IMMUNE CELL POPULATIONS AND THEIR PHENOTYPES IN B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA

Summary of Thesis

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Project co-financed by European Social Fund through Sectorial Operational Programme for Human Resources Development 2007 - 2013 Priority Axis 1 "Education and training in support for economic growth and society development based on knowledge “ Key Area of Intervention 1.5 "Doctoral and postdoctoral research support”
Project Title: “Universitary partnership for improving quality and interdisciplinarity of medical doctoral research through scholarships -DocMed.net Contract Code: POSDRU/107/1.5/S/78702
Beneficiary: University of Medicine and Pharmacy “Iuliu Hațieganu” Cluj Napoca
Partner 1: University of Medicine and Pharmacy “Grigore. T. Popa” Iași

-2014-
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Abbreviations

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<th>Description</th>
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<tr>
<td>B-CLL</td>
<td>B Cell chronic lymphocytic leukemia</td>
</tr>
<tr>
<td>MBL</td>
<td>Monoclonal B cell lymphocytosis</td>
</tr>
<tr>
<td>EM</td>
<td>Effector memory</td>
</tr>
<tr>
<td>CM</td>
<td>Central memory</td>
</tr>
<tr>
<td>TH</td>
<td>T helper cells</td>
</tr>
<tr>
<td>TFH</td>
<td>T follicular helper cells</td>
</tr>
<tr>
<td>TC</td>
<td>Cytotoxic T cells</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cells</td>
</tr>
<tr>
<td>PC</td>
<td>Plasma cells</td>
</tr>
<tr>
<td>MLPA</td>
<td>Multiplex Ligation dependent Probe Amplification</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of designation</td>
</tr>
<tr>
<td>CXCR</td>
<td>Chemokine receptor</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>ADN</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ARN</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>APS</td>
<td>Automated population separator</td>
</tr>
<tr>
<td>del</td>
<td>Deletion</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
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</table>

Doctoral thesis is illustrated with 17 tables and 102 figures.
Figure numbers from the abstract are concordant to those from the thesis.
Reference numbers are concordant to the extenso.
All research accomplished during the doctoral studies could be completed due to my status as fellow in the project „Doctoral fellowships for competitive PhD students in the European research area“, POSDRU/107/1.5/S/78702.
Chapter 4. Motivation and objectives of the doctoral thesis

B-cell chronic lymphocytic leukemia (B-CLL) is one of the most common hematologic malignancies that occurs mainly in the elderly. However, its manifestation is not restricted to this age group and it is far from being homogeneous. Of a particular interest appears to be the fact that, although B-CLL is well acknowledged as a pathological entity and it has been studied for decades, it continues to be an incurable disease in those patients who require therapeutic intervention. The fact that some B-CLL patients progress to fulminant and immediate terminal disease, while others can survive for years without even requiring treatment is unprecedented in oncology.

The origin of the B-CLL malignant clone has not been yet identified. Studies attempting to establish the origin of the clone in various malignancies usually focus on the relationship between the pathology and normal, corresponding circumstances. While in the case of the majority of hematological malignancies, in general, and of chronic B-cell lymphoproliferations, in particular, the cellular origin of the malignant clone has been identified, in B-CLL, due to the particular cell phenotype (an asynchronous mixture of molecules suggestive of either naive or memory B cells), no normal cell equivalent has been found. B-CLL stands as a unique entity also through its genetic background, because none karyotype aberration has been associated with. Additionally, although a normal karyotype may be found at debut, during the disease progression several genetic anomalies may accumulate due to the genomic instability, some of them having prognostic value.

The immune system of B-CLL patients has defective functions. This is rather to be expected, since the B cell is an important participant in adaptive immune responses. For this reason, most patients with B-CLL are detected having hypogammaglobulinemia, and those who are not, will acquire this deficiency later during therapy. The combined result would be an increased risk of infection, which remains the main cause of death during this disease. Therefore, the immune status in B-CLL becomes a key feature with clinical value in selecting appropriate therapy and controlling its consequences, since most drugs used also have immunomodulatory effects (positive or negative). Intrinsic immune defects linked to the disease and mostly to the advanced stages are expected to be soon identified. These defects, in addition to those induced by the treatment have repercussions that extend over a large period of time.

This observation was one of the most intriguing hypotheses: the immune system, by means of its multiplet networks of cells and molecules, behaves differently when mounting a specific response within the context of B-CLL. The extent of this particular response and the factors that can act as suppressors in some circumstances remain to be fully understood.

In this context, the current PhD study aimed at the following:

- phenotypic individualization of B lymphocytes in B-CLL in relation to both, B cell chronic lymphoproliferations and normal B cell phenotype, using large panels of molecules.
- defining a strategy for identification and characterization of residual normal B lymphocytes from B-CLL patients with active disease.
- analysis of intrinsic defects and of those therapeutically induced in the normal B lymphoid compartment of B-CLL patients in advanced stages of disease.
- genotypic identification of B cell clonal aberrations in B-CLL using molecular biology techniques.
- definition of a straightforward and reproducible strategy to identify and characterize subpopulations of T lymphocytes and NK cells involved in the immune response by flow cytometry and application of this strategy in the study of patients with B-CLL.
- analysis of the distribution of total T lymphocytes, T helper CD4+, cytotoxic T CD8+ and of the NK lymphocytes, and of their phenotypic/functional characteristics in patients with B-CLL correlated with the aggressiveness of the disease.
- evaluation of the presence of TCRαβ T cell clonal expansion of B-CLL patients by molecular biology techniques.
Chapter 5. Materials and methods

5.1. The material used in the doctoral study

During the doctoral study peripheral blood samples, obtained with the informed consent from patients with B-CLL were used. Two groups of patients from two different sources were included into the study: patients admitted to the Hematology Clinic of the St. Spiridon Hospital/Regional Institute of Oncology, Iasi and B-CLL patients coming from 49 centers of Spain and investigated in a larger research project conducted by the Cytometry Service, University of Salamanca, Spain.

B-CLL patients included into the phenotypic and genotypic study of clonal B lymphocytes and T/NK lymphocytes.

The inclusion criterion was the diagnosis of B-CLL, regardless of the disease stage, as our study aimed to investigate different disease stages in a comparative manner.

The group of patients consisted of 58 patients with B-CLL. This group was divided into subgroups: 43 patients for the evaluation of T lymphoid subpopulations by flow cytometry and 50 patients for the detection of TCR clonality. The genetic complexity of the malignant clone was evaluated in all 58 patients.

B-CLL patients included in the study of normal circulating B lymphocytes.

The inclusion criterion was the diagnosis of patients with active B-CLL requiring immediate therapeutic intervention.

The group of patients consisted of 191 B-CLL patients in advanced stages of the disease (Binet B / C): 113 untreated and 78 patients at the beginning of a new line of treatment (48 following the first treatment line, 30 patients following the second line or after more than two lines of therapy). The results obtained from this latter study were compared with a pre-existing database including 77 healthy controls.

5.2-5.6 Methods used in the doctoral study

The multiparameter flow cytometry allows the fast multiparametric analysis of fluorescently labelled cells. The intensity measurement of optical signals is achieved through a complex system that combines optical, fluidic and electronic components, in order to link the optical signals with cellular parameters of interest. The immunofluorescence staining involves the preparation of a cell suspension, its incubulation with fluorescent monoclonal antibody, lysis of red blood cells, followed by several washing steps to remove excess antibodies. In some protocols, red cell lysis can be carried out before the incubation with fluorochrome monoclonal antibodies.

In the study of T/NK lymphocytes the following monoclonal antibodies, with specificity for various membrane markers were used: CD3, CD4, CD8, CD56, CD94, CD57, CD25, CD127, CD27, CD28, CD45RA, CCR7, CXCR5, CXCRI3, CCR4. The expression of the chemokine receptors CCR7, CXCR5, CXCR3, CCR4 and CD38 was assessed in the malignant clone. The cytometry study was performed using mononuclear cells separated by density gradient centrifugation of samples collected from B-CLL patients.

For the study of normal B-lymphocytes the following monoclonal antibodies specific to cell surface molecule were used: IgM, IgG, IgA, CD19, CD20, CD5, CD27, CD38, and CD45. The cytometry study was carried out using peripheral blood samples processed by macrovolum red blood cell lysis.

For the study of genetic aberrations within the malignant clone and of the TCR beta chain clonality molecular biology techniques were used. The first step was the extraction of genomic DNA from samples of mononuclear cells from patients with B-CLL. For the study of genetic aberrations in the B lymphoid clone the MLPA technique was used (probe mixes P037 and P038, MRC Holland). In order to identify the TCR gene clonality the IdentiClone™ (TCRB + TCRG T-Cell Clonality Assay) kit for Beckman Coulter CE/NAT Platforms was used. The method involved detection of specific PCR amplification for several determined gene regions and migration of these amplicons by capillary electrophoresis. Results were issued as histograms related to amplification.

The statistical analysis of the percentage and absolute values, mean, median and rank for all continuous variables were performed using SPSS (SPSS 15 Inc, Chicago, IL). Statistically significant
differences between groups were calculated using the Mann-Whitney U test for continuous variables, with p≤0.05 as indicative value.

Chapter 6. Clinical and biological characteristics of patients with B-CLL and their correlation with the size of B cell clonal expansion

6.1. Introduction

B cell chronic lymphocytic leukemia represents the result of a progressive accumulation of clonal B lymphocytes, the main symptoms being leukocytosis with lymphocytosis, lymphadenopathy, hepatomegaly, splenomegaly, and blockage produced by bone marrow infiltration of malignant cells (identified by altered hemoglobin and platelets values). Neutropenia and hypogammaglobulinemia observed in patients with B-CLL are determinants of the high susceptibility of these individuals to different infections (the main cause of death in patients with B-CLL).

6.2. Material and method

The study included 58 patients diagnosed with B-CLL in the Hematology Clinic of St. Spiridon Hospital / Regional Institute of Oncology. The study received the approval of the ethics committee, patient data were used with informed consent. In the database developed during this doctoral study each patient received an internal identifier. For each patient information on a number of biochemical parameters relevant to the disease were extracted from their medical records.

6.3. Results

6.3.1. General characterization of the group of patients with B cell chronic lymphocytic leukemia

Patients included into the study had elevated leukocyte numbers due to the presence of a circulating mass of monoclonal B lymphocytes with a median of 49,278 cells/µL, and a distribution between a minimum of 1798 cells/µL and a maximum of 451,380 cells/µL.

Between the number of clonal B lymphocytes and the hemoglobin concentration a statistically significant inverse correlation was observed (p <0.001 and r² = 0.61), as represented in Fig. 6.1.

Fig. 6.1. Correlation of clonal B lymphocyte numbers with the serum hemoglobin value in patients with B-CLL.

Lactate dehydrogenase (LDH) showed a statistically significant positive correlation (p <0.001 and r² = 0.48) with the number of clonal B lymphocytes (Fig. 6.2).
6.3.2. Characterization of the patient group using the Rai classification

Most patients (50%) were included in Rai stages 1/2 (29 patients), 32% (19/58 patients) were enrolled in Rai stage 3/4 and only 16% (9/56 patients) in Rai stage 0.

6.3.3. Association of CLL clonal B lymphocytes with nodal infiltration and organomegaly

In terms of clinical manifestations, 36% (21/58) of the patients were diagnosed with fever, weight loss and night sweats, signs gathered under the name of B symptoms (Table 6.1).

Table 6.1. The frequency of clinical manifestations in B-CLL patients group

<table>
<thead>
<tr>
<th></th>
<th>Present</th>
<th></th>
<th>Absent</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nr</td>
<td>%</td>
<td>Nr</td>
<td>%</td>
</tr>
<tr>
<td>B Symptoms</td>
<td>21</td>
<td>36</td>
<td>35</td>
<td>60</td>
</tr>
<tr>
<td>Cervical LN</td>
<td>39</td>
<td>67</td>
<td>17</td>
<td>29</td>
</tr>
<tr>
<td>Supraclavicular LN</td>
<td>31</td>
<td>53</td>
<td>25</td>
<td>43</td>
</tr>
<tr>
<td>Axillary LN</td>
<td>24</td>
<td>41</td>
<td>32</td>
<td>55</td>
</tr>
<tr>
<td>Inguinal LN</td>
<td>22</td>
<td>38</td>
<td>34</td>
<td>59</td>
</tr>
<tr>
<td>LN &gt;5cm</td>
<td>11</td>
<td>19</td>
<td>45</td>
<td>78</td>
</tr>
<tr>
<td>LN &gt;10 cm</td>
<td>6</td>
<td>10</td>
<td>50</td>
<td>86</td>
</tr>
<tr>
<td>Hepatomegaly</td>
<td>24</td>
<td>41</td>
<td>32</td>
<td>55</td>
</tr>
<tr>
<td>Splenomegaly</td>
<td>28</td>
<td>48</td>
<td>28</td>
<td>48</td>
</tr>
</tbody>
</table>

The presence of hepatomegaly, splenomegaly and the presence of axillary, inguinal, and greater than 5 cm lymph nodes chains, were found to be associated with increased numbers of circulating B-clonal lymphocytes. The difference was not significant when the number of clonal CLL B lymphocytes and the presence of cervical and supraclavicular adenopathy were compared. Both the presence of hepatomegaly and splenomegaly is associated with significantly higher numbers (p <0.001) of circulating B lymphocytes compared to patients with no organomegaly, and correlated to each other (due to the fact that, most often, hepatomegaly and splenomegaly are simultaneously present).

6.4. Discussion

Clinical and biological parameters evaluated on a routine base in patients with B cell chronic lymphocytic leukemia, exhibits a distinct alteration represented by leukocytosis with persistent lymphocytosis in patients’ peripheral blood samples. The heterogeneity described in the literature
in this haematological malignancy was also observed in the current study from the beginning, following a simple analysis of total leukocyte distribution and clonal cell distribution. This is the reason why this parameter represents one of the consensus criteria for the diagnosis of B-CLL. These criteria were defined since 1988, based rather on the clinical experience of working groups than on the precise identification of the malignant clone. In light of this experience, various groups of authors recommend various threshold values of absolute lymphocyte count for the diagnosis of B-CLL, such as 11 000 B cells / μL or 5000 B cells / μL, the most common used threshold value nationally. Cases with values lower than this threshold, associated with minimal clinical symptoms (adenopathy, anemia, cytopenias) are also considered and classified as B-CLL, although, until recently, these patients were diagnosed with small lymphocytic lymphoma.

Most patients included in this study presented for diagnoses at an intermediate (Rai1-2) or advanced (Rai3-4) stage of disease, which illustrates that the preventive medicine at regional level is reinforced by the reality where the patient presents to the doctor only when the clinical manifestations are obvious.

At the time of diagnosis, anemia and thrombocytopenia may be present in about 20% of cases, aspects also observed in our study groups, when low hemoglobin (<10-11 g / dL) and platelets (<100 000 / μl) levels were recorded in about 25% and 45% of patients, respectively.

Chapter 7. Phenotypic and genotypic characteristics of clonal and residual, normal B cells in B-CLL

7.1. Interpretation of Euroflow panels – a way for differential diagnosis of B cell chronic haematological malignancies

7.1.1. Introduction

The current WHO classification recognizes about 40 types of lymphoma with B, T or NK cell origin. Out of these, approximately 97% are classified in 6 groups: diffuse large B-cell lymphoma (DLBCL- 37%), follicular lymphoma (FL-29%), mucosa associated lymphoid tissue lymphoma (MALT-9%), mantle cell lymphoma (MCL-7%), B chronic lymphocytic leukemia (B-CLL-12%), splenic marginal zone lymphoma (SMZL-2%), lymphoplasmacytic lymphoma (1%).

Chronic lymphocytic leukemia B is phenotypically distinctive in comparison to other chronic lymphoid proliferations of B cell origin. An accurate immunophenotypic analysis carried out for the diagnosis of B-CLL relies mainly on an interpretation strategy able to exclude other diagnostic entities.

Purpose: To illustrate the method used for accurate differential diagnosis of mature B cell haematological malignancies based on large panels of makers. The current section plan to review molecules necessary to achieve differential diagnosis between different diagnostic entities within chronic lymphoproliferative syndromes and explain the gating strategy for the analysis of multiparameter data files.

7.1.2. Material and method

Seven peripheral blood samples from patients with suspected and further confirmed B-cell chronic lymphoproliferation were used. The samples were processed in the Cytometry Service of the University of Salamanca, Spain. An extended panel of makers (the EuroFlow standard panel published on the website of the consortium) was applied: B-cell markers: CD19, CD20 and CD45. B cell characterization markers: CD5, CD10, CD23, CD79b, CD200, CD43, CD31, LAIR1, CD11c, IgM, CD81, CD103, CD95, CD22, CXCR5, CD49d, CD62L, CD39, HLA-DR, CD27. The expression analysis of these molecules was performed using a specially developed software in the consortium (Infinicyt, Cytognos, Spain).

7.1.3. Results

B lymphocytes were identified using lineage specific molecules and cell distribution on dot-plot graphs plotted on small size coordinate (forward scattered light - FSC) versus low intracellular complexity (side-scattered light SSC).
It can be noticed that the pathological B cells have a distinct phenotype in B-CLL when compared to other chronic B-cell lymphoproliferations and normal B cells. The phenotype of B-CLL malignant cells can be summarized as: CD19 +, CD20 + dim, CD22 + dim, CD5 +, CD23 +, CD27 +, CD10-, CD79b + dim, CD81 + dim, CD200 +, CD43 +, IgM + dim, CD103-. These molecules are crucial for the diagnosis of B-CLL, while the other molecules in the panel varies from one individual to another and provide information on prognosis (CD38, CD49d) or the expected B–cell migration behaviour to secondary lymphoid organs (CXCR5, CD62L). The HLA-DR molecule is consistently expressed by the B-CLL cells, although their reduced antigen-presenting capacity it is well known.

7.1.4. Discussion

Laboratory investigations have an important role in the diagnosis of patients with hematologic malignancies, in assessing the prognostic role of various factors and evaluation of treatment efficacy. Currently, immunophenotyping is widely acknowledged as a key provider of all necessary information to characterize all aspects mentioned above. Despite the high resolution of the technique, this still remains quite subjective, depending on the operator’s experience, in both the data files and the use of reagents that provide the best separation between positive and negative cell populations. EuroFlow represents a novel concept, involving new acquisition and analysis tools, optimal combinations of reagents, optimal methods for samples processing, novel softwares for data analysis, enabling international standardization and eliminating the huge gaps frequently observed between different diagnostic centres. The rational use of these resources can produce similar and, importantly, perfectly reproducible results.

We considered the introduction of this chapter in the doctoral thesis, as these complex and intricate panels, although difficult to interpret in the absence of experience, should preferably serve as a guide in implementing this type of analysis strategy in any Romanian flow-cytometry laboratory focused on diagnosis.

7.2-7.3. CD38 and chemokine receptors CCR7, CXCR5, CXCR3, CCR4 expression in the malignant clone and the association with clinical and biological parameters

7.2/1. Introduction

These chapters frame the study of clone of B cells behaviour in patients with CLL regarding the expression of CD38 molecule (in two circumstances: as assessment of any percentage of expression and association with clinical and biological parameters and using a threshold value previously identified value as having prognostic significance) and on the expression of chemokine receptors among which CXCR3 is reported in the literature as having prognostic value.

7.2/3.2. Material and method

The study was performed by means of the multiparameter flow cytometry technique, using peripheral blood samples and density gradient separated cells, and monoclonal antibodies needed to identify and characterize clonal B cells: CD38, CCR7, CXCR5, CXCR3, CCR4.

7.2/3.3. Results

Using the 30% threshold value for defining the patients with a CD38 positive malignant clone we noticed that more than 50% of patients positive for CD38 (marker associated with a poor prognosis when present) manifest adenopathies with distinct localizations (cervical, supraclavicular, axillary, inguinal) when compared to the CD38 negative lot., while 60% have organomegaly (hepatosplenomegaly or spleno-megaliths, $p = 0.048$ and $p = 0.05$), as presented in Figure 7.15. However, the statistically significant differences noticed between patients with lymphadenopathy over 5 / 10 cm and all others in terms of the frequency of CD38 positivity fis maintained ($p = 0.006$ and $p = 0.005$), according to the cut-off value of 30%.
Expression of CCR7 and CXCR5 chemokine was relatively homogeneous on clonal B lymphocytes from the patients investigated in this study. The expression of CCR4 receptor, although heterogeneous, showed no statistically significant differences when different stages of the disease were considered. CXCR3 expression had a median of 43% of the entire study group, with a heterogeneous distribution in the range of 0-100%. The expression of CXCR3 in the Rai0 stage had a median frequency of 26% on the B cells from patients of this group, whereas in the Rai1, Rai2, and Rai3 stages frequencies of 59%, 45%, and 51% have been identified. Interestingly, at Rai4 stage a drastic reduction of cells expressing CXCR3 was measured, with a median value of 4%, but with a higher heterogeneity within the group.

No statistically significant differences were found (p > 0.05) when the percentage of CCR4 or CXCR3 receptor positivity in malignant B lymphocytes was compared between groups of patients with lymphadenopathy (in any of the investigated areas) or organomegaly and those in which any of these events is absent. Although apparently CCR4 + B cell percentages were higher in patients with no adenopathy, the statistical significance was not reached.

Classifying patients according to the positivity for CD38 (molecule considered a reliable prognostic factor) and linking this particular set of data with those obtained from the study of chemokine receptor expression, showed no statistically significant differences between the 2 groups (Table 7.3).

**Fig. 7.15.** The number of CD38+ patients (with more than 30% CD38 positive clonal B lymphocytes) in the context of clinical manifestations.
Table 7.3. CXCR3 and CCR4 receptor expression in CD38+/− B-CLL patient groups.

<table>
<thead>
<tr>
<th></th>
<th>CXCR3 expression</th>
<th>CCR4 expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD38+ (&gt;30%)</td>
<td>Mean±SD</td>
<td>Median</td>
</tr>
<tr>
<td></td>
<td>37±32 %</td>
<td>42%</td>
</tr>
<tr>
<td></td>
<td>53±18%</td>
<td>54%</td>
</tr>
<tr>
<td>CD38− (&lt;30%)</td>
<td>Mean±SD</td>
<td>Median</td>
</tr>
<tr>
<td></td>
<td>46±30%</td>
<td>41%</td>
</tr>
<tr>
<td></td>
<td>55±18%</td>
<td>59%</td>
</tr>
</tbody>
</table>

A positive correlation between clonal B lymphocytes expressing CXCR3 and T helper lymphocytes with a potential Th1 profile (CXCR3 +) was identified and presented in Figure 7.27.

![Correlation between CXCR3 expression in the malignant B cell clone and the expression of the same receptor in the population of T helper cell of the same patients.](image)

Fig. 7.27. Correlation between CXCR3 expression in the malignant B cell clone and the expression of the same receptor in the population of T helper cell of the same patients.

7.2-4. Discussion

Expression of CD38 molecule on clonal B lymphocytes of CLL had been shown to be correlated with a poor outcome in the case of B-CLL patients. In most cases, the expression of this molecule is not uniform throughout the B lymphoid clone and studies comparing CD38 + lymphocytes vs CD38- lymphocytes of the same individual demonstrates that the cells within the first category (CD38 +) are more active and more likely to enter the cycle cell than those CD38- N. Chiorazzi and collaborators identified a rate of 1% of clonal cells that are renewed daily, using the deuterium incorporation method to measure cell kinetics in patients with CLL. This fraction consists of clonal cells that express CD38, the cells that have recently left the solid lymphoid tissues 12. The same heterogeneous distribution characterizes the group investigated by us.

Previous studies conducted on numerous cohorts of B-CLL patients mention a various incidence of CD38 positivity for which is presented in Table 7.2. The results obtained in the current study are comparable as incidence to those from larger studies.
Table 7.2. The frequency of CD38 positivity in patients with B-CLL mentioned in the literature.

<table>
<thead>
<tr>
<th></th>
<th>Domingo-Domenec \textit{et al.} \textsuperscript{2002\textsuperscript{303}}</th>
<th>Damle \textit{et al.} \textsuperscript{1999\textsuperscript{301}}</th>
<th>Del Poeta \textit{et al.} \textsuperscript{2001\textsuperscript{304}}</th>
<th>Chevallier \textit{et al.} \textsuperscript{2002\textsuperscript{305}}</th>
<th>Hamblin \textit{et al.} \textsuperscript{2002\textsuperscript{306}}</th>
<th>Heintel \textit{et al.} \textsuperscript{2001\textsuperscript{307}}</th>
<th>Gentile \textit{et al.} 2005</th>
<th>Jakob \textit{et al.} 2006\textsuperscript{309}</th>
<th>Present study</th>
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<tr>
<td>No</td>
<td>155</td>
<td>47</td>
<td>168</td>
<td>123</td>
<td>145</td>
<td>81</td>
<td>242</td>
<td>102</td>
<td>50</td>
</tr>
<tr>
<td>Freq CD38</td>
<td>19%</td>
<td>36%</td>
<td>30%</td>
<td>27%</td>
<td>42%</td>
<td>19%</td>
<td>14%</td>
<td>29%</td>
<td>28%</td>
</tr>
</tbody>
</table>

Del Poeta \textit{et al.}\textsuperscript{304}, noticed that 86% of CD38 + patients (defined based on the 30% threshold) out of a group of 168 B-CLL patients had lymphadenopathy and / or splenomegaly. In our study we found a comparable percentage (60-70%), although we conducted our study by type of adenopathy and organomegaly.

Data from the literature\textsuperscript{321} show that the expression of CCR4 receptor is significantly increased in CD38 + patients, while in the patient group investigated in the current study, this difference does not reaches statistical significance (Table 7.3). A possible explanation is that our patient group size was too small for studying such heterogeneously expressed molecules. On the other hand, a publish study noteda significant difference between the CD38+ and CD38-patients , with mean positivity percentages of 53% and 34%, but with a standard deviation lower than that obtained in our study (5% standard deviation in the published report, whereas in the current study the standard deviation was 18%).

The role of chemokines in the pathogenesis of hematological neoplasies is not very well defined. Obviously, the expression of chemokines over time was studied in multiple subtypes of lymphoma, with special focus on the microenvironment, although it is unclear whether malignant cells are the ones responsible for creating the the microenvironment or the other way around.

Some studies claim that CXCR3 is relatively well expressed by clonal B cells of CLL patients, in contrast to normal B cells and its interaction with specific ligands, chemokines IP-10 and MIG, leads to cellular migration \textsuperscript{333}. This study demonstrates that low levels of expression of CXCR3 receptor are associated with advanced and aggressive disease in CLL, although heterogeneity is clearly demonstrated. In our study we decided to operate not with fluorescence values but with percentages of positivity in the malignant clone, since the intensity of expression can be altered by cryopreservation, and nosignificant differences were identified within the group of studied B-CLL patients.

7.4. Genotype of pathologic B lymphocytes in LLC

7.4.1. Introduction

Unlike other hematologic malignancies, B cell chronic lymphocytic leukemia is a very heterogeneous disease, in terms of its genetic background. Some genetic aberrations are known to influence the further course (aggressive or indolent) of the disease\textsuperscript{346, 347}. A hierarchical association was demonstrated between several genetic abnormalities and the clinical outcome in B-CLL patients: deletions in the short arm (p) of chromosome 17 and the long arm (q) of chromosome 11 are associated with reduced survival, trisomy 12 and normal karyotype, with an intermediate prognosis, while deletions within the long arm (q) of chromosome 13, as a single aberration are associated with a favourable outcome \textsuperscript{348, 349}. Lately, a number of studies have identified additional chromosomal phenotypes presenting a high genomic complexity, including recurrent translocations (often unbalanced), aberrant complex karyotypes and even single nucleotide polymorphisms (SNP), and most importantly, their association with the progression of the disease\textsuperscript{350-353}.

7.4.2. Material and method

The lot consisted of a number of 58 patients with B-CLL in different stages. For the purpose of this study, the MLPA technique was used, based on the polymerase chain reaction (PCR), which can
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quantify at the same time up to 45 different genomic targets in one experiment by specific amplification of specific hybridized probes. The commercial MRC Holland SALSA MLPA CLL P037 and P038 kits were used, accordingly to the manufacturer’s instructions.

7.4.3. Results
The results generated in the current section of the study are schematically presented in Table 7.4.

7.4.4. Discussion
A total of 49 patients (67%) had simple or complex genetic aberrations. Of these, the aberrations identified in previous studies as the most common were also found in our patient group: 13q deletion (33%), 11q deletion (16%), trisomy 12 (16%) and 17p deletion (7%). The high frequency of 13q deletion, the similar frequency of del11q and trisomy 12 and the low frequency of 17p deletion were observed in studies on much larger groups of patients (79-325 patients). The similar results obtained in our study can be explained by the homogeneous distribution of disease stages within the patient group (Table 7.5).

Table 7.4. The frequency and type of genetic abnormalities identified in patients with B-CLL according to their Rai stages

<table>
<thead>
<tr>
<th>The type of cytogenetic aberration</th>
<th>Rai0</th>
<th>Rai1/2</th>
<th>Rai3/4</th>
</tr>
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<tbody>
<tr>
<td>Normal genotype</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trisomy 12</td>
<td>3</td>
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<td></td>
</tr>
<tr>
<td>del13q14 RB1+</td>
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<td></td>
</tr>
<tr>
<td>del13q14 RB1-</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Normal genotype</td>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trisomy 12</td>
<td>4</td>
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</tr>
<tr>
<td>del13q14 RB1+</td>
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</tr>
<tr>
<td>del13q14 RB1-</td>
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<tr>
<td>del13q14 RB1+ and mutation SF3B1</td>
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<tr>
<td>del13q14 RB1+ and mutation NOTCH1</td>
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<td>Mutation SF3B1</td>
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<tr>
<td>del6q21-q26</td>
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<tr>
<td>Normal genotype</td>
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<td>del13q14RB1+ and duplication8q24 and mutation NOTCH1</td>
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Table 7.5. The frequency of genetic defects in patients with B-CLL compared with previously published data.

<table>
<thead>
<tr>
<th></th>
<th>Dohner et al. 165</th>
<th>Durak et al. 363</th>
<th>Bullinger et al. 364</th>
<th>Stilgenbauer et al. 365</th>
<th>Dewald et al. 194</th>
<th>Glassman et al. 366</th>
<th>Giertlova et al. 367</th>
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<td>11%</td>
<td>25%</td>
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<td>23%</td>
<td>17%</td>
<td>16%</td>
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<tr>
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<td>15%</td>
<td>10%</td>
<td>12%</td>
<td>25%</td>
<td>11%</td>
<td>19%</td>
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<tr>
<td>Del17p</td>
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<td>6%</td>
<td>3%</td>
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<td>6%</td>
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<td>78%</td>
<td>81%</td>
<td>77%</td>
<td>64%</td>
<td>45%</td>
<td>67%</td>
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</table>

7.5. Characterization of residual normal B-lymphocytes in B-CLL patients with active disease

7.5.1. Introduction: Because the expanded population of malignant B cells gradually occupy niches on which the normal B cell compartment relies, in patients with B-CLL normal B lymphocytes can be found in smaller numbers than in healthy subjects. However, there is a lack of very detailed studies on the distribution of maturing/ functional B cell subpopulations in this disease. Identification of these subsets of normal B lymphoid cells and the precise separation from malignant B lymphocytes is often a challenge, requiring quite extensive experience in flow cytometry (obviously morphologically these subsets cannot be defined). This study aims to investigate the quantitative and qualitative defects within the lymphoid B cell compartment in patients with B-CLL with active disease (requiring therapeutic intervention). Also, this study provides an effective panel and a strategy for a rapid and sensitive analysis of normal B lymphoid subsets that can be applied to the study of other B lymphoid diseases (immunodeficiency, autoimmune diseases) or in any other context in which the evaluation of the B lymphoid compartment is necessary.

7.5.2. Material and Method: This study included 191 patients with B-CLL in B or C Binet stage. Patients originate from 49 hospitals across Spain, and the samples were processed and analysed in the Laboratory of Cytometry from Salamanca, Spain. Also, in order to perform a comparative study information from a database with 77 healthy controls was used.

From all B-CLL patients, 113 were at the initial diagnosis, before starting any therapy, while 78 patients had gone through at least a full line of therapy. In this last category, 48 patients came after the first line of treatment, while 30 patients had undergone two or more lines of therapy. From these patients, peripheral blood samples collected on EDTA were analysed by multiparameter flow cytometry. The following fluorescent monoclonal antibodies were used: CD45, CD19, CD20, CD5, CD27, CD38, IgM, IgG, IgA. In order to analyze a large number of cells (5x10^6 cells) a macrovolume red blood cell lysis technique was used, described in the Materials and methods section. Data acquisition was performed using a flow cytometer FACSCantoII, FACSDiva software. For data analysis and interpretation the Infinicyt software was used (Cytognos, Spain).

7.5.3. Results

Normal B cells were sub-classified into the following sets:
- immature: CD19+ CD20++ CD5+ CD27- CD38+ smIgM +
- naive: CD19+ CD20+++ CD5- CD27- CD38- smIgM +
- memory: CD19+ CD20++ CD5- CD27+ CD38- grouped in IgM or IgG or IgA subclasses.
- newly formed plasma cells (PC): CD19+ CD20+/ CD5- CD27++ CD38++ subclassified in IgM or IgG or IgA or Ig negative.
Overall, normal B lymphocytes are, in terms of relative numbers and absolute counts, reduced compared to healthy controls (Fig. 7.40).

As it can be noticed from Figure 7.41., the patients with B-CLL present a B lymphoid compartment dominated by memory (68%±29% vs 31%±12%, p<0.001) and immature cells (10%±19% vs 5%±4%, p<0.001), with a significant reduction of naive cells (14%±19% vs 64%±12%, p<0.001). Recently produced peripheral plasma cells present a very heterogeneous distribution and, apparently, with out statistically significant differences between the two groups (8%±20% vs 2%±3%, p>0.05 for CLL patients versus control subjects). In absolute numbers, all normal B lymphocyte subsets are significantly reduced in peripheral blood of B-CLL patients compared with healthy controls: immature B cells 6±17 vs 9±9 cells/µl, p<0.001), naive (15 ± 63 vs 101 ± 57 cells / µl, p <0.001), memory (46 ± 76 vs 52 ± 39 cells / µl, p <0.001) and plasma cells (5 ± 25 VS3 ± 7cells / µl, p <0.001).

Given the fact that in the group of patients with B-CLL the treatment status was variable, we decided to investigate the impact of treatment on normal B lymphocytes. Following the separation of patients according to the number of lines of treatment received until the initiation of the study, we found drastically reduced percentages of normal B lymphocytes . This feature was present regardless of the number of lines of treatment received, as a common denominator of the entire group (Figure 7.42). The percentage of normal circulating B lymphocytes was significantly reduced in patients with B-CLL compared with controls (2.5% ± 1.4%), whether they are untreated (0.2% ± 0.4%, p <0.001), treated with one line of treatment (0.2% ± 0.6%, p <0.001) or
more than one line of treatment (0.35% ± 1.17%, p <0.001). Both groups of patients showed significantly lower percentage of B cells compared with untreated CLL patients (p = 0.005 and p = 0.003 for patients treated with one line and ≥2 lines of treatment, respectively).

The polarization in normal B lymphoid compartment is represented in Figure 7.43. Immature B lymphocytes appear significantly reduced compared with healthy controls only in the subgroup of treated patients (median 1.5% vs 2%, p <0.001). Naive B lymphocytes are significantly decreased in all subgroups of B-CLL patients compared with controls (median 1.5% and 3.8% and 1.6% vs 4.7%, p <0.001 for all 3 subgroups vs normal), but memory B lymphocytes appear significantly expanded in patients with B-CLL (81% and 73% and 56% vs 32%, p <0.001 for untreated and treated subgroup with 1 line and 2 line treatment vs control). Regarding the proportion of normal plasma cells within the B lymphoid compartment, no statistically significant difference was noticed between different study groups (1.1% and 2.8% and 1.7% vs 1.3%, p> 0.05).

**Fig. 7.42.** The relative distribution of total normal B lymphocytes in groups of B-CLL patients with different therapeutic status (*p<0.05 vs controls; † p<0.05 vs untreated ).

**Fig. 7.43.** Polarization of B lymphoid compartment in subgroups of B-CLL patients according to treatment vs control group status (*p<0.05 vs controls; † p<0.05 vs untreated ).

In absolute values, normal B lymphocytes were reduced in patients with B-CLL, but visibly affected by therapy (Figure 7.44). Normal B lymphocytes were reduced in patients with untreated B-CLL (median 49 cells / µl, p <0.001), treated with 1 line (16 cells / µl, p <0.001), or treated with ≥2
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lines (13 cells / μl, p <0.001) compared with healthy controls (139 cells / μl). Also, the differences were significant when comparing different groups of B-CLL patients with an obvious decreased values in patients who have undergone one or more lines of therapy (p <0.001 in all combinations). When different B lymphoid subsets were compared, significant decreased values were obtained, compared with controls. A dramatic decrease was observed in the naïve B cell subset, which had a median of 90 cells / μl, while the B-CLL cells from treated patients had only 3 cells / μl, the B-CLL group treated with 1 line had 0 cells / μl and in the B-CLL subgroup treated with ≥2 lines 0.35 cells/ μl (p <0.001) were obtained. However, the subset of memory B cells presented no significant differences when values obtained from the untreated B-CLL subgroup (36 cells / μl) were compared with those from controls (44 cells / μl), but B-CLL patients treated either with 1 line and with more than two lines presented a severe decrease (6 or 9 cells / μl, p <0.001). Obviously, the group of treated patients showed less memory B cells compared to the untreated subgroup of B-CLL. As absolute numbers of circulating cells / μl, plasma cells were significantly reduced in all batches of B-CLL patients (0.6 and 0.3 and 0.2 cells / μl, p <0.001 for untreated B-CLL, treated with 1 line and that treated with 2 or more lines) compared with control (1.8 cells / μl).

**Fig. 7.44.** Distribution of absolute values of total normal B lymphocytes and circulating B cell subsets in patients with B-CLL with different therapeutic status vs control (*p<0.05 vs controls; † p<0.05 vs untreated).

Then we evaluated the distribution of memory cells with different isotype subclasses. When the relative frequencies of each isotype in the memory compartment was assessed, we did not found statistically significant differences. Converting these relative frequencies to absolute numbers of circulating cells / μl, we identified significantly reduced values of all memory cells regardless of the isotype in patients who had received prior therapy compared with untreated patients. Regarding peripherals tumor burden (numbers of circulating clonal B cell / μl) there were no statistically significant differences between CLL patients previously treated and those untreated (Figure 7.46).
Fig. 7.46. Comparative chart of absolute numbers of circulating B lymphocytes in patients with or without treatment prior study.

7.5.4. Discussion

As mentioned in previous chapters, most patients with B-CLL die as a consequence of severe infections or secondary malignancies. While in the latter case, the cellular immunity is directly involved, in the case of infections, mostly bacterial, the humoral immunity has the main role. Obviously, in most cases of B-CLL, the tumour burden is huge, thus, the distribution of normal cells is rarefied and the encounter with antigen specific cells is hindered. Also, the treatment, either chemotherapy, or immunotherapy, or both, combined, creates an even greater imbalance. The magnitude of this imbalance in lymphoid B cell compartment has not been quantified, for various reasons, including the lack of sufficiently sensitive methods. When appropriate combinations of molecules are used in optimized panels, multiparameter flow cytometry enables to concomitantly identify malignant cells and normal, residual lymphocytes.

To our knowledge, no simmilar data can be found in the literature. The reduced proportion of normal B lymphocytes in B-CLL patient is repeatedly reported, however no information on the polarization of normal B lymphoid compartment in patients with CLL can be found. Among the few studies published on this topic, we mention the research on normal B lymphoid compartment in patients with monoclonal B lymphocytosis. In this study significantly reduced absolute numbers of normal circulating B lymphocytes were calculated in MBL patients, either when the total B cell compartment was considered, or in various B cells subsets. Their results indicate the magnitude of humoral intrinsic defect in a pre / leukemia, pre / LLC condition.

In the current study severely decreased values were recorded, especially in the naïve B cells compartment, possibly because these patients were in an advanced stage of the disease, with a massive bone marrow infiltration preventing normal B lymphopoiesis. Subsets of memory and circulating immature B cells, although they were significantly reduced compared with healthy individuals, seemed less affected. This observation suggests that the alteration processes of applied therapies are irreversible and explains the high frequency of infections in treated patients. Marrow lymphopoiesis is maintained at low levels compared both to healthy individuals, and to patients untreated. De novo formation of effector B cells (plasma cells) do not differ between patient groups with and without treatment, but it is decreased compared to the control group.

The decreasing percentages and absolute numbers of normal B lymphocytes appear to be early events in the emergence of B-CLL, as it can be observed at the stage of pre-leukemia MBL, and the more obvious the more advanced MBL (increased numbers of clonal B lymphocytes but not yet meeting the criteria for LLC). The decrease is mainly due to the reduced immature and naive cells. The hypothesis emerging from this observation is that clonal B cells infiltrate the bone marrow, having a suppressive effect on normal B lymphopoiesis and the inverse correlation between the number of clonal and normal B lymphocyte could have a prognostic role. The current study captures these alterations of the normal B lymphoid compartment and document their aggravation due to therapy. Furthermore, in the case of B-CLL patients, not only immature and naïve B cells are significantly reduced compared to healthy controls, but also normal memory B lymphocytes investigated in accordance with specific isotype. Extended defects in all immunoglobulin subclasses (IgM, IgG and IgA) were identified. A dynamic assessment of these defects during therapy the their comparison between different types of therapies, will generate data important for the understanding of the manifestation of these events in vivo. The corroboration of these information with clinical data will
provide a concise picture, showing the impact of therapy on normal residual cells and on the period necessary for their recovery.

Chapter 8. Phenotypic and genotypic profile of T and NK lymphocytes in B-CLL

8.1. Introduction

Although the lineage of the malignant clone is of B cell type, significant qualitative and quantitative defects of T lymphocytes were equally described in patients with B-CLL. Compared to healthy individuals lower percentages, but higher absolute values of T and NK circulating cells have been reported in the peripheral blood of B-CLL patients. Both single positive (CD4 + or CD8 +) subsets can be found in increased numbers, while the ratio between them is subunitary in some patients, especially in those with an advanced stage of the disease.

It was recently suggested that chemokine receptors expressed by memory and / or effector cells are equally important for their migration towards the inflamed anatomical sites or for their recirculation between distinct immune sites. The polarization of T helper to a specific pattern (CCR4) was associated with Th2- cytokine type secretion, while CXCR3 was frequently associated with a Th1 response. The current study intended to perform the characterisation of different maturing and functional T and NK lymphoid subsets in samples from patients in different stages of the disease. Also, the study aims to characterize the TCR Vbeta region.

8.2. Material and method

Peripheral blood mononuclear cells (PBMC) separated by density gradient from 43 B-CLL patients were taken into account. Distinct subsets of T CD4 + (naive, central memory, memory peripheral / effector, regulatory, follicular-TFH, CXCR3 and / or CCR4 +), T CD8 + (naive + memory effector senescent CD57 +) and NK lymphoid cells (according to the expression of CD57 and / or CD94 +) were identified and compared between different Rai stages.

For the molecular biology study of T cell receptor (IdentiClone™ TCRB Clonality Assay – Beckman Coulter CE/NAT Platforms) PBMC were used from 50 patients with B-CLL in various Rai stages (10 in Rai0, 14 in Rai1 13 in Rai2, 5 in Rai3 and 8 in Rai 4).

8.3. Results

8.3.1. The distribution of T lymphocytes (total and subsets) and NK lymphocytes in samples from patients with B-CLL

Eloquently illustrated in Figure 8.1, a statistically significant expansion of T lymphoid compartment was found when Rai0 (mean of 2244 ± 723 cells / µL, median 2419 [768-3168] cells / µL) was compared toRai1 (mean of 3784 ± 1846 cells / µL, median 3639 [1100-7348] cells / µL, p = 0.03 vs Rai0), Rai2 (mean of 5252 ± 2195 cells / µL, median 5615 [2086-9090] cells / µL, p = 0.001 vs Rai0), and Rai3 stages (mean of 12,736 ± 7368 cells / µL, median 12656 [4655-24223] cells / µL, p = 0.002 vs Rai0).

8.3.2. CD4 / CD8 ratio in patients with B-CLL

Since there are studies that observed changes in the CD4 / CD8 ratio in patients with B-CLL, we decided to investigate this relation in patients from our group with CLL, according to their Rai stage. In the group of patients studied we did not identify statistically significant differences (p> 0.05) between the different Rai stages. However, there is a sharp drop in Rai4 (median 0.7 [0.3 to 6]) with regards to Rai0 (1.45 [0.73 to 3.84]), Rai1 (1.15 [0.52- 8]), Rai 2 (1.53 [0.54 to 3.6]) or Rai3 (1.09 [0.5 to 2.23]).
8.3.3. Circulating CD4 + T lymphoid compartment in patients with B-CLL

The classification on maturation stages of CD4 + T lymphocytes allowed us to develop a database and to perform a comparative evaluation of CD4 + T cell subsets in patients with different stages of B-CLL. In the study group, no statistically significant differences were found when the presence of naive CD4 + T cells from different Rai stages was evaluated. There were differences identified in absolute numbers of circulating cells from different stages of the disease in the case of the following subsets: memory, effector and follicular Th cells (Figure 8.5).

8.3.4. The polarization of T helper CD4 + circulating lymphoid compartment in B-CLL patients

Generally, the group of patients with B-CLL showed mean values of percentages of CXCR3 expression in the CD4 + compartment of 31 ± 16%, with a median of 33%. CCR4 expression was of 23 ± 8% for T helper lymphocytes, while co-expression of these receptors was found in 13 ± 10% of CD4 + T lymphocytes. The relative frequency of CD4 + T cells expressing the CXCR3 receptor increases progressively and in a statistically significant manner from stage Rai0 up to Rai4 (p <0.05) (Fig. 8.9).
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8.3.5. Distribution of regulatory T lymphocytes in the studied group

In the B-CLL group of patients studied by us, absolute numbers of circulating regulatory T lymphocytes progressively increased in advanced stages of the disease, except the Rai4 stage compared to stage Rai0. The distribution and the statistically significant differences are illustrated in Figure 8.13.

8.3.12. The clonality of T cell receptor (TCR) in B cell chronic lymphocytic leukemia

During the current study, five 5 patients with clonal T cell expansion, belonging to different categories of disease (2 patients in Rai0, 2 patients in Rai1 and 1 patient in Rai4) were identified. Within the investigated patient group oligoclonal T cell expansions were identified in 11 patients at different stages of B lymphoid disease: two patients in Rai0, 6 patients in Rai1, and 3 patients in Rai4.

It should be of note that from the stages Rai2 and Rai3 no clonal expansions were identified (neither monoclonal, nor oligoclonal). The remaining patients had a Vbeta polyclonal repertoire.
8.4. Discussion

Understanding the biological basis of clinical heterogeneity of B cell chronic lymphocytic leukemia remains limited. B-CLL pathogenesis is complex and can apparently be explained by B cell intrinsic pathological features (discussed in detail in the previous chapter), but also by the information provided by the microenvironment in which the malignant clone survives and self-maintains, thereby evading from immune anti-tumour responses. The first descriptions of these T lymphoid abnormalities in patients with B-CLL date more than 20 years ago, but the progress in elucidating the specific relations of malignant lymphoid cells and their “normal” immune microenvironment was rather slow, due to the constant discoveries of new molecules and cellular interactions 

Using new technologies and consensus, a general agreement emerged on the fact that, depending on the number of molecules used in the study, the more molecules, the more subsets of T lymphoid cells with distinct phenotype can be identified. It is also increasingly clear how difficult the staining and the identification of all circulating populations can become when large combinations of markers are used. Absolute numbers of total circulating T lymphocytes were reported as being normal or often increased in patients with B-CLL, any T lymphocytopenia is only relative. T lymphoid expansion is due equally to the expansion of CD4 + and CD8 + T lymphocytes, with a ratio reported to be reversed and having a prognostic value in advanced stages of the disease 

Consistent with the observations resulted from our study; the expansion of CD4 + helper T lymphocytes in B-CLL cells is due to an augmentation of mature T cells with a phenotype suggestive for an activated status, cells that either have already encountered the antigen, or are generated as a result of an ongoing immune response to the antigen at the time of sample collection (T memory or effector cells). Naïve CD4 + T lymphocytes are found significantly reduced in absolute numbers when compared to healthy controls from the early stages of the disease. The current study provided additional observations, that memory and effector T lymphocytes show gradually increased absolute numbers in relation with progression to more advanced disease stages. Therefore, we can be lead to the assumption that, during the pathology progression, a chronic stimulation correlated with the disease stage occurs. Not only central and peripheral memory T cells, but also follicular circulating T lymphocytes, defined by the CXCR5 receptor expression are found to have increased numbers in B-CLL patients. The identification of helper follicular circulating T cells in the peripheral blood has been confirmed as being relevant in the case of autoimmune diseases, such as lupus or rheumatoid arthritis.

In our study we observed a statistically significant and progressive increase (when any of the advanced stages were compared to the Rai0 stage, considered the earliest stage of the disease) of the CD4 + CXCR3 + subset of T lymphoid cells, associated with a Th1 profile and a decrease of the CD4 + CCR4 + T cell subset associated with a Th2 profile type. Studies of gene expression in T lymphocytes from patients with B-CLL reveal a number of changes induced by B cell leukemic clone in these cell compartments. A reduced expression of genes involved in major signalling pathways involved in the differentiation of CD4 + Th1 and Th2 cell subtypes was identified within the CD4 + T cell compartment. The authors suggested that, at a functional level, the identified changes should result in a reduced ability to perform differentiation into Th1 cells. Previous studies have captured a shift of the balance towards a Th2 profile. These results do not reproduce the observed gene expression defects by means of a direct analysis of T lymphocytes from B-CLL patients, but identify the altered expression of chemokine receptors, including an increased expression of the CXCR3 and CCR4 receptors. Also it should be of note that, in previous, already reported studies, significant changes were found when comparing the data to those obtained from healthy controls, while in our study a comparative evaluation between different B-CLL stages was intended.

Using the CD4 + CD25 + high phenotype to identify regulatory T lymphocytes, Beyer et al. have found a high percentage of regulatory T lymphocytes in patients with B-CLL compared with healthy donors and a correlation of their frequency with the Binet stages of the disease. Similarly, Giannopoulos et al. identified high percentages of CD25 + high FOXP3 + regulatory T lymphocytes and a correlation with the Binet stage, but not with other prognostic factors, such as the expression of CD38 and ZAP70 in CLL. Jak et al. have identified regulatory T lymphocytes by the following phenotype: CD4 + CD25 + high CD127 low and showed higher absolute circulating numbers in patients with B-CLL, reporting that the percentage of regulatory T lymphocytes increases with the Rai stage of the disease. Evaluation of the frequency of regulatory T cells in patients with different prognosis can be useful in interpreting the immune mechanisms responsible for the progression of the disease.
The exact mechanisms of in vivo T cell clonal/ oligoclonal expansion in patients with B-CLL are unclear. There are data that infirm the anti-tumour specificity, so that further justifications are required. For example, especially among CD8+ T lymphocytes, expansions can be related to age \(^{433, 434}\), sometimes can be caused by viral chronic infections \(^{435}\). However, a clonal expansion due to age would not explain the increase observed within the T lymphocyte populations (CD8+ and, less frequently, CD4 + T cells) in B-CLL patients with where these values increase in parallel with the expansion of the B lymphoid clone.

Our study open further research directions, one of them being the more in-depth analysis of B-CLL related T cell clonality and how is this translated in clinical manifestations. Questions remain to be answered. When the T cell clonal expansion becomes clinically manifested? It is known that B-CLL patients frequently manifest autoimmune diseases and immunodeficiencies as a consequence of this primary malignancy. The T cell clonal expansion involves only the memory compartment or is linked to an immediate T cell effector? Clonal / oligoclonal expansion in B-CLL is a normal physiological condition related to the patients’ age? To answer all these questions additional studies are needed, studies targeting CD4 and CD8 subsets on larger groups of thoroughly investigated patients.

**Chapter 9. Conclusions**

1. The study of phenotypically aberrant cells by flow cytometry, using EuroFlow panels, allows a specific and sensitive identification of clonal populations and the implementation of an accurate differential diagnosis of patients with B-cell chronic lymphoproliferations.
2. The frequency of genetic aberrations identified in the B lymphoid clone in our group of patients is similar to overall frequencies described in the literature. When these aberrations were associated with the clinical behaviour and disease course, those patients with indolent diseases were found to carry either normal genotypes or unique genetic alterations, while the majority of patients in an advanced stage of the disease had complex genetic aberrations.
3. Residual normal B lymphocytes are reduced in terms of relative and absolute numbers in B-CLL patients manifesting an active disease (advanced stages, requiring immediate treatment) when compared with healthy individuals of the same age. This decrease mainly affects the naïve cell compartment.
4. Polarization within the normal B lymphoid compartment of patients with B-CLL is oriented to memory (probably pre-existing memory) with a possible inhibition of the bone marrow hematopoietic function, due to its massive infiltration with pathological cells.
5. The treatment affects on a long-term the normal B lymphoid compartment, our study revealing that intrinsic defects of the disease are augmented in treated B-CLL patients, regardless of the line number or type of treatment received.
6. Our study developed an accessible and reproducible strategy, allowing the identification by multiparameter flow cytometry of the maturing and functional lymphoid cell stages, either of B (immature, naïve, memory, plasma cells) or T ( naïve, memory and effector, regulatory, follicular helper, with Th1 or Th2 profile) type. This strategy can be applied to other diseases affecting the immune system (such various types of immunodeficiencies).
7. Total circulating T lymphocytes are found in significantly and progressively increased absolute numbers in patients with B-CLL, augmentation attributed to both major subsets of T cells: CD4 + T helper and CD8 + cytotoxic T cells.
8. In the T lymphoid compartment, a selective T cell expansion, associated to the disease aggressiveness, was identified, involving memory, effector, regulatory, and T follicular helper T lymphocytes, but not naïve T helper lymphocytes.
9. The expression profile of CXCR3 and CCR4 chemokine receptors on the T lymphocytes membrane is modified in correlation to the stage of the disease, suggesting a polarization to a Th1 type of response.
10. These changes within the T lymphocyte compartment gradually augment as the disease progresses from the stage Rai0 to the stage Rai3, while for the Rai4 stage a different profile was described.
11. In our study, the expansion of T lymphocytes is not associated with the preferential use of any single family of TCRVβ chain, in most cases an either oligoclonal, or polyclonal recombination being identified.

12. Our study highlights the complexity of the immune system in the context of an oncologic disease and represents an illustration of a complex, interdisciplinary research approach, at various cellular and molecular levels, normal and pathological, of patients diagnosed with a frequent, relatively well studied hematologic disease, but still incurable: B cell chronic lymphocytic leukemia.

References


Original papers published during the doctoral study

Georgiana E. Grigore, Iuliu C. Ivanov, Mihaela Zlei, Angela Dascalescu, Roxana Popescu, Tudor Petreus, Eugen Carasevici, Specific Associations Between Clinical Signs, Immune Cells, Disease Genetic Background and Burden in a Group of Patients with B-Cell Chronic Lymphocytic Leukemia, Romanian Review of Laboratory Medicine, 2014; 22(1):79-92 (ISI)


Georgiana E. Grigore, Angela Dascalescu, Mihaela Zlei, Iuliu C. Ivanov, Catalin Danaila, Tudor Petreus, Eugen Carasevici, Rai stage-related changes within T/NK cell populations from B-CLL patients, Romanian Review of Laboratory Medicine, 2013; 21(3):321-331 (ISI)