MASTER THESIS

MOLECULAR BASIS OF HUMAN DISEASES

«EVALUATION OF THE QUANTITATIVE AND FUNCTIONAL CHARACTERISTICS OF MYELOID DERIVED SUPPRESSOR CELLS (MDSCs) IN PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA (B-CLL) AND ASSOCIATED HYPOGAMMAGLOBULINEMIA»

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ΖΑΒΙΤΣΑΝΟΥ ΚΩΝΣΤΑΝΤΙΝΑ

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1. Summary

Chronic lymphocytic leukemia (CLL) is the most common blood cancer among adults in the Western World and is characterized by excessive production of pathological, clonal B-lymphocytes of high CD23, CD5, and CD19 co-expression in peripheral blood, bone marrow and lymph nodes. Hypogammaglobulinemia is a common finding in CLL; it predisposes to infections and exacerbates the complications of the disease. However, the mechanism of its emergence is not well-defined yet. Myeloid Derived Suppressor Cells (MDSCs) are immature cells of myeloid origin, divided into two subpopulations, the polymorphonuclear (PMN)-MDSCs and the monocytic (M)-MDSCs and through various mechanisms they execute their immunosuppressive and tumorigenic function. The aim of the study was to investigate any possible involvement of MDSCs in CLL-associated hypogammaglobulinemia by evaluating their quantitative and functional characteristics. The proportion of PMN-MDSCs and M-MDSCs in untreated CLL patients with and without hypogammaglobulinemia and in healthy individuals was evaluated by flow cytometry in the low-density fraction of the peripheral blood mononuclear cells. Subsequently, the immunosuppressing function of MDSCs was studied by T-cell suppression assays in conditions with and without their presence. The study revealed that CLL patients display significantly increased proportion of peripheral blood PMN-MDSCs and M-MDSCs compared to healthy individuals. This upregulation occurs in patients with both normal and low Ig levels without significant differences for each MDSC subpopulation. Similarly, MDSCs from both CLL patient groups showed a statistically stronger but similar T-lymphocyte suppressive activity compared to MDSCs from healthy subjects. Therefore, MDSCs' expansion and suppressive function does not represent the main pathogenetic mechanism for the occurrence of hypogammaglobulinemia in CLL. Finally, this study strengthens the connection between MDSCs and CLL and supports the emerging role of these cells as potential biomarkers in malignant-neoplastic diseases.

1. Summary (in Greek) –Περίληψη

Η χρόνια λεμφοκυτταρική λευχαιμία (ΧΛΛ) είναι η συχνότερη αιματολογική νεοπλασία στους ενηλίκους και χαρακτηρίζεται από την υπερβολική παραγωγή παθολογικών, κλωνικών Β-λεμφοκυττάρων υψηλής CD23, CD5, CD19 συνέκφρασης στο περιφερικό αίμα, μυελό των οστών και στους λεμφαδένες. Η υπογαμμασφαιριναιμία αποτελεί συχνό εύρημα στη ΧΛΛ, προδιαθέτει σε λοιμώξεις και επιτείνει τις επιπλοκές της νόσου. Ο μηχανισμός εμφάνισής της όμως δεν είναι ακόμα καλά διευκρινισμένος. Τα μυελικά κατασταλτικά κύτταρα (MDSCs) αποτελούν ένα ετερογενή πληθυσμό ανώριμων μυελικών κυττάρων, διακρίνονται σε δύο κύριους υποπληθυσμούς, τα πολυμορφοπυρηνικά (PMN-MDSCs) και τα μονοκυτταρικά (M-MDSCs) και η κύρια δράση τους συνίσταται στην καταστολή του ανοσοποιητικού συστήματος και στην παθογένεια νεοπλασματικών νοσημάτων μέσω ποικίλων μηχανισμών. Στόχος αυτής της μελέτης ήταν να διερευνηθεί εάν και κατά πόσο αριθμητικές και λειτουργικές διαταραχές στους πληθυσμούς των μυελικών κατασταλτικών κυττάρων συμβάλλουν στην εμφάνιση της υπο-γ-σφαιριναιμίας σε ασθενείς με ΧΛΛ. Στην έρευνα αυτή αξιολογήθηκε η αριθμητική αναλογία των PMN-MDSCs και M-MDSCs σε ΧΛΛ ασθενείς με και χωρίς υπογαμμασφαιριναιμίαστους οποίους δεν χορηγούνταν θεραπεία καθώς και σε υγιή άτομα, με τη χρήση κυτταρομετρίας ροής στο κλάσμα χαμηλής πυκνότητας των μονοπύρηνων κυττάρων του περιφερικού αίματος. Επακολούθως μελετήθηκε η κατασταλτική δράση των MDSCs με πειράματα πολλαπλασιασμού των Τ-λεμφοκυττάρων σε συνθήκες με και χωρίς την παρουσία τους. Τα αποτελέσματα αποκάλυψαν ότι τόσο οι ασθενείς με υπογαμμασφαιριναιμία όσο και οι ασθενείς με φυσιολογικά επίπεδα γάμμα-σφαιρινών παρουσιάζουν στατιστικά σημαντική αύξηση στα ποσοστά M-MDSCs και PMN-MDSCs σε σχέση με τα φυσιολογικά άτομα, χωρίς όμως να διαφέρουν σημαντικά μεταξύ τους για κάθε υποπληθυσμό. Ομοίως, τα MDSCs και των δύο ομάδων ασθενών ΧΛΛ παρουσίασαν στατιστικά ισχυρότερη αλλά παρόμοιακατασταλτική δράση των Τ-λεμφοκυττάρων σε σχέση με τα MDSCs των υγιών ατόμων. Η επέκταση και δράση των MDSCs φαίνεται επομένως πως δεν αντιπροσωπεύει τον κύριο παθογενετικό μηχανισμό της εμφάνισης υπογάμμασφαιριναιμίας στη ΧΛΛ. Ωστόσο, η μελέτη αυτή ενισχύει την σύνδεση που υπάρχει μεταξύ των MDSCs και της ΧΛΛ και στηρίζει τον αναδυόμενο ρόλο αυτών των κυττάρων ως πιθανών βιομαρτύρων σε νεοπλασματικές ασθένειες.

Contents

2. Chronic lymphocytic leukemia (CLL)

Chronic lymphocytic leukemia (CLL) is the most common blood cancer among adults in the Western World. The incidence rate in Europe and US is estimated around 4 to 6 cases per 100.000 people every year and it is significantly increased by age, with males been affected more frequently than females (ratio of 1.5-2:1). The median age at diagnosis lies between 67 and 72 years, although there are cases of diagnosed CLL in individuals of 55 years old or younger (Howlader et al. 2016). According to Cancer Net Statistics, CLL has a higher survival rate than many other malignancies. The five-year survival rate is around 83%, whereas in those over the age of 75, the five-year survival rate drops to less than 70%.

CLL is characterized as a lymphoproliferative disorder, consisting of excessive pathological, monomorphic B-lymphocytes in the peripheral blood (PB), bone marrow (BM) and lymphoid organs (Figures 1&2) (Scarfò, Ferreri, and Ghia 2016). CLL lymphocytes are clonal B cells that have an immunophenotypic profile of CD19 expression, CD5 and CD23 co-expression, along with a low density of CD20 (Swerdlow et al. 2008).

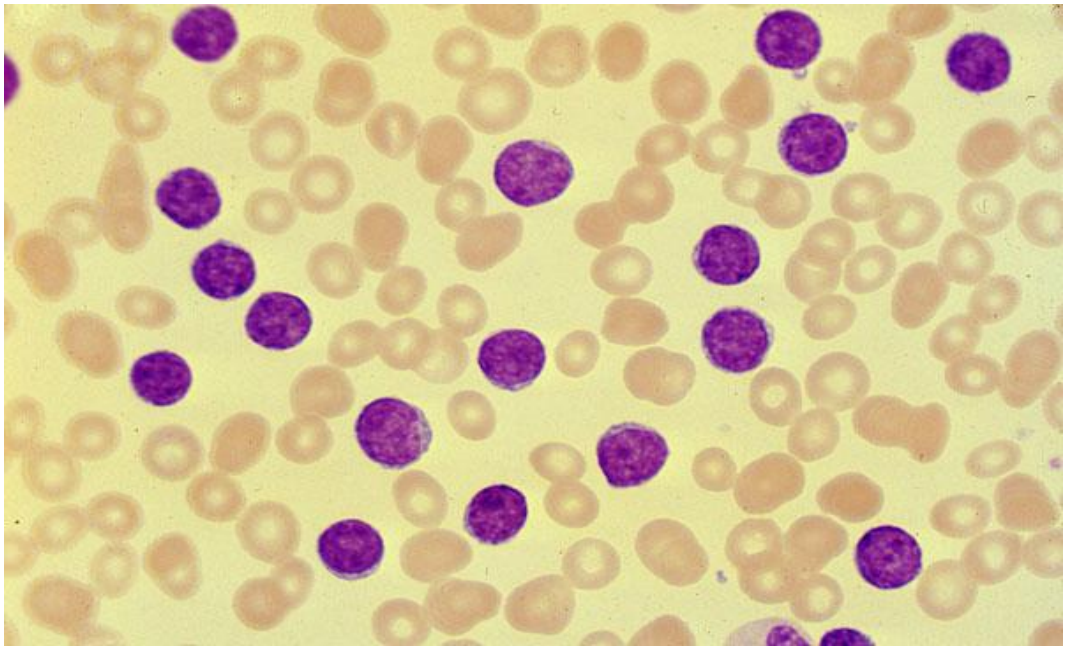

Figure 1: Chronic lymphocytic leukemia B-cells in peripheral blood (Medical Press)

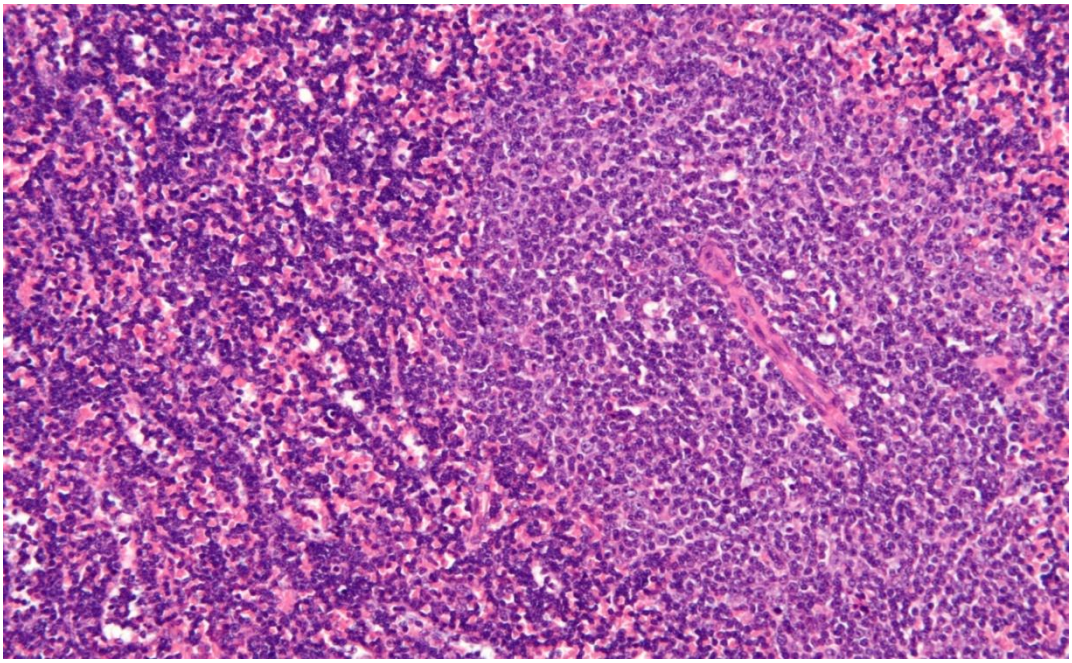

Figure 2: Micrograph of a lymph node affected by B-CLL showing a characteristic proliferation (center of image), composed of larger, lighter-staining, B- cells, H-E stain, [(Schmid and Isaacson 1994)Histopathology 24 (5): 445-51]

Genomic aberrations:

The ability of CLL to generate clonal B cells lacks a single driving mutation. The primary leukemogenic event in CLL is often initiated by the loss or addition of large chromosomal material followed by later additional mutations as it was presented in large cohort studies of CLL patients' genomic landscape (Landau et al. 2015). According to Hallek 2017, the most frequent alterations in CLL are the deletion 13q, deletion 11q, trisomy 12 and deletion 17p. In more detail, deletions on the long arm of chromosome 13 (13q14), represent the single most frequent cytogenetic alteration in CLL, occurring in 55% of all cases with miRNAs 15a and 16–1, found in the region, being very crucial in CLL leukemogenesis. Deletions on the long arm of chromosome 11 (11q) are detected in 25% of patients with late disease stages and in 10% of patients with early stage disease. The deletion 11q23 leads to the elimination of the DNA damage response kinase ATM, resulting in quick disease progression, and reduced overall survival (OS). Fortunately, some of the 11q deletion drawbacks can be conquered by the use of chemo-immunotherapy. Trisomy 12 is observed in 10%-20% of CLL patients; however, the genes that are responsible for the causality of the disease are still unknown. Deletions on the short arm of chromosome 17 (17p) are found in 5%-8% of chemotherapy-naïve patients and lead to significant resistance against genotoxic chemotherapies that cannot be overcome. Mutations in the 17p13

band result in the loss or alteration of TP53 tumor suppressor gene, which is associated with very poor prognosis. In addition, many studies (Landau et al. 2015; Puente et al. 2011; Quesada et al. 2011) have revealed a large number of mutated genes in CLL patients including NOTCH1, MYD88, TP53, ATM, SF3B1, FBXW7, POT1, CHD2, RPS15, IKZF3, ZNF292, ZMYM3, ARID1A, and PTPN11. Finally, many proteins are greatly involved in CLL, including those in the DNA damage signaling and DNA repair pathways, as well as in the RNA processing and export, in the MYC activity, and the MAPK signaling pathway (Hallek 2017). As for the immunogenetic aberrations, it is reported that the IG gene, that encodes for the surface immunoglobulins that are part of the B cell receptor and are essential for the survival and function of the B-cell, is unmutated in about half of the CLL cases with an almost identical sequence to the germ-line (percentage of identity of the heavy chain variable gene, IGHV, to the corresponding germ-line gene is >98%), whereas in the other half of cases somatic mutations in the gene are present (identity <98%). The IGHV mutational status (mutated vs. unmutated) is considered to be an independent prognostic marker, since unmutated cases usually follow a more aggressive clinical course (Crombie and Davids 2017; Hallek 2017). The later finding supports the idea that an antigenic-trigger could be a tumor driver in CLL since the process of somatic hypermutation takes place after antigen encounter in the lymph node microenvironment (Packham et al. 2014).

B-cell survival:

Survival of CLL cells depends on their microenvironment in the lymph nodes, marrow and other tissues, which is composed of cellular components such as macrophages, T cells and stroma follicular dendritic cells and is influenced by interactions with non-malignant cells. These cells provide stimuli necessary for the activation of survival and pro-proliferative signaling pathways in transformed cells. The chemokines, cytokines, and angiogenic factors that are produced by the microenvironment's cells interact with leukemic cells via surface receptors or adhesion molecules and support their survival (Hallek 2017). Furthermore, CLL cells have acquired characteristics of prolonged survival and defect programmed cell death (apoptosis). These include: (a) expression of survival receptors and their ligands, b) expression of autocrine survival pathways which are leukemia cell specific, (c) defects in plasma membrane receptor cell signaling, triggered by death receptors such as Fas- and TRAIL and finally (d) constitutively active survival signaling pathways such as NFkappaB and PI3K/Akt (Figure 3) (Pedersen and Reed. 2004). Of critical importance to the B-cell survival is also a member of the Tec family of kinases, Bruton's tyrosine kinase (BTK). BTK is essential for the chemokine-mediated homing and adhesion of B cells, as well as the

activation of several constitutively active pathways of CLL-cell survival (Grant et al. 2013).

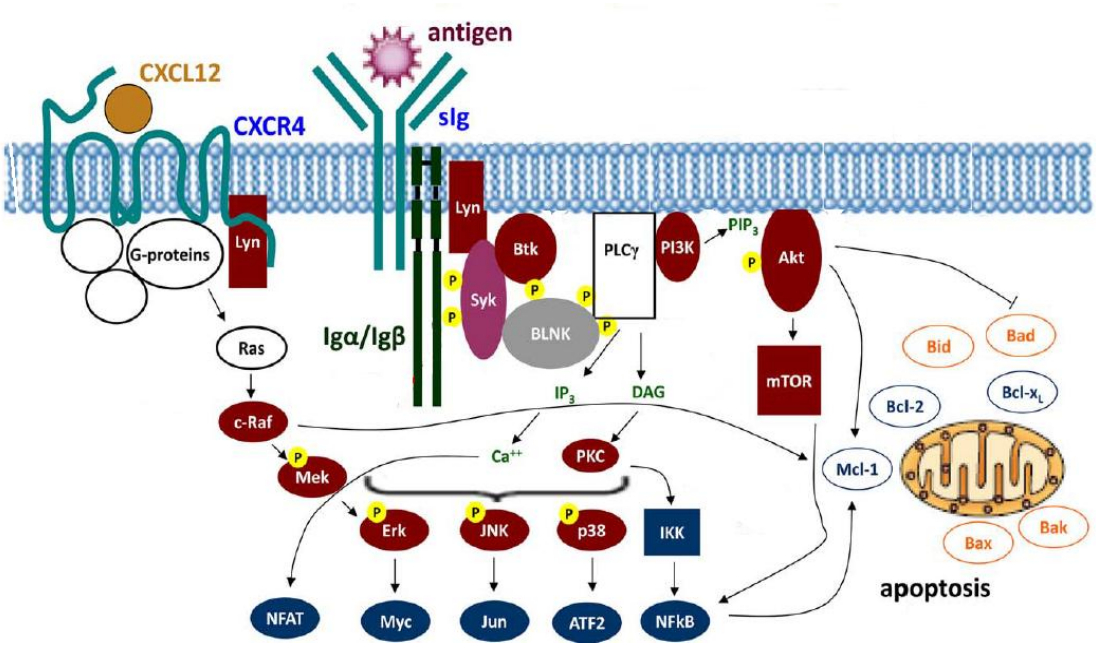

Figure 3: CLL surviving signaling pathways (Hallek 2017)

Diagnosis:

In the majority of cases, CLL is diagnosed through a simple blood test and confirmed with immunophenotyping. The diagnosis of CLL requires: the presence of 5000 monoclonal B lymphocytes /mL in the peripheral blood for the duration of at least 3 months and the co-expression of the T-cell antigen CD5 and the B-cell surface antigens CD19, CD20, and CD23. The expression of surface immunoglobulins (Ig), and CD20-CD19-CD79b, is lower compared to normal B cells and restricted to either kappa or lambda expression of immunoglobulin light chains which indicates B cell clonality. Lastly, phenotypically these malignant B lymphocytes are small, mature with a narrow border of cytoplasm and a dense nucleus with a partially aggregated chromatin and a lacking discernible nucleoli (Hallek 2017).

Clinical presentation:

The majority of CLL patients are asymptomatic at stage of diagnosis, whereas some experience lymphadenopathy and/or splenomegaly. Some of the rare symptoms of the disease include: fever of unknown origin (<38 C^0) for a couple of weeks without any cause of infection, weight loss of more than 10% within 6 months and night sweats. In more advanced stages of the disease, symptoms such as fatigue to physical exercise due to anemia and bone marrow infiltration may appear, while bleeding caused by low platelet count is considered rarer. In general, chronic lymphocytic leukemia is a disease with vulnerability to infections (Morrison 2010). Bacterial infections of the upper and lower respiratory and urinary tract are the manifestations most frequently reported. As the disease progresses the complications get worse leading to death. The infections in CLL are caused due to defects in the T-cell subsets and neutrophil-monocyte functions, along with hypogammaglobulinemia. The latter might occur over the disease course and is more likely to be present in advanced stages (Morrison 2010). CLL is also linked to autoimmune diseases such as autoimmune hemolytic anemia (10–25%), immune-thrombocytopenia (ITP, 1–5%), pure red cell aplasia (PRCA) and autoimmune neutropenia (<1% and 0.2% of cases, respectively) (Hodgson et al. 2011).

Staging and treatment:

There are two widely accepted clinical staging systems for the classification of CLL, the modified Rai staging system and the Binet staging system (Figure 4). The first, defines the disease as low-risk when lymphocytosis is present with more than 30% of leukemia cells in the blood and/or marrow, as intermediate-risk when patients present lymphocytosis, enlarged nodes in any site, splenomegaly and/or hepatomegaly, and finally as high-risk when patients are diagnosed with disease-related anemia (as defined by a hemoglobin (Hb) level <11 g/dl) or thrombocytopenia (as defined by a platelet count of <100 3 109/L). The Binet staging system is based on the number of involved areas (head and neck, including the Waldeyer ring, axillae, groins, including superficial femoral, palpable spleen and palpable enlarged liver), defined by the presence of enlarged lymph nodes (>1 cm in diameter) or organomegaly, and on whether there is anemia or thrombocytopenia. "The Binet staging system defines stage A as Hb ≥10 g/dL and platelets ≥100 x 10^9/L and up to 2 of the above involved; stage B as Hb≥10 g/dL and platelets≥ 100 x 10^9/L and organomegaly greater than that defined for stage A (3 or more areas of nodal or organ enlargement) and stage C as Hb of <10 g/dL and/or a platelet count of <100 x 10^9/L" (Hallek 2017).

Staging systems for CLL

RAI SYSTEM

Rai stage	Modified Rai stage (risk)	Clinical characteristics	Median survival (yr)
0	Low	Lymphocytosis in peripheral blood and bone marrow only	> 10
I	Intermediate	Lymphocytosis and enlarged lymph nodes	6
II		Lymphocytosis and enlarged spleen and/or liver	
III	High	Lymphocytosis and anemia (hemoglobin < 11 g/dL)	2
IV		Lymphocytosis and thrombocytopenia (platelets < 100×10^9/L)	

BINET SYSTEM

Binet stage	Clinical characteristics	Median survival (yr)
A	Hemoglobin level ≥ 10 g/dL, platelet count ≥ 100×10^9/L, and < 3 areas involved	> 7
B	Hemoglobin level ≥ 10 g/dL, platelet count ≥ 100×10^9/L, and ≥ 3 areas involved	< 5
C	Hemoglobin level < 10 g/dL, platelet count < 100×10^9/L, or both (independent of areas involved)	< 2

Figure 4: Staging systems for CLL (cancer network-home of journal oncology)

As for the treatment of CLL there are quite a few options available. Usually low risk, and intermediate risk patients do not receive treatment, but they are supervised. However, if treatment is of necessity, hematologists can choose between the different options available depending on the CLL case. These include single agents such as cytostatic agents, monoclonal antibodies (anti CD20), agents targeting B-cell receptor signaling, BCL-2 inhibitors, and immunomodulatory drugs. Furthermore, combination chemotherapy, chemo-immunotherapy and combination of targeted agents are also of use (Hallek 2017).

Hypogammaglobulinemia:

Hypogammaglobulinemia is the most common immune deficiency detected in CLL (Freeman et al. 2008) with an incidence rate that varies between studies (up to 85%) (Baliakas et al. 2014). Hypogammaglobulinemia exists in 25% of newly diagnosed CLL patients and approximately 25% of CLL patients with normal gamma globulin levels at diagnosis will subsequently develop hypogammaglobulinemia on long-term

follow-ups (Parikh et al. 2015). It is defined as the non-efficient production of gamma globulins (IgG, IgM, and IgA) in the blood. Globulins are proteins found in the blood that are responsible for the organism's protection and exist in three types; alpha, beta and gamma. Gamma globulins, otherwise known as immunoglobulins, are produced by plasma cells (differentiated B lymphocytes) and represent the humoral immune response. There are 5 different isotypes IgM, IgA, IgG, IgE, and IgD and 4 IgG subclasses IgG1, IgG2, IgG3, and IgG4. These immunoglobulins are produced in varying amounts when needed for an immune response to bacteria, viruses, and toxins. In hypogammaglobulinemia, the lower antibody levels lead to an impaired immune system.

Hypogammaglobulinemia in CLL becomes more pronounced with longer disease duration and advanced-stage disease and patients that are diagnosed with it, have an increased risk of recurrent infections, such as recurrent bacterial infections, often involving the respiratory tract, and increased incidence of parasitic gastrointestinal infections (Sun et al. 2015). However, the presence of hypogammaglobulinemia does not appear to impact overall survival (Parikh et al. 2015). Hypogammaglobulinemia can be caused by either a primary or secondary immunodeficiency. Primary immunodeficiency occurs due to mutations in the genome. A common mutation leading to hypogammaglobulinemia is the one that affects the BTK gene. This gene is needed for the growth and maturity of B-cells. The elimination or the defect of the gene leads to immature B cells, that don't produce enough antibodies (Grant et al. 2013). Secondary immunodeficiency is associated with hematologic malignancies. For CLL, the mechanisms responsible for the hypogammaglobulinemia remain unclear. However it is believed that it occurs due to defective functioning of the non-clonal differentiated (CD5)-negative B cells (Parikh et al. 2015). Attention has been given to the inadequate T cell help, which is believed to be undermined by the interaction between T cells and CLL B cells in the secondary lymph nodes. Another possibility to explain the anergy of T cells is that they are exposed to cytokines (e.g. TGF-β, IL-10, or IL-6) secreted by CLL B cells that impair their function. Moreover, these cytokines may also have an impact on the development and differentiation of non-malignant B cells in the BM (Parikh et al. 2015). Similarly, other investigators have reported an increase in T-cell suppressor activity, as well as a decrease in the capacity of enriched T cells to induce differentiation of normal B lymphocytes (Wadhwa and Morrison. 2006)

3. Myeloid derived suppressor cells

Characteristics and Function:

Myeloid Derived Suppressor Cells (MDSCs) are immature cells of myeloid origin with immunosuppressive properties that are accumulated during chronic inflammation and tumor progression. MDSCs are divided into two subpopulations, the polymorphonuclear (PMN)-MDSCs and the monocytic (M)-MDSCs, characterized by the immunophenotype HLA-DR$^{low/-}$CD11b^{+}CD33^{+}CD15^{+} and HLA-DR$^{low/-}$ CD11b^{+}CD33^{+}CD14^{+} respectively (Umansky et al. 2016). However, it should be noted that there is another subset of MDSCs with a Lin^{-} (CD3, CD14, CD15, CD19, CD56) HLA-DR^{-}CD33^{+} immunophenotype that contains mixed groups of MDSCs, comprising of more immature progenitors. These cells are called early-stage MDSCs (e-MDSC) and exist in such small numbers that are often forgotten or not studied (Chesney, Mitchell, and Yaddanapudi 2017).

MDSCs derive from the hematopoietic BM precursor cells and acquire strong immunosuppressive and tumorigenic activities. These functions include: the elimination of essential amino acids for T cell proliferation, such as arginine and cysteine; the production of nitric oxide (NO) and reactive oxygen species (ROS) that causes the nitration of T cell receptors (TCR) (Umansky et al. 2016); the production of indoleamine-2,3-dioxygenase (IDO), an enzyme that catalyzes the first and rate-limiting step in the kynurenine pathway, causing depletion of tryptophan and production of the catabolic product kynurenine, which can slow the growth of T cells, impair their survival and promote Treg generation (Jitschin et al. 2018); the production of chemokines responsible for T cell migration and T cells and NK apoptosis; the production of interleukin (IL)-10 and transforming growth factor (TGF)-1 that both inhibit T-cell functions and induce Treg activation; the expression of programmed death-ligand 1 (PD-L1) which can interact with PD-1 receptor expressed on T cells and reduce their activity; the reduction of the TCR-chain expression, which makes TCR-mediated antigen recognition more difficult; the secretion of angiogenic factors promoting tumor neovascularization and finally the production of growth factors, matrix metalloproteinases and cytokines that can stimulate tumor growth and activate Tregs (Umansky et al. 2016).

Generation, Expansion and Activation:

MDSCs are generated from the pathologic modulation of myelopoiesis which is induced by constantly produced inflammatory mediators under chronic inflammation in the tumor microenvironment. The generation and expansion of MDSCs is a complex process that requires the presence of two major signals. The first is responsible for the production of immature myeloid cells with no terminal differentiation and the second is responsible for their immunosuppressive function (Figure 5) (Chesney, Mitchell, and Yaddanapudi 2017). The first group of signals is mostly driven by tumor-derived growth factors. Tumor cells are able to produce a variety of growth factors and inflammatory mediators including granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), stem cell factor (SCF), vascular endothelial growth factor (VEGF), transforming growth factor (TGF), tumor necrosis factor (TNF), IL-1, IL-6, and IL-10 that generate MDSCs' production. In addition, various chemokines such as CCL2, CCL3, CCL4, CCL5, CXCL1 and CXCL8 are responsible for the migration of the previously generated MDSCs into the tumor site where the second group of signals takes place (Umansky et al. 2016). The second group of signals is mostly driven by the tumor microenvironment and tumor stromal cells that produce inflammatory cytokines such as interferon (IFN)-γ, IL-1β, IL-4, IL-13, TNF-β, toll-like receptor (TLR) ligands, PGE2 (Umansky et al. 2016) and is mediated by transcription factors STAT1, STAT6 and nuclear factor (NF)-κB as well as by elevation of cyclooxygenase (COX)-2 activity (Gabrilovich 2017). Finally, Bruton's tyrosine kinase, a key component of B cell receptor signaling and B-cell development has recently been found to be highly expressed by MDSCs, revealing its role in the generation, maturation, trafficking and function of these myeloid cells (Stiff et al. 2017). All the factors mentioned above are able to stimulate MDSCs' generation, enrichment and activation leading to the inhibition of T cell functions, the antitumor reactivity of dendritic cells and natural killers cells and the stimulation of Tregs (Umansky et al. 2016).

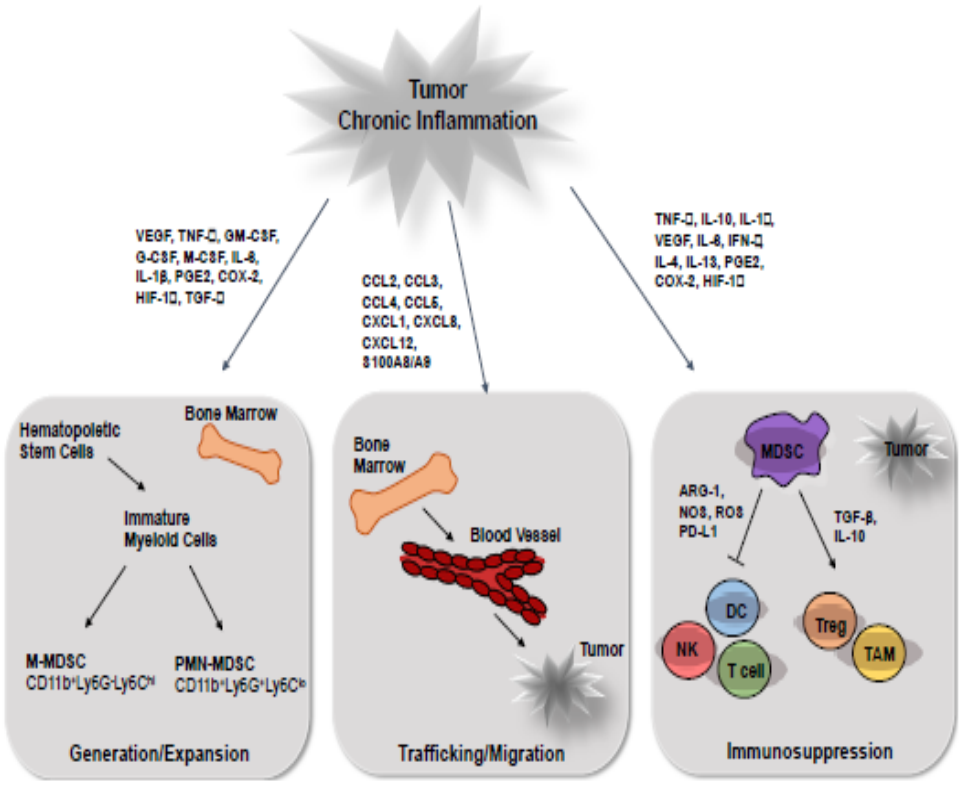

Figure 5: Chronic inflammatory factors stimulate myeloid-derived suppressor cells' (MDSCs) generation, migration and activation of immunosuppressive functions at the tumor site. (Umansky et al. 2016)

MDSCs stimulate tumor progression:

Apart from their immunosuppressive activity, MDSCs enhance tumor progression (Figure 6). MDSCs have been implicated in tumor neovascularization and tumor neoangiogenesis with the production of VEGF and basic fibroblast growth factor (b-FGF). Furthermore, MDSCs were demonstrated to promote tumor invasion by producing metalloproteinases (MMPs) which enable matrix degradation and chemokines that create a pre-metastatic environment. Metastasis is also promoted by MDSCs through their fusion with tumor cells (Umansky et al. 2016). Finally, S100A8/A9 inflammatory proteins aside from attracting MDSCs into the tumor microenvironment and enhancing their immunosuppressive activity, also promote the activation of MAPK and NF-κB signaling pathways in tumor cells, stimulating thereby tumor progression.

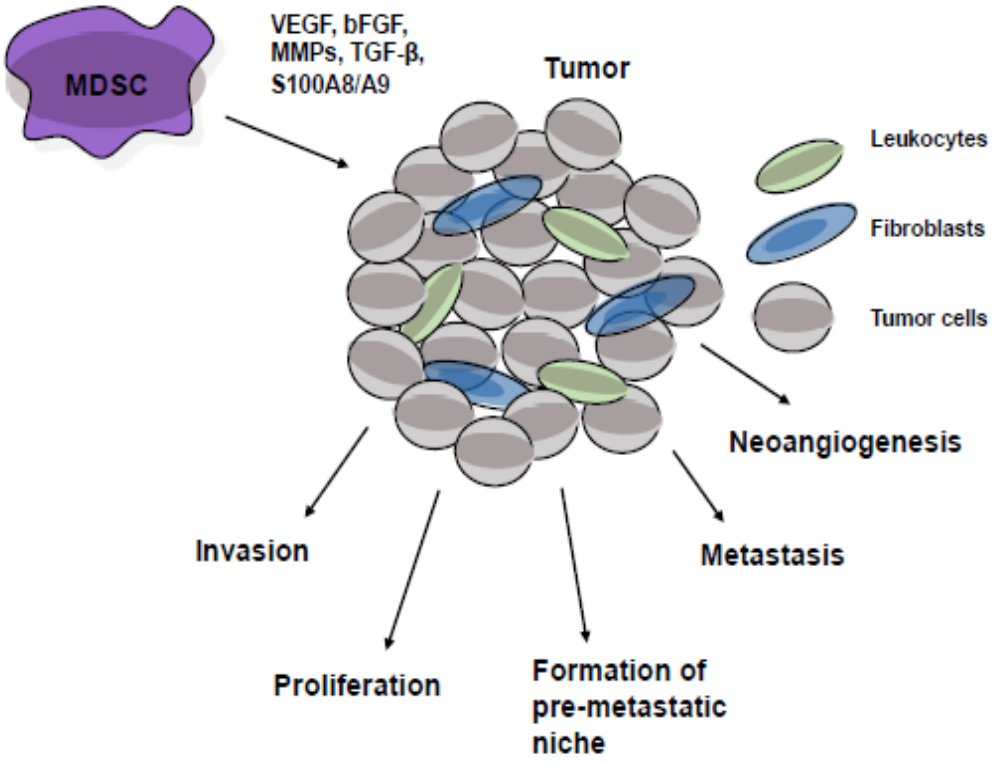

Figure 6: MDSCs support tumor development, metastasis, invasion, proliferation and neovascularization through the secretion of MMPs, VEGF, TGF-β, etc. (Umansky et al. 2016)

MDSCs at the tumor site can also differentiate into tumor associated macrophages (TAMs), which have a tumor-promoting phenotype and can up-regulate the expression of either arginase or iNOS and produce several suppressive cytokines (Gabrilovich and Nagaraj 2009). This differentiation from M-MDSCs into TAMs is accompanied by the upregulation of anti-apoptotic molecules c-FLIP and A1, as well as the ARG1 enzyme (Figure 7). TAMs have a relatively high expression of F4/80, intermediate expression of Ly6C, low expression of S100A9 protein and express higher levels of IRF8, a marker of terminal macrophage differentiation compared to M-MDSCs (Figure 7) (Bronte et al. 2016). Together MDSCs and TAMs contribute to nonspecific T-cell suppression in the tumor microenvironment.

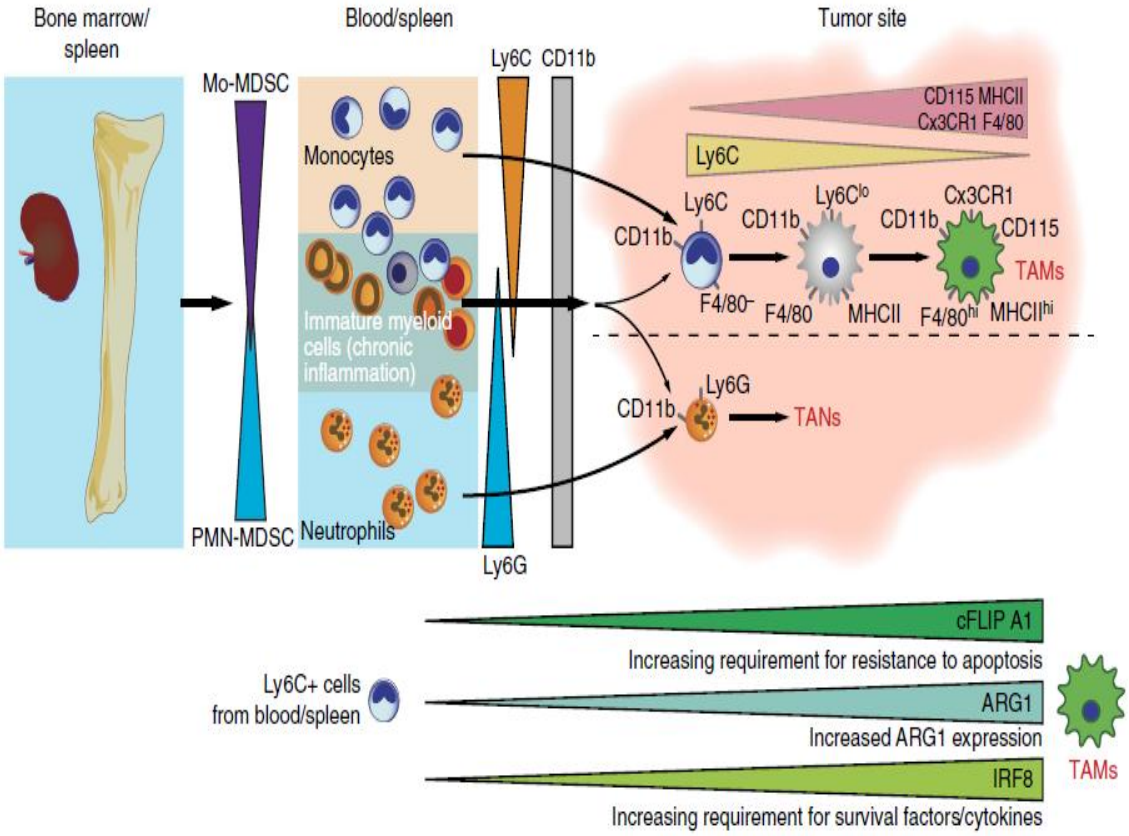

Figure 7: Overview of MDSC involvement in myeloid cell differentiation in cancer: Within solid tumors M-MDSC differentiate into TAMs through intermediate steps where Ly6C is progressively downregulated and MHCII, F4/80 and CX3CR1 are upregulated (Bronte et al. 2016)

Inhibition of MDSC's immunosuppressive activity:

In recent years, MDSCs have been implicated in the resistance to anticancer therapies as well as the inhibitory effect of chemotherapy on the immune system.
Since MDSCs are cells that are involved in the inhibition of immune responses and support of tumor progression, their elimination seems to be of much importance in order to ameliorate the clinical outcome of cancer patients. Several strategies are currently being tested in order to therapeutically target these cells. These strategies mainly involve the elimination of MDSCs, the deactivation of MDSCs, and the skewing of myelopoiesis away from the accumulation of MDSCs (Marvel and Gabrilovich 2015). In more detail, studies have already shown the successful elimination of MDSCs with low doses of chemotherapy. Several trials of testing low-dose chemotherapy to increase immune responses have been conducted, and

others are still ongoing. In murine models, gemcitabine, has effectively depleted MDSCs populations, resulting in decreased tumor growth and prolonged survival of cancer patients (Suzuki et al. 2005). In addition, cisplastin and 5-fluoro-uracil can eliminate MDSCs and in that way increase $CD8^+$ T cell responses (Ugel et al. 2012). As for the second strategy, MDSCs can be functionally inactivated by targeting their suppressive properties. ROS and NOS are essential components of MDSC suppressive machinery. NRF2 can modulate the expression of several antioxidant enzymes that scavenge ROS and NO, and its upregulation by a synthetic triterpenoid can downregulate MDSCs' ROS production (Nagaraj et al. 2010). To continue with, tadalafil, a phosphodiesterase-5 (PDE-5) inhibitor, has been reported to decrease arginase and INOS expression by MDSCs and resulted in the upregulation of tumor specific T cells (Califano et al. 2015). As for diverting myelopoiesis away from generating MDSCs and into differentiated and mature cells; STAT3 targeting (Nefedova et al. 2005), as well as treatment with all-trans-retinoic acid (ATRA) have shown to drive MDSCs' differentiation into dendritic cells (DCs) (Kusmartsev et al. 2003).

Myeloid derived suppressor cells in the context of CLL:

In recent years, there has been a rising interest in the investigation of the contribution of MDSCs in the pathogenesis/pathophysiology of cancer and especially of hematologic diseases. In lymphoid malignancies, in which CLL is included, MDSCs seem to play a key role in the disease's pathophysiology, progression and outcome with their immunosuppressive properties (Lin et al. 2011; Jitschin et al. 2014; Betsch et al. 2018; Romano et al. 2015).

Despite their characterized mechanisms and well understood connection to cancer, the information available on MDSCs in the context of CLL is still insufficient. It was not until recently that a few publications revealed the connection between MDSCs and CLL. MDSC numbers expand in CLL and correlate with the number of the B malignant cells while suppress the T-cell responses (van Attekum, Eldering and Karter 2017).
Furthermore, statistical analyses revealed that $CD14^+HLA\text{-}DR^{low/-}$ cells were significantly associated with the clinical stage of the disease in CLL patients, with their frequency being significantly higher in advanced stages compared to early CLL stages, indicating that these cells may participate in the progression of CLL (Liu et al. 2015). In addition, total MDSCs and T cells are highly connected, with a significant correlation between $CD4^+$ T cells and M-MDSCs, and between $CD8^+$ T cells and PMN-

MDSCs, resulting in the reduction of both naïve $CD4^+$ differentiation and T-cell responses (Ferrer et al. 2016).

A very recent publication revealed that $CD11b^+CD33^+CD14^+HLA\text{-}DR^{low}$ monocytic cells (M-MDSCs), that were previously discovered to be increased in the peripheral blood of untreated CLL patients, were able to suppress T-cell proliferation and IFNγ production through IDO (Jitschin et al. 2018). In more detail, CLL cells that are protected by stroma cells have the ability to enhance the generation of IDO^{high} MDSCs that can suppress T-cell activation and proliferation while providing help in the conversion of naïve T cells into Tregs. The later suppress CD8 T cells that are responsible for targeting CLL cells (Figure 8) (Zirlik 2014). Finally, CLL cells induced IDO^{hi} MDSCs from healthy donor monocytes revealing an immunosuppressive network among CLL cells, MDSCs, and Tregs (Jitschin et al. 2018).

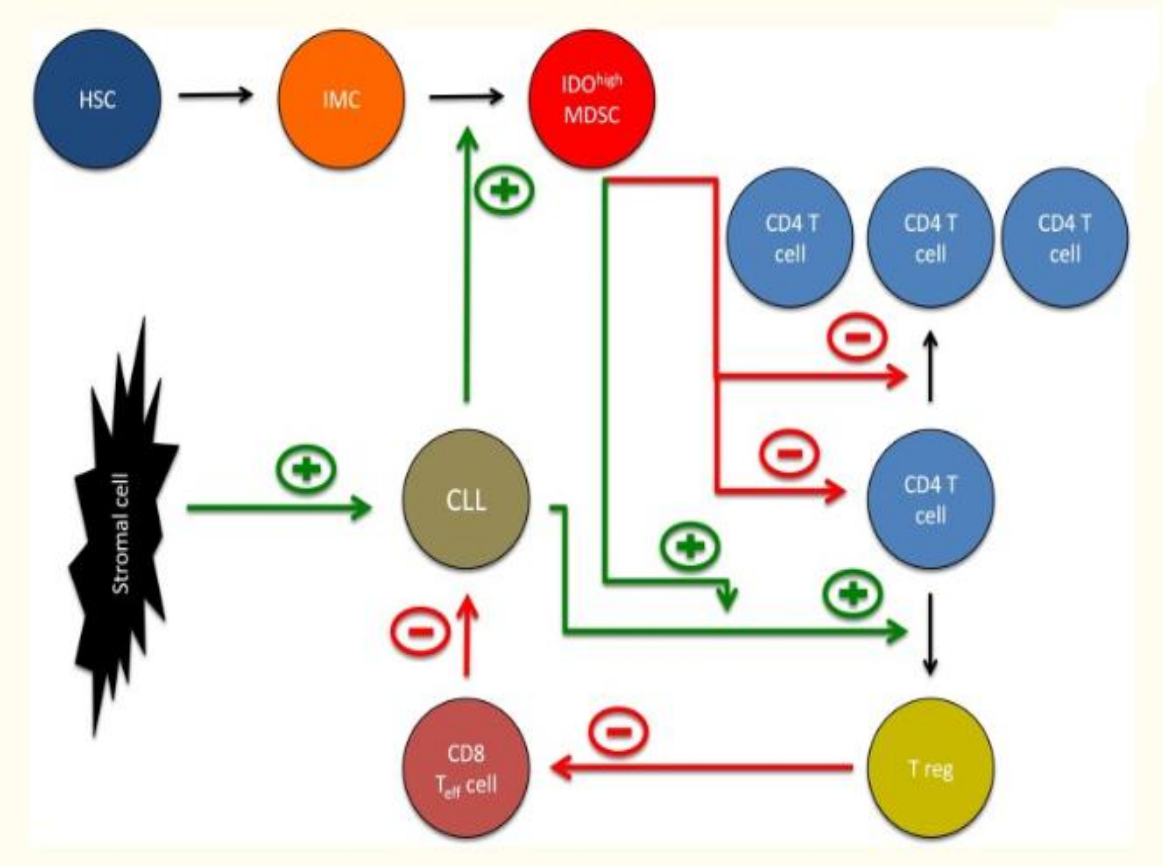

Figure 8: The role of IDO^{high} in the microenvironment of CLL. Hematopoietic stem cells give rise to immature myeloid cells (IMCS) that further differentiate into myeloid derived suppressor cells (MDSCs). IDO^{high} MDSCs promote CLL cells survival and suppression of the immune system responses by inhibiting T cell activation and proliferation, by promoting the conversion of naïve CD4 T cells into Tregs and by suppressing CD8 T cells. (Zirlik 2014)

4. Aim

Recent publications have provided important information on the connection between MDSCs and CLL. Many aspects of their connection have been revealed, highlighting the role of MDSCs in the disease's progression. However, no conclusive evidence on the correlation between the low immunoglobulin (Ig) levels that follow CLL in the majority of cases and the normal B-cell has been documented and overall the underlying mechanisms implicated in the Ig defect remain unclear.

The aim of the study is to investigate the possible involvement of MDSCs in CLL-associated hypogammaglobulinemia by evaluating the proportion of PB PMN-MDSCs and M-MDSCs and their suppressive activity in CLL patients with and without hypogammaglobulinemia.
This study will contribute to a better understanding of the pathophysiological mechanisms that govern the disease and may highlight new biomarkers and/or therapeutic targets for the disease and its complications.

5. Materials and methods

A) Patients and Controls

69 patients from the outpatient Clinic of the Hematology Department of University Hospital of Heraklion diagnosed with chronic lymphocytic leukemia (CLL) were enrolled in the study. They had not received any treatment for the disease and had either normal levels of gamma globulins (44 patients) or low levels of at least one of the immunoglobulins IgG, IgM or IgA (25 patients) (Figure 9). The control group was also acquired at the same hospital and consisted of 18 hematologically normal individuals of matched sex and age with the CLL patients. All patients had a routinely whole blood test and differential measurements.

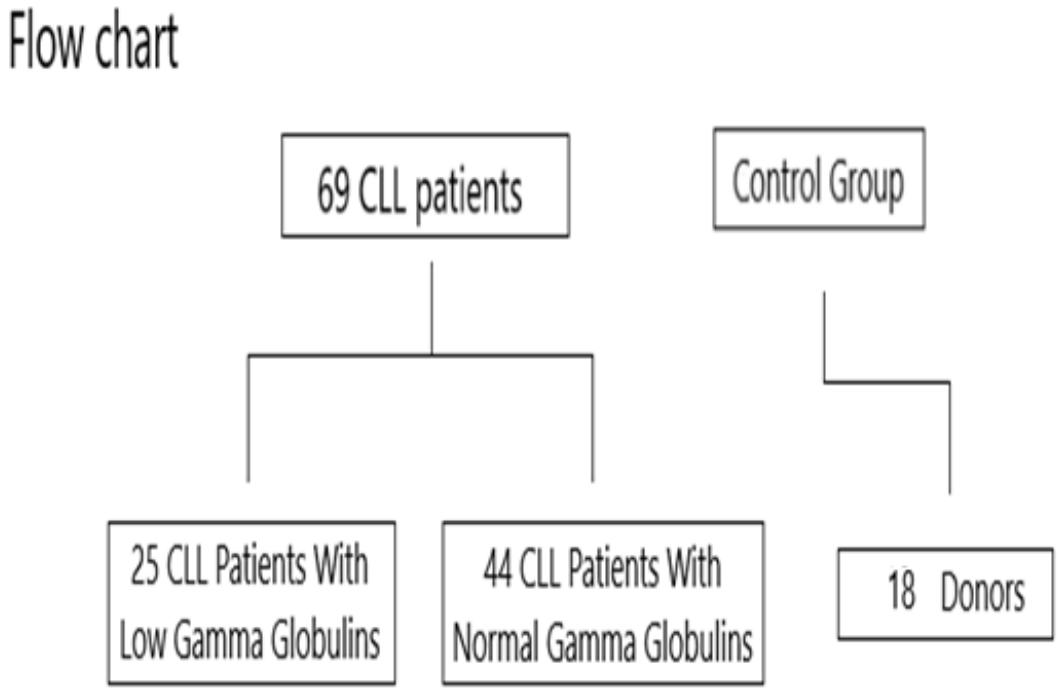

Figure 9: Flow chart of the number and characteristics of the study's participants

B) Peripheral Blood Mononuclear Cells (PBMC) isolation:

In order to estimate the number of peripheral blood MDSCs (PMN-MDSCs and M-MDSCs) in the different groups of our study (CLL patients with and without hypogammaglobulinemia and healthy individuals) we proceeded with the immunophenotyping of PBMCs as follows:

- PB samples were collected in tubes containing EDTA.
- Samples were diluted 1:1 with plain RPMI 1640 Medium Gibco™ (*THERMO-FISHER SCIENTIFIC).*
- 7-8 ml of diluted sample were layered over 4 ml of Ficoll-centrifuge gradient (*Lymphoprep™- STEMCELL TECHNOLOGIES)* in 15 ml centrifuge tube
- Cells were centrifuged at RT for 25-30 min, at 1800 rpm/360 g, with no brakes
- The mononuclear cell layer was transferred to a clean 50 ml tube.
- Cells were washed with PBS, added up to 15 ml and centrifuged at RT for 5 min, at 1600 rpm/280 g, with brakes. Supernate was discarded and cell pellet was reconstituted with PBS added up to 10 ml depending on the density of cells in each sample.
- Viable cells were counted by using Trypan Blue and a hemocytometer.

C) Immunophenotyping

To quantify the numbers of both PMN-MDSCs and M-MDSCs the following procedure was performed**:**

- 10^6 cells (PBMCs)in a volume of 100 µL were added in flow cytometry (FC) 5 ml tubes
- 40 µl Fc blocking reagent [γ-Globulins, Human: From Cohn Fraction II, III Approx. 99% (electrophoresis) G4386-1G/*SIGMA*] was added to avoid unspecific binding since it blocks unwanted binding of antibodies to human Fc receptor-expressing cells.
- Samples were incubated for 10 min at 4°C in the dark.

- The precise volume of anti-human monoclonal antibodies conjugated with the appropriate fluorochrome (BECKMAN COULTER Life Sciences) was added according to the manufacturer's instructions for the detection of MDSCs with the following combinations:
- CD33-PC7 (clone: D3HL60.251) / CD15-PC5 (clone: 80H5) / DR-ECD (clone: Immu-357) / CD14-PE (clone: RMO52) / CD11b-FITC (clone: Bear1)
- Samples were incubated for 20 min at 4°C in the dark.
- Cells were washed with Flow Cytometry buffer (0.5% FBS in PBS)
- 500 µl PFA 1% were added to preserve cell viability.
- Cells were analyzed on a flow cytometer (Beckman Coulter Cytomix FC500)

In addition, in order to ensure that nothing has changed over time regarding the diagnosis of CLL, the percentage of $CD45^+$, $CD3^+$, $CD4^+$, $CD8^+$, $CD57^+$ and $CD19^+$ cells in the PB of our patients collected in EDTA-containing tubes was measured by flow cytometry, as follows:

- The precise volume of anti-human monoclonal antibodies conjugated with the appropriate fluorochrome (*BECKMAN COULTER Life Sciences*) was added in FC 5ml tubes according to the manufacturer's instructions with the following combinations:
  - CD45-PC7 (clone: J33) / CD3-ECD (clone: UCHT1) / CD4-PC5 (clone: 13B8.2) / CD8-PE (clone: B9.11)
  - CD45-PC7 (clone: J33) / CD57-FITC (clone: NC1) / CD19-PE (clone: J3-119)
- 100 µl of blood was added to each of the FC tubes
- Samples were incubated for 15 min at RT in the dark
- Cells were washed with Flow cytometry buffer (0.5% FBS in PBS)
- Lysis of red blood cells was performed in a Q-PREP, (Epics Coulter) according to the manufacturer's instructions
- 500 µl PFA 1% was added to preserve cell viability
- Cells were analyzed on a flow cytometer (Beckman Coulter Cytomix FC500))

Flow cytometry principals:

Flow cytometry is a technique used to detect and measure physical and chemical characteristics of cells or particles and works based on the following principals; According to Thermo Fisher Scientific (Figure 10), when a sample containing fluorescently labeled cells is uptaken by the instrument, it is mixed with a physiological buffer called sheath fluid and it is transferred to the flow chamber, where cells within the sample line up to a single-file stream, which is a critical step for single-cell analysis. One by one, cells pass through a narrow channel called the interrogation point where they meet the laser of the cytometer. When the laser light beam illuminates a single cell, some of the light will strike physical structures within the cell, causing the light to scatter. The forward scatter (FSC) provides information about the size of the cell, whereas the side scatter (SSC) gives information about the complexity of the cell (granules). The light from the laser that hits the cells nearly simultaneously excites all fluorophores linked with antibodies, previously attached on the surface or the interior receptors of cells, producing a fluorescence emission. All of this light is collected by the different light detectors depending on the wave lengths of the scattered light and is processed by the electronics component of the flow cytometer. After passing through the interrogation point, the cells are no longer of need and are placed into the waste container with the assistance of the fluidics system. Data acquired by the sensors is collected and combined to build a comprehensive picture of the sample.

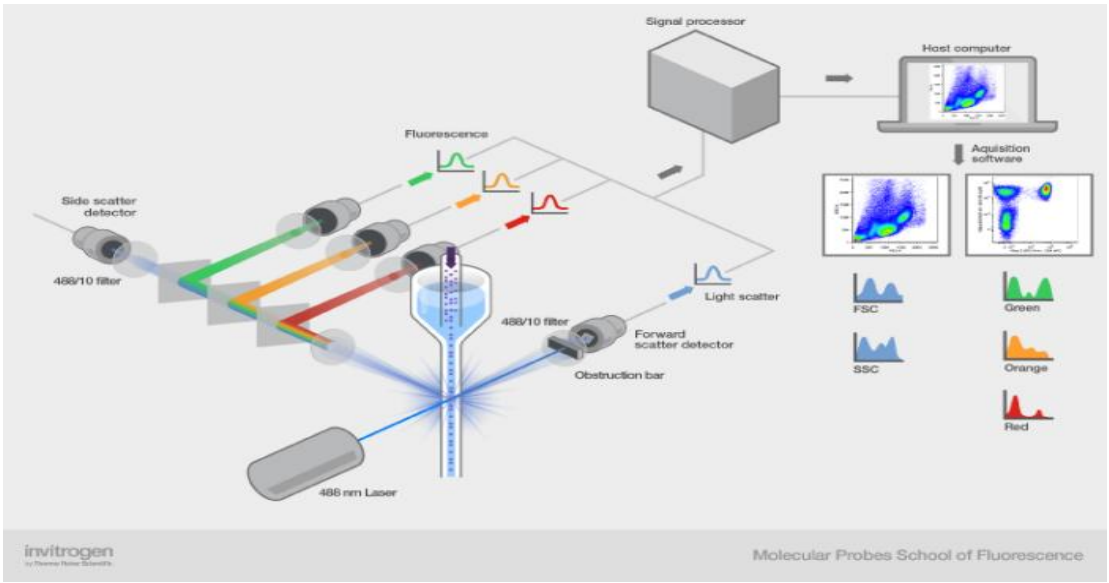

Figure 10: The working parts of the flow cytometer (explained in the text above)-(image acquired from Thermo Fisher Scientific)

Flow Cytometry analysis strategy

After performing flow cytometry for all samples, the analysis of MDSCs was performed by **Kaluza Flow Cytometry Analysis Software and** follows a back to back gating technique (Figure 11):

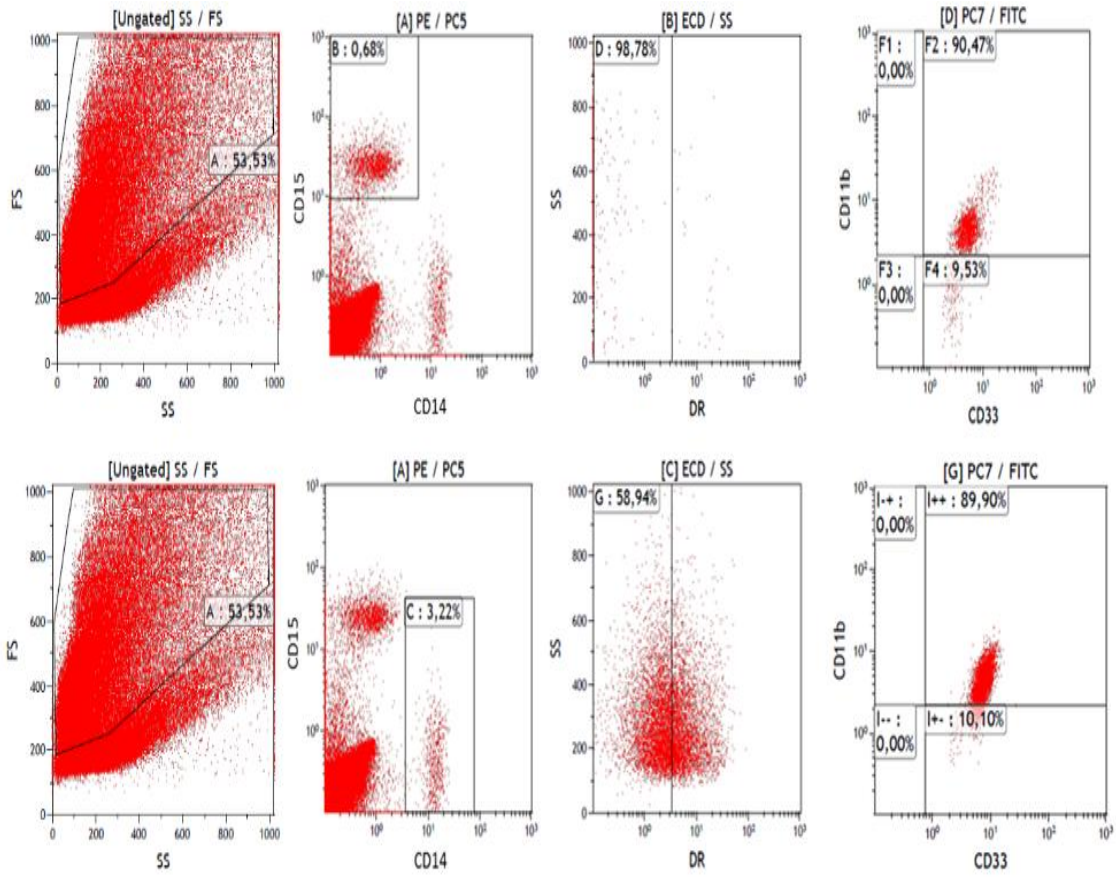

Figure 11: A representation of the FACS analysis for both MDSC populations: For the PMN-MDSC population (upper graphs): From total PBMCs, gate the **living population (A).** From the (A) population, gate the cells expressing the **CD15 marker (B).** From the (B) population, gate the **$DR^{-/low}$** cells **(D)**. From the (D) population, gate the cells expressing **both the CD11b and CD33 markers**. This final population is the PMN-MDSCs population existing in the total PBMCs sample. For the M-MDSC population (lower graphs): From total PBMCs, gate the **living population (A).** From the (A) population, gate the cells expressing the **CD14 marker (C).** From the (C) population, gate the **$DR^{-/low}$** cells **(G).** From the (G) population, gate the cells expressing **both the CD11b and CD33**. This final population is the M-MDSCs population existing in the total PBMCs sample.

D) T-Cell suppression assay

In order to estimate the suppressive activity of PB MDSCs (PMN-MDSCs and M-MDSCs) in the two different study groups (with and without hypogammaglobulinemia) we proceeded with a T-cell suppression assay.

The suppression of T-cell proliferation by MDSCs was estimated by comparing the proliferation by means of CFSE staining of the anti-CD3/anti-CD28 activated $CD3^+$ cells in PBMCs, to the sample depleted for $CD33^+$ cells, after 3 days in culture (Figure 12). The PBMCS depleted of $CD33^+$ cells is expected to be devoid of MDSCs.

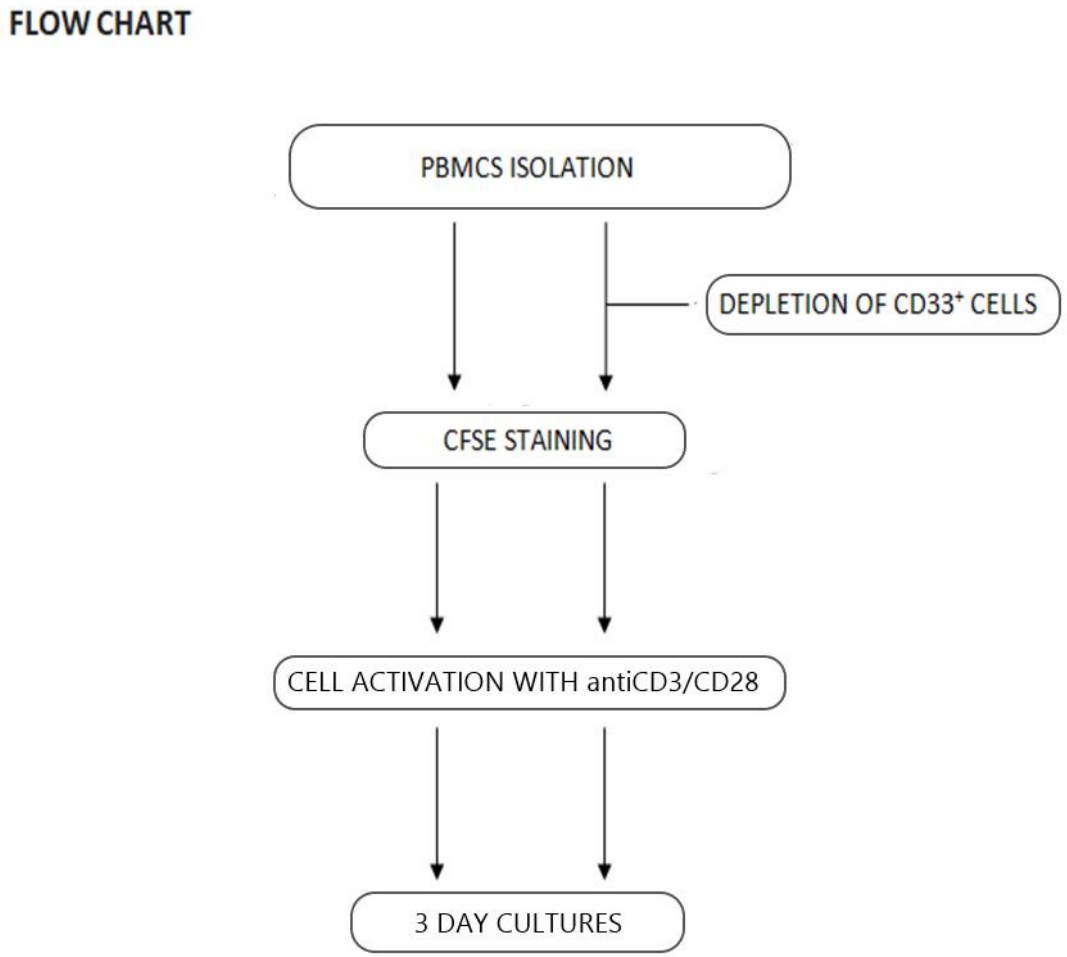

Figure 8: Flow chart of the process followed for the qualitative experiments

i. PBMCisolation:

- PB samples were collected in EDTA containing tubes
- Samples were diluted 1:1 with plain RPMI 1640 Medium Gibco™ (*THERMO-FISHER SCIENTIFIC)*
- 7-8 ml of diluted sample were layered over 4 ml of Ficoll (centrifuge gradient) (*Lymphoprep™- STEMCELL TECHNOLOGIES)* in a 15 ml centrifuge tube.
- Cells were centrifuged at RT for 20min, at 1600 rpm/280 g with no brakes
- The mononuclear cell layer was transferred to a clean 50 centrifuge tube
- Cold MINIMACS buffer was added up to 30 ml

  [500ml of MINIMACS buffer contains 50ml ACD, 25ml BSA (Bovine Albumin Fraction V Solution 7.5% Gibco™ - Life Technologies), 20ml NaHCO3 (Sodium Bicarbonate Solution 7.5% Gibco™ - Life Technologies) and 405ml PBS]
- Cells were centrifuged at RT for 5 min at 1600 rpm/280 g brakes on and supernate was discarded.
- Cell pellet was resuspended with the appropriate volume of MINIMACS buffer (10-20 mL) depending on the density of cells in each sample.
- Viable cells were counted by using Trypan Blue and a hemocytometer
- Up to $4*10^6$ PBMCs were stored in RPMI/10%FBS in 4^oC for later use (part a)
- Up to $4*10^6$ PBMCs stored in 1ml PBS/5%FBS in 4^oC for later use (part b)
- Up to $20\text{-}30*10^6$ PBMCs stored in MINIMACS buffer for later use (part c)

ii) PBMCs CD33 depletion by Immunomagnetic sorting:

- $20\text{-}30*10^6$ PBMCs in MINIMACS buffer (part c) were centrifuged at RT, for 5 min, at 1600 rpm/280 g brakes on and supernate was discarded.
- Cells were incubated with 40-60μl (depending on the number of PBMCs) of anti-CD33 antibody conjugated with magnetic microbeads (*MACS Miltenyi Biotec*-clone AC104.3E3) for 15 minat 4°C according to the manufacturer's instructions.
- Cells were washed with MINIMACS buffer and Centrifuged at 10^oC, for 5 min, at 1600 rpm/280 g, brakes on

- Cells were resuspended in 1ml of MINIMACS buffer and passed through an LD column, previously washed with 2ml of MINIMACS buffer. The LD column is used in order to better eliminate the unwanted cell populations.
- The column was washed once with 1ml of MINIMACS buffer
- The $CD33^-$ cells that flew through the LD column were gathered in a clean tube, resuspended and counted by using Trypan Blue and a hemocytometer
- Up to $4*10^6$ $CD33^-$ cells were stored in RPMI/10%FBS, in 4^oC for later use (part d)
- Up to $4*10^6$ $CD33^-$ cells were stored in 1ml PBS/5%FBS in 4^oC for later use (part e)

iii) CFSE staining

CFSE staining principals:

To monitor lymphocyte proliferation, with minimal disruption to cell viability and function we use an intracellular fluorescent dye, carboxyfluorescein succinimidyl ester (CFSE), which has the ability to stably label molecules within cells, with each cell division resulting in a sequential halving of fluorescence. Initially, carboxyfluorescein diacetate succinimidyl ester is non-fluorescent, owing to the presence of two acetate groups, but these groupings result in the compound being highly membrane permeant and thereby enables the dye to rapidly flux across the plasma membrane of cells. Once inside a cell, however, the acetate groups are rapidly removed by intracellular esterases to yield the highly fluorescent CFSE that is trapped inside cells owing to its reduced membrane permeability. Some of the CFSE conjugates are highly stable and remain inside cells. It is these fluorescent conjugates that persist within cells that are diluted between daughter cells following cell division, which allows lymphocyte proliferation to be monitored by flow cytometry (Figure 13). Usually, lymphocyte proliferation can be monitored by flow cytometry for up to eight divisions before CFSE fluorescence is decreased to the background fluorescence of unlabeled cells (Quah, Warren, and Parish 2007).

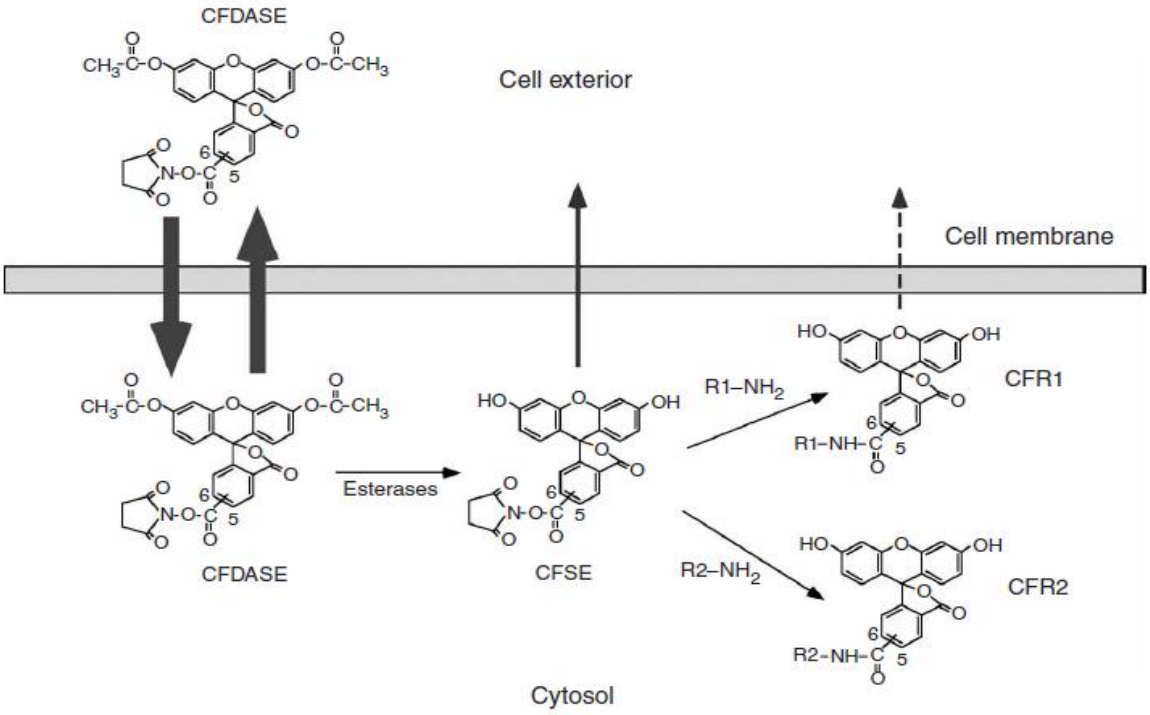

Figure 9: A schematic representation of the various molecular events that occur during the labeling of cells with CFDASE. "CFR1, carboxyfluorescein conjugated molecule that is lost from the cell; CFR2, carboxyfluorescein conjugated molecule that is retained by the cell." (Quah, Warren, and Parish 2007)

CFSE staining procedure:

- Up to$4*10^6$ PBMCs (part a) and $4*10^6$ $CD33^-$ cells (part d) were diluted in 1ml PBS/5%FBS each.
- Cells were incubated for 10 min, with 100µl of CFSE (2 µM) (*Cell-Trace CFSE Proliferation Kit-Thermo Fisher Scientific)* in 37 ^{0}C
- 8-12 ml of PBS/5% FBS was added to stop the reaction
- Cells were centrifugedat RT, 5 min, 1600 rpm/280 g, brakes on and supernate was discarded.
- Cells were resuspended in PBS/5% FBS and counted by using Trypan Blue and a hemocytometer
- 8-12ml were added and cells were washed by centrifugation at RT, 5 min, 1600 rpm/280 g, brakes on
- CFSE-stained PBMCs and $CD33^-$ cells were resuspended in RPMI/10%FBS

iv) Evaluation of autofluorescence, and CFSE staining with flow cytometry for both PBMCs and CD33$^-$ cells (day 0)

To evaluate the fluorescence baseline of both CFSE-unstained PBMCs and CFSE-unstained CD33-depleted PBMCs as well as the success of CFSE staining of both PBMCs and CD33-depleted PBMCs, all cells subpopulations were stained with fluorescent monoclonal antibodies-conjugated with fluorochromes other than FITC *(BECKMAN COULTER*) according to the manufacturer's instructions and the previously described method, with the following combinations:

CD3-PC5 (UCHT1 clone) / CD25-PE (B1.49.9 clone) / or CD33-PE (D3HL60.251 clone) and IgG1 Mouse-PC5 / PE Isotype Control (clone: 679.1Mc7)

v) Cell activation:

$0.25*10^6$ cells per well from each sample (PBMCs andCD33$^-$ cells were cultured in a 48 well-plate with 17 μl of the activating factors antiCD3/antiCD28 (STEMCELL TECHNOLOGIES–ImmunoCult^{TM} Human CD3/CD28 T-Cell Activator) in 0.5 ml final volume of RPMI/10 % FCS for 3 days.

Cultures:

	PBMCs (MDSCs INCLUDED) W/O ACTIVATION	PBMCs (W/O CD33$^+$ CELLS) W/O ACTIVATION
UNSTAINED CELLS	A	D
CFSE STAINED CELLS	B	E
	PBMCs (MDSCs INCLUDED) WITH ANTICD3/ANTICD28 ACTIVATION	PBMCs (W/O CD33$^+$ CELLS) WITH ANTICD3/ANTI28 ACTIVATION
CFSE STAINED CELLS	C	F

Table 1: Culture conditions

The experiment ran in duplicates.

The difference in the proliferation between CFSE stained PBMCs and CD33 depleted PBMCs following anti-CD3/CD28 activation was expected to be due mostly by MDSCs.

vi) Evaluation by Flow Cytometry

After 3 days in culture, cells were stained with fluorochrome conjugated antibodies and evaluated byflow cytometry.

A & D: Unstained PBMCs and CD33^{-}cells w/o activation, Day 3 to evaluate if culture conditions induce autofluorescence.

Cells were stained with fluorescent antibodies *(BECKMAN COULTER*): CD3-PC5 (UCHT1 clone) / CD25-PE (B1.49.9 clone) and IgG1 Mouse-PC5 Isotype Control (clone: 679.1Mc7) / IgG1 Mouse-PE Isotype Control (clone: 679.1Mc7)

B & E: CFSE stained PBMCS and CD33- cells w/o activation, Day3, to evaluate if culture conditions induce proliferation regardless of activating factors.

Cells were stained with fluorescent antibodies *(BECKMAN COULTER*): CD3-PC5 (UCHT1 clone) / CD25-PE (B1.49.9 clone) and IgG1 Mouse-PC5 Isotype Control (clone: 679.1Mc7) / IgG1 Mouse-PE Isotype Control (clone: 679.1Mc7)

C & F: CFSE stained PBMCs and CD33-cells activated by anti-CD3/CD28, Day3 to compare proliferation with the respective CFSE stained cell population on Day0). Cells were stained with fluorescent antibodies *(BECKMAN COULTER*): CD7-PE (8H8.1 clone) andIgG1 Mouse-PE Isotype Control (clone: 679.1Mc7)

Proliferation analysis:

The suppressive activity of MDSCs amongst the study groups (with, without hypogammaglobulinemia and controls) was estimated by using **FSC Express 6 Plus Research Edition proliferation analysis program**.

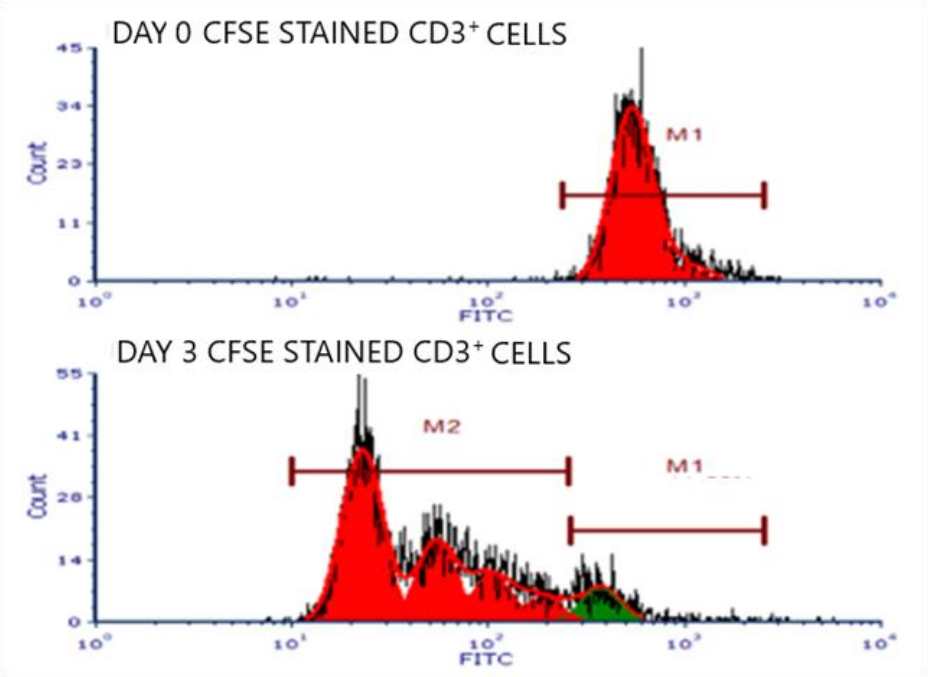

Figure 14: A representative image of the qualitative experiments' analysis method.

The proliferation diagrams are created by gating the $CD3^+$ population out of both CFSE stained PBMCs and $CD33^-$ samlpes on DAY 0, establishing in this way the time zero resting population of cells (M1) and by gating the $CD3^+$ population out of both CFSE stained PBMCs and $CD33^-$ samlpes on DAY 3, depicting in that way the percentage of the proliferating cells (M2) as well as the cells that stayed in the resting condition (no-division) (Figure 14).

E) Statistical analysis:

The statistical analysis for all experiments was performed by the **Graph-Pad Prism 6 statistical program**. For the immunophenotyping the statistical differences in the MDSCs numbers amongst the study's groups were calculated using the student's T test (parametric), whereas for the T-cell suppression assay the statistical differences in the T-cell proliferation amongst groups were calculated using two-way ANOVA test. Correlation tests (Person's test) were also performed in order to reveal any possible connection between patient's qualitative characteristics (i.e. age and CLL stage with the presence of hypogammaglobulinemia).

6. Results

A) Age and CLL stage correlation with hypogammaglobulinemia

As mentioned before, hypogammaglobulinemia in CLL becomes more pronounced with longer disease duration and advanced stage disease. In our study, the incidence rate of hypogammaglobulinemia was around 36%, which is approximately 1 out of 3-4 patients. No correlation between the CLL stage and the presence of hypogammaglobulinemia was revealed from our study (Table 2).

Correlation test	CLL status (low or normal Ig levels) vs. CLL stage
Pearson r	-0,07742
P (two-tailed)	0,6091
Significant? (alpha = 0.05)	No

Table 2: Correlation between CLL status and CLL stage of total CLL patients

Furthermore, no correlation between age and IgG, IgM and IgA levels of total patients was revealed (Table 3).

Correlationtest	Age vs IgG	Age vs IgM	Age vs IgA
Person r	-0,0546	-0,08761	-0,189
P (two-tailed)	0,6683	0,4842	0,1317
Significant? (alpha = 0.05)	No	No	No

Table 3: Correlation between age and IgG, IgM and IgA levels of total CLL patients

B) M-MDSCs number differences amongst groups:

The proportion of M-MDSCs was significantly higher in total CLL patients (0.95% ± 0.60%, median 0.81%) compared to healthy subjects (0.72% ± 0.71%, median 0,52%) (P=0,0075) (Figure 15).

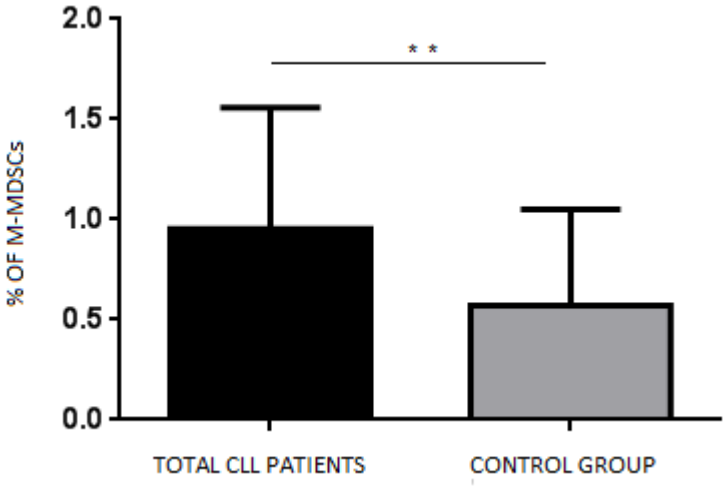

Figure 15: M-MDSCs number differences between total CLL patients and control group. Asterisks denote the statistical significance (P=0,007) between the aforementioned groups.

Both patient groups, namely those with normal and those with low Ig levels, displayed significantly increased proportion of M-MDSCs (0.97% ± 0,61%, median 0.82% and 0.96% ± 0.61%, median 0.81%, respectively) compared to healthy subjects (P=0.008 and P=0.02 respectively). No statistically significant difference was found in the proportion of M-MDSCs between patients with normal or low gamma globulins (Figure 16).

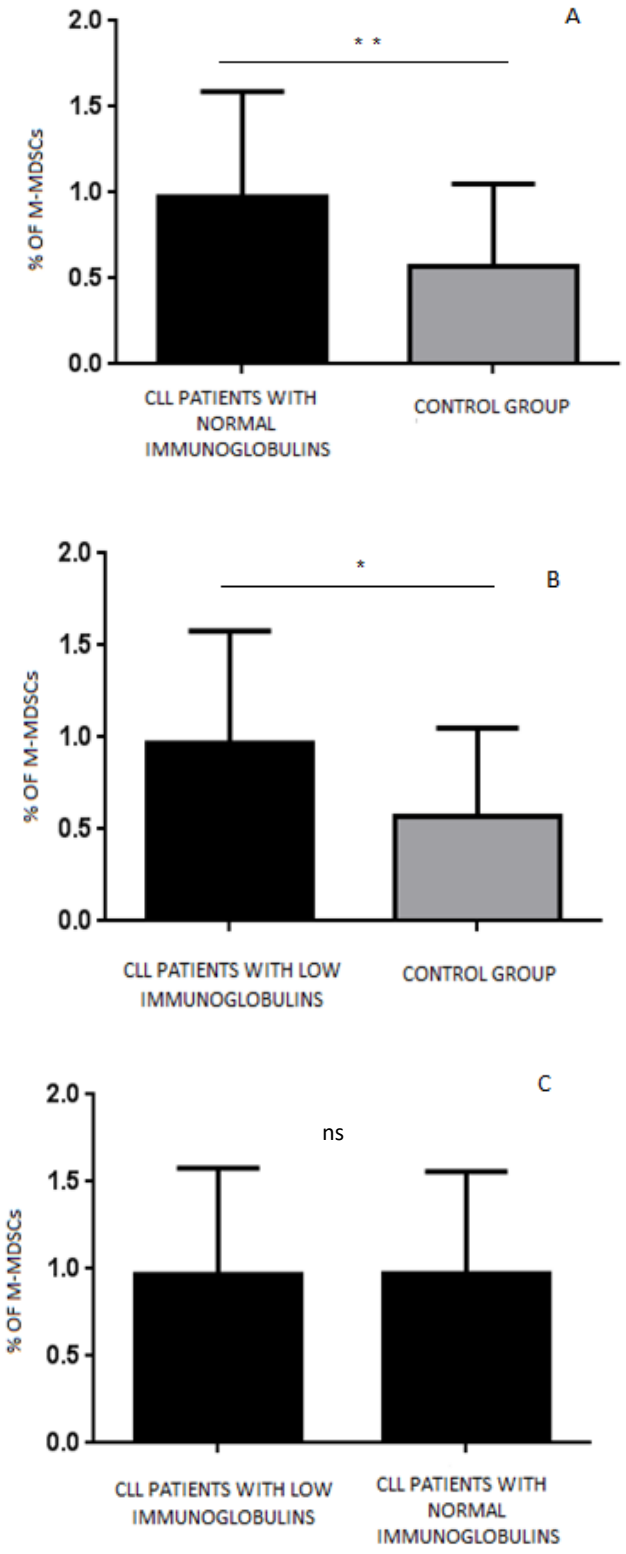

Figure 16: M-MDSCs number differences among: CLL patients with normal levels of immunoglobulins and control group (A), CLL patients with hypogammaglobulinemia and control group (B), CLL patients with normal levels of immunoglobulins and CLL patients with hypogammaglobulinemia (C). Asterisks denote the statistical significance (P=0.008, P=0.02 and P=ns respectively) between the aforementioned groups.

C) PMN-MDSCs number differences amongst groups:

The proportion of PMN-MDSCs was significantly higher in all CLL patients (1.24% ± 0.92%, median 1.05%) compared to healthy subjects (0.11% ± 0.21%, median 0,02%) (P<0.0001) (Figure 17).

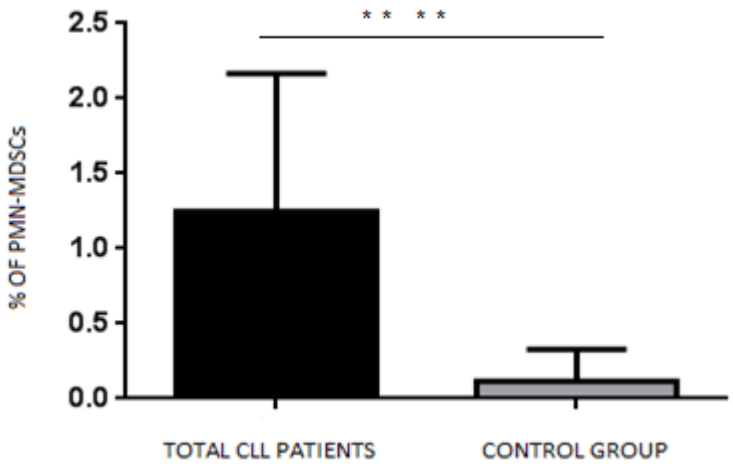

Figure 17: PMN-MDSCs number differences between total CLL patients and control group. Asterisks denote the statistical significance (P=0.0001) between the aforementioned groups.

Patients with normal gamma globulin levels as well as patients with hypogammaglobulinemia displayed increased proportion of this cell population (1.25% ± 0.97%, median 1.02% and 1.27% ± 0.86%, median 1.05% respectively) compared to healthy controls (P<0.0001 and P<0.0001 respectively). No statistically significant difference was found in the proportion of PMN-MDSCs between patients with normal or low Ig levels (Figure 18).

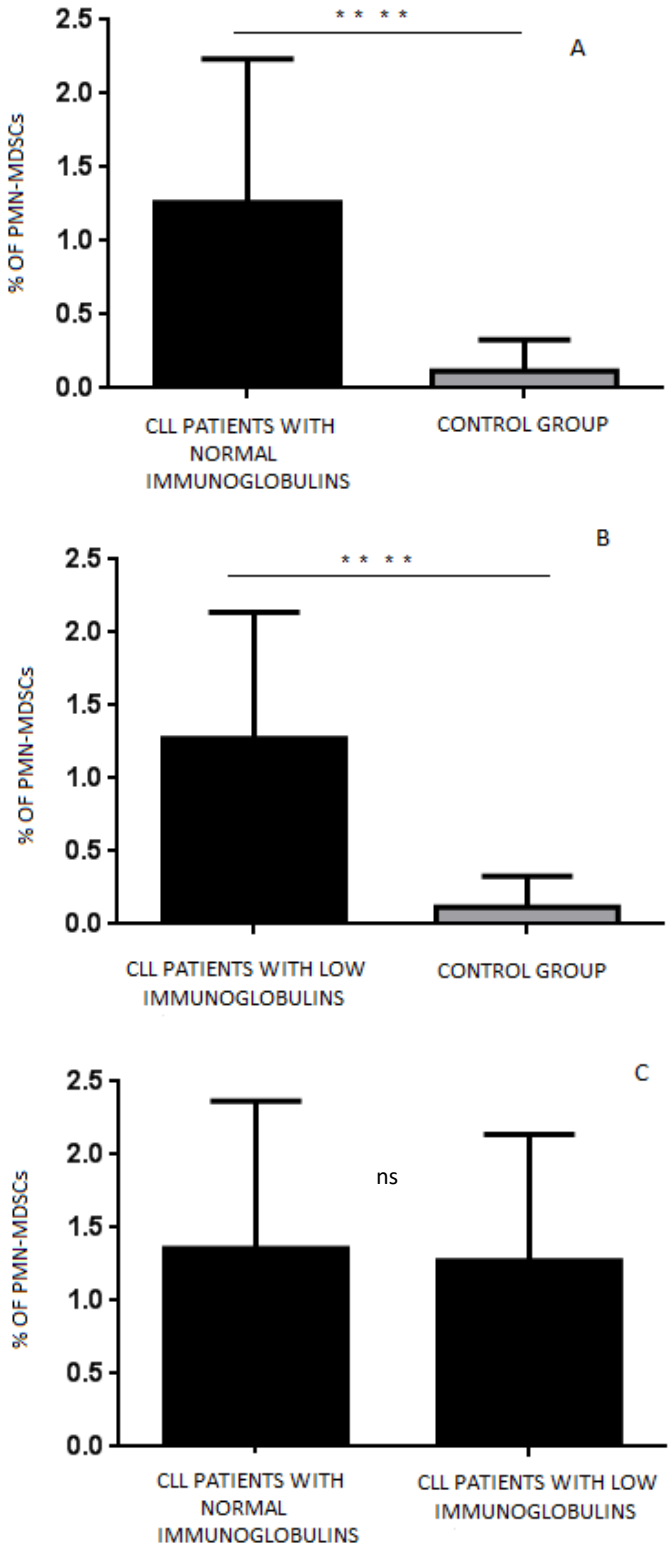

Figure 18: PMN-MDSCs number differences among: CLL patients with normal levels of immunoglobulins and control group (A), CLL patients with hypogammaglobulinemia and control group (B), CLL patients with normal levels of immunoglobulins and CLL patients with hypogammaglobulinemia (C). Asterisks denote the statistical significance (P=0.0001, P=0.0001 and P=ns respectively) between the aforementioned groups.

D) PMN-MDSCs vs. M-MDSCs:

When comparing the two subpopulations of MDSCs in CLL, the results quite differ from those of the control group. The M-MDSCs in the control group are higher in number than the PMN-MDSCs (0.72% ± 0.71%, median 0,52% and 0.11% ± 0.21%, median 0,02% respectively). However, in CLL, PMN-MDSCs are significantly upregulated compared to the M-MDSCs (1.24% ± 0.92%, median 1.05% and 0.95% ± 0.60%, median 0.81% respectively).

E) Double positive ($CD14^+/CD15^+$) MDSCs number differences amongst groups:

As for the double positive ($HLA\text{-}DR^{low/-}CD11b^+CD33^+CD14^+CD15^+$) subset of MDSCs it was revealed that total CLL patients displayed decreased proportion of this cell population compared to healthy individuals (0.001% ± 0.003, median 0 and 0.004% ± 0.006, median 0.002 respectively) (P=0,045) (Figure 19).

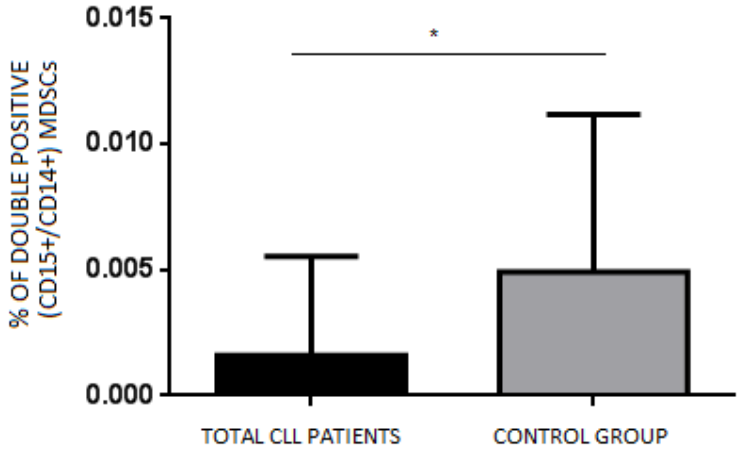

Figure 19: Double positive ($CD14^+/CD15^+$) MDSCs number differences between total CLL patients and control group. The asterisk denotes the statistical significance (P=0.04) between the aforementioned groups.

It was also observed that the percentage of these cells was lower in patients with normal immunoglobulin levels as well as in patients with hypogammaglobulinemia (0.001% ± 0.004, median 0 and 0.001% ± 0.003, median 0 respectively) compared to the control group (P=0,042 and P=0,047 respectively). Finally, no statistically significant difference was found in the proportion of double positive ($CD14^+/CD15^+$) between patients with normal or low Ig levels (Figure 20).

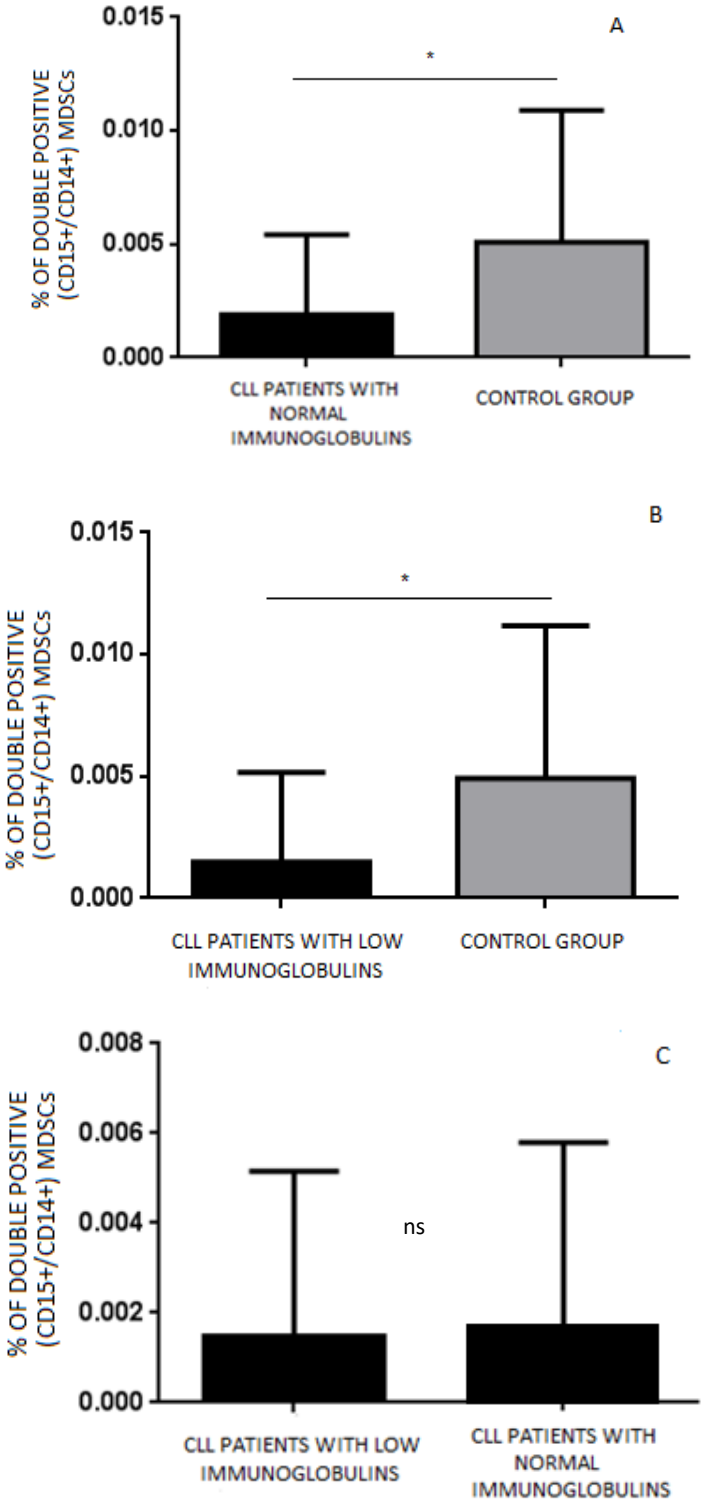

Figure 20: Double positive ($CD14^+$ $CD15^+$)MDSCs number differences among: CLL patients with normal levels of immunoglobulins and control group (A), CLL patients with hypogammaglobulinemia and control group (B), CLL patients with normal levels of immunoglobulins and CLL patients with hypogammaglobulinemia (C). Asterisks denote the statistical significance (P=0.04, P=0.04 and P=ns respectively) between the aforementioned groups.

F) T-Cell Proliferation Assay

15 T-cell proliferation assays have been conducted in order to reveal the function of MDSCs in the context of CLL. Blood samples from 11 untreated CLL patients were processed, 6 of those were patients with low levels of at least one of the immunoglobulins IgG, IgM, IgA, while the rest 5 had normal levels of gamma globulins. Blood samples of 4 hematologically healthy individuals of matched sex and age with the CLL patients were also processed in the lab as the control group (Figure 21).

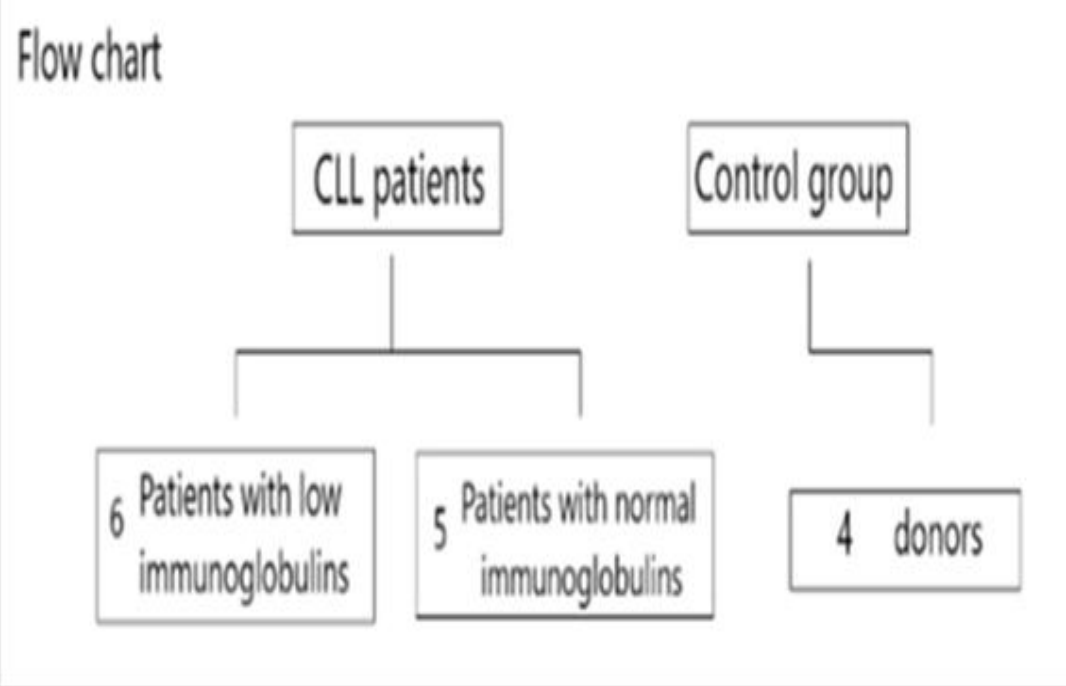

Figure 21: Flow chart of the number and characteristics of the study's participants

MDSCs suppression of T-Cell proliferation:

After ensuring that for all experimentsthe culture conditions do not induce autofluorescence or proliferation regardless of activating factors, we preceded with the evaluation of the T-Cell suppression by MDSCs, which was estimated by comparing the proliferation of the activated by anti-CD3/anti-CD28 $CD3^+$ cells in PBMCs, to the sample depleted for $CD33^+$ cells, after 3 days in culture. Also the proliferation index was measured as a method of assessing proliferation. The proliferation index was calculated as the sum of the cells in all generations including the parental, divided by the computed number of original parent cells theoretically present at the start of the experiment.

The results of the proliferation analysis indicate that MDSCs (within the $CD33^+$cell population) are able to suppress the proliferation of T-cells ($CD3^+$) in all our groups (Figures 22-24). The proliferation of T cells in cultures with total PBMCs was clearly decreased compared to the proliferation of T-cells in cultures with $CD33^+$ depleted PBMCs. That indicates that the depletion on the $CD33^+$ cells, which confirms the absence of MDSCs was indeed the major contributor to the higher T-cell proliferation.

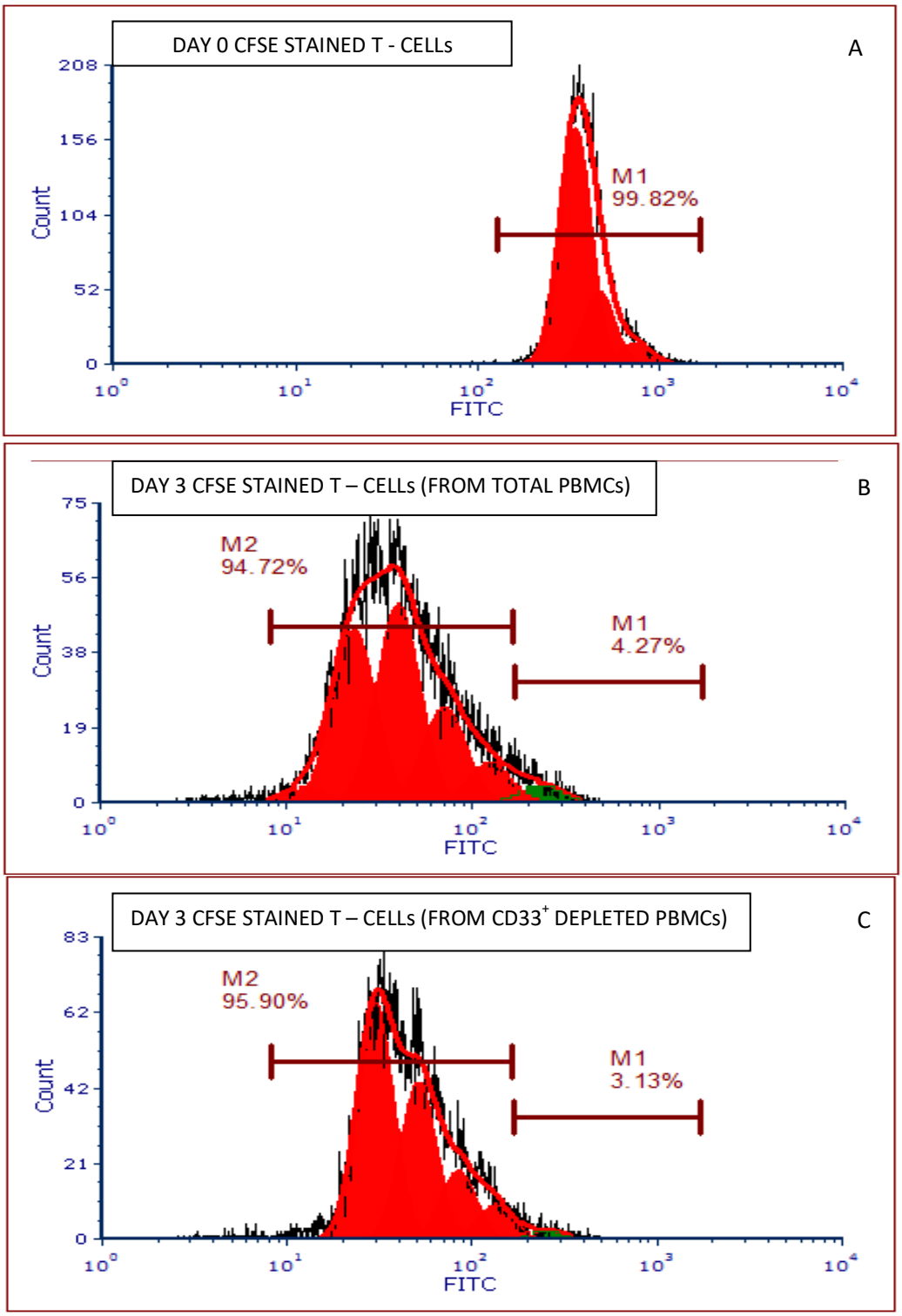

Figure 22: A representative figure of the control group's T-cell proliferation analysis – Elimination of CD33$^+$ cells (MDSCs) from PBMCs (C) improves the percentages of proliferating T-cells (M2), while decreases the percentages of resting cells (M1) in comparison to the sample involving all PBMCs (B) (proliferation index (C)=6,8 and proliferation index (B)=5,6).

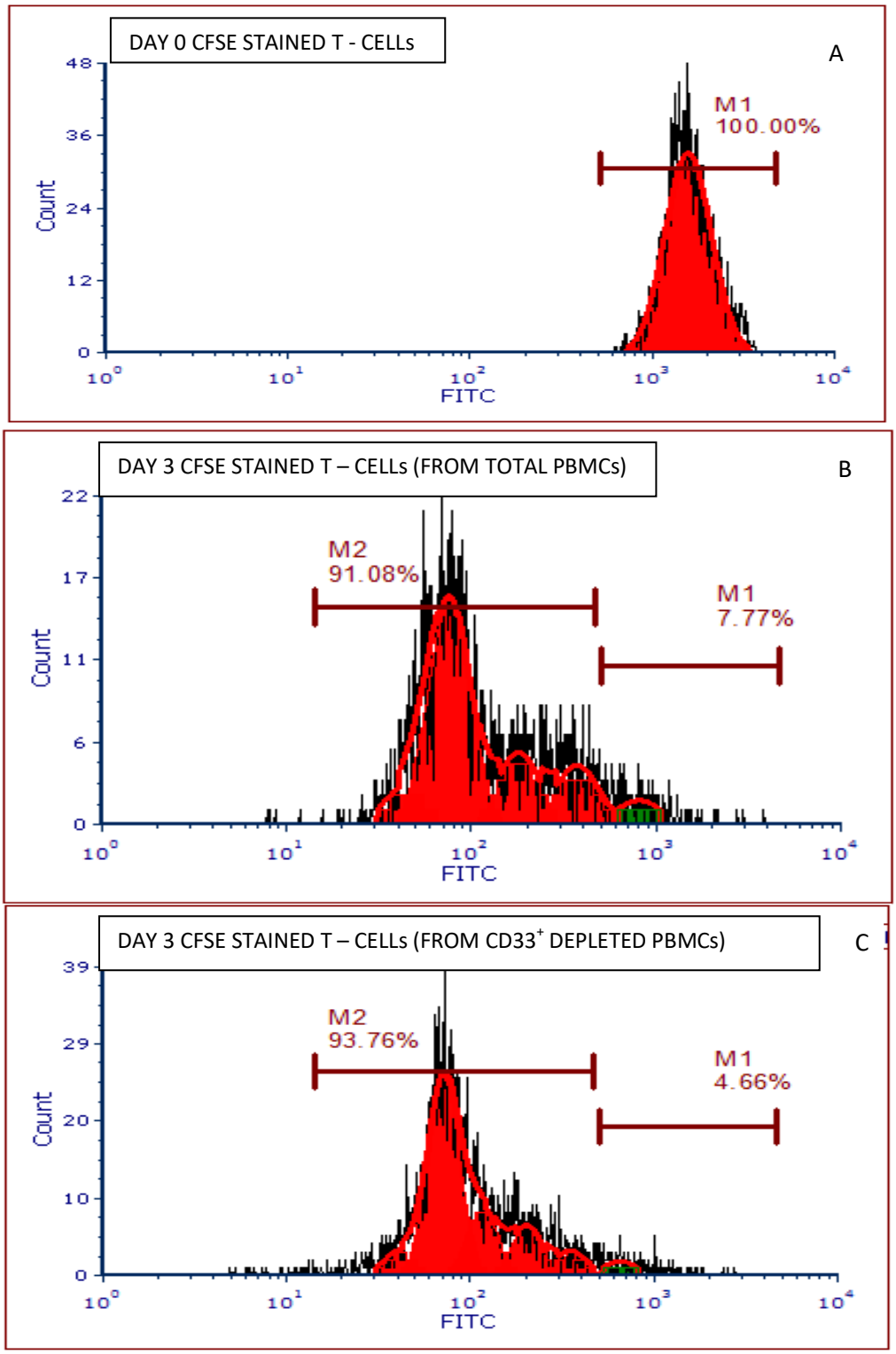

Figure 23: : A representative figure of the CLL with normal levels of immunoglobulins group's T-cell proliferation analysis – Elimination of CD33^{+} cells (MDSCs) from PBMCs (C) improves the percentages of proliferating T-cells (M2), while decreases the percentages of resting cells (M1) in comparison to the sample involving all PBMCs (B) (proliferation index (C)=5,7 and proliferation index (B)=7,3).

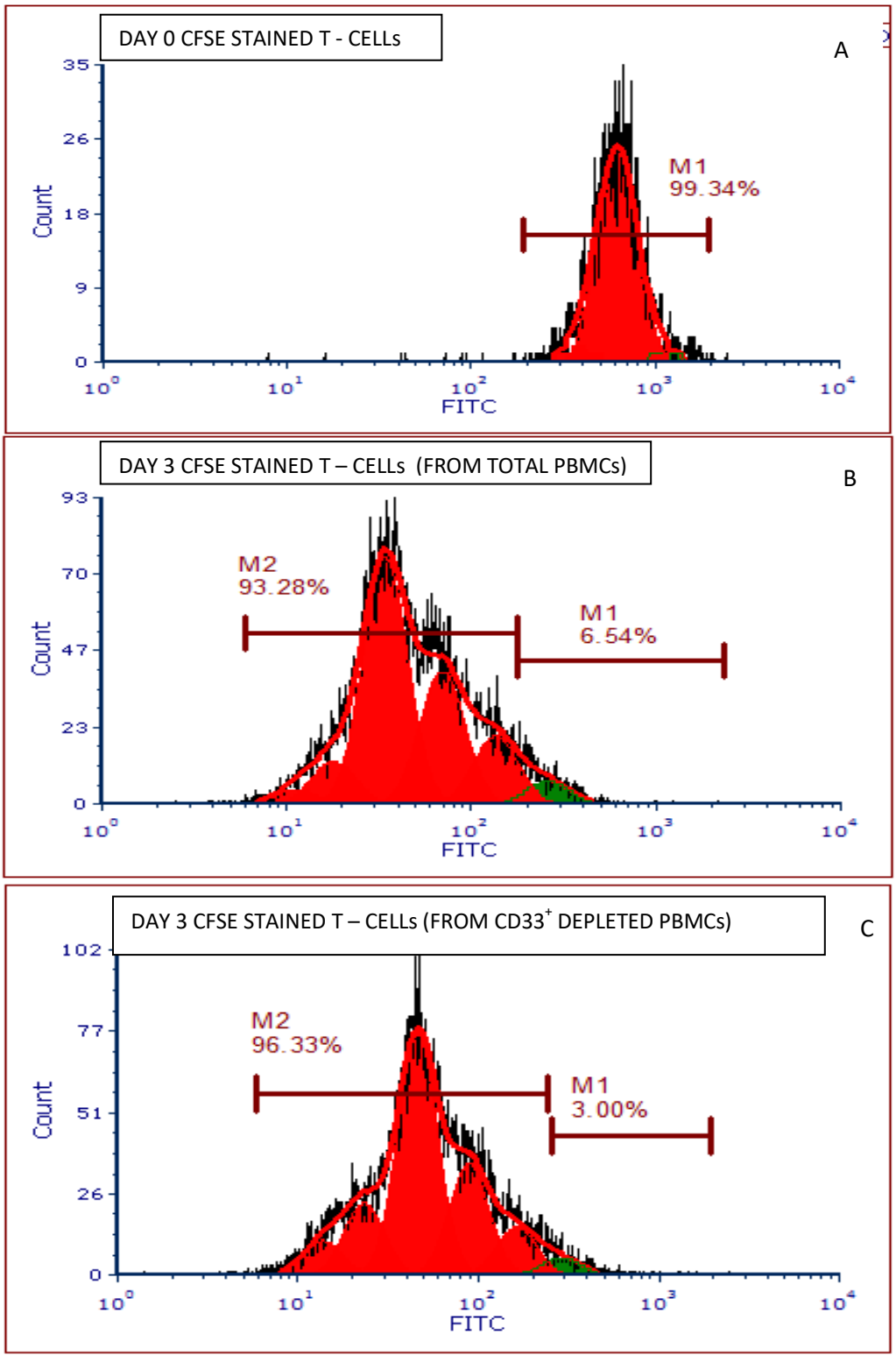

Figure 10: : A representative figure of the CLL with low levels of immunoglobulins group's T-cell proliferation analysis – Elimination of CD33^{+} cells (MDSCs) from PBMCs (C) improves the percentages of proliferating T-cells (M2), while decreases the percentages of resting cells (M1) in comparison to the sample involving all PBMCs (B) (proliferation index (C) =4,3 and proliferation index (B) = 5,5).

In more detail, the higher percentage of proliferating T-cells that was visible in the culture condition bearing $CD33^-$ cells compared to the one with total PBMCs was observed through the stronger T-cell proliferation, the existence of more cell generations and the higher cell generation peaks.

The differences in the percentages of proliferating T-cells between total and $CD33^-$ depleted PBMCs were found to be statistically significant amongst all groups (P=0.03) (Figure 25). The suppression of T-Cells in the two CLL groups was higher compared to the control group. This was clearly shown by the statistically lower differences in the percentages of proliferating T-Cells between total PBMCs and $CD33^-$ depleted PBMCs in the control group and the higher differences in the percentages of proliferating T-Cells between total PBMCs and $CD33^-$ depleted PBMCs in the CLL groups (CLL with low immunoglobulins -> T-cell proliferation in total PBMCs and in $CD33^-$ cells: 90,3% ± 2.3 and 93,3% ± 2.6 respectively, CLL with normal immunoglobulins -> T-cell proliferation in total PBMCs and in $CD33^-$ cells: 91,2% ± 3.4 and 93,9% ± 3 respectively, Control group -> T-cell proliferation in total PBMCs and in $CD33^-$ cells: 94,9% ± 1,1 and 95,9% ± 1,3 respectively).
Regarding the CLL groups, the T-Cell suppression that was observed in patients with low Ig levels was similar to the T-Cell suppression of those with normal Ig levels. This was supported by the similar difference in the T-Cell proliferation between PBMCs and $CD33^-$ depleted PBMCs in both CLL groups (Figure 25).

The mean percentage of proliferating T-cells in total PBMCs as well as in $CD33^-$ depleted PBMCs was statistically higher in the control group compared to the mean percentage of proliferating T-cells in the respective conditions in the CLL subgroups (P=0.02) (Figure 25). No statistical difference was observed in the mean percentage of proliferating T-cells in the condition of total PBMCs between CLL patients with low and normal Ig levels. The same result (non- statistically different) was acquired after comparing the mean percentage of proliferating T-cells in the condition of $CD33^-$ depleted PBMCs between CLL patients with low and normal Ig levels (Figure 26).

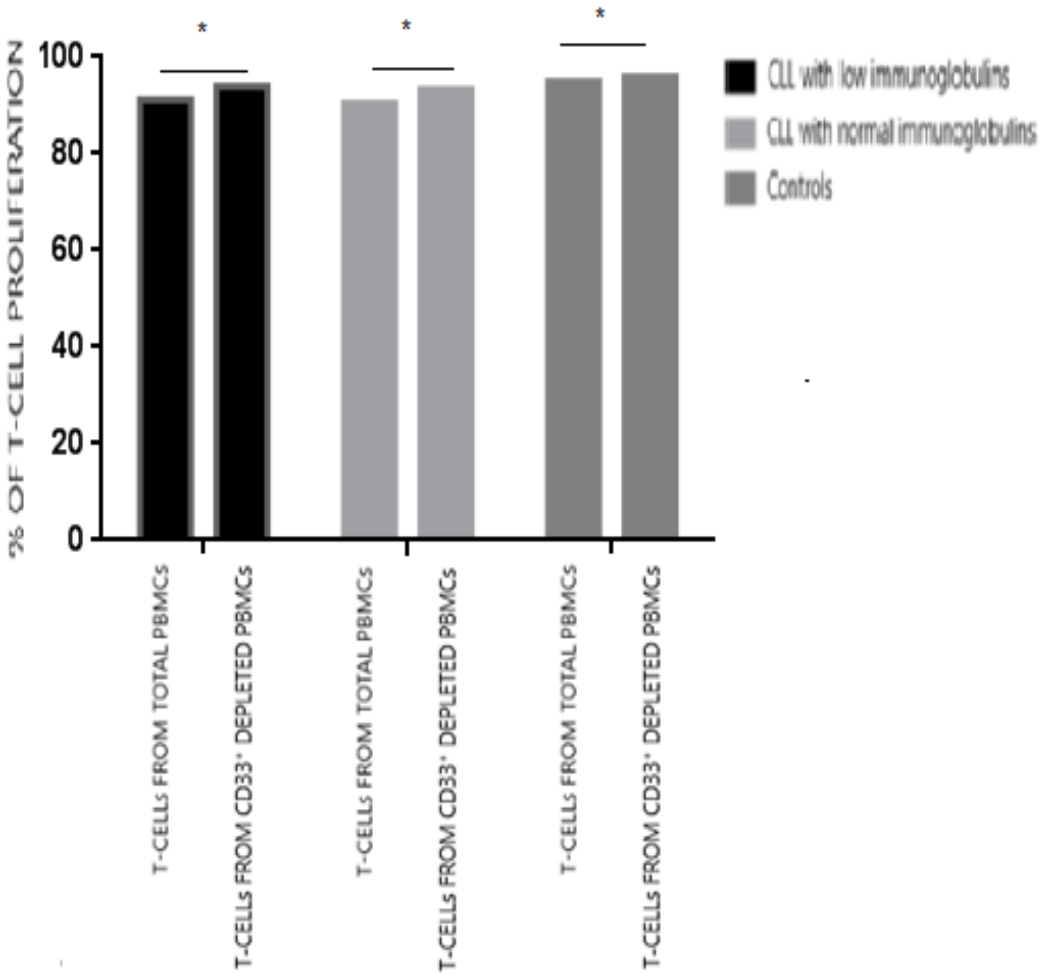

Figure 25: Comparison of the T-cell proliferation differences between total PBMCs and CD33^{+} depleted PBMCs amongst CLL patients with normal levels of immunoglobulins, CLL patients with hypogammaglobulinemia and control group. Asterisks denote the statistical significance (P=0.03) between the aforementioned groups.

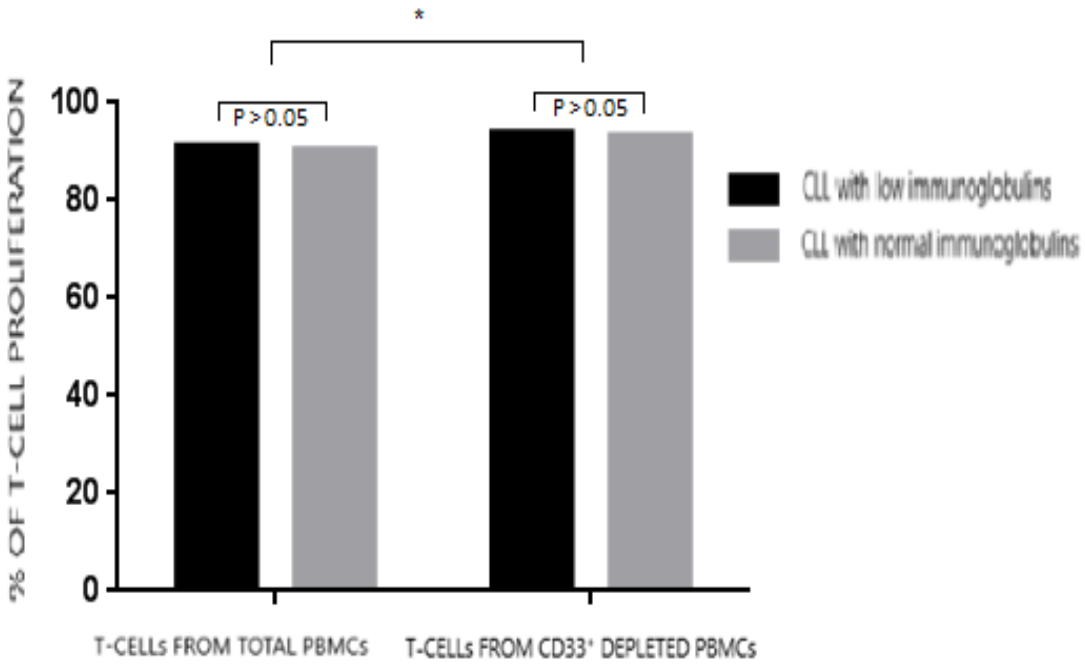

Figure 26: Comparison of the T-cell proliferation differences between total PBMCs and CD33$^+$ depleted PBMCs between CLL patients with normal levels of immunoglobulins and CLL patients with hypogammaglobulinemia. Asterisk denotes the statistical significance (P=0.03) between the aforementioned groups.

Similar results were obtained by the comparison of the T-cell proliferation index differences between total PBMCs and CD33$^+$ depleted PBMCs amongst CLL patients with normal levels of immunoglobulins, CLL patients with hypogammaglobulinemia and control group. The proliferation index is the average number of divisions that all cells have undergone after they had been stained by a cell proliferation dye (CFSE). The proliferation index was as expected higher in the CD33$^+$ depleted PBMCs condition for all groups, since the depletion of MDSCs results in a higher T-cell proliferation (P=0.005). The mean proliferation index of T-cells in the control group in both conditions (total PBMCs and CD33$^+$ depleted PBMCs) was statistically higher compared to those in the CLL groups (P=0.01) (Figure 27). No statistical difference was observed in the mean proliferation index of each condition between the two CLL groups (Figure 27).

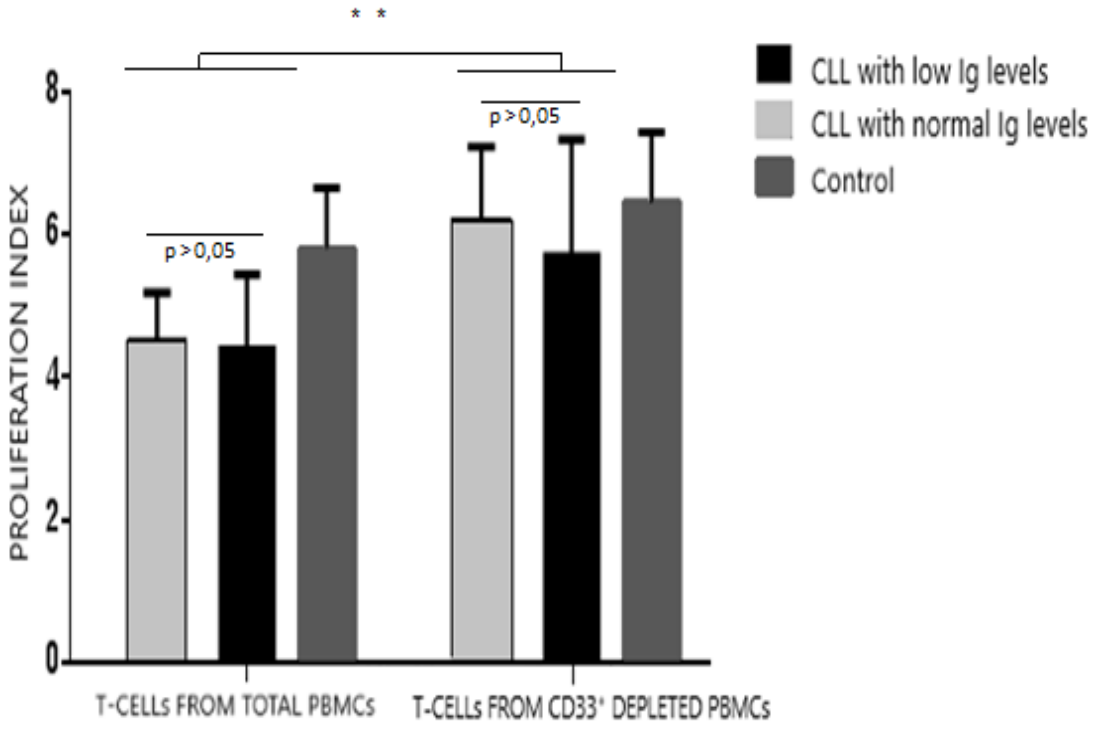

Figure 27: Comparison of the T-cell proliferation index differences between total PBMCs and CD33^{+} depleted PBMCs amongst CLL patients with normal levels of immunoglobulins, CLL patients with hypogammaglobulinemia and control group. Asterisks denote the statistical significance (P=0.005) between the aforementioned groups.

7. Discussion

In recent years, there has been a rising interest in the investigation of the contribution of MDSCs in the pathogenesis/pathophysiology of cancer and especially of hematologic diseases. This particular interest comes from the fact that MDSCs derive from the hemopoietic stem cells, which are primarily affected in many myeloid malignancies. Regarding CLL, which is a lymphoid malignancy, the involvement of MDSCs in the disease progression is mostly supported by their immunosuppressive properties rather than their participation in the malignant population (Lin et al. 2011; Jitschin et al. 2014; Betsch et al. 2018; Romano et al. 2015). Recent publications have provided strong information on the MDSCs' expansion, immunosuppressive function and involvement in the CLL's progression, but no conclusive evidence still exist on the underlying mechanisms implicated in the Ig defects that follow CLL in the majority of cases (A. D. Hamblin and T. J. Hamblin 2008; van Attekum, Eldering and Karter 2017; Liu et al. 2015; Zirlik 2014)
This study aimed to investigate the possible involvement of MDSCs in CLL-associated hypogammaglobulinemia by evaluating the proportion of (PB) PMN-MDSCs and M-MDSCs and their suppressive activity in CLL patients with and without hypogammaglobulinemia.

Our study revealed that CLL patients display significantly increased proportion of PB PMN-MDSCs and M-MDSCs compared to healthy individuals. This upregulation of MDSCs' numbers in the context of CLL was also observed in previous studies (van Attekum, Eldering, and Kater 2017; Zirlik 2014; Jitschin et al. 2018). However, we showed that this upregulation occurs in patients with both normal and low Ig levels without significant differences. Furthermore, the comparison of the two subpopulations of MDSCs in CLL, revealed that the M-MDSCs in the control group are higher in number than the PMN-MDSCs, whereas, in CLL, PMN-MDSCs are significantly upregulated compared to the M-MDSCs. This finding is also supported by other studies, which reveal that the PMN-MDSC subpopulation represents more than 80% of all MDSC in most cancer types (Gabrilovich 2018). Regarding the double positive MDSCs (HLA-DR$^{low/-}$CD11b^{+}CD33^{+}CD15^{+}CD14^{+}), their proportion in the peripheral blood of all CLL patients, regardless of Ig status, was downregulated compared to the control group. This down-regulation that was noticed does not seem to be of great importance, since this population already exists in very small numbers, and it is unlikely to have any effect on the disease. So far, the origin of CD14^{+}CD15^{+} MDSCs remains unclear, however, it is believed that they might derive from an immature precursor in the bone marrow or from CD14^{+} monocytes (Busch et al. 2014).

The upregulation and down-regulation of different MDSC subsets seems to be connected with the type of the hematological malignancy. For example, the double positive ($CD14^+CD15^+$) MDSC population has been reported to be upregulated and able to suppress $CD4^+$ and $CD8^+$ T cell proliferation in Multiple Myeloma (MM) patients, the $CD11b^+/CD14^-/CD33^+$ MDSC population was expanded in Chronic Myeloid Leukemia (CML) patients (Damuzzo et al. 2015), while the M-MDSC and PMN-MDSC subpopulations were found to be upregulated in CLL patients (Ferrer et al. 2016), with the $CD11b^+CD33^+CD14^+HLA\text{-}DR^{low}$ population being stronger reviewed.

Regarding the functional characteristics of MDSCs, it was observed that, the disparity in the percentage of T-cell proliferation between samples with $CD33^-$ depleted PBMCs and total PBMCs was significantly higher in both CLL groups, namely the ones with low and normal Ig levels (although with no statistical differences between them) as opposed to the control group that showed lower differences, indicating that depletion of MDSCs was indeed a major contributor to the higher T-cell proliferation and that in CLL, MDSCs are not only upregulated in number but are also activated and fully functional as immunosuppressors. The low difference in the T-cell proliferation between total and $CD33^-$ depleted PBMCs in the control group can be explained by the previously found small numbers of MDSCs and indicates that in normal conditions MDSCs are not strongly immunosuppressive, since there is absence of strong signals required for their activation. In addition, the higher difference in the T-cell proliferation between total and $CD33^+$ depleted PBMCs in both CLL patients with normal levels of immunoglobulins and in CLL patients with hypogammaglobulinemia that was observed in all experiments is supported by the previously found increased number of MDSCs in the blood of CLL patients and indicates that in CLL MDSCs receive strong signals that cause the activation of their immunosuppressive properties.

The presence of hypogammaglobulinemia in CLL becomes more pronounced with longer disease duration and advanced stage disease. All patients that were enrolled in the study were untreated for the disease, which means that were in the first stages of CLL, with most of them diagnosed at stage 0 or 1. As a result, no correlation between the CLL stage and the presence of hypogammaglobulinemia was revealed from our study. Also, no correlation between age and the presence of hypogammaglobulinemia was observed, which means that the reduction in the immunoglobulin levels is not likely to be connected with the age of the patient, but with the characteristics of each CLL case. However, this can only be applied to the group of people that entered the study and may not reflect CLL patients in general.

Conclusively, CLL patients with normal Ig and low Ig levels experience a similar upregulation in the number of MDSCs and a similar degree of T-cell suppression, suggesting that the hypogammaglobulinemia that is present in most CLL cases seems to be triggered or caused by other factors rather than the accumulation and suppressive activity of MDSCs. Even though it is concluded that MDSCs' expansion does not represent the main pathogenetic mechanism for the Ig defect in CLL, it is not clear if their number upregulation and function may offer a supportive role and contribute even a little in this effect. More specific experiments need to be conducted, possibly in a larger scale of participants, in order to clarify their exact role in CLL's hypogammaglobulinemia.

Our research's results contribute to the few available publications that implicate the role of MDSCs in CLL. The increase in the number as well as the immunosuppressive function of PB MDSCs' that was observed in our CLL patients is of high importance, since it can be correlated with various aspects of the disease, such as cancer progression, clinical outcome, CLL staging, treatment efficacy and can provide information on the unique characteristics of each CLL case.

MDSCs are cells with a lot of potential and can offer great insights on the status of different malignancies. In recent years, MDSCs have been recognized as important immune regulators, potential biomarkers and even therapeutic targets in cancer and other diseases (Gabrilovich 2017; Veglia et al. 2018). A few studies have already proposed their use as biomarkers of cancer progression and responsiveness to therapy, since there are findings on the close association between MDSCs accumulation and patients' clinical outcome and on their involvement in the resistance to anticancer therapies (Suzuki et al. 2005; De Sanctis et al. 2016). The latter also suggests their targeting as an attractive strategy for improving the efficacy of concurrent therapies, as well as their potential as a novel strategy treatment following their elimination (Marvel and Gabrilovich 2015; Chesney, Mitchell, and Yaddanapudi 2017). Regarding the hematologic malignancies, a number of therapies currently used, have already been reported to impact the number and function of MDSCs. To name a few, treatment with all-trans-retinoic acid (ATRA) has shown to drive MDSCs' differentiation into dendritic cells (DCs) (Kusmartsev et al. 2003) and the pyrimidine nucleoside analog gemcitabine, has effectively depleted MDSCs populations in murine models, resulting in decreased tumor growth and prolonged survival of cancer patients (Suzuki et al. 2005). Novel therapies have been targeting pathways in malignant cells that are also implicated in the regulation of MDSCs, with representative examples being the PI3K and Jak/Stat signaling pathways which are therapeutic targets in lymphomas as well as major signaling pathways in MDSCs (Trikha and Carson 2014).

Finally, despite the increasing knowledge on the biology of MDSCs, a large number of questions regarding their generation, expansion, circulation and precise role in neoplastic disorders, especially in malignant and immune-mediated hematologic disorders, need to be answered still. One of the greatest issues facing MDSCs nowadays lies in the methods of their assessment, which still need to be homogenized, since there is variability in how MDSCs have been measured and reported in different studies, which only leads to inconsistencies and erroneous conclusions (Duffy et al. 2013). Lastly, the identification of more specific and unique markers for MDSCs is of need in order for them to be better characterized.

Our future goal is to further explore MDSCs by studying their quantitative and functional characteristics in CLL patients before and after treatment, and by researching their role as prognostic markers of patients' survival.

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