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4 Reduced expansion of CD94/NKG2C⁺ NK cells in chronic lymphocytic leukemia 5 and CLL-like monoclonal B cell lymphocytosis is not related to increased HCMV 6 seronegativity or *NKG2C* deletions

7 **Running tittle:** 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 ($\geq 20x10^9$/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.

55 **INTRODUCTION**

56 Chronic lymphocytic leukemia (CLL) is a B cell malignancy typically associated with a
57 significant perturbation of the immune system. Direct interaction of clonal B cells and
58 circulating T cells induces a T cell tolerance that is crucial for disease development[1]. Functional
59 alterations in circulating T cells affect proliferation, motility, immune synapse formation and
60 cytotoxicity, and skewing toward a Th2 phenotype[2,3]. Dysregulated NK-cell-mediated immune
61 responses also contribute to the evasion of CLL cells from immune-mediated destruction. In
62 this regard, NK cells from patients with CLL show decreased expression of several NK cell
63 activating receptors, such as NKp30, NKp46 and NKG2D[4-8], and defective degranulation
64 responses[7] which are related to an impaired antibody-dependent cell mediated cytotoxicity[6].
65 Clinical CLL-like monoclonal B cell lymphocytosis (MBL) is a pre-leukemic condition of CLL
66 defined as the presence of a clonal population of B lymphocytes in peripheral blood (0.5 to
67 $<5 \times 10^9$/L) with a phenotype consistent with CLL, in the absence of other clinical features of the
68 disease. It shows a progression rate to CLL requiring therapy of 1.1% per year[9,10]. Remarkably,
69 although modifications in the T cell population are detectable in MBL[11-14], some studies
70 suggested that major T cell alterations are mainly detected after transition to CLL[13,15]. Thus
71 far, little is known about the NK cell compartment in MBL individuals.

72 On the other hand, despite their immune suppression state and their increased susceptibility
73 to infections, CLL patients exhibit preserved responses to human cytomegalovirus (HCMV).
74 Actually, the accumulation of HCMV-specific T cells prompted by the chronic viral infection is
75 more pronounced in CLL patients than in age-matched controls[16,17]. Similarly, HCMV induces
76 a persistent reconfiguration of the NK cell compartment which is characterized by an adaptive
77 expansion of a subset displaying high levels of the CD94/NKG2C activating NK cell receptor
78 specific for HLA-E[18,19]. CD94/NKG2C recognizes HLA-E bound to conserved nonamers from
79 the leader sequences of other HLA class I molecules[20], also present in the HCMV UL40
80 protein[21,22]. Notably, the magnitude of this effect is quite variable among $HCMV^+$ individuals
81 and a hemizygous deletion of the *NKG2C* gene has been associated to reduced expansions

82 of CD94/NKG2C^{+} NK cells[23-25]. Immunocompromised patients show an expansion of
83 CD94/NKG2C^{+} NK cells inversely correlated to the efficacy of the T cell response against the
84 virus, suggesting that this subset might act as a compensatory mechanism in anti-viral
85 defense[19]. In this line, a role of CD94/NKG2C^{+} cells in controlling HCMV infection in kidney
86 transplantation recipients has been recently reported[26]. To the best of our knowledge, the
87 expression of NKG2C in MBL and CLL has been poorly investigated. Two studies suggested
88 that HCMV^{+} CLL patients show expanded CD94/NKG2C^{+} NK cells as described in healthy
89 populations[8,27]. Regarding other hematological malignances, NK cells from patients with acute
90 myeloid leukemia showed a reduced expression of CD94/NKG2C among several other NK
91 cell-activating receptors[28]. The potential mechanisms by which HCMV infection could drive a
92 global shift in NK subsets of CLL patients are still unknown.

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

97 # MATERIAL AND METHODS

98 ## Patients and samples

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

113 ## NK cell receptor analysis by flow cytometry

114 Immunophenotypic analysis was performed in fresh peripheral blood from 24 MBL and 37 CLL
115 patients. Blood samples were pretreated with saturating concentrations of human aggregated
116 Igs to block FcgR and then labeled with the following surface antibodies: CD3 (PerCP-Cy5.5),
117 CD56 (FITC) and CD45 (APC) (BD Biosciencies, San Diego, CA), and unlabeled non-
118 commercial Ab against MYC used as negative control, NKG2C, NKG2A, ILT2, CD161, CD57
119 and a mixture of anti-KIR antibodies (clones detailed in Supplemental Methods). Non-
120 commercial Abs were produced in the laboratory and analyzed by indirect
121 immunofluorescence staining, using PE-conjugated F(ab')2 polyclonal goat antimouse Ig
122 (Jackson Inmmunoresearch, West Grove, PA) as secondary Ab. Data acquisition was

123 performed in a BD FACSCanto II cytometer and analyzed using the FACSDiva software (BD
124 Biosciences) and FlowJo (FlowJo LLC, Ashland, OR). The percentage of NK cells, defined as
125 $CD56^{+}CD3^{-}$ lymphocytes, expressing each NKR was recorded as detailed in Supplemental
126 Figure 2.

127 **_NKG2C_ genotyping**

128 Genotyping was assessed on DNA from peripheral blood granulocytes (N=105), peripheral
129 blood mononuclear cells (PBMCs) (N=9), $CD3^{+}$ (N=4) or $CD19^{+}$ (N=17) lymphocytes. DNA was
130 extracted using a Gentra Puregene extraction kit (QIAGEN, Hilden, Germany). *NKG2C*
131 zygosity was assessed as previously described[29] and detailed in Supplemental Methods.

132 **Cell isolation and RNA extraction**

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

139 **_NKG2C_ gene expression analysis**

140 Microarray experiments were performed on RNA from $CD56^{+}CD3^{-}$ cells. Briefly, 30 ng of total
141 RNA were retrotranscribed into cDNA and amplified (Ovation® Pico WTA System V2, NUGEN,
142 Redwood City, CA, USA). The cDNA product was purified (MinElute Reaction Cleanup Kit,
143 QIAGEN) and hybridized on a GeneChip Human Gene 2.0 ST array (ThermoFisher, Santa
144 Clara, CA, USA) following manufacturer's instructions. After quality assessment, *NKG2C* gene
145 expression (also named *KLRC2*) was obtained through standard procedures
146 (RMA normalization and ComBat batch effects correction). Linear gene expression intensity
147 values for *NKG2C* were registered.

148 ## Analysis of tumor HLA-E and HLA-G expression by flow cytometry

149 Cryopreserved PBMCs from 8 CLL patients with increased absolute lymphocyte count (ALC)
150 levels (≥20x10^9 cells/L) were further characterized. Cells were thawed and labeled with DAPI
151 and with the following surface antibodies: CD45 (Alexa Fluor 700, eBioscience), CD19 (FITC,
152 BD Biosciences), CD5 (PerCP-Cy5.5, BD Biosciences), CD20 (APC, BD Biosciences), HLA-
153 E (PE, Miltenyi Biotec) and isotype control (PE, eBioscience). For the analysis of HLA-G
154 expression on tumor cells, unlabeled antibodies against HLA-G (clone G233) (Invitrogen) and
155 MYC (clone 9E10, used as negative control) were employed, followed by indirect
156 immunofluorescence staining with PE-Cy7 goat anti-mouse IgG (minimal x-reactivity)
157 (Biolegend). Data acquisition was performed in a LSR Fortessa instrument (BD Biosciences)
158 and analyzed using FlowJo. Mean fluorescence intensity (MFI) values were evaluated as
159 shown in Supplemental Methods and in Supplemental Figure 3.

160 ## Statistical analysis

161 Comparison between groups was performed with Chi-square or Fisher exact tests for discrete
162 variables, while the Mann-Whitney test was used for continuous variables. Linear regression
163 analysis and Pearson correlations were used to assess the relationship between quantitative
164 variables. Time to first treatment (TFT) was defined as the time from diagnosis to the beginning
165 of treatment and was analyzed using Kaplan-Meier plots and the log-rank test. Statistical
166 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 (0.85x10^9/L vs. 0.57x10^9/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

193 (29/35 cases) did not differ from our study group (83% vs 85%, P=0.772). Our patients showed
194 a significant lower median percentage of NKG2C^{+} NK cells (4.6% vs. 12.2%, P=0.012). A
195 similar trend was observed when only HCMV^{+} individuals were considered (7.3% vs. 12.7%,
196 P=0.168) (Figure 1). A significantly positive correlation between *NKG2C* gene expression and
197 the percentage of NK cells that were positive for NKG2C was disclosed (r_P=0.68, P<0.001)
198 (Supplemental Figure 7A), suggesting that the increase in NKG2C^{+} NK cells is also
199 accompanied by augmented *NKG2C* gene expression levels.

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

202 *NKG2C* zygosity was assessed in 135 patients to ascertain if the frequency of germinal
203 genomic deletions could explain the diminished NKG2C expression observed in our cohort.
204 *NKG2C*$^{+/+}$, *NKG2C*$^{+/del}$ and *NKG2C*$^{del/del}$ individuals represented 56%, 37% and 7% of the
205 studied cohort respectively; these frequencies were comparable to those detected in the
206 general population[23,29]. In line with previous reports, among 54 patients with available data on
207 NKG2C surface receptor expression, *NKG2C*$^{+/+}$ cases (N=26) showed significantly increased
208 proportion of NKG2C^{+} NK cells compared to hemizygous individuals (N=22; median: 8.4% vs
209 3.6%, P=0.014). As expected, NKG2C was undetectable in the six patients with homozygous
210 deletions. Remarkably, cases with very low (<2%) or undetectable NKG2C expression were
211 also found among *NKG2C*$^{+/-}$ (10/22, 45%) and *NKG2C*$^{+/+}$ (3/26, 12%) individuals (Figure 2),
212 ruling out a relation between the decreased expression with *NKG2C* deletion in these cases.
213 Similar differences in *NKG2C* gene expression intensity according to *NKG2C*$^{+/+}$, *NKG2C*$^{+/del}$
214 and *NKG2C*$^{del/del}$ genotypes were also observed (Supplemental Figure 7B).

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

217 Next, to evaluate if the expression of some of these NKR could be affected by the magnitude
218 of clonal B cell expansion, CLL patients were categorized in two groups according to their

219 lymphocytosis. CLL patients with an ALC ≥20x10^9 cells/L exhibited a significant lower median
220 percentage of NK cells expressing NKG2C compared to CLL patients with an ALC <20x10^9
221 cells/L (1.8 vs. 8.1% respectively, P=0.029) (Figure 3), besides no differences in HCMV
222 serology or *NKG2C* zygosity were observed (Supplemental Table 2). CLL patients with an ALC
223 ≥20x10^9 cells/L showed a significant lower median percentage of NKG2C$^+$ NK cells compared
224 to the independent control cohort, although differences did not reach statistical significance
225 when considering only HCMV$^+$ individuals (6/7 patients with an ALC ≥20x10^9 cells/L with
226 available HCMV serostatus, 86%) (Figure 1). No significant differences were observed for the
227 expression of other NKR (Figure 3).

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

235 As expected, with a median follow-up of 62 months (0-76), none of the subjects diagnosed
236 with MBL required treatment and only two of them (8%) died. As for CLL, after a median follow-
237 up of 55 months (1-77), seven patients received therapy (19%) and six were dead (16%) at
238 last follow-up. Interestingly, those CLL patients with a percentage of NKG2C$^+$ cells below the
239 median (low NKG2C, <7%) exhibited an inferior TFT (five-year treatment-free survival: 67%
240 vs. 94%, P=0.037) (Figure 4). Similarly, CLL patients with high ALC (≥20x10^9/L) also displayed
241 shorter TFT (five-year treatment-free survival: 61% vs. 89%, P=0.039). With the exception of
242 CD38, none of the other known prognostic factors in CLL mentioned before showed significant
243 differences concerning TFT (Figure 4, Supplemental Table 4).

244 **Tumor B cells show high HLA-E and variable HLA-G expression**

245 CD94/NKG2C recognizes HLA-E bound to nonamers from the leader sequence of other class
246 I molecules. Among them, the leader peptide from HLA-G, reported to be aberrantly expressed
247 on tumor cells from some CLL patients[30], confers the highest affinity to the interaction. On that
248 basis, the expression of HLA-E and HLA-G on clonal B cells was analyzed in a subset of our
249 patients (Supplemental Table 5). HLA-E expression was detected in all cases, whereas the
250 expression of HLA-G differed among patients (median percentage of positive cells: 12.4%,
251 range: 0.5-56.4). Of note, one patient showed a bimodal distribution of HLA-G expression
252 being detected in 56.4% of the clonal B cell population and displayed a particularly aggressive
253 clinical course (Supplemental Figure 3). As expected, the percentage of clonal B cells
254 expressing HLA-G positively correlated with HLA-G ΔMFI values (r_P=0.87, P=0.005) and
255 relative HLA-G MFI values (r_P=0.71; P=0.050). No significant correlations were observed
256 between relative HLA-E and HLA-G expression on tumor cells and the percentages of NKG2C$^+$
257 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)[4-8]. 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 $NKG2C^{+/\mathrm{del}}$ and $NKG2C^{del/\mathrm{del}}$ genotypes assessed in our MBL/CLL cohort were comparable to those previously defined in two Spanish cohorts of

healthy donors[23,29]. Moreover, as in health conditions, a significantly higher median percentage of CD94/NKG2C+ 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+ 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+ NK cell subsets associated with HCMV infection are lower in elderly HCMV+ donors (>70 years) compared to younger ones (18-35 years)[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+ NK cells.

In line with the aforementioned, we found that CLL patients with high ALC levels displayed a significant lower percentage of CD94/NKG2C+ 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^{9} cells/L, leading to defective NK cytotoxicity[31]. In our study, reduced proportions of NKG2C+ 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[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

312 HLA-G molecules. Prior investigations reported that binding of HLA-E to the HLA-G signal
313 peptide-derived nonamer confers to the HLA-E complex a high affinity for the CD94/NKG2C
314 receptor triggering NK cell activation[38,39] and promoting NKG2C receptor internalization[40]. All
315 these findings potentially suggest that a persistent exposure to tumor cells bearing HLA-E,
316 particularly upon HLA-G co-expression such as that confirmed in a subset of CLL patients with
317 high ALC, could lead to chronic CD94/NKG2C$^+$ NK cell activation and exhaustion, underlying
318 their reduced numbers in circulation. It is also worth mentioning that a decreased gene
319 expression of NK cell activating receptors was reported in CLL but not in small lymphocytic
320 lymphoma, a CLL variant with identical immunophenotypic features but without peripheral
321 blood lymphocytosis[6], concluding that NK cell receptor and function may be markedly
322 influenced by exposure to peripheral blood tumor burden. Additionally, we observed that low
323 NKG2C expression in NK cells from CLL patients was significantly associated with shorter TFT
324 at a cutoff level of 7%. Since the number of CLL patients in our study is limited, the selected
325 cutoff value should be corroborated in a lager validation cohort. Regardless of which is the
326 optimal cutoff, NKG2C internalization could potentially be a consequence of the increased
327 exposure to tumor cells in patients with high lymphocytosis and, therefore, the poorer outcome
328 could be the result of their increased ALC levels.

329 To the best of our knowledge, CD94/NKG2C expression on NK cells from CLL has been
330 previously addressed in two studies which, in accordance to our findings, described an
331 expansion of CD94/NKG2C$^+$ NK cells driven by HCMV[8,27]. Notably, low median percentages
332 of NK cells (CD56$^+$CD3$^-$) expressing NKG2C in 24 CLL patients were also reported by
333 Petersen et al (3.1% [range: 0.2-43.2] in HCMV$^+$ vs. 2.1% [range: 0.5-2.6] in HCMV$^-$)[27]. Hofland
334 et al. assessed 41 CLL patients with higher lymphocytosis than our cohort (median ALC:
335 93x10^9 cells/L; range: 13-359) and found similar low levels of NKG2C in different NK
336 subpopulations according to CD56 and CD16 expression[8]. In contrast to our study, the HCMV$^+$
337 control group studied by Hofland et al. exhibited a reduced CD94/NKG2C$^+$ NK cell population
338 compared to HCMV$^+$ CLL patients, although differences were not significant. Intrinsic

339 characteristics of the different control groups of the two studies, together with the different
340 methodologies employed, may account for these differences.

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

354 **Conflicts of interest**

355 No potential conflicts of interest were disclosed.

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496 **TABLES**

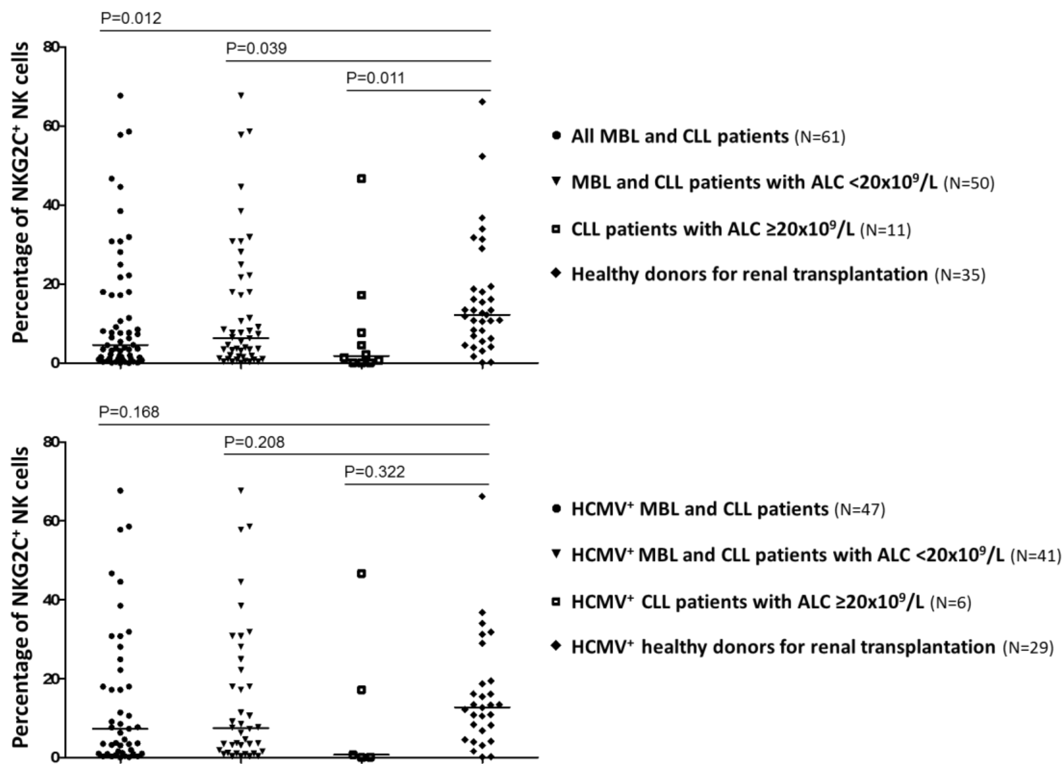
497 **Table 1.** Age, gender, *NKG2C* genotype, lymphocyte counts and NKG2C expression on NK and T cells comparing HCMV positive and negative
498 MBL and CLL patients.

	All MBL and CLL patients N=61*	**HCMV$^-$ MBL and CLL** N=8	**HCMV$^+$ MBL and CLL** N=47	**P-value** (HCMV$^-$ vs HCMV$^+$)
Age	73 (43-89)	68 (43-81)	75 (50-89)	0.056
Male	35 (57.4%)	3 (37.5%)	28 (59.6%)	0.276
***NKG2C* zygosity**				0.482
+/+	27/55 (49.1%)	2/7 (25.0%)	22/42 (52.4%)	
+/del	22/55 (40.0%)	4/7 (50.0%)	17/42 (40.5%)	
del/del	6/55 (10.9%)	1/7 (12.5%)	3/42 (7.1%)	
Absolute lymphocyte count (x10^9/L)	9.9 (1.8-84.6)	8.4 (2.0-62.0)	9.5 (1.8-66.5)	0.738
Absolute clonal B cells (x10^9/L)	6.4 (0.5-76.1)	5.4 (0.7-57.0)	6.0 (0.5-63.1)	0.780
Percentage of NK cells (CD3$^-$ CD56$^+$)	8.0 (0.9-28.0)	8.5 (2.8-15.0)	8.0 (1.0-28.0)	0.811
Absolute NK cell count (x10^9/L)	0.6 (0.1-9.3)	0.5 (0.2-9.3)	0.6 (0.1-3.3)	0.504
Percentage of NKG2C$^+$ NK cells	4.6 (0-67.7)	2.9 (0-8.1)	7.3 (0-67.7)	0.176
Percentage of T cells (CD3$^+$ CD56$^-$)	29.8 (3.3-62.3)	37.9 (17.2-57.0)	30.7 (3.3-62.3)	0.346
Absolute T cell count (x10^9/L)	2.4 (0.9-29.1)	2.4 (0.9-29.1)	2.4 (0.9-17.3)	0.962
Percentage of NKG2C$^+$ T cells	1.8 (0.3-17.4)	2.5 (1.2-11.7)	1.9 (0.3-17.4)	0.219

*Serology for HCMV was not available in 6 patients.
Values are given as median (range) or number (%).

499 **FIGURES**

500



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502

503 **Figure 1.** Distribution of NK cells ($CD56^{+}CD3^{-}$) expressing NKG2C in the whole MBL and

504 CLL cohort, in MBL and CLL patients considering ALC (high ALC: ≥20x10^{9} cells/L) and

505 in the non-CLL cohort previously assessed by our group. Results from all the included

506 patients and healthy controls (A) or restricted to $HCMV^{+}$ individuals (B) are shown.

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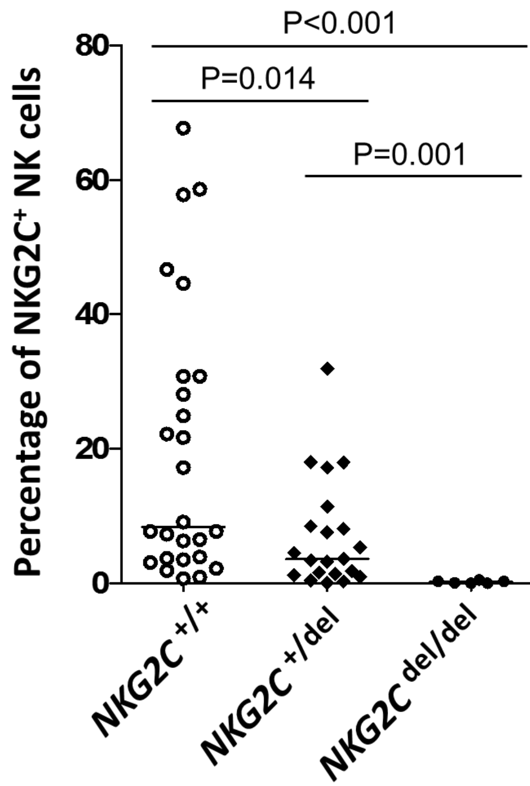

509

510 **Figure 2.** Distribution of NK cells (CD56$^+$CD3$^-$) expressing NKG2C in CLL and MBL

511 patients showing *NKG2C*$^{+/+}$, *NKG2C*$^{+/\mathrm{del}}$ and *NKG2C*$^{del/\mathrm{del}}$ genotype.

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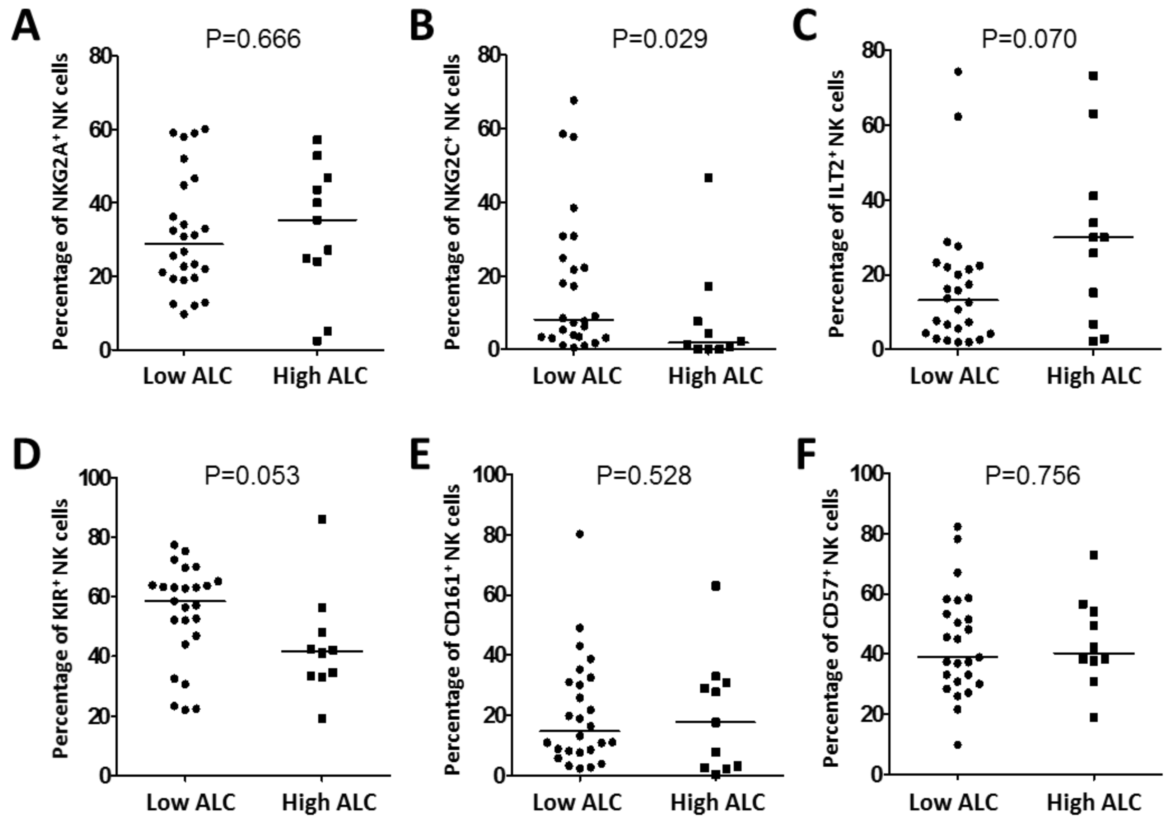

514

515 **Figure 3.** Comparison of the percentage of NK cells ($CD56^+CD3^-$) expressing NKG2A
516 (A), NKG2C (B), ILT2 (C), KIR (D), CD161 (E) and CD57 (F) in CLL patients with lower
517 absolute lymphocyte count (ALC) levels (Low ALC; $<20x10^9$ cells/L) compared to those
518 with increased ALC levels (High ALC; $\geq 20x10^9$ cells/L).

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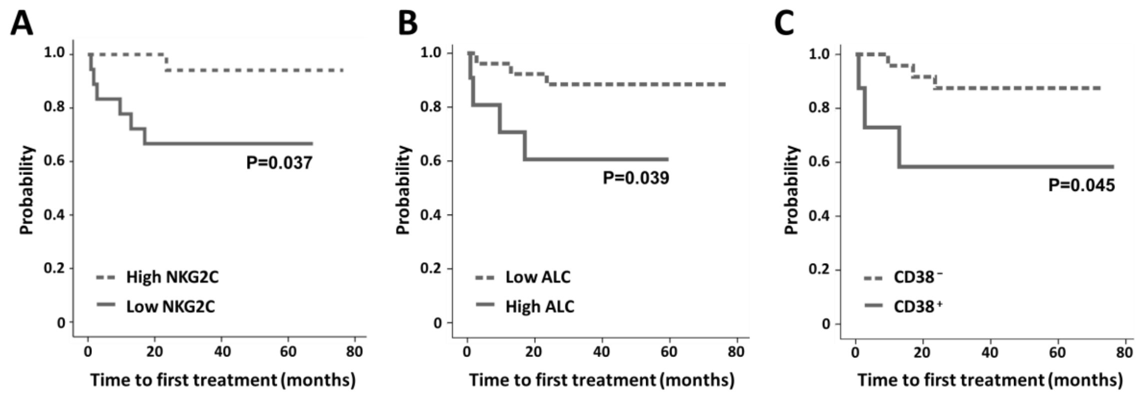

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522 **Figure 4.** Kaplan-Meier plots for TFT in CLL patients with low (<7%) or high (≥7%)
523 percentage of NKG2C⁺ NK cells (A), high (≥20) or low (<20x10^9 cells/L) ALC values (B),
524 and expression of CD38 in clonal B cells according to the previously established cut-off
525 of 30% (C).

526