Reduced expansion of CD94/NKG2C+ NK cells in chronic lymphocytic leukemia and CLL-like monoclonal B cell lymphocytosis is not related to increased HCMV seronegativity or NKG2C deletions

Running title: Reduced NKG2C+ NK cells in CLL and MBL

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ABSTRACT

Introduction: Dysregulated NK-cell-mediated immune responses contribute to tumor evasion in chronic lymphocytic leukemia (CLL), although the NK cell compartment in CLL-like monoclonal B cell lymphocytosis (MBL) is poorly understood. In healthy individuals, human cytomegalovirus (HCMV) induces the expansion of NK cells expressing high levels of CD94/NKG2C NK cell receptor (NKR) specific for HLA-E. Methods: We analyzed the expression of NKG2A, NKG2C, ILT2, KIR, CD161 and CD57 in 24 MBL and 37 CLL. NKG2C was genotyped in these patients and in 81 additional MBL/CLL, while NKG2C gene expression was assessed in 26 cases. In 8 CLL patients with increased lymphocytosis (≥20x10⁹/L), tumor HLA-E and HLA-G expression was evaluated. Results: NKR distribution did not significantly differ between MBL and CLL patients, although they exhibited reduced NKG2C⁺ NK cells compared with a non-CLL group (4.6% vs. 12.2%, P=0.012). HCMV⁺ patients showed increased percentages of NKG2C⁺ NK cells compared with HCMV⁻ (7.3% vs. 2.9%, P=0.176). Frequencies of NKG2C deletions in MBL/CLL were similar to those of the general population. Low/undetectable NKG2C expression was found among NKG2C⁻⁻ (45%) and NKG2C⁻⁺ (12%) patients. CLL cases with increased lymphocytosis displayed especially reduced NKG2C expression (1.8% vs. 8.1%, P=0.029) and tumor cells with high HLA-E (>98%) and variable HLA-G expression (12.4%, range: 0.5-56.4). CLL patients with low NKG2C expression (<7%) showed shorter time to first treatment (P=0.037). Conclusion: Reduced percentages of CD94/NKG2C⁺ NK cells were observed in CLL and MBL patients independently of HCMV serostatus and NKG2C zygosity, particularly in CLL patients with increased lymphocytosis, which could potentially be related to the exposure to tumor cells.

Key words: Chronic lymphocytic leukemia; Monoclonal B cell lymphocytosis; NKG2C; Human cytomegalovirus; HLA-E.
INTRODUCTION

Chronic lymphocytic leukemia (CLL) is a B cell malignancy typically associated with a significant perturbation of the immune system. Direct interaction of clonal B cells and circulating T cells induces a T cell tolerance that is crucial for disease development1. Functional alterations in circulating T cells affect proliferation, motility, immune synapse formation and cytotoxicity, and skewing toward a Th2 phenotype2,3. Dysregulated NK-cell-mediated immune responses also contribute to the evasion of CLL cells from immune-mediated destruction. In this regard, NK cells from patients with CLL show decreased expression of several NK cell activating receptors, such as NKp30, NKp46 and NKG2D4-8, and defective degranulation responses7 which are related to an impaired antibody-dependent cell mediated cytotoxicity6.

Clinical CLL-like monoclonal B cell lymphocytosis (MBL) is a pre-leukemic condition of CLL defined as the presence of a clonal population of B lymphocytes in peripheral blood (0.5 to \(<5x10^9/L\)) with a phenotype consistent with CLL, in the absence of other clinical features of the disease. It shows a progression rate to CLL requiring therapy of 1.1% per year9,10. Remarkably, although modifications in the T cell population are detectable in MBL11-14, some studies suggested that major T cell alterations are mainly detected after transition to CLL13,15. Thus far, little is known about the NK cell compartment in MBL individuals.

On the other hand, despite their immune suppression state and their increased susceptibility to infections, CLL patients exhibit preserved responses to human cytomegalovirus (HCMV). Actually, the accumulation of HCMV-specific T cells prompted by the chronic viral infection is more pronounced in CLL patients than in age-matched controls16,17. Similarly, HCMV induces a persistent reconfiguration of the NK cell compartment which is characterized by an adaptive expansion of a subset displaying high levels of the CD94/NKG2C activating NK cell receptor specific for HLA-E18,19. CD94/NKG2C recognizes HLA-E bound to conserved nonamers from the leader sequences of other HLA class I molecules20, also present in the HCMV UL40 protein21,22. Notably, the magnitude of this effect is quite variable among HCMV+ individuals and a hemizygous deletion of the NKG2C gene has been associated to reduced expansions.
CD94/NKG2C+ NK cells. Immunocompromised patients show an expansion of CD94/NKG2C+ NK cells inversely correlated to the efficacy of the T cell response against the virus, suggesting that this subset might act as a compensatory mechanism in anti-viral defense. In this line, a role of CD94/NKG2C+ cells in controlling HCMV infection in kidney transplantation recipients has been recently reported. To the best of our knowledge, the expression of NKG2C in MBL and CLL has been poorly investigated. Two studies suggested that HCMV+ CLL patients show expanded CD94/NKG2C+ NK cells as described in healthy populations. Regarding other hematological malignances, NK cells from patients with acute myeloid leukemia showed a reduced expression of CD94/NKG2C among several other NK cell-activating receptors. The potential mechanisms by which HCMV infection could drive a global shift in NK subsets of CLL patients are still unknown.

In the present study we aimed to identify possible variations in the distribution of NK cell receptors (NKR) from patients with MBL and CLL which could be related to their clinical characteristics and viral infections, with special interest on the modulation of the CD94/NKG2C+ subset in the context of HCMV infection.
MATERIAL AND METHODS

Patients and samples

The study cohort included 61 patients from Hospital del Mar and Hospital Vall d’Hebron (Barcelona), 24 of them had been diagnosed with clinical CLL-like MBL and 37 were treatment-naïve CLL. Most of the CLL patients were recruited at initial stages, being 87% (32/37) at Binet stage A. The main demographic, clinical and biological characteristics of the cohort are summarized in Supplemental Table 1. To elucidate the strength of the results, an independent cohort of 81 MBL and CLL patients was also genotyped for NKG2C zygosity. Moreover, the percentage of NKG2C+ NK cells by flow cytometry was compared with a non-CLL cohort of 35 subjects recruited as donors for renal transplantation previously assessed by the group with available HCMV serostatus and demographics (age and gender) data. Distribution of patients among the different experiments is detailed in Supplemental Figure 1. Written informed consent was obtained from all patients prior to the extraction of peripheral blood samples used in subsequent analyses. The study was performed in accordance with national and international guidelines (Professional Code of Conduct, Declaration of Helsinki) and approved by the Ethics Committee of Hospital del Mar (2011/4317/I).

NK cell receptor analysis by flow cytometry

Immunophenotypic analysis was performed in fresh peripheral blood from 24 MBL and 37 CLL patients. Blood samples were pretreated with saturating concentrations of human aggregated Iggs to block FcgR and then labeled with the following surface antibodies: CD3 (PerCP-Cy5.5), CD56 (FITC) and CD45 (APC) (BD Biosciences, San Diego, CA), and unlabeled non-commercial Ab against MYC used as negative control, NKG2C, NKG2A, ILT2, CD161, CD57 and a mixture of anti-KIR antibodies (clones detailed in Supplemental Methods). Non-commercial Abs were produced in the laboratory and analyzed by indirect immunofluorescence staining, using PE-conjugated F(ab’)2 polyclonal goat antimouse Ig (Jackson Immunoresearch, West Grove, PA) as secondary Ab. Data acquisition was
performed in a BD FACSCanto II cytometer and analyzed using the FACSDiva software (BD Biosciences) and FlowJo (FlowJo LLC, Ashland, OR). The percentage of NK cells, defined as CD56+CD3- lymphocytes, expressing each NKR was recorded as detailed in Supplemental Figure 2.

**NKG2C genotyping**

Genotyping was assessed on DNA from peripheral blood granulocytes (N=105), peripheral blood mononuclear cells (PBMCs) (N=9), CD3+ (N=4) or CD19+ (N=17) lymphocytes. DNA was extracted using a Gentra Puregene extraction kit (QIAGEN, Hilden, Germany). NKG2C zygosity was assessed as previously described and detailed in Supplemental Methods.

**Cell isolation and RNA extraction**

In 26 of the subjects evaluated by flow cytometry (14 MBL and 12 CLL), fresh peripheral blood was also subjected to Ficoll density gradient centrifugation. Purified CD56+CD3- cells were isolated from PBMCs based on positive selection methods employing immunomagnetic beads (CD3 and CD56 MicroBeads) and autoMACS technology (Miltenyi Biotec, Bergisch Gladbach, Germany) and were stored at -80°C in 1% BME RLT-plus buffer (QIAGEN). RNA was finally extracted following the RNeasy Plus Mini Kit protocol (QIAGEN).

**NKG2C gene expression analysis**

Microarray experiments were performed on RNA from CD56+CD3- cells. Briefly, 30 ng of total RNA were retrotranscribed into cDNA and amplified (Ovation® Pico WTA System V2, NUGEN, Redwood City, CA, USA). The cDNA product was purified (MinElute Reaction Cleanup Kit, QIAGEN) and hybridized on a GeneChip Human Gene 2.0 ST array (ThermoFisher, Santa Clara, CA, USA) following manufacturer’s instructions. After quality assessment, NKG2C gene expression (also named KLRC2) was obtained through standard procedures (RMA normalization and ComBat batch effects correction). Linear gene expression intensity values for NKG2C were registered.
Analysis of tumor HLA-E and HLA-G expression by flow cytometry

Cryopreserved PBMCs from 8 CLL patients with increased absolute lymphocyte count (ALC) levels (≥2×10⁹ cells/L) were further characterized. Cells were thawed and labeled with DAPI and with the following surface antibodies: CD45 (Alexa Fluor 700, eBioscience), CD19 (FITC, BD Biosciences), CD5 (PerCP-Cy5.5, BD Biosciences), CD20 (APC, BD Biosciences), HLA-E (PE, Miltenyi Biotec) and isotype control (PE, eBioscience). For the analysis of HLA-G expression on tumor cells, unlabeled antibodies against HLA-G (clone G233) (Invitrogen) and MYC (clone 9E10, used as negative control) were employed, followed by indirect immunofluorescence staining with PE-Cy7 goat anti-mouse IgG (minimal x-reactivity) (Biolegend). Data acquisition was performed in a LSR Fortessa instrument (BD Biosciences) and analyzed using FlowJo. Mean fluorescence intensity (MFI) values were evaluated as shown in Supplemental Methods and in Supplemental Figure 3.

Statistical analysis

Comparison between groups was performed with Chi-square or Fisher exact tests for discrete variables, while the Mann-Whitney test was used for continuous variables. Linear regression analysis and Pearson correlations were used to assess the relationship between quantitative variables. Time to first treatment (TFT) was defined as the time from diagnosis to the beginning of treatment and was analyzed using Kaplan-Meier plots and the log-rank test. Statistical analyses were performed using SPSS v.22 software (SPSS Inc., Chicago, IL, USA).
RESULTS

**MBL individuals show a reduced NK cell count compared to CLL patients**

As expected, CLL patients showed a significant increased leukocytosis, lymphocytosis and median absolute clonal B cell count compared to MBL individuals (Supplemental Table 1, Supplemental Figure 4). Regarding NK cells (defined as CD56+CD3- lymphocytes), a significant positive correlation was observed between the clonal B cell count and the absolute number of NK cells ($r_p=0.50$, $P<0.001$). Indeed, NK cells were significantly increased in CLL compared to MBL in terms of median absolute NK counts ($0.85\times10^9/L$ vs. $0.57\times10^9/L$, $P=0.002$). On the other hand, NK cells from CLL patients represented a lower median percentage within total lymphocytes ($5.5\%$ vs. $10\%$, $P=0.003$) and a significant negative correlation was observed between the clonal B cell count and the percentage of NK cells ($r_p=-0.28$, $P=0.033$). Finally, the NK:clonal B cell ratio was decreased in CLL patients ($0.07$ vs. $0.24$, $P<0.001$) (Supplemental Figure 4).

**NK cells from MBL and CLL patients display reduced NKG2C expression, although response to HCMV is preserved**

Regarding the expression of the inhibitory and activating surface NKR studied, we did not detect any significant difference between MBL and CLL patients: NKG2A ($31.4\%$ vs. $30.8\%$, respectively), NKG2C ($3.6\%$ vs. $6.8\%$), ILT2 ($18.0\%$ vs. $15.8\%$), KIRs ($54.4\%$ vs. $52.7\%$), CD161 ($16.1\%$ vs. $16.4\%$) and CD57 ($40.4\%$ vs. $38.9\%$) (Supplemental Figure 5). As our MBL and CLL cohort was highly enriched in patients seropositive for HCMV ($85\%$, 47/55 with available data), we assessed if NKG2C expression was modulated by HCMV serology. In this regard, HCMV+ patients showed an increased median percentage of NK cells expressing NKG2C than HCMV−, although statistical significance was not achieved ($7.3\%$ vs. $2.9\%$ respectively, $P=0.176$) (Table 1, Supplemental Figure 6). Next, we compared the expression of NKG2C in our MBL/CLL cohort with a non-CLL control cohort of donors for renal transplantation that were slightly younger (median age: 60 years, range: 43-73; $P<0.001$), whose HCMV seropositivity
(29/35 cases) did not differ from our study group (83% vs 85%, P=0.772). Our patients showed a significant lower median percentage of NKG2C⁺ NK cells (4.6% vs. 12.2%, P=0.012). A similar trend was observed when only HCMV⁺ individuals were considered (7.3% vs. 12.7%, P=0.168) (Figure 1). A significantly positive correlation between NKG2C gene expression and the percentage of NK cells that were positive for NKG2C was disclosed (r=0.68, P<0.001) (Supplemental Figure 7A), suggesting that the increase in NKG2C⁺ NK cells is also accompanied by augmented NKG2C gene expression levels.

Frequencies of NKG2C germinal genomic deletions in MBL and CLL are comparable to those found in the general population

NKG2C zygoty was assessed in 135 patients to ascertain if the frequency of germinal genomic deletions could explain the diminished NKG2C expression observed in our cohort. NKG2C⁺/⁺, NKG2C⁺/del and NKG2C⁻/⁻ del individuals represented 56%, 37% and 7% of the studied cohort respectively; these frequencies were comparable to those detected in the general population. In line with previous reports, among 54 patients with available data on NKG2C surface receptor expression, NKG2C⁺/⁺ cases (N=26) showed significantly increased proportion of NKG2C⁺ NK cells compared to hemizygous individuals (N=22; median: 8.4% vs 3.6%, P=0.014). As expected, NKG2C was undetectable in the six patients with homozygous deletions. Remarkably, cases with very low (<2%) or undetectable NKG2C expression were also found among NKG2C⁺/⁻ (10/22, 45%) and NKG2C⁻/⁻ (3/26, 12%) individuals (Figure 2), ruling out a relation between the decreased expression with NKG2C deletion in these cases. Similar differences in NKG2C gene expression intensity according to NKG2C⁺/⁺, NKG2C⁺/del and NKG2C⁻/⁻ del genotypes were also observed (Supplemental Figure 7B).

Low NKG2C expression in CLL patients is associated with higher lymphocytosis and shorter time to first treatment

Next, to evaluate if the expression of some of these NKR could be affected by the magnitude of clonal B cell expansion, CLL patients were categorized in two groups according to their
lymphocytosis. CLL patients with an ALC ≥20x10⁹ cells/L exhibited a significant lower median percentage of NK cells expressing NKG2C compared to CLL patients with an ALC <20x10⁹ cells/L (1.8 vs. 8.1% respectively, P=0.029) (Figure 3), besides no differences in HCMV serology or NKG2C zygosity were observed (Supplemental Table 2). CLL patients with an ALC ≥20x10⁹ cells/L showed a significant lower median percentage of NKG2C⁺ NK cells compared to the independent control cohort, although differences did not reach statistical significance when considering only HCMV⁺ individuals (6/7 patients with an ALC ≥20x10⁹ cells/L with available HCMV serostatus, 86%) (Figure 1). No significant differences were observed for the expression of other NKR (Figure 3).

Besides ALC, differences in NKR expression among patients stratified according to known prognostic factors in CLL (CD38 and ZAP-70 expression, 13q deletion, trisomy 12 and mutational status of the IGHV gene) were assessed. In this regard, no differences were either found for NKG2C nor other NKR, except for a significant higher percentage of CD57⁺ NK cells in patients with trisomy 12 (Supplemental Table 3). Notably, differences regarding the presence of deletions in 17p (TP53) or 11q (ATM) could not be analyzed due to their scarce representation in our cohort.

As expected, with a median follow-up of 62 months (0-76), none of the subjects diagnosed with MBL required treatment and only two of them (8%) died. As for CLL, after a median follow-up of 55 months (1-77), seven patients received therapy (19%) and six were dead (16%) at last follow-up. Interestingly, those CLL patients with a percentage of NKG2C⁺ cells below the median (low NKG2C, <7%) exhibited an inferior TFT (five-year treatment-free survival: 67% vs. 94%, P=0.037) (Figure 4). Similarly, CLL patients with high ALC (≥20x10⁹/L) also displayed shorter TFT (five-year treatment-free survival: 61% vs. 89%, P=0.039). With the exception of CD38, none of the other known prognostic factors in CLL mentioned before showed significant differences concerning TFT (Figure 4, Supplemental Table 4).

Tumor B cells show high HLA-E and variable HLA-G expression
CD94/NKG2C recognizes HLA-E bound to nonamers from the leader sequence of other class I molecules. Among them, the leader peptide from HLA-G, reported to be aberrantly expressed on tumor cells from some CLL patients\(^{30}\), confers the highest affinity to the interaction. On that basis, the expression of HLA-E and HLA-G on clonal B cells was analyzed in a subset of our patients (Supplemental Table 5). HLA-E expression was detected in all cases, whereas the expression of HLA-G differed among patients (median percentage of positive cells: 12.4%, range: 0.5-56.4). Of note, one patient showed a bimodal distribution of HLA-G expression being detected in 56.4% of the clonal B cell population and displayed a particularly aggressive clinical course (Supplemental Figure 3). As expected, the percentage of clonal B cells expressing HLA-G positively correlated with HLA-G \( \Delta \text{MFI} \) values (\( r_p=0.87, P=0.005 \)) and relative HLA-G MFI values (\( r_p=0.71; P=0.050 \)). No significant correlations were observed between relative HLA-E and HLA-G expression on tumor cells and the percentages of NKG2C\(^+\) NK cells.
DISCUSSION

CLL is known to impair NK cell immune responses in terms of defective antibody-dependent cell mediated cytotoxicity and decreased expression of some NK cell activating receptors (e.g. NKp30, NKp46 and NKG2D)\(^4,\,^d\). Nonetheless, studies assessing the NK cell phenotype in MBL and CLL are scarce and additional NKR which could be relevant for tumor-associated immune responses deserve attention. This is the case for NKR known to be modulated by HCMV infection, whose characterization in MBL and CLL patients could shed light on the relationship between this viral infection and potential differences in NK cell activity between both entities. Moreover, it is unclear at which stage of disease progression NK cell dysfunction occurs. To elucidate these questions, we have characterized the distribution of several NKR in MBL and CLL, focusing on the adaptive CD94/NKG2C\(^+\) subset which differentiates and expands in response to HCMV.

In accordance with previous studies, our findings evidenced that CLL patients showed an NK cell count significantly higher than MBL subjects, although the NK cell population represented a higher proportion in the latter\(^5,\,^31\). Moreover, no significant differences in the expression of any of the NKR assessed were detected between MBL and CLL groups, which suggests that none of them would be associated with the transition from MBL to CLL.

With regard to CD94/NKG2C expression, both entities showed a low median percentage of CD94/NKG2C\(^+\) NK cells compared with the frequencies described in healthy populations\(^32\). Consequently, additional studies were performed to assess factors that could underlie the low/undetectable NKG2C expression observed in some MBL/CLL patients and its potential relationship with the disease development. As described in healthy individuals\(^18,\,^24\), CD94/NKG2C\(^+\) NK cells appeared increased in HCMV\(^+\) compared to HCMV\(^-\) patients, although differences did not reach statistical significance in our cohort. NKG2C gene deletion has also been associated with the numbers of CD94/NKG2C\(^+\) NK cells as well as with surface receptor levels\(^23,\,^25\). In this regard, the frequencies of NKG2C\(^\text{n/del}\) and NKG2C\(^\text{del/del}\) genotypes assessed in our MBL/CLL cohort were comparable to those previously defined in two Spanish cohorts of
healthy donors\textsuperscript{23,29}. Moreover, as in health conditions, a significantly higher median percentage of CD94/NKG2C\textsuperscript{+} NK cells was found in patients conserving both alleles. These results ruled out the possibility that the distribution of NKG2C genotypes might account for the reduced numbers of CD94/NKG2C\textsuperscript{+} NK cells in MBL and CLL. Aging is another variable that may affect the frequency, phenotype and distribution of NK cells. In this regard, as the proportions of CD94/NKG2C\textsuperscript{+} NK cell subsets associated with HCMV infection are lower in elderly HCMV\textsuperscript{+} donors (>70 years) compared to younger ones (18-35 years)\textsuperscript{33}, a potential bias in NKG2C expression associated with the advanced age of our cohort was addressed. Despite this study was limited by the unavailability to perform parallel NKR analyses on healthy age-matched individuals, we confirmed lower NKG2C expression in our patients compared with a non-CLL cohort previously assessed. Although this control group was slightly younger, its median age was close to 70 years and minor age effects would be expected. Indeed, when the comparison was restricted to younger MBL and CLL patients (≤70 years), differences in NKG2C expression with the non-CLL cohort were similarly observed (data not shown). In all, these results indirectly support that besides the advanced median age of CLL patients, other intrinsic characteristics of the tumor are contributing to the limited expansion of NKG2C\textsuperscript{+} NK cells.

In line with the aforementioned, we found that CLL patients with high ALC levels displayed a significant lower percentage of CD94/NKG2C\textsuperscript{+} NK cells, and no differences in terms of HCMV serology or NKG2C genotype. In this regard, Costello et al detected decreased expression of the natural cytotoxicity receptors NKp30/NCR3 and NKp44/NCR2 only in CLL patients with an ALC>30x10\textsuperscript{9} cells/L, leading to defective NK cytotoxicity\textsuperscript{31}. In our study, reduced proportions of NKG2C\textsuperscript{+} NK cells were also associated with increased ALC values. Additionally, we found that clonal B cells of CLL patients with high ALC showed high HLA-E expression, some of them also expressing HLA-G molecules. Of note, previous studies demonstrated HLA-G expression on CLL cells and proposed that this molecule may be involved in the escape of CLL cells from immunosurveillance\textsuperscript{30,34-37}. As far as we know, this is the first time that the highly specific G233 clone was employed, which is mandatory to definitely confirm that CLL tumor cells can express
HLA-G molecules. Prior investigations reported that binding of HLA-E to the HLA-G signal peptide-derived nonamer confers to the HLA-E complex a high affinity for the CD94/NKG2C receptor triggering NK cell activation\textsuperscript{38,39} and promoting NKG2C receptor internalization\textsuperscript{40}. All these findings potentially suggest that a persistent exposure to tumor cells bearing HLA-E, particularly upon HLA-G co-expression such as that confirmed in a subset of CLL patients with high ALC, could lead to chronic CD94/NKG2C\textsuperscript{+} NK cell activation and exhaustion, underlying their reduced numbers in circulation. It is also worth mentioning that a decreased gene expression of NK cell activating receptors was reported in CLL but not in small lymphocytic lymphoma, a CLL variant with identical immunophenotypic features but without peripheral blood lymphocytosis\textsuperscript{6}, concluding that NK cell receptor and function may be markedly influenced by exposure to peripheral blood tumor burden. Additionally, we observed that low NKG2C expression in NK cells from CLL patients was significantly associated with shorter TFT at a cutoff level of 7%. Since the number of CLL patients in our study is limited, the selected cutoff value should be corroborated in a lager validation cohort. Regardless of which is the optimal cutoff, NKG2C internalization could potentially be a consequence of the increased exposure to tumor cells in patients with high lymphocytosis and, therefore, the poorer outcome could be the result of their increased ALC levels.

To the best of our knowledge, CD94/NKG2C expression on NK cells from CLL has been previously addressed in two studies which, in accordance to our findings, described an expansion of CD94/NKG2C\textsuperscript{+} NK cells driven by HCMV\textsuperscript{8,27}. Notably, low median percentages of NK cells (CD56\textsuperscript{+}CD3\textsuperscript{-}) expressing NKG2C in 24 CLL patients were also reported by Petersen et al (3.1\% [range: 0.2-43.2] in HCMV\textsuperscript{+} vs. 2.1\% [range: 0.5-2.6] in HCMV\textsuperscript{-})\textsuperscript{27}. Hofland et al. assessed 41 CLL patients with higher lymphocytosis than our cohort (median ALC: 93x10\textsuperscript{9} cells/L; range: 13-359) and found similar low levels of NKG2C in different NK subpopulations according to CD56 and CD16 expression\textsuperscript{8}. In contrast to our study, the HCMV\textsuperscript{-} control group studied by Hofland et al. exhibited a reduced CD94/NKG2C\textsuperscript{+} NK cell population compared to HCMV\textsuperscript{+} CLL patients, although differences were not significant. Intrinsic
characteristics of the different control groups of the two studies, together with the different methodologies employed, may account for these differences.

In summary, we demonstrated that MBL/CLL patients do not differ in HCMV serostatus nor NKG2C zygosity compared with the general population. We also definitely confirmed that CLL tumor B cells are able to express HLA-G molecules. Further exploratory analyses of CD94/NKG2C+ NK cell percentages (comparisons with a non-CLL cohort and between CLL patients with high or low ALC levels) suggested that exposure to tumor B cells may be related to reduced CD94/NKG2C+ NK cells in leukemic patients. Additionally, recent studies demonstrated an association between HLA-G expression and NKG2C internalization\textsuperscript{40}, therefore a similar effect could be expected in CLL. Since HCMV and NKG2C zygosity have a strong impact on NKG2C expression, we made an effort to collect these data in our patients. Nonetheless, other factors including subclinical infections and other immune or pathological conditions could have an impact on NKG2C. Additional studies in larger cohorts of patients together with functional assays are mandatory to definitely understand the interactions between the HLA-E:G peptide complex and CD94/NKG2C+ NK cell dynamics in CLL.
Conflicts of interest

No potential conflicts of interest were disclosed.

REFERENCES


### Table 1. Age, gender, NKG2C genotype, lymphocyte counts and NKG2C expression on NK and T cells comparing HCMV positive and negative MBL and CLL patients.

<table>
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<th>All MBL and CLL patients</th>
<th>HCMV+ MBL and CLL</th>
<th>HCMV+ MBL and CLL</th>
<th>P-value (HCMV+ vs HCMV-)</th>
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<td></td>
<td>N=61*</td>
<td>N=8</td>
<td>N=47</td>
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<tr>
<td>Age</td>
<td>73 (43-89)</td>
<td>68 (43-81)</td>
<td>75 (50-89)</td>
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<tr>
<td>Male</td>
<td>35 (57.4%)</td>
<td>3 (37.5%)</td>
<td>28 (59.6%)</td>
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<tr>
<td>NKG2C zygosity</td>
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<tr>
<td>+/+</td>
<td>27/55 (49.1%)</td>
<td>2/7 (25.0%)</td>
<td>22/42 (52.4%)</td>
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<td>+/del</td>
<td>22/55 (40.0%)</td>
<td>4/7 (50.0%)</td>
<td>17/42 (40.5%)</td>
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<td>del/del</td>
<td>6/55 (10.9%)</td>
<td>1/7 (12.5%)</td>
<td>3/42 (7.1%)</td>
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<tr>
<td>Absolute lymphocyte count (x10⁹/L)</td>
<td>9.9 (1.8-84.6)</td>
<td>8.4 (2.0-62.0)</td>
<td>9.5 (1.8-66.5)</td>
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<td>Absolute clonal B cells (x10⁹/L)</td>
<td>6.4 (0.5-76.1)</td>
<td>5.4 (0.7-57.0)</td>
<td>6.0 (0.5-63.1)</td>
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<td>Percentage of NK cells (CD3-CD56+)</td>
<td>8.0 (0.9-28.0)</td>
<td>8.5 (2.8-15.0)</td>
<td>8.0 (1.0-28.0)</td>
<td>0.811</td>
</tr>
<tr>
<td>Absolute NK cell count (x10⁹/L)</td>
<td>0.6 (0.1-9.3)</td>
<td>0.5 (0.2-9.3)</td>
<td>0.6 (0.1-3.3)</td>
<td>0.504</td>
</tr>
<tr>
<td>Percentage of NKG2C+ NK cells</td>
<td>4.6 (0.6-67.7)</td>
<td>2.9 (0.8-1.1)</td>
<td>7.3 (0-67.7)</td>
<td>0.176</td>
</tr>
<tr>
<td>Percentage of T cells (CD3-CD56+)</td>
<td>29.8 (3.3-62.3)</td>
<td>37.9 (17.2-57.0)</td>
<td>30.7 (3.3-62.3)</td>
<td>0.346</td>
</tr>
<tr>
<td>Absolute T cell count (x10⁹/L)</td>
<td>2.4 (0.9-29.1)</td>
<td>2.4 (0.9-29.1)</td>
<td>2.4 (0.9-17.3)</td>
<td>0.962</td>
</tr>
<tr>
<td>Percentage of NKG2C+ T cells</td>
<td>1.8 (0.3-17.4)</td>
<td>2.5 (1.2-11.7)</td>
<td>1.9 (0.3-17.4)</td>
<td>0.219</td>
</tr>
</tbody>
</table>

*Serology for HCMV was not available in 6 patients.
Values are given as median (range) or number (%).
Figure 1. Distribution of NK cells (CD56<sup>+</sup>CD3<sup>-</sup>) expressing NKG2C in the whole MBL and CLL cohort, in MBL and CLL patients considering ALC (high ALC: ≥2×10<sup>9</sup> cells/L) and in the non-CLL cohort previously assessed by our group. Results from all the included patients and healthy controls (A) or restricted to HCMV<sup>+</sup> individuals (B) are shown.
Figure 2. Distribution of NK cells (CD56^+CD3^-) expressing NKG2C in CLL and MBL patients showing NKG2C^{+/+}, NKG2C^{+/-del} and NKG2C^{del/del} genotype.
Figure 3. Comparison of the percentage of NK cells (CD56⁺CD3⁻) expressing NKG2A (A), NKG2C (B), ILT2 (C), KIR (D), CD161 (E) and CD57 (F) in CLL patients with lower absolute lymphocyte count (ALC) levels (Low ALC; <20x10⁹ cells/L) compared to those with increased ALC levels (High ALC; ≥20x10⁹ cells/L).
Figure 4. Kaplan-Meier plots for TFT in CLL patients with low (<7%) or high (≥7%) percentage of NKG2C⁺ NK cells (A), high (≥20) or low (<20×10⁹ cells/L) ALC values (B), and expression of CD38 in clonal B cells according to the previously established cut-off of 30% (C).