UNIVERSITÀ POLITECNICA DELLE MARCHE

**DIPARTIMENTO SCIENZE DELLA VITA E DELL'AMBIENTE**

Corso di Laurea Magistrale
Biologia Molecolare e Applicata

# OTTIMIZZAZIONE DI UN PANNELLO CITOFLUORIMETRICO MULTICOLORE PER IL FENOTIPO DI CELLULE T REGOLATORIE IN EMATOLOGIA

# OPTIMISING MULTICOLOUR FLOWCITOMETRY FOR DEEP PHENOTYPING OF TREGS IN HEMATOLOGY

Tesi di Laurea Magistrale di:
Giada Marrone

Relatore:
Chiar.mo Prof.
Attilio Olivieri

Correlatore:
Shahram Yaser Kordasti

**Sessione estiva Luglio 2022**
**Anno Accademico 2021 - 2022**

***INTRODUCTION***

## *1. Human Immune System*

All the organisms have an immune system that protects them from possible foreign attacks caused by pathogens, viruses or many forms of external macromolecules (Figure 1). It can be considered as a network composed by many elements which collaborate exercising a defence against any attack. The immune system is activated to eliminate foreign antigens (known as non-self) managing to discriminate the antigens of body's own cells (known as self), in fact dysfunction of this ability to identify foreign cells from own cells can cause an autoimmune disease such as GVHD (Graft-versus-Host Disease), which will be discussed later.

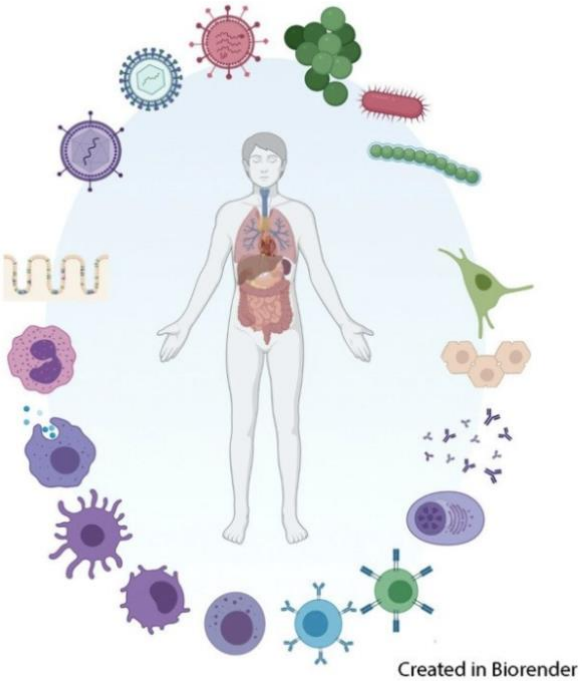


***Figure 1:*** *Human body exposed to pathogens adapted by Karolinska Institute.*

The immune system includes two types of response: the innate one and the adaptative one. Both have Humoral and cell-mediated components.

### *1.1 Innate Immune System*

The first one (also called *natural* or *native immunity*) is important in the early stages of infections before the adaptive response is established, indeed it is based on mechanisms that are already active before infection.

The innate response is regarded as a signal that stimulates the adaptive response, it can be compared to an early wake-up call; when a microbe or a damaged cell gets in touch with the organism, the innate immunity receptors recognize structures connected to the cell to be eliminated.

The main components of innate immunity are anatomical barriers which included physical, chemical and biological defences; for example, the epithelial surface covered by the skin represents a physical barrier acting against foreign organisms, in fact desquamation of the skin can remove bacteria or microbial agents.

An example for chemical barriers is enzymes such as Lysozyme secreted by saliva that as mentioned there are also biological barriers which include the genitourinary and gastrointestinal tract thanks to the commensal flora that competes with pathogens to maintain balance.

Other cells involved in the innate system include phagocytes such as macrophages, neutrophils and DC best known ad Dendritic cells; they identify the pathogens and eliminate them through a process called phagocytosis. According to it, the cells involved in this process incorporate the presumed pathogen or particle forming a structure named phagosome which is one of the components of another structure, the phagolysosome, composed by the phagosome and the lysosome; when the pathogen is included in the phagolysosome is killed by the action of enzymes.

Neutrophils and Dendritic cells are also involved in innate immune system; the first one originates from stem cells in the bone marrow and together with basophils and eosinophils form part of PMNs (polymorphonuclear cells); they play an important role because they are the first responders of inflammatory cells to migrate towards the site of inflammation to control it. Dendritic cells are involved in the innate immune system as well, acting as an intermediary with the adaptive immune system; they can be found in the external environment exposing foreign antigen, which is kept in contact with T cells of the immune system. Once activated, they interact with T and B cells in the lymph node, and they contribute to initiate the adaptative immune response.

### *1.2 Adaptive Immune System*

The Adaptive immune system can be considered as a subsystem of the immune one; this response is more specific because it is activated later in connection with the infection; it is able to recognize and respond to many microbial and non-microbial substances, defined as antigens. This immunity consists in two types: active and passive; the active immunity acts when someone is exposed to a foreign antigen, it reacts forming her own defences starting from the lymphocytes T and B.

The passive one consists in that induced from birth, inherited from the mother or passively induced through seroprophylaxis[1].

Adaptive immunity creates an immunological memory after the first reaction to a pathogen, useful for a future attack from the same pathogen, it leads a sort of specific response.

Cells that act here are white blood cells known as lymphocytes T and B, they carry out the main activities to protect an organism: antibody responses and cell-mediated immune responses. These cells seem to be identical one to another until they are activated, B cells are involved in humoral immune

[1] Seroprophylaxis: It is a prevention of disease by injection of serum containing preformed antibodies against the microrganism

response, whereas T cells are the most important cells in cell-mediated immune response.

The major functions of acquired immune system are recognition of "non-self" antigens during the antigen presentation, generation of responses to eliminate pathogens or infected cells, development of the immunological memory.

### *1.2.1 Humoral immunity*

Part of adaptive immunity is called Humoral immunity response (HIR) mediated by circulating molecules such as antibodies which recognize and help to eliminate antigens.

The antibodies involved here are produced by B lymphocytes transformed in plasma cells; they act following two different lines of defence known as active and passive. The active is based on the activity of B lymphocytes produced after stimulation by an antigen, they proliferate in effector cells producing antibodies integrating the action of T lymphocytes; after the infection, cells called "memory cells" remain acting faster in case of a new infection.

The passive one is based on the antibodies derived from another man or animal immune serum.

## *2. T cells*

T cells are one of the important white blood cells of the immune system, better known as lymphocytes which play a central role in the adaptive immune response.

T cells are born from hematopoietic stem cells, located in the bone marrow, after that they start to develop migrating to the thymus to mature, so they are called T cells. After the migration to the thymus, the precursor cells mature in different types of T cells, but the differentiation also continue after they have left this gland. They can be distinguished from other lymphocytes by the presence of a T cell receptor (TCR) on their surface. [1]

### *2.1 TCR development*

During T cell maturation is important to make a functional T cell receptor because each T lymphocytes express a different receptor on his surface allowing the immune system to recognize different types of pathogens.

It is composed by two components: the alpha and beta chains; they both contain elements designed to produce a wide variety of TCRs.

At first, T cells attempt to create a functional beta chain testing it against a mock alpha chain; then they attempt to create a functional alpha chain. Once a working TCR has been produced, T cells then must show it recognizing the

body's MHC complex[2] (positive selection) and it does not react to self-proteins (negative selection). [2]

### *2.2 T cells maturation*

Firstly, T cells are defined as Thymocyte [3] related to their maturation in the thymus; they are produced as stem cells in the bone marrow and reach the thymus with blood. The process which transforms thymocytes into mature T cells is called Thymopoiesis according to either negative or positive selection. This selection is so important to set up the thymocytes into mature T cells able to respond to pathogens keeping tolerance toward body's own antigens; the positive one selects cells able to bind MHC class I or II molecules with weak affinity, it is functional to eliminate T cells with inability to bind MHC.

Negative selection eliminates thymocytes with strong affinity for self-peptides or MHC; this is not 100% effective, so many T cells could be escape reaching the circulation but in some cases, they are stopped by autoreactive mechanisms to avoid the autoimmunity reactions.

This thymocytes are characterised by the expression of different molecules surface depending on the stage of maturation. The first thymocyte stage is the double negative one (negative for both CD4 and CD8), which can be divided

[2] MHC or major histocompatibility complex: It is a locus on the vertebrate DNA containing a set of polymorphic genes that code for cell surface proteins. [19] The function is to bind peptide fragments derived from pathogens and display them on the cell surface for recognition by the appropriate T cells [20]

into other four substages; the next phase is the double positive (positive for both CD4 and CD8). The last is a single positive stage (positive for either CD4 or CD8). [4]

There are two major types of T cells: helper T cell and cytotoxic T cell; as suggested the name the first one "helps" other cells of the immune system, whereas cytotoxic T cells kill infected cells or cancer cells. Both are characterised by the presence of a protein in their surface, CD4[3] for helper T cell and CD8 for cytotoxic T cell.

### *2.3 CD4 T cell*

CD4 helper T cells (Th) are involved in assisting other lymphocytes during their maturation for example in B lymphocytes development into plasma cells and memory B cells, or in cytotoxic T cells activation and macrophages.

CD4 primarily classification is based on their helper activity: Th1, Th2, Th17, Treg (T regulatory cells), Tfh (T follicular helper cells) with different cytokines profiles.

These cells are classified as CD4+ T cells. They are activated when they are presented with peptide antigens by MHC class II molecules which are expressed in the surface of antigen-presenting cells (APCs); once activated they

[3] CD or cluster of differentiation: It is a protocol used for the identification and investigation of cell surface molecules providing targets for immunophenotyping of cells. They can act in numerous ways, often acting as receptors or ligands important to cell.

divide rapidly and secrete cytokines that regulate or assist the immune response. [5]

They can be also identified through flow cytometry marker into four subpopulations based on the expression of CD45RA and CCR7 (CD197): CD4 Naïve (CD45RA+CCR7+), CD4 Central Memory (CM) - (CD45RA-CCR7+), CD4 Effector Memory (EM) – (CD45RA-CCR7-), CD4 T Effector Memory re-expressing RA (TEMRA) -(CD45+CCR7-).

### *2.4 CD8 T cell*

Cytotoxic T cells are the main lymphocytes that eliminate virus-infected cells, tumor cells or damaged cells. They are defined by the expression of the CD8 marker on their surface, thanks to which they can be recognized.

Cytotoxic T cells recognize their targets by binding to short peptides with MHC class I molecules, on the surface of cells. They also produce the cytokines IL-2 and IFN (gamma), these can be influencing the NK cells and macrophages. [6]

These cells have to be activated depending on interactions between molecules expressed on their surface and molecules on the surface of the antigen-presenting cell (APC); two signals are considered for the activation: the first involves an interaction between CD8 co-receptor and the class I MHC molecule to stabilize the signal, whereas the second signal has costimulators (CD80 and

CD86) for the activation and it can be assisted by cytokines released by the stimulation of T helper cells.

CD8 T cells can be also classified in the same way of CD4 after the exposition of the antigen into: CD8 Naïve, CD8 Central Memory, CD8 Effector Memory and CD8 Effector Memory re-expressing RA.

### *3. T regulatory cell*

CD4+ T cells are divided into regulatory T cells (Treg) and T helper cells. Treg cells are defined as suppressor T cells, they are a subpopulation of T cells that modulate the immune system maintaining tolerance to self-antigens and prevent autoimmune disease; they are classified as immunosuppressive cells that generally downregulate induction and proliferation of effector T cells.

Treg cells can be recognized by the expression of different biomarkers on their surface: CD4, CD25 (highly expressed) and FOXP3, they derive from the same lineage as naïve CD4, indeed it is too difficult discern them from effector CD4+. [7]

The identification of the transcriptional factor, known as forkhead box P3 (FOXP3) is essential for the development, maintenance and functionality of these cells; humans that lack functional FOXP3 can develop IPEX[4]

[4] IPEX: It is one of the autoimmune polyendocrine syndromes. It manifests with autoimmune enteropathy, psoriasiform, eczematous dermatitis, nail dystrophy; It is an attack from the body's own immune system

(immunodysregulation, polyendocrinopathy and enteropathy, X-linked syndrome) which is a severe autoimmune disease that develops early in infancy.

After activation, Naïve Treg cells differentiate into Effector, they migrate to the tumor site where they carry out the suppressive action.

### *3.1 Mechanisms of Treg cell's function*

From a functional point of view, the potential suppression of Treg cells can be divided into four way of action (figure 2): suppression by inhibitory cytokines, suppression by cytolysis, suppression by metabolic disruption and suppression by modulation of dendritic cell (DC)[5] maturation or function.

against the body's own tissues and organs. The condition indicates the loss of CD4+ CD25+ Treg cells, sequentially there is T cells activation, FOXP3 levels decrease and then Treg cells don't develop.

[5] Dendritic cells (DC): they are antigen-presenting cells (APC) of the human immune system. Their main function is to process the antigen and to present it on the cell surface to the T cells. DCs act as messengers between the innate and adaptive immune system; they are present in tissues that are in contact with the external environment, such as skin and the inner lining of nose, lungs, stomach and intestines. They can also be found in an immature state in blood. [21]

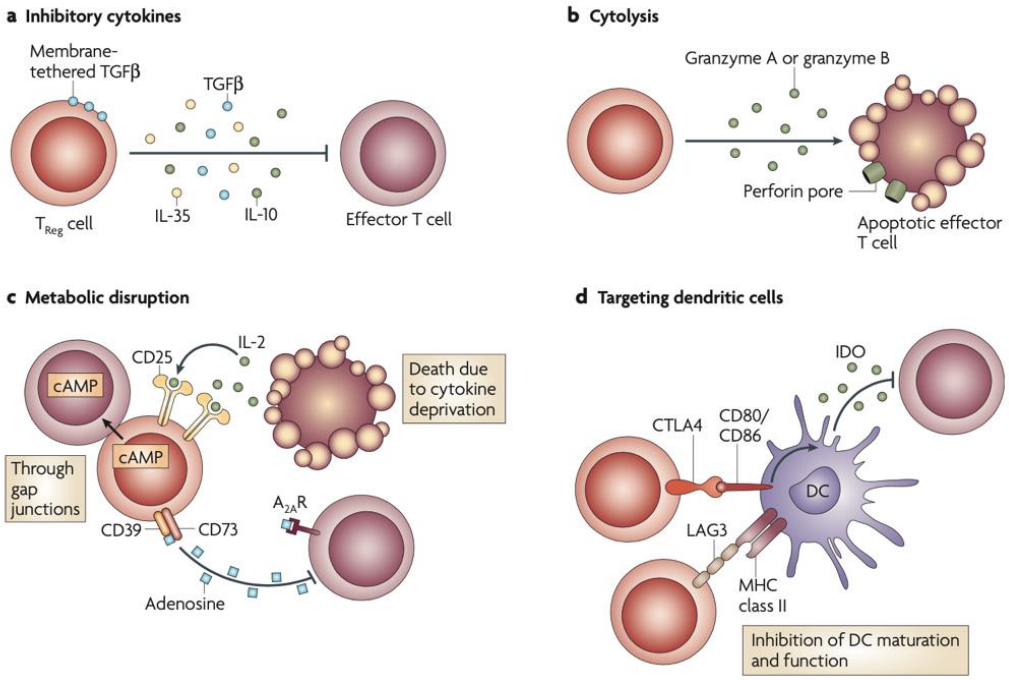


***Figure 2:*** *functional mechanisms of T regulatory cells.*

*Adapted by "How regulatory T cells work" - Nature*

*a. Suppression by inhibitory cytokines:*

Treg cells produce many inhibitory cytokines, including Transforming grow factor beta, Interleukin 35 and Interleukin 10; they also induce other cell types to express Interleukin-10. [8]

*b. Suppression by cytolysis:*

It is mediated by the secretion of Granzymes[6] A and B, especially Granzyme B which can induce apoptosis to the effector cells.

[6] Granzymes: family of serine proteases found in the cytoplasmic granules of cytotoxic T lymphocytes and natural killer cells. They bind target cells through perforin pores, cleave and activate intracellular caspases, resulting in target-cell apoptosis.

c. *Suppression by metabolic disruption:*

There are two recently proposed intracellular or extracellular mechanisms which release adenosine nucleosides with the expression of ectozymes[7] CD39 and CD73 that induce the generation of this adenosines suppressing effector T-cell through the activation of the adenosine receptor 2A. It is demonstrated that binding this 2A receptor, not only inhibit effector T cell- functionality, but also to enhance the generation of Treg cells by inhibiting IL-6 expression while promoting TGFbeta secretion. [9]

d. *Suppression by targeting Dendritic cells:*

Treg cells can also modulate the maturation and function of DCs, which are required for the activation of effector T-cells.

Regulatory T cells and Dcs develop interactions.

## 4. ***Treg in blood cancer***

The role of Treg cells seems to be fundamental in tumor progression and suppression antitumor activity [10]; most of the studies report that the main suppressive function of these cells is increased in cancer patients compared to Healthy Donor, because they are involved in the cancer development and progression by inhibiting antitumor activity. In clinical trial metastasis are the

[7] Ectozymes: enzymes that are outside of the cell membrane; they cleave extracellular substrate. These are typically tethered to the outside of the cell by a transmembrane domain.

most critical aspect of tumor progression, Treg cells in Peripheral Blood could support cancer cells during metastasis. In the early stage of the tumor development the higher number of T regulatory cells is associated with the tumor size. [11]

There are different mechanisms involved in the tumor progression: inhibition of costimulatory signals by CD80 and CD86 expressed by dendritic cells through cytotoxic T-lymphocyte antigen-4, interleukin (IL)-2 consumption by high-affinity IL-2 receptors with high CD25 (IL-2 receptor α-chain) expression, secretion of inhibitory cytokines, metabolic modulation of tryptophan and adenosine, and direct killing of effector T cells.

Many studies report that the increased percentage of these cells is related also to a poor prognosis.

### *4.1 Treg in Lymphoma*

Lymphomas is one of the blood cancers that affects the lymphatic system organs. When lymphoma grows out of the control, it can spread different parts of the body. [12] Treg cells represent an important modulator to interact between lymphomas and the host microenvironment. There are two types of lymphoma cancer:

- Non – Hodgkin's Lymphoma (NHL) with several subtypes; the most frequent are diffuse large B – cell lymphoma (DLB-CL) and follicular lymphoma (FL)
- Hodgkin's Lymphoma (HL)

The first is more common than the second.

Based on the Treg cells characteristics, there are 4 different roles involved in Lymphoma (figure 3):

1. *Suppressor Tregs:* they act the suppression of CD8+ T cell, similar in various lymphoma and myeloid malignancies
2. *Malignant Tregs:* FoxP3 as a selective marker for a subset of adult T cell leukemia/lymphoma (ATLL) and cutaneous T-cell lymphoma (CTCL) suggest that they can be also malignant
3. *Direct tumor killing Tregs:* B cells lymphoma can be target cells for Tregs suppressive cytotoxic suggesting that Tregs are also tumor cells killers
4. *Incompetent Tregs*: as reduced infiltration of Tregs, mostly rTreg in angioimmunoblastic T-cell lymphoma (AITL), contributing to the autoimmune symptoms

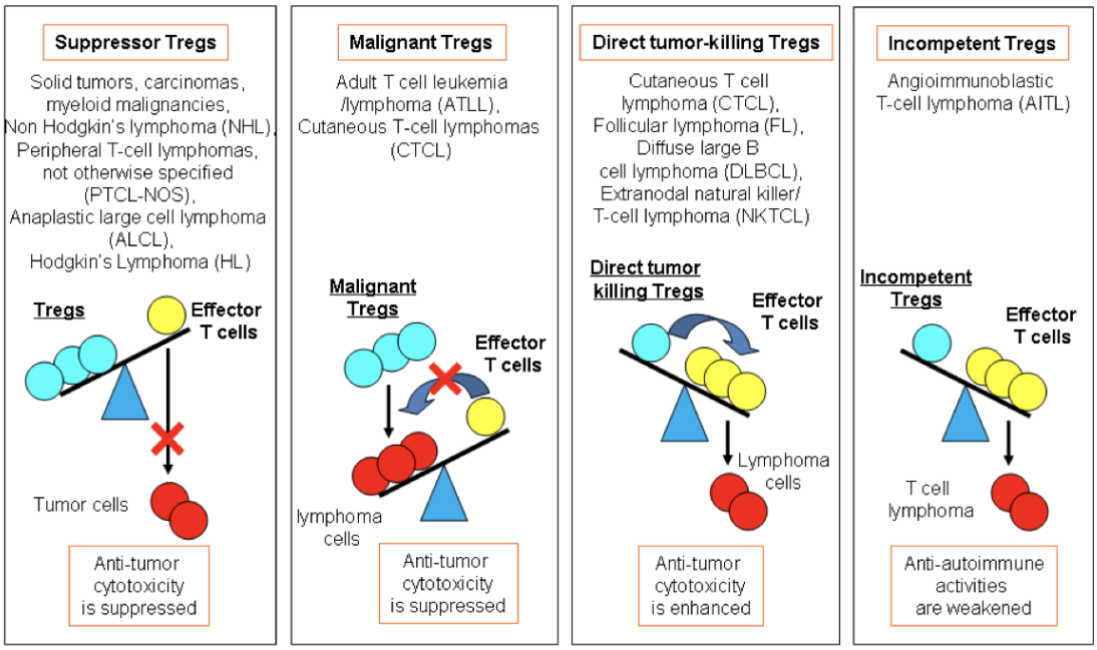


***Figure 3:*** *Four types of Treg' functions have been identified in patients with lymphoma. Adapted by Journal of Hematology and Oncology*

### *4.2 Treg in myeloid malignancies - AML (Acute myeloid Leukemia)*

AML is a common hematopoietic disease characterized by and high relapse rate and neoplastic myeloid hematopoietic precursor cells an impaired production of normal hematopoiesis.

It is a severe and aggressive disease who's clinical and laboratory picture depends on three conditions: insufficient production of mature and therefore norm functioning blood cells; infiltration of tissues and organs by leukemic

cells; finally, release of chemical mediators by tumors cells. Anemia manifests as asthenia, easy fatigability, heart palpitation and dyspnea. Platelet deficiency results in hemorrhagic manifestations while insufficient granulocyte production is reflected by increased infectious risk, mostly bacterial in nature. [13]

The first attempt to classify AML dates to the 1970s with a classification called "French – American – British (FAB). It has undergone several revisions over time, most recently in the late 1990s which were necessary include some new technologies.

FAB classification distinguishes 8 subtypes of AML (M0 to M7) based on the morphologic and cytochemical features. The M0 and M forms are the only ones in which additional immunophenotypic aspects are considered. Necessary conditions for the diagnosis of AML are:

- A percentage of blasts in the BM ≥ 30% of total mononuclear cells.
- Positivity for the enzyme myeloperoxidase (MPO) or Sudan B black (SBB).

The FAB classification has been progressively replaced by the WHO classification, emerged in 2001, to integrate diagnostic and therapeutic advances.

Different studies compared Peripheral blood and bone marrow of AML patients to healthy donor and the number of T regulatory cells is increased.

Other studies show that circulating CD4+CD25+ Treg cells were higher compared with healthy controls and complete remission (CR) patients.

### *4.3 Treg in Chronic myeloid Leukemia (CML)*

CML is a hematological disease occurs when a pluripotent stem cell is transformed in malignant one and it is subjected to myeloproliferation leading to the overproduction of mature and immature granulocytes. Its progression goes with different stages (from anorexia, weight loss to splenomegaly, pallor, fever).

Studies found that Treg cells have an increased number in this disease, demonstrating a role in the pathogenesis of this disease; CML is an immunogenic tumor, so this increased proportion may induce tolerance of T cells that target tumor cells. [14]

Additionally, a higher percentage of CD4+ CD25 high FoxP3+ cells was found in untreated CML patients compared to healthy controls.

### *4.4 Treg in Myelodysplastic Syndromes (MDS)*

Myelodysplastic syndrome is a bone marrow failure disorders where the body isn't capable to form normal blood cells in the bone marrow.

In a normal condition, BM produce:

- Red blood cells
- White blood cells
- Platelets

In a pathological situation it doesn't make enough healthy cells, instead there is a proliferation of abnormal cells not fully developed. [15] [16]

Myelodysplastic syndromes are classified using the World Health Organization (WHO) system into:

- Low risk MDS, with an International Prognostic Scoring Score ≤3.5 points; characterized mainly by anemia
- High risk MDS, with an International Prognostic Score >4.5 – 6, in this case the blast cells do not develop into normal red cells, white cells and platelets, often causing severe deficits

One of the features of MDS is the presence of autoimmune diseases of T cell mediated inhibition of hematopoiesis; Regulatory T cells play an important role in this case:

1. Expansion of Tregs may inhibit immune responses against the dysplastic clone, facilitating disease progression
2. Low numbers of Tregs could be associated with low-risk MDS

### *4.5 Treg in Myeloproliferative Neoplasms (MPNs)*

Myeloproliferative Disorders are blood cancer disease that begin with an abnormal mutation in stem cells in the bone marrow. [17] This mutation leads to hyperproliferation of different combinations of white cells, red cells and platelets, such as:

- Essential Thrombocythemia (ET), a rare blood disease where the bone marrow produces too many platelets, they could be lead a thrombus or a blood clot in vessels
- Myelofibrosis (MF), a rare disorder where abnormal blood cells and fibers build up in the bone marrow
- Polycythemia Vera (PV), a pathological condition in which too many red blood cells are made in the BM together with an elevated number of white blood cells and platelets

Studies demonstrate that the quantity of Treg cells is not different from different myeloproliferative disorders (MPD) and the normal control, but T regulatory cells dysfunction was observed in MF patients; this could explain

why some patients with MF are more inclined to develop autoimmune phenomenon.

### 5. *Treg in myeloid malignancies – Chronic Myelomonocytic Leukemia (CMML)*

Chronic Myelomonocytic Leukemia is a rare blood disease in which there are too many monocytes[8] in the blood, called "*Monocytosis*" (>1x10^9/L monocytes) in PB.

It could persist for at least three months and secondary causes must be excluded.

The World Health Organization (WHO) includes CMML in the category of "myelodysplastic/myeloproliferative disorders" (MDS/MPN). [18]

It can be divided into 3 types:

- *Type 0* – less than 2% blast cells in the blood and less than 5% blast cells in the bone marrow
- *Type 1* – 2 to 4% blast cells in the blood and 5-9% in the bone marrow
- *Type 2* – 5 to 19% blast cells in the blood and 10-19% in the bone marrow

In CMML, the bone marrow produces abnormal monocytes, not fully developed so they can't work in a normal way. Sometimes, there is also an

[8] Monocyte: a type of white blood cell

increased number of blast cells. The abnormal blood cells stay in the bone marrow, or they could be destroyed before they get into the bloodstream, so the BM could become full of these abnormal cells as the result of this overproduction.

Patients with CMML are often elderly with general symptoms such as fever, fatigue, weight loss and other symptoms depending on the type of MDS/MPN development, they might include:

- Tiredness
- Infections
- Skin rashes or lumps

Diagnosis is based on the blood count examination of BM aspirate and genetic-molecular studies. in CMML there are many gene changes; the first regarded half of all the people in the pathological condition, is the TET2 which makes a protein that controls how many monocytes the stem cells make.

Other genes involved are RAS, ASXL1 and SRSF2; many people with CMML have more than one gene change.

Juvenile CMML deserves a separate discussion. It is an aggressive clonal disorder in childhood, characterized by proliferation of monocytic and granulocytic lineages; approximately 90% of patients have somatic or germline mutations of the PTPN11, KRAS, NRAS, CBL or NF1 genes.

## *AIM*

The aim of this study is to optimize a Treg Panel for Flowcytometry to monitor myeloid malignancies, in particular Chronic Myelomonocytic Leukemia, comparing T cell and Treg subpopulation in CMML patients versus Healthy Donor through Peripheral Blood samples and Bone Marrow samples.

Phenotypic markers of this disease have been selectively studied as a starting point for future analysis.

Comparisons are based on the flowcytometry analysis between HD samples and CMML samples

## *MATERIALS AND METHODS*

### *1. Patients*

Peripheral blood (PB) samples were obtained from 11 CMML patients with a median age of 73 ± 8 (range 59- 86) *Table1*.

Bone Marrow (BM) samples were obtained from 9 patients with a median age of 78 ± 8 (range 66 –89) *Table 2*.

As negative controls, 4 PB Healthy Donor samples were used with a median age of 70 ± 8.

| Patient | Gender | Age | Diagnosis | Sample |
|---|---|---|---|---|
| 1 | M | 77 | CMML | PB |
| 2 | M | 75 | CMML | PB |
| 3 | F | 59 | CMML | PB |
| 4 | M | 72 | CMML | PB |
| 5 | M | 73 | CMML | PB |
| 6 | M | 64 | CMML | PB |
| 7 | M | 66 | CMML | PB |
| 8 | M | 74 | CMML | PB |
| 9 | F | 73 | CMML | PB |
| 10 | M | 84 | CMML | PB |
| 11 | F | 86 | CMML | PB |

***Table 1**: Peripheral blood samples from CMML patients*

| Patient | Gender | Age | Diagnosis | Sample |
|---|---|---|---|---|
| 1 | M | 72 | CMML | BM |
| 2 | F | 78 | CMML | BM |
| 3 | M | 66 | CMML | BM |
| 4 | M | 78 | CMML | BM |
| 5 | M | 81 | CMML | BM |
| 6 | M | 89 | CMML | BM |
| 7 | M | 76 | CMML | BM |
| 8 | F | 84 | CMML | BM |
| 9 | M | 77 | CMML | BM |

***Table 2**: Bone Marrow samples from CMML patients*

### *2. Peripheral blood mononuclear cell (PBMC) isolation*

PBMCs were obtained using a Ficoll-based gradient cell separation technique: 20 ml of citrate anti-coagulated blood was taken from each patient and was carefully added onto 15 ml of Ficoll Lymphosep – Lymphocyte Separation Medium in a 50 ml Falcon tube and then centrifuged at 1520 rpm for 30 minutes at room temperature, with the brake off.

The layer of PBMCs is between a layer of Plasma and a layer of Ficoll medium (Figure 4).

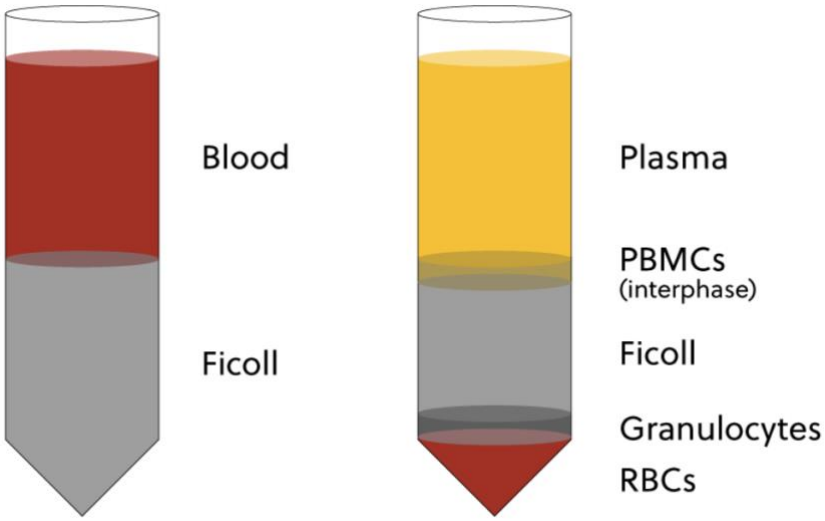


***Figure 4**: PBMC isolation*

After aspiration with sterile serological pipette, PBMCs were transferred into 50 Falcon tube, washed twice in a final volume of 30 ml using PBS (Phosphate-buffered saline) and centrifuged at 2000 g for 10 minutes (brake on).

After each wash, the supernatant was discarded, and the final cell pellet was re-suspended in 10 ml of PBS.

Cells were counted using a hemocytometer and frozen.

### *3. Cells staining*

After thawing, PBMCs were stained using kit: DURAClone IM T cell subsets and DURAClone IM Treg by Beckman Coulter Life Sciences. T cell Kit is composed by 10 monoclonal antibodies used to identify extracellular markers (Antigens) in the surface of different T cell subpopulations present in whole blood, peripheral blood and bone marrow.

Treg kit contains 8 different monoclonal antibodies in each tube allowing the possibility to discriminate different Treg cell subsets binding different surface and intracellular markers.

The instrument used to acquire the samples is the *Cytometer DxFlex* from Beckman Coulter Life Sciences.

Data are analyzed with *CytExpert* Software and *KALUZA* Software.

### 4. *T cell Panel*

| T CELL PANEL | | |
|---|---|---|
| **Specificity** | **Excitation / Emission** | **Fluorochrome** |
| CD45 | 405 nm / 528 nm | Krome Orange |
| CD3 | 633 nm / 775 nm | APC – A750 |
| CD4 | 633 nm / 650 nm | APC |
| CD57 | 405 nm / 455 nm | PB |
| CD279 (PD1) | 488 nm / 692 nm | PC5.5 |
| CD45RA | 488 nm / 523 nm | FITC |
| CD197 (CCR7) | 488 nm / 575 nm | PE |
| CD28 | 488 nm / 613 nm | ECD |
| CD27 | 488 nm / 760 nm | PC7 |
| CD8 | 633 nm / 720 nm | A700 |

***Table 3**: T cell Panel*

The markers used in the table to differentiate T cell population and subpopulation are:

- CD45 is a protein tyrosine phosphatase receptor type C, also known as PTPRC; it can be present in various isoforms on all differentiated hematopoietic cells (except erythrocytes and plasma cells). It is useful to recognize preliminarily T cell Lymphocytes.
- CD3 is a protein complex composed by distinct polypeptide chains epsilon, gamma, delta and zeta; this is one of the main features to identify T cell lineage therefore anti-CD3 antibodies can be used effectively as T cell markers.

- CD4 is a co-receptor for the TCR (T cell Receptor) widely studied together with CD3 chains and CD8 co-receptor. The CD4 antibody is useful to discriminate T – Helper cells and T – Cytotoxic cells because they both express TCR, as CD4 is specific to T-helper cells while CD8 is expressed on T-cytotoxic cells.
- CD57 antigen is commonly used to determine the "senescent cells" or terminally differentiated which reduce their capacity to proliferate and are involved in altered functionality. It is expressed on T cells and NK cells that may also show a high cytotoxic potential.
- CD279 (PD1 – Programmed Death) expression is often associated to T cell; It plays an important role by down-regulating the immune system and promoting self-tolerance suppressing T cell inflammatory activity.
- CD45RA is an isoform of CD45 (also present in another one CD45RO), is useful to define T cell subpopulations of CD4 and CD8. It is expressed in naïve as well as effector human T cell population.
- CD197 (CCR7) is another specific marker used to distinguish the subpopulation of CD4 and CD8.
- CD27 is an important T cell costimulatory receptor belonging to the TNF receptor family, together with CD28 belonging to the B7 receptor family allow identification of more or less differentiated cells.

- CD8 expression is specific for Lymphocytes T cytotoxic; CD8+ T cells recognize peptides presented by MHC Class I molecules, when they bind the antigen and become activated, they can kill cells with three different mechanisms.

### *3.1.1 Samples preparation*

PBMCs are thawed in a water bath at 37° C, then they are resuspended in FBS (Fetal Bovine Serum) and, after a wash in centrifuge for 5 minutes, there was a resuspension in PBS and then the cells are transferred to the DURAClone IM T cell tubes.

They are incubated, protected from light, for 15 minutes – during this phase the cells with the relative antigen can bind the dry Antibodies in the tubes – after another wash in the centrifuge for 5 minutes and a resuspension with PBS, the samples are ready for the acquisition.

### *3.1.2 Gating strategy*

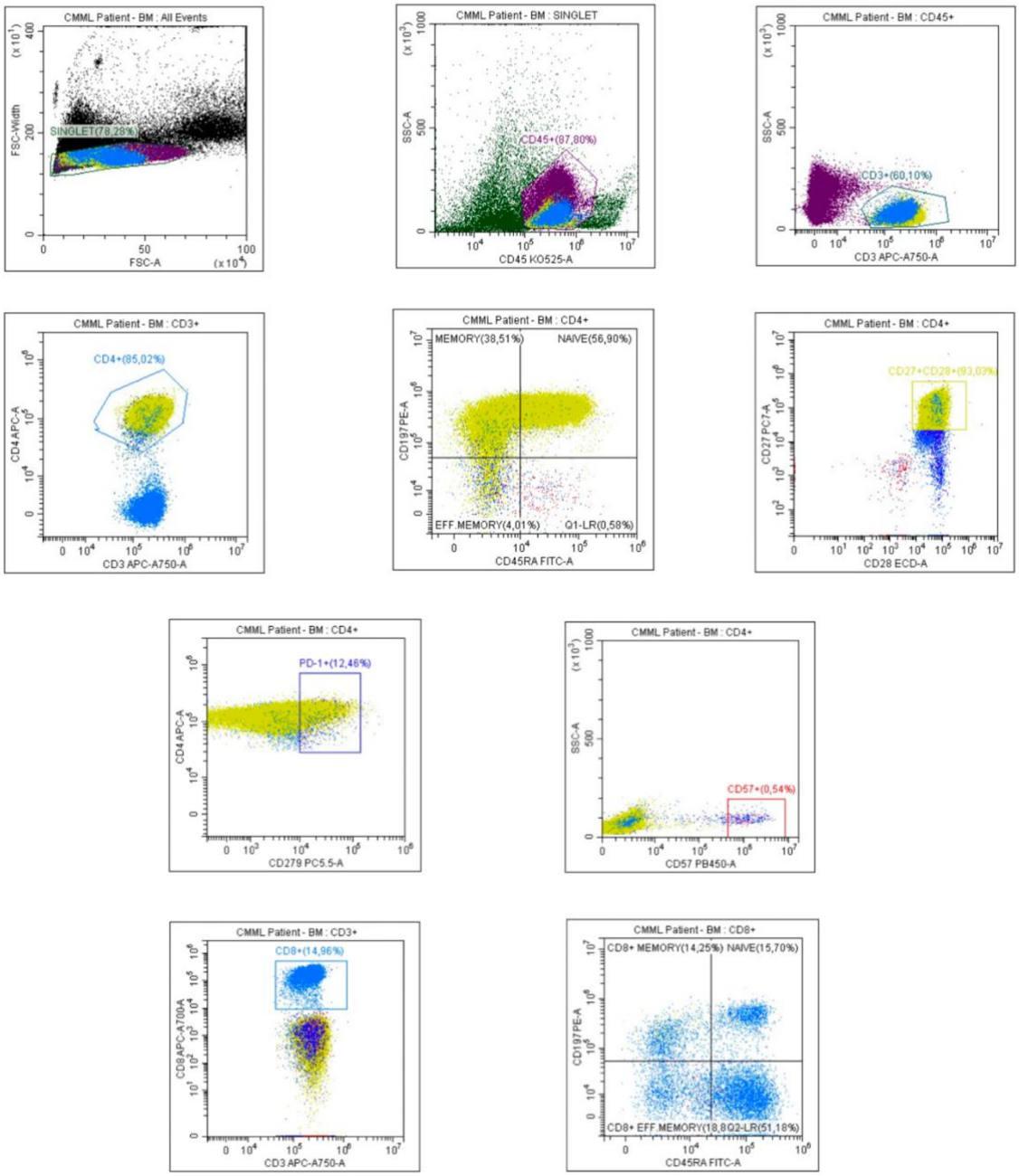


***Figure 5**: T cell gating strategy*

T cell gating strategy is based on different plots (Figure 5): the first is used to show that the analysis is fine, then is useful to discriminate “Singlets” from “Doublets”. A doublet is a *single event* that consists of 2 independent particles, so the cytometer classified these particles as a single event because they passed through the laser detection very close one to another. The gate used to distinguish these cells from the good one is called “Singlets” obtained between SSC and FSC – side scatter and forward scatter - so the Analysis goes on based on the singlets. Now T cells are recognized based on the expression of CD45 (CD45 +), after that it is important to determine CD3+ and then CD4+ and relative subsets with two different markers CD197 (CCR7) and CD45RA: CCR7+ CD45RA+ are CD4 Naïve; CCR7+ CD45RA- are CD4 T cell Memory; CCR7- CD45RA+ are CD4 T cell Effector. Using the other two markers CD27 and CD28 it can be possible to study the stage of differentiation and activation of T cells. The analysis ends with the study of CD8 T cells, and their subpopulations identified with the same markers used for CD4 T cells, CCR7 and CD45RA: CCR7+ CD45RA+ are CD8 Naïve; CCR7+ CD45RA- are CD8 T cells Memory; CCR7- CD45RA+ are CD8 T cell Effector.

### *3.2 Treg Panel*

The markers used in these tubes are involved in the identification and proliferation stages of T regulatory cells. The kit includes two differents tubes because one contains surface antibodies and the other one is useful for the intracellular staining (FoxP3 and Helios).

| Treg PANEL | | |
|---|---|---|
| **Specificity** | **Excitation / Emission** | **Fluorochrome** |
| **CD3** | 633 nm / 783 nm | APC |
| **CD45RA** | 468-509 nm / 504-541 nm | FITC |
| **CD45** | 405 nm / 528 nm | Krome Orange |
| **CD4** | 486 nm / 580 nm | PC7 |
| **CD25** | 488 nm / 575 nm | PE |
| **CD39** | 488 nm / 692 nm | PC 5.5 |
| **FoxP3** | 633-638 nm / 665 nm | Alexa Fluor647 |
| **Helios** | 405 nm / 455 nm | Pacific Blue |

***Table 4**: Treg Panel*

The Table 4 shows specific marker to study T regulatory cells including the other shared with T cell Panel:

- CD25 is the alpha chain of IL2 receptor, it is one of the hallmarks used to identify Treg cells
- CD39 is an ectozyme with a suppressive function by its ATPase activity important to convert pro-inflammatory extracellular ATP to adenosine – a potent immunosuppressive factor
- FoxP3 or forkhead box P3 is a transcription factor expressed by Treg cells, it is considered one of their main markers
- Helios is a member of Ikaros family useful to distinguish naturally thymic-derived Tregs from those peripherally induced from naïve CD4+ T cells.

### *3.2.1 Sample preparation*

Cells are thawed in a water bath at 37° C, after a wash in centrifuge with PBS and 2% FBS, the pellet is resuspended in FBS and added to the tube 1.

Then the cells are washed another time with PBS and treated with Fixative Reagent and later with Permeabilizing Buffer to prepare cells for being transferred to the tube 2. Cells are now washed twice and ready for the acquisition.

### *3.2.2 Gating strategy*

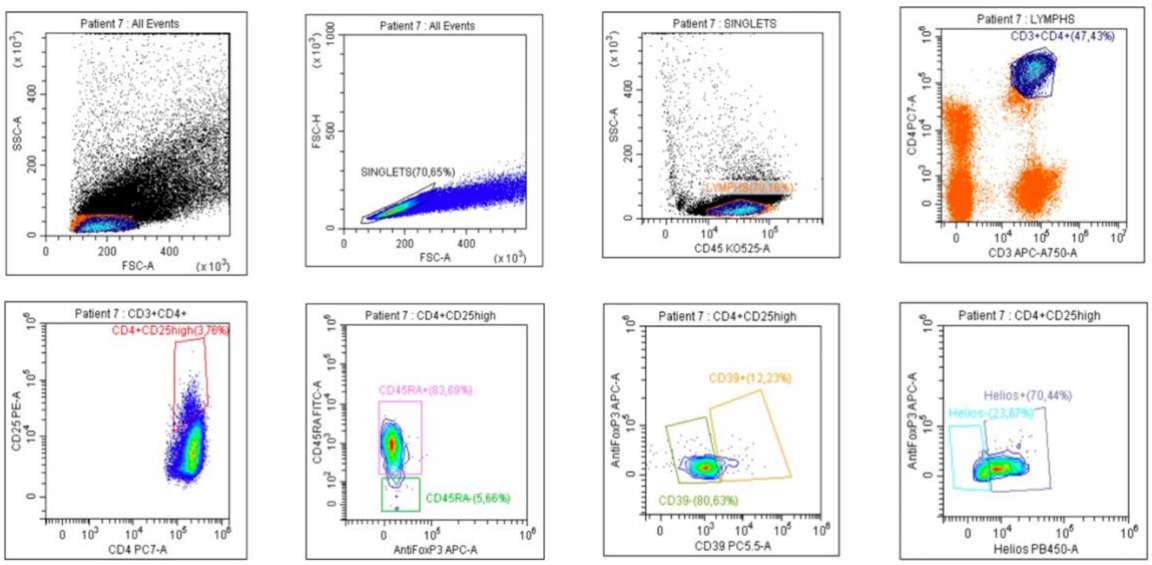


***Figure 6:*** *Treg gating strategy*

The analysis (Figure 6) starts with the "singlets" gate, followed by Lymphs CD45+.

Now it is important to determine CD3+CD4+ T cells to continue the identification of T regulatory cells with the phenotype CD4+CD25high and FoxP3+.

The following markers were used to identify Treg subpopulation: CD45RA, CD39 and Helios.

## *RESULTS*

### *1. T cell results*

11 PB samples were compared with 9 BM samples, to find any differences between T cells and Treg subsets in HD and CMML samples.

T Lymphocytes were studied from the differentiation and depletion point of view, based on the analysis of the subpopulation of CD4 and CD8.

Results are analyzed with T Test.

The values are standardized with a P value between 0,01 and 0,05 (Figure 7)

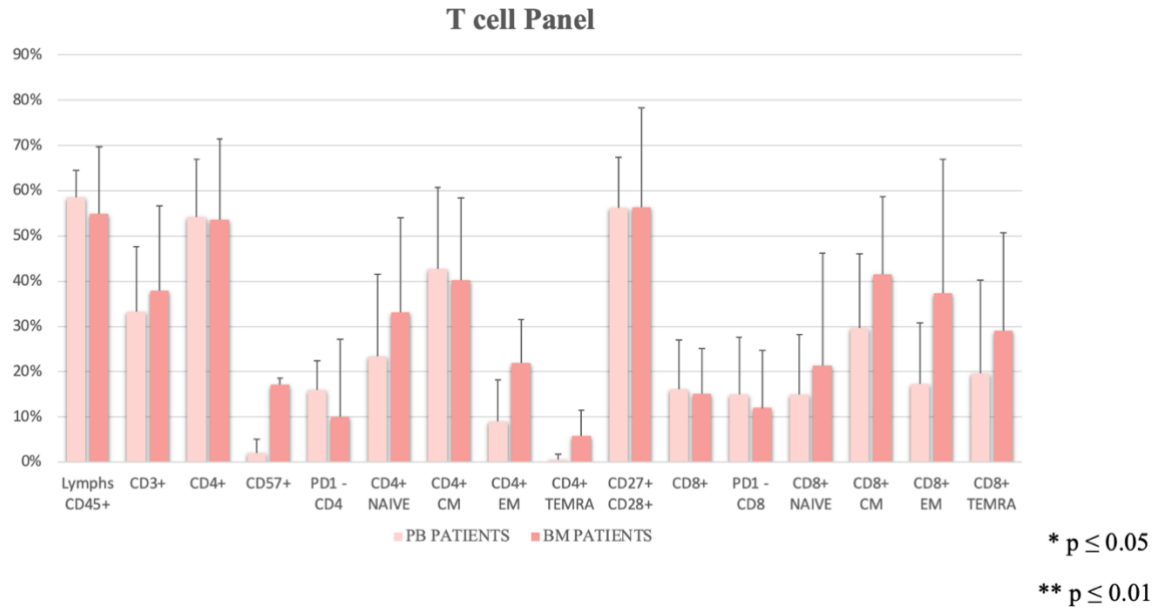


***Figure** 7: Results of the comparison between PB of CMML patients and BM of CMML patients, based on the T cell subsets.*

As figure 1 showed, there is no statistically significant difference between Peripheral Blood and Bone Marrow analyzing T cell and subpopulation through their specific antigen markers.

The same comparison done between PB of patients and PB of 4 Healthy Donor (Figure 8).

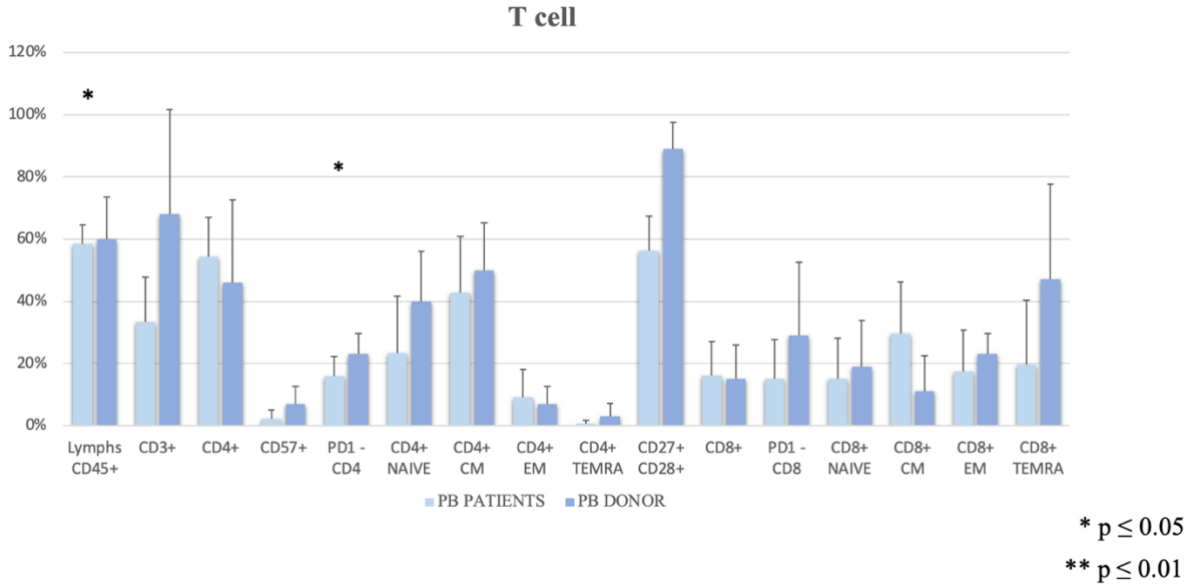


***Figure 8**: comparison between PB of CMML samples and PB of HD samples, through the T cell subsets.*

Results showed that there are significantly differences in CD45+ lymphocytes *(p value < 0.01)* with a higher level in HD samples and in the

PD1 marker *(p value: 0,027)* for CD4, by indicating that CD4 T cells are less exhausted in patients compared to HD.

### 2. *Treg results*

Samples of PB and BM from CMML patients are analyzed based on T regulatory cells and the subpopulation, to find differences between them.

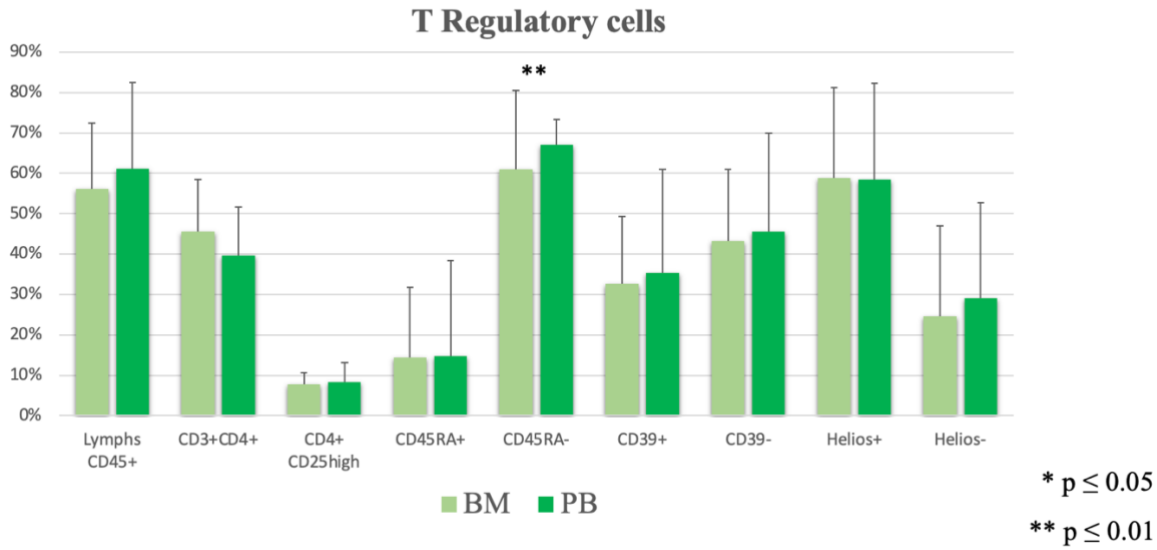


***Figure 9:*** *Comparison between Treg subsets in Peripheral Blood and Bone Marrow CMML samples*

Comparing PB CMML samples and BM CMML samples (Figure 9), there is no statistically difference between these 2 groups in terms of the number of CD45RA+ Tregs.

However, CD45RA- Tregs are increased in PB samples and decreased in BM samples *(p value <0,01)*.

There aren't other significant statistically differences in the number of Treg cells.

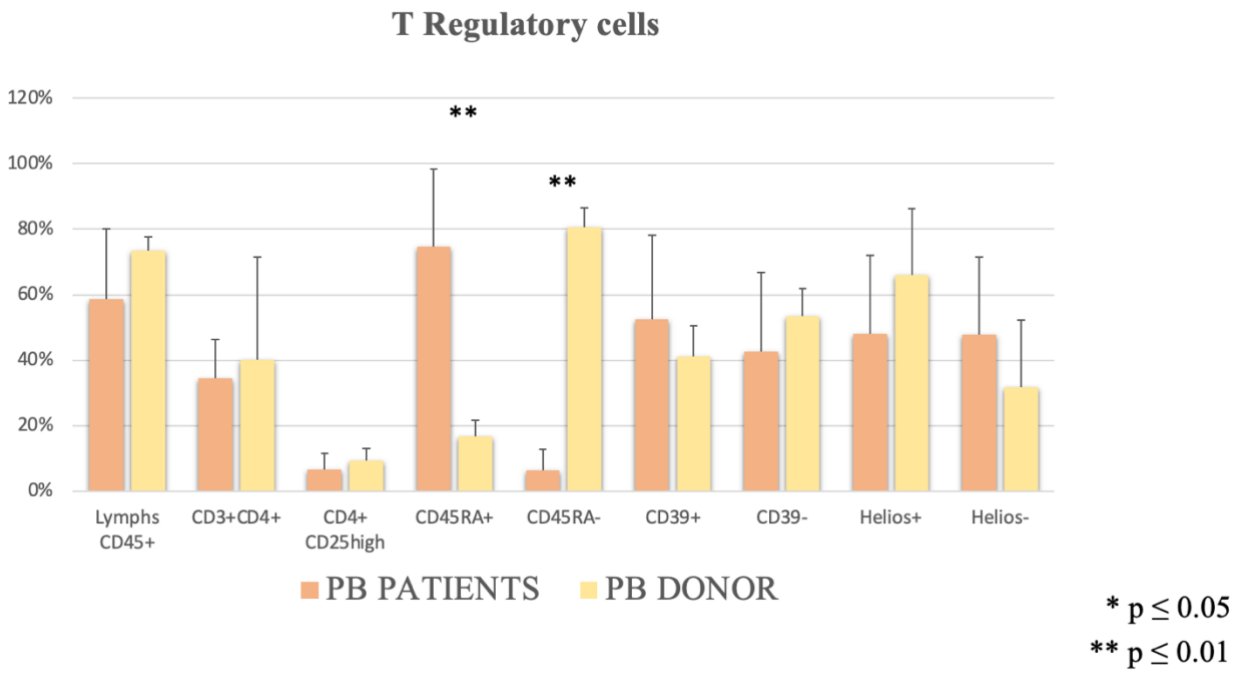


***Figure 10:*** *comparison between PB of CMML patients and PB of HD, analyzing Treg subsets.*

The analysis between PB CMML patients and PB of HD (Figure 10) show a statistically significant difference in CD45RA+ and CD45RA- Treg subsets.

The population of CD45RA+ is increased in PB of patients compared to HD (*p value < 0.01).*

CD45RA- Treg subset are reduced in the PB of patients compared to HDs *(p value <0.01).*

## *DISCUSSION*

The aim of this project was to optimize multicolor flowcytometry panels to investigate the number of Tregs and T cells and their subsets. The study involved patients with Chronic Myelomonocytic Leukemia, a hematopoietic stem cell disorder with features of MDS/MPN disorders.

To better study the hematological response in this disorder, the attention is focused on the T helper (CD4) and cytotoxic cells (CD8) that mediate immune response during an inflammatory pathway.

These different responses are analyzed with flowcytometry monitoring the expression of various specific markers.

Firstly, peripheral blood samples are compared with Bone Marrow samples of patients analyzing T cell subsets of CD4+ and CD8+ and there wasn't statistically significant data that show differences between these two types of samples.

This comparison also involved Treg cells and related subsets (CD45RA+ Naïve Treg cells, CD45RA- Memory Treg cells).

In the Bone Marrow samples the number of Treg cells is lower than in the peripheral blood.

The study continues with another comparison between peripheral blood of CMML patients and Peripheral Blood of healthy donor analyzing T cell subset in relation of their proliferation and differentiation; data are not statistically significant except for Lymphs CD45+ which are higher in the HD.

The *Programmed Death 1 marker (PD1)* indicated that CD4 T cells are less exhausted in patients compared to healthy donor.

Treg cells response between peripheral blood of CMML samples and healthy donor showed an increased level of CD45RA+ Treg in patients indicating the presence of many Naïve Treg cells.

The comparison shows that CD45RA- Tregs (Memory phenotype) are reduced in the peripheral blood of patients with CMML which suggests they were exposed to an inflammatory environment as shown before (Kordasti et al, Blood, 2016).

**Future studies** could be extended analyzing Bone Marrow samples from healthy donor according to the Ethical Committee, then they could be compared to the Bone Marrow samples of CMML patients by finding differences in T cell immune response.

# Bibliography


[1] J. A. L. J. R. M. R. k. W. P. Alberts B, T cells and B cells derive their names from the organs in which they develop. T cells develop [mature] in the thymus, and B cells, in mammals, develop [mature] in the bone marrow in adults or the liver in fetuses., New York: Garland Science, 2002.

[2] C. Janeway, Immunobiology, Garland Science, 2012.

[3] thymocyte, Dorland's Medical Dictionary.

[4] R. S. Mitchell, V. Kumar, A. K. Abbas and N. Fausto, Robbins Basic Pathology, Philadelphia: Saunders, 2007.

[5] B. B. Gutcher I, APC-derived cytokines and T cell polarization in autoimmune inflammation, 2007.

[6] A. S. AN, "Lymphocytes". Advanced Hematology in Integrated Cardiovascular Chinese Medicine, Elsevier, 2020.

[7] C. W, "Tregs in immunotherapy: opportunities and challenges", August 2011.

[8] W. C. K. T. B. K. W. Y. V. K. C. R. S. D. B. R. V. D. Collison LW, "The inhibitory cytokine IL-35 contributes to regulatory T-cell function", November 2007.

[9] P. E. e. a. Zarek, A2A receptor signaling promotes peripheral tolerance by inducing T-cell anergy and the generation of adaptive regulatory T cells, 2008.

[10] H. N. Yoshihiro Ohue, Regulatory T (Treg) cells in cancer: Can Treg cells be a new therapeutic target?, 2019.

[11] C. X. Y. L. C. Z. Z. Z. L. C. Y. W. Ruihua Fang, Significance of peripheral blood Tregs in tumor: a narrative review, 2020.

[12] J. W. a. X.-Y. Ke, The Four types of Tregs in malignant lymphomas, 2011.

[13] C. Z. T. T. T. Z. R. W. F. H. C. Z. M. H. a. D. M. Min Wang, Increased Regulatory T Cells in Peripheral Blood of Acute Myeloid Leukemia Patients Rely on Tumor Necrosis Factor (TNF)-α–TNF Receptor-2 Pathway, June, 2018.

[14] H. B. A. I. Asman M. Zabran, Prognostic value of regulatory T cells in newly diagnosed chronic myeloid leukemia patients, 2013.

[15] W. I. J. H. D. D. L. B. B. A. G. L. M. W. W. J. P. M. F. F. G. J. M. Shahram Y Kordasti, CD4+CD25high Foxp3+ regulatory T cells in myelodysplastic syndrome (MDS), 2007.

[16] A. Giagounidis, Current treatment algorithm for the management of lower-risk MDS, 2017.

[17] M. P. C. C. M. P. D. A. S. M. M. B. M. M. M. Y. S. Y. S. M. P. D. J.-C. W. M. Shams A Shakil, T Regulator Cells (Treg) In Patients with Myelofibrosis, 2010.

[18] E. S. a. R. Itzykson, How I treat chronic myelomonocytic leukemia, 2017.

[19] H. P, Notes on the Dr Snell's observations concerning the H-2 locus polymorphism, 1970.

[20] T. P. W. M. e. a. Janeway CA Jr, Immunobiology: The Immune System disease, New York: Garland Science, 2001.

[21] R. M. Steinman and Z. A. Cohn, "Identification of a Novel Cell Type in Peripheral Lymphoid Organs of Mice: I. Morphology, Quantification, Tissue Distribution, The Journal of Experimental Medicine, 1973.

[22] H. N. Yoshihiro Ohue, "Regulatory T (Treg) cells in cancer: Can Treg cells be a new therapeutic target?," 2019. [Online].