SUPPLEMENTARY APPENDIX

Host virus and pneumococcus-specific immune responses in high-count monoclonal B-cell lymphocytosis and chronic lymphocytic leukemia: implications for disease progression

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SUPPLEMENTARY METHODS

Controls, patients and samples. Healthy donors (controls; n=91) and MBL^{lo} individuals (n=71) were prospectively recruited from the general population, at Primary Health Care Centers of Salamanca between 2007 and 2009, as part of a study examining the prevalence of MBL -through high-sensitive flow cytometry- from a cohort of 639 healthy adults aged >40y with normal peripheral blood cell counts[17]. In turn, MBL^{hi} (n=29) and newly-diagnosed untreated CLL (n=58) patients were prospectively recruited at the Hematology Service of University Hospital of Salamanca (Salamanca, Spain) between 2008 and 2011. None of the subjects included in the study had been treated for CLL and had received any other drug interfering with the immune response (i.e. steroids and antibiotics) for 3 months prior to sample collection.

EDTA-anticoagulated PB samples, as well as plasma specimens -obtained by sequential centrifugation for 10 min at 800g and at 2000g for another 5 min-, were collected from each subject at recruitment, after informed written consent had been given. The study was approved by the local Ethics Committee of the University Hospital of Salamanca, and conducted in accordance with the Declaration of Helsinki. Plasma specimens were immediately stored at -80ºC in the Spanish DNA Bank (NUCLEUS Service, University of Salamanca, Salamanca, Spain and ISCIII, *Ministerio de Economía y Competitividad*, Madrid, Spain) until used, while whole PB samples were immediately processed, as described below.

Measurement of soluble plasma levels of anti-viral and streptococcus pneumoniae (pneumococcus)-specific antibodies. Exposure to cytomegalovirus (CMV), Epstein-Barr virus (EBV), influenza A and B viruses, and pneumococcus were measured by immunoenzymatic-based approaches, including either enzyme-linked immunosorbent (ELISA) or chemiluminescent immune assays, using commercially available kits, as detailed below. In

brief, CMV-specific IgM and IgG plasma levels, as well as IgM and IgG antibodies to viral capsid antigen (VCA) and IgG antibodies against Epstein-Barr nuclear antigen 1 (EBNA-1) were all quantified by CMIA (chemoluminiscence microparticle immunoassay; Architect® system, Abbott, Wiesbaden, Germany). In turn, conventional ELISA approaches were employed to quantify the plasma levels of IgM and IgG antibodies specific for both influenza A and influenza B (VIRCELL S.L., Granada, Spain) and to evaluate the IgG levels specific for the pneumococcal capsular polysaccharide (PCP) antigen prepared from a mixture of 23 *Streptococcus pneumoniae* serotypes (1-5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F and 33F) that represent ≈80% of the commonly found virulent serotypes (**VaccZyme™** PCP IgG, The Binding Site Inc, Birmingham, UK). All measurements were performed strictly following the recommendations of the manufacturer, as well as the criteria, controls and cut-off values used to define the status for each infection (information on each serologic assay, as well as the analytical sensitivity and specificity of the different assays used are below [Supplementary Table 2]. For those samples showing Ig plasma levels above or below the ranges established, a lower or greater dilution was made with the manufacturer's diluents, in order to bring the final concentration into the analytical range of the corresponding calibration curve.

In parallel to the quantitation of the microorganism-specific antibody plasma levels, total IgM, IgG and IgA plasma levels were systematically measured using nephelometry. None of the subjects had received Ig preparations before (total and specific) Ig plasma levels were investigated.

Vaccination data. Information about the vaccination status of controls and patients, as regards influenza viruses and *Pneumococcus,* was obtained from the *"Gerencia Territorial de Sanidad"* vaccination database (*Junta de Castilla y León*, Valladolid, Spain). For this purpose, data from the previous nine years before closing the present study (2006-2015) was collected.

Analysis of influenza A- and influenza B-specific IgM and IgG and *S.pneumoniae*-specific IgG plasma levels was restricted to those subjects who had not been vaccinated against influenza and *S.pneumoniae,* respectively, during the 9-year period prior to the study.

Statistical analyses. Mean values and their standard deviation (SD), median and range, as well as the 25^{th} and 75^{th} percentiles were calculated for each continuous variable under study; for categorical variables, frequencies were used. Appropriate tests were used for comparison of continuous variables among ≥2 independent groups (the Kruskal-Wallis test and analysis of variance were used for continuous variables which showed a non-normal and normal distribution, respectively) and/or between two independent groups (the Mann-Whitney U and the Student's *t* tests for non-parametric and parametric data, respectively); the Pearson's $\chi 2$ and Fisher exact tests were applied for categorical variables. For all statistical analysis, the SPSS software program (SPSS 19.0, SPSS software, IBM, Armonk, NY) was used. P-values ≤ 0.05 were considered to be associated with statistical significance.

SUPPLEMENTARY TABLES

SUPPLEMENTARY TABLE 1

Combinations of fluorochrome-conjugated antibodies used for the immunophenotypic analysis of peripheral blood samples by flow cytometry

Panel A: LST (EuroFlow®)(Lymphocyte Screening Tube) performed in all subjects studied. For MBL^{lo}≥ $5x10^6$ PB cells were evaluated, while for MBL^{hi} and CLL ≥ $5x10^5$ cells were analyzed.

Fluorochrome	PacB	OC515	FITC	PE	PerCPCy5.5	PECy7	APC	APCH7
Marker	CD20/ CD4	CD45	CD8/ anti-Lambda	CD56/ anti-Kappa	CD5	CD19/ anti-TCRγδ	CD3	CD38
Clone	2H7/ RPA-T4	GA90	UCHT4/ Polyclonal	C5.9/ Polyclonal	L17F12	J3-119/11F2	SK7	HB7
Manufacturer	eBioscience/ BD	Cytognos	Cytognos	Cytognos	BD	Beckman Coulter /BD	BD	BD

Panel B: Single 8-color 9- antibody combination used for the specific characterization of clonal B cells in MBL^{lo} (≥ of $5x10^6$nucleated cells analyzed/case).

Fluorochrome	PacB	BV510	FITC	PE	PerCPCy5.5	PECy7	APC	APCH7
Marker	CD20	CD27	CD5	CD305	CD79b	CD19	CD3/ anti-Kappa	anti-Lambda
Clone	2H7	L128	UCHT2	DX26	3A2-2E7	J3-119	SK7/ Polyclonal	Polyclonal
Manufacturer	eBioscience	BD	BD	BD	BD	Beckman Coulter	BD/ Cytognos	Cytognos

Panel C: Single 8-color antibody combination used for the characterization of clonal B cells in MBL^{hi} and CLL (≥ of $5x10^6$ nucleated cells analyzed/case).

Fluorochrome	PacB	OC515	FITC	PE	PerCPCy5.5	PECy7	APC	APCH7
Marker	CD20	CD45	CD23	CD305	CD5	CD19	CD200	CD43
Clone	2H7	GA90	EBVCS5	DX26	L17F12	J3-119	OX-104	1610
Manufacturer	eBioscience	Cytognos	BD	BD	BD	Beckman Coulter	BD	BD

Abbreviations (alphabetical order): **Ab,** antibody; **Alexa750,** Alexa Fluor®750; **APC,** Allophycocyanin; **APCH7**, Allophycocyanin-Hilite®7; **BD,** Becton/Dickinson Biosciences®; **BV515,** Brilliant Violet™ 510; **CLL,** chronic lymphocytic leukemia; **MBL^{lo},** low-count monoclonal B lymphocytosis; **MBL^{hi},** high-count monoclonal B lymphocytosis; **OC515,** Orange Cytognos™ 515; **FITC,** fluorescein isothiocyanate; **PacB,** Pacific Blue™; **PE,** phycoerythrin; **PECy7,** phycoerythrin-cyanine7; **PerCPCy5.5,**peridinin chlorophyll cyanine5.5.

SUPPLEMENTARY TABLE 2

Technical features of commercially available kits used for the detection of pathogen -specific plasma immunoglobulin (Ig) levels

Pathogen	Ig detected isotype	Antigen	Platform	Sensitivity	Commercial Source
Cytomegalovirus (CMV)	IgM	AD169 strain	Architect®	<0.85 Index	ABBOT Diagnostics
	IgG	AD169 strain	Architect®	<0.6 AU/ml	ABBOT Diagnostics
Epstein-Barr Virus (EBV)	IgM	VCA	Architect®	<0.50 RLU	ABBOT Diagnostics
	IgG	VCA	Architect®	<0.75 RLU	ABBOT Diagnostics
	IgG	EBNA	Architect®	<0.01 S/CO	ABBOT Diagnostics
Influenza A	IgM	VR-822™strain	ELISA	<0.5 O.D	Vircell
	IgG	VR-822™strain	ELISA	<0.5 O.D	Vircell
Influenza B	IgM	VR-791™strain	ELISA	<0.5 O.D	Vircell
	IgG	VR-791™strain	ELISA	<0.5 O.D	Vircell
Pneumococcus	IgG	anti-PCP*	ELISA	<0.07 O.D	Binding Site

*Pneumococcal Capsular Polysaccharide. This kit is prepared for the identification of 23 different serotypes of Pneumococcus (serotypes 1-5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F and 33F).

Abbreviations (alphabetical order): **AU,** arbitrary units; **EBNA,** Epstein-Barr virus nuclear antigen; **ELISA,** enzyme-linked immunosorbent assay; **Ig,** immunoglobulin; **RLU,** relative light units; **O.D,** optical density; **S/CO,** sample/cut-off; **VCA,** viral capside antigen.

SUPPLEMENTARY TABLE 3

Distribution of CMV, EBV and Influenza virus seropositive healthy donors, MBL^{lo} and MBL^{hi} subjects and CLL patients

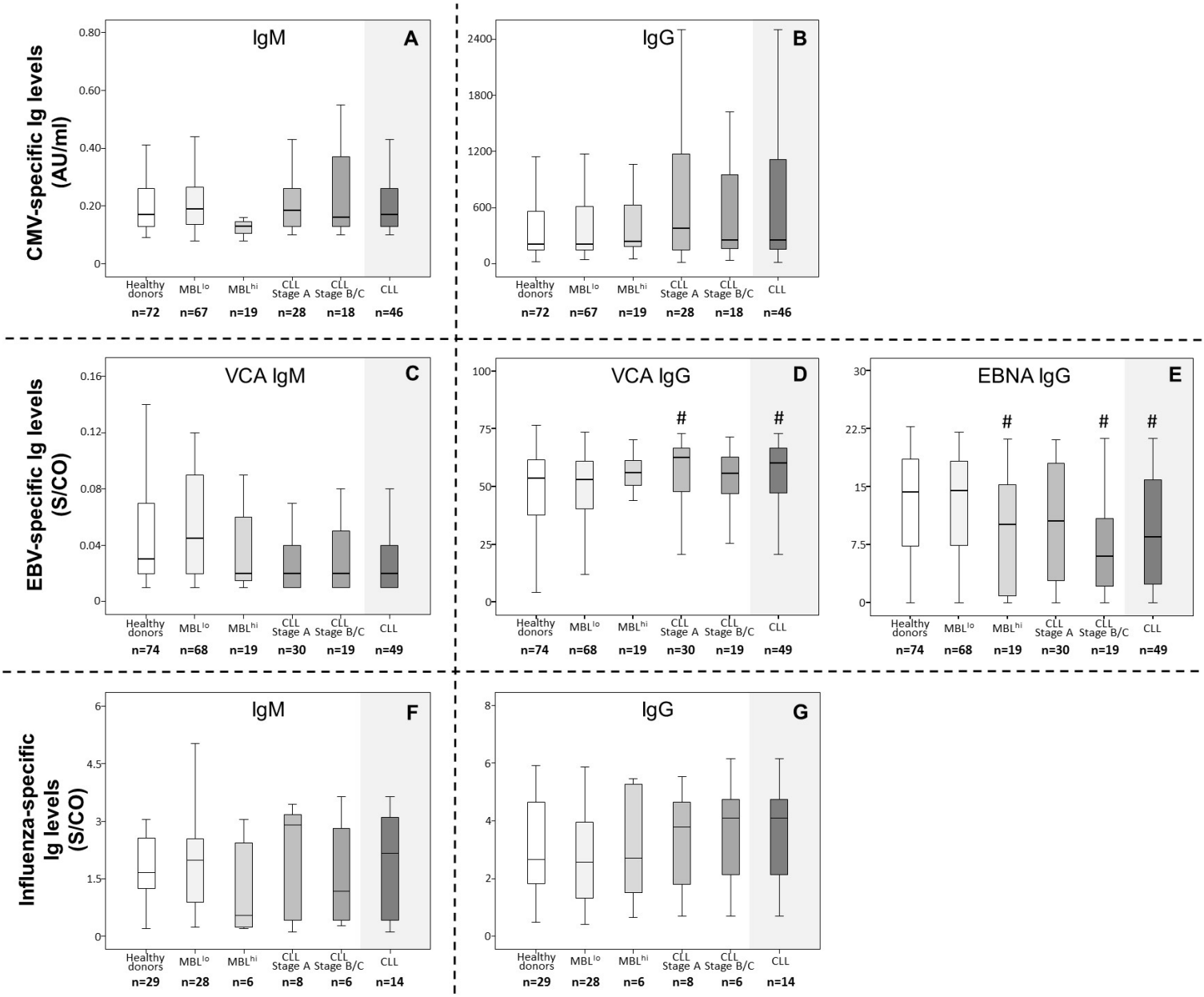
	Healthy donors	MBL^{lo}	MBL^{hi}	CLL Stage A	CLL Stage B/C	CLL
Cytomegalovirus+ (CMV)	72 (97%)	67 (99%)	19 (91%)	28 (93%)	18 (95%)	46 (94%)
Epstein-Barr Virus+ (EBV)	74 (100%)	68 (100%)	19 (91%)	30 (100%)	19 (100%)	49 (100%)
Influenza+ (A or B strain)	42 (57%)	34 (50%)	7 (33%)	12 (70%)	10 (100%)	22 (82%)
Cases vaccinated against Influenza						
	13 (18%)	6 (8.8%)	1 (4.8%)	4 (24%)	4 (40%)	8 (30%)

Cut-off values for seropositivity were established according to the recommended values provided by the corresponding manufacturers of each reagent kit. No statistically significant differences ($p>0.05$) among the groups for any pathogen-specific Ig plasma levels. Abbreviations (alphabetical order): **CLL**, chronic lymphocytic leukemia; **Ig**, immunoglobulin; **MBL^{lo}**, low-count monoclonal B lymphocytosis; **MBL^{hi}**, high-count monoclonal B lymphocytosis.

SUPPLEMENTARY FIGURES

SUPPLEMENTARY FIGURE 1

CMV-, EBV- and Influenza-specific IgM and IgG in MBL and CLL patients vs. healthy controls



Panels A and B: CMV (AD169 strain)-specific IgM and IgG plasma levels in the different groups of individuals analyzed. Panels C, D and E: VCA-EBV-specific IgM and IgG plasma levels and EBNA-EBV-specific IgG plasma levels. Panels F and G show Influenza- (both strains, A and B) specific IgM and IgG titers in plasma for the different groups of subjects investigated. Only seropositive subjects for each pathogen are included in this figure; in addition, in panels F and G, the data presented corresponds only to subjects who referred no previous vaccination against influenza. Notched boxes represent 25th and 75th percentile values; the lines in the middle correspond to median values (50th percentile), whereas vertical lines represent the highest and lowest values that are neither outliers nor extreme values. # $p<0.05$ vs healthy donors and MBLlo cases.

Abbreviations (Alphabetical order): **AU,** arbitrary unit; **CLL,** chronic lymphocytic leukemia; **CMV,** Cytomegalovirus; **EBNA,** Epstein-Barr Nuclear Antigen; **EBV,** Epstein-Barr virus; **Ig;** Immunoglobulin, **MBL^{hi},** high-count monoclonal B lymphocytosis; **Ig,** immunoglobulin; **MBL^{lo},** low-count monoclonal B lymphocytosis; **VCA,** Viral Capside Antigen.