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Specific Associations Between Clinical Signs, Immune Cells, Disease Genetic Background and Burden in a Group of Patients with B-Cell Chronic Lymphocytic Leukemia

Asocieri specifice între simptomatologia clinică, celulele non-maligne, defectele genetice și încărcătura tumorală la pacienți cu leucemie limfocitară cronică B

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Abstract

Traffic of tumor- and normal cells through the peripheral blood (PB) of patients with B-cell chronic lymphocytic leukemia (B-CLL) to the lymph nodes (LN) or spleen/ liver sites is governed by specific changes in surface and intracellular molecule expression. The study aims to investigate the potential association between different lymphocyte subsets, chemokine receptors or genetic alterations and specific clinical signs in a group of B-CLL patients. Forty-three patients were included in the study. The expression of CCR7, CXCR5, CXCR3, CCR4, CD3, CD4, CD8, CD27, CD28, CD45RA, CD25, CD127, CD38 was tested by multiparameter flow cytometry. Genetic alterations were determined by MLPA. We found increased frequency of CD38+ B-CLL cells directly correlated with the presence of LN>5cm. CXCR5 and CCR7 are homogenously expressed by monoclonal B-CLL cells. CCR4+ B-CLL cell frequency is found to be lower in the PB of patients presenting particular LN involvement. Heterogeneous and complex genetic alterations were found and only the presence of trisomy 12 associated with less frequent axillary LN involvement. We also report a significant increase in the frequency of total T cells and T cell subsets (effector- and central memory CD4+ T cells, regulatory T cells, follicular T helper cells, distinct functional CD8+ T cells) with the occurrence of specific clinical manifestations. Chemokine receptor expression on circulating CD4+ T cell subsets was augmented in connection to some specific LN locations. Consequently, clinical manifestations in B-CLL are linked to both, factors intrinsic to the monoclonal B cells, and external influences coming from the microenvironment.

Keywords: B-CLL, genetic lesions, microenvironment.

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Rezumat

Traficul limfocitelor maligne și normale prin sângele periferic (PB) către ganglioni, splina și ficat este guvernat de modificări specifice ale nivelurilor de expresie de suprafață/ intracelulară a unor molecule. Studiul își propune să investigheze asocierea dintre diferite subseturi limfocitare, receptori pentru chemokine sau aberații genetice și anumite simptome clinice la pacienți cu LLC-B. Patruzeci și trei de pacienți au fost incluși în studiu. Prin citometrie în flux multiparametrică a fost testată expresia pentru CCR7, CXCR5, CXCR3, CCR4, CD3, CD4, CD8, CD27, CD28, CD45RA, CD25, CD127, CD38. Defectele genetice au fost determinate prin MLPA. Am observat o frecvență crescută a celulelor CD38+ clonale corelată cu infiltrarea ganglionilor >5cm. CXCR5 și CCR7 sunt omogen exprimați de celulele de LLC-B. Frecvența celulelor B CCR4+ este redusă la pacienții cu un anumit tip de infiltrare ganglionară. Am detectat aberații genetice heterogene și complexe și trisomia 12 este mai frecventă la pacienții ce nu prezintă ganglioni axilari. De asemenea, frecvența limfocitelor T totale și a unor subseturi (memorie efector și centrală, T reglatorii, T helper foliculare, diferite subseturi funcționale CD8+) este crescută doar concomitent cu prezența anumitor manifestări clinice. Expresia receptorilor pentru chemokine la nivelul limfocitelor T CD4+ este crescută în conexiune cu anumite localizări ganglionare. În concluzie, manifestările clinice la pacienții cu LLC-B sunt în strânsă legătură atât cu factori intrinseci limfocitelor B clonale, cât și de influențe externe, furnizate de semnale provenite din micromediu.

Cuvinte cheie: LLC-B, leziuni genetice, micromediu

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Introduction

Chronic lymphocytic leukemia (B-CLL) is a complex immune disease, characterized by high circulating numbers of monoclonal B cells. The expansion of these B-cells is associated with severe defects of T-cell responses and homeostasis. Accordingly, we and others have documented proofs of a systemic immune dysregulation in CLL patients, including increased circulating T cell numbers with disease stage, imbalanced Th1/Th2 profiles, increased circulating numbers of regulatory phenotypes, and reduced B-cell capacity for antigen presentation, among others, which may lead to immunodeficiency or autoimmune manifestations related to the disease [1, 2]. Additionally, hepato- and splenomegaly and the occurrence of enlarged lymph nodes (LN) with various localizations are considered the most frequent clinical manifestations in B-CLL patients [3]. Trafficking of B-CLL cells between different compartments, through peripheral blood (PB), is governed by changes in surface and intracellular molecules and by micro-environmental signals

and interactions [4-6]. Such interactions contribute to the regulation of proliferation/ apoptosis events within the malignant clone and provide conditions for the acquisition of genomic alterations [7, 8].

The study of phenotypic changes and specific microenvironmental interactions in B-CLL, currently partially understood, may provide relevant information for the behavior of malignant B-cells and may contribute to the design of novel therapeutic strategies, as current therapies do not effectively reach the residual disease pool in secondary lymphoid organs and bone marrow [6].

Our study aims to investigate whether there is a preferential migration of distinct lymphocyte populations from B-CLL patients towards spleen, liver, or different LN sites. Also the study intended to find specific linkage between the tissue involvement and factors intrinsic to the disease: genetic aberrations, chemokine receptor expression by the tumor clone, and circulating T cell subsets considered representative components of the tumor microenvironment in B-CLL.

Material and methods

Patients and samples

The group of subjects included in the present study consisted of 43 patients (27 males and 16 females) hospitalized and diagnosed with B-CLL in the Hematology Clinic, St Spiridon Hospital/ Regional Institute of Oncology, Iasi, Romania. Six to nine mL of PB were harvested on EDTA after informed consent was obtained from each individual, in accordance to the Local Ethical Committee. Clinical data were recorded: the presence of organomegaly (spleen and liver) and LN involvement (all LN chains larger than 2 cm were taken into account, and cervical, supraclavicular, axillary and inguinal localization).

Flow cytometry studies

Circulating B-CLL cells were identified and quantified by multicolour flow cytometry in fresh PB samples using the standard stain-lyse and wash procedure. The monoclonal antibody combination used included: CD19, CD20, CD22, CD5, CD23, CD10, CD38, kappa, lambda, IgM, IgD, IgG. A total number of 1×10^5 events per tube was acquired and analysed. For the analysis of T cell subsets and chemokine expression on B-CLL and T-cells, peripheral blood mononuclear cells (PBMCs) were separated by density

gradient centrifugation with Histopaque (Sigma) and stored at -150°C in freezing medium (RPMI/ FCS/ DMSO) until use. The cells were then thawed, washed twice in PBS and stained with anti-CCR7, CXCR5, CXCR3, CCR4, CD3, CD4, CD8, CD27, CD28, CD45RA, CD25, CD127 (BD Biosciences - San Jose, CA). Data acquisition (2×10^6 events per tube) was carried out using a BD FACS Aria III cytometer and data analysis performed using the Infinicyt software (Cytognos, Salamanca, Spain).

Cell subsets identification

Monoclonal B cells were identified by their particular phenotype: CD19+ CD20+low CD5+ CD23+ and light chain restriction (either kappa or lambda). CD4+ CD8- T lymphocytes were grouped according to their phenotypic differentiation (*Table I*) in naive (N), central memory (CM), effector memory (EM), follicular (TFH), and regulatory (Tregs) CD4+ T cells. Further Th1 and Th2 associated profiles were identified according to their expression of CXCR3 or CCR4, respectively. Four subsets were identified within CD4- CD8+ T cell population, according to CD27 and CD28 expression (*Table I*): Tc1 (containing the naive and memory cytotoxic pool), and three effector types of cytotoxic T cells: Tc2, Tc3, Tc4.

Table I. Phenotype of circulating T cell subsets in B-CLL patients

		CCR7	CD45RA	CD27	CD28	CXCR5	CD25	CD127
CD4 T cell subsets	naive	+	+	na				
	CM	+	-	na				
	EM	-	-	na				
	TFH	+	-	na		+	na	
	T reg	na		+/-	+/-	na	+++	-/+
CD8 T cell subsets	TC1	na		+	+	na		
	TC2	na		-	+	na		
	TC3	na		+	-	na		
	TC4	na		-	-	na		

na-not assessed; CM-central memory; EM-effector memory; TFH-follicular helper T cells, TC-cytotoxic T cell, T reg – regulatory T cells

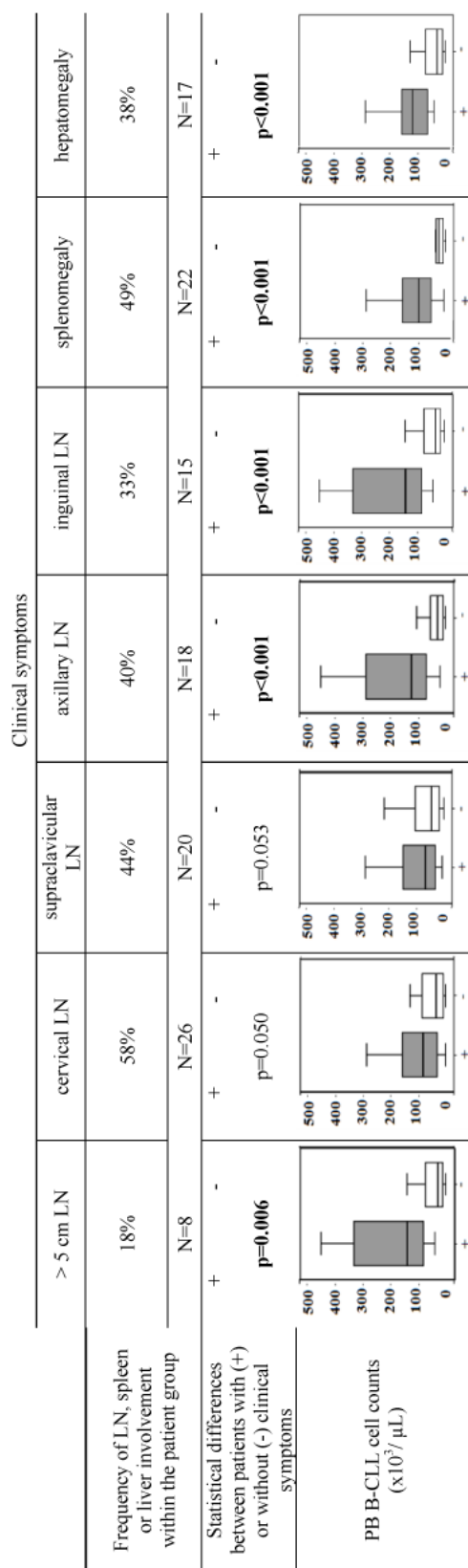


Figure 1. B-CLL absolute counts - association with lymph node and organomegaly involvement

Counts (cells x 10³/μL) of circulating B-CLL cells in the PB of patients with or without clinical manifestations. Boxes extend from the 25th to the 75th percentiles; the line in the middle and the vertical lines represent median value and both the 10th and 90th, respectively. N-number of patients; PB-peripheral blood; LN-lymph node

Molecular biology analysis

For the MLPA analysis (Multiplex Ligation-Dependent Probe Amplification) two commercial kits were used (SALSA MLPA P037 and P038 CLL, MRC Holland). DNA was extracted from PBMCs (5x10⁶ cells) using a Promega kit-Wizard Genomic DNA Purification kit, then diluted to a final concentration of 40 ng/μL. Five μL of DNA were used for each reaction following the standard protocol indicated by MRC Holland and previously described in detail [9]. In each of the runs at least one normal (non B-CLL) DNA specimen was tested. The hybridization, ligation and PCR amplification were performed on a SensoQuest Lab cyler (Biomedical Electronics), and the capillary electrophoresis on CEQ 8000 (Beckman Coulter) with GenomeLab DNA size standard 600 (Beckman Coulter). Data interpretation was performed using Coffalyser. Net MLPA data analysis software, 2013 version.

Statistical Methods

Mean values, and standard deviation, median and range values were calculated for all continuous variables, and relative frequencies were determined for categorical variables using the SPSS statistical software (SPSS 21 Inc., Chicago, IL). Statistical significance of the differences observed between patients was tested by the Mann-Whitney *U* and the chi-square tests for non-paired continuous variables and for categorical variables, respectively.

Results

Association of B-CLL cell accumulation with LN involvement and organomegaly

The assessment of LN, spleen, and liver enlargement within the B-CLL patient group investigated revealed a clinically heterogeneous image (Figure 1). The presence of enlarged spleen, liver, and LN (for axillary, inguinal, and larger than 5 cm LN, but not for cervical or supraclavicular locations), was found to be associated with significantly higher numbers of circulating B-CLL cells (Table 1). Thus, in contrast to patients with no such symptoms, the following

absolute counts of circulating B-CLL (cells/ μL) were found significantly increased in patients having splenomegaly or axillary, inguinal, cervical, supraclavicular, and larger than 5 cm adenopathies (Table I).

Chemokine receptors expression on malignant cells in B-CLL patients grouped based on LN involvement and organomegaly

In terms of chemokine receptors, malignant clones from B-CLL patients investigated were characterized by a heterogeneous expression pattern for CXCR3 (1-100% positivity) and CCR4 (13-87% positivity) (Figure 2), while CXCR5 and CCR7 were homogeneously present within the entire B-CLL group. When the proportion of positivity for each receptor was comparatively assessed based on the presence of LN, spleen, and liver involvement, CCR4 was expressed on a significantly lower proportion of B-CLL cells in patients with supraclavicular ($48\% \pm 19\%$ vs $62\% \pm 14\%$, $p=0.01$) and inguinal ($46\% \pm 16\%$ vs $60\% \pm 16\%$, $p=0.01$) LN involvement, regardless the LN size. No significant difference was found in terms of CXCR3 expression.

Expression of CD38 on malignant B cells was found to be significantly increased in pa-

tients having LN larger than 5 cm ($39\% \pm 27\%$ vs $12\% \pm 23\%$, $p=0.03$), with no significant differences linked to the LN localizations. None of the above phenotypic characteristics of B-CLL cells were significantly associated with organomegaly.

Genotypic alterations of B-CLL cells in patients grouped based on LN involvement and organomegaly

When genotypic alterations within PBMCs of B-CLL patients were assessed by MLPA, copy number alterations were found in 28 of 43 B-CLL cases (65%), including 17 cases with simple (40%) and 11 cases with complex (25%) abnormalities (Table II). The most frequent alterations were represented by deletion of 13q14 – del(13q) (17 cases-40%), followed by del(11q) (7 cases-16%) and trisomy12 (+12) (6 cases-14%). The presence of the genetic lesions was not significantly associated with either adenopathies or organomegaly in our group, except the lower frequency of axillary LN involvement in patients with +12 ($p=0.03$) (Figure 3). No association was found between the chemokine receptors (or CD38) expression on B-CLL cells in patients with del(11q), +12, or del(17p) (Figure 4).

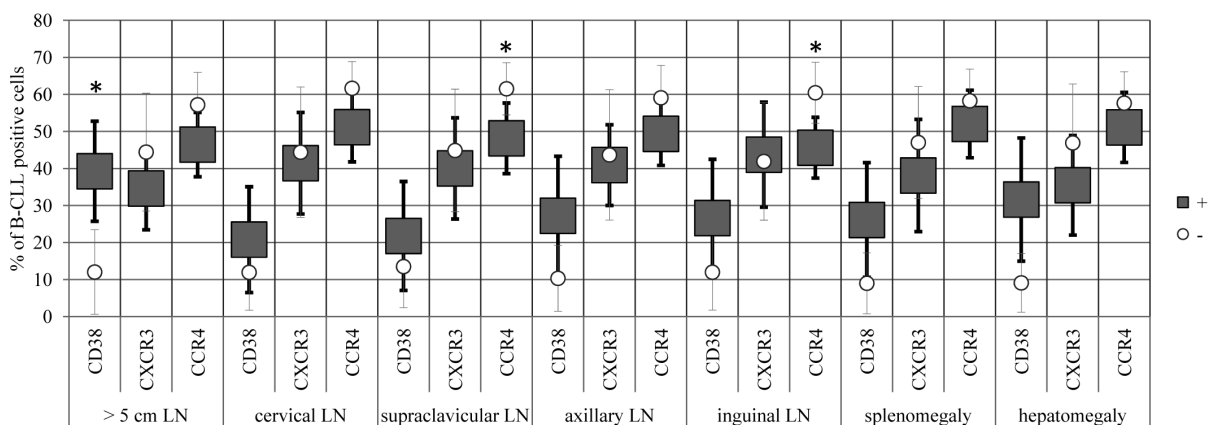


Figure 2. Chemokine receptor (CXCR3 and CCR4) and CD38 expression by monoclonal B cells in B-CLL patients with different clinical manifestations

Frequency of CXCR3+, CCR4+ and CD38+ B-CLL cells in the PB of patients with or without LN/organomegaly involvement. Mean values \pm standard deviation calculated for proportion of positivity in patients with (dark squares) or without (blank circles) clinical signs. * $p < 0.05$ for the comparison between presence or absence of symptoms. PB-peripheral blood; LN-lymph node

Association of PB T cells and adenopathies/organomegaly in B-CLL patients

The presence of adenopathies was found to be linked to higher numbers of circulating total T cells (cells/ μ L) in B-CLL patients having enlarged cervical ($p=0.004$), axillary ($p<0.001$), inguinal ($p=0.003$), and >5 cm LN ($p=0.04$) (Figure 5). Total T cell counts were also increased

($p=0.05$) in patients presenting enlarged supraclavicular LN (Supplementary data available in the online issue). Some LN locations were associated with significantly higher numbers of total CD4+ T-cells (cells/ μ L), including: cervical ($p=0.004$), axillary ($p=0.004$) and inguinal ($p=0.02$) sites. Patients with adenopathies also presented higher numbers of total PB CD8+ T

Table II. Genetic alterations identified by MLPA analysis and their frequency in B-CLL patients

		N	%
Simple copy number alterations (N=17)	del(13q)	8	19
	+12	5	12
	del(11q)	3	7
	del(17 p)	1	2
Complex alterations (N=11)	del(13q) and del(11q)	2	5
	del(13q) and del(17p)	1	2
	+8q and mutated NOTCH1	1	2
	+2p	1	2
	+12	1	2
	mutated NOTCH1	2	5
	mutated SF3B1	1	2
	del(11q) and +8q	1	2
Varia	del(13q) and +8q and del(17p) and mutated SF3B1	1	2
	del(13q) and RB1 loss	8	19
	biallelic loss of 13q	1	2

del-deletion; tris-trisomy; dupl-duplication; N-number of patients

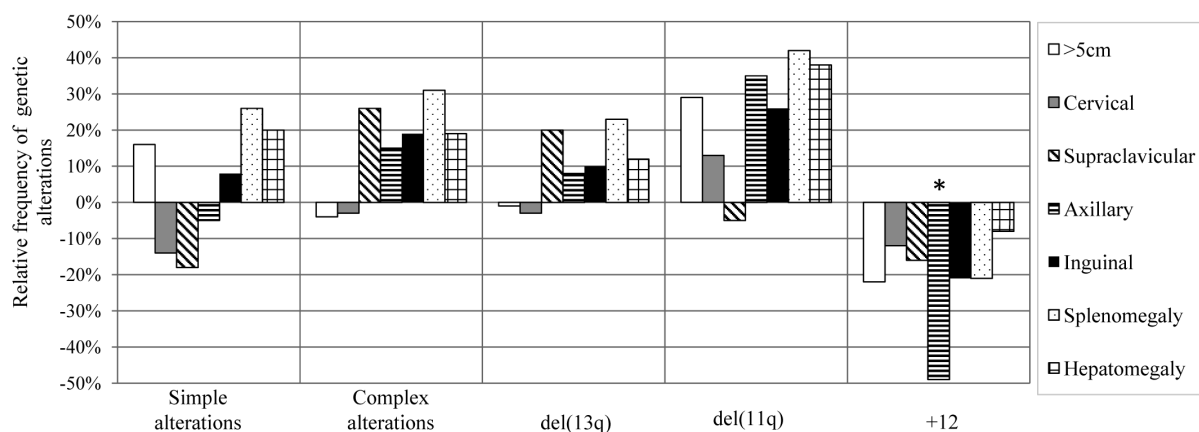


Figure 3. Relative frequency of genetic alterations in relation to the occurrence of clinical manifestations.

The relative frequency was calculated by subtracting the percentages of patients presenting the genetic defect and clinical symptoms from those with the genetic defect and without clinical symptoms * $p<0.05$ for the comparison between presence or absence of symptoms.

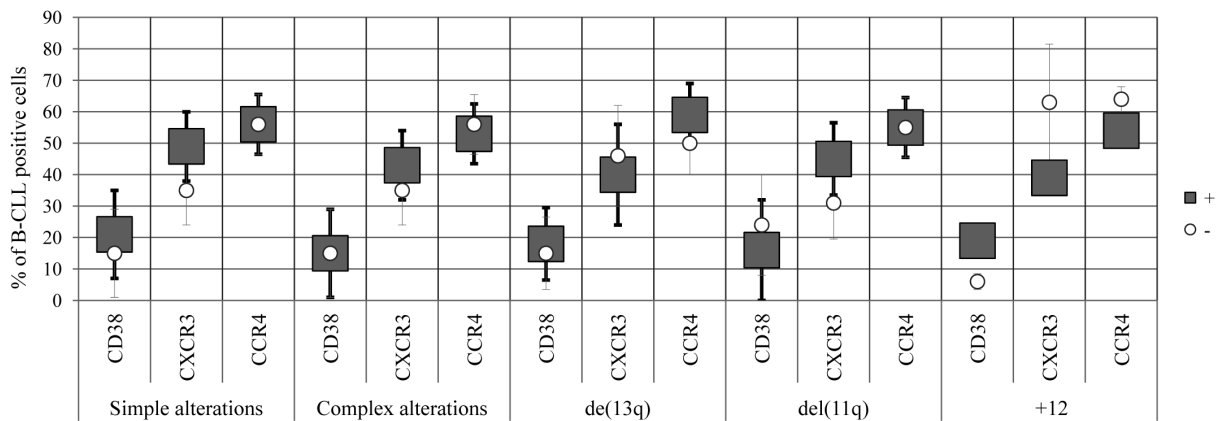


Figure 4. Frequency of CXCR3+, CCR4+ and CD38+ B-CLL cells in the PB of patients with or without cytogenetic alterations.

Mean values±standard deviation calculated for proportion of chemokine receptor and CD38 positivity in patients with (dark squares) or without (blank circles) genetic defect. PB-peripheral blood.

cells (cells/ μ L), including those with: cervical ($p=0.01$), supraclavicular ($p=0.02$), axillary ($p=0.001$), and inguinal ($p=0.001$) involvement.

When the CD4+ T-cells were classified according to the main maturation subsets, numbers of circulating EM cells (cells/ μ L) were found to be significantly higher in patients with cervical ($p=0.002$), supraclavicular ($p=0.043$), axillary ($p<0.001$) and inguinal involvement ($p=0.003$). Significantly higher numbers (cells/ μ L) of CM T-cells were found in patients with enlarged inguinal ($p=0.04$) and axillary ($p=0.05$) LN. Higher regulatory CD4+ T cell counts associated with cervical ($p=0.004$) and axillary LN enlargement ($p=0.017$), meanwhile TFH were only significantly increased in those patients with cervical LN ($p=0.02$).

When the CD8+ T-cells were classified according to the main functional subsets, significantly higher counts of Tc1 and Tc3 cells (cells/ μ L) were found in patients with enlarged cervical (Tc1: $p=0.002$; Tc3: $p=0.006$), supraclavicular (Tc1: $p=0.024$; Tc3: $p=0.022$), axillary (Tc1: $p<0.001$; Tc3: $p=0.004$), inguinal (Tc1: $p<0.001$; Tc3: $p<0.001$), and > 5cm LN (Tc1: $p=0.007$; Tc3: $p=0.02$). Also, the effector subset appears

to be terminally differentiated (CD8+ CD57+ T cells) and seen mostly in patients presenting supraclavicular, inguinal and axillary enlarged lymph nodes (data not shown).

No statistically significant differences were observed between total circulating T-cells, total or subsets CD4+ T cells in B-CLL patients and organomegaly. In contrast, two T CD8+ subsets (Tc1 and Tc3) were increased in absolute counts (cells/ μ L) in patients with splenomegaly (Tc1: $p=0.01$; Tc3: $p=0.02$) and hepatomegaly (Tc1: $p=0.008$; Tc3: $p=0.02$).

Chemokine receptors on T cells in B-CLL patients

The association between CXCR3 and CCR4 expression in the CD4+ T cell subsets and the presence of LN was investigated. A significantly higher proportion of CXCR3+ cells was detected in the EM subset in patients with cervical ($50\%\pm 21\%$ vs $33\%\pm 23\%$; $p=0.03$), axillary ($56\%\pm 20\%$ vs $35\%\pm 23\%$; $p=0.005$), and inguinal LN ($57\%\pm 20\%$ vs $34\%\pm 22\%$; $p=0.003$), but not in those with supraclavicular LN involvement. A higher CXCR3 expression on TFH in patients with inguinal ($57\%\pm 23\%$ vs $42\%\pm 22\%$;

Counts (cells/ μ L) of circulating T cell subsets defined by their maturational/functional phenotype in PB samples from LB-CLL patients with (grey boxes) or without (white boxes) LN/organomegaly involvement. Boxes extend from the 25th to the 75th percentiles; the line in the middle and the vertical lines represent median value and both the 10th and 90th, respectively. * $p < 0.05$ for the comparison between presence or absence of symptoms. PB=peripheral blood; LN=lymph node.

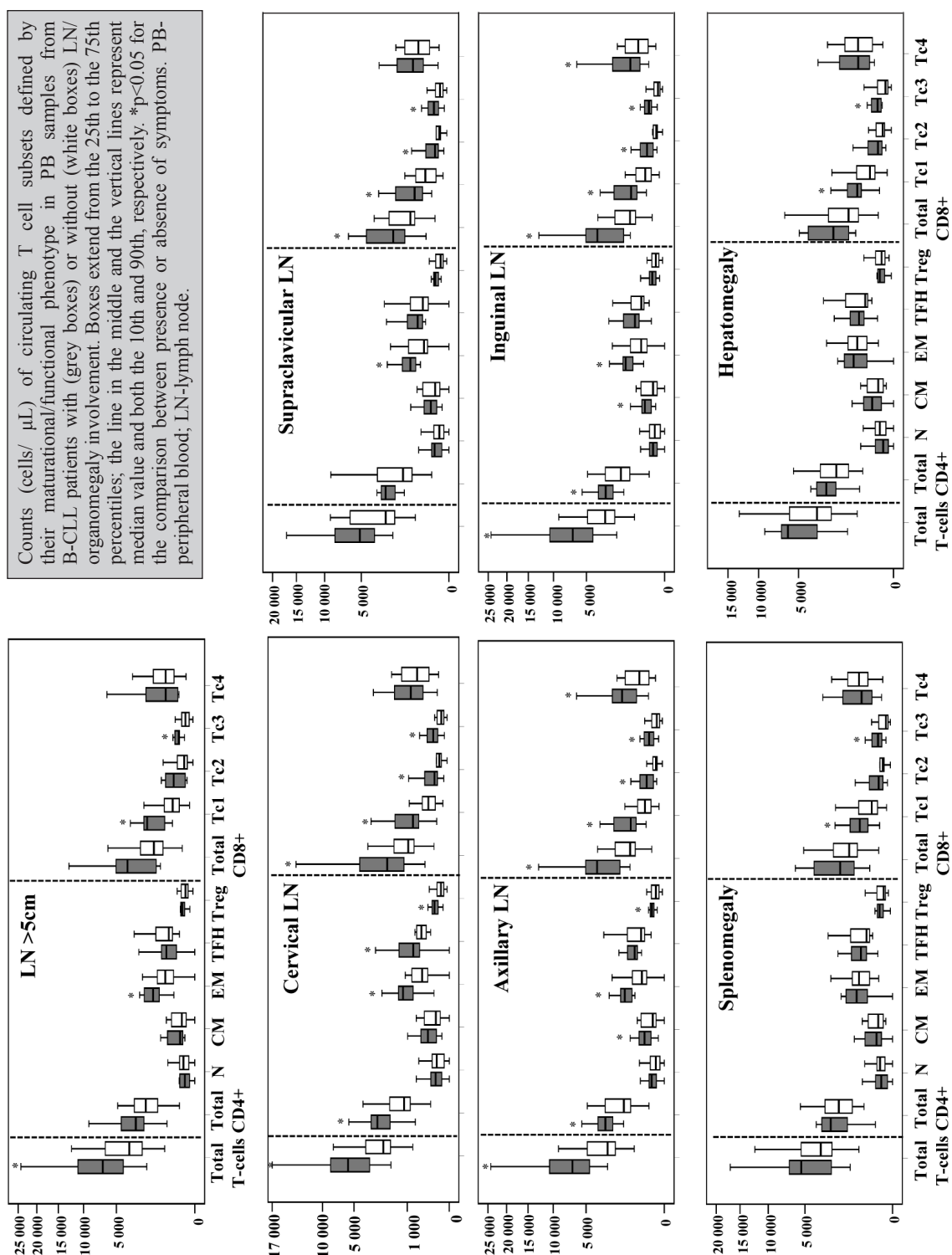


Figure 5. Specific T cells subsets in the context of enlarged LN and hepato/splenomegaly in B-CLL patients.

$p = 0.02$) and supraclavicular ($57\% \pm 17\%$ vs $41\% \pm 25\%$; $p = 0.04$) was recorded. No significantly differences in CXCR3+ expression were detected for CM (data not shown). The frequency of CD4+ T-cell subsets expressing CCR4 was not significantly associated with the presence of adenopathies (data not shown).

Discussions

We documented here connection particularities between tumour microenvironment, genetic background, and tumour burden in a group of B-CLL patients.

Data from the literature point to chemokine receptors as crucial players in the development and progression of lymphoid neoplasms [6, 10-19]. B- and T-cell circulation to LN has been proven to be strictly regulated by a combination of different chemokines (e.g. CXCR3, CXCR5, CCR7) [20, 21] and adhesion molecules such as CD38 [22] responsible not only for lymphocyte homing to, but also for the specific positioning within the follicles of LN, spleen and Peyer patches, inflammation sites, and linked with the process of extravasation through HEV-high endothelial venules [20, 21, 23, 24].

In our study the proportion of CCR4 positive B-CLL cells was found to be reduced in patients with particular clinical signs of LN/ organ involvement in comparison to those lacking such symptoms. While CCR4 has been previously reported to be over-expressed in some haematological malignancies [11-13] and solid tumors with a high metastatic potential [14-16], others, in line with our results, showed that, in B-CLL cells, CCR4 expression may be absent or significantly reduced when compared to normal B cells from elderly donors [25]. Hence, one may quickly infer that CCR4 does not qualify as a reliable diagnostic or therapeutic target for B-CLL patients. Nevertheless, such conclusion should be considered with caution, as high proportion of

CCR4 positive B-CLL cells and high intensity of their CCR4 expression have been documented to correlate with poor prognostic-associated factors (ZAP-70, CD38, Ki67) [25]. Besides, there are at least two other instances that may motivate the choice of using CCR4 blockage as a therapeutic alternative in B-CLL. One is the finding that CD40/CD40L – activated B-CLL secrete CCL17 and CCL22 which in turn stimulate the CCR4 expression on T cells that continue to provide proliferating signals for B cells [8]. The other is connected with the expression of CCR4 on regulatory T cells, therefore it may be used as a novel target for immunotherapy (depletion of Treg cells) in patients with different cancers [13].

The expression of CCR4 on T lymphocytes was also investigated in our study as a marker of Th2 helper phenotype (in contrast to CXCR3, associated with the Th1 phenotype) [26] but no significant association of CCR4 expression on any CD4+ T-cell subsets with the presence of adenopathies was established. Instead, a significantly higher proportion of CXCR3+ cells within the EM CD4+ T cell subset was found in the majority of patients with LN involvement and a CXCR3+ TFH subset significantly increased in circulation when adenopathies were present. If looking from the Th cytokine profile point of view, our results are in contradiction to data available in the literature suggesting a gradual change in composition of the T cell microenvironment from a Th1 to a Th2 phenotype with the evolution of the disease, in order to secure the selective advantage for tumor growth [27, 28]. Based on intracellular cytokines presence, a very recent study found a link between high proportions of circulating Th2 cells in B-CLL patients and the expression of the poor prognostic factor ZAP-70 [29]. If considering CXCR3 as a homing marker towards inflamed tissues, the noted expanded frequency of T cells expressing this chemokine receptor associated with LN involvement is not surprising.

The expression of CXCR3 on the malignant clone was previously reported to be of prognostic value for B-CLL patients [28]. In our study CXCR3 proportion within the B-CLL pool was found to be lower (not significantly, though) in the occurrence of LN/ organ involvement. In line with our results, only patients with early stage B-CLL showed over-expression of CXCR3 [6, 17] and low CXCR3 expression was associated with advanced stage and poorer prognosis in B-CLL patients [6, 30]. Physiologically, CXCR3 is expressed in a small subset of normal circulating B lymphocytes (such as memory B cells, normal plasma cells, but not naive B cells), while in the vast majority of LN and splenic B cells it has been reported to be absent [31, 32]. In lymphoproliferative disorders, others found CXCR3 expression on tumor cells in nearly all [31] or all [33] cases of B-CLL, but not in follicular or mantle lymphoma and high-grade lymphomas. The high variability of CXCR3 expression reported on normal/ malignant B-cells in various studies has been attributed to the instability of CXCR3 molecule during sample processing for flow cytometry detection [34] which may also explain the lack of statistical significance in our study, despite the obvious tendency. In solid tumors, a contrasting situation (over-expression associated with tumor growth and metastasis) have been documented [18, 19, 35, 36] while inhibitors of CXCR3 have been already tested efficient for their inhibitory role of metastases in colon cancer [35] and osteosarcoma [36].

Other two LN homing receptors, CCR7 and CXCR5, were found to be expressed on all B-CLL cells in our patient group, as previously described [20, 37]. Their expression apparently determines the nodal dissemination pattern and the erased LN architecture observed in patients with chronic lymphoproliferative disorders and LN involvement [37]. CCL19 and CCL21 secreted by HEV (high endothelial venules) and accessory cells are responsible for extravasation

of CCR7+ cells (B, T or dendritic cells) and for directing the B cells towards T cell areas [38]. Interestingly, we found a direct correlation between CM T cells (identified by their memory phenotype and CCR7 positivity) and certain LN stations (inguinal and axillary), but not for spleen or liver enlargement, although it is clear that CD4+ T cells normally enter these organs, using probably other entering mechanisms. CXCR5 is also expressed by follicular T helper cells which we found increased especially in advanced Rai stages, in line with other studies [39].

A very recent study showed the connection between the expression of ZAP-70 in B-CLL patients, reduction of naïve T helper subsets, and the expansion of EM T cell subsets [29]. Although we found no differences and no associations between the circulating naïve T cells and the presence or absence of any secondary lymphoid organs involvement, an association between increased numbers of effector T cells and all LN chains enlargements was revealed. Whether this means that in the patients presenting certain LN chains the effector/central memory cells are produced in these locations, or whether these cells are not sequestered in these locations, it has to be investigated. We also found increased circulating numbers of T regs in advanced B-CLL and when cervical or axillary enlarged LN are present. Further studies need to be performed in order to define the meaning of the increased numbers of circulating T regs and the association with these specific lymphatic stations. There are studies suggesting that chemokines such as CCL3 and CCL4, secreted by the very B-CLL clone, are capable to recruit T regs, in order to ensure a proximate source of survival signals [40]. The precise role played by T regs in B-CLL is not fully elucidated, but recent studies prove that CD4+ T regs have the capacity to inhibit polyclonal B cells and effector T cells, downregulating the anti-tumoral response in B-CLL [41]. CD8+ T cells are found to be increased in our patient group whenever

the clinical manifestations are present. In previous studies CD8 lymphocytosis correlates with disease progression and may also reflect a clonal expansion in B-CLL patients [42].

CD38 expression on B-CLL cells from our patient group was found to be increased regardless the LN chain involved and strongly related to LN larger than 5 cm. The CD38 expression by B-CLL cells is considered of prognostic importance, a hallmark of a recent activation process [43, 44]. In addition to its association with the B-CLL cell proliferative activity, it has been documented that leukemic cells from the bone marrow and the LN express higher amount of this molecule, suggesting that CD38 could be a molecular compass driving B-CLL cells to specialized niches [22].

Genetic lesions are common events in B-CLL, mostly in advanced stages of the disease and having unmutated IgVH. This may be explained by the polyreactivity of the BCR that determines an increased sensitivity to antigen contact, leading to an increased proliferation rate, which in turns raises the chance for the formation and accumulation of genetic aberrations [45]. The most frequent copy number variation in B-CLL, also found in our study, is the deletion of 13q14, either as single aberrancy, or in combination. Del(13q14) alone confers a good prognostic and is related to indolent disease, while in combination with other genetic lesions it is a marker of accumulated or acquired gene copy number variations lesions, for it is known that miR15/16 as part of the 13q deletion and that targets the Bcl2 expression, contributes to the pathogenesis of the disease [46]. We found no association between the presence of this DNA lesion, regardless the implication of RB1 gene (implicated in cell cycle control and chromosomal instability) [47] and none of the organ involvement studied in our group. Trisomy 12 is considered an intermediate prognostic factor in B-CLL patients. In our group, this genetic le-

sion was more prevalent in patients presenting axillary LN involvement. Also it is well known that trisomy 12 along with NOTCH1 mutations identifies a group of patients with poor prognostic [48], but due to the size of our investigated group such analysis was not possible.

Conclusions

We demonstrated here that some specific functional T cell subsets associate with specific LN stations. Understanding the complicated interactions between the malignant cells and the surrounding factors is very important in finding the solutions for re-establishing the equilibrium. Further extended studies need to be performed in order to reveal the clinical application of such observations.

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Abbreviations

B-CLL = B- chronic lymphocytic leukemia
 LN = lymph node
 CM = central memory
 EM = effector memory
 TFH = follicular T helper cells
 Tc = cytotoxic T cells
 T reg = regulatory T cells
 MLPA = Multiplex Ligation-Dependent Probe Amplification
 CD = cluster of designation
 PB = peripheral blood
 min = minimum
 max = maximum
 del(13q) = deletion of 13q14

del(11q) = deletion of 11q

+12 = trisomy12

del(17p) = deletion of 17p

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