Online Supplementary Methods* section for more detailed descriptions).

Statistical methods. For all statistical analyses the SPSS software program (SPSS 20.0, IBM SPSS Statistics, IBM, Armonk, NY, USA) was used.

Results

Distribution and immunophenotypic features of B-cell clones. A total of 228 B-cell clones were identified. These corresponded to 143 B-cell clones (89 CLL, 11 non-CLL, 39 CLL-like MBL and 4 non-CLL-like MBL clones) from monoclonal cases and 85 B-cell clones (26 CLL, 14 non-CLL, 40 CLL-like MBL and 5 non-CLL-like MBL clones) from multiclonal cases (Supplementary Table 2). The complete immunophenotypic and cytogenetic features of the individual clones of multiclonal cases are summarized in Supplementary Table 3.

In 26/41 multiclonal cases, all coexisting B-cell clones showed a CLL-like phenotype, while in 11 of the remaining 15 cases, at least one CLL-like B-cell population coexisting with another non-CLL aberrant B-cell population was identified. In the remaining 4 cases, two distinct non-CLL-like B-cell clones were found (Supplementary Table 3). The distribution of all CLL/non-CLL and CLL-like MBL/non-CLL-like MBL clones analyzed (from all monoclonal and multiclonal cases considered together) in the distinct diagnostic categories was as follows: 27 B-cell clones corresponded to CLL-like MBL^{lo}, 52 to CLL-like MBL^{hi}, 115 to CLL, 5 to non-CLL-like MBL^{lo}, 4 to non-CLL-like MBL^{hi} and 25 to non-CLL B-CLPD (Supplementary Table 2). The precise diagnoses of the B-cell clones from B-CLPD patients other than CLL are specified in the footnote of Supplementary Table 2.

Overall size and BCR features of B-cell clones from multiclonal versus monoclonal MBL, CLL and other B-CLPD cases. The relative and absolute median number of peripheral blood clonal B-cells was significantly lower in multiclonal than in monoclonal cases (13% vs 45% and 2,692 cells/µL vs 9,115 cells/µL, respectively; *P*=0.001). Of note, the absolute median number of CLL-like MBL^{hi} and CLL B-cell clones were also significantly lower in multiclonal than in monoclonal cases (1,254 vs 2,464 cells/µL and 9,113 vs 18,600 cells/µL, respectively; P=0.004 and P=0.02) (Supplementary Figure 1). In contrast, the absolute median number of peripheral blood CLL-like MBL^{lo} B-cell clones was significantly higher in multiclonal than in monoclonal cases (79 vs 1 cells/ µL, P=0.002). No significant differences were found in the clone size between non-CLL like and non-CLL B-cell clones in multiclonal vs monoclonal cases (Supplementary Figure 1). In addition, the frequency of CLL-like MBL B-cell clones was

significantly higher in multiclonal than in monoclonal cases (47% vs 27%, respectively; *P*=0.002), whereas the frequency of CLL B-cell clones was higher in monoclonal vs multiclonal subjects (62% vs 31%, respectively; *P*=0.001). CLL B-cell clones from multiclonal and monoclonal CLL patients showed a similar distribution in Binet stage A vs Binet stages B/C (*P*>0.05). Of note, non-CLL B-cell clones were present at higher frequencies in multiclonal vs monoclonal cases (17% vs 8%, respectively; *P*=0.04) (Table 1).

Regarding BCR features, a similar distribution of IGHV mutated and IGHV unmutated B-cell clones was found in multiclonal vs monoclonal cases − 51/85 (60%) vs 84/139 (60%) and 34/85 (40%) vs 55/139 (40%), respectively; Table 1 −. Despite this, the percentage of alignment of IGHV aminoacid sequences among B-cell clones from multiclonal cases (n=3,560 two by two comparisons of clonal IGHV aminoacid sequence) was slightly higher than that obtained among B-cell clones from monoclonal cases (n=8,891 comparisons): median of 52% vs 50%, respectively; (*P*=0.001; Table 1).

Cytogenetic features of B-cell clones from multiclonal versus monoclonal MBL and B-CLPD cases. The frequency of CLL-like MBL and CLL clones from multiclonal cases that showed cytogenetic alterations was significantly lower than that found among CLL-like MBL and CLL clones from monoclonal cases: 27/66 (41%) vs 77/128 (60%), respectively (*P*=0.02). Likewise, the proportion of CLL-like B-cell clones showing coexistence of ≥2 cytogenetic alterations was also significantly lower in multiclonal than in monoclonal cases − 8/66 (12%) vs 32/128 (25%); *P*=0.047 −; this was specially true among B-cell clones from CLL patients − 2/26 (8%) vs 29/89 (33%), respectively; *P*=0.03 – (Table 2).

Regarding each specific cytogenetic alteration, only a decreased frequency of CLL-like B-cell clones with del(13q14) involving the *RB1* gene and a lower percentage of del(13q14)$^{+}$ cells was found in multiclonal vs monoclonal cases − frequency of del(13q14)$^{+}$ clones − of 5% vs 15% with a median of del(13q14)$^{+}$ cells of 55% vs 86%, respectively; *P*=0.01) (Table 2). Of note, these differences were mostly due to the lower frequency of B-cell clones with del(13q14) (4% vs 19%, *P*=0.01) found among CLL clones from multiclonal vs monoclonal cases (Table 2).

No statistically significant differences were observed in the cytogenetic patterns of non-CLL B-cell clones from multiclonal vs monoclonal cases, which is probably due to the relatively

low number of non-CLL clones included in the study; the precise cytogenetic alterations found in non-CLL / non-CLL-like MBL cases are shown in Supplementary Tables 3 and 4. In turn, the overall cytogenetic features of non-CLL like B-cell clones from multiclonal (N=19; 3 non-CLL MBL^{lo}, 2 non-CLL MBL^{hi}, 14 non-CLL B-cell clones) vs monoclonal (N=15; 2 non-CLL MBL^{lo}, 2 non-CLL MBL^{hi}, 11 non-CLL B-cell clones) subjects were similar, as regards both the frequency of cytogenetically altered clones (6/19, 32% and 6/15, 40%) and the percentage of cases with ≥2 genetic alterations − 2/19 (11%) vs 2/15 (13%) – (Supplementary Tables 3 and 4).

Molecular characteristics of the BCR of B-cell clones from multiclonal versus monoclonal MBL and B-CLPD cases. The molecular profile of the BCR of CLL-like MBL^{lo}, MBL^{hi} and CLL B-cell clones and of B-cell clones other than CLL from multiclonal vs monoclonal cases was very similar (Table 3 and Supplementary Table 5). No statistically significant differences in multiclonal vs monoclonal VH gene usage were found for most groups. Despite this general behavior, CLL-like MBL^{hi} B-cell clones from multiclonal cases less frequently showed usage of the DH1, DH4 and DH7 gene families than B-cell clones from monoclonal cases; in addition, JH6 genes were also less frequently used by CLL B-cell clones from multiclonal vs monoclonal cases (Table 3). Overall, 33 functional IGHV gene rearrangements were identified from which 12 (V4-34, V3-23, V3-48, V3-30, V1-69, V3-21, V4-39, V3-33, V3-11, V3-53, V1-2, V3-7) were highly represented among the B-cell clones (≥5% of all B-cell clones corresponding to ≥4 and ≥5 B-cell clones sharing the same IGHV gene in multiclonal and monoclonal cases, respectively) (Figure 1A). Interestingly, 11 of these IGHV genes were found at similar frequencies within the clones of multiclonal vs monoclonal cases, while the V3-33 gene was typically associated with multiclonal cases (6% vs 1%, P=0.03). Regarding IGHD genes, no significant differences were observed between B-cell clones from multiclonal and monoclonal cases, the D3-3, D5-12, D3-10, D6-19, D2-15, and D2-2 genes being the most frequently used and shared by both groups of B-cell clones (Figure 1B). Among IGHJ genes, significant differences were only observed for the JH6 gene, which was more frequently used in monoclonal cases (40% vs 26%, P= 0.03) (Figure 1C).

Except for slightly longer LCDR3 sequences of the IGKV and IGLV genes found among B-cell clones from multiclonal versus monoclonal cases, specially among CLL-like MBL^{hi} clones

(Table 3), no other significant differences were found in the molecular characteristics of the immunoglobulin light chain genes, neither among CLL-like nor non-CLL like B-cell clones from multiclonal vs monoclonal cases (Table 3 and Supplementary Table 5). Regarding IGKV and IGLV genes, only the VK1-33 gene was associated with multiclonal cases (6% vs 0%, P=0.02) (Figure 1D)

Molecular features of phylogenetically related BCRs of B-cell clones from multiclonal cases. Thirty-two of the 85 B-cell clones from individual multiclonal cases were phylogenetically closely related and had exactly the same IGHV family (IGHV3 in 28 B-cell clones and IGHV4 in 4 B-cell clones) (Figure 2). Of note, this subgroup of B-cell clones frequently showed IGHV3 gene usage (28/85, 33%) and they displayed shorter HCDR3 sequences than other (multiclonal and monoclonal) B-cell clones − 13 (6-25) vs 17(9-26) and 16 (8-32) aminoacids; *P*=0.001 and *P*=0.004, respectively −; in addition, they also showed a higher frequency of del(13q14.3) compared to B-cell clones from multiclonal cases expressing phylogenetically unrelated IGHV families (41% vs 17%, respectively; *P*=0.05). Moreover, a slightly higher frequency of multiclonal cases whose coexisting clones were cytogenetically altered was found among phylogenetically closely related clones vs phylogenetically unrelated clones from multiclonal cases (53% vs 34%, respectively; *P*=0.06). Interestingly, a trend towards an increased percentage of IGHV mutated B-cell clones among phylogenetically related B-cell clones from multiclonal cases compared to other B-cell clones from multiclonal cases, was also found (70% vs 54%, respectively; *P*=0.1). Interestingly, most of the co-existing phylogenetically related clones had a CLL-like phenotype (10/16 cases, identified in Supplementary Table 3 with the ¶ symbol), while in 4/16 multiclonal cases, one CLL-like B- cell clone coexisted with one non-CLL B-cell clone, (2 marginal zone lymphomas, 1 lymphoma of mucosa-associated lymphoid tissue and 1 hairy cell leukemia clones from cases 29, 32, 37 and 38, also identified in Supplementary Table 3 with the ¶ symbol). In a minority of cases (2/16), the two co-existing phylogenetically related clones were both non-CLL like, their phenotype being consistent with follicular lymphoma (case 16¶, in Supplementary Table 3) and lymphoma of mucosa-associated lymphoid tissue (case 34¶, in Supplementary Table 3), respectively.

Homology of the HCDR3 region between B-cell clones coexisting in multiclonal cases vs non-coexisting (monoclonal) B-cell clones. The HCDR3 aminoacid sequence from coexisting B-cell clones had the same length or it just differed in one aminoacid in 8/41 multiclonal cases analyzed (19%) (Table 4A). The homology of all these case-paired HCDR3 regions was calculated as the number of identical aminoacids or aminoacids with an analogous side-chain polarity (excluding the anchor second-CYS104 (**C**_) and the J-TRP 118 (_**W**) aminoacid positions that delineate the HCDR3 region) divided by the corresponding HCDR3 length (Table 4A). It is worth noting that the aminoacid composition of HCDR3 sequences of the same length (±1 aminoacid) that belonged to the same or evolutionary, highly-related, VH families (e.g. VH3-48, VH3-21, VH3-11)[33] (n=57) from monoclonal cases (Supplementary Table 6) showed a tendency towards a lower homology than that of multiclonal cases: median of 37% (range: 11% to 71%) vs 50% (range: 26% to 64%), respectively; (P=0.1). Since stereotyped sequences are widely represented in CLL,[34] we further analyzed the frequency of stereotyped HCDR3 sequences in multiclonal cases (Table 4B) vs. monoclonal cases (Table 4C). Interestingly, the number of multiclonal cases showing the same or highly similar stereotyped HCDR3 sequences was significantly higher than that of monoclonal cases: 8/41 (19%) vs 11/143 (8%), respectively, (P=0.001). Furthermore, the aminoacid composition of HCDR3 sequences from monoclonal cases with stereotyped HCDR3 sequences showed clearly less identical and/or conserved positions than those found among multiclonal cases (underlined aminoacids in Tables 4B and 4C).

Discussion

Multiclonal expansions of phenotypically aberrant B-cell clones (MBL^{lo}) have been reported as frequently present in the general population[35]; of note, multiclonal expansions of immunophenotypically normal B-cells can also be found in non-malignant diseases, such as autoimmune disorders and inflammatory responses against several infectious agents (e.g. Helicobacter pylori, hepatitis C virus).[36,37] Whether clonal expansions of aberrant B-cells found in otherwise healthy individuals (MBL^{lo}) reflect a prominent reactive process against potent antigenic stimuli with unknown clinical relevance, or they represent an early (multi)clonal manifestation of a BCR-dependent neoplastic event, still remains to be established. In this regard, it should be noted that between 30% and 40% of such cases show cytogenetic changes shared by MBL and CLL, e.g. del(13q). Of note, among other large structural chromosomal alterations, clonal mosaicism involving del(13q14) has also been recently found in peripheral blood cell populations from otherwise healthy individuals, particularly among subjects with more advanced age (around 2-3% in the elderly), but its potential relationship with MBL and CLL remains unknown.[38,39] Compared to the typical (monoclonal) MBL and B-CLPD, coexisting B-cell clones from multiclonal MBL and B-CLPD may potentially have a greater probability of interacting with common immunological determinants. However, there is still little information about the potential existence of shared BCR features in cases showing ≥2 coexisting B-cell clones vs monoclonal cases.

In the present study, we analyzed for the first time the molecular and cytogenetic features of a large group (n=85) of coexisting, but unrelated, B-cell clones from a series of 41 multiclonal MBL and B-CLPD cases, in comparison to 143 monoclonal cases. Overall, the former clones more frequently showed cytogenetic and hematological features which are typical of the earliest MBL stages and/or initial phases of CLL.[18,36,37] Accordingly, B-cell clones from multiclonal cases more frequently corresponded to MBL cases, whereas B-cell clones from monoclonal cases were more frequently found to correspond to overt CLL. Of note, these findings do not contradict the apparent discrepancy between such association and our previous observation among CLL patients of a worse clinical outcome for multiclonal cases carrying non-CLL clones,[11] as this latter study was restricted to overt CLL cases. In addition, multiclonal

cases were also associated with lower clonal B-cell counts in peripheral blood, a lower number of cytogenetically altered clones, particularly of those carrying del(13q), and a decreased frequency of clones with ≥2 alterations. Of note, clonal expansions of non-CLL like B-cell clones were also more frequently observed in multiclonal than in monoclonal cases, such expansions corresponding mainly to indolent lymphomas (e.g. marginal zone lymphoma) which have been associated with chronic immune responses.[40,41]

Altogether, these results support the notion that the presence of multiple B-cell clones in the same individual more closely reflects the earlier stages of the disease. If this holds true and chronic antigen stimulation is involved in the onset of MBL and B-CLPD -as it has been recently suggested for MBL, based on epidemiological studies-,[42] it could be hypothesized that B-cell clones coexisting in multiclonal cases would show more closely related BCR features than B-cell clones from monoclonal cases. In this regard, our results point out the existence of a slightly higher level of HCDR3 homology among B-cell clones from multiclonal vs monoclonal cases. In fact, in around one fifth of all multiclonal cases, the co-existing B-cell clones showed a high homology in their HCDR3 aminoacid sequences; this also hold true when we compared the homology of the HCDR3 sequences of these multiclonal against those of monoclonal cases whose B-cell receptors were restricted to the same and/or ontogenetically related IGHV families. In addition, the frequency of stereotyped HCDR3 was also higher in multiclonal vs. monoclonal cases. Such more closely related BCR features would be found independently of whether common antigens or superantigens are specifically involved, although the former would potentially lead to a higher HCDR3 homology, whereas superantigens could contribute to a greater frequency of usage of specific IGHV, IGHD and/or IGHJ genes.[13,43]

In the present study, we found a similar frequency of IGHV gene usage between coexisting multiclonal and non-coexisting monoclonal B-cell clones in association with a lower frequency of DH1, DH4 and DH7 as well as JH6 families in multiclonal vs monoclonal B-cell clones. Overall, these results suggest that no single antigen or superantigen is involved in common in MBL and B-CLPD. This is further supported by the relatively low percentage of alignment (≈50%) of the IGHV aminoacid sequences observed among the different clonal B-cell populations analyzed, since such potential antigens -including superantigens- would require interaction with highly conserved sites at the IGHV/HCDR3 regions of the BCR.[44] Interestingly

however, the higher representation of DH1, DH4, DH7 and JH6 IGH gene segments in monoclonal vs multiclonal B-cell clones, together with the slightly higher levels of HCDR3 homology observed among coexisting (multiclonal) vs non-coexisting (monoclonal) B-cell clones from MBL, CLL and other B-CLPD cases, would indicate that still non-random selection of specific HCDR3, DH and JH segments could exist in the MBL and CLL repertoire of both multiclonal and monoclonal cases, which could reflect antigen-driven selection and expansion of specific B-cell clones, both at the MBL and/or CLL stages.[45]

In this regard, based on the phylogenetic proximity of their BCR, we could further identify within the B-cell clones from multiclonal cases, a considerably represented subgroup of B-cell clones showing preferential usage of IGHV3 genes and shorter HCDR3 sequences carrying a significantly higher number of IGHV mutations vs the unrelated clones. These results further support the involvement of a common antigen, at least in this specific subset of cases.[46] Interestingly, these "phylogenetically-related" B-cell clones coexisting in multiclonal cases showed a significantly higher frequency of del(13q) than B-cell clones expressing other IGHV genes. These observations further suggest that the BCR features of this subset of coexisting multiclonal B-cell clones could also contribute to determine the probability and/or type of cytogenetic progression occurring at the earliest stages of the disease, as previously suggested by our group[18] and others.[47,48] Further long-term, longitudinal studies are required to confirm this hypothesis, since multiple productive IGHV gene rearrangements may also underline clonal drift leading to selection for more aggressive clones whose proportions would change over time.[49]

In summary, based on the molecular features of the BCR and the cytogenetic profile of B-cell clones from the multiclonal vs monoclonal MBL, CLL and other B-CLPD cases here analyzed, it may be concluded that multiclonality is typically associated with early stages of B-CLPD, at the same time it appears to more closely reflect an antigen-driven nature of MBL and B-CLPD, with potential involvement of multiple and diverse antigenic determinants.

AUTHORSHIP AND DISCLOSURES

AH and AR-C performed research, data analysis, and wrote the manuscript; IC helped with the execution of laboratory work and sequencing alignment analysis; AWL and WGN conducted analysis and validation of data; QL performed computational research and contributed to manuscript preparation; MG and EC provided patient samples and clinical data; AP retrieved relevant clinical information from patient records; JA and AO designed research, analyzed the data and wrote the manuscript. All authors read, approved the manuscript and reported no potential conflicts of interest.

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Table 1. Peripheral blood (PB) B-cell counts and BCR features of multiclonal vs. monoclonal B-cell clones from B-cell chronic lymphoproliferative disorders (B-CLPD) and monoclonal B cell lymphocytosis (MBL) cases.

	Multiclonal B-cells n=85 clones	Monoclonal B-cells n=143 clones	TOTAL n=228 clones
N. of PB clonal B cells(x10^6/L)*	**2,692 (0.6-156,168)[a]**	**9,115 (0.1-369,288)**	5,530 (0.1-369,288)
% of PB clonal B cells from WBC*	**13% (0.1%-89%)[a]**	**45% (0.002%-97%)**	35% (0.001%-97%)
CLL like MBL^{lo} B-cell clones	14/85 (16%)	13/143 (9%)	27/228 (12%)
CLL like MBL^{hi} B-cell clones	**26/85 (31%)[a]**	**26/143 (18%)**	52/228 (38%)
CLL B-cell clones	**26/85 (31%)[a]**	**89/143 (62%)**	113/228 (50%)
CLL-stage A clones	12/20 (60%)	53/89 (60%)	65/109 (60%)
CLL-stage B/C clones	8/20 (40%)	36/89 (40%)	44/109 (40%)
Non-CLL like MBL B-cell clones	5/85 (6%)	4/143 (3%)	9/228 (4%)
Non-CLL B-cell clones	**14/85 (17%)[a]**	**11/143 (8%)**	25/228 (11%)
IGHV mutated CLL-like B-cell clones	40/66 (61%)	76/128 (59%)	116/194 (60%)
IGHV mutated non-CLL-like B-cell clones	11/19 (58%)	8/15 (53%)	19/34 (56%)
% alignment of IGHV aa sequences between coexisting B-cell clones*	51% (38%-79%)	NA	51% (38%-79%)
% alignment of IGHV aa sequences between each B-cell clone and the other clones*	**52% (31%-100%)[a]**	**50% (29%-100%)**	51% (29%-100%)

Results expressed as number of B-cell clones and percentage between brackets or as *median value (range). PB, peripheral blood; WBC, white blood cells; CLL, chronic lymphocytic leukemia/small lymphocytic lymphoma; MBL^{lo}, low count monoclonal B-cell lymphocytosis; IGHV, immunoglobulin heavy chain variable region genes; CLL, chronic lymphocytic leukemia/small lymphocytic lymphoma; MBL^{hi}, clinical monoclonal B-cell lymphocytosis; aa, aminoacids. NA, not appropriate. [a]Statistically significant differences ($P < 0.05$) found between clones from multiclonal *vs* monoclonal cases. Information about the parameters included in this table is separately displayed for CLL-like vs non-CLL-like clones in Supplementary Table 7.

Table 2. **Cytogenetic features of CLL-like MBL^{lo}, MBL^{hi} and CLL B-cell clones from monoclonal *vs* multiclonal cases.**

Cytogenetic alterations	MBL^{lo} clones		MBL^{hi} clones		CLL clones		TOTAL	
	Multiclonal	**Monoclonal**	**Multiclonal**	**Monoclonal**	**Multiclonal**	**Monoclonal**	**Multiclonal**	**Monoclonal**
	n=14	**n=13**	**n=26**	**n=26**	**n=26**	**n=89**	**n=66**	**n=128**
No. of cytogenetically altered clones	2/14 (14%)	6/13 (46%)	13/26 (50%)	13/26 (50%)	12/26 (46%)	58/89 (65%)	**27/66 (41%)[b]**	**77/128 (60%)**
No. of clones with ≥2 alterations	1/14 (7%)	1/13 (8%)	5/26 (19%)	2/26 (8%)	**2/26 (8%)[a]**	**29/89 (33%)**	**8/66 (12%)[b]**	**32/128 (25%)**
Type of cytogenetic changes								
No. of del(13q)$^+$ clones (%)	2/14 (14%)	5/13 (38%)	7/26 (27%)	8/26 (31%)	8/26 (31%)	36/89 (40%)	17/66 (26%)	49/128 (38%)
*% del(13q)$^+$ cells **	46% (19%-73%)	86% (22%-96%)	74% (15%-98%)	38% (21%-99%)	93% (30%-96%)	80% (47%-99%)	84% (10%-98%)	79% (18%-99%)
No. of del(13q14.3)$^+$ clones (%)	2/14 (7%)	4/13 (31%)	7/26 (27%)	8/26 (31%)	8/26 (31%)	35/89 (39%)	17/66 (26%)	47/128 (37%)
*% del(13q14.3)$^+$ cells **	(19%-73%)	78% (22%-96%)	65% (15%-98%)	38% (21%-99%)	81% (30%-96%)	73% (5%-99%)	80% (15%-98%)	71% (5%-99%)
No. of del(13q14)$^+$ clones (%)	0/14 (0%)	1/12 (8%)	2/26 (8%)	1/26 (4%)	**1/26 (4%)[a]**	**17/89 (19%)**	**3/66 (5%)[b]**	**19/127 (15%)**
*% del(13q14)$^+$ cells **	-	86% (-)	57% (15%-98%)	96% (-)	**95% (-)**	**79% (47%-99%)**	**55% (10%-98%)[b]**	**86% (47%-99%)**
No. of trisomy 12$^+$ clones (%)	0/14 (0%)	1/13 (8%)	6/26 (23%)	5/26 (19%)	2/26 (8%)	17/89 (19%)	8/66 (12%)	23/128 (18%)
*% trisomy 12$^+$ cells **	-	59% (-)	87% (19%-95%)	84% (80%-93%)	84% (75%-93%)	76% (33%-97%)	87% (41%-95%)	80% (33%-97%)
No. of t(14q32)$^+$ clones (%)	0/12 (0%)	0/10 (0%)	2/26 (8%)	0/26 (0%)	1/26 (4%)	10/89 (11%)	3/64 (5%)	10/125 (8%)
*% t(14q32)$^+$ cells **	-	-	42% (31%-52%)	-	98% (-)	82% (18%-94%)	72% (28%-98%)	59% (18%-94%)
No. of del(11q)$^+$ clones (%)	0/12 (0%)	0/11 (0%)	1/26 (4%)	1/26 (4%)	1/26 (4%)	7/89 (8%)	2/64 (3%)	8/126 (6%)
*% del(11q)$^+$ cells **	-	-	93% (-)	20% (-)	91% (-)	57% (21%-98%)	92% (91%-93%)	57% (20%-98%)
No. of del(11q22.3)$^+$ clones (%)	0/12 (0%)	0/11 (0%)	1/26 (4%)	1/26 (4%)	1/26 (4%)	6/89 (7%)	2/64 (3%)	7/126 (6%)
*% del(11q22.3)$^+$ cells **	-	-	93% (-)	20% (-)	91% (-)	70% (24%-98%)	92% (91%-93%)	68% (20%-98%)
No. of del(11q23)$^+$ clones (%)	0/12 (0%)	0/11 (0%)	0/26 (0%)	0/26 (0%)	0/26 (0%)	3/89 (3%)	0/64 (0%)	3/126 (2%)
*% del(11q23)$^+$ cells **	-	-	-	-	-	32% (21%-64%)	-	40% (24%-64%)
No. of del(17p13.1)$^+$ clones (%)	0/13 (0%)	0/12 (0%)	0/26 (0%)	0/26 (0%)	0/26 (0%)	5/89 (6%)	0/65 (0%)	5/127 (4%)
*% del(17p13.1)$^+$ cells **	-	-	-	-	-	44% (33%-88%)	-	44% (33%-88%)

Results expressed as number of clones with cytogenetic changes from all clones in the corresponding group (percentage) or as *median values of altered cells/clone (range). In seven clones (1 multiclonal MBL^{lo}, 3 monoclonal and 3 multiclonal CLL clones) biallelic del(13q14.3) was detected, and polysomy was found in 1 multiclonal CLL clone. Statistically significant differences found between multiclonal *vs* monoclonal B-cell clone groups for [a]CLL clones (P=0.01) and [b]all (total) clones (P=0.01). CLL, chronic lymphocytic leukemia/small lymphocytic lymphoma; MBL, monoclonal B-cell lymphocytosis.

Table 3. Molecular characteristics of the B-cell receptor (BCR) of chronic lymphocytic leukemia (CLL)-like monoclonal B-cell lymphocytosis (MBL)^{lo}, MBL^{hi} and CLL B-cell clones from monoclonal versus multiclonal cases.

	MBL^{lo} clones		MBL^{hi} clones		CLL clones		TOTAL	
	Multiclonal n= 14	**Monoclonal n= 13**	**Multiclonal n= 26**	**Monoclonal n= 25**	**Multiclonal n= 26**	**Monoclonal n= 87**	**Multiclonal n= 66**	**Monoclonal n= 126**
HCDR3 length* (N. of aa)	15 (6-22)	13 (11-20)	16 (8-23)	17 (9-26)	17 (11-26)	18 (8-32)	17 (6-26)	17 (8-32)
VH families								
VH1	2/14 (14%)	2/13 (15%)	3/26 (12%)	4/25 (16%)	4/26 (15%)	25/87 (29%)	9/66 (14%)	31/125 (25%)
VH3	9/14 (65%)	9/13 (69%)	15/26 (58%)	16/25 (64%)	11/26 (42%)	36/87 (41%)	35/66 (53%)	61/125 (49%)
VH4	2/14 (14%)	2/13 (15%)	6/26 (23%)	3/25 (12%)	9/26 (35%)	23/87 (27%)	17/66 (26%)	28/125 (22%)
VH2, VH5, VH6	1/14 (7%)	0/13 (0%)	2/26 (8%)	2/25 (8%)	2/26 (8%)	3/87 (3%)	5/66 (7%)	5/125 (4%)
DH families								
DH1, DH4, DH7	3/14 (21%)	2/13 (15%)	**1/26 (4%)[a]**	**6/24 (25%)**	6/26 (23%)	11/86 (13%)	10/66 (15%)	19/123 (16%)
DH2	2/14 (14%)	3/13 (23%)	5/26 (19%)	7/24 (29%)	4/26 (15%)	15/86 (17%)	11/66 (17%)	25/123 (20%)
DH3	3/14 (21%)	2/13 (15%)	9/26 (35%)	7/24 (29%)	13/26 (50%)	36/86 (42%)	25/66 (38%)	45/123 (37%)
DH5	2/14 (14%)	3/13 (23%)	6/26 (23%)	2/24 (8%)	3/26 (12%)	9/86 (11%)	11/66 (17%)	14/123 (11%)
DH6	4/14 (29%)	3/13 (23%)	5/26 (19%)	2/24 (8%)	0/26 (0%)	15/86 (17%)	9/66 (13%)	20/123 (16%)
JH genes								
JH1, JH2, JH3, JH5	3/14 (21%)	2/13 (15%)	7/26 (27%)	7/24 (29%)	9/26 (35%)	14/86 (16%)	19/66 (29%)	23/123 (19%)
JH4	6/14 (43%)	7/13 (54%)	13/26 (50%)	8/24 (33%)	10/26 (38%)	34/86 (40%)	29/66 (44%)	49/123 (40%)
JH6	5/14 (36%)	4/13 (31%)	6/26 (23%)	9/24 (38%)	**7/26 (27%)[a]**	**38/86 (44%)**	**18/66 (27%)[a]**	**51/123 (41%)**
LCDR3 length* (N. of aa)	9 (8-13)	10 (8-10)	**10 (8-12)[a]**	**9 (7-12)**	10 (8-15)	9 (5-12)	**10 (8-15)[a]**	**9 (5-12)**
VK families								
VK1	0/6 (0%)	1/4 (25%)	6/14 (43%)	3/12 (25%)	6/12 (50%)	23/48 (48%)	12/32 (38%)	27/64 (42%)
VK2, VK5, VK6	1/6 (17%)	0/4 (0%)	1/14 (7%)	3/12 (25%)	2/12 (17%)	8/48 (17%)	4/32 (13%)	11/64 (17%)
VK3, VK4	5/6 (83%)	3/4 (75%)	7/14 (50%)	6/12 (50%)	4/12 (33%)	17/48 (35%)	16/32 (50%)	26/64 (41%)
JK genes								
JK1, JK3, JK5	3/6 (50%)	1/4 (25%)	5/14 (36%)	7/12 (58%)	5/12 (42%)	21/47 (45%)	13/32 (40%)	29/63 (46%)
JK2	2/6 (33%)	2/4 (50%)	8/14 (57%)	3/12 (25%)	4/12 (33%)	10/47 (21%)	14/32 (44%)	15/63 (24%)
JK4	1/6 (17%)	1/4 (25%)	1/14 (7%)	2/12 (17%)	3/12 (25%)	16/47 (34%)	5/32 (16%)	19/63 (30%)
Vλ families								
Vλ3	2/3 (67%)	NA	1/7 (14%)	0/7 (0%)	2/7 (29%)	8/25 (32%)	5/17 (29%)	8/32 (25%)
Other	1/3 (33%)	NA	6/7 (86%)	7/7 (100%)	5/7 (71%)	17/25 (68%)	12/17 (71%)	24/32 (75%)
Jλ genes								
Jλ1	1/3 (33%)	NA	0/7 (0%)	0/7 (0%)	4/7 (57%)	6/21 (71%)	5/17 (29%)	6/28 (21%)
Other	2/3 (67%)	NA	7/7 (100%)	7/7 (100%)	3/7 (43%)	15/21 (29%)	12/17 (71%)	22/28 (79%)
IGHV mutational status								
Mutated IGHV	9/14 (64%)	8/11 (73%)	17/26 (65%)	20/25 (80%)	14/26 (54%)	46/86 (54%)	40/66 (61%)	74/122 (61%)
Unmutated IGHV	5/14 (36%)	3/11 (27%)	9/26 (35%)	5/25 (20%)	12/26 (46%)	40/86 (47%)	26/66 (39%)	48/122 (39%)

Results expressed as number of B-cell clones from all clones in the corresponding group (percentage) or as *median (range). CLL, chronic lymphocytic leukemia/small lymphocytic lymphoma; MBL, monoclonal B-cell lymphocytosis; BCR, B-cell receptor; HCDR3, heavy chain complementarity-determining region 3; LCDR3, light chain complementarity-determining region 3; aa, amino acid. NA, not analyzed; [a] statistically significant differences found between groups of clones from multiclonal vs monoclonal cases ($P \leq 0.03$).

Table 4. Multiclonal cases with coexisting B-cell clones sharing HCDR3 features (A). Multiclonal (B) and monoclonal (C) cases with B-cell clones showing stereotyped HCDR3 aminoacid sequences.

A. Multiclonal cases with coexisting B-cell clones showing identical or one aa differing length of HCDR3 regions, and analogous composition of aa in some parallel positions.

Multiclonal Case ID	VH families	AA composition of HCDR3 (length)	% homology[#]
8A	V1-3	**C**_ARDRVVIIPDTTTINWFDP_**W** (19)	26
8B	V3-53	**C**_ATHPTNIYTRWPYVSDMDV_**W** (19)	
11A	V3-23	**C**_ANRGETRGMDV_**W** (11)	54
11B	V3-48	**C**_VRDGFHYYGFDI_**W** (11)	
14A	V4-34	**C**_ARGPDRLYSGSYTRFDY_**W** (17)	47
14B	V4-34	**C**_ARREDDNFWSGFYMDV_**W** (16)	
22A	V3-74	**C**_ARDLDGSGSGVFDW_**W** (14)	64
22B	V4-59	**C**_ARGWRSTDSYYGMDV_**W** (15)	
29A	V3-48	**C**_VRELWFGNGGDY_**W** (12)	42
29B	V3-15	**C**_ATAGQGSADFLY_**W** (12)	
31A	V3-33	**C**_ARGELLHNWFDP_**W** (12)	58
31B	V3-23	**C**_AKDGFPYYGFDI_**W** (12)	
32A	V4-39	**C**_ARQTGWLAPSDY_**W** (12)	54
32B	V4-34	**C**_ARRDSSGWYYFDY_**W** (13)	
33A	V2-26	**C**_AGTNIPRQFDFWSGSSPNWFDP_**W** (22)	32
33B	V3-53	**C**_ARAGGYCNSGSCRGAPRWYFDL_**W** (22)	

Aminoacids with analogous side-chain polarity (highlighted in gray): case 8 (I, V and F, M), case 11 (A, V and M, F), case 14 (L, F), case 22 (L, W; S, T and F, M), case 29 (A, V), case 31(L, F and H, Y), case 32 (T, S), case 33 (W, C) (EMBL-EBI Cambridge, UK). All cases had coexisting B-cell clones with CLL-like phenotype, except case 29 (one B-cell clone corresponded to a MALT lymphoma-like phenotype), cases 32 and 33 (both had one B-cell clone with a MZL-like phenotype). MALT, lymphoma of mucosa-associated lymphoid tissue; MZL, marginal zone lymphoma. [#]Number of aminoacids with analogous side-chain polarity (excluding the delineating **C**_ and _**W** positions)/HCDR3 length*100

B. Multiclonal cases with B-cell clones showing stereotyped HCDR3 sequences.

Multiclonal Cases ID	V(D)J rearrangement	AA composition of HCDR3
1	V3-30(D3-9)J6	**C**_AKYGGVKLRYFDWLLYGDYYYGMDV_**W**
2	V3-30(D3-9)J6	**C**_AKYGGVKLRYFDWLLYGDYYYGMDV_**W**
9	V3-23(D5-12)J6	**C**_ANRGETRGMDV_**W**
15	V3-23(D3-22)J6	**C**_ANRGESWGMDV_**W**
21	V3-21(D2-2)J6	**C**_ARDANGMDV_**W**
35	V3-21(D2-2)J6	**C**_ARDANGMDV_**W**
22	V3-74(D3-10)J4	**C**_ARDLDGSGSGVFDW_**W**
40	V3-21(D4-23)J4	**C**_ARDLDGGNSVFDC_**W**

Cases 1 and 2; 9 and 15; 21 and 35; 22 and 40 showed a highly similar HCDR3 sequence; the underlined aminoacids were different. All the listed B-cell clones had a CLL-like phenotype.

C. Monoclonal cases with B-cell clones showing stereotyped HCDR3 sequences.

Monoclonal Cases ID	V(D)J rearrangement	AA composition of HCDR3
117	V3-7(D3-3)J4	**C**_VRENELWSGGWGLDG_**W**
134	V3-7(D3-3)J4	**C**_VRENEFWSGGWGLDG_**W**
207	V4-39(D2-2)J6	**C**_ARHRLGYCSSTSCYYYYYGMDV_**W**
208	V4-39(D2-2)J6	**C**_ARHRLGYCSSTSCYYYYYGMDV_**W**
210	V4-39(D2-2)J6	**C**_ARDRLGYCSSTSCYYYYYGMDV_**W**
187	V4-b(-)J4	**C**_ARSWIQLWSEFDY_**W**
215	V4-b(D5-5)J4	**C**_ARAWIQLWSDFDY_**W**
180	V1-2/D6-19/J4	**C**_ARLQWLGISHFDY_**W**
204	V1-2/D6-19/J4	**C**_ARAQWLVLENFDY_**W**
196	V4-34/D3-16/J6	**C**_VRGYPSDYTERRYYYYGLDV_**W**
198	V4-34/D4-23/J6	**C**_ARGYGSTGETRRYYYYGMDV_**W**

Cases 117 and 134; 207, 208 and 210; 187 and 215; 180 and 204; 196 and 198 showed a highly similar HCDR3 region; the underlined aminoacids were different. All the listed B-cell clones had a CLL-like phenotype.

Legend to Figures

Figure 1. Frequency of IGHV (panel A), IGHD (panel B), IGHJ (panel C) and both IGKV and IGLV (panel D) genes in multiclonal and monoclonal CLL versus non-CLL like B cell clones. Diagrams show the relative frequency of each IG gene in multiclonal compared to monoclonal B-cell clones (black and white bars, respectively). *Statistically significant differences were found between the multiclonal vs monoclonal subgroups ($P < 0.05$).

Figure 2. Sequence distance cladogram of IGHV gene usage in CLL-like and non-CLL like B-cell clones from multiclonal (dark colored bars in the outside circle) and monoclonal (light colored bars in the outside circle) cases. Five major branches were found in the sequence distance cladogram (i.e VH1, VH5, VH3, VH2-VH6, VH4). B-cell clones from individual multiclonal cases are represented by numbers; from them, those phylogenetically closely related B-cell clones, which share the same IGHV family, are specifically identified by bold numbers in the inner part of the circle and the symbol ¶. Of note, B-cell clones from multiclonal cases 14¶, 16¶ and 35¶ belong to closely located sub-branches of the cladogram, having their IGHV sequences an aminoacid identity of 79%, 76% and 69%, respectively. In turn, B-cell clones from the multiclonal case 32¶ belong to the VH4 major branch with IGHV sequences whose aminoacid identity is of 69%. Finally, the other B-cell clones from multiclonal cases – cases 2¶, 11¶, 15¶, 18¶, 19¶, 27¶, 29¶, 31¶, 34¶, 37¶, 38¶ and 40¶– belong to the VH3 major branch, having IGHV sequences with an aminoacid identity which is > 60% (–68%, 73%, 73.4%, 61%, 79%, 70%, 63%, 77%, 69.9%, 70%, 72% and 68.4%–, respectively).

Figure 1

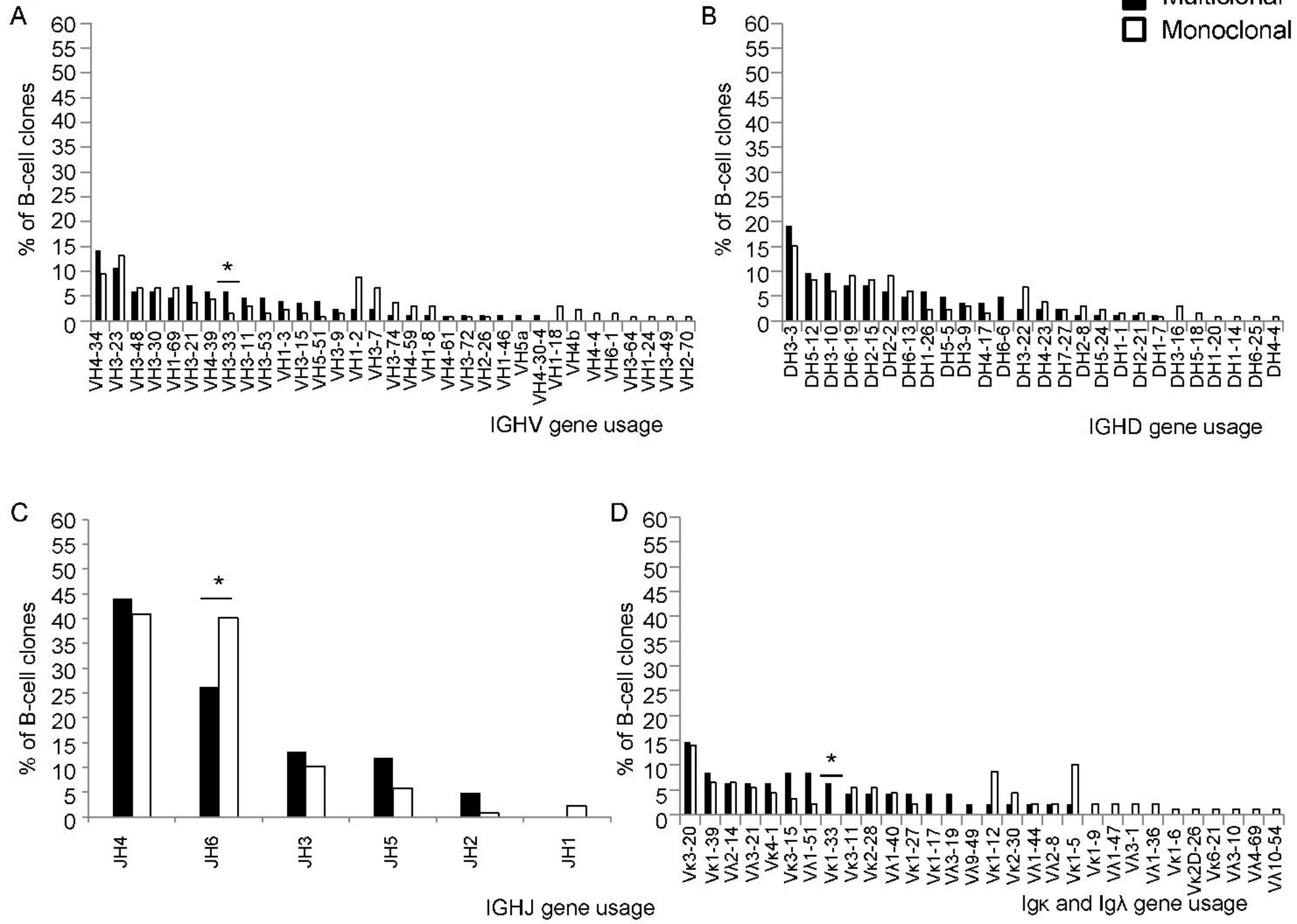
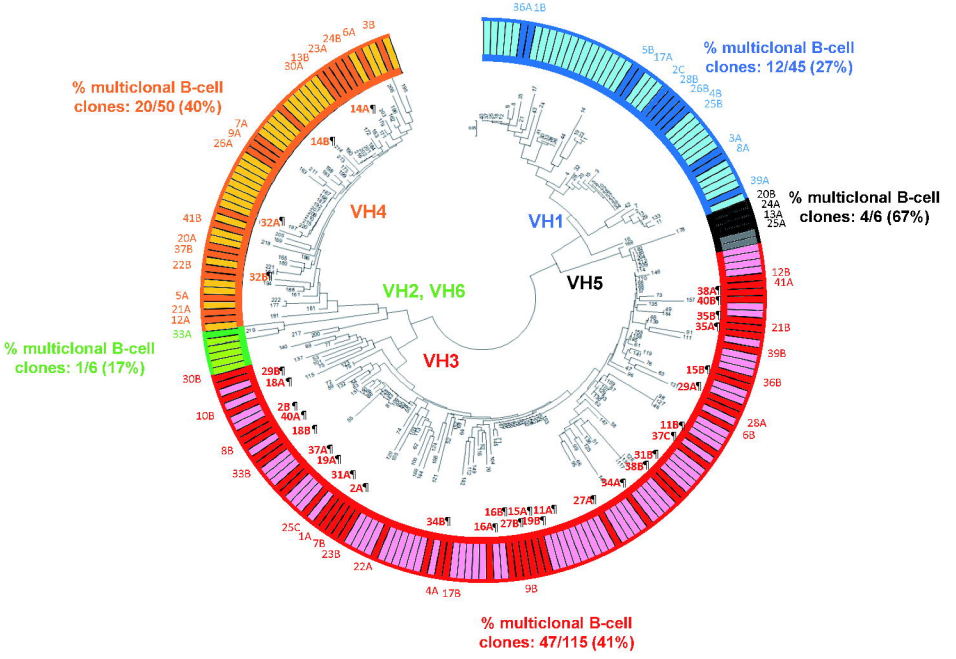

A
% of B-cell clones
IGHV gene usage
VH4-34
VH3-23
VH3-48
VH3-30
VH1-69
VH3-21
VH4-39
VH3-33
VH3-11
VH3-53
VH1-3
VH3-15
VH5-51
VH3-9
VH1-2
VH3-7
VH3-74
VH4-59
VH1-8
VH4-61
VH3-72
VH2-26
VH1-46
VH5a
VH4-30-4
VH1-18
VH4b
VH4-4
VH6-1
VH3-64
VH1-24
VH3-49
VH2-70
B
Multiclonal
Monoclonal
% of B-cell clones
IGHD gene usage
DH3-3
DH5-12
DH3-10
DH6-19
DH2-15
DH2-2
DH6-13
DH1-26
DH5-5
DH3-9
DH4-17
DH6-6
DH3-22
DH4-23
DH7-27
DH2-8
DH5-24
DH1-1
DH2-21
DH1-7
DH3-16
DH5-18
DH1-20
DH1-14
DH6-25
DH4-4
C
% of B-cell clones
IGHJ gene usage
JH4
JH6
JH3
JH5
JH2
JH1
D
% of B-cell clones
Igκ and Igλ gene usage
Vκ3-20
Vκ1-39
Vλ2-14
Vλ3-21
Vκ4-1
Vκ3-15
Vλ1-51
Vκ1-33
Vκ3-11
Vκ2-28
Vλ1-40
Vκ1-27
Vκ1-17
Vλ3-19
Vλ9-49
Vκ1-12
Vκ2-30
Vλ1-44
Vλ2-8
Vκ1-5
Vκ1-9
Vλ1-47
Vλ3-1
Vλ1-36
Vκ1-6
Vκ2D-26
Vκ6-21
Vλ3-10
Vλ4-69
Vλ10-54

Figure 2


% multiclonal B-cell clones: 12/45 (27%)
% multiclonal B-cell clones: 4/6 (67%)
% multiclonal B-cell clones: 47/115 (41%)
% multiclonal B-cell clones: 1/6 (17%)
% multiclonal B-cell clones: 20/50 (40%)
VH1
VH2, VH6
VH3
VH4
VH5

SUPPLEMENTARY MATERIAL

SUPPLEMENTARY METHODS

Patients: diagnostic criteria for MBL. MBL was defined by the presence of small clones of aberrant B-cells in the peripheral blood (PB), with a clonal B-cell count below the threshold for diagnosis of CLL (< 5.0 × 10^9 cells/L) (*Br J Haematol*. 2005;130(3):325-32). A MBL case was subclassified as "low count" MBL (MBL^{lo}) when the absolute number of clonal B-lymphocytes was less than 200 cells/μL in PB and as "high count" MBL (MBL^{hi}) when this number ranged between ≥200 and <5,000 clonal CLL-like B-cells/μL of PB.[18]

Immunophenotypical analyses. Immunophenotypic studies to screen for the presence and full characterization of clonal B-cell populations were performed on erythrocyte-lysed PB samples according to procedures which have been previously described in detail.[18,25] PB white blood cells (WBC) were systematically stained with the following monoclonal antibody (MAb) combinations following the EuroFlow recommendations:[26,27] 1) CD20-Pacific Blue (PacB), CD45-Pacific Orange (PacO), CD8-fluorescein isothiocyanate (FITC) plus anti–SmIgλ-FITC, CD56-phycoerythrin (PE) plus anti–SmIgκ-PE, CD4-peridin chlorophyll protein-cyanin 5.5 (PerCPCy5.5), CD19-PE-cyanin 7 (PECy7), CD3-allophycocyanin (APC) and CD38-Alexa Fluor 700 (AF700) – EuroFlow lymphocyte screening tube (LST) –;[27] 2) CD20-PacB, CD45-PacO, CyBcl2-FITC, CD23-PE, CD19-PerCPCy5.5, CD10-PECy7, CD5-APC and CD38-AF700, and; 3) CD20-PacB, anti–SmIgλ-FITC, anti–SmIgκ-PE, CD19-PerCPCy5.5, CD10-PECy7 and CD5-APC. All cases showed a clonal (imbalanced SmIgκ:SmIgλ ratio of >3:1 or <1:3) and/or an aberrant $CD5^+$ B-cell population for the above MAb combinations[18]; in every case, the phenotypic study was extended with additional 5- and 6-color stainings, as reported elsewhere.[28]

Data acquisition for ≥5x10^6 leucocytes/tube was performed in FACSCanto II flow cytometers (BD) using the FACSDiva software (V6.1; BD). Instrument setup, calibration and daily monitoring were performed according to the EuroFlow protocols.[26] For data analysis, the Infinicyt™ software (Cytognos SL, Salamanca, Spain), was used. The minimum number of clustered events required to define a B-cell population was of 50 cells.

Purification of B-cell populations. In all cases studied, each sIg light chain restricted and phenotypically aberrant B-cell population identified was purified in a FACSAria II flow cytometer (BD). In those samples (n=41) containing ≥2 aberrant B-cell populations, discrimination among them was based on their distinct patterns of expression for ≥1 of the B-cell markers analyzed, as described elsewhere.[29] The clonal nature

of each FACS-purified B-cell population (purity: 98%±0.8%) was assessed by both cytogenetic and molecular techniques, as described below.

Cytogenetic and molecular studies. The presence of those cytogenetic alterations commonly associated with CLL and other non-CLL B-CLPD was investigated by multicolor interphase fluorescence *in situ* hybridization (iFISH) on slides containing FACS-purified and fixed aberrant B-cells, as previously described in detail.[18,30] The following DNA probes purchased from Vysis Inc. (Downers Grove, IL, USA) allowed the detection of gains/losses and/or chromosomal translocations involving specific genes and chromosomal regions: CEP6 DNA probe conjugated with spectrum orange (SO), CEP12 DNA probe conjugated with SO, LSI ATM (11q22.3) conjugated with SO, LSI MLL (11q23) dual color probe, LSI p53 (17p13.1) conjugated with SO, LSI13/RB1 gene (13q14) conjugated with spectrum green, LSI D13S25 (13q14.3) conjugated with SO, LSI BCL6 (3q27) dual color, LSI MALT1 (18q21) dual color, LSI IgH (14q32) dual color, LSI IGH/CCN1 t(11;14)(q13;q32.3) dual color, LSI IGH/bcl2 t(14;18)(q32;q21) dual color probe and IGH/MYC/CEP8 (8q24) tri-color probe.

In parallel, analysis of the patterns of rearrangement of the immunoglobulin heavy chain variable region genes (IGHV) and immunoglobulin K (IGKV) and λ (IGLV) light chain genes was performed for each FACS-purified B-cell clone. Extraction and purification of genomic DNA, PCR amplification, as well as sequencing, and analysis of V, (D), J gene sequences were performed following well-established protocols, which have been described in detail elsewhere.[18,31,32] Forward (F) and reverse (R) sequences were aligned into a single resolved sequence and then aligned with germline sequences using the IMGT database and tools (http://imgt.org). For MBL^{lo} clones, whole genomic amplification (WGA) was performed prior to analysis, using the Replig^{R}UltraFast Mini kit (Qiagen, Valencia, CA) as per the recommendations of the manufacturer. For each FACS-sorted B-cell population, only in-frame rearrangements were evaluated. Sequences containing >2% deviation from the germline sequence were considered as being somatically mutated.

Each deduced "IMGT/V-QUEST aminoacid (aa) sequence" corresponding to individual IGHV gene sequences from purified B-cell clones from both monoclonal and multiclonal cases was aligned using the bioinformatic tools available at the web services of the European Bioinformatics Institute (EMBL-EBI Cambridge, UK). More than 12,400 alignments of IGHV aminoacid sequences, with a coverage ranging from framework region (FR) 1 to the HCDR3 region (both regions included) were obtained for all B-cell clones (a total of 8,891 alignments within the monoclonal and 3,560 alignments within the multiclonal

groups of cases).Then, the percentage of alignment of IGHV aminoacid sequences obtained after two-by-two comparisons between the distinct B-cell clones, was calculated for every pair of B-cell clones. Finally, each single paired-alignment obtained -8,891 and 3,560 in monoclonal vs. multiclonal cases, respectively- was included in a final database, to calculate the median and range of the total IGHV aminoacid alignment percentages and to calculate the statistical significance of their differences observed between the two groups (p-values).

To investigate the level of phylogenetic relationship among IGHV aminoacid sequences corresponding to distinct clones from multiclonal cases, as well as monoclonal cases, a sequence distance tree was built using the neighbor-joining method implemented in the freely available Molecular Evolutionary Genetic Analysis (MEGA) software (version 5.2, http://www.megasoftware.net).[33] Examination of the different branches of the sequence distance tree allowed the distinction of multiclonal cases whose clones had IGHV aminoacid sequences phylogenetically closer than others. Thus, sequences in the same major branch were guaranteed to exhibit ≥50% aminoacid identity (from FR1 to HCDR3, both regions included).[33] As might be expected, sequences in sub-branches emerging from the same major branch exhibit even more aminoacid identity, ranging from 60% to 99%. In our analysis, those co-existing B-cell clones with IGHV aminoacid sequences that belonged to the same major branch with >60% aminoacid identity or belonged to close located sub-branches were assumed to be "phylogenetically" related sequences.

Those HCDR3 regions with an identical length or a length differing in one aminoacid were (case-paired) analyzed using the EMBL-EBI tools to determine those positions which had an identical or conserved composition in terms of "hydropathy", "volume" and "chemical characteristics" as outlined in the IMGT classification of aminoacids (J Mol Recognit. 2004;17:17-32).

Statistical methods. Comparisons between groups were performed with either the nonparametric Kruskal-Wallis and Mann-Whitney U tests (for continuous variables) or the Pearson's $\chi 2$ and Fisher exact tests (for categorical variables) using the SPSS software/version 20.0 (IBM SPSS Statistics, IBM, Armonk, NY, USA). *P* values < 0.05 were considered to be associated with statistical significance.

Supplementary Table 1. Age and gender features of subjects included in the study.

		Diagnostic subgroups					
		CLL and CLL-like MBL			Non-CLL B-CLPD and non-CLL MBL		
	Age/Gender Features	**MBL^{lo}**	**MBL^{hi}**	**CLL**	**MBL^{lo}**	**MBL^{hi}**	**Non-CLL**
Cases	Monoclonal (n=143)	13 (9%)	26 (18%)	89 (62%)	2 (1%)	2 (1%)	11 (8%)
	Age (years)*	66±13 (49-84)	69±13 (37-89)	69±13 (35-89)	65 & 95	79 & 80	71±9 (53-84)
	Male/Female	4/9	15/11	50/39	1/1	0/2	7/4
	Multiclonal (n=41)	2 (5%)	8 (19.5%)	23 (56%)	- (0%)	- (0%)	8 (19.5%)
	Age (years)*	77 & 83	76±6 (65-82)	75±9 (57-89)	-	-	74±9 (56-81)
	Male/Female	1/1	5/3	16/7	-	-	6/2

Age values expressed as *media ± one standard deviation (range) when n>2. MBL, monoclonal B-cell lymphocytosis; CLL, chronic lymphocytic leukemia/small lymphocytic lymphoma; B-CLPD, B-cell chronic lymphoproliferative disorders other than CLL or MBL.

Supplementary Table 2. Distribution of subjects included in the study and their corresponding CLL and non-CLL like B cell clones, according to diagnosis.

		Diagnostic subgroups					
		CLL and CLL-like MBL			Non-CLL B-CLPD and non-CLL MBL*		
		MBL^{lo}	MBL^{hi}	CLL	MBL^{lo}	MBL^{hi}	Non-CLL
Cases	Monoclonal (n=143)	13 (87%)	26 (76.5%)	89 (80%)	2 (100%)	2 (100%)	11 (58%)
	Multiclonal (n=41)	2 (13%)	8 (23.5%)	23 (20%)	- (0%)	- (0%)	8 (42%)
	Total (n=184)	15	34	112	2	2	19
B cell clones	Monoclonal (n=143)	13 (48%)	26 (50%)	89 (77%)	2 (40%)	2 (50%)	11 (44%)
	Multiclonal (n=85)	14 (52%)	26 (50%)	26 (23%)	3 (60%)	2 (50%)	14 (56%)
	Total (n=228)	27	52	115	5	4	25

CLL, chronic lymphocytic leukemia/small lymphocytic lymphoma (n=115 clones); MBL, monoclonal B-cell lymphocytosis (n=88 clones: 79 CLL-like MBL clones and 9 non-CLL-like MBL clones); B-CLPD, B-cell chronic lymphoproliferative disorders other than CLL (n=25 clones).
*Patients other than CLL included the following diagnoses: HCL, hairy cell leukemia (n=1 clone); MZL, marginal zone lymphoma (n=17 clones); MALT, lymphoma of mucosa-associated lymphoid tissue (n=7); MCL, mantle cell lymphoma (n=3 clones); FL, follicular lymphoma (n=4 clones); DLBCL, diffuse large B-cell lymphoma (n=1 clones); LPL, lymphoplasmacytic lymphoma (n=1 clone).
The precise diagnosis of multiclonal cases (CLL vs non-CLL) were based on consistent clinic-biological features, according to the WHO 2008 criteria[23]

Supplementary Table 3. Diagnosis, differential immunophenotypic/IGHV features and cytogenetic alterations of the coexisting aberrant B-cell populations from multiclonal MBL, CLL and other B-CLPD cases (n=41).

Case ID	Phenotype of population 1 (% from WBC; compatible diagnosis) V(D)J rearrangement-MS†	iFISH	Phenotype of population 2 (% from WBC; compatible diagnosis) V(D)J rearrangement-MS†	iFISH*
1	FSC/SSC^{lo} CD19+κ+ CD5+ CD20^{lo} (62.9%; **CLL**) V3-30(D3-9)J6-UM	**ND**	FSC/SSC^{int} CD19+κ^{hi} CD5- CD20+ (4.1%; **Non-CLL-like MBL^{hi}MZL**) V1-2(D5-5)J4-UM	**ND**
2¶	FSC/SSC^{lo}κ^{lo} FMC7^{lo} CD23+ CD5+ CD43^{lo} CD11c^{lo} (33%; **CLL**) V3-30(D3-9)J6-UM	**+12** (93%) **del(11q22.3)** (91%)	FSC/SSC^{lo}κ^{lo} FMC7^{lo} CD23+ CD5+ CD43+ CD11c+ (21%; **CLL-like MBL^{hi}**) V3-23(D2-15)J4-M	**+12** (95%) **del(11q22.3)** (93%)
3	FSC/SSC^{lo} CD19+ κ^{lo} CD5+ CD79b^{lo} FMC7- (26.5%; **CLL**) V1-3(D1-26)J3-M	**ND**	FSC/SSC^{int} CD19^{lo}λ+ CD5^{het} CD79b-FMC7+ (25.7%; **CLL**) V4-34(D2-15)J3-M	**ND**
4	FSC/SSC^{int}κ^{lo} CD20^{lo} CD79b^{lo}CD5+ (20.6%; **CLL**) V3-53(D3-22)J6-UM	**+12** (49%) **polysomy**	FSC/SSC^{lo}λ^{lo} CD20^{lo} CD79b^{lo}CD5+ (0.6%; **CLL-like MBL^{lo}**) V1-69(D3-3)J3-UM	**ND[a]**
5	FSC/SSC^{lo}λ^{lo}CD20^{lo} CD5+ CD43+ CD23+ (44.2%; **CLL**) V4-34(D3-3)J4-M	**biallelicdel(13q14.3)** (99%)	FSC/SSC^{lo}κ^{hi}CD20+ CD5- CD43-CD23- (1.4%; **Non-CLL-like MBL^{lo}MZL**) V1-8(D3-3)J5-UM	**ND**
6	FSC/SSC^{lo}κ^{lo} CD20^{lo} FMC7- CD5+ CD23+ (33%; **CLL**) V4-34(D4-23)J2-M	**del(13q14.3)** (96%)	FSC/SSC^{lo}λ^{lo} CD20^{lo} FMC7- CD5+ CD23+ (10.6%; **CLL-likeMBL^{hi}**) V3-11(D6-19)J4-M	**del(13q14.3)** (96%)
7	FSC/SSC^{lo}CD19+ κ^{lo} CD20^{lo} CD5+ (34.6%; **CLL**) V4-39(D3-3)J6-UM	**ND**	FSC/SSC^{lo}CD19+ λ^{lo} CD20^{lo} CD5+ (9.4%; **CLL-likeMBL^{hi}**) V3-33(D3-9)J4-M	**ND**
8	FSC/SSC^{lo} CD19^{lo} CD43- CD5+ CD25+ IgM+ CD27+ CD11c+/het (50.2% **CLL**) V1-3(D5-5)J5-M	**ND**	FSC/SSC^{lo} CD19+ CD43+ CD5^{hi} CD25^{hi} IgM^{hi} CD27^{hi} CD11c+ (5% **CLL-like MBL^{hi}**) V3-53(D2-8)J6-M	**ND**
9	FSC/SSC^{lo}κ+ CD5+ CD20^{lo} CD43- (12.6%; **CLL-like MBL^{hi}**) V3-23(D5-12)J6-UM	**+12** (87%)	FSC/SSC^{lo}κ+ CD5+ CD20^{lo} CD43+ (3%; **CLL-like MBL^{hi}**) V4-39(D6-13)J5-UM	**+12** (93%)
10	FSC/SSC^{lo}λ^{lo} CD22^{lo} CD23+ CD5+ (54.6%; **CLL**) Not found	**del(13q14.3)** (96%)	FSC/SSC^{lo}κ^{lo} CD22^{lo} CD23+ CD5+ (4.1%; **CLL-like MBL^{hi}**) V3-72(D2-2)J5-M	**ND**
11¶	FSC/SSC^{lo} CD19+ κ^{lo} CD5+ CD79b^{lo} FMC7- (0.6%; **CLL-like MBL^{lo}**) V3-23(D5-12)J6-UM	**ND**	FSC/SSC^{lo} CD19+ λ+ CD5+ CD79b^{lo} FMC7- (1.6%; **CLL-like MBL^{lo}**) V3-48(D5-12)J6-M	**del(13q14.3)**(19%) **+12** (41%)
12	FSC/SSC^{lo}CD19+λ^{lo} CD5+ CD20^{lo} (89%; **CLL**) V4-39(D1-7)J3-UM	**ND**	FSC/SSC^{lo}CD19+ κ^{lo} CD5+ CD20^{lo} (0.1%; **CLL-like MBL^{lo}**) V3-21(D6-13)J4-UM	**ND**
13	FSC/SSC^{lo}CD19+ κ^{lo} CD20^{lo} FMC7-CD5+ (49.9%; **CLL**) V5-51(D2-15)J4-UM	**ND**	FSC/SSC^{lo}CD19+ λ^{lo} CD20^{lo} FMC7-CD5+ (40.8%; **CLL**) V4-34(D3-10)J4-UM	**ND**
14¶	FSC/SSC^{lo}λ+d CD20^{lo} CD5+ CD22^{lo} CD23+ FMC7- (84%; **CLL**) V4-34(D1-26)J4-UM	**biallelicdel(13q14.3)** (85%);	FSC/SSC^{lo}κ^{lo}CD20$^{lo/het}$ CD5+ CD22^{lo} CD23+ FMC7- (0.7%; **CLL-like MBL^{hi}**) V4-34(D3-3)J6-M	**del(13q14.3)** (83%)
15¶	FSC/SSC^{lo}CD19^{lo}κ+ CD5- (11%; **CLL-like MBL^{hi}**) V3-23(D3-22)J6-UM	**ND**	FSC/SSC^{lo}CD19+ λ^{lo}CD5+ (1.1%; **CLL-like MBL^{lo}**) V3-11(D2-15)J4-M	**ND[a]**
16¶	FSC/SSC^{hi} CD19^{lo}CD38++CD10+ cBcl2+ sIg- cλ+ (20.6%; **Non-CLL FL**) V3-11(D1-1)J3-M	**t(14q32)**(95%); **t(14;18)** (96%); **+18q21** (95%) **+8q24** (92%)	FSC/SSC^{lo} CD19+CD38-CD10-λ+ (2.5%; **Non-CLL FL**) V3-23(D5-12)J6-UM	**t(14;18)** (90%); **+8q21** (90%) **+18q21** (87%)
17	FSC/SSC^{lo}CD19+ λ+ CD5+ CD20^{lo} CD11c- (59.3%; **CLL**) V1-46(D3-3)J4-UM	**ND**	FSC/SSC^{lo} CD19+ Ig- CD5- CD20^{hi} CD11c^{hi} (0.5%; **Non-CLL-like MBL^{lo}MZL**) V3-53(D1-26)J4-M	**ND**

18¶	FSC/SSC^{lo}CD19+ CD5+ λ^{lo} (15.6%; **CLL-like MBL^{hi}**) V3-15(D3-3)J4-M	**ND**	FSC/SSC^{lo}CD19+ CD5+ κ^{lo} (10.1%; **CLL-like MBL^{hi}**) V3-30(D5-12)J4-M	**+12** (19%); **del(13q14.3)** (32%)
19¶	FSC/SSC^{lo}CD19+λ^{lo} CD20^{lo} CD5+ (44%; **CLL**) V3-30(D5-12)J4-M	**del(13q14.3)** (80%)	FSC/SSC^{lo}CD19+ κ^{lo}CD20^{lo} CD5+ (0.3%; **CLL-like MBL^{lo}**) V3-23(D6-19)J6-UM	**biallelic del(13q14.3)** (73%)
20	FSC/SSC^{lo}CD19+ κ^{lo}CD20^{lo} CD5+ (35.6%; **CLL**) V4-34(D3-10)J6-M	**+12** (75%);	FSC/SSC^{lo}CD19+ λ^{lo} CD20^{lo} CD5+ (0.5%; **CLL-like MBL^{lo}**) V5-a(D6-19)J6-UM	**ND[a]**
21	FSC/SSC$^{lo}\kappa^{hi}$ FMC7+ CD5- CD23- CD43- (5.2%; **Non-CLLMZL**) V4-61(D7-27)J4-M	**ND**	FSC/SSC$^{lo}\kappa^{lo}$ FMC7- CD5+ CD23+ CD43+ (2.3%; **CLL-like MBL^{lo}**) V3-21(D2-2)J6-M	**ND**
22	FSC/SSC$^{lo}\kappa$+ CD5+ CD20^{lo} CD79b^{lo} (6.4%; **CLL-like MBL^{lo}**) V3-74(D3-10)J4-M	**ND[a]**	FSC/SSC$^{lo}\kappa^{lo}$CD5+ CD20^{lo} CD79b- (0.6%; **CLL-like MBL^{lo}**) V4-59(D3-10)J6-M	**ND**
23	FSC/SSC^{lo}CD19+ κ^{lo} CD22^{lo} CD23+ CD5+ (11.5%; **CLL-like MBL^{hi}**) V4-34(D5-24)J4-M	**ND**	FSC/SSC^{lo}CD19+ λ^{lo} CD22^{lo} CD23+ CD5+ (3.8%; **CLL-like MBL^{hi}**) V3-33(D6-19)J2-M	**ND**
24	FSC/SSC$^{lo}\kappa^{lo}$ CD20^{lo} FMC7- CD5+ CD23+ (21.1%; **CLL-like MBL^{hi}**) V5-51(D5-5)J4-M	**+12** (93%)	FSC/SSC$^{lo}\lambda^{lo}$ CD20^{lo} FMC7- CD5+ CD23+ (8.7%; **CLL-like MBL^{hi}**) V4-30-4(D2-2)J4-M	**ND**
25	FSC/SSC^{lo}CD19+ κ^{lo} CD5^{het} CD20^{lo} (17.8%; **CLL-like MBL^{hi}**) V5-51(D3-3)J4-UM	**+12**(76%); **t(14q32)** (52%)	FSC/SSC^{lo}CD19+ λ^{lo} CD5^{lo} CD20^{lo} (2.6%; **CLL-like MBL^{hi}**) V1-69(D6-13)J6-UM	**ND**
26	FSC/SSC$^{lo}\kappa$+ CD5+ CD20^{lo} CD43+ (23%; **CLL-like MBL^{hi}**) V4-39(D3-3)J5-UM	**del(13q14)** (15%); **del(13q14.3)** (15%)	FSC/SSC$^{lo}\kappa^{lo}$ CD5+ CD20^{lo} CD43- (3.7%; **CLL-like MBL^{hi}**) V1-69(D5-5)J4-UM	**ND**
27¶	FSC/SSC$^{lo}\kappa^{lo}$CD20^{lo} CD79b^{lo} CD5+ (10%; **CLL-like MBL^{hi}**) V3-7(D5-12)J3-M	**del(13q14)** (98%); **del(13q14.3)** (98%)	FSC/SSC$^{lo}\lambda^{lo}$CD20^{lo} CD79b^{lo} CD5+ (0.6%; **CLL-like MBL^{lo}**) V3-23(D6-6)J4-M	**ND**
28	FSC/SSC^{lo}CD19+ λ+ CD5+ CD20^{lo} CD22- (68%; **CLL**) V3-48(D4-17)J4-M	**del(13q14)** (95%); **del(13q14.3)** (87%)	FSC/SSC^{lo}CD19+ κ+ CD11c+ CD5^{het} CD20^{hi} CD22+ (1.1%; **Non-CLL-like MBL^{hi}MZL**) V1-69(D6-6)J4-M	**ND**
29¶	FSC/SSC$^{lo}\kappa^{lo}$ CD19^{lo}, CD20^{lo} CD79b- CD43+ (36.4%; **CLL**) V3-48(D3-10)J4-M	**ND**	FSC/SSC$^{lo}\kappa^{hi}$ CD19+, CD20^{hi} CD79b+ CD43- (16.7%; **Non-CLLMALT**) V3-15(D4-17)J4-M	**del(17p13)** (91%)
30	FSC/SSC$^{lo}\lambda^{lo}$FMC7^{lo} CD5+ CD79b- CD23+ CD43+ (33.7%; **CLL**) V4-34(D3-10)J4-UM	**t(14q32)** (98%)	FSC/SSC$^{lo}\lambda$+ CD5- CD11c- FMC7^{hi}CD79b^{het} CD23- CD43- (8.4%; **Non-CLL MALT**) V3-15(D6-6)J6-M	**ND**
31¶	FSC/SSC$^{int}\kappa^{lo}$ CD23+ CD5+ CD11c+ (24%; **CLL**) V3-33(D2-15)J5-UM	**ND**	FSC/SSC$^{lo}\kappa^{lo}$ CD5+ CD23^{het} CD11c^{lo} (20%; **CLL**) V3-23(D5-12)J3-M	**ND**
32¶	FSC/SSC^{int}CD19+ κ+ CD20+ CD5- (17.7% **Non-CLLMZL**) V4-39(D6-19)J4-M	**ND**	FSC/SSC^{lo}CD19+ κ^{lo} CD5+ CD20^{lo} (3.2%; **CLL-like MBL^{hi}**) V4-34(D6-19)J4-M	**ND**
33	FSC/SSC^{lo}CD19+dλ^{lo} CD5+ CD20^{lo} (65.3%; **CLL**) V2-26(D3-3)J5-M	**del(13q14.3)** (81%)	FSC/SSC^{lo}CD19$^{hi}\lambda$+ CD5+ CD20^{hi} (12.5%; **Non-CLL MZL**) V3-53(D2-15)J2-M	**t(14q32)** (28%)
34¶	FSC/SSC$^{lo}\lambda$+ CD19+ CD20^{hi} CD22+ CD38- CD11c- CD25+ (65.8%; **Non-CLLMALT**) V3-7(D2-21)J4-M	**ND**	FSC/SSC$^{lo}\kappa$+ CD19+ CD20^{hi} CD22+ CD38- CD11c- CD25+ (13.2%; **Non-CLLMALT**) V3-23(D2-2)J2-M	**ND**
35¶	FSC/SSC$^{lo}\kappa^{lo}$ CD20^{lo} FMC7- CD5+ CD23+ (55.3%; **CLL**) V3-9(D3-3)J3-UM	**ND**	FSC/SSC$^{lo}\lambda^{lo}$ CD20^{lo} FMC7- CD5+ CD23+ (9.8%; **CLL-likeMBL^{hi}**) V3-21(D2-2)J6-M	**ND**
36	FSC/SSC$^{int}\kappa^{hi}$CD5+ CD11c+ FMC7^{lo} (46%; **Non-CLL MZL**) V1-2(D6-6)J5-UM	**ND**	FSC/SSC$^{lo}\kappa^{hi}$ CD5- CD11c- FMC7^{hi} (40.9%; **Non-CLLMALT**) V3-48(D1-26)J4-UM	**ND**
37¶	FSC/SSC^{hi} CD103+ CD25+ CD11c+ (21%; **Non-CLLHCL**) V3-30(D3-3)J5-UM	**NA**	FSC/SSC$^{lo}\lambda^{lo}$CD5+ (0.8%; **CLL-like MBL^{lo}**) V3-11(D4-17)J6-M	**ND[a]**
38¶	FSC/SSC^{lo}CD19$^{lo}\lambda$+ CD5+ CD20+ CD23- (38.6%; **Non-CLL MZL**) V3-21(D6-13)J6-UM	**+3q27** (89%)	FSC/SSC^{lo}CD19$^{hi}\kappa^{lo}$ CD5+ CD20^{lo} CD23+ (6.3%; **CLL-likeMBL^{hi}**) V3-48(D3-3)J3-M	**del(13q14.3)** (18%)
39	FSC/SSC$^{lo}\kappa^{lo}$ CD19+, CD20^{lo} CD79b- CD43+ (24%; **CLL-like MBL^{hi}**) V1-3(D3-3)J6-UM	**ND**	FSC/SSC$^{lo}\lambda^{lo}$ CD19+ CD20^{lo} FMC7- CD5+ CD23+ (14%; **CLL-like MBL^{hi}**) V3-9(D1-26)J4-M	**del(13q14.3)** (65%); **t(14q32)** (31%)

40¶	FSC/SSC$^{lo}\kappa^{lo}$CD20^{lo} CD5+ CD22^{lo} CD23+ FMC7- (46.7%; **CLL**)	**biallelicdel(13q14.3)** (95%)	FSC/SSC$^{lo}\lambda^{lo}$CD20$^{lo/het}$ CD5+ CD22^{lo} CD23+ FMC7- (43.1%; **CLL**)	**del(13q14.3)** (30%)
	V3-33(D3-10)J6-M		V3-21(D4-23)J4-M	
41	FSC/SSC$^{int}\lambda^{lo}$ CD5+ CD19+ CD11c- (87.1%; **Non-CLL MCL**)	**t(11;14)** (97%)	FSC/SSC$^{lo}\lambda$+ CD11c^{hi} CD19^{hi} (1.3%; **Non-CLL-like MBL^{lo}MZL**)	**ND**
	V3-21(D3-3)J6-UM		V4-34/D3-10/J5-M	

CLL, chronic lymphocytic leukemia/small lymphocytic lymphoma; HCL, hairy cell leukemia; LPL, lymphoplasmacytic lymphoma; MZL, marginal zone lymphoma; FL, follicular lymphoma; MCL, mantle cell lymphoma; MALT, B-cell lymphoma of mucosa-associated lymphoid tissue. + indicates antigen expression at normal levels; -, absence of expression; het, heterogeneous antigen expression; hi, high antigen expression; lo, low antigen expression; int, intermediate scatter; c, cytoplasmatic antigen expression. †MS, mutational status (UM, unmutated; M, mutated), *FISH was performed on interphase nuclei from FACS-purified cells; ND: no chromosomal alterations detected by iFISH for the probes studied; NA: not analyzed. An additional B-cell clone (**population 3)** was detected by interphase FISH in **case 2** (FSC/SSC$^{lo}\lambda^{lo}$ CD23^{lo} CD5^{lo} CD43^{lo} CD11c^{lo}; 0.7%, **CLL-like MBL^{lo}**; **ND^{a}**), **case 25** (FSC/SSC^{lo}CD19+ κ+ CD5^{hi} CD20^{lo}; 2.4%, **CLL-like MBL^{hi}**; **+12**(66%)) and **case 37** (FSC/SSC$^{lo}\kappa^{lo}$ CD5+; 0.2%, **CLL-like MBL^{lo}**; **ND^{a}**)^{a}Thepercentage of B-cells from this subpopulation was very low and only allowed some iFISH probes (13q14, 13q14.3, 17p13.1 and 11q22.3). The presence of genetic abnormalities in one population was always evaluated in the other coexisting population.

¶Cases whose clones had IGHV aa sequences phylogenetically closer than those found in the rest of multiclonal cases.

Supplementary Table 4. Cytogenetic features of non-CLL like B-cell clones from monoclonal cases.

Clone type	Type of cytogenetic changes / % aberrant B-cells analyzed by iFISH
Non-CLL-like MBL^{hi} (MZL)	trisomy 12^{+} (80%); and t(14q32)$^{+}$ (35%)
Non-CLL (FL)	del(13q14.3)$^{+}$ (18%); 3 copies of IGH gene (85%)*
Non-CLL (FL)	t(14;18)$^{+}$ (89%); and t(8;14)$^{+}$ (78%); polysomy
Non-CLL (MCL)	t(11;14)$^{+}$ (93%)
Non-CLL (MALT)	t(14q32)$^{+}$ (94%)
Non-CLL (MZL)	t(14q32)$^{+}$ (94%); 3 and 4 copies of IGH gene (53% and 37%, respectively)

Only cytogenetically altered clones are shown; cytogenetically non-altered clones from monoclonal cases included non-CLL MZL (n=5; 2 MBL^{lo}, 1 MBL^{hi} and 2 MZL), MALT-lymphoma (n=1), MCL (n=1), DLBCL (n=1) and LPL (n=1). CLL, chronic lymphocytic leukemia/small lymphocytic lymphoma; MBL, monoclonal B-cell lymphocytosis; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; LPL, lymphoplasmacytic lymphoma; MCL, mantle cell lymphoma; MZL, marginal zone B-cell lymphoma; MALT, B-cell lymphoma of mucosa-associated lymphoid tissue; * t(14;18)$^{+}$ by molecular studies; ND, not detected ; NA, not analyzed.

Supplementary Table 5. Molecular characteristics of the B-cell receptor (BCR) of non-CLL B-cell clones from both MBL and B-CLPD other than CLL.

	Non-CLL B-cell clones n=34 clones	
	Multiclonal	**Monoclonal**
HCDR3 length* (N. of aa)	14 (8-26)	16 (7-22)
VH families		
VH1	4/19 (21%)	3/15 (20%)
VH3	12/19 (63%)	8/15 (53%)
VH4	3/19 (16%)	2/15 (13%)
VH5	0/19 (0%)	1/15 (7%)
VH6	0/19 (0%)	1/15 (7%)
DH families		
DH1, DH4, DH7	5/19 (26%)	2/14 (14%)
DH2	3/19 (16%)	4/14 (29%)
DH3	4/19 (21%)	2/14 (14%)
DH5	2/19 (11%)	5/14 (36%)
DH6	5/19 (26%)	1/14 (7%)
JH genes		
JH1, JH2, JH3, JH5	7/19 (37%)	3/15 (20%)
JH4	8/19 (42%)	7/15 (47%)
JH6	4/19 (21%)	5/15 (33%)
LCDR3 length* (N. of aa)	9 (9-11)	9 (8-12)
VK families		
VK1	4/11 (36%)	3/12 (25%)
VK2	2/11 (18%)	1/12 (8%)
VK3-4	5/11 (46%)	8/12 (67%)
JK genes		
JK1, JK3, JK5	5/11 (42%)	5/11 (45%)
JK2	2/11 (33%)	2/11 (21%)
JK4	4/11 (25%)	4/11 (34%)
Vλ families		
Vλ3	1/3 (33%)	1/1 (100%)
Other	2/3 (67%)	0/1 (0%)
Jλ genes		
Jλ2	2/3 (67%)	1/1 (100%)
Jλ3	1/3 (33%)	0/1 (0%)

Results expressed as number of B-cell clones from all clones in the corresponding group (percentage) or as *median (range). Non-CLL, clones mimicking or compatible with B-cell chronic lymphoproliferative disorders other than chronic lymphocytic leukemia; BCR, B-cell receptor; HCDR3, heavy chain complementarity-determining region 3; LCDR3, light chain complementarity-determining region 3; aa, aminoacid

Supplementary Table 6. **Monoclonal cases with B-cell clones sharing HCDR3 sequences of the same length (± 1 aminoacid) and belonging to identical or evolutionary highly-related VH families.**

Monoclonal Cases ID	VH families	HCDR3 length	AA composition of HCDR3	(% homology)[#]
149	V3-48	8	**C**_SRRGRLDI_**W**	(25)
158	V3-11	8	**C**_ARGSYFDY_**W**	
66	V3-7	9	**C**_ARGRYVYDI_**W**	(44)
136	V3-7	9	**C**_ARGGWYGDY_**W**	
113	V3-74	9	**C**_ARQLDMYSL_**W**	(11)
121	V3-64	9	**C**_AVDRTGMDV_**W**	
92	V3-23	12	**C**_AKGRQLWSYLDY_**W**	(33)
153	V3-23	12	**C**_AKDGFTKDVFDI_**W**	
16	V1-2	13	**C**_ARGLNTDYGAFDI_**W**	(31)
18	V1-2	13	**C**_ARAQWLVLENFDY_**W**	
201	V4-34	13	**C**_ARGFHWGGYYLDF_**W**	(23)
206	V4-34	13	**C**_ATNSRESQGWFDP_**W**	
190	V4-34	14	**C**_APARYYDFSAPIDY_**W**	(29)
214	V4-34	14	**C**_ARVIGDKGGYYLTY_**W**	
205	V4-59	14	**C**_ARGPDISGWNGLDY_**W**	(50)
218	V4-61	14	**C**_AKRYGDHGEGWFDP_**W**	
124	V3-23	14	**C**_AKFYDDIQPNAFDI_**W**	(29)
145	V3-23	14	**C**_AFHCCRISCYGVDF_**W**	
24	V1-2	15	**C**_ARDLEMRYSQGSFDS_**W**	(60)
35	V1-2	15	**C**_GRDVELRYWQGYFDL_**W**	
125	V3-7	16	**C**_ASALRYLPYADTAFDL_**W**	(31)

147	V3-7	16	**C**_GSQCSTTSCPSSISEY_**W**	
105	V3-23	16	**C**_TKDPRDTGYGGDAFDY_**W**	(35)
142	V3-23	17	**C**_AKDRTLATVIQKDTFDI_**W**	
142	V3-23	17	**C**_AKDRTLATVIQKDTFDI_**W**	(35)
104	V3-23	18	**C**_AKDLPSTYNWNSGGAFDI_**W**	
151	V3-30	17	**C**_ASGSMIGGVILPPGFDY_**W**	(18)
120	V3-30	18	**C**_TRPHCSMSSCSWNDAFAI_**W**	
132	V3-30	19	**C**_AKIGMAGDFLEFRYYGMDV_**W**	(37)
150	V3-30	19	**C**_ANRGDTSGLGTCCQGIGDS_**W**	
96	V3-21	20	**C**_ARHHPVRESSATGHYYGMDV_**W**	(45)
155	V3-48	20	**C**_ARSPGYDFWSGYPDYYGMDV_**W**	
196	V4-34	20	**C**_VRGYPSDYTERRYYYYGLDV_**W**	(40)
202	V4-34	20	**C**_ARGLIGAYGSGSYYPFPFDY_**W**	

(Continuation)

Monoclonal Cases ID	**VH families**	**HCDR3 length**	**AA composition of HCDR3**	**(% homology)[#]**
198	V4-34	20	**C**_ARGYGSTGETRRYYYYGMDV_**W**	(50)
202	V4-34	20	**C**_ARGLIGAYGSGSYYPFPFDY_**W**	
155	V3-48	20	**C**_ARSPGYDFWSGYPDYYGMDV_**W**	(71)
130	V3-48	21	**C**_ARDYDFWSGYYSYYYYYGMDV_**W**	
96	V3-21	20	**C**_ARHHPVRESSATGHYYGMDV_**W**	(35)
114	V3-21	21	**C**_AREGGLGYCSSTSCYTTLFDY_**W**	
130	V3-48	21	**C**_ARDYDFWSGYYSYYYYYGMDV_**W**	(48)
114	V3-21	21	**C**_AREGGLGYCSSTSCYTTLFDY_**W**	

22	V1-69	21	**C**_AREVVYGVAGTYYYYYYGMDV_**W**	(67)
39	V1-69	21	**C**_ARDTGLMTNWGYYYYYYYMDV_**W**	
22	V1-69	21	**C**_AREVVYGVAGTYYYYYYGMDV_**W**	(48)
31	V1-69	21	**C**_ARGGNYDYIWGSYRPNDAFDI_**W**	
31	V1-69	21	**C**_ARGGNYDYIWGSYRPNDAFDI_**W**	(43)
39	V1-69	21	**C**_ARDTGLMTNWGYYYYYYYMDV_**W**	
143	V3-30	21	**C**_ARGPNVSHTYYYDNSGSHFDY_**W**	(28)
138	V3-30	22	**C**_ARDLKTAYYDFWSGYYGDGMDV_**W**	
114	V3-21	21	**C**_AREGGLGYCSSTSCYTTLFDY_**W**	(42)
110	V3-21	22	**C**_ARDRRNGNFDWLEDPLYNWFDP_**W**	
114	V3-21	21	**C**_AREGGLGYCSSTSCYTTLFDY_**W**	(42)
157	V3-21	22	**C**_ARGRLSAWLLMEGIYYYYGMDV_**W**	
110	V3-21	22	**C**_ARDRRNGNFDWLEDPLYNWFDP_**W**	(36)
157	V3-21	22	**C**_ARGRLSAWLLMEGIYYYYGMDV_**W**	
110	V3-21	22	**C**_ARDRRNGNFDWLEDPLYNWFDP_**W**	(41)
93	V3-11	22	**C**_ARDPRYYDFWSGYYLPDDKFDY_**W**	
157	V3-21	22	**C**_ARGRLSAWLLMEGIYYYYGMDV_**W**	(32)
93	V3-11	22	**C**_ARDPRYYDFWSGYYLPDDKFDY_**W**	
26	V1-69	22	**C**_ATTTITIFGVVTVYYYYYGMDV_**W**	(32)
34	V1-69	22	**C**_ARGSSTYYYDSSVYGVAEYFQH_**W**	
26	V1-69	22	**C**_ATTTITIFGVVTVYYYYYGMDV_**W**	(50)
27	V1-69	23	**C**_ARADGGYDFWSGYSTVNYYGMDV_**W**	
34	V1-69	22	**C**_ARGSSTYYYDSSVYGVAEYFQH_**W**	(23)
27	V1-69	23	**C**_ARADGGYDFWSGYSTVNYYGMDV_**W**	

129	V3-11	23	**C**_ARDRRDDFWSGYRIYYYYYGMDV_**W**	(48)
141	V3-48	23	**C**_ARDNTANDIVVVPADYYYYGMDV_**W**	
27	V1-69	23	**C**_ARADGGYDFWSGYSTVNYYGMDV_**W**	(69)
15	V1-69	24	**C**_ARAEQYYDFWSGHKGVDYYYYMDV_**W**	

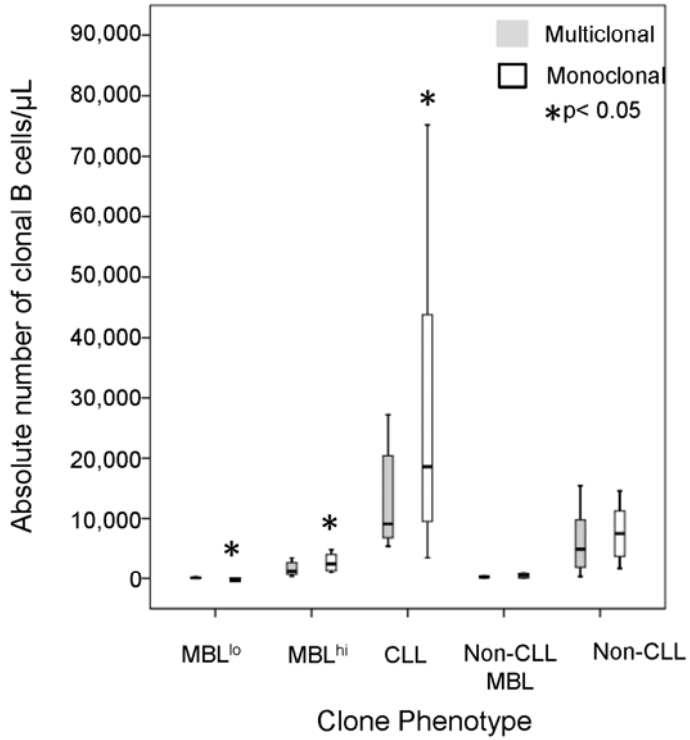
Aminoacids with analogous side-chain polarity (highlighted in gray): L,F; A,V; I,L; K,R; L,V; L,M; L,C; T,S; A,I; D,E; I,M; V,C; L,F; M,F; I,V; H,Y; I,C; F,W. [#]Number of aminoacids with analogous side-chain polarity (excluding the delineating **C**_ and _**W** positions)/HCDR3 length*100.

Supplementary Table 7. Peripheral blood (PB) B-cell counts and BCR features of multiclonal vs. monoclonal CLL-like and non-CLL-like B-cell clones

	CLL-like B-cells		Non-CLL-like B-cells	
	Multiclonal B-cells n=66 clones	**Monoclonal B-cells** n=128 clones	**Multiclonal B-cells** n=19 clones	**Monoclonal B-cells** n=15 clones
N. of PB clonal B cells (x10^6/L)*	**2,675 (0.6-71,485)[a]**	**10,956 (0.1-369,288)**	4,375 (85-156,168)	4,771 (54-41,221)
% of PB clonal B cells from WBC*	**13% (0.1%-89%)[a]**	**45% (0.001%-97%)**	13% (0.5%-87%)	41% (1%-73%)
MBL^{lo} B-cell clones	**14/66 (21%)[a]**	**13/128 (10%)**	3/19 (16%)	2/15 (13%)
MBL^{hi} B-cell clones	**26/66 (39%)[a]**	**26/128 (20%)**	2/19 (10%)	2/15 (13%)
CLL B-cell clones	**26/66 (39%)[a]**	**89/128 (69%)**	NA	NA
CLL-stage A clones	**12/66 (18%)[a]**	**53/128 (41%)**	NA	NA
CLL-stage B/C clones	**8/66 (12%)[a]**	**36/128 (28%)**	NA	NA
Non-CLL B-cell clones	NA	NA	14/19 (74%)	11/15 (73%)
IGHV mutated B-cell clones	40/66 (61%)	76/124 (61%)	11/19 (58%)	8/15 (53%)
% alignment of IGHV aa sequences between coexisting B-cell clones	51% (38%-79%)	NA	62% (46%-76%)[#]	NA
% alignment of IGHV aa sequences between each B-cell clone and the other clones	**52% (31%-100%)[a]**	**50% (29%-100%)**	**51% (32%-89%)[a]**	**49% (33%-86%)**

Results expressed as number of B-cell clones and percentage between brackets or as *median value (range). PB, peripheral blood; WBC, white blood cells; CLL, chronic lymphocytic leukemia/small lymphocytic lymphoma; MBL^{lo}, low count monoclonal B-cell lymphocytosis; IGHV, immunoglobulin heavy chain variable region genes; CLL, chronic lymphocytic leukemia/small lymphocytic lymphoma; MBL^{hi}, clinical monoclonal B-cell lymphocytosis; aa, aminoacids. NA, not appropriate.[#]Both coexisting B-cell clones showed Non-CLL-like phenotype (n=4 cases) or the majority B-cell clone showed Non-CLL-like phenotype (n=4 cases). [a]Statistically significant differences ($P < 0.05$) found between clones from multiclonal *vs* monoclonal cases.

Supplementary Figure 1



	MBL^{lo}		MBL^{hi}		CLL		Non-CLL MBL		Non-CLL	
Absolute N. of clonal B-cells/µL	Multiclonal	Monoclonal	Multiclonal	Monoclonal	Multiclonal	Monoclonal	Multiclonal	Monoclonal	Multiclonal	Monoclonal
Median	79	1	1,254	2,464	9,113	18,600	266	465	4,932	7,500
Range (min-max)	(0.6-250)	(0.1-112)	(346-3,458)	(986-4,851)	(5,412-71,485)	(3,507-369,289)	(85-440)	(54-890)	(300-156,168)	(1,728-41,221)
p-value	**.002**		**.004**		**.02**		NS		NS	

Supplementary Figure 1. Absolute number of CLL-like MBL^{lo}, CLL-like MBL^{hi}, CLL, non-CLL MBL and non-CLL B-cell clones per µL of peripheral blood in multiclonal vs monoclonal cases distributed according to diagnosis. Boxes extend from the 25th to the 75th percentiles, the lines in the middle represent median values (50th percentile). Vertical lines represent the highest and lowest values that are not outliers or extreme values (being outliers and extreme values those values that lie more than 1.5- and 3- fold the length of the box). The adjacent table compiles the median number and range of each subgroup of CLL-like and non-CLL like clonal B-cells/µL of peripheral blood and the exact P-values obtained after comparing multiclonal vs monoclonal cases (Mann-Whitney U test) for the MBL^{lo}, MBL^{hi} and CLL subgroups**.** NS: no statistical significant differences were detected (P≥0.05).