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

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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**	

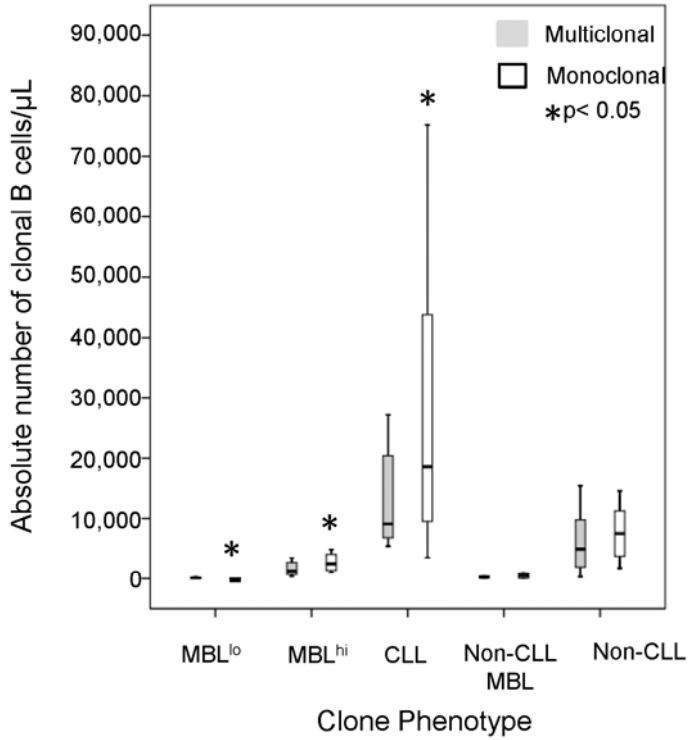
Aminoacids with analogous side-chain polarity (highlighted in gray): L,F; A,V; I,L; K,R; L,V; L,M; L,C; T,S; A,I; D,E; I,M; V,C; L,F; M,F; I,V; H,Y; I,C; F,W. [#]Number of aminoacids with analogous side-chain polarity (excluding the delineating **C**_ and _**W** positions)/HCDR3 length*100.

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).