Universidade de Aveiro Departamento de Química
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Catarina Morgado Simões

Frequência e atividade funcional das células T Vδ1 em Leucemia Linfocítica Crónica e Linfocitose B Monoclonal

Frequency and functional activity of Vδ1 T cells in Chronic Lymphocytic Leukaemia and in Monoclonal B Cell Lymphocytosis

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, ramo de Bioquímica Clínica, realizada sob a orientação científica do Professor Doutor Artur Augusto Paiva, Coordenador da Unidade de Gestão Operacional de Citometria (UGOC) dos Hospitais da Universidade de Coimbra – Centro Hospitalar e Universitário de Coimbra (CHUC) e da Professora Doutora Maria do Rosário Gonçalves Reis Marques Domingues, Professora Associada com Agregação do Departamento de Química da Universidade de Aveiro

Apoio Institucional:

Dedico este trabalho aos meus pais e aos meus avôs.

o júri

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agradecimentos

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"Não sou nada. Nunca serei nada. Não posso querer ser nada. À parte isso,

tenho em mim todos os sonhos do mundo"

Álvaro de Campos

Palavras-chave

Células T Vdelta1, células T gamma delta, leucemia linfocítica crónica, linfocitose B monoclonal, atividade citotóxica, atividade efetora, imunoterapia

resumo

A leucemia linfocítica crónica (LLC) é uma doença linfoproliferativa crónica de células B caracterizada pela proliferação descontrolada de linfócitos B patológicos. É uma doença de curso geralmente indolente, mas que pode em alguns casos progredir rapidamente e necessitar de tratamento. Com vista a melhorar o prognóstico destes doentes, imunoterapias baseadas em células T têm vindo a ser desenvolvidas, com especial interesse numa subpopulação minoritária dos linfócitos T: células T γδ. Estas células representam, normalmente, menos de 10% dos linfócitos T circulantes, apresentando características distintas dos linfócitos αβ, o que lhes confere vantagem quando selecionadas para terapia celular. Dentro deste grupo de células, os linfócitos T Vδ1 parecem exibir atividade citotóxica contra células B de LLC, e como tal podem vir a ser utilizadas em protocolos de imunoterapia anti-tumoral. No entanto, o comportamento destas células *in vivo* é francamente desconhecido tanto em situações de normalidade, como de doença. O objetivo deste projeto foi estudar a frequência das células Vδ1 em sangue periférico e avaliar, através de estudos imunofenotípicos, a sua atividade efetora e citotóxica (expressão de CD27, CD69 e granzima B) em casos de LLC, de linfocitoses B monoclonais (LBM) e num grupo controlo. Os resultados mostraram uma expansão do compartimento efetor em todas as subpopulações de linfócitos T, particularmente nas células TCD8+ e nas células T Vδ1, o que se correlacionou com um aumento destas células a expressar granzima B desde o grupo controlo até ao grupo de LLC com estádio mais avançado da doença. A percentagem de células a expressar o marcador de ativação CD69 foi marcadamente mais alta nas células T Vδ1, mas no grupo controlo. Assim, os resultados obtidos sugerem que as células TVδ1 parecem apresentar um fenótipo efetor citotóxico, com características semelhantes às células TCD8+ e restantes subpopulações de células T γδ, o que parece evidenciar que conjuntamente com outras células, as células TVδ1 podem contribuir para uma atividade na resposta antitumoral contra células B de LLC.

keywords

Vdelta1 T cells, gamma delta t cells, chronic lymphocytic leukaemia, monoclonal B cell lymphocytosis, cytotoxic activity, effector activity, immunotherapy

abstract

Chronic lymphocytic leukaemia (CLL) is a B-cell chronic lymphoproliferative disease characterized by an uncontrolled proliferation of pathological B lymphocytes. This disease is normally of indolent progression, but it may, in some cases, progress rapidly and require treatment. In order to improve the prognosis of these patients, T-cell based immunotherapies have been developed, with special interest in a minor subpopulation of T lymphocytes: γδ T cells. These cells represent, normally, less than 10% of circulating T lymphocytes and exhibit distinct characteristics comparing to αβ T lymphocytes providing them with advantages when selected for T cell therapy. Within this group, Vδ1 T lymphocytes seem to display cytotoxic activity against B cells from CLL, conferring to these cells the ability to be used in antitumoral immunotherapies. However, the behaviour of these cells *in vivo* is frankly unknown in both normal and disease situations. The objective of this project was to study the frequency of Vδ1 T cells on peripheral blood and to evaluate, through immunophenotypic studies, their effector and cytotoxic activity (expression of CD27, CD69 and granzyme B) in CLL, monoclonal B cell lymphocytosis (MBL) and controls. Our results disclosed an expansion of effector compartment in all subpopulations of T lymphocytes, particularly for CD8+ T cells and Vδ1 T cells, which correlated with an increase in these cells expressing granzyme B from control group to advanced stages of CLL. CD69 expression was markedly higher in Vδ1 T cells, in the control group. In conclusion, the results obtained suggest that Vδ1 T cells appear to present an effector cytotoxic phenotype, with similar characteristics to CD8 + T cells and the remaining γδ T cells, which might point to a contribution to an antitumor response against CLL cells, mediated by the interplay between these cells.

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[b] – P<0,05, comparing individually this group with HC-MBL and controls group.
[c] – P<0,05, comparing individually this group with Binet A CLL group.
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[a] – P<0,05, comparing this group with group control

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[a] – P<0,05, comparing individually this group with Binet A CLL group and control group.

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[a] – P<0,05, comparing individually this group with control group.

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[a] – P<0,05, comparing individually this group with control group.

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values ≤ 0,05 for all T cell subsets. LC-MBL, on the other hand, only presented statistical significant differences in CD4+ T cells and CD4+CD8+ T cells.
[a] – P<0,05, comparing individually this group with control group.

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Graphic 21 - Percentage of CD27+ T cells Granzyme B+ - T cells belonging to CD27+ compartment are represented in terms of percentage that are positive for the expression of granzyme B. CD4+ T cells display lower frequency of cells expressing granzyme B in all groups understudy. The other T cells subpopulations did not present any specific pattern concerning to the frequency of T cells expressing granzyme B.
[a] – P<0,05, comparing individually this group with control group.
[b] – P<0,05, comparing individually this group with LC-MBL group.

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[a] – P<0,05, comparing individually this group with control group.

Graphic 23 - Percentage of CD27+ T cells expressing CD69 marker – T cell subpopulations displayed a heterogeneous expression of CD69 marker, but with higher frequencies of T cells expressing this marker observed for both Vδ1 T cells and other γδ T cells subpopulations.

IV. LIST OF ABBREVIATIONS

CLPDs: Chronic Lymphoproliferative Disorders
WHO: World Health Organization
CLL: Chronic Lymphocytic Leukaemia
NHL: Non-Hodgkin Lymphomas
B-CLPDs: B-cell Chronic Lymphoproliferative Disorders
BCR: B-Cell Receptor
GCR: Germinal Centre Reaction
SHM: Somatic Hypermutation
CSR: Class Switch Recombination
EBV: Epstein-Barr Virus
HHV8: Human Herpes Virus 8
MBL: Monoclonal B Cell Lymphocytosis
FISH: Fluorescent In Situ Hybridization
SLL: Small Lymphocytic Lymphoma
HC-MBL: High Count Monoclonal B Cell Lymphocytosis
LC-MBL: Low Count Monoclonal B Cell Lymphocytosis
CHOP: Cyclophosphamide, Hydroxydaunorubicin, vincristine (Oncovin®) and Prednisolone
R-CHOP: Rituximab, Cyclophosphamide, Hydroxydaunorubicin, vincristine (Oncovin®) and Prednisolone
IFN-γ: Interferon Gamma
MHC: Major Histocompatibility Complex
TNF-α: Tumour Necrosis Factor Alpha
TRAIL: TNF-Related Apoptosis-Inducing Ligand
T-regs: Regulatory T Cells
ACT: Adoptive Cell Transfer
TCR: T Cell Receptor
CAR: Chimeric Antigen Receptor
TIL: Tumour Infiltrating Lymphocyte
HLA: Human Leukocyte Antigen
HSC: Hematopoietic Stem Cell
DN: Fouble Negative
DP: Double Positive
SP: Single Positive
Tnfrsf7: Tumour Necrosis Factor Receptor Superfamily, member 7
APC: Antigen Presenting Cell
TLR: Tool-Like Receptor
TRAFS: TNF Receptor Associated Factors
PAMPs: Pathogen Associated Molecular Patterns
DAMPs: Danger-Associated Molecular Patterns
PRR: Pattern Recognition Receptors
HMBPP: (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate
IPP: Isopentenyl Pyrophosphate
BN3A1: Butyrophilin 3A1
MCP-1: Monocyte Chemoattractant Protein 1
RANTES: Regulated on Activation, Normal T Cell Expressed and Secreted
MIP-1α: Macrophage Inflammatory Protein-1α
MIP-1β: Macrophage Inflammatory Protein-1β

DNAM-1: DNAX Accessory Molecule-1
MDSCs: Myeloid-Derived Suppressor Cells
NCR: Natural Cytotoxicity Receptor
DOT-cells®: Delta One T cells
LST: Lymphoid Screening Tube
CRP: C-Reactive Protein
ESR: Erythrocyte Sedimentation Rate
FITC: Fluorescein Isothiocyanate
PB: Pacific Blue
APC: Allophycocyanin
PE: Phycoerythrin
PE-Cy™7: Phycoerythrin-Cyanin 7
PerCP-CY™5.5: Peridinin chlorophyll protein-cyanine 5.5
APC-H7: Allophycocyanin-Hilite 7
PO: Pacific Orange

INTRODUCTION

INTRODUCTION

1. B-CELL CHRONIC LYMPHOPROLIFERATIVE DISORDERS: AETIOLOGY, PATHOFISIOLOGY AND THERAPEUTIC APPROACHES

1.1. AETIOLOGY AND PATHOFISIOLOGY OF B-CELL CHRONIC LYMPHOPROLIFERATIVE DISORDERS

Chronic lymphoproliferative disorders (CLPDs) are malignancies of the lymphoid lineage (B, T or NK cells) that are characterized by an uncontrolled proliferation of abnormal lymphocytes. They are a heterogeneous group of diseases that vary enormously in their clinical progression, from indolent to aggressive evolutions. CLPDs are classified, by the World Health Organization (WHO) classification of lymphoid neoplasm, based on a wide variety of morphologic, immunologic and clinical findings, depending on the different stages of maturation and differentiation that these affected cells are. Of note, they are not steady diseases, frequently progressing from one to another, and are often associated with genetic abnormalities that may or may not be correlated with a poor prognosis(1).

According to *Harrison's Manual of Medicine*, about 75% of all lymphoid leukaemia and 90% of all lymphomas are of B-cell origin(2). Chronic lymphocytic leukaemia (CLL) is the most prevalent form of leukaemia in Western countries(2,3) and the Non-Hodgkin lymphomas (NHL) account for about 62% of all lymphoid malignancies(2). CLL is more prevalent in male and in older people, however the aetiology of typical CLL is, so far, unknown(3). On the other hand, NHL are normally associated with people suffering from primary or secondary immunodeficiencies and are frequently associated with an exposure to infectious agents(2,4).

The development of B-cell chronic lymphoproliferative disorders (B-CLPDs) is associated with dysfunctions occurring in the several steps occurring in maturation and differentiation of B cells. Normal B-cell development occurs by a series of steps that lead to the formation of mature, naïve B cells that express a B-cell receptor (BCR) complex at their surface. BCR is composed by a membrane-bound immunoglobulin of one isotype (IgD, IgM, IgA, IgG or IgE), structure responsible for antigen recognition, and a heterodimer cofactor: CD79 (Igα/Igβ), responsible for signal transduction (as CD3 in T cells), acting through interactions with intracellular tyrosine kinases through their

cytoplasmic tails(5). The surface immunoglobulin is composed of 4 polypeptide chains: 2 heavy and 2 light chains that are produced by somatic V(D)J recombination, via RAG1 and RAG2, of the respective loci.

As illustrated in figure 1, B-cell maturation starts with rearrangement of D and J gene segments of the immunoglobulin heavy chains leading to the formation of pro B-cells. Further V gene segment rearrangement and pairing with a surrogate light chain lead the B cell to the state of pre-BCR. Opposing to D and J gene segments rearrangement, V gene segment rearrangement occurs only in one allele(5). If the BCR produced is functional, rearrangement of the second allele is stopped, a process called allelic exclusion, and the cell reaches the phase of pre-B cell. If not functional, the cell tries second allele rearrangement and if this process doesn't produce a functional BCR, the cell is targeted for apoptosis. When the B cell reaches the pre-B cell state, rearrangement of the light chain initiates. Light chain rearrangement occurs only in V and J segments starting with κ loci and with λ loci rearrangement only occurring if rearrangement of κ loci is not functional, a process called isotype exclusion. At the end of this phase, if both chains form a stable and functional BCR, immature B-cell phase is reached. At this point all B cells have immunoglobulins of IgM isotype and the cell is tested for autoreactivity. If autoreactive, the cell tries further rearrangements that, if not successful, lead to B-cell anergy and apoptosis. If the cell doesn't present autoreactivity it is now considered a mature, naïve B cell. Mature B cells leave the bone marrow, populate secondary lymphoid tissues and circulate in peripheral blood(4,5).

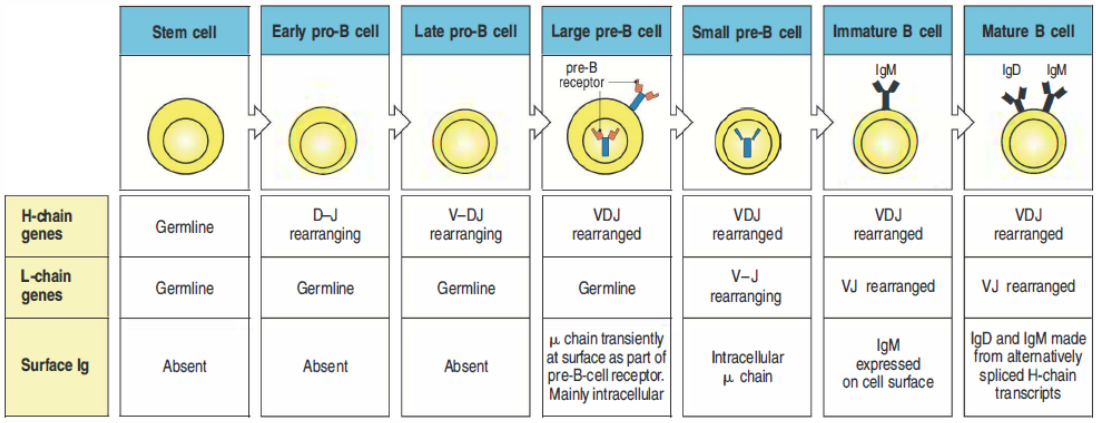


Figure 1 – Maturation steps in B lymphocytes. In this figure, it is illustrated the series of steps that begin with a hematopoietic stem cell and finish with a mature, naïve B cell, demonstrating the changes occurring in the genes encoding the immunoglobulin's heavy and light chains that will be expressed at the surface of B cells. After several V(D)J rearrangement steps, a mature, naïve B cells is produced, expressing a B cell with a BCR that includes the surface-bounded immunoglobulin and a heterodimer cofactor: CD79. From Murphy KM. *Janeway's Immunobiology*. 8th ed. Taylor & Francis Group; 2011. 888 p.(5).

As they encounter an antigen, B cells migrate to the T cell zones of secondary lymphoid organs where CD4+ helper T cells stimulate further B cell proliferation. Some B cells turn into plasma cells, that produce antibodies with low antigen-specificity (first response to antigens) and others migrate with T cells to the B cell follicles, where they initiate the germinal centre reaction (GCR), demonstrated in figure 2. The germinal centre is composed by a dark zone where occurs intense proliferation and somatic hypermutation (SHM) of B cells and a by light zone where B cells are put together with follicular helper T cells plus follicular dendritic cells and where occurs class switch recombination (CSR). These two zones are surrounded by a marginal and a mantle zones. The processes occurring in GCR aim to increase BCR affinity to the specific antigen. In SHM a series of proliferation/genetic alterations occur in the already rearranged immunoglobulin heavy chain variable region (IGHV) genes, leading to the formation of B cells with altered affinity to the specific antigen. In CSR, the immunoglobulins expressed at the surface of B cells can, through genetic expression of different chains, turn into other isotype, a process that is associated with modification of the effector functions performed by these cells(6). Of note, B cells don't necessarily modify their immunoglobulin class. These mechanisms of selection preclude the survival of the B cells with increased affinity to the antigens and lead those with less affinity to the antigen to targeted cell death. After rounds of proliferation, genetic modifications and positive selections, the B cells expressing the best properties are divided in two subgroups: plasma cells that now secrete antibodies with high-antigen specificity and memory B cells, that allow a faster response in a second encounter with the antigen. Although this process is much more complex than what it is described in this review, this resume allows the understanding that these processes are associated with an intense proliferation and a high degree of genetic modifications. Even though all these processes are tightly regulated, sometimes the regulatory mechanisms fail and that's the point where disease might begin. A more detailed description of this process can be read in the chapter written by Seifert and colleagues(4).

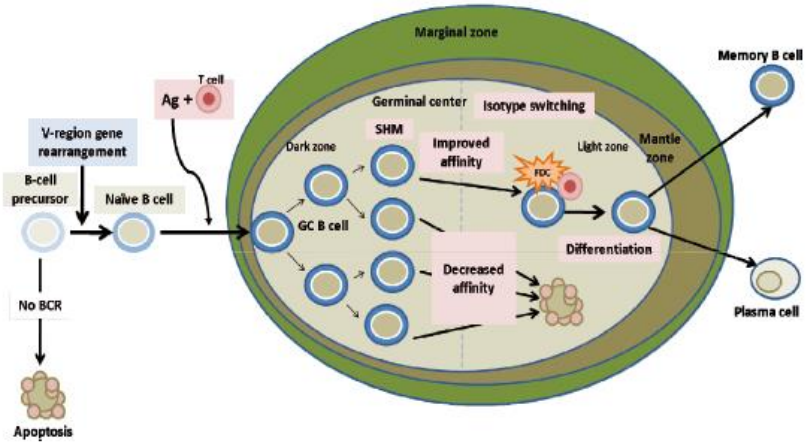


Figure 2 - Germinal Centre Reaction. In this figure, it is represented the structure of a germinal centre: dark zone, where proliferation and SHM occurs, light zone where CSR occurs, mantle and marginal zones. In the processes occurring in germinal centre reaction (occurring after antigen encountering), B cells are prone to acquire genetic mutations that might lead to the development of a B-CLPD. From Zainuddin N. *Molecular Genetic Analysis in B-cell Lymphomas: A focus on the p53 pathway and p16 INK4a*.,2010(7)**.**

Although some B-CLPD may present pathological B cells with a phenotype of naïve/unmutated B cells (CD27-), as occurs in unmutated CLL(3), most of them present a phenotype of memory/mutated B cells (CD27+). As stated before, the transformation of naïve B cells to memory and effector B cells occur in GCR, suggesting that the transformation of normal B cells to pathological B cells occurs somewhere in the reactions that occur in the germinal centre. It is now believed that this increased incidence of CD27+ pathological B cells is associated with the:

(1) Intense proliferation of B cells in the dark zone: The rapid and intense proliferation of B cells in germinal centre might be associated with an increased rate of errors in replication that might surpass cell cycle checkpoints allowing the proliferation of cells with DNA damage. TP53 deletion is associated with this failure as this gene produces p53, a tumour suppressive protein that targets damaged cells to apoptosis. In cases where this genetic alteration is present, the capability of tumour progression is augmented. Thus, deletion of TP53 gene is associated with poor prognosis in B-CLPD, but also in almost all types of cancers(8–10), as it will be further explained.

(2) The genetic modifications that occur in V(D)J recombination (pre-GCR), SHM and CSR can give rise to harmful mutations that can lead to B-cell transformation. A frequent observation in these malignancies is a translocation involving an Ig locus and a proto-oncogene. Since Ig loci are associated with

strong expression, the translocation of a proto-oncogene, that starts to be under the control of Ig loci promoters, results in an extensive expression of this oncogene with the consequence of "out of control" proliferation of the cells carrying this mutation. An example of these translocations is Bcl-2 translocation in follicular lymphoma that is encountered in about 80-90% of these lymphomas and it is associated with an augmented survival of the mutated cells, that would normally suffer apoptosis, increasing their potential to acquire even more genetic alterations(11).

Besides that, alterations in chromatin remodelling and deletions of micro-RNAs have also been found in malignant cells of CLL and in B-cell lymphomas, both associated with a deregulation of tumour suppressor genes expression and consequently with an increased chance of tumour progression(12,13). Despite these facts, B-CLPD might have another origin not related to B-cell development. As stated above, these malignancies might be associated with exposure to infectious pathogens. Among these, Epstein-Barr Virus (EBV), Human Herpes Virus 8 (HHV8) and hepatitis C virus are the most frequently reported. These viruses infect B cells and express some proteins that mimic B cell components, such as BCR or CD40, a receptor expressed in B cells that engages to CD40 ligand expressed in activated T cells(14), providing these cells with increased survival signals and favouring their selection in GCR(15). Some viruses might even rescue B cells that were already marked for apoptosis and, through the expression of the receptors stated above, provide them the ability to survive. They can act as chronic stimulators of these cells providing critical survivor signals allowing their survival and proliferation(16).

Despite the enormous variety of B-CLPD described in the literature, this project will only rely in the phenotype of Vδ1 T cells in two entities: CLL and in monoclonal B cell lymphocytosis (MBL), entities that will be further discussed in the next topics.

1.2. CHRONIC LYMPHOCYTIC LEUKEAMIA

CLL is, as stated previously, the most prevalent form of leukaemia in Western countries. It is more prevalent in men and in older people (median age of diagnosis: 72 years) with an incidence of 4.2:100.000/year, an incidence that increases in older ages(3). Phenotypically, pathological CLL cells present with positive expression of CD5 antigen, a marker that is expressed in T cells and absent in normal B cells. Besides that, pathological B cells also present with a dim expression of CD20 antigen, positive

expression of CD23, CD43 and CD200 (strong expression), are negative for CD10 and CD79b expression is considered dim to negative. CD38 expression is variable and its positivity had been correlated with a poor prognosis of the disease, being this possible linked to an increased resistance to the chemotherapeutic agents used in the treatment of the disease(17). These cells are restricted to one of the immunoglobulin light chains, being clonal to kappa or lambda chains (with weaker expression when compared to normal B cells). To establish a diagnosis of CLL, the patient might present more than 5000 pathological B cells per μL of peripheral blood, being the clonally confirmed by flow cytometry. A blood smear is also used to identify the malignant cells. In order to distinguish from other entities that might also express CD5 antigen, such as mantle cell lymphoma, markers as FMC7 and CD23 might be useful, as well as Fluorescent In Situ Hybridization (FISH) studies to find specific translocations and other mutations related with B-cell lymphomas. When the pathological B cells represent less than 5000 cells per μL of peripheral blood, we might be in the presence of a small lymphocytic lymphoma (SLL) or a MBL, as discussed in the next issue(3).

To predict the median survival for patients with CLL two staging systems are used: Rai and Binet staging systems, being the Binet staging system the most used in European countries. These staging systems classify the patients in different groups of risk, taking in account the number of lymphocytes, thrombocytes and haemoglobin levels, data available in a full blood count, but also the presence of ganglionic involvement (lymphadenopathies) and organ damage (organomegaly), observed by a CT scan(3). This risk stratification allows physicians to take decisions about the treatment to apply. The two staging systems are represented in figure 3. It is important to highlight that despite the fact that patients are classified at the moment of the diagnosis, a patient follow-up is absolutely necessary, as the disease might progress, becoming of higher risk and perhaps suitable for being treated, an evolution that might be correlated with the presence of genetic abnormalities that are frequently related to CLL, as it is discussed next.

Stage	Definition	Median survival
Binet system		
Binet A	Hb ≥ 10.0 g/dl, thrombocytes ≥100 × 10^9/l, <3 lymph node regions	>10 years
Binet B	Hb ≥ 10.0 g/dl, thrombocytes ≥100 × 10^9/l, ≥3 lymph node regions	>8 years
Binet C	Hb < 10.0 g/dl, thrombocytes <100 × 10^9/l	6.5 years
Rai system		
Low risk		
Rai 0	Lymphocytosis >15 × 10^9/l	>10 years
Intermediate risk		
Rai I	Lymphocytosis and lymphadenopathy	>8 years
Rai II	Lymphocytosis and hepatomegaly and/or splenomegaly with/without lymphadenopathy	
High risk		
Rai III	Lymphocytosis and Hb < 11.0 g/dl with/without lymphadenopathy/organomegaly	6.5 years
Rai IV	Lymphocytosis and thrombocytes <100 × 10^9/l with/without lymphadenopathy/organomegaly	

The overall survival times included in this table were adapted and have changed during the past 30 years [7].
Binet's lymphoid areas consist in: lymphadenopathy either uni- or bilateral in (1) cervical, (2) axillary, (3) inguinal areas, (4) spleen, (5) liver.
Hb, haemoglobin.

Figure 3 - Staging Systems for CLL – Both Binet and Rai staging systems predict the median survival of patients diagnosed with CLL and are the standardized method to evaluate cases of CLL in terms of the severity of the disease. Binet classification takes in account haemoglobin levels, absolute count of thrombocytes and ganglionic involvement. On the other side, Rai staging system, besides the parameters referred for Binet classification, also takes in account the absolute number of lymphocytes. The involvement of the liver and spleen (organomegaly) is separated from involvement of the lymph nodes (lymphadenopathy), which does not occur in Binet classification. From Eichhorst B, Robak T, Montserrat E, Ghia P, Hillmen P, Hallek M, Buske C, ESMO Guidelines Committee. *Chronic lymphocytic leukaemia: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up.* Ann Oncol Off J Eur Soc Med Oncol. 2015 Sep;26(Suppl 5):v78-84.(3).

Several genetic mutations are associated with patients' prognosis in CLL, such as del(17p), del(11q), trisomy 12 (Tris12) and del(13q), ordered in terms of their contribution to disease severity(18). Deletion of the 17p13 (affecting 3-8% of patients at the time of the diagnosis), is one of the most important genetic alterations associated with tumour progression, not only for CLL patients, but for all types of cancer. This occurs because TP53 gene loci, responsible for the encoding of p53 protein, is present in chromosome 17, band 17p13.1. p53 protein, a tumour suppressor protein, acts as regulator of the cell cycle, arresting cells from progressing through it if any detectable DNA damage is present. If this protein is absent (deletion) or not fully functional (mutated), mutated cells are able to surpass the cell cycle checkpoints and escape programmed cell death, allowing the proliferation of cells carrying these mutations. These events lead to

the accumulation of cells with DNA damage, which might contribute to the increased resistance of pathological cells to normal chemotherapeutic regimens. As demonstrated by Gonzales et colleagues, patients with CLL and concomitant del(17p) reported the worst prognosis, with poorer overall response rates and shorter progression-free survival and overall survival, when subjected to a specific chemotherapeutic regimen(19). This data enhances the importance of treating patients with del(17p) as a high-risk group, despite the Binet and Rai classification that these patients might be classified to, as patients with del(17p) are also prone to develop Richter's Syndrome, a clinical situation where a CLL quickly progresses to an aggressive lymphoma.

Similarly, deletion of 11q23 (5-20% of CLL patients) is also associated with a protein that is related to the recognition of DNA damage. In this case, the protein is ATM serine/threonine kinase, a protein encoded by the ATM gene located in chromosome 11, band 11q22.3, that senses DNA damage and signals it to p53 protein, allowing cell cycle arrest. In cases of deleted or defected ATM protein, cells with DNA damage surpass cell cycle checkpoints, escaping apoptosis and, consequently, resistance to chemotherapeutic regimens is promoted. Patients with del(11q) present clinically with extended lymphadenopathies and other markers of poor prognosis, such as unmutated IGHV genes(8). Trisomy 12, the third most common abnormality in CLL, is a genetic alteration where an extra copy of chromosome 12 is present. This mutation has been associated with the observation of atypical morphology in the blood smear, atypical immunophenotype and once more with the detection of unmutated IGHV genes. Another finding encountered is the concomitant existence of mutations in Notch 1, a protein also associated with cell-fate determination.

To conclude, deletion of 13q14, or del(13q) is the genetic abnormality most encountered in CLL cases (>50% of the cases) and it is associated with the best prognosis among the genetic alterations addressed in this chapter. Although bigger deletions might include some important gene regions contained in this chromosome, the majority of these deletions, do not produce any functional alterations that might lead to a worse response by the patients carrying this mutation(8). In fact, Döhner et all, showed median survivals higher in these patients compared to patients with a normal karyotype(18), when this mutation is present alone, without the concomitant occurrence of other abnormalities. These evidences clarify the implications of genetic features in the treatment of CLL and provide us with the ability to understand the need for a more targeted therapy, mainly in cases of chemotherapeutic resistance, as will be explained throughout this introduction.

1.3. MONOCLONAL B CELL LYMPHOCYTOSIS AND SMALL LYMPHOCYTIC LEUKAEMIA

As referred above, when the absolute number of pathological B cells is lower than 5000 cells per μL of peripheral blood, we might be in the presence of a MBL or a SLL. SLL is diagnosed when this number of pathological B cells is accompanied with lymphadenopathy and/or splenomegaly. In absence of lymphadenopathy, organomegaly, cytopaenias and clinical symptoms, the patient is diagnosed with a MBL. According to the latest guidelines of WHO for the classification of lymphoid neoplasms, MBL can be divided in high-count MBL (HC-MBL) and low-count MBL (LC-MBL). HC-MBL is characterized by the presence of 500-5000 pathological B cells per μL of peripheral blood, being a stage similar to Binet A/Rai 0, requiring yearly follow-up and presenting with great chances of progression to Binet A CLL. On the other hand, LC-MBL presents with less than 500 pathological B cells per μL of peripheral blood, and has an "extremely limited, if any, chance of progression", not requiring routine medical follow-up. Of note, MBL are not necessarily of typical CLL type, although in this project we only focused on these ones(1). Patients with MBL clones are not treated since the size of the clone and the absence of clinical symptoms don't justify treatment. If the disease progresses it might become suitable for treatment, a topic discussed next.

1.4. THERAPEUTIC APPROACHES IN B-CLPD

Current approaches on B-CLPD treatment still relay on chemotherapeutic agents. The combinatorial approaches used depend not only on the type of haematological malignancy, but also on the disease staging. Based on this evidence, clinical staging classifications are crucial in treatment's decision, as occurs in CLL with Binet and Rai staging systems(3). In asymptomatic patients and with an indolent clinical course, it is admissible to maintain a watchful waiting approach. In the case of symptomatic patients and/or evidence of end-organ damage and/or bulky disease, depending on the type of malignancy (combinatorial regimens for each type of B-CLPD are reviewed in the respective guidelines of treatment)(3,20–23), chemotherapeutic agents alone or in combinatorial regimens are the preferential treatment. Among the combinatorial regimens, it can be emphasized the CHOP treatment that consists of: cyclophosphamide, hydroxydaunorubicin, vincristine (Oncovin®) and prednisolone or the R-CHOP regimen that combines CHOP with Rituximab, an anti-CD20 monoclonal antibody, which was one of the first targeted drugs to molecular targets, and completely changed the prognosis

of all B-cell NHL. Although treatment with R-CHOP compared to CHOP have been associated with better clinical responses(24), these treatments are not specific for tumour cells and therefore they are always associated with some side effects. These treatments target not only neoplastic cells but also normal cells, leading the patient to a state of immunosuppression that is associated with innumerous side effects, such as increased risk of infections, feeling sick and tired, hair loss and others. Besides that, chemotherapeutic regimens are associated with a wide range of clinical responses, varying from complete remissions to no effect at all. Of note, these treatments are also associated with disease relapse after a period of remission(25). Other therapeutic approaches include radiotherapy, autologous and allogenic stem cell transplantation, the last one being used in only few cases due to the high risk of death.

The main side effects of current cancer treatment are, as seen above, related to the lack of specificity of antitumoral treatments. Several studies on tumour progression throughout the years uncovered the fact that tumour evolution is mainly associated with an immune deregulation. A myriad of factors provides the tumour with a potent ability to turn the immune system to its side, allowing it to escape immune surveillance and finally progress without strains, in a process known as tumour escape, a step of cancer immunoediting. Switching the balance back to the immune system and make it capable of fighting the cancer again is the main goal of cancer immunotherapy. However, this requires a specific knowledge about the process of tumour immunoediting, described in the next chapter, with the purpose of enhance the best antitumor properties offered by the immune system.

2. THE THEORY OF TUMOR IMMUNOEDITING

Hanahan and Weinberg (26) redefined, in 2011, the hallmarks of cancer, attributing ten features that characterize the ability of the tumour to subsist in the host: a) sustained proliferative signalling, b) evasion from growth suppressors, c) avoid immune destruction, d) enabling replicative immortality, e) tumour-promoting inflammation, f) activating invasion and metastasis, g) inducing angiogenesis, h) genome instability and mutation, i) resisting cell death and j) deregulation of cellular energetics. These features demonstrate that somehow, when the host's immune system attempts to eradicate tumour cells and avoid cancer progression, some mechanisms fail allowing the tumour to grow and acquire the capability to control the immune system and use it in its own favour with

the establishment of an immunosuppressive tumour microenvironment that blocks the action of the immune system against tumour cells.

The relationship between the immune system and cancer progression has been firstly proposed by Paul Ehrlich in 1909. This author proposed that tumour development was probably supressed by the host immune system, supported on the fact that if some mechanism of protection didn't exist, the development of carcinomas would occur more frequently. Fifty years later, Burnet and Thomas proposed the immunosurveillance hypothesis: Burnet postulated that tumour cell-specific antigens would be recognized as foreign molecules and, somehow, they would trigger an immune response against them that would lead to tumour regression, similar to what happened in transplant rejection while Thomas assumed that the cells of the body might be constantly under genetic alterations/mutations and in some way the immune system was capable of eliminating the cells carrying these mutations(27). However, due to the lack of the techniques at the time, this theory could only have started to be demonstrated several years later. Later studies indeed showed the existence of tumour immunosurveillance. However, the concept was enlarged to include the definition of cancer immunoediting that involves three distinct phases: elimination, equilibrium and escape(28), phases described in figure 4.

Elimination phase matches the phase described in immunosurveillance theory, being associated with the mechanisms that act to control tumour growth. These mechanisms are mainly associated with interferon gamma (IFN-γ) expression and cytolytic capacity provided by the immune system. IFN-γ mediated tumour regression is achieved by several mechanisms, one of which is by direct action of IFN-γ in cancer cells with upregulation of tumour immunogenicity by an increased major histocompatibility complex (MHC) class I expression and consequently increased antigen processing and presentation, allowing the recognition and effector responses mediated by CD8+ αβ T lymphocytes. IFN-γ has also effects on other immune cells promoting the expression of CD4+Th1 T cells, increasing the production of IFN-γ and tumour necrosis factor alpha (TNF-α), Th1 type cytokines. Besides the effects on adaptive immune system, this cytokine also activates cytocidal activity in macrophages(29). Another key molecule in tumour elimination process is perforin. Perforin is associated with tumour killing properties, by creating pores in the cytoplasmic membrane allowing the release of granules containing granzymes, serine proteases that mediate the death of tumour cells by inducing programmed cell death in the targeted cells. Another mechanism associated with tumour regression is the apoptosis mediated by TNF-related apoptosis-inducing

ligand (TRAIL)(28). Of note, is the importance of γδ T cells on these mechanisms. These cells target transformed cells by recognition of different molecules from those recognized by αβ T cells, being also a main source of IFN-γ in human body. They are endowed with potent cytotoxic mechanisms that are suitable for tumour growth control. The pathways by which these cells recognize these ligands and their functions in tissue homeostasis and tumour immunosurveillance are described in chapter 4.

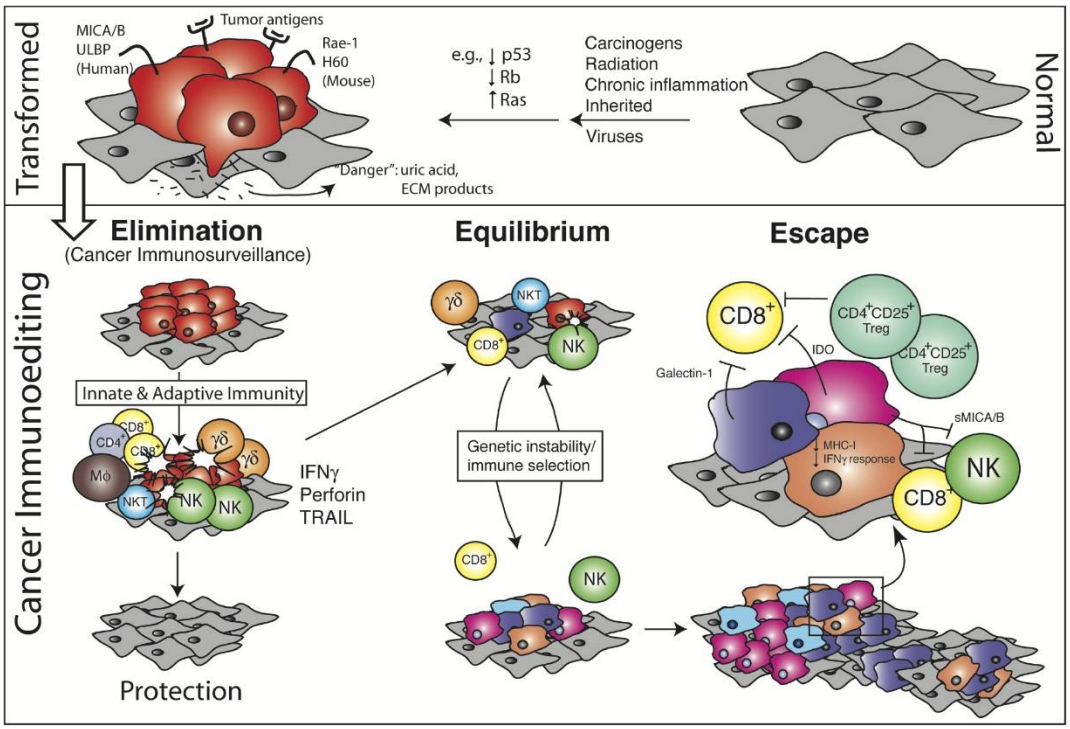


Figure 4 – The process of tumour immunoediting. The three phases of tumour immunoediting are represented in this figure: elimination, equilibrium, and escape. After being exposed to several tumour-inducing agents, a cell might pass from a normal state (grey cells) to a transformed state (red cells). Elimination matches the phase described in immunosurveillance theory and represents the stage where the immune system can detain the proliferation of transformed cells, by mechanisms involving the production of Th1 type cytokines and by cytotoxic mechanisms, leading to the apoptosis of transformed cells. When the accumulation of genetic mutations is widespread, and transformed cells proliferate in extended proportion, tumour reaches the phase of equilibrium, that although not clinically detectable, already emerges as a state where disease easily progresses. When the tumour finally acquires the ability to grow without the influence of immune system, through recruitment of immunosuppressive populations to tumour site and by reducing its immunogenicity, becoming unrecognized by the immune system, the tumour enters the escape phase and becomes clinically detectable. From Dunn GP, Old LJ, Schreiber RD. The Immunobiology of Cancer Immunosurveillance and Immunoediting. Immunity. 2004 Aug;21(2):137–48. (28)

Although these mechanisms are quite effective in preventing tumour progression, some cancer cells can evade this recognition and killing processes. This ability to evade immune system recognition is associated with the appearance of new cell's variants that were produced due to multiple genetic mutations. One common feature of these tumour cells is their reduced immunogenicity that provide them the ability to proliferate without

being recognized as foreign by the immune system. At this moment, tumour enters in the equilibrium phase. This stage represents the phase in which the tumour acquired the ability to survive without being attacked by the immune system but is not yet capable of being clinically detected. As these cells continue to acquire mutations associated not only with the ability to stay unrecognized but also with sustained proliferative signals and capability of angiogenesis, they become finally free of the immune control, being capable of proliferate without strains. At this stage the tumour finally becomes clinically detectable, entering the escape phase, the final step of tumour immunoediting(27).

In this last phase tumour growth precedes unrestrained by the immune pressure and a few features characterize this state: unresponsiveness to IFN-γ, downregulation of antigen processing and presentation (reduced expression of MHC molecules) and overexpression of immunosuppressive cytokines that contribute to tumour's immunosuppressive microenvironment, such as inhibitors of T cell response. Together, these mechanisms contribute to the ability of the tumour to supress the action of the immune system, giving rise to a state where the tumour controls the immune system and not the other way around(29). Of note, it also occurs an upregulation of immunosuppressive T cell populations in tumour microenvironment, such as regulatory T cells (T-regs), a subset of T cells that express CD4, CD25 and the transcription factor FOXP3. Impaired dendritic maturation is also observed.

All these observations clearly clarify that the idea of controlling the immune system and switch the control back to the immune system seems very promising and led to a rise in the interest on immunotherapeutic approaches. However, complete understanding of tumour immunoediting biology is absolute necessary to achieve treatments capable of modulating the immune system in the initial phases of this process and that don't produce even more damage. Emerging from this belief T cells based therapies are one of the most prominent promises of cancer immunotherapy with some already reported results which are going to be described in the next section.

3. IMMUNOTHERAPY FOR CANCER TREATMENT: T CELL BASED THERAPIES

Due to the substantial side effects of chemotherapy, mainly due to the lack of specificity of those treatments, associated with overall immune system suppression, several efforts have been made with the aim of address cancer specific therapeutic

approaches. Immunotherapy has been gaining widely recognition supporting on the view that the patient's own immune system can be manipulated in order to overcome tumour immunosuppressive microenvironment and induce tumour regression. Immunotherapy includes different approaches, such as adoptive cell transfer (ACT) that consists in transferring cells into the patient that have been originated from the patient himself (autologous) or from another donor (allogenic). The purpose of this therapy is to provide the patient with the immune cells capable of fighting their cancer cells. Among these therapies, adoptive T-cell therapy has been widely studied to stop cancer progression in solid and haematological tumours.

T cell based therapies are currently of two types: therapies that use T cells with rearranged/genetically modified T cell receptors (TCR) or chimeric antigen receptor (CAR) T cells, illustrated in figure 5. In the first one, the structure of TCR is maintained, increasing only the specificity of the antigen recognition by modifying the expression of the TCR chains. To obtain the desired antigen specificity several methods can be used: the first one consists in isolate tumour infiltrating lymphocytes (TILs), that have tumour antigen specificity, expand them *ex vivo* and finally reinfuse them into the patient. Although quite reliable, it is not so easy to use this method because a tumour biopsy is needed to isolate TILs. Another approach consists in immunize transgenic mice expressing human leukocyte antigen (HLA) molecules with cancer cells, collect the T cells produced and amplify them *ex vivo*. Finally, TCR can be genetically modified, with the purpose of obtaining a sequence that has the higher specificity for tumour antigens and the best properties for tumour cell killing(30). Although these approaches are quite promising they still present some problems, such as the fact that these modified TCRs still require antigen presentation by MHC molecules, a mechanism that is, as stated above, downregulated in cancer. Although not discussed in this chapter, it is important to state that this requirement for antigen processing and presentation by MHC molecules is only needed for αβ T cells, as γδ T cells present MHC-unrestricted recognition which make them more suitable for this type of treatment(31), a subject that will be detailed in section 4. Besides that, optimized expansion protocols need to be developed allowing not only the selection of T cells with the best properties for tumour cells' killing but also their expansion to numbers suitable for ACT. This is particularly important in the use of TILs where different subsets of lymphocytes are present, not necessarily all with antitumor properties (heterogeneity of TIL will be discussed in chapter 6). Furthermore, immunosuppressive signals in tumour microenvironment, mainly mediated by Tregs

might trigger patients' irresponsiveness to treatment. This last feature is the main reason by which immunotherapeutic approaches often fail. The most prominent side effect in TCR based therapies is, however, "on target off tumour reactivity". This effect consists in TCR specific binding to the target antigen but in tissues other than the tumour which reflects the lack of tumour specificity of the chosen antigens. This phenomenon leads to several treatment-associated side effects such as described by Morgan and colleagues (32) that in 2013 reported the appearance of neurological toxicity with the use of a TCR based therapy that aimed to target Melanoma-associated antigen MAGE-A3 peptide to the treatment of melanoma. The T cells used in this experiment, however, also recognized MAGE-A12, a peptide expressed in normal human brain tissue, leading to the side effects observed. Another example can be seen in the study of Linette et. al (33) that reported lethal cardiac toxicity in a TCR-based treatment for multiple myeloma and melanoma. This TCR also aimed to target MAGE-A3, but later studies revealed that muscle protein Titin was also affected. These studies clarify that these therapies require a careful study and strong evidence that the target antigen is tumour-specific and doesn't bind to any other cell or tissue in the body.

With the purpose of overcome the antigen presentation issue and the lack of specificity of the target antigen, a new technology named CAR-T cells have gained special interest. CAR-T cells are T cells that are modified in structure comprising an extracellular antibody-like domain, a transmembrane domain that binds CAR to the signalling machinery of T cell and an intracellular T cell signalling domain. The antibody-like domain aims to recognize antigens like the immunoglobulins expressed in the BCR of B cells (overcoming the antigen processing and presentation by MHC molecules), associated with the downstream effects of T cell signalling(30). The first generation of CAR-T cells, although very promising was associated with a highly incidence of side effects. This was mainly related with the choice of the targets used. For example, in the treatment of B-cell haematological conditions, the desired goal is to kill malignant B lymphocytes, while normal B cells are maintained. However, since the first generation of CAR-T cells had only one target antigen, in this case, CD19 antigen was established as the desired target as it is a B-cell specific antigen. As expected, Grupp and colleagues (34) reported the death of normal and pathological cells, a case of "on target off tumour reactivity", as CD19 is expressed not only in pathological but also in normal B cells. With the intention of overcome this reported side effect, second generation of CAR-T cells are now being developed with the aim to increase tumour specificity by targeting two

antigens at the same time (bispecific antibodies), enhancing a strong response only in cells expressing both antigens at the same time. In the treatment of B-CLPD this might imply that besides CD19, a specific antigen only expressed in malignant cells needs to be targeted, which will allow a specific treatment for each patient depending on the phenotype expressed by their pathological B cells.

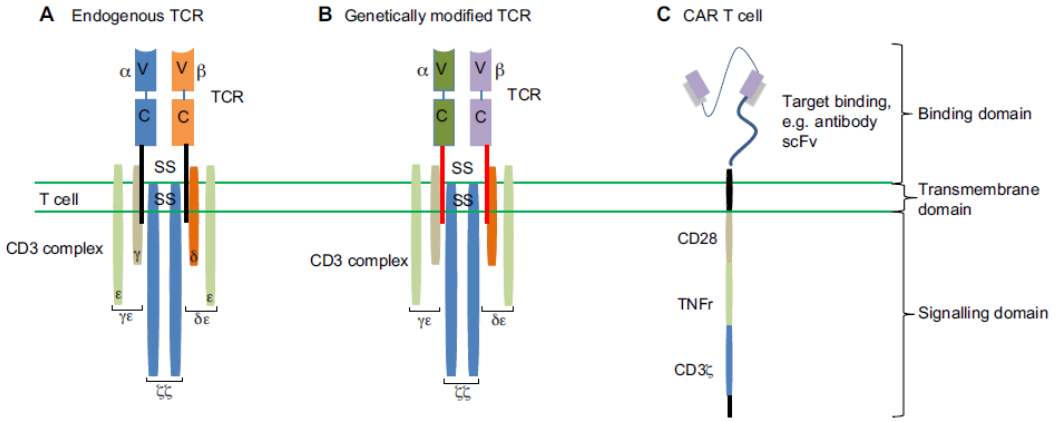


Figure 5 - T cell based therapies. (A) A endogenous TCR is represented. (B) Genetically modified TCR. The structure of TCR is maintained changing only TCR specificity (represented by α and β chains with different colours, comparing to figure A. (C) CAR-T cells: the binding domain resembles an immunoglobulin-like recognition, but the signalling domain still leads to T-cell like signal transduction and effector functions. From Sharpe M., Mount N., *Genetically modified T cells in cancer therapy: opportunities and Challenges*, 2015(30)

γδ T cells, a subset of T lymphocytes, are emerging from T-cell based therapy interest. These cells represent about 1 to 10% of circulating T lymphocytes. They differ from αβ T lymphocytes, the other subtype of T lymphocytes, by the expression of a TCR with γ and δ chains, opposing to the α and β chains expressed in αβ T cells and by the absence of CD4 and CD8 expression, molecules involved in MHC restricted recognition of processed antigens in αβ T lymphocytes. Due to the lack of expression of these markers, these cells recognize antigens by a MHC-unrestricted recognition, an important feature in cancer immunotherapy since tumour cells can surpass immune surveillance mechanisms by downregulate the expression of MHC molecules. It is now widely known that these cells recognize another type of antigens different from those recognized by αβ T cells, such as lipids and stress-associated molecules produced upon bacterial and microbe infections, but also in cellular stress as occurs in malignant cells/malignant transformation. They are placed in the bridge between innate and adaptive immune systems, providing a response with different kinetics and with functions that make them strong candidates for cancer immunotherapy, as will be described in the next chapter.

4. IMMUNOTHERAPY FOR CANCER TREATMENT: GAMMA DELTA T LYMPHOCYTES

4.1. MATURATION AND THYMIC DIFFERENTIATION, STRUCTURE, TCR REPERTOIRE AND TISSUE DISTRIBUTION OF GAMMA DELTA T CELLS

Hematopoietic cells arise from a common hematopoietic stem cell (HSC) that give rise to lymphoid and myeloid progenitors. The lymphoid progenitor can be committed to B, T, or NK lineage. As the other subsets of T lymphocytes, γδ lymphocytes arise from the T cell progenitor, a common double negative (DN) precursor (CD4-CD8-) that seeds in the thymus to continue maturation. In humans, thymopoiesis develop over four DN, a double positive (DP) and a single positive (SP) stages. DN1 stage is characterized by the expression of CD44 and the absence of CD25 expression and during this phase the main event occurring is thymocyte's proliferation. As they proliferate, thymocytes pass through the DN2 stage where CD25 and c-kit (CD117) expression is upregulated and DN3 phase where thymocytes lose the expression of c-kit and reduce CD44 expression(35). As thymocytes enter the DN2 stage, TCR chains rearrangement initiates, as the recombinase genes RAG1 and RAG2 start to be expressed. At this stage rearrangement of TCRd, TCRg and TCRb genes occur giving rise to cells expressing TCRγδ or a pre-TCR, a TCR with a rearranged β chain and an invariant α chain (TCRa rearrangement occurs lately in thymopoiesis), if a successful rearrangement occurs. T cells expressing functional TCRγδ or a pre-TCR proliferate and proceed to DN4 stage where these cells become CD25 negative (35,36). The transition of the T cells expressing a pre-TCR to the DN4 stage is called β-selection and leads to the initiation of α chain rearrangement. At this point, it was reasonable to assume that T cells expressing a TCRγδ were committed to be γδ T lymphocytes and T cells expressing a pre-TCR were committed to be αβ T lymphocytes. However, it is important to highlight that a T cell expressing a pre-TCR, even though expressing a functional β chain, is not yet committed to be an αβ T lymphocyte as this occurs only when α chain locus rearranges, accompanied by γ chain locus deletion, δ chain locus silencing and progression to DP phase, the hallmark of αβ lineage commitment(37). Still widely unknown are the signals that lead to the maturation of one or another type of T lymphocyte but studies have reported that TCR signal strength, rather than TCR type, this is the TCR gene rearrangement, determinates T cell fate with stronger TCR signals favouring γδ T cell development and weaker signals favouring commitment

to αβ lineage(38,39). At the end of the DN4 stage, γδ T cells leave the thymus while αβ development continues with positive and negative selection processes (explains why γδ T cells don't express CD4 and CD8 molecules). After leaving the thymus, γδ T cells, that present a tissue tropism, are allocated to different tissues. As they reach these specific tissues they become tissue resident γδ lymphocytes and their properties are intimately related to the function they perform in it, functions that are mediated upon γδ T cell activation, after they encounter with an antigen.

γδ T lymphocytes can be classified according to the V gene that they express after V(D)J recombination of their γ and δ chains. According to the Vδ gene usage, human γδ T cells can be classified in: Vδ1 T cells that are predominant in intestines (small intestine and colon), dermis, spleen, liver and epithelia, in general, Vδ2 that are the predominant γδ population in peripheral blood and secondary lymphoid organs and Vδ3, a residual population of γδ T cells that is present in liver and gut epithelium(40). These cells present a limited TCR V gene germ-line repertoire compared to αβ T lymphocytes as can be seen in table 1, where the number of segments in V genes for γ and δ T cell chains (14 and 3, respectively) are significantly lower compared to the α and β V gene segments (50 and 57, respectively).

Table 1 - TCR Multigene families in humans. In this table it is represented the number of V, D, J and C gene segments for each TCR chain. The differential expression of these chains impacts on the repertoire expressed by T cells. Adapted from Kindt TJ, Osborne BA, Goldsby RA, *Kuby Immunobiology* - Sixth Edition, 2006(41)

Gene	V gene segments	D gene segments	J gene segments	C gene segments
α chain	50	-	70	1
β chain	57	2	13	2
δ chain	3	3	3	1
γ chain	14	-	5	2

They are similar in structure to αβ lymphocytes as they also express the T-cell co-receptor CD3, that allows the signalling cascade after antigen stimulation. This need is due to the short intracytoplasmic tail of the TCR that is not capable of inducing an intracellular signal responses. The general structure of a γδ T lymphocyte is represented in figure 6.

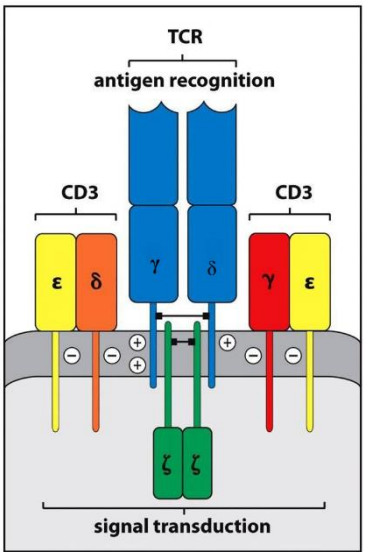


Figure 6 - Schematic representation of gamma delta TCR complex. It is represented a γδ TCR complex composed by the TCR expressing γ and δ chains (blue) and the CD3 co-receptor composed by one CD3γ (red), one CD3δ (orange), two CD3ε (yellow) and two CD3$_\zeta$ (green) chains, responsible for signal transduction. Without this co-receptor, T cells were incapable of inducing a response against the antigens, as the intracytoplasmic tails of TCR (blue) are very short. Adapted from Peter Parham, *The Immune System*, 3th Edition, 2009(42)

Regarding their absence of CD4 and CD8 expression, is very feasible to assume that these cells recognize different antigens than those recognized by αβ T lymphocytes. The ligands discovered so far for these cells, in the context of microbe infection and tumour immunosurveillance are described in a further topic.

4.2. MARKERS OF ACTIVATION AND T CELL FUNCTION: CD27 AND CD69

Concerning the expression of activation markers by γδ T cells, in this chapter will only be mentioned the two markers evaluated in this project: CD27 and CD69. For T lymphocytes to become activated, proliferate and differentiate into effector T cells, specific antigen recognition mediated by TCR binding must occur (antigen recognition by γδ T cells is discussed below). However, co-stimulatory receptors must act alongside to provide the signals needed for these processes to occur. CD27 antigen is one example of these co-stimulatory receptors, acting together with CD70 to provide the signals for T cell activation and differentiation.

CD27 gene, present on chromosome 12, band 12p13.31, codes for the CD27 antigen, a member of the TNF-receptor superfamily, previously called tumour necrosis factor receptor superfamily, member 7 (Tnfrsf7). This antigen works as a co-stimulatory receptor, alongside other co-stimulatory receptors, such as CD28, providing signals not

only for T, but also for B lymphocyte activation. CD27 acts through the interaction with its ligand CD70 (also referred as Tnfsf7 or CD27L), molecule expressed by activated antigen presenting cells (APC). CD70 is a cytokine encoded by CD70 gene, present in chromosome 19, band 19p13.3. The expression of CD70 and other ligands on APC, such as dendritic cells, B and T lymphocytes, is promoted by antigen and Toll-like receptors (TLR) stimulation of these cells. Activated APC also produce cytokines that modulate the effector differentiation of T lymphocytes(43).

It is well described that in αβ T cells CD27 is constitutively expressed on naïve T cells and that upon TCR engagement, T cells upregulate CD27 expression. CD27 binds to CD70 leading to the NF-κB and JNK pathways activation, which is mediated by the binding of the cytoplasmic tail motifs of CD27 with TNFR associated factors (TRAFS), providing surviving signals (anti-apoptotic signals) to T cells, promoting their proliferation, and the induction of cytokines and chemokines production. After this transient upregulation, CD27 is cleaved from the cell surface, as it is not needed for T cell effector functions (effector T cells are CD27- T cells)(44). On the other side, the interactions that lead to γδ T cell activation and differentiation are still unclear, but the knowledge so far point to a similar mechanism. Up to now, two γδ T cells subsets were identified concerning to their CD27 expression: a counterpart that expresses CD27 and IFN-γ and a subset that don't express CD27 but produces IL-17(45). The mechanisms leading to T cell activation and differentiation seem to mimic αβ T cells for CD27 antigen, as CD28 is generally absent on these cells. In flow cytometry, CD27 allows, alongside other CD markers, for example, CD28/CCR7/CD45RA, to divide T cells in four compartments: naïve, central memory, effector memory and effector T cells in αβ T cells, but for γδ T cells, only three subsets were reported so far: naïve and central memory (CD27+) and effector T cells (CD27-).

CD69 gene, present in chromosome 12, band 12p13.31 encodes for the CD69 antigen, a member of the calcium dependent lectin superfamily of type II transmembrane receptors. It is the earliest inducible cell surface glycoprotein acquired during lymphoid activation. Its upregulation is mediated by TCR engagement in T lymphocytes, crosslinking of surface immunoglobulin in B cells and by IL-2, IL-7, IL-12, IFN-γ or CD16 stimulation on NK cells. It is also inducible expressed in monocytes, neutrophils and some thymocytes and constitutively expressed on other subset of thymocytes and platelets. After TCR engagement, CD69 expression is upregulated and although natural ligands for CD69 receptor are not known, studies with monoclonal antibodies against

CD69 antigen showed that this binding mediates Ca^{2+} mobilization, increases T cell proliferation, increases the expression of IL-2R α chain (soluble CD25) and increases cytokine production (IL-2, TNF-α and IFN-γ). It is then clear that CD69 in T lymphocytes is involved in their activation and proliferation (46,47).

In flow cytometry, CD69 antigen in very useful to identify recently activated T lymphocytes, mainly the ones that have not yet expressed other activation markers. These two activation markers will allow, in this project to define, together with granzyme B the effector functions of T cells, as will be further discussed in the objectives of the project.

4.3. LIGAND RECOGNITION BY GAMMA DELTA T CELLS

Mature naïve γδ T cells are activated upon contact with an antigen. However, as stated above, the antigens that these cells recognize are different from the peptide antigens recognized by αβ T lymphocytes. These cells recognize infected and stressed cells in a way similar to innate immune cells that recognize the pathogens by their expression of pathogen associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) through pattern recognition receptors (PRR). In these cases the molecules recognized are stress associated ligands expressed by cells under cellular stress(48). Opposing to αβ T cells that have a well-defined set of recognized antigens, γδ T cells antigens and the mechanisms by which they are recognized are unknown.

The best studied ligands of γδ T cells, more specifically for Vδ2Vγ9 T cells, are phosphoantigens. These cells sense alterations on the levels of the isoprenoid metabolites and are preferentially stimulated by (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP), a microbial antigen produced in the non-melavonate pathway of isoprenoid biosynthesis and by isopentenyl pyrophosphate (IPP), a self-metabolite produced in melavonate pathway of isoprenoid biosynthesis(49). Although these are widely recognized ligands of these cells, the exact mechanisms by which this process occurs are still unknown. Recently, Hong Wang and Craig T. Moritah(50) showed that this mechanism didn't occur by direct binding of these antigens to TCR, but it is mediated by a molecule that senses the changes on the levels of these molecules and by some mechanism not fully understood leads to TCR signalling and activation. So far, these authors assign butyrophilin 3A1 (BN3A1) protein the role of mediator of this interaction, which has an intracellular domain known as B30.2 domain that changes its conformation upon changes in phosphoantigens levels and signals these changes to TCR. The same discoveries were performed by Sandstrom and colleagues(51). These evidences support

the role of γδ T cells as key mediators on inflammatory response since the recognition of bacteria and microbes is based on their expression of HMBPP and engagement of γδ T cells lead to the effector functions that trigger elimination of infected cells. Nevertheless, this recognition is also very important in antitumor activity performed by these cells since many tumours have a deregulated melavonate pathway leading to the accumulation of IPP which can be recognized by γδ T cells, generating an antitumor response.

Engagement of TCR also occurs with CD1 superfamily molecules that have also been widely proposed as being one of γδ T lymphocytes ligands. These molecules are non-classic MHC molecules that bind to lipid antigens and present them to αβ and γδ T lymphocytes. CD1d has been recognized as being a molecule linked to Vδ1 and Vδ3 recognition, but not for Vδ2 subset of γδ T cells. Human Vδ1+ T cells can recognize phospholipid antigens presented by CD1a, CD1c or CD1d(52,53). The importance of this molecule is more evident concerning to the function of γδ T cells in cases of infection as several authors have stated that some infectious agents might produce sulfatide-like compounds that are presented by CD1 superfamily to γδ T cells(54,55). These evidences lead to the idea that some infectious agents and stressed cells might produce lipid molecules that can be presented by CD1 superfamily to γδ T cells allowing further responses to those antigens.

It is important to note that γδ T cells response is not only mediated by TCR binding, but it might also be mediated by NKG2D receptor, a receptor associated with NK cells that it is also expressed in γδ T cells. These receptors allow the recognition of MICA, MICB, UL16 binding protein and other ULBP, stress ligands expressed in malignant cells but not in normal cells. MICA and MICB are non-classic MHC cell surface glycoproteins that are recognized by NKG2D receptor, but also by the TCR of Vδ1 T cells. NGK2D is also capable of recognizing the ULBP family, a cell surface marker of transformed and stressed cells. NKG2D ligand binding subunit is coupled to DAP 10 or DAP 12 subunits allowing the downstream signalling mechanisms and effector functions to occur(48). Recognition by TLR have also been associated with γδ T cell response. These receptors are PRR that can recognize conserved molecules derived from microbes. By expressing TLR at their surface γδ T lymphocytes are also capable of inducing effector responses against infected cells(56). Although other molecules have been described to be ligands of these cells, all of them fall in the classification of stressed/infected cells' associated molecules. It is still however unknown the tumour-specific antigens that bind to γδ T cells in the context of tumour-infiltrating γδ T

lymphocytes. Knowledge of the specific antigens that these cells bind within the tumour microenvironment would allow genetic engineering of these T cells in order to use then in T cell based therapy. The main ligands of Vδ1 and Vδ2 T cells are represented in figure 7.

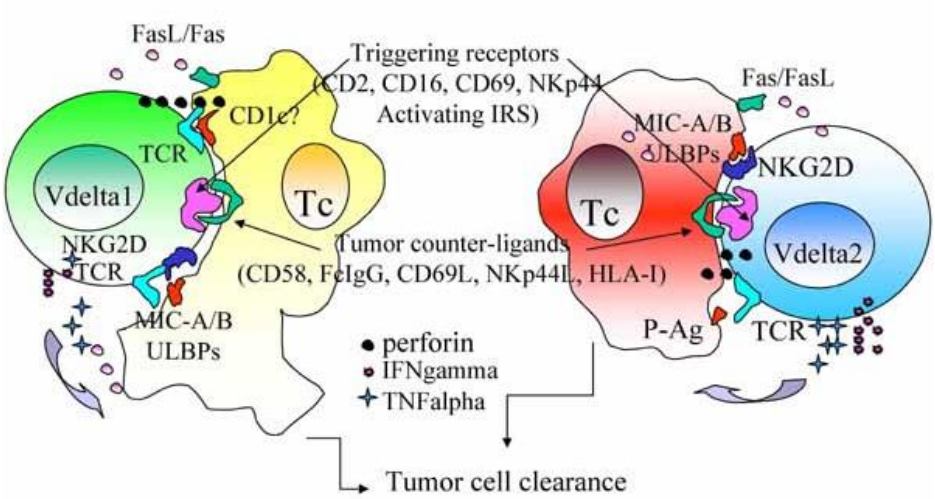


Figure 7 - Antigen Recognition by γδ T cells. Vδ1 T cells (green), recognize infected/malignant cells (yellow) by recognition of CD1 molecules via TCR engagement or MICA/MICB and ULBP molecules through NGK2D receptor (and in less proportion by TCR). Vδ2 T cells (blue), on the other side, recognize foreign molecules (red) by recognition of phosphoantigens via TCR and MICA/MICB and ULBPs through NGK2D receptor. In both cases, this recognition leads to the production of Th1 type cytokines and perforin, mechanisms that as seen in the tumour immunoediting theory aim to eradicate abnormal cells. From Zocchi MR, Poggi A. Role of gammadelta T lymphocytes in tumor defense. Front Biosci. 2004 Sep 1;9:2588–604. (57)

4.4. GAMMA DELTA T CELLS FUNCTIONS

As described previously, γδ T cells are mainly tissue resident and therefore they are thought to act as a first line of defence in microbial infections and stress responses to other agents, contributing to tissue homeostasis. They are also involved in cancer immunosurveillance, particularly in localized tumours or haematological malignancies. These cells present a myriad of functions that vary among different γδ T cells subsets and these functions can be classified as effector or regulatory ones, depending on the final contribution to the immune response.

Tissue-resident γδ T cells directly encounter the antigens. However, γδ T cells that infiltrate tumours microenvironment need to be attracted to the tumour site. Among the chemoattractant molecules mediating this attraction are: monocyte chemoattractant protein 1 (MCP-1), Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES), macrophage inflammatory protein-1α and macrophage inflammatory protein-1β (MIP-1α and MIP-1β). When they reach the tumour site, these cells are able

to recognize the antigens described previously, through TCR and through co-stimulatory receptors such as NGK2D, but also by DNAX Accessory Molecule-1 (DNAM-1) and CD16, being this one responsible for antibody dependent cellular cytotoxicity(58).

Concerning to the effector functions of these cells, direct antigen recognition triggers the innate cytotoxic activity of these cells which can be mediated by the engagement of a death receptor/ligand pathway, such as Fas/Fas ligand, TRAIL/TRAIL ligand and CD95/CD95 ligand pathways that work by transmission of death signals to the targeted cells or it can also be mediated by a cytolytic granule pathway, such as the perforin/granzymes pathway(40). Perforin acts by inducing the formation of pores in the cell membrane and granzyme induces apoptosis of the targeted cell. Granzyme B, for example, is responsible for apoptosis by different mechanisms all leading to the activation of caspases cascade. This serine protease is known to cleave BID and truncated BID moves to the mitochondria, acting synergistically with BAX and BAK to induce the release of pro-apoptotic proteins, permeabilization of the mitochondrial outer membrane and release of cytochrome c. These events lead to the formation of apoptosome, activation of caspase 9 and downstream effector caspases, all events leading to the death of the targeted cell(59).

Besides their cytotoxic activity, γδ T cells also produce cytokines and chemokines, which might exhibit an effector or regulatory role. Concerning to effector functions, these cells produce Th1 type cytokines, such as INF-γ and TNF-α. IFN-γ production is associated with an enhancement of MHC class I expression leading to an increased response by CD8+ T cells, a T cell subset endowed with potent cytolytic responses. This cytokine can also induce inhibition of angiogenesis, a key factor in tumour growth(40,58).

These cells also produce Th17 type cytokines, such as IL-17. If the production of Th1 type cytokines are an excellent indicator for the value of γδ T cells in antitumor response, the production of IL-17 by γδ T lymphocytes doesn't reach consensus. IL-17 producing γδ T lymphocytes are thought to be the main source of this cytokine in innate immune response. Although the Th17 cells produce IL-17, this production is delayed by antigen presentation. Consequently, the innate immune system needs to have a source of IL-17 to target neutrophils to initiate maturation and to act in acute phases of infection and this increased production seems to be mediated by γδ T cells(60). Due to the rapid response, it is believed that this is not an adaptive response, being this activation triggered by recognition of PAMPS and DAMPS and not by specific antigen recognition. However, in the tumour microenvironment, immunosuppressive signals provided through

expression of Th17 driving cytokines can target γδ T cells to produce IL-17 which have been correlated with protumour activity and tumour growth due to an enhanced angiogenesis(61). Tumour progression associated with TILs producing IL-17 seems to be correlated to the recruitment of immunosuppressive cells, such as myeloid-derived suppressor cells (MDSCs) or small peritoneal macrophages, which can promote angiogenesis, tumour cell growth and inducible T-regs differentiation(40). These data highlight two important questions: the first one is if all the functions of γδ T cells are favourable for controlling tumour progression? If not should γδ T cells be modulated when proliferated *ex vivo* for adoptive transfer purposes?

Denoting from γδ T cell ability to produce regulatory responses stands the production of Th2 type cytokines, like IL-4, IL-10 and TGF-β. These cytokines are produced by the γδ T regulatory cells and are responsible for the inhibition of the recruitment of other cell lineages to the tumour/infection site. These cytokines block the ability of γδ T cells to interact with dendritic cells, NK cells and provide B cell help, having the role of controlling the inflammatory responses(58). These interactions occur, for example, with Vδ2 subset that can interact with B cells by producing CXCL13, a B lymphocyte chemoattractant, regulating the organization of B cells within the follicles of lymphoid tissues, allowing them to finish its differentiation process and recruitment of these cells to the tumour/infection site. γδ T cells can also express CXCR5 acquiring a T follicular helper phenotype, providing B cell help, as described in GCR, particularly in the presence of IL-21, the cytokine that enhances T follicular helper responses allowing CSR to IgE isotype(62). γδ T cells can also interact with dendritic cells. Since these cells fail to recognize the antigens that are only recognized by the NGK2D receptor, γδ T cells can mimic dendritic cells acting as APC presenting antigens to cells of the immune system. Activation of NK cells is mediated by CD137/CD137 ligand interactions(58).

This brief resume of the functions of γδ T cells showed that these cells are endowed with some interesting qualities that make them attractive for their use in ACT. However, it is important to denote that an equilibrium between the regulatory and effector functions of these cells is needed with the intention of achieve the better clinical responses. Evidences from the use of γδ T cells as immunotherapeutic approaches to tumours and the main problems encountered so far are described below.

5. GAMMA DELTA T CELLS AND THEIR USE IN IMMUNOTHERAPY

As γδ T cells are a minor population of T lymphocytes in human body, optimized expansion protocols of these cells are needed to use them as a therapeutic option. So far, two therapeutic approaches have been used using these cells: adoptive transfer of γδ T cells that were proliferated *ex vivo* and *in vivo* stimulation and proliferation of this T cell subset.

In vivo stimulation and proliferation of γδ T lymphocytes has been restricted to Vδ2Vγ9 subset. Since it is known that phosphoantigens, such as IPP, are the main ligands of this T cell subset, aminobiphosphates have been used to improve this response. These compounds inhibit cholesterol synthesis, resulting in the accumulation of IPP which further stimulates γδ T cell production(63). Meraviglia S. et al (64) published in 2010 the results of a Phase I clinical trial of *in vivo* manipulation of Vδ2Vγ9 T cells with zoledronate and low-dose IL-2 in patients with advanced breast cancer. In this study ten females with metastasized tumour that were resistant to chemotherapy were evaluated. Five patients reported some flu-like symptoms and some developed erythema in the injection site. Three patients died before month 3 after injection and among the remaining seven, three died before reaching one year after therapy. Although these results seem quite negative, an interesting observation was made: the patients that survived presented sustained levels of Vδ2Vγ9 T cells with increased expression of activation markers CD69 and HLA-DR, while the patients that died had a decline in these cells numbers. Two patients achieved stable disease and one achieved partial remission. Their Vδ2Vγ9 T cells after month 12 were characterized by an increasing number of effector function cells, associated with the production of IFN-γ.

From this study and from others of the same type, valuable lessons can be taken: the response to this treatment widely relies in the ability to sustain the levels of these cells in the body (which in Vδ2Vγ9 subset has been difficult to achieve) and in the capability of them to acquire an effector phenotype after injection. It is also clear that these therapies are only possible with efficient protocols of expansion that allows great production of these cells to use in ACT, which implies precise knowledge on the ligands of these cells (a limitation in the use of Vδ1 subset). So far, due to their relative abundance in peripheral blood and due to the established expansion protocols (highly related to the approaches used in *in vivo* expansion), Vδ2Vγ9 T cells have been widely studied with the aim to apply them in cancer immunotherapy. However, some limitations have been encountered, such as difficulty to expand these cells in numbers suitable for ACT and failure to

maintain these increased levels of γδ T cells after injection. The main problems encountered are reviewed in reference (65).

Vδ1 is, as mentioned before, a subset of γδ T lymphocytes and represent about 10 to 30% of circulating γδ T lymphocytes(66). These cells are less studied than Vδ2Vγ9 T cells due to the lack of suitable protocols for expansion and differentiation since these cells are less prevalent in peripheral blood. However, while Vδ2Vγ9 T cells are mostly on peripheral blood, Vδ1 T cells are the major γδ population in tumour infiltrates, which suggests a role of these cells in antitumor responses. These cells are less susceptible to T cell exhaustion and activation-induced cell death, allowing the levels of these cells to remain stable weeks/years after injection, contrary to Vδ2Vγ9 T cells (67).

Bruno Silva Santos and co-workers have been made the most recent advances in the use of Vδ1 in immunotherapy. In 2011 they were able to selectively induce, *in vitro*, on Vδ1 T cells, the expression of the natural cytotoxicity receptor (NCR) NKp30 for recognition of lymphoid leukaemia cells, a natural killer receptor involved in diverse effector functions of γδ T cells(68) and in 2016 they were able to achieve a formula composed by different TCR agonists and cytokines capable of expand Vδ1 T cells expressing NCR receptors (specially NKp30) and NKG2D receptor with use of IL-4 (proliferation) and IL-15 (differentiation). A two-step expansion was needed due to the observation that simultaneous administration of IL-4 and IL-15 prevented the expression of NCR and consequently the capacity of Vδ1 T cells to induce cytotoxicity. These expanded cells were able to specifically kill leukaemia cells line *in vitro*. Evaluation of *in vivo* ACT using a xenograft model of human CLL revealed a "striking enrichment of Vδ1+ T cells in all tissues" with the histological analysis demonstrating that the T cells injected could be recovered from all of those tissues (maintenance of the injected Vδ1+ T cells). Analysis of the phenotype of these cells revealed a marked production of IFN-γ and TNF-α in the absence of IL-17 production which, based on the properties of γδ T cells described so far, seems to be an excellent marker for their use as T cell based immunotherapy. Bruno Silva Santos et al proved the efficacy of adoptive Delta One T cells (DOT cells®) immunotherapy at pre-clinical level and now their aim is to pursue clinical trials with the purpose of proving that Vδ1 T cell subset might be more suitable in the treatment of haematological malignancies than Vδ2Vγ9 T cell subset.

6. GAMMA DELTA T CELLS AS PROGNOSTIC MARKERS IN TUMORS

Despite the important role of γδ T cells in cancer immunotherapy, the role of γδ T cells produced endogenously should not be underscored. As stated earlier, TILs are a heterogeneous population, varying from effector to immunosuppressive leukocytes. Due to this heterogeneous presentation, the number of TILs has never been accepted as prognostic factor in human cancers since populations with antagonistic functions might be present at the same time within tumour microenvironment(69). Although diverse subsets of TILs have been correlated with good prognosis (reviewed in (69)) in this review the focus will be in γδ TILs. A meta-analysis study preformed in 2015(70) analysing the expression signatures of tumours with overall survival outcomes across 39 malignancies revealed "several broadly favourable prognostic T cell signatures, including γδ and CD8 T cells", proposing a favourable prognosis related to the number of γδ TILs. However, in this study it was also reported that "regulatory T-cells and macrophages can confer good or poor prognosis depending on context". This might be associated with an equilibrium between the immunosuppressive functions that totally block the immune response (poor prognosis) *vs* the immunosuppressive functions that by blocking a state of continuous inflammation prevent the tumour growth (good prognosis). A study in human breast cancer (71) reported an overall poor prognosis related to the number of γδ T cells in tumour infiltrates. However, it is important to denote that most of the TILs in those cases were γδ T regulatory lymphocytes which might be correlated with tumour progression functions.

Indeed, it can be denoted that the relationship between the number of TILs and the prognosis of malignancies is not straightforward and a lot of research still needs to be done before establishing a prognosis value to this parameter. It might come to knowledge in future studies that the type of TILs and not the quantity of TILs might be the best prognosis factor in the evaluation of cancer progression.

OBJETIVES

OBJECTIVES

Concerning to this issue, this project relies on the belief that the number and phenotype of Vδ1+ T cell subset presented in peripheral blood of patients with CLL, and MBL are different from those observed in healthy individuals. To prove this hypothesis an assessment of the number and phenotype of Vδ1+ T cells was performed. For that purpose, we evaluated the frequency of Vδ1+ T cells expressing or not CD27, allowing the discrimination between non-effector and effector cells; expressing the cytotoxic marker granzyme B and the activation marker CD69. The obtained results will be correlated with disease staging.

Moreover, the same evaluation was performed in other T cell subpopulations, like: CD4; CD8, double positive CD4/CD8 and Vδ1- γδ T cells, in order to understand if the alterations observed in Vδ1+ T cells also occurred in other T cells subpopulations, evidencing a broad antitumor activity against abnormal B cells or if this activity is more restricted to a specific T cell subpopulation, predicting the advantages of its selection and *ex vivo* expansion to use in T cell based immunotherapy.

METHODS

METHODS

1. STUDY GROUPS

In this study, the peripheral blood samples of fifty-eight individuals, collected in tubes K3E K3EDTA, were analysed. The samples belonged to fifty patients diagnosed with CLL or MBL of CLL type, and eight controls. The samples were collected since October of 2016 until March of 2017. Regarding to their origin, five patients' samples were from Centro Hospitalar Tondela – Viseu (Hospital de São Teotónio, Viseu), four were from Centro Hospitalar do Baixo Vouga (Hospital Infante D. Pedro, Aveiro) and forty-nine were from Centro Hospitalar e Universitário de Coimbra (Hospitais da Universidade de Coimbra and Hospital Geral de Coimbra – Hospital dos Covões).

The age of the individuals ranged between 45 and 92 years old, being the mean age 71±11 years old, following the subsequent gender distribution: 33 males **(56,90%)** and 25 females **(43,10%)**. These individuals were included in five groups according to the diagnosis performed by clinical haematologists: BINET B AND C CLL and BINET A CLL, according to CLL Binet staging system, HC-MBL and LC-MBL, according to WHO guidelines for classification of lymphoid neoplasms and a group control. The median age and gender distribution encountered in the different groups understudy are described in table 2.

2. CRITERIA FOR INCLUSION AND EXCLUSION OF PATIENTS

Were admitted to this study peripheral blood samples from patients that presented, by flow cytometry, B cells with CLL phenotype (CLL phenotype is described in introduction), despite the size of the clone (CLL or MBL), and with a diagnosis confirmed by clinical haematologists. Samples were from the time of the diagnosis or prior to patient's treatment. Previous chemotherapeutic treatment for CLL or for other malignancy was a factor for exclusion. Patients with B cells of CLL phenotype but with clinical courses that do not correlate with CLL symptoms were also excluded (for example, patients with SLL). Assessment of B cells' phenotype was performed using standardized Euroflow® panels for flow cytometric immunophenotyping of normal,

Table 2 - Characterization of patients by study groups. Characterization of the patients in the different groups understudy according to mean age, gender distribution, incidence of genetic features in malignant B cells, absolute counts of leukocytes, lymphocytes, neutrophils and platelets, and haemoglobin levels.

	BINET B AND C CLL	BINET A CLL	HC-MBL	LC-MBL	CONTROLS
Number of individuals (n)	9	26	10	5	8
Mean Age	76±4	70±12	69±15	68±8	76±7
Gender Distribution (M=Male, F=Female)	M (n=4) F (n=5)	M (n=14) F (n=12)	M (n=8) F (n=2)	M (n=2) F (n=3)	M (n=5) F (n=3)
Absolute Count of Leukocytes (x 10^9/L)	89,90±116,35	39,90±56,75	11,37±2,36	7,59±2,03	8,46±1,70
Absolute Count of Lymphocytes (x 10^9/L)	83,94±110,78	33,24±57,02	5,99±1,90	2,40±1,03	1,50±0,51
Absolute Count of Neutrophils (x 10^9/L)	4,67±4,77	5,74±2,44	4,37±1,57	4,10±0,99	6,03±1,96
Haemoglobin (g/dL)	12,36±2,16	13,73±1,67	13,08±2,49	13,58±0,79	14,26±0,99
Platelets (x 10^9/L)	122,44±65,12	196,27±75,66	211,80±65,76	204,60±36,68	198,25±52,78
Genetic features	2 del(17p) 1 del(13q)	1 del(17p) 3 del(13q) 1 Tris12	-	-	-

reactive and malignant leukocytes(72), with resource to Lymphoid Screening Tube (LST) and tube 2 of B-CLPD diagnosis panel, whose monoclonal antibodies are described in table 3. These tests were performed in hospital daily routine, and confirmation of CLL phenotype was made upon clinical reports given by the Unit of Operational Management in Cytometry.

3. CLINICAL DATA COLLECTION

The necessary data to risk stratification was collected from several platforms used in public hospitals and included a full blood count (to access absolute count of leukocytes, lymphocytes, neutrophils and platelets, and haemoglobin levels), a CT scan (to evaluate ganglionic involvement), and several biochemical tests, such as: β2-microglubulin (for risk assessment), C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR), for evaluation of infectious states. The presence of genetic abnormalities (del(17p), del(13q), del(11q) and Tris12) were also assessed. Clinical haematologists of the different hospitals performed the diagnosis and risk stratification classification based on the guidelines described in the introduction. The data related to full blood counts and genetic features (although the last one was not available for all patients) is described in table 2. The remaining results, because they were unavailable for many patients were only considered for evaluating the disease severity and they were not described in table 2.

4. QUANTIFICATION AND PHENOTYPIC CARACTHERIZATION OF LYMPHOCYTE POPULATIONS

The identification of the different lymphocyte populations and the evaluation of their phenotype was performed by multicolour flow cytometry with recourse to three tubes: two tubes of Euroflow® panels: LST and Tube 2 of B-CLPD panel, as described previously, and a third specific tube designed for this project, further referred as Vδ1 tube, that included TCRVδ1, CD45, CD3, CD27, CD4, CD8, CD69 and granzyme B. The fluorochromes chosen for each monoclonal antibody, as well as the clones used, are described in table 3.

Table 3 - Table of monoclonal antibodies, respective fluorochromes and clones used in LST, tube 2 B-CLPD and Vδ1 tubes. In this table it is described the monoclonal antibody combinations in terms of fluorescence for the three tubes used in this project.

Fluorochrome	LST Tube	Tube 2 B-CLPD	Vδ1 Tube
Fluorescein Isothiocyanate (FITC)	CD8/LAMBDA (Mix)	CD23 (clone MHM6)	CD3 (clone UCHT1)
Pacific Blue (PB/V450)	CD20/CD4 (clone L27/ clone RPA-T4)	CD20 (clone L27)	CD4 (clone RPA-T4)
Allophycocyanin (APC)	CD3 (clone SK7)	CD200 (clone OX104)	TCRVδ1 (clone REA173)
Phycoerythrin (PE)	CD56/KAPPA (Mix)	CD10 (clone ALB1)	GRANZYME B (clone GB11)
Phycoerythrin-Cyanin 7 (PE-Cy™7)	CD19/TCRγδ (clone J4.119/ Clone 11F2)	CD19 (clone J4.119)	CD69 (clone L78)
Peridinin chlorophyll protein-cyanine 5.5 (PerCP-CY™5.5)	CD5 (clone L17F12)	CD79b (clone 3A2-2E7)	CD27 (clone L128)
Allophycocyanin-Hilite 7 (APC-H7)	CD38 (clone HB7)	CD43 (Clone 1G10)	CD8 (clone SK1)
Pacific Orange (PO)	CD45 (clone 2D1)	CD45 (clone 2D1)	CD45 (clone 2D1)

Using the LST tube, we could assess the lymphocyte's percentage, compare these values to the ones observed in Vδ1 tube and evaluate the T cell subpopulations distribution (in percentage): CD4+, CD8+, CD4+CD8+ and γδ T cells (with CD3, CD4, CD8, CD5 and TCR γδ monoclonal antibodies), as represented in figure 8.

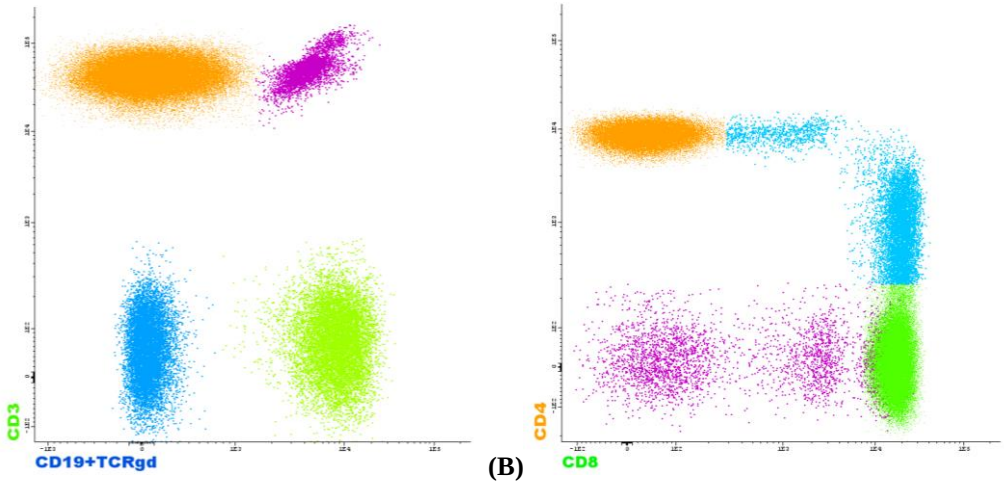


Figure 8 - (A) B, T and NK Lymphocytes Distribution and (B) T cell subpopulations distribution. (A) In figure A it is represented a dot plot comparing CD3 expression vs CD19 and TCRγδ expression. Regarding CD3+ cells, we observe two populations (orange and purple), representing αβ T cells and γδ T cells, respectively, as purple cells also display expression of TCRγδ marker. The population that is CD19+, accompanied by the absence of CD3 expression is a population of B cells (green). Cells that do not express either CD3, CD19 or TCRγδ markers are NK cells (blue). **(B)** In figure B, in a dot plot comparing CD4 and CD8 expression, the CD3 + T cells are separated in their subpopulations. CD4+T cells (orange) and CD8+ T cells (green) represent the major populations in peripheral blood samples, as CD4+CD8+T cells (blue) and γδ T cells (purple) represent the minor subpopulations present in peripheral blood. Vδ1 T cells are not represented in this figure, as their percentage were only available in Vδ1 tube.

This tube, as a lymphoid screening test, also allows to obtain the percentage of pathological B cells (by analysis of the CD5, CD20, CD19 and kappa and lambda light chains monoclonal antibodies). By combining the LST with tube 2B of B-CLPD panel we are able to confirm the CLL phenotype (with CD19, CD20, CD5, CD38, CD23, CD79b, CD10, CD200 and CD43), as represented in figure 9. By analysing the percentages of these cells in peripheral blood, absolute counts of T, B (total and pathological) and NK cells, as well as CD4+, CD8+, CD4+CD8+ and γδ T cells were calculated using the data available in full blood counts provided by the hospital database. Vδ1 T cells percentage was assessed with Vδ1 tube and its absolute count in peripheral blood was also obtained.

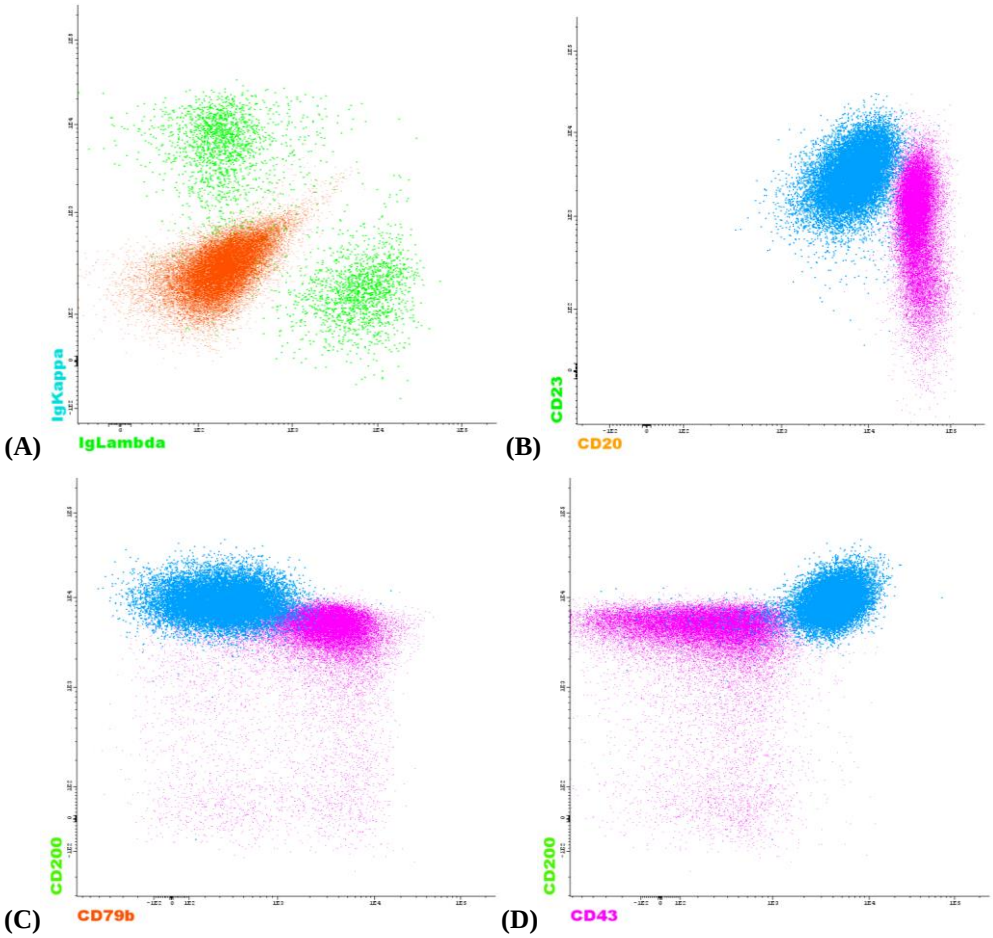


Figure 9 - Dot plots displaying normal and pathological B cells. (A) B cells in LST tube. In this plot we compare expression of Kappa and Lambda chains in B cells. It can be seen that normal B cells (green) express these chains in a normal Kappa/Lambda ratio and present a stronger expression comparing to pathological B cells (orange) that are clonal to Kappa light chain and present a weaker expression of this marker when compared to normal B cells. **(B), (C)** and **(D)** By comparing different markers of tube 2 of B-CLPD the phenotype of CLL cells can be observed: dim expression of CD20, CD23+, CD200++, CD43+ and CD79b dim to negative (blue cells).

5. PHENOTYPIC CARACTHERIZATION AND EVALUATION OF EFFECTOR AND CYTOTOXIC FUNCTIONS OF T CELL SUBPOPULATIONS

Using Vδ1 tube, we were able to separate the different T cell subpopulations: CD4+, CD8+, CD4+CD8+, Vδ1 T cells and other Vδ1-γδ T cells. After this identification, each T cell subpopulation was assessed in terms of total percentage of CD69 and granzyme B expression. Each T cell subset was further divided in their CD27+ and CD27- counterparts, being each compartment assessed for its expression of CD69 and granzyme B markers.

In order to perform this experiment, were pipetted 250 µL of a peripheral blood sample to a 12x75 mm propylene tube together with the following monoclonal antibodies: FITC Mouse Anti-Human CD3 (Beckman Coulter, cat. nº. A07746), V450 Mouse Anti-

Human CD4 (BD Horizon™, material nº. 560345), PE-Cy™7 Mouse Anti-Human CD69 (BD Biosciences, cat. nº. 335792), PerCP-Cy™5.5 Mouse Anti-Human CD27 (BD Biosciences, cat. nº. 656643), V500-C Mouse Anti-Human CD45 (BD Biosciences, cat. nº. 655873), APC-H7 Mouse Anti-Human CD8 (BD Biosciences, cat. nº. 641400) and APC Recombinant Human, Anti-Human Vδ1 (Miltenyi Biotec, order nº 130-100-519). The mixture was mixed in the vortex and incubated 10 minutes in the dark after which it was added 100µL of Fix and Perm Solution A of FIX & PERM™ Cell Permeabilization Kit (ThermoFisher, cat. nº. GAS003). The solution was mixed once again in the vortex. The sample was subjected to 10 more minutes of incubation. After this step, the mixture was washed with 2 mL of PBS and centrifuged 5 minutes at 1500G. After the centrifugation, the supernatant was discarded and 100µL of Fix and Perm Solution B of FIX & PERM™ Cell Permeabilization Kit (ThermoFisher, cat. nº. GAS004), alongside PE Mouse Anti-Human Granzyme B (BD Pharmingen™, cat. nº. 561142) were added to the mix with an incubation time of 20 minutes. After this incubation time, the mixture was washed 2 times, using once again 2 mL of PBS and centrifuged 5 minutes at 1500G. After the two washes, were added to the sample 100µL of PBS after which the acquisition on BD FACS Canto II (BD Bioscienses; San José, CA, USA) was performed. The FCS files were produced by the software BD FACSDiva (v6.1.2; BD) and the file was analysed with Infinicyt® v1.8 (Cytognos, S.L., Salamanca, Spain).

6. STATISTICAL ANALYSIS

The results were statistically analysed using IBM SPSS Statistics 23 (SPSS, version 23.0, Armonk, NY, USA) using dispersion and boxplot graphics. Columns bars graphics were performed in Microsoft Office Excel, using the values of mean and standard deviation calculated by SPSS. The statistical tests used were non-parametric tests: Kruskal-Wallis and Mann Whitney-tests. The P value established as significant was $P<0,05$.

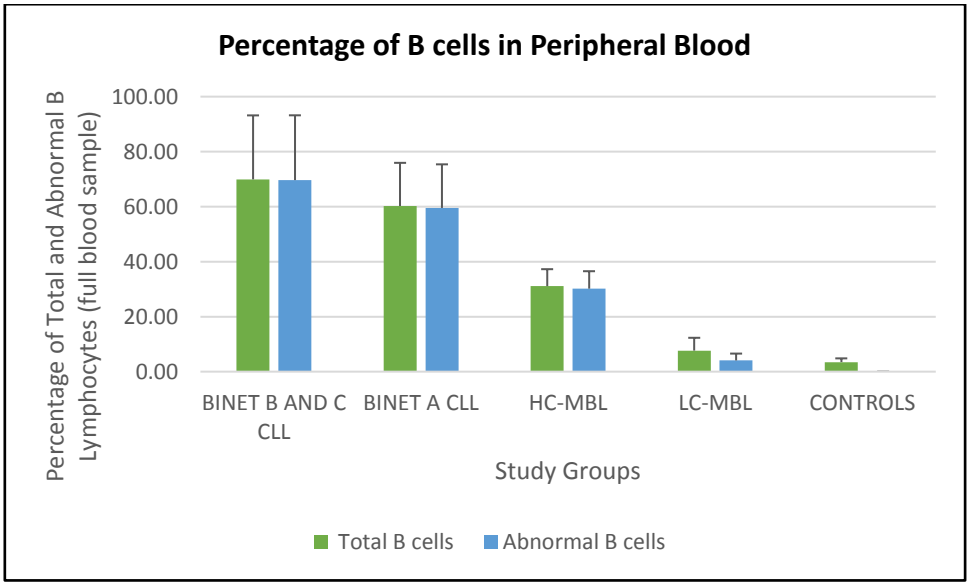
RESULTS

RESULTS

1. PERCENTAGES AND ABSOLUTE COUNTS OF B, T AND NK LYMPHOCYTES IN FULL BLOOD SAMPLES

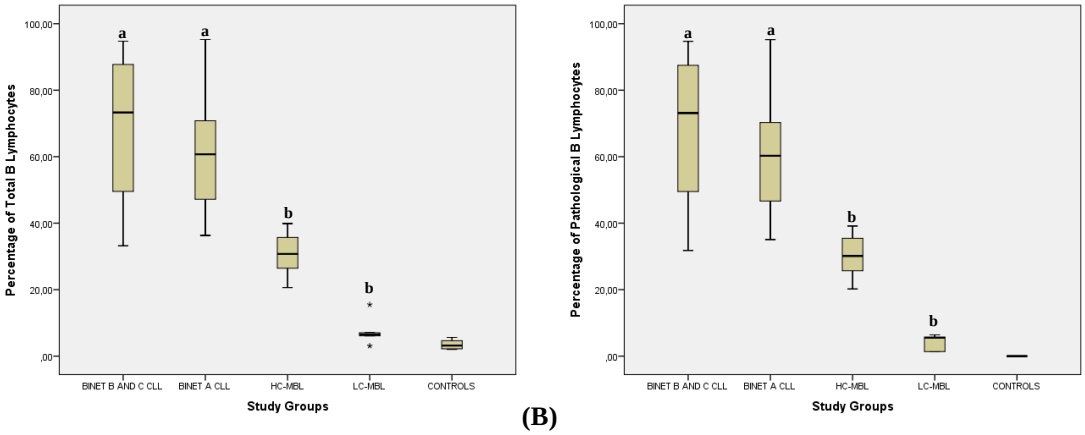
1.1. PERCENTAGE AND ABSOLUTE COUNTS OF B CELLS IN PERIPHERAL BLOOD

The percentage of total and pathological B cells in the whole peripheral blood samples are represented in graphic 1. It can be observed an increasing percentage of total B cells and abnormal B cells from controls to advanced stages of CLL (P<0,001, for both variables, when comparing all groups understudy).



Graphic 1 - Percentage of Total and Abnormal B lymphocytes on patient's full peripheral blood samples – In this graphic it can be observed an increasing percentage of not only B lymphocytes in general (green), but also the increasing proliferation of abnormal B lymphocytes (blue) across all groups understudy, from controls to Binet B and C CLL group (P<0,001, for both variables, comparing all groups understudy).

Moving further to the comparison between the groups understudy, no statistical significant differences were observed in the percentage of total and pathological B cells when the Binet B and C CLL group was compared to the Binet A CLL group. On the other hand, when comparing individually these groups to the other entities understudy, both showed statistical significant differences across all groups. HC-MBL and LC-MBL presented statistical significant differences between them, but also when compared to all the other groups understudy (graphic 2A and 2B).



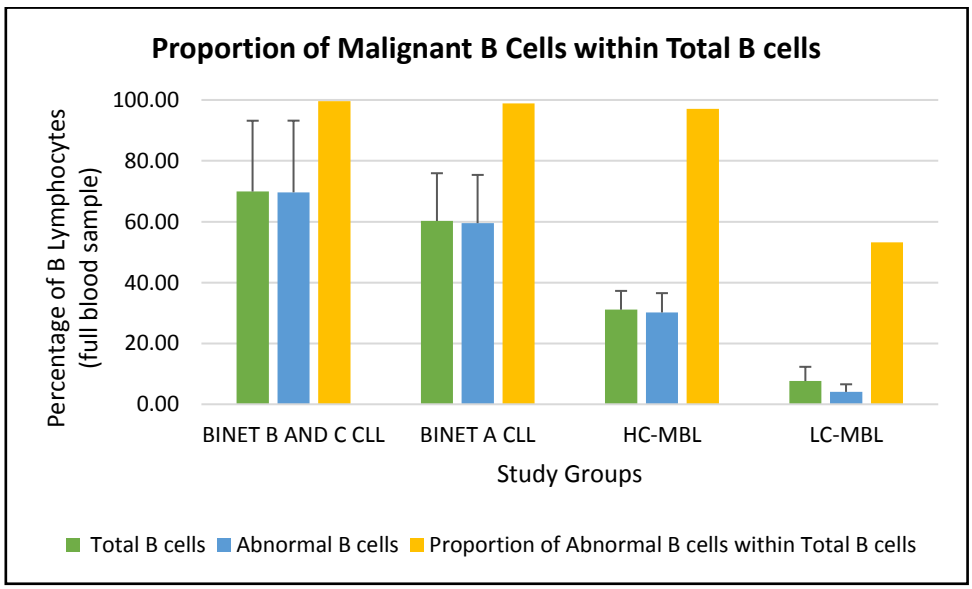
(A) **(B)**

Graphic 2 - (A) Percentage of Total B lymphocytes and (B) Percentage of Pathological B Lymphocytes in full peripheral blood samples – In these graphics it is evidenced a statistical significant increase in the percentage of (A) total and (B) pathological B cells from controls to Binet B and C CLL groups. Despite these observations, no statistical significant differences were observed when comparing both CLL groups.

[a] – P<0,05, comparing individually this group with both MBL and control groups.

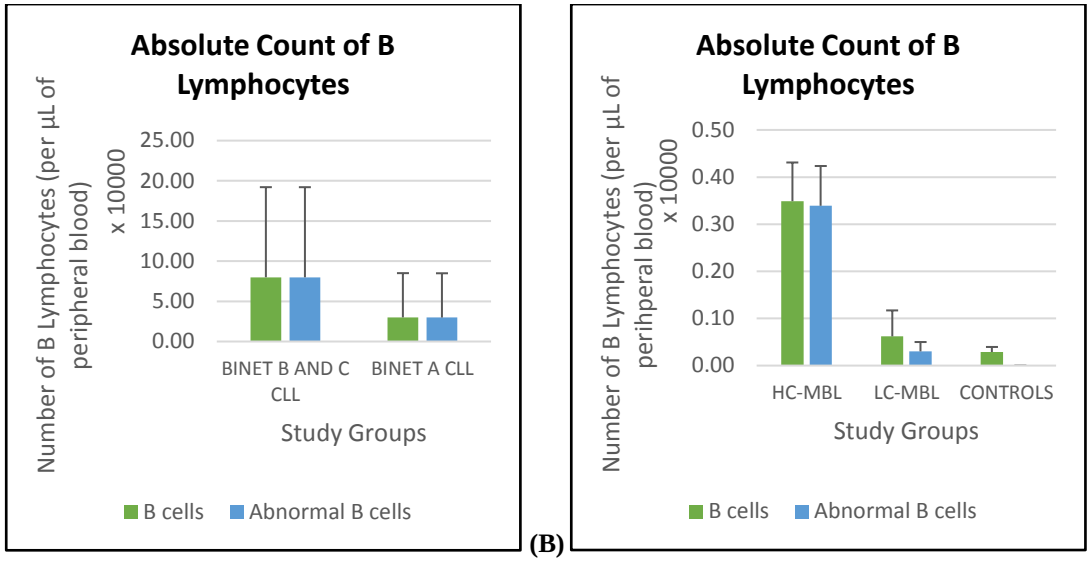
[b] – P<0,05, comparing individually this group with all groups understudy.

From the observation that pathological B cells comprised the majority of B cells with disease progression, analysis of the proportion of malignant B cells within total B cells was performed, revealing that this proportion in HC-MBL (97,10%) is very similar to the ones observed for Binet A CLL (98,82%) and Binet B and C CLL (99,63%) while in LC-MBL only about 50% of the B cells were pathological (53,2%), evidencing a high similarity between HC-MBL and CLL groups (graphic 3), in the proportion of malignant cells in peripheral blood.



Graphic 3 - Proportion of Abnormal B cells within Total B lymphocytes on patient's full blood samples – The analysis of the proportion (yellow) between the percentage of abnormal B lymphocytes (blue) and total percentage of B lymphocytes (green) evidences that in HC-MBL and both groups of CLL, the proportion is higher than 95%, contrasting to LC-MBL group, where this proportion is only 53,2%.

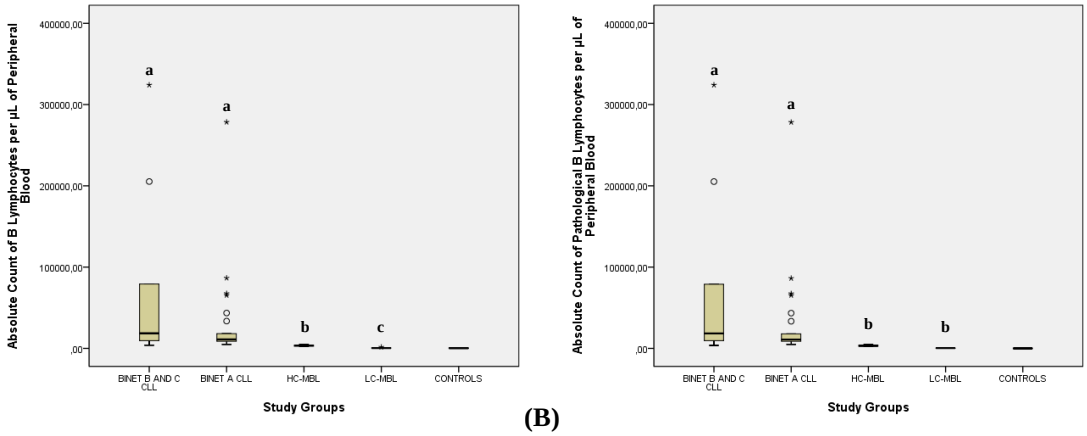
To conclude the analysis of B cells, the absolute counts of total and abnormal B lymphocytes (number of B lymphocytes per µL of peripheral blood) were also assessed. B cells exhibited an increase in their absolute counts, for both total and abnormal B-cell absolute counts from controls to advanced stages of the disease, with Binet B and C CLL group revealing mean values, for both variables, approximately three times higher than Binet A CLL group and twenty-four times higher when compared to HC-MBL group, demonstrating once again, the massive proliferation of pathological B cells, mainly in advanced stages of the disease (P<0,001, for both variables, when comparing all groups understudy). These results are evidenced in graphic 4.



Graphic 4 – Absolute count of total and abnormal B lymphocytes for (A) Binet B and C CLL and Binet A CLL and (B) for HC-MBL, LC-MBL and group control – In these graphics it can be observed the absolute counts of total B lymphocytes (green) and abnormal B lymphocytes (blue) in the different groups understudy, and the tendency for the increase of these values with disease progression. The mean absolute count of abnormal lymphocytes is near 24x higher in more advanced stages of the disease, comparing to HC-MBL, evidencing an exponential proliferation of B cells with disease progression.

By comparing the differences among groups individually (graphic 5), the same pattern observed for the percentage of total and pathological B cells could be observed for the absolute count of B lymphocytes, exception made for LC-MBL group that doesn't reach statistical significant differences in absolute count of total B lymphocytes when compared to control group (P=0,079). In resume, in our study we observed increasing percentages and absolute counts of total and pathological B cells, from group control to advances stages of CLL disease, with statistical significant differences among the groups understudy. It was also observed a similar percentage of pathological B cells among total B cells in HC-MBL and both groups of CLL, while LC-MBL presented a much lower

percentage of pathological B cells. The mean values and standard deviations for all these variables are described in table 4, in appendix.



(A) **(B)**

Graphic 5 – (A) Absolute count of Total B lymphocytes and (B) Absolute Count of Pathological B Lymphocytes in full peripheral blood samples – Both graphics evidence a statistical significant increase in the absolute counts for both total and pathological B cells from controls to Binet B and C CLL groups. Despite this evidence, no statistical significant differences were observed when comparing both CLL groups.

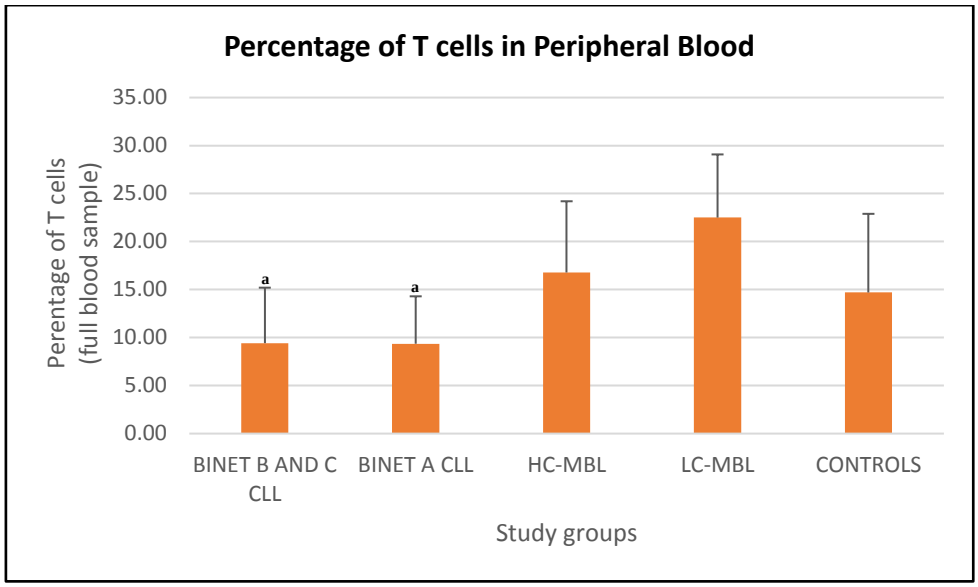
[a] – P<0,05, comparing individually this group with both MBL and controls groups.

[b] – P<0,05, comparing individually this group with all groups understudy.

[c] – P<0,05, comparing individually this group with all groups understudy, except for control group.

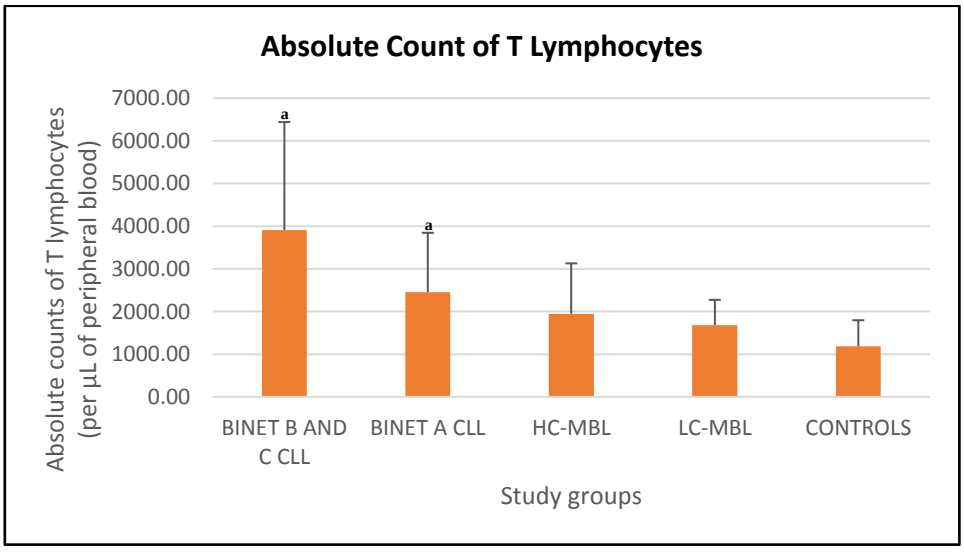
1.2. PERCENTAGE AND ABSOLUTE COUNTS OF T CELLS IN PERIPHERAL BLOOD

Moving from B cells to T cells, the total percentage of T cells in the whole sample was analysed (graphic 6), and an inverted progression, comparing to B lymphocytes, was observed, with CLL patients showing lower percentages of T cells when compared to the other entities understudy (P=0,002, comparing all groups understudy). Nevertheless, T cells in group control do not follow this pattern, exhibiting a percentage very similar to the ones observed for CLL groups (no statistical significant differences observed when CLL groups were compared with control group). These lower percentages, however, do not translate to lower absolute counts of T lymphocytes in CLL groups, as these are relative percentages and, due to the massive proliferation of B lymphocytes, T cells decrease their relative percentage in the whole blood but not their absolute counts (graphic 7). The absolute count of T cells (number of T lymphocytes per µL of blood) increases from controls to Binet B and C CLL group, as occurs in B lymphocytes, (p=0,014, comparing all groups understudy), however, in a much slower rate comparing to B cells.



Graphic 6 - Percentage of T lymphocytes on patient's full blood samples – Decreasing percentages of T cells can be seen from LC-MBL to Binet B and C CLL, passing through all the groups in between. Inverting this tendency is control group, showing a T cell percentage very similar to ones observed for CLL groups, but higher than the ones observed for these entities. The percentage of T cells in MBL groups are higher than those observed in control group which might point to an expansion of T cells in early stages of disease onset.
[a] – P <0,05, comparing individually this group with both MBL groups.

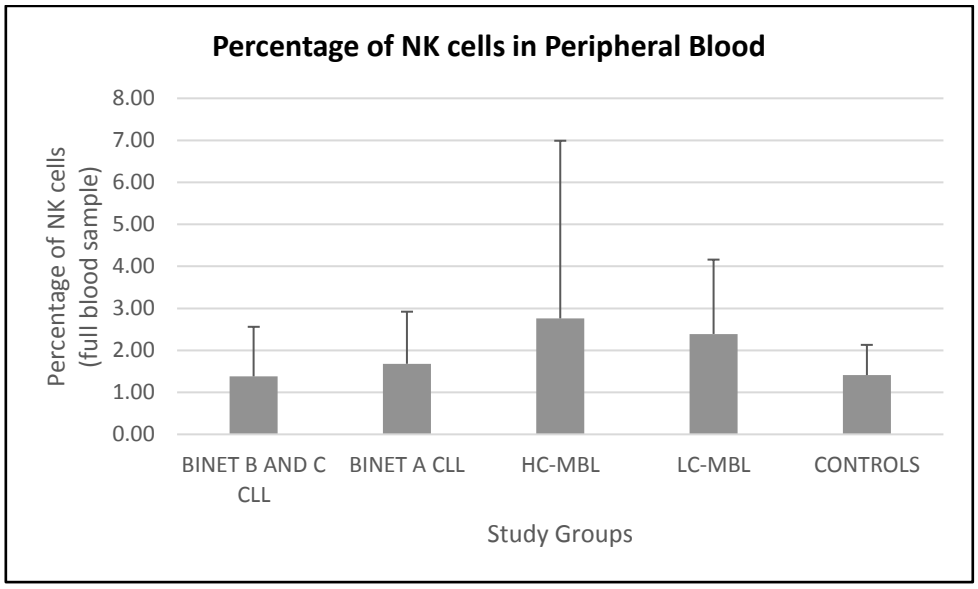
As occurred in B cells, T cells percentages and absolute counts among CLL groups did not present statistical significant differences. However, in these cases, MBL groups did not also present statistical differences among them. Both CLL groups displayed statistical significant differences in T cell percentages when confronted to MBL groups and in T cell absolute counts when compared to control group. The mean values and standard deviations for both variables are represented in table 4, in appendix.



Graphic 7 - Absolute count of T lymphocytes in all groups understudy – Absolute counts of T lymphocytes per µL of peripheral blood increases from group control to Binet B and C CLL which evidences an increased proliferation of T cells in advanced stages of the disease progression.
[a] – P <0,05, comparing individually this group with group control.

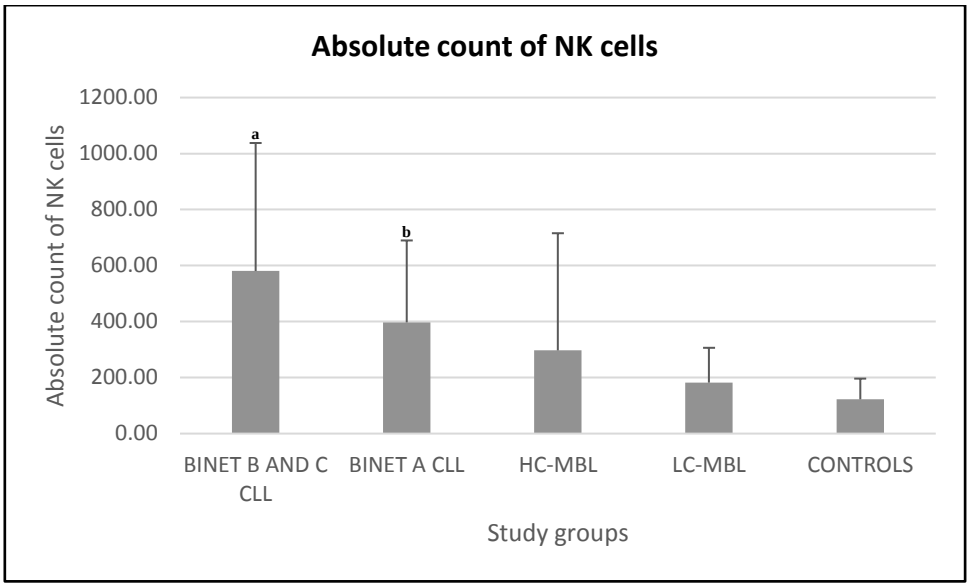
1.3. PERCENTAGE AND ABSOLUTE COUNTS OF NK CELLS IN PERIPHERAL BLOOD

To conclude the analysis of the distribution of lymphocytes' populations, graphic 8 displays the relative percentages of NK cells within total peripheral blood samples. Although displaying a large standard deviation in HC-MBL group, NK cells seem to follow a pattern that is similar to T lymphocytes, but with a less noticeable decrease in NK cells' percentage from LC-MBL to CLL groups, and without reaching statistical significant differences (P=0,767, when comparing all groups understudy).



Graphic 8 - Percentage of NK lymphocytes on patient's full blood samples – In the case of NK cells, the same pattern observed for T cells seemed to be present, with NK cells percentage increasing in a lower grade from Binet B and C CLL to LC-MBL. Though, NK cells percentage didn't present statistical significant differences when compared to the other groups understudy (P=0,767).

Regarding to NK cells' absolute counts (graphic 9), as spotted for the other lymphocytes' subsets, their absolute count (number of NK cells per μL of peripheral blood) also augmented from controls to Binet B and C CLL group (p=0,004, comparing all groups understudy).



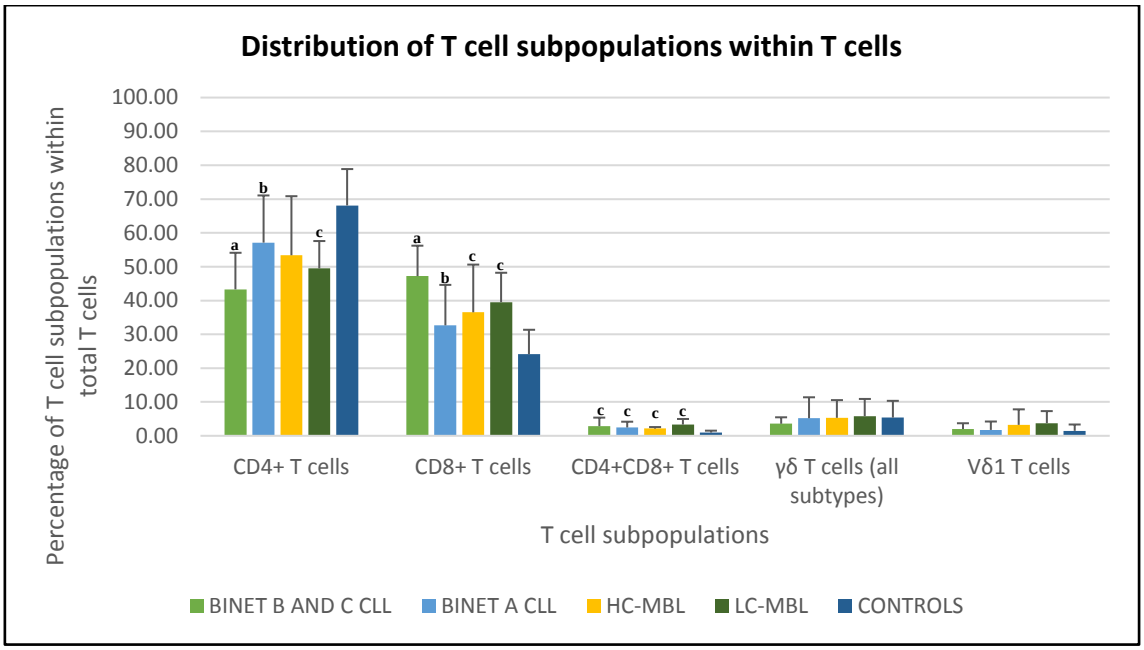
Graphic 9 - Absolute count of NK cells in all groups understudy – Absolute counts of NK cells per µL of peripheral blood increases from group control to advanced stages of the disease (p=0,004), as observed for the other subpopulations of lymphocytes.
[a] – P <0,05, comparing individually this group with control group.
[b] – P <0,05, comparing individually this group with HC-MBL and control group.

In terms of statistical significant differences among groups, those were not observed in NK cells percentage. In NK cells absolute counts, Binet A CLL group exhibited significant differences when compared to HC-MBL group and control group. On the other hand, Binet B and C CLL group only presented differences when compared to group control (graphic 9).

In resume, T cells and NK cells display decreasing percentages in full blood samples, opposing to what was observed for B lymphocytes, from controls to Binet B and C CLL. However, these lower percentages do not translate to decreased absolute counts and, as occurs in B cells, T and NK cells also increase their absolute counts from controls to CLL groups.

2. DISTRIBUTION OF T CELL SUBPOPULATIONS

From the observation that T cells absolute counts were increased from controls to CLL groups, further analysis of T cells subpopulations was performed, starting with T cells subsets distribution within T cells, in order to access which subpopulations were more expanded across these entities. The percentage of T cells subpopulations within total T cells can be seen in graphic 10.

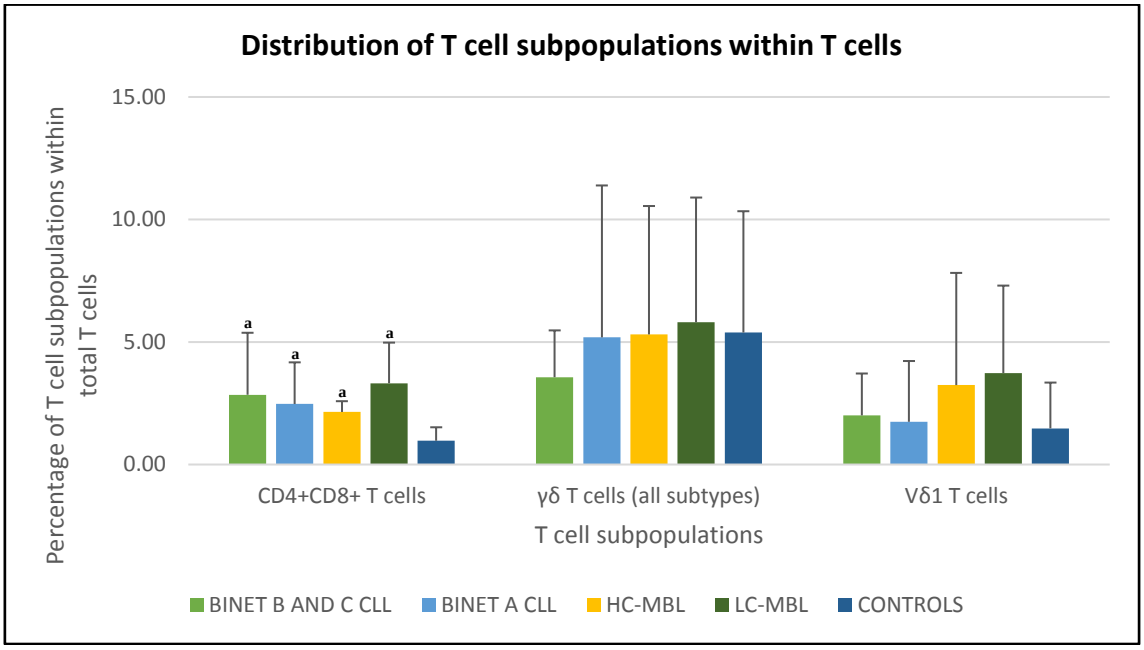


Graphic 10 – Distribution, in percentage, of T cell subpopulations within total T cells – CD4+ and CD8+ T cell subpopulations represent the majority of T cells, comprising 43,29% to 68,10% and 24,12% to 47,21% of T cells, respectively. The minor T cell subpopulations represent, for CD4+CD8+ T cells: 0,97% to 3,32% and for γδ T cells: 3,56% to 5,81%. CD4+, CD8+ and CD4+CD8+T cell subpopulations present with statistical significant differences among the studied groups (P<0,05, comparing all the entities understudy).
[a] – P <0,05, comparing this group with Binet A CLL and control group.
[b] – P <0,05, comparing this group to Binet B and C CLL and control group.
[c] – P <0,05, comparing this group to control group.

By analysing in first place the two major populations of T lymphocytes (CD4+ and CD8+ T cells), it could be observed that CD4+ T cells percentage decreases from controls to Binet B and C CLL group (p=0,01, comparing all groups understudy), which was accompanied by an increasing percentage of CD8+ T cells from controls to advanced stages of the disease (p=0,004, when comparing all groups understudy). CD4+ T cells percentage displayed statistical significant differences when comparing Binet B and C CLL group to Binet A CLL group, but also when these groups were individually compared to control group. This percentage was also significantly different for LC-MBL when this group was compared to group control. Concerning to CD8+ T cells percentage, the same statistical significant differences observed for CD4+ T cells apply to these cells, but in this T cell subpopulation, HC-MBL also presented statistical significant differences, when compared to the group control.

Moving forward to the distribution of the minor T cell subpopulations, CD4+CD8+ T cells in the peripheral blood presented a significant lower percentage in the group control, when compared with all groups understudy, with an increase in its percentage observed from controls to Binet B and C CLL (p=0,01, when comparing all

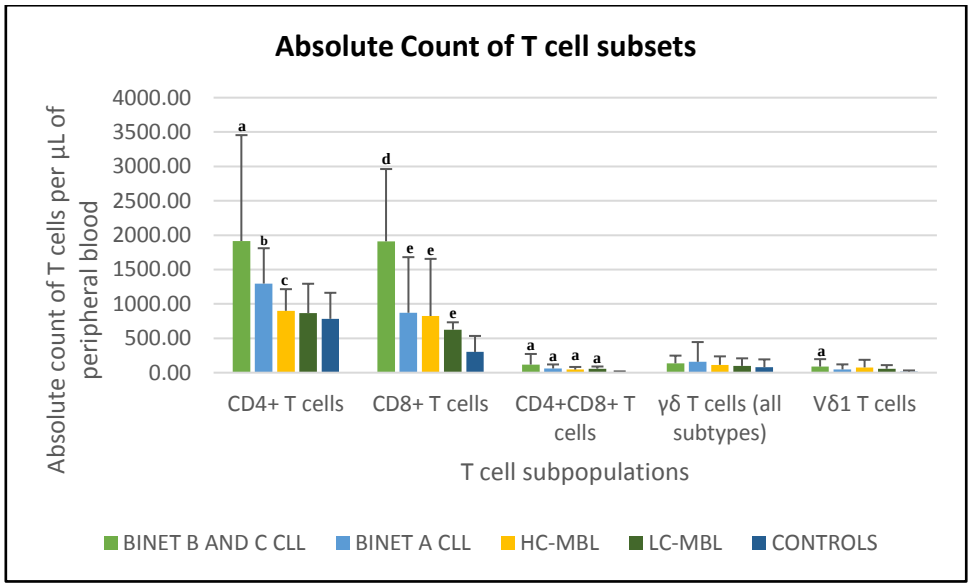
groups understudy). By analysing the percentages of γδ T cells and, particularly, of Vδ1 T cells, it could be observed that although showing similar percentages across all entities, total γδ T cells present their lowest percentage in Binet B and C CLL patients (p=0,990, when comparing all groups understudy). This lower percentage is however accompanied with an expansion of Vδ1 compartment, with a higher proportion of Vδ1 T cells among γδ T cells observed in this group. It was also exciting to observe that the higher percentages of Vδ1 T cells were observed for both MBL groups, with CLL groups presenting percentages very similar to the ones detected for the control group (p=0,572, comparing all groups understudy). No significant differences among groups were observed for both total γδ and Vδ1 T cells' percentages. The mean values and standard deviations for the distribution of T cell subpopulations are exhibited in table 5, in appendix.



Graphic 11 - Distribution, in percentage, of the minor T cell subpopulations within total T cells – CD4+CD8+ T cells displayed, in general, an increase in their percentage from controls to CLL groups (p=0,01). On the other hand, γδ T cells presented the same distribution across all entities, showing only a slight decrease in Binet B and C CLL group (p=0,990). Vδ1 T cells present with an augmented percentage in MBL groups comparing to the other groups, but with a distribution very similar among groups (p=0,572). [a] – P<0,05, comparing individually this group with group control

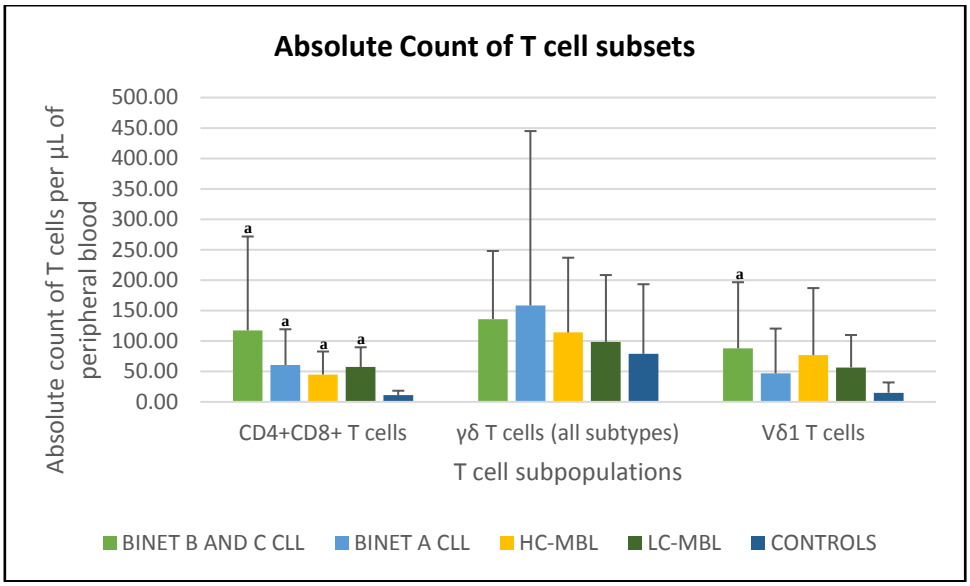
Regarding now to T cell subpopulations' absolute counts (graphic 12), as expected, as total T lymphocytes displayed an increase in their absolute counts from controls to CLL, the different T cell subpopulations also presented this pattern (mean values and standard deviations of absolute counts for all the T cell subpopulations are accessible in table 5, in appendix). However, an interesting observation that was made

was, that for Vδ1 T cells, this increasing tendency was not accompanied by Binet A CLL group, that presented a lower absolute count of Vδ1 T cells comparing to the other groups understudy. On the other hand, Binet B and CLL group displayed a statistical significant increase in Vδ1 T cells absolute counts comparing to the control group, evidencing a strike expansion of Vδ1 T cells in advanced stages of the disease.



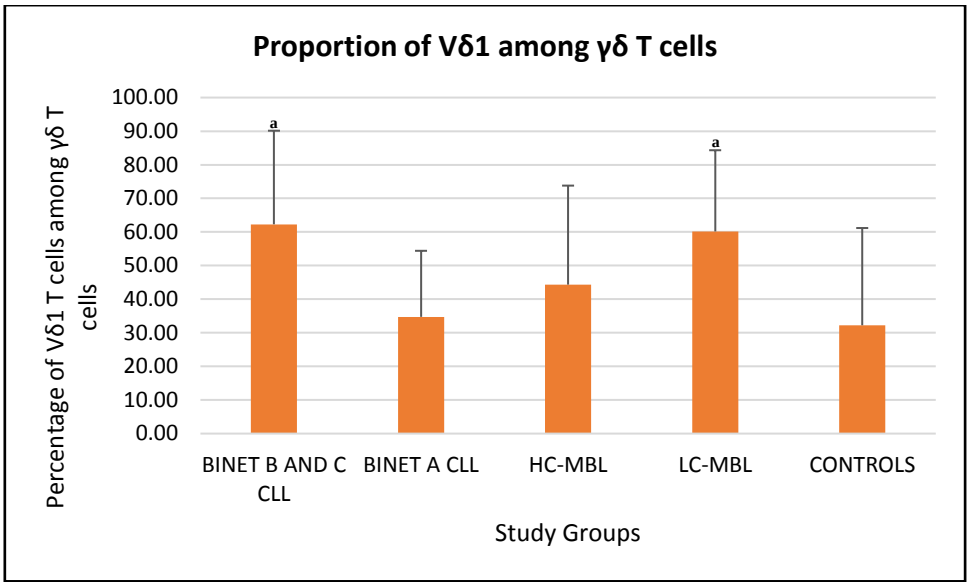
Graphic 12 - Absolute Count of T cell subpopulations per µL of peripheral blood – In this graphic we can observe, for all the groups understudy, an increase in absolute counts of all subsets of T cells, except for Vδ1 T cells that present a deviation from this increasing tendency for Binet A CLL group. Vδ1 T cells displayed, in Binet B and C CLL a statistical significant increase in their absolute counts when compared to control group.
[a] – P<0,05, comparing individually this group with control group.
[b] – P<0,05, comparing individually this group with HC-MBL and controls group.
[c] – P<0,05, comparing individually this group with Binet A CLL group.
[d] – P<0,05, comparing individually this group with all groups understudy.
[e] – P<0,05, comparing individually this group with Binet B and C CLL and control group.

As it is clarified in graphic 13, Vδ1 T cells present with their lowest absolute count in Binet A CLL (exception made for group control) which is completely contrary to the fact that absolute count of total γδ T cells is the highest in Binet A CLL. These data suggest that the expansion of γδ T cells observed for Binet A CLL is not attributed to a Vδ1 expansion, contrary to Binet B and C CLL where this expansion is above 60%, a result spotted in graphic 14. It can also be noticed that, exception made for Binet A CLL, the remaining groups behave the same way as the other T cell subpopulations, with increasing Vδ1 T cells absolute counts observed with disease progression.



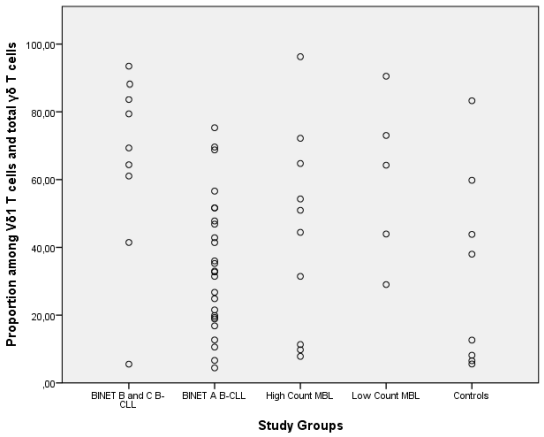
Graphic 13 - Absolute Count of T cell subpopulations per µL of peripheral blood (minor subpopulations) – In this graphic it can be observed that CD4+CD8+ T cells and γδ T cells in general increase their absolute counts from controls to CLL groups. However, a different pattern is observed for Vδ1 T cells, with Binet A CLL groups showing lower absolute counts, when compared to the other disease entities.
[a] – P<0,05, comparing this group with group control

Lastly, we were interested to further analyse this particular observation of heterogeneity in Vδ1 T cells and for this reason, we tried to understand the pattern associated with the percentage of Vδ1 T cells among γδ T cells, which would allow us to easily verify if these cells were expanded when compared to the other γδ T cells and in which entities that was verified. We observed that control group showed the lowest proportion of Vδ1 T cells among γδ T cells (32,23±28,95). These values were very similar to the ones observed for BINET A CLL and HC-MBL (34,74±19,66 and 44,33±29,47, respectively). However, for LC-MBL and BINET B and C CLL were observed values above 50% which demonstrated an expansion of Vδ1 T cells, comparing to the other γδ subpopulations in these groups (60,16±24,17 and 62,28±27,86, respectively). Binet B and C CLL group, as well as LC-MBL group presented statistical significant differences for this variable when compared to Binet A CLL and control groups.



Graphic 14 – Proportion of Vδ1 T cells among total γδ T cells – Control group, alongside, Binet A CLL group evidenced the lowest expansion of Vδ1 T cell subset. On the other hand, LC-MBL and Binet B and C CLL demonstrate values higher than 50%, displaying a higher prevalence of Vδ1 T cells in proportion to total γδ T cells.
[a] – P<0,05, comparing individually this group with Binet A CLL group and control group.

Using a dispersion graphic (graphic 15), it could be detected that almost all Binet B and C CLL cases analysed in this study presented with huge expansions of Vδ1 T cells, with Vδ1 T cells comprising, for most of them (n=7), the major γδ T cell subpopulation in the peripheral blood. These results oppose to more heterogeneous distributions on the other groups understudy, which evidences that in advanced stages of the disease the major γδ T cell population present in peripheral blood is Vδ1 T cells, reflecting an expansion of this subpopulation, that occurs with disease progression.



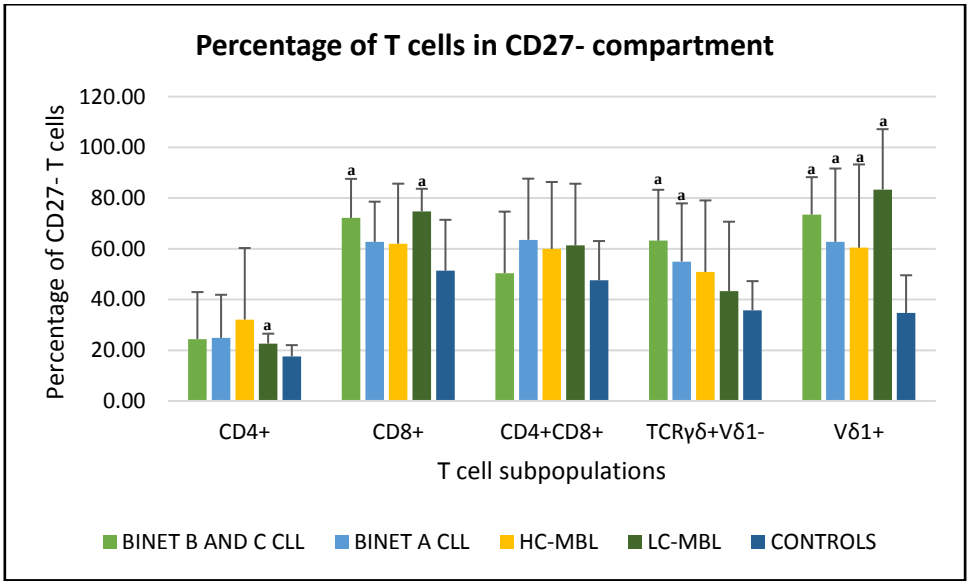
Graphic 15 - Percentage of Vδ1 T cells among γδ T cells observed for each individual enrolled in this study: In this graphic it can be evidenced that the majority of patients belonging to Binet B and C CLL group presented proportions of Vδ1 T cells among γδ T cells higher than 50%, which suggests an expansion of this subpopulation in advanced stages of the disease.

3. DISTRIBUTION OF T CELLS IN CD27 COMPARTMENTS

After being capable of separating T cell subpopulations, we tried to understand how they behave in the different stages of the disease, evaluating their cytotoxic profile (expression of granzyme B) and their activation and effector status (with CD69 and CD27 markers, respectively).

3.1. CD27 EXPRESSION IN T CELL SUBSETS

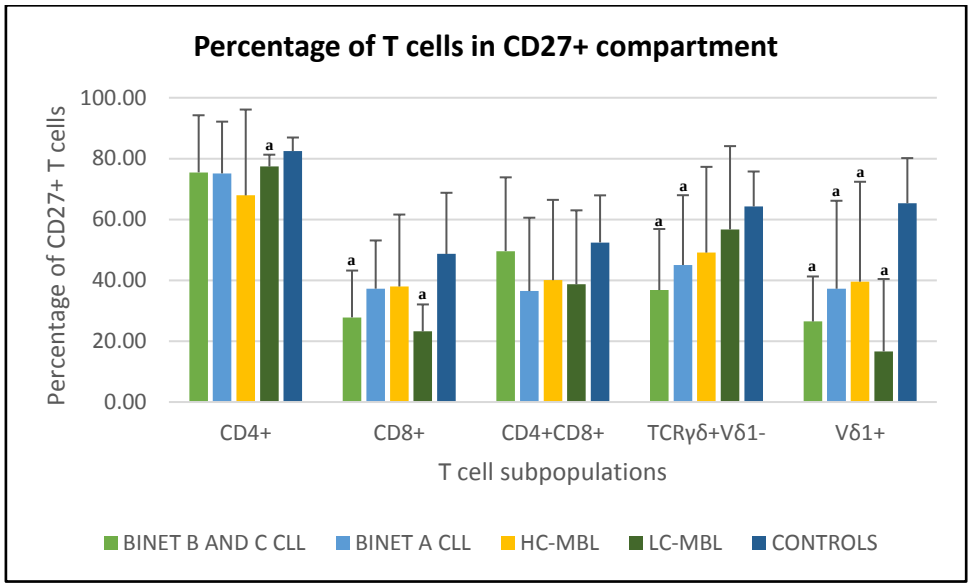
In this section, graphics concerning percentages of T cells subsets that are negative for CD27 expression (graphic 16) and positive for CD27 expression (graphic 17) are presented. As it can be observed in graphic 16, in all T cells subsets, group control presents the lowest percentage of T cells in CD27- compartment, and, with the exception of some deviations, this percentage increases from controls to Binet B and C CLL groups (P=0,020 for Vδ1 T cells, and no statistical significance differences observed for the other groups, comparing all groups understudy: CD4+: P=0,719; CD8+: P=0,130; CD4+CD8+: P=0,399 and Vδ1-γδ T cells: P=0,127).



Graphic 16 - Percentage of CD27- T cells in T cell subpopulations – CD27- T cells compartment (effector compartment) increases from controls to Binet B and C CLL, with statistical significant differences observed for Vδ1 T cells (P=0,020, comparing all groups understudy). When compared with group control, Vδ1 T cells exhibit statistical differences in Binet B and C CLL group, Binet A CLL and both MBL groups.
[a] – P<0,05, comparing individually this group with control group.

Concerning these results, we turned our observation to the comparison of the differences observed among the groups understudy for each T cell subpopulation. For CD4+ T cells, no statistical significant differences were observed for CD27 expression among the studied groups (exception made for LC-MBL, when this group was compared

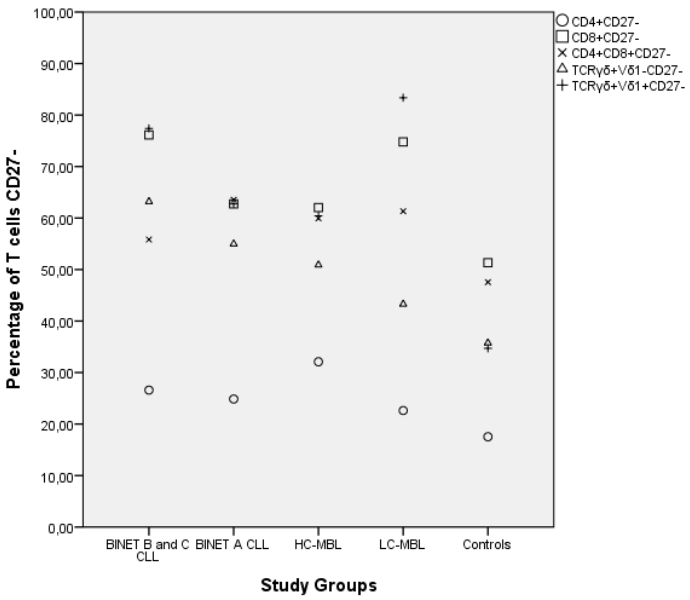
to control group). For CD8+ T cells, statistical significant differences were observed between the control group and Binet B and C CLL and LC-MBL groups, while CD4+CD8+ T cells did not display any statistical significant difference in the frequency of CD27- T cells among groups. For γδ T cells, Binet B and C CLL and Binet A CLL groups demonstrated differences when compared to control group, an observation that was also made for Vδ1 T cells. This last subset also presented differences when both MBL groups were compared to control group. The graphic representing the percentage of T cells in CD27+ compartment follows completely in the exact opposite way of what was observed in CD27- compartment, with decreasing percentages of T cells in CD27+ compartment from controls group to Binet B and C CLL.



Graphic 17 - Percentage of CD27+ T cells in T cell subpopulations – CD27+ T cells compartment (naïve and central memory compartment) decreases from controls to Binet B and C CLL, exception made for some MBL groups. The same significant differences observed for CD27- compartment apply for CD27+ compartment.
[a] – P<0,05, comparing individually this group with control group.

In conclusion, it can be seen in graphic 18 that among all groups understudy, the T cell subpopulations showing higher percentages of T cells in CD27- compartment are CD8+ T cells and Vδ1 T cells, followed by other γδ T cells and CD4+CD8 T cells. CD4+ T cells comprise the group with the lowest percentage of T cells in effector compartment. These data suggest a remarkable resemblance between CD8+ T cells and Vδ1 T cells in terms of the frequency of these cells expressing CD27 activation marker and gives evidence for an expansion of CD27- compartment in all groups understudy, comparing with T cells belonging to CD27+ compartment (exception made for CD4+ T cells, where CD27+ T cell compartment is expanded comparing to CD27- compartment). Vδ1 T cells, alongside other γδ T cells displayed differences in the frequency of cells expressing CD27

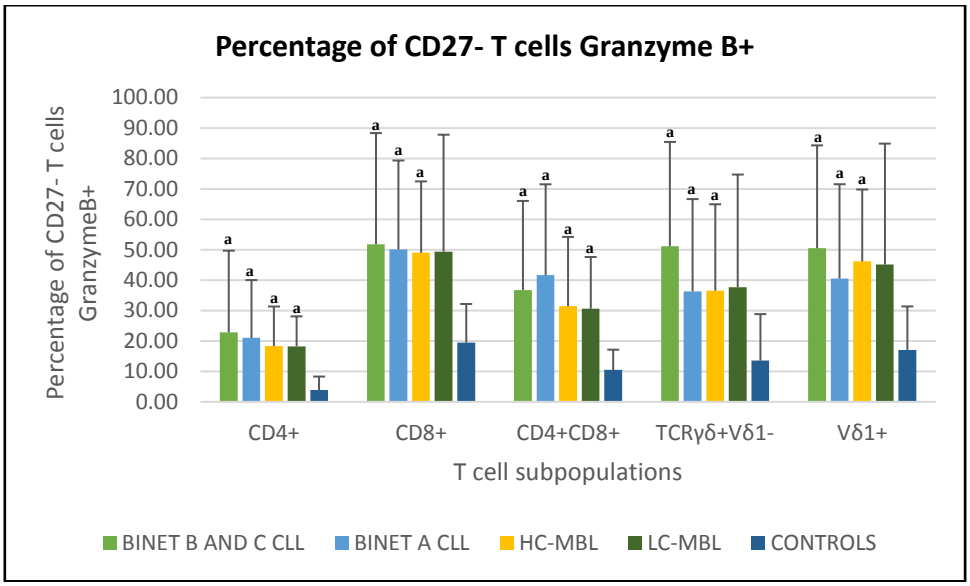
marker when compared to control group, which evidences an increase in effector functions with disease progression, a profile that points to an augmented frequency of γδ effector T cells in advanced stages of the disease.



Graphic 18 - Dispersion of the mean values of percentage of T cells in the different T cell subpopulations in the CD27- effector compartment – CD8+ T cells (squares), alongside Vδ1 T cells (plus), represent the T cell subsets with higher percentage of T cells in the CD27- compartment, followed by CD4+CD8+ T cells (cross) and γδ T cells (triangle), in different orders, according to the group understudy. Finally, CD4+ T cells (circles) exhibit the lowest expansion of CD27- compartment, with mean values under 40%, indicating that the major compartment represented in these cells is CD27+ compartment.

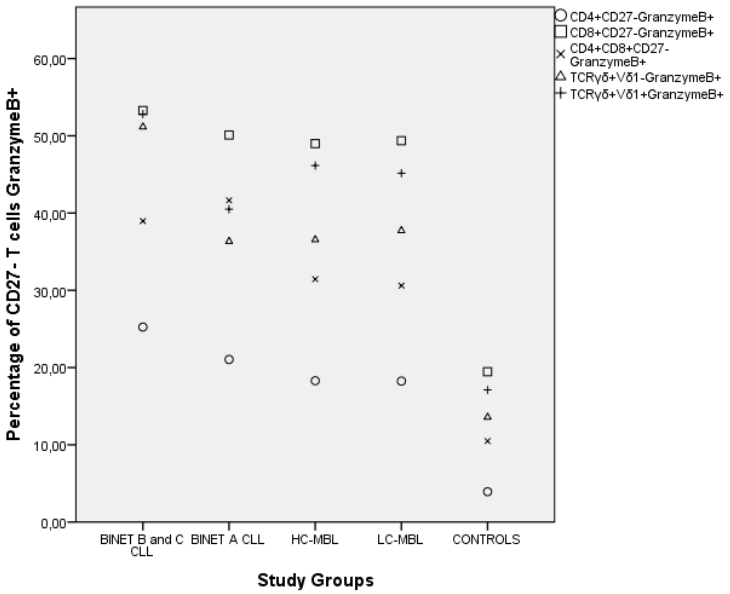
3.2. GRANZYME B EXPRESSION IN CD27- AND CD27+ T CELL SUBSETS

In this segment, results concerning cytotoxic activity of T cells are presented. In graphic 19, it is represented the frequency of CD27- T cells granzyme B+. In all T cells subsets, control group displayed the lowest frequency of cells expressing granzyme B, increasing from controls to Binet B and C CLL, that although not showing statistical differences among groups, except for CD4+CD8+T cells (CD4+: p=0,071; CD8+: p=0,118; CD4+CD8+: p=0,048; γδ T cells: p=0,188 and Vδ1 T cells: p=0,126), clearly revealed statistical significant differences when opposed to control group. Further analysis of the differences among the groups understudy revealed that all T cells subpopulations in Binet B and C CLL, Binet A CLL and HC-MBL groups displayed statistical significant differences when compared to control group, revealing an increasing cytotoxic profile with disease progression. For LC-MBL, the differences were only significant for CD4+ T cells (p=0,013) and CD4+CD8+ T cells (p=0,008).



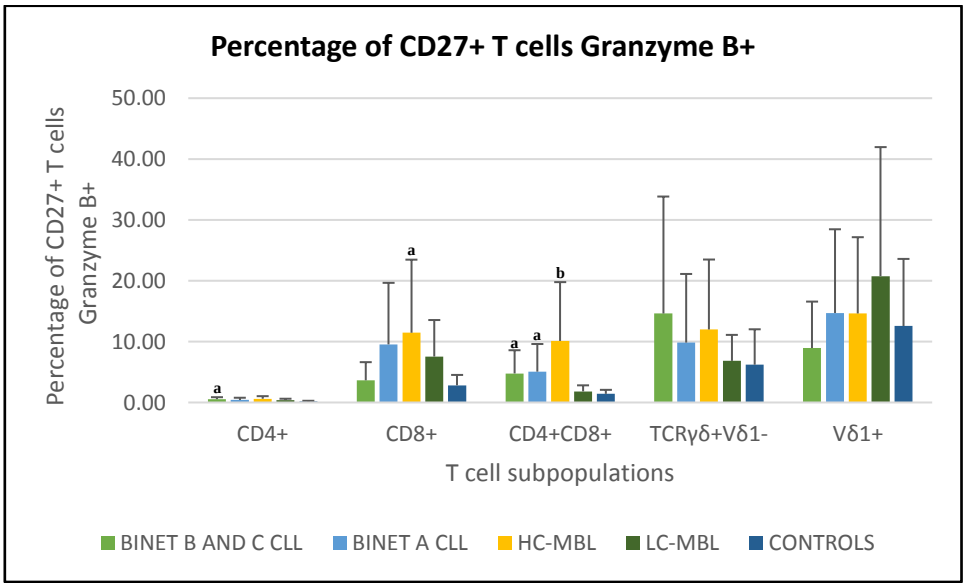
Graphic 19 - Percentage of CD27- T cells Granzyme B+ - T cells belonging to CD27- compartment are represented in terms of percentage that are positive for the expression of granzyme B. In all T cell subsets, controls have the lowest frequency of T cells expressing granzyme B and this frequency increases from controls to Binet B and C CLL. Statistical significant differences were observed when the different groups were compared to the control group, with Binet B and C, Binet A and HC-MBL showing P values ≤ 0,05 for all T cell subsets. LC-MBL, on the other hand, only presented statistical significant differences in CD4+ T cells and CD4+CD8+ T cells.
[a] – P<0,05, comparing individually this group with control group.

Regarding these results, a dispersion graphic was performed (graphic 20) evidencing once more the differences among the groups understudy and the control group. In this graphic, it can be seen that the highest mean of cells expressing granzyme B belonged to CD8+ T cells, followed by Vδ1 T cells (except for Binet A CLL). Of note, other γδ T cells follow Vδ1 T cells with lower percentages of cells expressing granzyme B, being followed by CD4+CD8+ T cells and CD4+ T cells. As occurred with distribution in CD27 compartments, CD8+ T cells and Vδ1 T cells display a similar behaviour, representing the CD27- T cells with higher percentages of cells expressing granzyme B. This observation allows the conclusion that Vδ1 T cells, besides displaying an increase in effector compartment also display an increased cytotoxic profile with disease progression, that is very similar to CD8+ T cells and might point to an effective antitumor response mediated by these cells.



Graphic 20 - Dispersion of the mean values of the percentage of the different CD27- T cells subpopulations in terms of granzyme B expression – In this graphic, two observations are very clear: the first one is related to the significant differences observed between the groups understudy and control group in terms of the frequency of T cells granzyme B+. On the other hand, we can observe that the higher percentages of T cells expressing granzyme B belonged to CD8+T cells (squares), alongside Vδ1 T cells (plus), followed by γδ T cells (triangles) and CD4+CD8+T cells (cross). CD4+ T cells (circles) display the lowest percentage of granzyme B expression.

Analysis of granzyme B expression in CD27+ T cells evidences much lower percentages of T cells expressing this marker. CD4+ T cells mainly do not present CD27+ cells expressing granzyme B, while the other T cell populations displayed very heterogenous percentages, which did not allow to conclude about a tendency in CD27+ T cells, although once again Vδ1 T cells seem to display a pattern similar to CD8+ T cells.

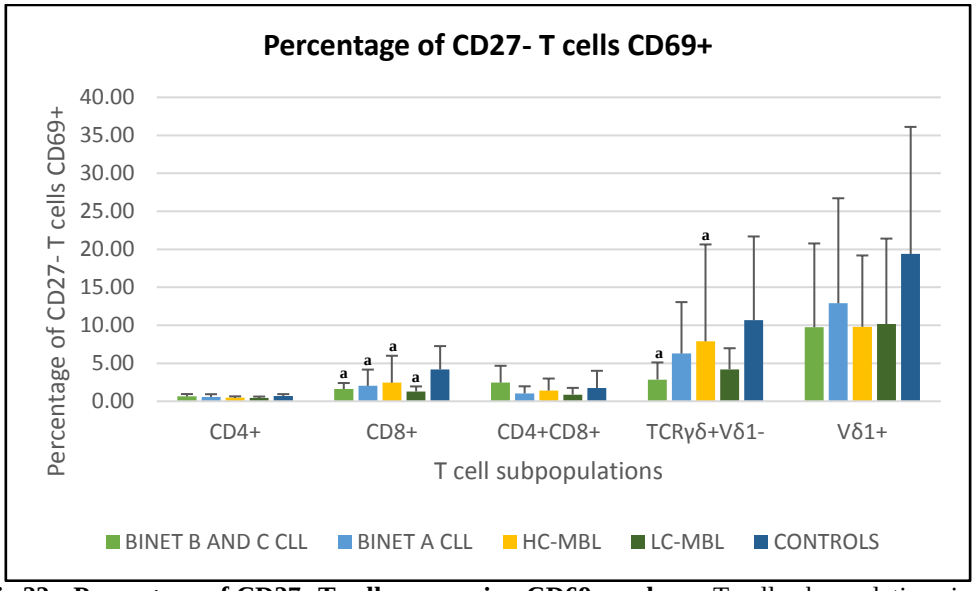


Graphic 21 - Percentage of CD27+ T cells Granzyme B+ - T cells belonging to CD27+ compartment are represented in terms of percentage that are positive for the expression of granzyme B. CD4+ T cells display

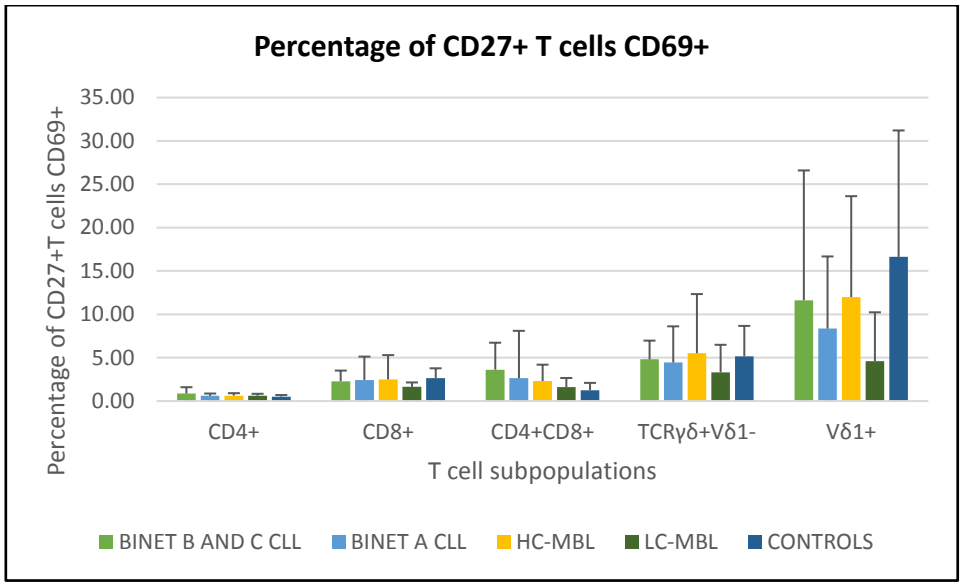
lower frequency of cells expressing granzyme B in all groups understudy. The other T cells subpopulations did not present any specific pattern concerning to the frequency of T cells expressing granzyme B.
[a] – P<0,05, comparing individually this group with control group.
[b] – P<0,05, comparing individually this group with LC-MBL group.

3.3. CD69 EXPRESSION IN CD27- AND CD27+ T CELL SUBSETS

In this final section, we observed the percentage of CD27- T cells (graphic 22) and CD27+ (graphic 23) expressing the early activation marker CD69. The results observed in this marker were very heterogeneous. For CD27- T cells, in all T cell subpopulations (except for CD4+CD8+ T cells), we observed the highest percentages of cells expressing CD69 in control group, but without an evident pattern among groups. For CD27+ T cells, this observation was only detected for Vδ1 T cells. Despite these observations, a curious fact observed is that Vδ1 T cells, alongside other γδ T cells displayed the higher percentages of T cells expressing CD69 activation marker, suggesting a more activated phenotype of these cells compared to αβ T lymphocytes.



Graphic 22 - Percentage of CD27- T cells expressing CD69 marker – T cell subpopulations in control group presented the highest frequencies of cells expressing the CD69 marker, except for CD4+CD8+ T cells. Both γδ T cells subpopulations present with higher frequencies of cells expressing CD69 when compared to the other subpopulations understudy.
[a] – P<0,05, comparing individually this group with control group.



Graphic 23 - Percentage of CD27+ T cells expressing CD69 marker – T cell subpopulations displayed a heterogeneous expression of CD69 marker, but with higher frequencies of T cells expressing this marker observed for both Vδ1 T cells and other γδ T cells subpopulations.

The mean values and standard deviations for CD27, CD69 and granzyme B expression across groups are presented in table 6, in appendix.

DISCUSSION

DISCUSSION

1. INCREASED PERCENTAGE AND ABSOLUTE COUNTS OF B LYMPHOCYTES IN CLL

CLL is, as stated previously, a B-CLPD, this is a disease where intense proliferation of abnormal B lymphocytes occurs. By comparing the groups understudy, it was possible to observe that all variables related to B cells: percentage of total B lymphocytes, percentage of malignant B cells, absolute number of total B cells and absolute number of pathological B cells were increased from controls to Binet B and C CLL group, with statistical significant differences observed among the groups understudy. As stated in the introduction, although not taking in account the absolute number of lymphocytes, Binet staging system values other markers of disease progression such as the presence of cytopenias (anemia and thrombocytopenia) and the existence of lymphadenopathies and organ damage. Due to this fact, it is very plausible that we observe a significant increasing tendency in these variables, as we progress to states of higher severity of the disease(3). This increasing accumulation of B cells is due to the abnormal proliferation of pathological B cells (almost all B cells are abnormal in CLL groups and HC-MBL group), caused by an imbalance between birth and death rates of these cells(73), a disproportion that is related to deficiencies in the ability of the immune system in recognizing these cells as foreign cells, through the mechanisms described in introduction. This inability is mainly related to the genetic modifications that these affected cells acquired, since the most common genetic features associated with CLL are related to mutations that allow B cells with abnormalities to remain unrecognized by the immune system. These observations are in line with some recent studies that intended to propose a mechanism for pathophysiology of CLL, from Sutton et colleagues and Vardi et al., that attributed the beginning of CLL to a malignant alteration in a HSC or a normal B cell, giving rise to a small clone of CLL cells: LC-MBL, that further evolved due to the microenvironment that these cells were exposed to. These authors proposed that a normal B cell, subjected to continuous stimuli, such as ongoing antigen stimulation, and genetic harmful mutations, might suffer clonal expansion, eventually turning into a MBL population. Depending on the microenvironment and the stimuli given to this clone, MBL might continue stable throughout patients' life, or it might suffer additional genetic

lesions that would induce its proliferation and progression to a state of HC-MBL and later to a CLL state, as described in figure 10 (74,75).

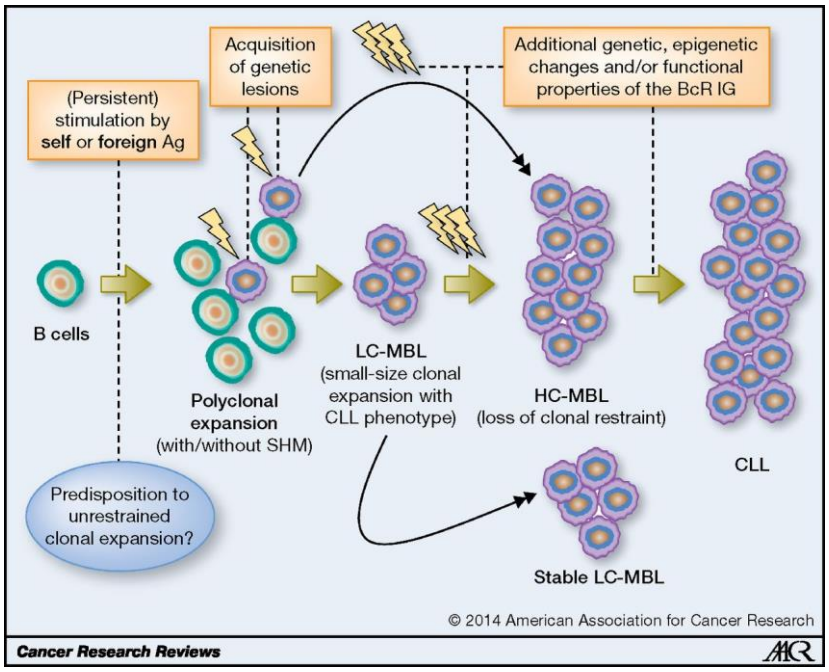


Figure 10 – Proposed model for CLL pathobiology. In this model, it is proposed that CLL arises from a HSC or a normal B cell that suffered continuous antigen stimulation or genetic mutations. These events triggered its polyclonal expansion, giving rise to a small clone of CLL cells (LC-MBL). Depending on the microenvironment experienced by the cell, this small clone might remain stable throughout patients' life or evolve to a HC-MBL or even to a state of CLL, if these cells acquire more genetic mutations and the ability to escape immune surveillance. From Vardi A, Agathangelidis A, Sutton L-A, Ghia P, Rosenquist R, Stamatopoulos K. Immunogenetic studies of chronic lymphocytic leukemia: revelations and speculations about ontogeny and clinical evolution. Cancer Res. 2014 Aug 15;74(16):4211–6.

A very interesting finding that seems to also be explained by these authors, was the proportion of pathological B cells among total B cells in peripheral blood in the different groups understudy. Focusing, in first place, on the two groups of MBL, we easily find a significant difference among groups, with LC-MBL group presenting only 53,2% of malignant B cells, while HC-MBL presented a percentage of 97,10% of pathological B cells. This is in agreement with WHO guidelines for classification of lymphoid neoplasms that classifies LC-MBL as a stage where disease progression is very unlikely to occur, while HC-MBL requires a routine year follow-up, as it has a greater chance to progress to a Binet A CLL state(1). To confirm this statement, we analysed the same proportion in the two groups of CLL, finding that the percentages are very similar to the ones observed for HC-MBL, with CLL cells comprising 98,82% for Binet A and 99,63% for Binet B and C CLL groups of all B cells on patient's peripheral blood. It is however important to remember that despite the proportion of malignant B cells, HC-MBL displays a significant lower absolute number of pathological B cells, not being considered

a disease stage, but rather a pre-disease state, as this increasing number is related to the development and progression of the disease, with lymphocytosis worsening faster, accompanied by the appearance of cytopenias, ganglionic involvement and organ damage, as evidenced in the biochemical data available for the patients selected for this study (Table 2).

2. DECREASED T AND NK CELLS PERCENTAGES AND INCREASED ABSOLUTE COUNTS OF T AND NK CELLS IN CLL PATIENTS

Accompanying B cell proliferation, in this study T cells and NK cells were also increased in their absolute counts from controls to Binet B and C CLL groups, although in a much slower rate when compared to B cells. T and NK cells percentages were, however, decreased in CLL groups, which is justified by the massive proliferation of B lymphocytes that in comparison to T and NK cells leads to a decreased proportion of T and NK cells in the whole blood sample. These observations are in line with several studies that reported decreased percentages and increased absolute counts of CD3+ (T) and CD56+ (NK) cells in CLL patients(76,77). The observation that lymphocytes' populations are increased in CLL groups has been made for several years, but the observation that both cell types presented with impaired functions have raised the interest in understanding which deficiencies occur in these cells in states of disease and how they affect the immune response in CLL patients. In fact, the most prominent common feature to CLL patients is immunosuppression, accompanied by an increase in their susceptibility to infectious agents and lack of tumour regression in response to antitumoral mechanisms triggered by patients' host immune system. This state of immunosuppression has been attributed to the observation that immune cells in CLL present with defects that impair their ability to perform responses against infectious agents and tumour cells, a phenomena reported as exhaustion of T cells in CLL(78).

Although falling off the scope of this project, it is remarkably interesting to cite a recent study, from Alexander W. MacFarlane and colleagues that described the NK cells changes in CLL and SLL. These authors have proposed that the ratio between $CD56^{bright}$ and $CD56^{dim}$ NK cells did not differ among these patients, however both cells presented with increased expression of CD27 marker in CLL patients, which is normally downregulated in mature NK cells. This data evidences an expansion of immature NK cells/reduction in mature NK cells in CLL groups comparing with controls. Together with this observation, it was also observed a significant reduction of NGK2D receptor

expression in $CD56^{dim}$ NK cells in CLL patients, and NK cells from CLL patients also presented with defects in degranulation, enhanced susceptibility to activation induced cell death and an impaired cytolytic function(79), which clearly reflects an weakened NK cell function in CLL patients, consequently implying an inability of these cells to recognize and kill cancer cells.

3. DISTRIBUTION OF T CELL SUBSETS WITHIN T CELLS AND T CELL SUBPOPULATIONS ABSOLUTE COUNTS

In addition to the fact that CD3+ T cells are significantly augmented in their absolute counts in CLL groups comparing to control group, as we have demonstrated for both Binet A and Binet B and C CLL groups, it has also been reported that CD8+ T cells are the subpopulation more likely responsible for this increase(80), as CD4+ T cells maintain their absolute counts more or less stable, while cytotoxic CD8+ T cells intensively proliferate. This is due to the fact that CD4+ T cells are more susceptible to cell death by Fas ligand-bearing effector cells, and consequently are more prone to suffer apoptosis(81).

In our study, we reported, for Binet B and C CLL a mean 2,5x increase in CD4+ T cells absolute count, while CD8+ T cells absolute count displayed an increase of 6x when comparing to the control group. Binet A CLL presented with a mean 1,7x increase for CD4+ T cell absolute counts and 3x increase for CD8+ T cell absolute count. Both results are in line with the results reported in the literature in terms of CD4+ and CD8+ expansion rates, providing data that supports the fact that CD8+ T cells are indeed expanded in peripheral blood of CLL individuals, which results in a fall of CD4+/CD8+ ratio (normal range between 1,0 and 3,0), an observation that has been reported to have an impact in disease prognosis. The impact of this reduction, in advanced stages of the disease, has not yet been established with some authors defending an augmented time to treatment and better prognosis when CD8+T cells are augmented, as they might preform antitumor responses against pathological cells(80), while other authors associate this decreased ratio with a worst prognosis, as CD8+T cells in CLL have been reported as exhausted T cells, with poor effector function, loss of proliferative capacity, impaired cytotoxicity, and reduced cytokine production(82), an observation that is accompanied by the observation that CD4+ regulatory T cells are also augmented in advanced stages of the disease, as it is described in tumour immunoediting process(28). In this study, and comparing the mean values for CD4+/CD8+ ratio, we observed a reduction of this ratio

from 3,19 in group control to 0,99 in advanced stages of the disease, which confirms the observation that CD4+/CD8+ ratio is effectively diminished in advanced stages of the disease (table 5, in appendix). However, it is curious to observe that this ratio is already significantly reduced when comparing MBL groups with control group, which evidences an augmented proliferation of CD8+ T cells since the onset of the MBL clone, reflecting earlier responses performed by the host's immune system in an attempt to restrain tumour growth.

Less reported in the literature are the changes in the minor subpopulations of T cells, but our data point to the fact that these cells are also likely to be increased in CLL patients. In our study we observed that in terms of percentage these cells do not present statistical significant differences among groups, with total γδ T cells assuming mean percentages equal in all groups, except for Binet B and C CLL group that revealed a slight decrease in this percentage. Contrary to these observations, in Vδ1 T cells, MBL groups displayed the highest percentages, with CLL groups presenting a Vδ1 distribution very similar to control group. The most remarkable observation was, however, that there was a Vδ1 expansion (proportion of Vδ1 T cells among γδ T cells), with values above 50% in Binet B and C CLL and LC-MBL, indicating that this population is the major γδ T cell subpopulation on peripheral blood in these patients. This observation evidences that in Binet B and C CLL, almost all patients present a significant expansion in Vδ1 T subset. This might indicate that Vδ1 T cell expansion occurs in advanced stages of the disease and might be related to an attempt of the immune system to overcome the progression and growth of CLL clone. This response might be mediated by Vδ1 T cells, since, at this point of the disease, response of αβ T lymphocytes is diminished, as tumour immunogenicity is decreased, as described in tumour immunoediting theory, leading to a decreased ability of αβ T lymphocytes in recognizing tumour cells. The observation that Vδ1 T cells are expanded in the beginning of the onset of the disease might be explained by an initial effort to control clone growth, in which the role of γδ T cells might be crucial(27). In terms of absolute counts, some studies report an increased absolute count of total γδ T cells in CLL patients, an observation that was also evident in our study(83). A study from Alessandro Poggi, reported lower absolute counts of Vδ1 T cells in controls, which is supported by our results, but also reported higher absolute counts of Vδ1 in low-risk CLL patients compared to intermediate/high risk CLL patients(84), which in our study was not observable with absolute counts of Vδ1 T cells reported higher in Binet B

and C CLL group. The progressive expansion of Vδ1 T cells, and γδ T cells since MBL groups was not described in literature before.

Regarding to CD4+CD8+ T cells, these cells displayed an increase in their percentage and absolute counts, an observation very common in cases of cancer states, where these cells are frequently increased. However, studies are not clear in terms of their function in immune system since these cells might preform a cytotoxic function (due to their CD8+ expression) or a regulatory effect (due to their CD4+ expression)(85). Despite this information, it is very clear, in our study, that these cells are increased in their percentages and absolute counts since earlier stages, with control group exhibiting a very low absolute count of CD4+CD8+ T cells when compared to the other groups understudy.

Taken together, the observation that some particular T cell subsets are expanded with disease progression, suggests that these cells might somehow participate in antitumor responses, exhibiting an effector cytotoxic phenotype against tumour cells, results that will be discussed in the next topic.

4. EXPRESSION OF CD27, GRANZYME B AND CD69 IN T CELL SUBPOPULATIONS

Regarding CD27 expression, T cells can be divided in two compartments: a CD27+ compartment that includes naïve T cells and central memory T cells and a CD27- compartment that includes effector memory T cells and effector T cells(44). As explained in the introduction, after encountering an antigen, T cells increase the expression of CD27 antigen and T cell proliferation is induced. As these cells turn into effector T cells, they lose the expression of CD27 and acquire an effector phenotype. In the T cell subsets studied in this project, it was observed an increasing percentage of CD27- T cells, from controls to CLL groups, evidencing an expansion of effector compartment occurring with disease progression. Interestingly, the T cell subset with highest percentages of CD27- T cells was CD8+ T cells, which is in consonance with the effector functions performed by these T cells, immediately followed by Vδ1 T cells, that displayed higher expansions of the effector compartment when compared to other γδ T cells. In fact, Vδ1 T cells followed the same pattern as CD8+T cells, resembling a very similar behaviour in terms of the frequency of T cells expressing CD27. This might suggest that both naïve CD8+ T cells and Vδ1 T cells are activated in order to become effector T cells and participate in antitumor responses against tumour cells. Another curious observation was, that contrary to these observations, CD4+ T cells have most of their cells in CD27+ compartment

which reveals a non-effector phenotype presented by these cells. An interesting thing to notice is that this CD27- compartment expansion occurs early in the progression of the disease. Few studies report the behave of T cells in MBL stages, comparing only high-risk and low-risk CLL groups with controls groups, and in this study, we were able to report that the expansion of effector compartment initiates soon in disease progression with MBL groups showing already elevated percentages of T cells in effector compartment, when compared to control groups, a statistical significant result observed for Vδ1+ T cells. This data enlightens the T cell role in fighting clone growth and stop disease progression, since the beginning of the onset of the disease.

We further tried to find an association between this phenotype and the cytotoxic effector functions displayed by T cells and we found that, indeed, granzyme B expression differed when comparing the CD27- compartment and CD27+ compartment, a normal observation since CD27- compartment is expected to display cells with effector functions, and consequently with higher frequency of T cells expressing granzyme B. As expected, we observed higher percentages of granzyme B+ cells in CD27- compartment, however, a quite fascinating finding was that MBL groups already reported frequencies of T cells expressing granzyme B significantly different from those observed in control group and very similar to the ones observed for CLL groups. This observation suggests that T cells' cytotoxic activity against CLL malignancy starts very early in the course of the disease. The higher frequencies of granzyme B+ cells were, once more, associated with CD8+ T cells, followed by Vδ1 T cells and other γδ T cells. These suggests a correlation between CD27 and granzyme B expression, with T cells subsets that display lower expression of CD27 being the ones that display the strongest cytotoxic activity. These results evidence cytotoxic effector functions of Vδ1 T cells, that are stronger when compared to other γδ T cells, and with a pattern very similar to the ones observed for CD8+ T cells. Further discussion in implications of these similarities are described below. Finally, in terms of CD69 expression we did not find any particular tendency, but we were curious by the fact that in both CD27+ and CD27- T cell compartments, Vδ1 T cells, alongside the remaining γδ T cells presented the highest percentages of activated T cells comparing to the much lesser activated αβ lymphocytes. As it was referred previously, γδ T cells recognize different ligands in the context of tumour recognition, which might explain their active effector phenotype and their elevated level of activation, and might indicate that these cells act together with CD8+ T cells by recognizing of other type of ligands in the context of tumour recognition. A curious factor that was seen throughout this project is the

remarkable heterogeneity of T cell subpopulations among the subjects, with complete different responses even within the same groups, which give clues to the need of selectively amplify the Vδ1 T cells with the best antitumor properties, when the aim is their use in tumour immunotherapy.

We were further interested in understanding with a closer look the features observed for each T cell subpopulation, suggesting an explanation for the observed phenotype for each T cell subtype, elucidations that are briefly described in the next topics.

4.1. CD4+ T CELLS

Starting with CD4+ T cells, the results presented in this study clarify four important features related to the differences encountered in these cells for the different groups understudy: (1) their relative percentage within T cells is decreased in advances stages of the disease while their absolute counts increased with disease progression, (2) there is a gradual increase of CD4+ T cells belonging to CD27- compartment from controls to advanced stages of the disease, although CD27+ T cells comprise in all groups the largest compartment, with more than 60% of CD4+ T cells belonging to it in all groups understudy, (3) there is a significant increase in the frequency of T cells producing granzyme B in CD4+ T cells belonging to CD27- compartment, which is visible since early stages of disease development, with MBL groups already showing statistical significant differences in granzyme B production when compared to control group. On the other hand, the frequency of CD4+CD27+ T cells expressing granzyme B is residual, with no significant differences observed among groups. The last feature encountered was that (4) CD69 expression is mainly equal in CD27- and CD27+ compartments and did not exhibit differences among groups. Taken together these observations evidence that in advanced stages of CLL occurs an expansion of CD4+ T cells, with progressive effector and cytotoxic activity, although its prevalence is lower when compared to T cells belonging to naïve/central memory compartment. CD69 expression is residual in both groups suggesting that CD4+ T cells are not becoming activated, which might explain the reduced transition of CD27+ T cells to effector CD27- T cells. These results are in line with the work of Porakishvili et colleagues that reported a significant increase in CD4+ T cells with cytotoxic activity (increased expression of perforin and granzyme B) in advanced stages of CLL, with perforin positive T cells representing up to 50% of total CD4+ T cells. Further analysis of the phenotype of these perforin positive T cells revealed

that their expression of CD45RO was augmented, a marker expressed in more mature T cells. The observation that these cells presented with a more effector-like phenotype is in line with our evidence that CD27- compartment increases in later stages of the disease. However, it is important to retain the thought that expansion of CD27- compartment can also be associated with an increase of effector memory T cells instead of effector T cells, a deviation of T cells normal track that had been correlated with a more aggressive course of the disease(85), as these cells are associated mainly with regulatory functions. These facts would explain the observation that CD4+ T cells are frequently reported as having impaired function in CLL.

These authors also studied the expression of CD69 and HLA-DR in these cells and there was no evidence of significant differences when these cells were compared to the perforin negative T cells, in controls and CLL groups, demonstrating that CD4+ T cells do not present elevated activation markers in CLL and MBL, as our results confirm. Further studies from these authors evidenced that these cells can indeed kill *ex vivo* malignant cells by perforin mediated mechanisms which might enlighten that the proliferation of CD4+ T cells might endow an attempt of host immune system of fighting tumour cells. However, it is important to refer that CD4+ T cells expression in CLL has been related to a more regulatory like phenotype, which doesn't correlate with antitumour effector functions, but the markers available in this study do not allow the distinction of regulatory T cells subsets.

4.2. CD8+ T CELLS

Concerning to CD8+ T cells, in this study, they represented the T cell subpopulation with a more noticeable proliferation rate, with increasing percentages and absolute counts from controls to CLL groups. Even though this population is frequently reported as a pool of exhausted T cells, this is, CD8+ T cells present frequently in CLL with decrease ability to proliferate, diminished cytokine production and impaired cytotoxic activity(78), our results evidenced a marked attempt of CD8+ T cells to exhibit a cytotoxic profile, as most of these cells belonged to effector compartment, with increasing percentages of CD27- T cells from controls to advanced stages of the disease. This is accompanied by an increased cytotoxic activity, with the frequency of these cells expressing granzyme B being augmented since earlier stages of the disease. In fact, CD8+ T cells presented a marked effector cytotoxic phenotype since MBL groups, which evidences an attempt of CD8+ T cells to fight CLL cells since the onset of the clone.

Although these observations point to a very efficient cytotoxic phenotype displayed by CD8+ T cells, this might not result in effective tumour cell killing. Riches et al. and Ramsay et al. reported that despite the augmented granzyme B expression by CD8+T cells in advanced stages of the disease, they are incapable of preforming cytotoxic functions because in CLL, CD8+T cells present an impaired ability to form the immune synapse with CLL cells, related to alterations in the polarization of the actin cytoskeleton in the site of antigen binding(86,87). This evidences that the defect is not in the quantity of granzyme B expressed but in its ability to induce cell mediated apoptosis in the targeted cell. Several studies also report an increased expression of inhibitory receptors, such as PD-1, in CD8+ T cells, however our results didn't allow to take conclusions in terms of phenotype expressed by CD8+ exhausted T cells.

In conclusion, our data seems to evidence an attempt of antitumor response by the host immune system mediated by CD8+ T cells, however, we did not have enough data to conclude if these cells could effectively accomplish their cytotoxic effect. Further studies with leukemic cell lines would evidence if these cells are indeed capable of fighting CLL cells. CD8+ T cells display the higher frequency of T cells expressing granzyme B when comparing to the other T cell subpopulations, however, Vδ1 T cells and other γδ T cells display equally raised levels of granzyme B expressing CD27- T cells which might elucidate an important role of γδ T cells in host immune response in disease states as will be further discuss. In terms of CD69 expression, CD8+ T cells presented decreasing percentages of activated T cells in both CD27- and CD27+ T cells, as we progressed to advanced stages of the disease which might support that T cells are progressively acquiring an effector phenotype, with loss of CD69 expression.

4.3. Vδ1 T CELLS AND OTHER γδ T CELLS

γδ T cells, contrary to what was observed for other T cells displayed percentages that were very similar across all groups, with a slight decrease in Binet B and C CLL, but their absolute counts, as occurred for the other T cell subpopulations displayed an increase from controls to CLL patients. Vδ1 T cells, on the other hand, displayed the higher percentages in MBL stages, and absolute counts evidenced an increase in Vδ1 T cells from controls to CLL groups (Binet A is an exception, displaying lower absolute counts comparing to the other groups understudy). As occurred in other T cell subpopulations, both groups displayed an increasing percentage of CD27- effector T cells, from controls to advanced stages of the disease, which was accompanied by increasing cytotoxic

activity (increased frequency of T cells expressing granzyme B). These cells displayed a behaviour very similar to CD8+ T cells which might indicate that these cells, together with CD8+ T cells, play an important function in antitumor response. Taking in account this information, it can be recognized that probably these two subsets are working on tumour cell killing by recognition of different antigens expressed by CLL cells. Vδ1 T cells, alongside γδ T cells, displayed the highest mean values of T cells expressing CD69, when compared to the other T cell subpopulations, which reveals an increased state of activation displayed by these cells. Bruno Silva Santos reported that freshly isolated Vδ1 T cells of CLL patients displayed lower expression of CD27 and values of CD69 that were very variable among individuals, a feature that was also encountered in our study(66). These data reveal a tendency of γδ T cells to participate in antitumor response, however it is important to analyse if the markers of T cell exhaustion exhibited by CD8+ T cells are present in Vδ1 T cells and other γδ T cells, which would allow to conclude if these cells are preforming the effector functions matching to the phenotype they express.

5. USE OF Vδ1 T CELLS IN ADOPTIVE CELL THERAPY

In this study, and as can be evidenced by the standard deviations presented in all the parameters evaluated, were observed very different responses among patients, particularly for Vδ1 T cells, and other γδ T cells, not only among the groups understudy but within the same group. This observation was already made by Bruno Silva Santos that published a heat map with the differences of Vδ1 T cells that were freshly isolated from CLL patients and compared them with the selectively amplified Vδ1 T cells (achieved by the formula described in introduction). This author also encountered heterogeneity in Vδ1 T cells isolated from CLL patients, as occurred in our work(66). These data evidences that all T cells, and mainly Vδ1 T cells need to be selectively amplified when the purpose is its use for cancer immunotherapy, but the observation that Vδ1 T cells displayed an effector cytotoxic phenotype very similar to CD8+ T cells, together with the observation that previous studies reported that Vδ1 T cells seem to display a phenotype of less exhausted T cells reinforces the promising effects of this T cell subpopulation in the treatment of CLL.

CONCLUSIONS

CONCLUSIONS

Vδ1 T cells, alongside the remaining γδ T cells, and CD8+ T cells, display an effector cytotoxic behaviour that seems to correlate with an antitumor response against tumour cells. These cells are increased in their absolute counts since earlier stages of disease progression and this increase is associated with an expansion of effector compartment and an augmented cytotoxic activity, from controls to advanced stages of the disease.

Nevertheless, since CD8+ T cells in CLL are reported as exhausted T cells, with inability to perform the expected antitumor effector functions, it is important to understand if Vδ1 T cells reflect a phenotype that translates in active tumour cells killing activity, and, because of that, further studies are needed to evaluate if these cells are achieving the functions that correlate with their effector phenotype.

Reaching these conclusions is imperative due to the fact that effector cytotoxic phenotype of Vδ1 T cells is observed since the beginning of the disease which might reveal a broadly favourable use of Vδ1 T cells in immunotherapeutic approaches to CLL. Our study reported for the first time an antitumor immune response since MBL groups, evidencing that the immune systems tries to fight cancer cells since the beginning of the onset of the disease.

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APPENDIX

Table 4 - Mean and standard deviation values for lymphocytes' distribution. In this graphic, it is represented the mean values and standard deviations for percentage and absolute counts of total and abnormal B lymphocytes, T lymphocytes and NK cells.

	BINET B AND C CLL	BINET A CLL	HC-MBL	LC-MBL	CONTROLS
Total B cells (%)	69,91±23,27	60,24±15,69	31,08±6,18	7,65±4,66	3,47±1,36
Pathological B cells (%)	69,65±23,56	59,53±15,85	30,18±6,34	4,07±2,50	0,00±0,00
Absolute count of Total B cells	79943,90±112146,19	30005,60±55068,68	3488,27±821,06	619,04±551,57	287,81±106,94
Absolute count of Pathological B cells	79880,35±112160,36	29850,76±55069, 63	3390,69±844,12	300,95±198,56	0,00±0,00
T cells (%)	9,42±5,78	9,36±4,94	16,78±7,42	22,50±6,58	14,70±8,19
Absolute count of T cells	3911,40±2529,99	2452,28±1392,95	1946,39±1182,06	1681,63±592,62	1158,84±610,40
NK cells (%)	1,38±1,18	1,68±1,24	2,76±4,23	2,39±1,77	1,41±0,72
Absolute count of NK cells	580,60±457,12	397,11±292,63	297,01±418,52	182,05±124,31	122,36±73,97

Table 5 – Mean values and standard deviations for percentages and absolute counts of T cells subpopulations in the different groups understudy.

	BINET B AND C CLL	BINET A CLL	HC-MBL	LC-MBL	CONTROLS
CD4+ (%)	43,29±10,82	57,12±13,94	53,38±17,46	49,48±8,10	68,10±10,76
CD4+ (Abs)	1914,75±1540,28	1295,89±514,12	899,63±314,63	866,61±426,60	783,09±378,64
CD8+ (%)	47,21±9,00	32,65±11,97	36,62±14,10	39,51±8,70	24,12±7,23

CD8+ (Abs)	1910,01± 1051,84	871,54± 808,46	824,11± 830,96	625,76± 106,88	301,42± 232,67
CD4+CD8+ (%)	2,85±2,53	2,47±1,70	2,15±0,44	3,32±1,66	0,97±0,55
CD4+CD8+ (Abs)	117,44± 154,29	60,64± 58,67	45,01± 37,77	57,42± 32,31	11,30±7,05
Tota γδ T cells (%)	3,56±1,91	5,19±6,20	5,31±5,24	5,81±5,09	5,39±4,95
Total γδ T cells (Abs)	135,93± 111,97	158,37± 286,58	114,01± 122,85	98,56± 109,83	79,01± 114,30
Vδ1 T cells (%)	2,00±1,71	1,74±2,48	3,24±4,58	3,73±3,57	1,48±1,87
Vδ1 T cells (Abs)	87,94± 108,64	46,91± 73,55	76,83± 110,16	56,54± 53,33	15,03±16,91
Ratio CD4+/CD8+	0,99±0,46	2,26±1,73	1,81±1,05	1,34±0,52	3,19±1,49

Table 6 - Mean values and standard deviations for CD27, CD69 and Granzyme B expression in T cell subpopulations

	BINET B AND C CLL	BINET A CLL	HC-MBL	LC-MBL	CONTROLS
CD4+27- T cells (%)	24,42±18,51	24,86±17,01	32,09±28,21	22,62±3,91	17,54±4,46
CD8+27- T cells (%)	72,18±15,39	62,74±15,84	62,04±23,64	74,79±8,87	51,33±20,10
CD4+CD8+27- T cells (%)	50,41±24,25	63,55±24,11	59,95±26,39	61,33±24,33	47,56±15,49
TCRγδ+Vδ1-CD27- T cells (%)	63,20±20,07	54,97±22,94	50,89±28,18	43,29±27,39	35,74±11,50
Vδ1+CD27- T cells (%)	73,50±14,75	62,75±28,90	60,43±32,82	83,37±23,80	34,70±14,86
CD4+27+ T cells (%)	75,47±18,77	75,14±17,01	67,91±28,21	77,38±3,91	82,47±4,46
CD8+27+ T cells (%)	27,82±15,39	37,26±15,84	37,97±23,64	25,21±8,87	48,67±20,10
CD4+CD8+27+ T cells (%)	49,60±24,25	36,46±24,12	40,05±26,39	38,67±24,33	52,44±15,49

TCRγδ+Vδ1-CD27+ T cells (%)	36,80±20,07	45,03±22,94	49,11±28,18	56,71±27,39	64,26±11,50
Vδ1+CD27+ T cells (%)	26,47±14,80	37,24±28,91	39,57±32,82	16,63±23,80	65,30±14,86
CD4+27- T cells Granzyme B+ (%)	22,83±26,86	21,05±18,98	18,29±13,05	18,25±9,86	3,93±4,39
CD8+27- T cells Granzyme B + (%)	51,77±36,56	50,10±29,23	48,99±23,47	49,37±38,44	19,46±12,71
CD4+CD8+27- T cells Granzyme B+ (%)	36,76±29,28	41,64±29,86	31,44±22,77	30,60±17,01	10,49±6,67
TCRγδ+Vδ1-CD27- T cells Granzyme B+ (%)	51,14±34,29	36,34±30,30	36,56±28,37	37,73±36,98	13,57±15,27
Vδ1+CD27- T cells Granzyme B+ (%)	50,49±33,80	40,50±31,03	46,15±23,64	45,16±39,71	17,10±14,28
CD4+27+ T cells Granzyme B+ (%)	0,54±0,33	0,41±0,37	0,61±0,43	0,40±0,22	0,19±0,09
CD8+27+ T cells Granzyme B+ (%)	3,64±2,99	9,55±10,11	11,46±12,01	7,55±6,00	2,82±1,71
CD4+CD8+27+ T cells Granzyme B+ (%)	4,77±3,82	5,07±4,54	10,12±9,66	1,82±1,00	1,42±0,66
TCRγδ+Vδ1-CD27+ T cells Granzyme B+ (%)	14,62±19,22	9,84±11,28	12,00±11,49	6,84±4,27	6,21±5,82
Vδ1+CD27+ T cells Granzyme B + (%)	8,95±7,63	14,70±13,76	14,63±12,53	20,76±21,20	12,57±11,02
CD4+27- T cells CD69+ (%)	0,63±0,32	0,56±0,37	0,46±0,19	0,44±0,18	0,67±0,27
CD8+27- T cells CD69+ (%)	1,60±0,80	2,02±2,15	2,44±3,55	1,29±0,66	4,20±3,06
CD4+CD8+27- T cells CD69+ (%)	2,45±2,22	1,03±0,94	1,41±1,58	0,87±0,89	1,75±2,26
TCRγδ+Vδ1-CD27- T cells CD69+ (%)	2,86±2,25	6,28±6,77	7,89±12,75	4,20±2,78	10,68±11,01
Vδ1+CD27- T cells CD69+ (%)	9,75±11,02	12,89±13,83	9,80±9,39	10,17±11,24	19,38±16,73
CD4+27+ T cells CD69+ (%)	0,87±0,73	0,60±0,27	0,63±0,28	0,62±0,22	0,52±0,18
CD8+27+ T cells CD69+ (%)	2,28±1,24	2,44±2,69	2,50±2,80	1,64±0,51	2,66±1,12
CD4+CD8+27+ T cells CD69+ (%)	3,62±3,11	2,63±5,47	2,31±1,89	1,61±1,05	1,26±0,84

TCRγδ+Vδ1-CD27+ T cells CD69+ (%)	4,81±2,16	4,44±4,18	5,52±6,82	3,31±3,18	5,16±3,51
Vδ1+CD27+ T cells CD69+ (%)	11,62±14,98	8,36±8,32	11,99±11,64	4,62±5,62	16,63±14,58