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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ABSTRACT

Patients diagnosed with chronic lymphocytic leukemia (CLL) display a high incidence of infections due to an associated immunodeficiency that includes hypogammaglobulinemia. A higher risk of infections has also been recently reported for high-count monoclonal B-cell lymphocytosis, while no information is available in low-count monoclonal B-cell lymphocytosis. Here, we evaluated the status of the humoral immune system in patients with chronic lymphocytic leukemia (n=58), as well as in low- (n=71) and high- (n=29) count monoclonal B-cell lymphocytosis *versus* healthy donors (n=91). Total free plasma immunoglobulin titers and specific levels of antibodies against cytomegalovirus, Epstein-Barr virus, influenza and *S.pneumoniae* were measured by nephelometry and ELISA-based techniques, respectively. Overall, our results show that both CLL and high-count monoclonal B-cell lymphocytosis patients, but not low-count monoclonal B-cell lymphocytosis subjects, present with relatively high levels of antibodies specific for the latent viruses investigated, associated with progressively lower levels of *S.pneumoniae*-specific immunoglobulins. These findings probably reflect asymptomatic chronic reactivation of humoral immune responses against host viruses associated with expanded virus-specific antibody levels and progressively decreased protection against other micro-organisms, denoting a severe humoral immunodeficiency state not reflected by the overall plasma immunoglobulin levels. Alternatively, these results could reflect a potential role of ubiquitous viruses in the pathogenesis of the disease. Further analyses are necessary to establish the relevance of such asymptomatic humoral immune responses against host viruses in the expansion of the tumor B-cell clone and progression from monoclonal B-cell lymphocytosis to CLL.

Introduction

Chronic lymphocytic leukemia (CLL) is the most common leukemia in adults in Western countries. It is characterized by an expansion of 5×10^9/L or more clonal B lymphocytes in peripheral blood (PB) that co-express CD5, CD19, CD23 and CD200, together with abnormally low levels of CD20, CD22, CD79b and surface immunoglobulins (sIg).[1-4] CLL typically occurs in elderly patients and has a highly variable clinical course.[5] Despite the heterogeneous clinical outcome, the majority of CLL patients share a profound immune dysregulation which is already detected at the earliest stages of the disease, and that progressively becomes more severe during clinical observation, leading to patient death even in the absence of disease progression.[6] The precise mechanisms underlying such immune dysregulation in CLL are not fully understood; however, hypogammaglobulinemia has been identified as one of the major factors involved,[6-8] both in the immunodeficiency status and death of CLL patients.[9,10] Thus, hypogammaglobulinemia is present in up to 85% of patients. During the course of disease, a direct association has been reported between the stage and duration of disease and the severity of hypogammaglobulinemia.[11,12] As a result, infection is one of the most prevalent causes of morbidity and mortality in CLL.[13] Approximately 80% of CLL patients have infections during the course of the disease; such infections particularly involve the respiratory tract, pneumonia accounting for approximately 75% of all pulmonary complications in CLL.[14]

Recent studies have reported that subjects at earlier stages of the disease [e.g. high-count monoclonal B-cell lymphocytosis (MBL^{hi})] also have an increased risk of infections and a greater rate of infection-related deaths.[15] Thus, hospitalization due to infection is significantly more common among MBL^{hi} cases than in the general population (16% *vs.* 2.6% after a median follow-up period of 10 years, respectively), the overall frequency of infection in MBL^{hi} individuals being similar to that of newly-diagnosed CLL patients (18%).[15] Since vaccination represents an effective strategy to decrease the risk of infection in immunocompromised patients, the potential definition of optimal vaccination strategies in MBL^{hi} and CLL requires a more in depth and comprehensive understanding of the dysregulated immunological mechanisms in these patients.

In order to gain further insight into the nature, relevance and clinical significance of hypogammaglobulinemia in CLL and MBL patients, we evaluated the soluble levels of plasma antibodies specific for ubiquitous pulmonary infection-associated pathogens (i.e. influenza A and B viruses and *S.pneumoniae*) as well as other ubiquitous host pathogens, such as cytomegalovirus (CMV) and Epstein-Barr virus (EBV), in newly-diagnosed untreated CLL patients at different stages of the disease (Binet A *vs.* Binet B/C), pre-leukemic MBL^{hi}, and low-count MBL (MBL^{lo}) subjects *versus* a large group of age- and sex-matched healthy individuals from the same geographical area.

Methods

Controls and patients

A total of 249 individuals were prospectively studied between November 2007 to November 2012. These subjects were classified into four subgroups: healthy donors (controls; n=91), CLL-like MBL^{lo} (n=71), CLL-like MBL^{hi} (n=29), and newly-diagnosed previously untreated CLL patients (n=58). According to the World Health Organization (WHO) 2016 criteria,[16] MBL was diagnosed whenever less than $5x10^9$/L clonal B cells with a CLL phenotype were present in PB, in the absence of other signs of disease; otherwise, diagnosis of CLL was established. Within CLL, 32 patients were classified as early stage CLL (Binet A), while the remaining 26 corresponded to advanced-stage CLL (Binet B/C).[4] In turn, MBL^{lo} and MBL^{hi} cases were discriminated based on a cut-off value of less than $0.5x10^9$/L circulating clonal B cells with CLL-like phenotype, as described elsewhere.[17] Additional information about the inclusion and exclusion criteria for selection of controls and patients, as well as procedures for sample collection and storage are detailed in the *Online Supplementary Methods*. The study was approved by the local Ethics Committee of the University Hospital of Salamanca, and conducted in accordance with the Declaration of Helsinki.

Immunophenotypic studies

Immunophenotypic studies were performed on erythrocyte-lyzed PB samples, using a high-sensitive multicolor flow cytometry approach, previously described in detail.[18] For this purpose, PB white blood cells (WBC) were systematically stained with the monoclonal antibody (MAb) combinations detailed in *Online Supplementary Table S1*. For flow cytometry data analysis, the INFINICYT™ software (Cytognos S.L., Salamanca, Spain) was used. All cases showed a clonal-imbalanced surface membrane (Sm) immunoglobulin (Ig)-kappa : SmIg-lambda ratio of >3:1 or <1:3[19]and/or an aberrant $CD5^+$ CLL(-like) B-cell population. The minimum number of clustered events required to define an abnormal B-cell population was 50 cells or more.

Measurement of soluble plasma levels of anti-viral and *streptococcus pneumoniae* (*pneumococcus*)-specific antibodies

Exposure to CMV, 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 in the *Online Supplementary Methods* and *Online Supplementary Table S2*. Of note, 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 (*Online Supplementary Methods*). In each patient, total plasma levels of IgM, IgG and IgA were systematically measured in parallel by nephelometry.

Quantitation of CMV and EBV viral copy number in plasma

Detection and quantitation of CMV and EBV viral load in plasma was determined in a subset of 177 and 191 subjects, respectively, using commercially available kits: COBAS®AMPLiPrep/COBAS®TaqMan (Roche Diagnostics, Basel, Switzerland) and EBV R-gene® (BioMerieux, Verniolle, France), with strict adherence to the manufacturers' instructions.

Results

Clinical and laboratory features of MBL *versus* CLL patients

Overall, 249 individuals, including 119 males (48%) and 130 females (52%), with a mean age of 68±11 years were

studied; there was a similar distribution according to age across the different patient groups and controls. Interestingly, while females predominated among MBL^{lo} cases (male/female ratio 1:2), MBL^{hi} and CLL showed a significantly (P<0.01) higher male/female ratio (5:1 and 1.2:1, respectively) (Table 1). As expected, abnormal blood cell counts were found only in MBL^{hi} and CLL patients (but not in MBL^{lo}), including lower platelet counts and hemoglobin levels among stage B/C CLL. Likewise, the absolute number of PB clonal B cells/μl progressively increased from MBL^{lo} subjects to advanced-stage CLL patients (P<0.05). CLL patients also showed a greater frequency of IGHV unmutated cases (from 20% in MBL^{lo} to 26% in MBL^{hi}, 41% in CLL stage A and 64% in CLL stage B/C; P=0.04), whereas MBL^{lo} cases showed a significantly lower frequency of cytogenetically altered CLL-like clones compared to both MBL^{hi} and CLL (30% *vs.* 68% and 70%, respectively; P=0.002) (Table 1). Of note, all subjects were from the same geographical area (Province of Salamanca, Northwest-Central Spain) and, therefore, shared a similar antigen environment.

Soluble Ig plasma levels in MBL and CLL *versus* healthy controls

Whereas total Ig plasma levels were within the normal range in MBL^{lo} cases, they were significantly decreased in MBL^{hi} and CLL patients *versus* both controls and MBL^{lo} cases (Figure 1A). Interestingly, progressively lower levels of total plasma Igs were found from MBL^{hi} to stage A and stage B/C CLL cases, the latter two groups showing significantly lower amounts of plasma Igs *versus* MBL^{hi} cases (P=0.04 for stage A and P=0.02 for stage B/C) (Figure 1A). In more detail, none of the MBL^{lo} subjects presented with decreased total Ig plasma levels below the normal range, while the frequency of hypogammaglobulinemia increased from MBL^{hi} to early- and advanced-stage CLL patients: 7% *versus* 16% and 19%, respectively (P<0.001) (Figure 1A). Of note, progressively lower levels of plasma Igs were observed from MBL^{hi} to advanced stage CLL also for each Ig isotype (Figure 1B, C and D), particularly for IgM and IgA. Thus, all except one MBL^{lo} case showed normal amounts of IgM, IgG and IgA plasma levels; in contrast, 17% of MBL^{hi} subjects, 38% stage A CLL and 46% stage B/C CLL patients had decreased amounts of plasma IgM (P<0.0001). In addition, 10% of MBL^{hi} cases had decreased IgA plasma levels *versus* 16% of stage A CLL and 46% of stage B/C CLL patients (P<0.0001); in turn, IgG plasma levels were decreased in 14% of MBL^{hi} cases and 24% of CLL patients (P>0.05) *versus* none of the MBL^{lo} subjects.

CMV-, EBV- and influenza-specific IgM and IgG plasma levels in MBL and CLL patients *versus* healthy controls

As expected for a Mediterranean country, more than 90% of adults analyzed here had been exposed to both CMV and EBV before sample collection, regardless of the

Table 1. Clinical and laboratory characteristics of controls *versus* monoclonal B-cell lymphocytosis subjects and chronic lymphocytic leukemia patients.

	Healthy donors (n=91)	MBL^{lo} (n=79)	MBL^{hi} (n=29)	CLL Stage A (n=32)	CLL Stage B/C (n=26)	CLL (n=58)	*P*
Age (years)	70 (43-87)	72 (43-95)	68 (52-85)	67 (45-85)	70 (41-85)	68 (41-85)	NS
Sex (M/F)	42% / 58%	35% / 65%	83% / 17%	56% / 44%	54% / 46%	55% / 45%	P<0.01[b]
Hemoglobin (g/L)	147 (106-181)	144 (99-177)	144 (130-190)	145 (120-174)	118 (88-164)	136 (88-174)	P<0.01[a]
N. of platelets x10^9/L	226 (90-388)	222 (119-262)	198 (85-386)	211 (112-408)	137 (67-271)	174 (67-408)	P<0.01[a]
N. of leukocytes/μl	6,200 (3550-11,240)	6,090 (3650-9400)	11,550 (7154-15,660)	27,310 (13,520-393,530)	53,880 (16,630-289,420)	34,920 (13,520-393,530)	P<0.01[ab]
N. of lymphocytes/μl	1678 (766-4124)	1774 (317-3749)	5250 (2291-9333)	18,591 (6469-381,409)	50,346 (12,779-282,098)	25.939 (6469-381,409)	P<0.01[ab]
N. of B lymphocytes/μl	138 (31-776)	139 (31-478)	3.097 (978-4773)	17,727 (5134-369,288)	41.493 (8176-276,367)	21.352 (5134-369,288)	P<0.01[ab]
N. of clonal B lymphocytes/μl	NA	0.731 (0024-82.24)	3.035 (921-4844)	17.686 (5065-369,288)	41.442 (8019-276,367)	21.130 (5065-369,288)	P<0.01[ab]
Mutational status (Mut/UMut)	NA	*80%/20%	74%/26%	59%/41%	36%/64%	49%/51%	P<0.05[c]
Cytogenetic alterations							
% cases altered	NA	*30%	68%	69%	71%	70%	P<0.01[c]
% cases del13q14(D13S25)	NA	*30%	39%	50%	39%	45%	NS
% cases trisomy cr. 12	NA	*6%	25%	6%	33%	18%	NS
% cases del11q22(ATM)	NA	*0%	7%	13%	13%	13%	NS
% cases del17p13(TP53)	NA	*0%	0%	0%	0%	0%	NS

Results expressed either as median (range) or as percentage of cases for continuous and categorical variables, respectively. The CLL group includes both CLL Binet stage A and CLL Stage B/C cases. [a]CLL *versus* all other groups. [b]MBL^{hi} *versus* all other groups. [c]CLL *versus* healthy individuals. CLL: chronic lymphocytic leukemia; F: female; M: male; MBL^{hi}: high-count monoclonal B lymphocytosis; MBL^{lo}: low-count monoclonal B lymphocytosis; Mut: mutated; NA: not applicable; ND: not determined; NS: no statistically significant differences detected (P>0.05); Umut: unmutated. *Sample size restricted to 23 subjects in which molecular and cytogenetic determinations were performed.

diagnostic subgroup (*Online Supplementary Table S3*). In virtually every case the pattern of plasma antibodies specific for both viruses was consistent with past infection (i.e. CMV- or EBV-specific IgG-positive and IgM-negative plasma antibodies). In contrast, variable percentages of cases from the different study groups showed influenza virus-specific plasma Igs for the strains evaluated (*Online Supplementary Table S3*); in most of these cases, the pattern observed also corresponded to past exposure to the viruses. From the whole series of subjects who showed influenza virus-specific plasma Igs (n=127), 36 reported that they had been vaccinated against influenza virus before their recruitment; no statistically significant differences were observed in the distribution of these subjects in the distinct groups of individuals under study (*Online Supplementary Table S3*). Those patients found to have been previously exposed to any of the viruses investigated (i.e. those who showed increased plasma levels of at least one of the virus-specific Ig tested) were further evaluated for the corresponding pathogen-specific Ig plasma levels.

Overall, plasma levels of pathogen-specific IgM and IgG antibodies did not follow the pattern observed for total IgM and IgG plasma levels in the different groups of subjects analyzed (Figure 1). Thus, there was no reduction of specific IgM and IgG against CMV, EBV viral capside antigen (VCA) and influenza A and B in MBL^{hi} and even in CLL patients *versus* both controls and MBL^{lo} (*Online Supplementary Figure S1*). Regarding CMV-specific IgM and IgG titers and the amount of plasma IgM antibodies against VCA-EBV and the influenza virus, no significant differences were actually observed among individuals of the different groups studied (e.g. controls, MBL^{hi} and CLL) (*Online Supplementary Figure S1A, C* and *F*). In contrast, VCA-EBV-specific IgG plasma levels were higher (P=0.01) in CLL patients *versus* both controls and MBL^{lo} cases (*Online Supplementary Figure S1D*). However, clear differences emerged (or they increased) when the ratio between the plasma levels of each of these pathogen-specific IgG antibodies (CMV-, VCA-EBV- and influenza-specific IgG) plasma levels and the overall amount of plasma IgG per subject/patient was considered (Figure 2). Thereby, the CMV-specific IgM/total IgM and CMV-, VCA-EBV-specif-

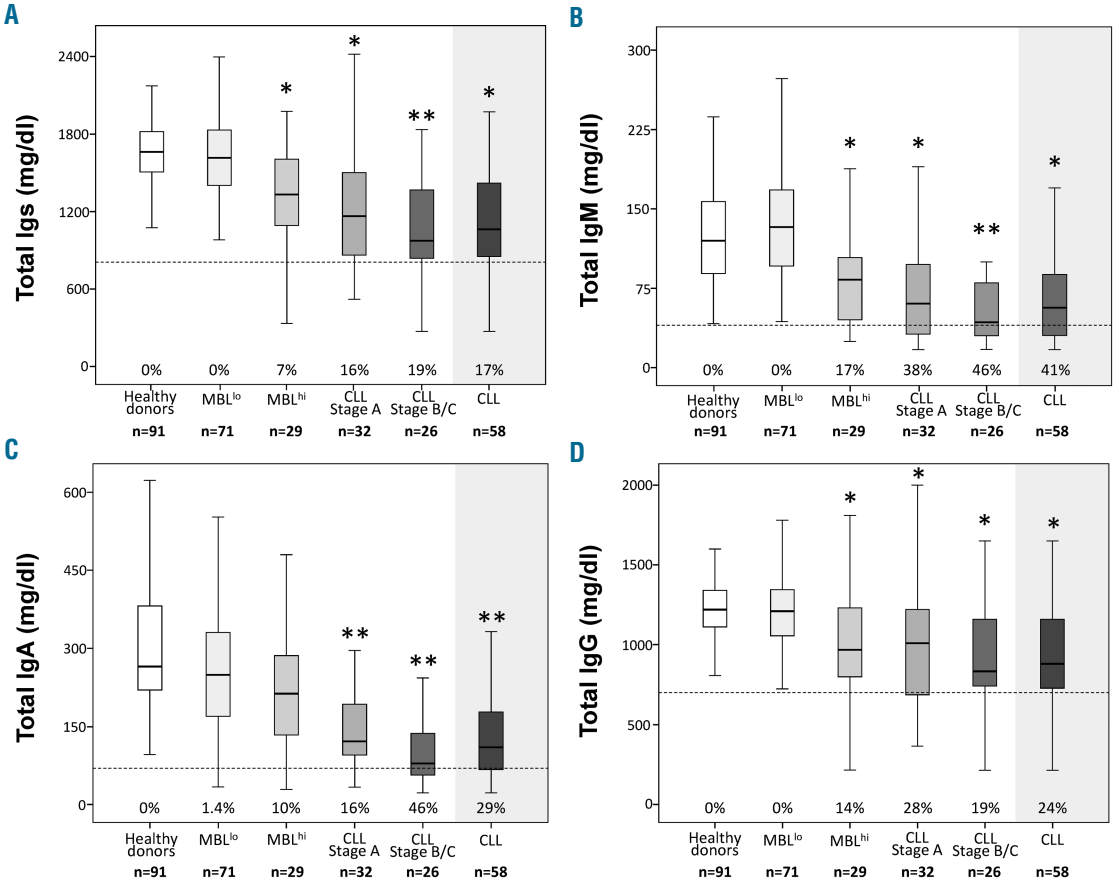

Figure 1. Soluble immunoglobulin immunoglobulin (Ig) plasma levels in monoclonal B lymphocytosis (MBL) and chronic lymphocytic leukemia (CLL) *versus* healthy donors. (A) The overall amount of plasma immunoglobulins (md/dl) determined by conventional nephelometry is shown for the different groups of individuals analyzed. (B-D) IgM, IgG and IgA plasma levels within the different groups of individuals studied, respectively. Notched boxes represent 25th and 75th percentile values; the lines in the middle correspond to median values (50th percentile) and vertical lines represent the highest and lowest values that are neither outliers nor extreme values. Vertical dotted lines represent the inferior limit value of normality for each immunoglobulin. Dotted lines represent the lower limit of normality for each immunoglobulin (40 mg/dl; 700 mg/dl; and 70 mg/dl). Numbers under dotted lines represent the percentage of cases with plasma levels of the corresponding immunoglobulin found to be below normal values. *P<0.05 *versus* healthy donors and MBL^{lo}; **P<0.05 *versus* healthy donors, MBL^{lo} and MBL^{hi}. MBL^{hi}: high-count monoclonal B lymphocytosis; MBL^{lo}: low-count monoclonal B lymphocytosis.

ic IgG/total IgG ratios were significantly higher in CLL ($P \leq 0.001$), particularly in stage B/C CLL cases ($P \leq 0.02$) *versus* healthy donors and MBLlo subjects. Likewise, the influenza-specific IgG/total IgG ratio tended to be higher ($P=0.056$) for CLL patients compared to healthy donors and MBL^{lo} cases (Figure 2). Of note, MBL^{hi} also showed significantly higher anti-VCA-EBV-specific IgG/total IgG plasma levels than controls and MBL^{lo} cases (Figure 2D). An exception to this general pattern was the EBNA-specific IgG plasma levels, which were found to be significantly reduced (*vs.* healthy donors) in both MBL^{hi} ($P=0.01$) and CLL patients ($P=0.002$), particularly in stage B/C CLL ($P=0.002$) (*Online Supplementary Figure S1E*).

S.Pneumoniae-specific IgG plasma levels in MBL and CLL *versus* healthy controls

As mentioned above, *S.pneumoniae*-specific IgG plasma levels were quantified in those subjects who reported no previous administration of anti-PCP (*Pneumococcal Capsular Polysaccharide*) vaccination (Figure 3). Their amount, as well as the pathogen specific IgG/total IgG ratio were within the normal range in all MBL^{lo} subjects and healthy controls analyzed (Figure 3A and 3). However, *S.pneumoniae*-specific IgG plasma levels were significantly reduced in MBL^{hi} and CLL patients *versus* both controls and MBL^{lo} (Figure 3A), in contrast to what was observed for virtually all viral pathogens described above, except the EBNA-specific IgG antibodies. Interestingly, no statistically significant differences were observed between MBL^{hi} and CLL as regards the overall amount of anti-*S.pneumoniae*-specific IgG plasma levels. Of note, the ratio between the anti-*S.pneumoniae*-specific IgG and total IgG plasma levels was similar among the distinct groups of subjects analyzed (Figure 3B), as both the *S.pneumoniae*-specific IgG and the overall IgG plasma levels directly correlated within each group of subjects analyzed.

CMV and EBV viral load and virus-specific Ig titers

Overall, viral load for CMV was studied in plasma from 177 subjects (53 controls, 56 MBL^{lo}, 22 MBL^{hi} and 46 CLL patients). No viral DNA was detected in any sample except 3 cases (1 MBL^{hi}and 2 CLL Binet A subjects), in which the viral load could not be precisely quantified, as

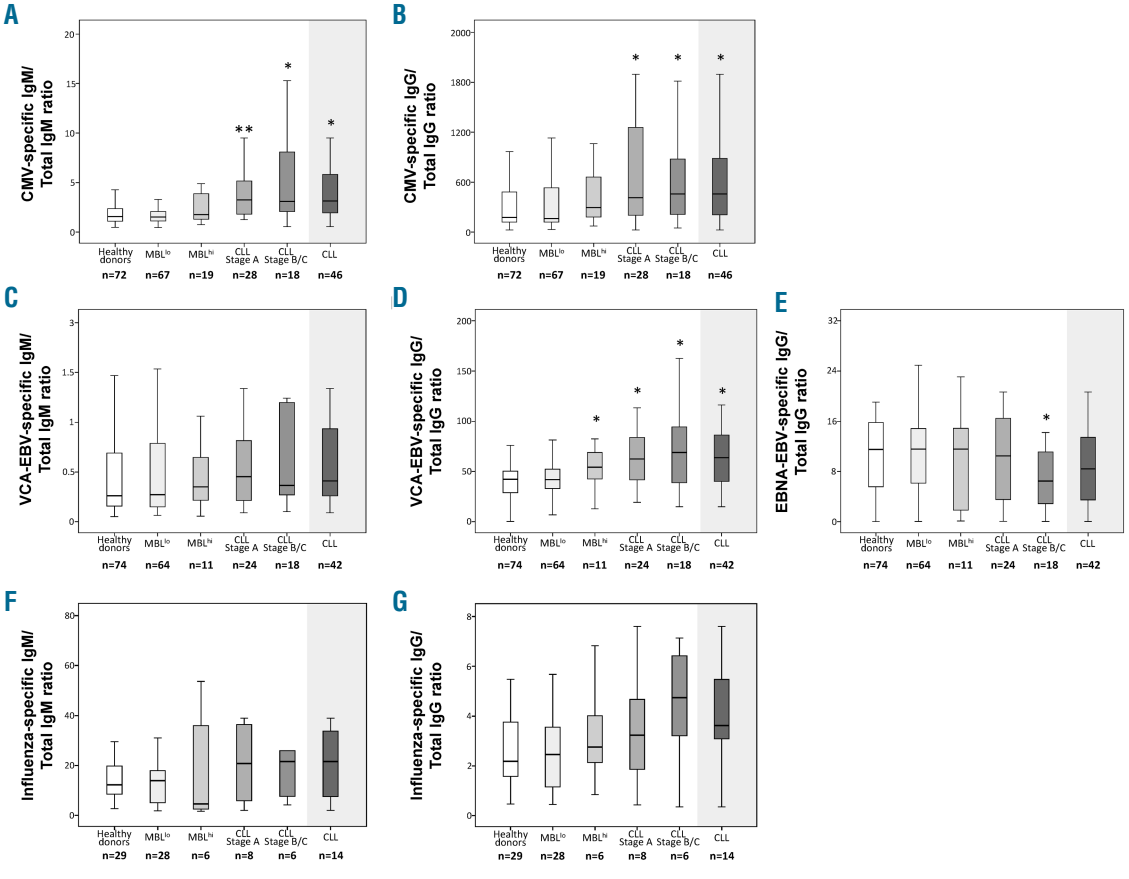

Figure 2. Ratio between pathogen-specific immunoglobulin (Ig) plasma levels and total immunoglobulin plasma levels per Ig isotype in monoclonal B lymphocytosis (MBL) and chronic lymphocytic leukemia (CLL) patients *versus* healthy subjects. (A and B) Ratio between cytomegalovirus (CMV)-specific IgM and IgG plasma titers and the overall plasma IgM and IgG levels, respectively. (C and D) Ratio between viral capside antigen (VCA)-Epstein-Barr virus (EBV)-specific IgM and IgG titers in plasma and the overall amount of IgM and IgG in plasma, respectively. (E) Anti Epstein-Barr nuclear antigen (EBNA)-EBV-specific IgG/total IgG plasma level ratio. (F and G) Influenza (strains A + B)-specific/total IgM and IgG ratios, respectively. Only data on seropositive subjects for each pathogen are included in this figure. (F and G) Data presented correspond 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. *$P<0.05$ *versus* healthy donors and MBL^{lo}; **$P<0.05$ *versus* healthy donors, MBL^{lo} and MBL^{hi}.

it was below the limit of quantification of the method (<137 IU/ml). In turn, EBV DNA load (measured in 191 samples: 57 controls, 59 MBL^{lo}, 23 MBL^{hi}, 53 CLL patients) was detected in plasma from 7 of 53 Binet A CLL patients (13%), while systematically undetectable in the other three groups (P<0.0001). No statistically significant differences in gender distribution, age, number of clonal B cells and EBV (VCA)-specific IgM and IgG titers were found between CLL cases with quantifiable EBV DNA in plasma *versus* negative CLL cases. Also, no statistical correlation was found between the number of EBV DNA copies (median of 3.6 DNA copies/μl; range 1.4-22.8 DNA copies/μl) and EBV-specifc immunoglobulin titers in plasma for those 7 EBV-viral load-positive CLL cases.

Discussion

Infection is one of the most frequent causes of death in CLL (approx. 30-50% of CLL patients).[8] Although the specific mechanisms underlying immune dysregulation in CLL are not fully understood[8], hypogammaglobulinemia, together with T-cell abnormalities, are common features of the CLL-associated immunodeficiency status, the former affecting up to 85% of the patients already at diagnosis or during the course of their disease.[9,10] The frequency and severity of hypogammaglobulinemia (at the expense of all major Ig isotypes) increase from MBL subjects to early and advanced stage CLL patients. Here, we confirm and extend on these observations. Thus, we show for the first time that total soluble Ig plasma levels are within normal values in MBL^{lo} subjects, regardless of the Ig isotype evaluated; in contrast, hypogammaglobulinemia was a relatively frequent feature of MBL^{hi} cases. Of note, the degree of decreased IgM and IgG plasma levels in MBL^{hi} was similar to that observed in stage A CLL. In a recent study, Glancy *et al*. have even reported a higher frequency of decreased IgG levels in MBL^{hi} (i.e. 7 of 24 MBL^{hi} cases, which represents a frequency of IgG hypogammaglobulinemia of 29%[20] *vs.* 14% in our series). This apparent discrepancy might be due to the fact that our series mostly comprised MBL^{hi} cases with lower numbers of clonal B cells studied at diagnosis, while 4 of 7 MBL^{hi} cases reported by Glancy *et al*. to have low IgG titers, had absolute lymphocyte counts more than 4x10^9/L. Nevertheless, it should be noted that we did not find any correlation between soluble Ig plasma levels and the number of clonal B cells in PB, within each group of subjects analyzed (*data not shown*). In contrast, a statistically significant direct correlation was found between total Ig plasma levels and the number of normal residual B cells among CLL patients (r=0.29, P=0.04). Therefore, presence of hypogammaglobulinemia in MBL^{hi} cases could also reflect a defective normal residual B-cell function and it might contribute to explain the near 3-fold increased frequency of infection observed among these subjects *versus* the general population of the same age and having the same comorbidities, to that of newly-diagnosed CLL.[15] Altogether, these findings suggest that antibody-related immunodeficiency might emerge before the onset of CLL, already at an MBL^{hi} state, preceding (or potentially favoring) malignant transformation and progression of the disease.

Despite a progressive reduction of (total) soluble Ig plasma levels from MBL to advanced CLL, similar levels of CMV-specific IgM and IgG, VCA-EBV-specific IgM and influenza-specific IgM and IgG were found among the five groups analyzed (i.e. healthy donors, MBL^{lo}, MBL^{hi}, early CLL and advanced stage CLL). Indeed, VCA-EBV-specific IgG levels were even increased in CLL patients *versus* healthy subjects. Furthermore, when the ratio between each of these Ab levels and the total plasma levels of the corresponding Ig isotype (IgM or IgG) were considered, progressively greater fractions of the above referred antigen-specific antibodies per-isotype were found from MBL^{hi} to stage A and stage B/C CLL patients, respectively. Our findings on the antibody levels against CMV confirm previous results on CLL reported by Vanura *et al*. who also showed that, despite progressive decay of total IgM and

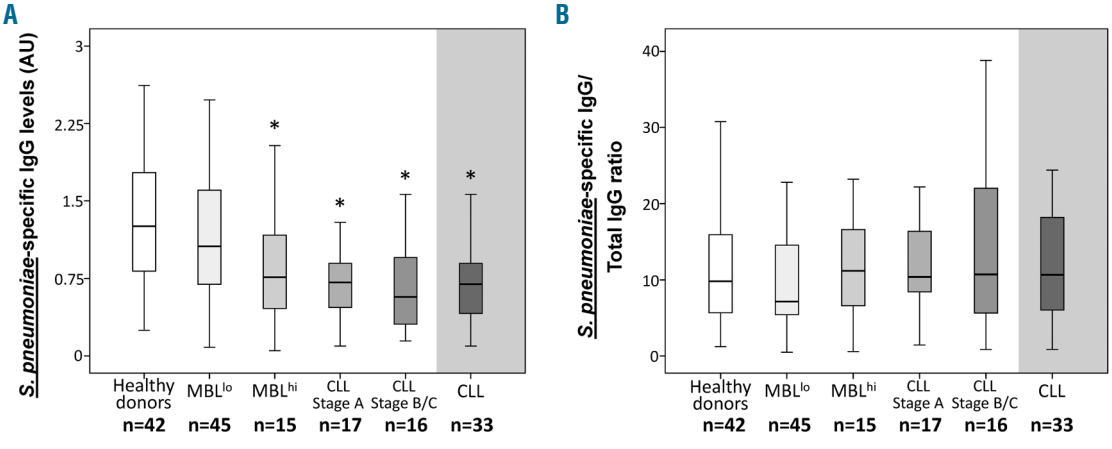

Figure 3. *Streptococcus pneumoniae*-specific IgG plasma levels in monoclonal B lymphocytosis (MBL) and chronic lymphocytic leukemia (CLL) patients *versus* healthy controls. (A) Titers of antibody-specific plasma levels against the pneumococcal polysaccharide antigen for each group of individuals analyzed. (B) Ratio between anti-pneumococcus-specific IgG and total IgG plasma levels for each group of subjects investigated. Only data from those subjects that did not receive vaccination against *S.pneumoniae* are displayed. Notched boxes represent 25th and 75th percentile values; the lines in the middle correspond to median values (50th percentile), while vertical lines represent the highest and lowest values that are neither outliers nor extreme values. *P<0.05 *versus* healthy donors and MBL^{lo}. MBL^{hi}: high-count monoclonal B lymphocytosis; MBL^{lo}: low-count monoclonal B lymphocytosis.

IgG subclasses, the CMV-specific immune response may be preserved even in CLL cases with advanced disease.[21] Here, we confirm and extend on these findings by showing for the first time that: i) this behavior is already detectable at the MBL^{hi} stage; and ii) it is also common to other antibody responses against EBV and the influenza virus in non-vaccinated individuals, despite the mechanisms by which influenza infects cells are completely different from those of CMV and EBV.[22-24] As mentioned above, we did observe decreased titers of EBV-specific IgG levels in both MBL^{hi} and CLL; interestingly, this was restricted to the antibody response against the EBNA-EBV antigen, but not the VCA-EBV antigen. The EBNA-EBV protein is located in the nucleus of infected host cells and it acts as a transcription factor for the virus, allowing for its replication inside the cell;[23] in contrast, the VCA-EBV protein is a structural component of the capside of the virus.[25] Therefore, the (humoral) immune response against the VCA-antigen might only occur if infected cells are lysed and active viral replications occurs. Therefore, our results suggest that like CMV, EBV probably undergoes a mild (undetectable) reactivation, whenever an immunodeficiency state has been acquired, but fully bloomed EBV and CMV infections can still be controlled, as reflected by the preserved production of specific antibodies against both viruses in MBL^{hi} and CLL patients[21] and the detection of quantifiable EBV DNA in plasma of CLL cases but not MBL. Long-term monitoring of virus-specific Ig plasma levels in CLL *versus* MBL *versus* healthy donors is required to validate this hypothesis.

In contrast to the general pattern found for the plasma levels of antibodies against the ubiquitous viruses here investigated, a significant reduction was observed in the plasma levels of pneumococcus-specific IgG from MBL^{hi} to stage B/C CLL, in parallel to the overall decrease in total IgG plasma levels. These findings further suggest that, while the antibody-mediated immune response against ubiquitous pathogens (e.g. human herpesviruses and influenza virus) is still preserved and the virus is actively controlled in immunocompromised MBL^{hi} and CLL patients, protection against other pathogens (i.e. pneumococcus) is progressively lost, placing these patients at risk of severe infection and death. In line with this hypothesis, CMV disease is infrequent among untreated CLL patients compared to other immunocompromised patients.[13,26] In contrast, CLL patients frequently present respiratory tract infections caused by encapsulated bacteria, particularly *Streptococcus pneumoniae* and *Haemophilus influenza*,[27] further supporting a unique dysregulation of immunesurveillance against infectious agents in MBL^{hi} and CLL.

To the best of our knowledge, no studies have been reported so far about the immune response profile against different pathogens in MBL^{lo} subjects. As no differences were detected in both total and pathogen specific Ig plasma titers in MBL^{lo} *versus* age-matched healthy subjects of the same geographical area, it might be expected that the antibody response of these subjects remains normal or at most little altered. Altogether, these findings suggest that the onset of dysregulated antibody-based immune responses might occur in the transition from MBL^{lo} to MBL^{hi} and CLL, being associated with a clinically silent reactivation of preserved T-cell dependent antibody responses against host viruses. If this holds true, and chronic baseline activation of antibody responses against host viruses occurs in MBL^{hi} and CLL patients, such a response could also potentially affect the tumor clone and contribute to its expansion and progression of the disease. In line with this hypothesis, it has been shown that most MBL^{lo} subjects show (oligoclonal) expansions of CD4^{+}/CD8^{+} double-positive T cells[28] which have a limited TCRvβ repertoire and participate in immune responses against chronic viral infections, particularly against CMV.[29] There is even stronger evidence to suggest that CLL evolves from repetitive activation of particular B-cell clones through B-cell receptor (BCR) triggering by conventional antigens,[30] which, in the light of the results reported here, increase in the CMV- and EBV-specific IgG/total Ig ratio in both MBL^{hi} and CLL patients. This might further suggest a potential role for ubiquitous viruses in the pathogenesis of the disease. Previous findings showing an association between the presence of CMV- and EBV-DNA in blood of CLL patients who express stereotyped IGHV4-34 BCRs[31] would further support this hypothesis. However, here we only analyzed a relatively limited number of cases within each study group, particularly within the MBL^{hi} group, and, therefore, further long-term longitudinal studies in MBL and CLL in larger series of subjects are necessary in order to elucidate the value of (total and pathogen-specific) Ig plasma levels, as a surrogate marker for a normal *versus* abnormal B-cell function, and to determine both the risk of progression from MBL to CLL and the potential need for adoption of specific active immunotherapy measures for patients at risk of life-threatening infections. In this regard, extensive research on the effectiveness of vaccines, particularly against influenza and *S.pneumoniae*, has been carried out in CLL, while there is limited information on MBL^{hi}.[15] Thus, response to vaccination against both polysaccharide (e.g. classical multivalent pneumococcal vaccines[32,33]) and protein antigens (e.g. tetanus toxoid and influenza virus[34,35]) has been shown to be associated with poor seroprotective responses in CLL, even after various doses. Such defective antibody responses have been related to a broad variety of immune defects including complement dysregulation, T-cell impaired function and altered antigen presentation, in addition to B-cell deficiency.[8,9,27,36,37] Because of this, vaccination of CLL patients early after diagnosis, and particularly even at the MBL stage when better responses might be expected,[8,33] has been proposed as a potentially effective strategy to improve serological immune protection of CLL patients against the most common pathogens. Parallel analyses focused on the humoral immunity and immune responses other than just the evaluation of plasma antibody levels are required to fully understand the uniqueness of the immunodeficiency status of MBL^{hi} and CLL patients.

In summary, we report on the existence of a significant and selective, defective antibody protection against *S.pneumoniae* in CLL which emerges already among MBL^{hi} to early stage CLL and worsens through progression of the disease. Such an immune defect might be associated with an active, but silent, response against host viruses such as CMV, EBV and influenza, for which preserved antibody serum levels are detected, even in advanced CLL. These results suggest that chronic viral re-activation might contribute to the preserved host virus-specific antibody titers through sustained immune responses, which might also favor parallel expansion of the tumor B-cell clone and progression from MBL^{hi} to CLL. Further studies in larger MBL and CLL patient cohorts with long-term follow up and

sequential serological analyses are necessary to confirm this hypothesis.

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