

## Rai stage-related changes within T/NK cell populations from B-CLL patients

### Modificări ale populațiilor celulare T/NK la pacienți cu LLC-B în diferite stadii Rai

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#### Abstract

*Background/aim:* T lymphocytes are important players of the immune response. B-CLL is characterized by several immune defects. Our study aims to characterize the distinct maturational and functional T/NK cell subsets within B-cell chronic lymphocytic leukemia disease Rai stages. *Patients and methods:* Peripheral blood mononuclear cells from 43 patients enrolled in the study (16 females and 27 males, aged 68±10, 8 Rai 0, 22 Rai 1/2 and 13 Rai 3/4) were analyzed by multiparameter flow cytometry. Distinct subsets within the CD4+ (naive, central memory, effector/peripheral memory, regulatory-Tregs, follicular-TFH, CXCR3+ and/or CCR4+), CD8+ (naive+memory, effector, senescent) and NK (CD57+ and/or CD94+) were identified and compared between disease Rai stages. *Results:* Total numbers of T lymphocytes increase with disease stage. Both CD4+ and CD8+ T cells are elevated in absolute counts. The majority of CD4+ T cells are antigen-experienced, with increased Tregs, TFH and CXCR3+ (Th1-associated profile) T cell counts. The CD8+ T cells expansion is due mostly to the senescent CD57+ subset. No significant difference within NK subsets was observed among different disease stages. *Conclusions:* B-CLL behaviour seems to be associated with increased numbers of TFH and Tregs. The therapeutic modulation of T cell response in B-CLL patients may play an important role in the disease behaviour and may be a key event compensating for the immunodeficiency occurring mostly in advanced stages of the disease.

**Keywords:** T/NK cells, B-CLL, chemokine receptors, regulatory phenotype, multiparameter flow cytometry.

#### Rezumat

*Obiectiv:* Limfocitele T se constituie în factori celulari importanți ai răspunsului imun. Leucemia limfocitară cronică B se caracterizează prin defecte ale sistemului imun. Studiul nostru își propune caracterizarea diferitelor subseturi maturative și funcționale limfoide T și NK la pacienți aflați în stadii distincte

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de boală. *Material și metoda:* au fost utilizate celule mononucleate separate în gradient de densitate de la 43 de pacienți cu LLC-B (16 femei, 27 bărbați, vârsta  $68 \pm 10$  ani, 8 în Rai0, 22 în Rai1/2 și 13 în Rai3/4) în vederea analizei prin citometrie în flux multiparametrică. Au fost identificate și comparate între diferite stadii Rai, subseturile limfoide T CD4+ (naive, memorie centrală, memorie periferică/efector, reglatorii, foliculare-TFH, CXCR3 și/sau CCR4+), CD8+ (naive+memorie, efectorii, senescente) și NK (CD57 și/sau CD94+). *Rezultate:* Limfocitele T totale circulante se găsesc în numere crescute la pacienți în stadii avansate ale bolii. Majoritatea limfocitelor T CD4+ sunt celule ce au întâlnit antigenul, cu subseturi crescute de tip reglator, folicular și CXCR3+ (asociat profilului Th1). Expansiunea limfocitelor citotoxice CD8+ este corelată cu un subset diferențiat terminal, senescent, CD57+. În ceea ce privește subseturile limfocitare NK, nu s-au identificat diferențe semnificative între diferite stadii ale bolii. *Concluzii:* Comportamentul leucemiei limfocitare cronice B pare a fi asociat cu o creștere în valori absolute a limfocitelor T CD4+ reglatorii și foliculare circulante, dar și cu expansiunea limfocitelor T citotoxice senescente. Modularea răspunsului T la pacienții cu LLC-B joacă un rol important în evoluția bolii și se constituie în eveniment cheie al compensării imunodeficienței caracteristice stadiilor avansate ale bolii.

*T/NK cells, B-CLL, chemokine receptors, regulatory phenotype, multiparameter flow cytometry.*

**Cuvinte cheie:** celule T/NK, leucemie limfatică cronică cu celule B, receptori pentru chemokine, fenotip reglator, citometrie în flux multiparametrică

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## Introduction

B-cell chronic lymphocytic leukemia (B-CLL) is the most common lymphoproliferative disorder among adults. The clinical course of B-CLL patients is highly variable. Some patients may live years without progression and need for treatment. However, a significant group of B-CLL patients (85%) present a severe immunodeficiency at diagnosis, manifested mostly as hypogammaglobulinemia (1). Hypogammaglobulinemia is associated with the stage and duration of the disease, and with reduced number of normal residual B cells (2).

The extent of the immune defect in B-CLL patients may have a tremendous importance, since the major cause of morbidity and mortality in B-CLL is infection-related (due to viral, bacterial or fungal antigens) (1).

Profound quantitative and qualitative defects of T cells have been also described in B-CLL. As compared to normal, decreased percentages, but elevated absolute counts of NK and total T cells have been reported (3). Both subsets of CD4+ and CD8+ T cells are increased, while the CD4+/CD8+ ratio is reversed

in some patients, mostly in advanced Rai stages (4-7). The phenotypic feature of both CD4+ and CD8+ cells in B-CLL patients correspond to the antigen-experienced memory and effector T cell type, but not to the naive T cell type (8). In line with these findings, a predominant T cell subtype associated with a cytotoxic effector function (CD45RA+ CD57+ CD27-) was identified within the expanded CD8+ compartment. This phenotype has been previously found in cytomegalovirus latent infections, also known as a replicative, senescence-associated T cell type (9, 10). High absolute counts of circulating regulatory T cells have been also reported in B-CLL patients, when compared to normal controls (11), mainly in advanced stages of the disease (12). Although these expanded populations have been correlated with the survival, the precise role of T cells and of the reported immunosuppressed status in B-CLL patients has not been clarified yet.

In contrast with phenotype-based studies showing increased cytotoxic T cell numbers, the analysis of the T cell response by *in vitro* stimulation showed increased IL-4 production by CD4+ and CD8+ T cells. These findings suggest

a Th2 biased pattern of response, mostly in patients with progressive disease (13, 14). In agreement with these studies, it has been recently suggested that chemokine receptors expressed by memory and effector T cells are equally essential for migration towards inflammation sites, recirculation through the immune sites (15, 16), or T helper polarization (17).

Altogether, these studies may lead to the hypothesis that T cell subsets from patients with distinct B-CLL stages present different chemokine receptors expression. Therefore, gaining additional insights into T cell differentiation in B-CLL, may facilitate our prediction of patient individual ability to respond to infectious agents or to mount an effective anti-tumor immune response. Additionally, such data may become useful in monitoring the immunomodulatory effects of specific therapies in B-CLL.

## Material and methods

### *Patients*

A total number of 43 patients (16 females and 27 males, aged  $68 \pm 10$ ) diagnosed with B-CLL were included in the present study. All patients were diagnosed according to the WHO criteria and hospitalized in the Hematology Clinic of St Spiridon Hospital/ Regional Institute of Oncology, Iasi. According to Rai staging system they were classified and grouped as follows: 8 Rai 0, 22 Rai 1/2 and 13 Rai 3/4. All samples were collected after informed consent was given by each individual, according to the Local Ethical Committee.

### *Multiparameter flow cytometry immunophenotypic studies*

Peripheral blood mononuclear cells (PBMC) were separated by density gradient centrifugation, using Histopaque (Sigma) as described elsewhere (18) and stored in freezing medium at  $-150$  Celsius degrees. PBMCs were thawed, washed and then labeled using a direct 6/7-color immunofluorescence stain-and-then-lyse technique, with the following antibody

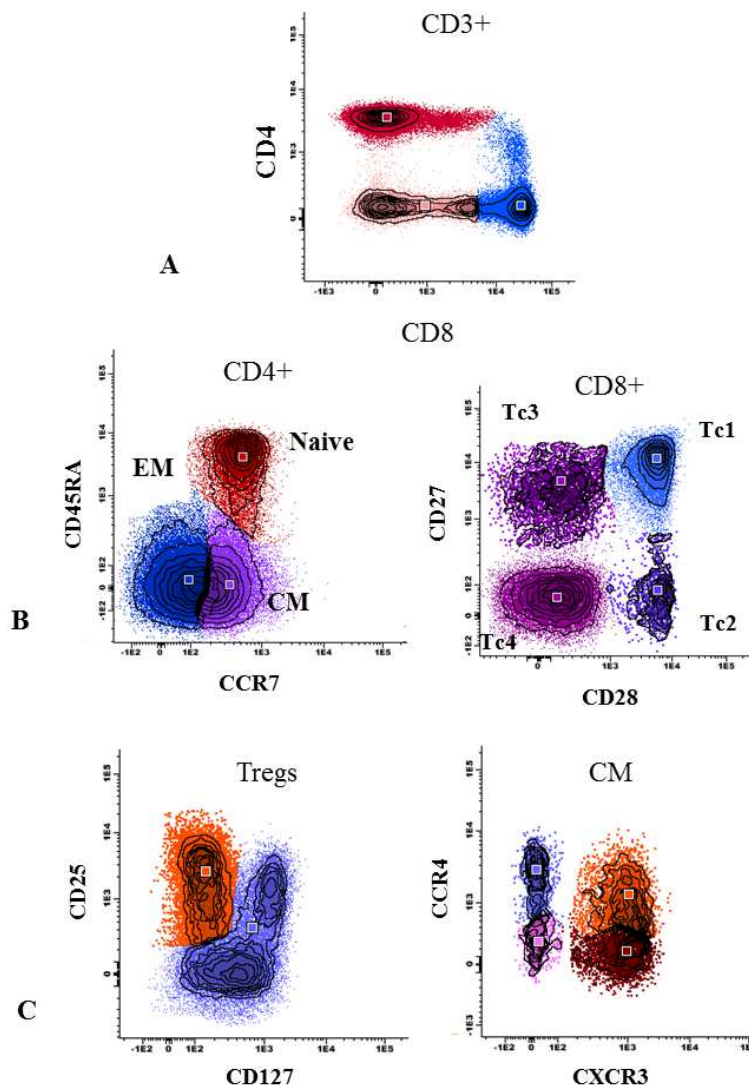
combination (Horizon Violet (HV450)/ fluorescein isothiocyanate (FITC)/ phycoerythrin (PE)/ peridinin chlorophyll protein-cyanin 5.5 (PerCP-Cy5.5)/ phycoerythrin-cyanin 7 (PE-Cy7)/ allophycocyanin (APC)/ APC-Cy7: CD127, CD28, CD27, CD25, CD3, CD8, CD4; CXCR5, CXCR3, CCR7, CD194, CD45RA, CD3, CD4; and -CD57, CD94, CD3, CD4, CD56, CD8; all antibodies were purchased from BD Biosciences (San Jose, CA). Data acquisition was performed in BD FACSCanto II and BD FACSAria III (BD Biosciences) cytometers, running on FACSDiva software (BD Biosciences). For T cell subsets a total number of  $2 \times 10^6$  events per tube were acquired. Data analysis was performed using Infinicyt software (Cytognos, Salamanca, Spain).

### *Cell subsets identification*

Monoclonal B cells were identified by their particular phenotype: CD19, CD20low, CD5+, CD23+, and light chain restriction (either kappa or lambda). CD3+ CD4+ CD8- T lymphocytes were classified according to their phenotypic differentiation (Figure 1) in naïve (CCR7+ CD45RA+ CD27+ CD28+), central memory (CM) (CCR7+ CD45RA- CD27+ CD28+), effector memory (EM) (CCR7- CD45RA- CD27+ CD28-/+), follicular (TFH) (CD4+CXCR5+) and regulatory CD4+ (CD25+high CD127-/+low) T cells. Further Th1 and Th2 profiles were identified according to their expression of CXCR3 or CCR4, respectively, as previously described (17). For CD8+ four subsets were identified according to CD27 and CD28 expression: CD27+ CD28+ (Tc1) containing the naïve and memory cytotoxic pool, and three effector types of cytotoxic T cells: CD27- CD28+ (Tc2), CD27+ CD28- (Tc3), CD27- CD28- (Tc4).

### *Statistical Methods*

The SPSS v21 statistical software package (SPSS Inc., Chicago, IL) was used for calculation of mean, standard deviation, median and range values of all continuous variables analyzed. The Mann-Whitney U test was used to estimate



**Figure 1. Identification of T cell subsets in peripheral blood of B-CLL patients.** A) Dot plot distribution of CD4+ (red), CD8+ (blue) and CD4- CD8- (pink) T cell subsets; B) Left: CCR7/ CD45RA expression distinguishes between naive, central memory (CM) and effector/ peripheral memory (EM) CD4+ T cells; right: Based on CD27/ CD28 expression four CD8+ subsets Tc1, Tc2, Tc3, Tc4 can be identified; C) Left: regulatory T cells (T regs) are identified as CD25+ CD127-/+low; right: CXCR3/ CCR4 expression on CD4+ CM T cells.

statistical significance of the differences observed between different experimental groups. In all cases,  $p$  value  $\leq 0.05$  was considered to be associated with statistical significance.

## Results

### *Lymphocyte distribution in the peripheral blood of B-CLL patients in different stages (Rai 0-4)*

Absolute counts of monoclonal B-CLL cells were significantly increased from early stage (Rai 0) to advanced disease Rai1/2 and Rai3/4 ( $p=0.012$  and  $p<0.001$ , respectively) (*Table 1*).

As compared to Rai 0, significantly higher numbers of total circulating T-cells are observed both in intermediate stage patients Rai1/2 ( $p=0.002$ ) and advanced disease Rai3/4 ( $p=0.02$ ). When compared to Rai 0, both Rai1/2 and Rai3/4 patients showed significantly increased absolute counts of CD4+ T helper cells ( $p=0.005$  for Rai0 vs Rai1/2 and  $p=0.03$  for Rai0 vs Rai3/4), cytotoxic CD8+ T cells ( $p=0.03$  and  $p=0.01$ , respectively) and CD4-CD8-/+low cells ( $p=0.03$  and  $p<0.001$ , respectively). The observed differences in both absolute counts/  $\mu\text{L}$  and percentages from total T cells of CD4+ and CD8+ T cells, were not statistically significant, when comparing Rai1/2 to Rai3/4 ( $p>0.05$ ). The percentages and absolute counts of CD4-CD8-/+ low T cells were significantly increased in Rai3/4 patients when compared to Rai1/2 ( $7\% \pm 5\%$  vs  $14\% \pm 6\%$ ,  $p=0.003$  and  $p=0.001$  for absolute counts – *Table 1*).

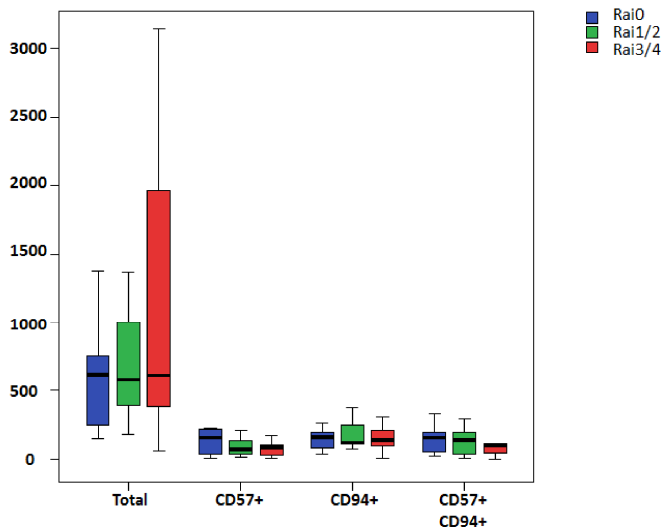
The percentages of NK cells from lymphocytes were found to be significantly decreased in Rai1/2 and Rai3/4 patients when compared with Rai 0 ( $4\% \pm 3.6\%$  vs  $1.66\% \pm 1.75\%$  or vs  $0.58\% \pm 0.3\%$ ;  $p=0.02$  or  $p<0.001$  for Rai 0 vs Rai1/2 or vs Rai3/4, respectively), but no differences in abso-

**Table 1. Distribution of lymphocytes in B-CLL patients according to Rai stage**

	Rai Stage		
	0	1/2	3/4
B-cells	18261±14316	93556±110257*	171474±143760*
T-cells	2244±723	4581±2117	8746±6571*
CD4+	1208±482	2404±1139*	3745±3083*
CD8+	916±408	1825±1146*	3858±3413*
CD4-CD8-	120±76	289±190*	1112±873*
NK cells	592±400	788±645	1052±950
Ratio CD4/CD8	1.7±1.1	1.88±1.7	1.33±1.5

Absolute counts in peripheral blood (cells/  $\mu$ L). \*= $p$ <0.05 as compared to Rai 0.

lute counts were observed ( $p$ >0.05). According to the expression of CD57 and CD94, four NK subsets could be identified: CD57+ CD94-, CD57+ CD94+, CD57- CD94+, and CD57- CD94-. Heterogeneous and comparable absolute numbers and percentages of all NK subsets were seen when comparing Rai0 vs Rai1/2 vs Rai3/4 ( $p$ >0.05), as in *Figure 2*.



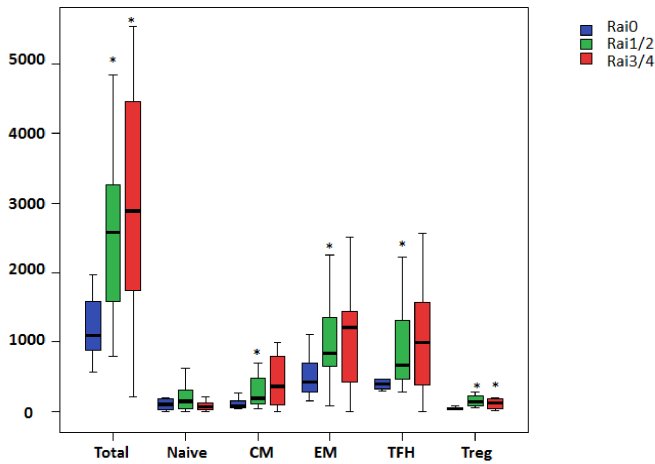
**Figure 2. NK cell subsets according to Rai stages in B-CLL patients.** Counts (cells/  $\mu$ L) of circulating NK cell subsets defined according to CD57 and CD94 expression in samples of peripheral blood from B-CLL patients in Rai 0 (blue), Rai 1/2 (green), and Rai 3/4 (red) stages. Boxes extend from the 25<sup>th</sup> to the 75<sup>th</sup> percentiles; the line in the middle and the vertical lines represent median value and both the 10<sup>th</sup> and 90<sup>th</sup>, respectively. \* $p$ <0.05 as compared to Rai 0.

**CD4+ and CD8+ T cell subsets distribution in the peripheral blood of patients with different B-CLL disease Rai stages (Rai 0-4)**

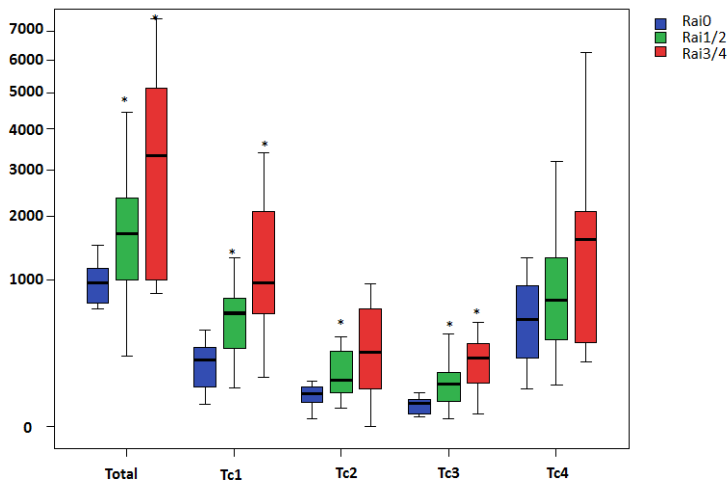
In the CD4+ compartment the following populations were monitored (*Figure 3*): naive, central memory (CM), effector/peripheral memory (EM), follicular helper CXCR5+ (TFH), and regulatory T cells (T regs).

As compared to Rai 0, T regs counts are increased in Rai1/2 (40±20 vs 170±149 cells/  $\mu$ L;  $p$ =0.001) and advanced Rai3/4 (40±20 vs 409±812;  $p$ =0.04) patients.

Patients in intermediary stages Rai1/2 showed significant and progressively increased absolute counts of TFH (390±73 vs 1042±817 cells/  $\mu$ L;  $p$ =0.001), CM (112±77 vs 298±226 cells/  $\mu$ L;  $p$ =0.028) and EM CD4+ T cells (508±329 vs 987±598 cells/  $\mu$ L;  $p$ =0.049), when compared to Rai0 patients. In contrast, although Rai 3/4 patients showed higher mean numbers of circulating CM (925 ±1406 cells/  $\mu$ L), EM (1334 ±1307 cells/  $\mu$ L), and TFH cells (1359±1543 cells/  $\mu$ L), these differences did not reach statistical significance. Similar absolute counts for naive CD4+ T cells were observed when comparing Rai0 to Rai1/2 and



**Figure 3. Distribution of CD4+ T cell subsets according to Rai stages in B-CLL patients.** Counts (cells/  $\mu\text{L}$ ) of peripheral blood CD4+ T cell subsets including: naïve, central memory (CM), effector/peripheral memory (EM), follicular helper (TFH), and regulatory (Treg) from B-CLL patients in Rai 0 (blue), Rai 1/2 (green), and Rai 3/4 (red). Boxes extend from the 25<sup>th</sup> to the 75<sup>th</sup> percentiles; the line in the middle and the vertical lines represent median value and both the 10<sup>th</sup> and 90<sup>th</sup>, respectively. \* $p < 0.05$  as compared to Rai 0.



**Figure 4. CD8+ T cell subsets according to different Rai stages in B-CLL patients.** Counts (cells/ $\mu\text{L}$ ) of circulating CD8+ T cell subsets identified as Tc1 (CD27+ CD28+), Tc2 (CD27- CD28+), Tc3 (CD27+ CD28-), and Tc4 (CD27- CD28-) from B-CLL patients in Rai 0 (blue), Rai 1/2 (green), and Rai 3/4 (red) stages. Boxes extend from the 25<sup>th</sup> to the 75<sup>th</sup> percentiles; the line in the middle and the vertical lines represent median value and both the 10<sup>th</sup> and 90<sup>th</sup>, respectively. \* $p < 0.05$  as compared to Rai 0.

Rai3/4 ( $198 \pm 316$  vs  $209 \pm 197$  vs  $110 \pm 145$  cells/ $\mu\text{L}$ ;  $p > 0.05$ ).

In the CD8+ compartment, four subsets were monitored according to CD27 and CD28 expression (Figure 4), as follows: Tc1 containing the naïve and memory cytotoxic pool, and three effector types of cytotoxic T cells: Tc2, Tc3, and Tc4. Rai1/2 patients showed significantly increased circulating numbers of Tc1 ( $214 \pm 146$  vs  $631 \pm 471$  cells/ $\mu\text{L}$ ;  $p = 0.006$ ), Tc2 ( $61 \pm 36$  vs  $155 \pm 134$  cells/ $\mu\text{L}$ ;  $p = 0.018$ ) and Tc3 ( $32 \pm 20$  vs  $121 \pm 105$  cells/ $\mu\text{L}$ ;  $p = 0.005$ ), but not Tc4 cells ( $609 \pm 451$  vs  $918 \pm 743$ ;  $p > 0.05$ ), when compared to Rai0.

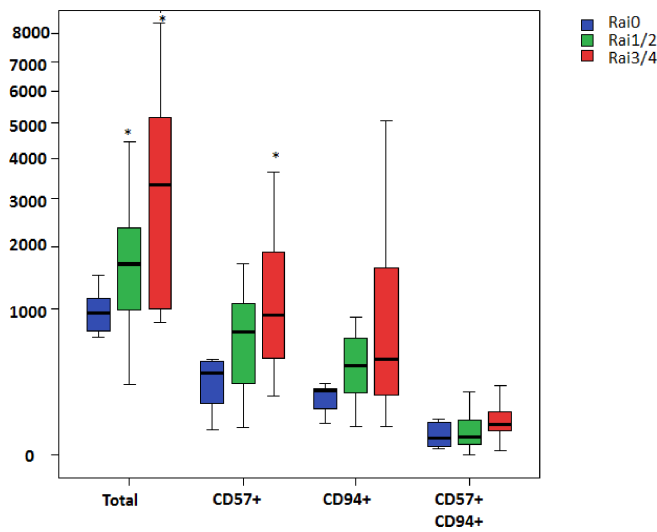
When comparing Rai0 to Rai3/4, the increase of absolute counts of Tc1 ( $214 \pm 146$  vs  $1407 \pm 1074$  cells/ $\mu\text{L}$ ;  $p < 0.001$ ) and Tc3 cells ( $32 \pm 20$  vs  $487 \pm 692$  cells/ $\mu\text{L}$ ;  $p = 0.001$ ) are statistically significant. Although in higher numbers in the peripheral blood of Rai3/4 patients, the Tc2 ( $61 \pm 36$  vs  $349 \pm 345$  cells/ $\mu\text{L}$ , for Rai0 vs Rai3/4) and Tc4 subsets ( $609 \pm 451$  vs  $1615 \pm 1629$  cells/ $\mu\text{L}$ , for Rai0 vs Rai3/4) did not reach statistical significance.

In the same CD8+ T cell compartment, the degree of senescence increases progressively with the Rai stage, according to CD57 expression:  $374 \pm 361$  vs  $732 \pm 526$  cells/ $\mu\text{L}$ ;  $p > 0.05$  for Rai 0 vs Rai1/2; and  $374 \pm 361$  vs  $1207 \pm 1111$  cells/ $\mu\text{L}$ ,  $p = 0.025$  for Rai 0 vs Rai3/4 (Figure 5).

**The expression profile of CXCR3 and CCR4 on CD4+CD8- T cells and on malignant B cells, according to B-CLL disease status**

In Rai 0 patients, the CXCR3 expression was found on





**Figure 5. Senescent and inhibitory cytotoxic CD8<sup>+</sup> T cell subsets according to distinct Rai stages in B-CLL patients.** Counts (cells/ $\mu$ L) of circulating CD8<sup>+</sup> T cell subsets identified by their CD57 and CD94 expression from B-CLL patients in Rai 0 (blue), Rai 1/2 (green), and Rai 3/4 (red) stages. Boxes extend from the 25<sup>th</sup> to the 75<sup>th</sup> percentiles; the line in the middle and the vertical lines represent median value and both the 10<sup>th</sup> and 90<sup>th</sup>, respectively. \* $p < 0.05$  as compared to Rai 0.

about 20% in all the different antigen-driven subsets: CM (20% $\pm$ 9%), EM (17% $\pm$ 16%), and FH (19% $\pm$ 14) (Figure 6).

In Rai1/2 and Rai3/4 patients all maturation CD4<sup>+</sup> T cell subsets were similar in terms of their CXCR3 – positivity: CM (35% $\pm$ 12% and 34% $\pm$ 18%;  $p = 0.003$  and  $p > 0.05$ ; for Rai 1/2 and Rai 3/4 vs Rai 0, respectively), EM (42% $\pm$ 15% and 36% $\pm$ 22%;  $p = 0.001$  and  $p = 0.05$ ) or FH (39% $\pm$ 15% and 32% $\pm$ 21%;  $p = 0.006$  and  $p > 0.05$ ; for Rai 1/2 and Rai 3/4 vs Rai 0, respectively).

In early disease Rai 0 patients, the percentage of CCR4<sup>+</sup> cells among CD4<sup>+</sup> CD8<sup>-</sup> T cells was found to be higher in the CM as compared to EM and FH subsets (40% $\pm$ 7% and 16% $\pm$ 6% and 20% $\pm$ 9%). As compared to Rai 0, the proportion of CCR4<sup>+</sup> T cells in the CM compartment was found significantly decreased in Rai1/2 and Rai3/4 patients (40% $\pm$ 7% vs 28% $\pm$ 12% and vs 21% $\pm$ 11%;  $p = 0.01$  and  $p = 0.001$ , respectively).

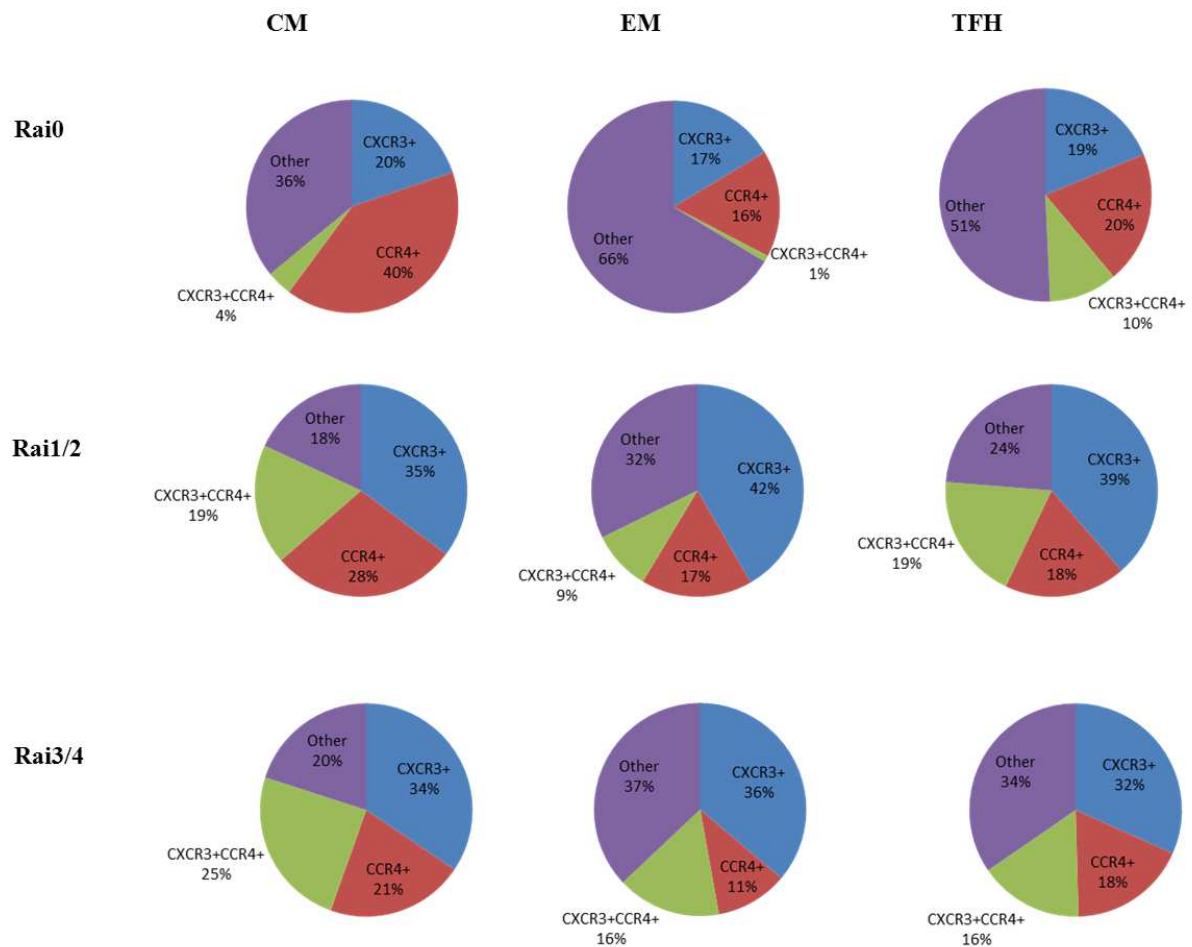
No significant differences were observed for proportion of CCR4<sup>+</sup> profile in EM (16% $\pm$ 6% and 17% $\pm$ 10% and 11% $\pm$ 8%;  $p > 0.05$ ; for Rai 0, Rai 1/2 and Rai 3/4, respectively) or TFH cells (20% $\pm$ 9% and 18% $\pm$ 9% and 18% $\pm$ 13%;  $p > 0.05$  for Rai 0, Rai 1/2 and Rai 3/4, respectively) when comparing the 3 groups.

The polarization towards co-expression of CXCR3 and CCR4 was found to be pronounced with disease evolution, in all maturation compartments: CM (4% $\pm$ 5% vs 19% $\pm$ 11% vs 25% $\pm$ 18%;  $p = 0.001$  and  $p = 0.02$ ), EM (1% $\pm$ 2% vs 9% $\pm$ 7% vs 16% $\pm$ 17%;  $p < 0.001$  and  $p = 0.007$ ) and TFH (10% $\pm$ 6% vs 19% $\pm$ 10% vs 16% $\pm$ 10%;  $p = 0.021$  and  $p > 0.05$ ), when comparing Rai0 with Rai1/2 and Rai3/4, respectively.

CCR7 and CXCR5 were homogeneously expressed on all monoclonal B-CLL cells. In contrast, an heterogeneous pattern of expression of CXCR3 and CCR4 was observed, although the percentage of cells expressing these receptors was not different ( $p > 0.05$ ) in Rai 0 (32% $\pm$ 27% and 56% $\pm$ 16%), Rai 1/2 (53% $\pm$ 30% and 55% $\pm$ 21%), and Rai3/4 (31% $\pm$ 28% and 55% $\pm$ 14%).

## Discussions

In the present study, we carried out a comparative analysis of T cell subsets in a cohort of 43 B-CLL patients. Both percentages and absolute counts of phenotypically distinct T cell subsets were calculated. In accordance to previous studies (8), in our group of B-CLL patients, increased numbers of total T cells and elevated CD4<sup>+</sup> and CD8<sup>+</sup> subsets were found. The noted increase had a progressive, stage-related pattern (from Rai 0 to Rai1/2 and Rai3/4) and was correlated with the tumor burden. However, the CD4<sup>+</sup>/CD8<sup>+</sup> ratio calculated in



**Figure 6. Chemokine receptors and Th polarization in the CD4+ T cell compartment.** The frequency of CXCR3 and CCR4 expressing cells on central memory (CM), effector/peripheral memory (EM) and follicular (TFH) CD4+ T cells from B-CLL patients in Rai 0, Rai 1/2, and Rai 3/4 stages.

our study was greater than 1, regardless of the disease stage, which is in disagreement with previously published studies (5). One explanation may lie in the small dimension of our patient group. Alternatively, we may assume that in our patients, the CD8+ T cell compartment is less skewed, since the decreased ratio is known to be mainly due to the expansion of the CD8+ compartment, rather than to the expansion of CD4+ T cells (10).

Within the CD4+ T cell compartment, we observed increased numbers of antigen-experienced central memory and effector memory

T cells, associated with the disease stage. However, there were no clear differences with regards to the naive T cell compartment.

Notable, we have also observed an expansion of TFH cell numbers in advanced stages. A recent study showed that the number of circulating TFH cells is increased in B-CLL when compared to normal subjects (19), but, as far as we know, the association with the disease stage has not been previously explored. TFH cells represent a recently described circulating T helper cell subset, associated with the occurrence of several autoimmune diseases (20-23). This find-



ing is not surprising, in light of the fact that autoimmune diseases are characterized by auto-antibodies production taking place predominantly within germinal centers. On the other hand, the expansion of a dysfunctional B cell compartment leads to an alteration of the architecture of secondary lymphoid organs. As a consequence, one of the most common risks in these patients is developing autoimmune complications.

We found elevated numbers of regulatory T cells (CD4<sup>+</sup> CD25<sup>+</sup> CD127<sup>-/+low</sup>) progressively increasing with the disease stage, from Rai0 to Rai1/2 and Rai3/4, in our group. Previous studies also described the expansion of this specific T cell compartment, a direct correlation with the disease stage, and an inverse correlation with non-regulatory T cell - mediated responses to viral or tumor antigens (11, 12, 24).

Although we could not determine whether the observed CD8<sup>+</sup> Tc1/Tc2 expansion was due to naive or to memory cells, we found that the CD28<sup>-</sup> subsets (Tc3 and Tc4), responsible for the effector cytotoxic function, follow the same trend. Moreover, CD57 expression, associated with terminally differentiated cells is also increased in CD8<sup>+</sup> T-cells, as previously observed (9), all these data supporting the hypothesis of a chronic antigenic stimulation in advanced stages of the disease. CD94 expression on T/NK cells, previously correlated with a reduced function of B-CLL-reactive cytotoxic T lymphocytes (25), has been also explored but no statistically significant differences in the proportion of NK or T CD8<sup>+</sup> cells expressing CD94 among the different disease groups was observed.

Data indicating the association between cytokine production and chemokine receptor expression on T cells have been increasingly accumulated during the last ten years. While, in some studies, cells lacking CXCR3 or CCR4 were shown unable to produce cytokines (26), associations between expression of CXCR3 and Th1/ cell-mediated immune responses, as well as between CCR4 expression and Th2/ humoral response have been demonstrated (17). In addition

to the chemokine expression, the maturation stage of T cells has been also associated with cytokine production. In this respect, CM cells were shown to secrete less cytokines than the corresponding EM cell subset (26). In the present study, we showed that the expansion of CD4<sup>+</sup> T cells in intermediate and advanced disease stages was associated with a higher proportion of T cells expressing CXCR3, as compared to early disease. This Th1-associated expansion was observed in both CM and EM CD4 subsets, together with the TFH cell compartment, supporting the increase of cytotoxic T CD8<sup>+</sup> cell numbers observed.

Previous studies found that CD4<sup>+</sup> and CD8<sup>+</sup> T cells of B-CLL patients with indolent disease exhibit a predominance of type 1 (IFN- $\gamma$ ) over type 2 (IL-4) cytokine production (27), when stimulated in vitro, while in advanced stages a switch to a type 2 (IL-4) T cell response occurs (28). In contrast, in our study, the expression pattern of chemokine receptors identified suggests an increasing tendency of the Th1 compartment, both in intermediate and advanced disease stages, when compared to early disease. Similarly, CCR4 – a suggested Th2 marker - was decreased in CM T cells from patients in advanced stages from our group. Noteworthy, increased frequency of CXCR3<sup>+</sup> CCR4<sup>+</sup> T-cells (subset associated with both Th1 and Th2 production) was also detected in advanced disease. However, when compared to earlier stages, the difference was not statistically significant, probably due to the heterogeneous behaviour of the T-cells in these patients.

In summary, our results show increased antigen-experienced CD4 and CD8 T cells in B-CLL patients with intermediate/advanced disease. This expansion is associated with an increased expression of Th1-related chemokine receptors within the CD4 compartment, while within the CD8 T cell compartment the expansion associates with the expression of CD57, a marker of terminally differentiated cells. Furthermore, the disease behaviour seems to be also associated with increased numbers of TFH and regulatory T cells.

The therapeutic modulation of T cell response in B-CLL patients may play an important role in the disease behaviour and may be a key event compensating the immunodeficiency characteristic for the advanced stages of the disease.

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### Conflicts of interest

The authors declare that there are no conflicts of interest concerning this paper.

### Abbreviations

B-CLL - B cell chronic lymphocytic leukemia  
 CD - cluster of differentiation  
 CM - central memory  
 EM - effector memory  
 FH - follicular helper  
 PBMC - peripheral blood mononuclear cells  
 Tc - cytotoxic T cells  
 Tregs - regulatory T cells

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