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Departamento de Química

Cátia Filipa Marques Rodrigues

Análise de células supressoras de linhagem mieloide (MDSC) e o seu papel no Imunograma de doentes oncológicos

Myeloid-derived suppressor cells (MDSC) analysis and their role in the Immunogram of cancer patients

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção de grau de Mestre em Bioquímica, realizada sob a orientação científica do Doutor Lúcio Lara Santos, Professor de Oncologia Cirúrgica e responsável pelo grupo de Patologia e Terapêutica Experimental do Departamento de Oncologia Cirúrgica do Instituto Português de Oncologia do Porto, Francisco Gentil E.P.E, do Doutor Bruno Miguel Rodrigues das Neves, Professor Auxiliar do Departamento de Ciências Médicas da Universidade de Aveiro e da Doutora Iola Melissa Fernandes Duarte, Investigadora Principal do CICECO – Instituto de Materiais de Aveiro, Departamento de Química da Universidade de Aveiro.

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Dedico esta dissertação à minha família.

o júri

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palavras-chave

Cancro, sistema imune, células supressoras de linhagem mieloide, imunoterapia, imunograma, citometria de fluxo

resumo

O cancro é uma das principais causas de morte em todo o mundo e inúmeros esforços têm sido realizados ao longo do tempo para melhor compreender a complexidade da doença neoplásica. O sistema imune desempenha um papel fundamental, tanto no reconhecimento e controle de tumores, como no seu desenvolvimento e progressão. Este duplo papel desempenhado pelo sistema imune designa-se por imunoedição tumoral e consiste em três fases – eliminação, equilíbrio e escape. Como uma das estratégias de escape ao sistema imune, os tumores induzem no seu microambiente a formação de células supressoras de linhagem mieloide (MDSC), células que possuem uma potente capacidade imunossupressora e que permitem o desenvolvimento e progressão da doença.

Nos últimos anos, a imunoterapia tem ganho grande relevância como estratégia de tratamento antitumoral. No entanto, uma das suas principais limitações reside na grande variabilidade de resposta de doente para doente, atendendo a que depende da condição e competência do sistema imune de cada indivíduo. Com o intuito de avaliar a potencial resposta à imunoterapia foi estabelecido um painel composto por sete classes de parâmetros designados no seu conjunto de imunograma, sendo que um deles é a avaliação das MDSC.

Neste contexto, o presente trabalho teve como objetivo a identificação, quantificação e o estudo da função das MDSC, com vista à posterior integração desta avaliação no imunograma de doentes oncológicos, a realizar no Serviço de Imunologia do IPO-Porto. A identificação e quantificação das MDSC do sangue foi realizada por citometria de fluxo, recorrendo a um painel multiparamétrico a 8 cores e utilizando amostras de sangue periférico total de 31 doentes oncológicos e de 12 dadores saudáveis para controlo. O grupo dos doentes apresentou maiores percentagens de ambos os subgrupos de MDSC (cerca de 12 vezes superiores no caso das PMN-MDSC e cerca de 1.1 vezes superiores no caso das M-MDSC), em relação ao grupo de dadores saudáveis. Para o estudo da função das MDSC foram avaliados por qPCR, após separação celular (*fluorescence-activated cell sorting* (FACS)) das PMN-MDSC e M-MDSC de 3 doentes oncológicos, os níveis transcricionais de *NOS2* e de *TGF-β*, duas moléculas características destas populações imunossupressoras. Foi também avaliada a expressão da cadeia zeta (CD247) nos linfócitos T e nas células NK, recorrendo a amostras de sangue periférico total de 10 doentes oncológicos e 7 dadores saudáveis, tendo sido obtidos rácios de intensidade

média de fluorescência (MFI) da expressão de CD247 menores no grupo dos doentes oncológicos para ambas as células T e NK. Assim sendo, foi comprovado que as MDSC estão presentes em maiores quantidades nos doentes oncológicos, nomeadamente naqueles cuja doença se encontra avançada ou em recaída, e que possuem características imunossupressoras. Uma vez que, indivíduos saudáveis possuem apenas quantidades vestigiais de MDSC, estas células podem ser utilizadas como alvo terapêutico sem possíveis efeitos secundários, particularmente em terapia combinada com inibidores de checkpoint imunológicos.

keywords

Cancer, immune system, myeloid-derived suppressor cells, immunotherapy, immunogram, flow cytometry

abstract

Cancer is one of the main causes of death worldwide and multiple efforts have been conducted over time to better understand the complexities of neoplastic disease. The immune system plays a fundamental role, not only in the recognition and control of tumors, but also in their development and progression. This dual role of the immune system is termed cancer immunoediting and consists of three phases – elimination, equilibrium and escape – the last one being the most thoroughly discussed in this work. As an escape mechanism to the immune system, tumors induce in their microenvironment the formation of myeloid-derived suppressor cells (MDSC), which are cells that have potent immunosuppressive activity and promote the development and progression of the disease.

In the last years, immunotherapy has gained great relevance as an antitumor treatment strategy. However, one of its main limitations is the large variability of patient-to-patient response, as it depends on the condition and competence of each individual's immune system. In order to evaluate the potential response to immunotherapy, a panel of seven classes of parameters, collectively called the cancer immunogram, was established, which includes the evaluation of MDSC.

In this context, the aim of the present work was to identify, quantify and assess MDSC function, with a view to integrate this evaluation in the cancer immunogram of oncological patients, which is under development in the Immunology Service of the IPO-Porto. The identification and quantification of blood MDSC was performed by flow cytometry, using an 8-color multiparametric panel and using samples of peripheral whole blood from 31 cancer patients and, as control, from 12 healthy donors. Higher percentages were obtained in the group of cancer patients for both subsets of MDSC (about 12 times higher for PMN-MDSC and about 1.1 times higher for M-MDSC), comparatively to the group of healthy donors. For the study of the MDSC function, following cell sorting (*fluorescence-activated cell sorting* (FACS)) of the PMN-MDSC and M-MDSC from 3 cancer patients, qPCR was employed to determine the transcriptional levels of *NOS2* and *TGF-β*, two characteristic molecules of these immunosuppressive populations. It was also evaluated the expression of the zeta chain (CD247) in both T lymphocytes and NK cells using peripheral whole blood samples from 10 cancer patients and from 7 healthy donors, and lower mean fluorescence intensity (MFI) ratios of expression of CD247 were obtained for both T- and NK-cells. Therefore,

it was shown that MDSC are present in higher levels in cancer patients, particularly in those with advanced state or relapsed disease and that these cells have immunosuppressive activity. Since healthy individuals have only trace amounts of MDSC, these cells can be used as a therapeutic target without possible side effects, particularly in combination therapy with immune checkpoint inhibitors.

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List of Abbreviations and Acronyms

APC	Antigen-Presenting Cell
ARG-1	Arginase-1
ATRA	All-Trans Retinoic Acid
BSA	Bovine Serum Albumin
CAR	Chimeric Antigen Receptor
CC	Colon Carcinoma
CCL2	C-C Motif Chemokine Ligand 2
cGMP	Cyclic Guanosine Monophosphate
COX	Cyclooxygenase
C-PTIO	Carboxy-2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide
CRC	Colorectal Cancer
CRP	C-Reactive Protein
CRS	Cytokine Release Syndrome
CTL	$CD8^+$ Cytotoxic T Cell
CTLA-4	Cytotoxic T Lymphocyte-associated Antigen-4
CXCL	C-X-C Motif Ligand
DC	Dendritic Cell
dH_2O	Distilled Water
DLBCL	Diffuse Large B-Cell Lymphoma
EDTA	Ethylenediamine Tetraacetic Acid
e-MDSC	Early-Stage Myeloid-Derived Suppressor Cell
FACS	Fluorescence-Activated Cell Sorting
FC	Flow Cytometry
FDA	Food and Drug Administration
FMO	Fluorescence Minus One
FOXP3	Forkhead Box Protein 3
GM-CSF	Granulocyte/Monocyte Colony Stimulating Factor
HSC	Hematopoietic Stem Cell
IAP	Integrin-Associated Protein

IARC	International Agency for Cancer Investigation
IDO1	Indolamine 2,3-dioxygenase-1
IFN-γ	Interferon-Gamma
IL	Interleukin
IMC	Immature Myeloid Cell
iNOS/NOS2	Inducible Nitric Oxid Synthase
irAE	Immune-Related Adverse Event
ITAM	Immunoreceptor Tyrosine-Based Activation Motif
ITIM	Immunoreceptor Tyrosine-Based Inhibition Motif
iT_{reg}	Inducible or Adaptive Regulatory T cell
LDH	Lactate Dehydrogenase
L-NMMA	NG-Methyl-L-Arginie
LOX-1	Lectin-Type Oxidized LDL Receptor 1
mAb	Monoclonal Antibody
M-CSF	Macrophage Colony-Stimulating Factor
MDSC	Myeloid-Derived Suppressor Cell
MFI	Mean Fluorescence Intensity
MHC-I	Major Histocompatibility Complex I
MM	Multiple Myeloma
M-MDSC	Mononuclear Myeloid-Derived Suppressor Cell
NaN_3	Sodium Azide
NK	Natural Killer cell
NO	Nitric Oxide
nor-NOHA	N-Hydroxy-nor-L-Arginine
Nrf2	Nuclear Factor Erythroid 2-Related Factor 2
NSCLC	Non-Small Cell Lung Carcinoma
nT_{reg}	Natural Regulatory T cell
PAP	Prostatic Acid Phosphatase
PBMC	Peripheral Blood Mononuclear Cell
PD-1	Programed Cell Death 1
PD-L1/2	Programed Cell Death Ligand 1/2
PMD-MDSC	Polymorphonuclear Myeloid-Derived Suppressor Cell

PNT	Peroxinitrite
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RT	Room Temperature
SCF	Stem-Cell Factor
SH2	Src Homology 2
SIRP-α	Signal Regulatory Protein-Alpha
STn	Sialyl-Tn antigen
TACA	Tumor-Associated Carbohydrate Antigen
TAM	Tumor-Associated Macrophages
TCR	T-Cell Receptor
TGF-β	Transforming Growth Factor-Beta
TIL	Tumor-Infiltrating Lymphocyte
TNF-α	Tumor Necrosis Factor-Alpha
TP53	Tumor Protein p53
T_{reg}	Regulatory T cell
TRUCK	T Cell Redirected for Universal Cytokine Killing
TSP-1	Thrombospondin-1
VEGF	Vascular Endothelial Growth Factor

Context

Cancer designates a set of diseases characterized by uncontrolled growth and spread of abnormal cells[1]. The interactions between the immune system and cancer development have been the object of numerous studies for many years now, leading to key discoveries about disease biology and important advances in cancer treatment[2].

Over the last decade, the concept of *"cancer immunoediting"* has emerged as a way to describe the many facets of the interactions between tumor cells and the immune system, from surveillance and elimination of malignant cells to immune escape and tumor spreading[3]. Tumor cells can evade the immune system by several mechanisms, for example, by the presence of high numbers of immunosuppressive cells, such as the myeloid-derived suppressor cells (MDSC).

Cancer immunotherapy, a set of approaches that aim to enhance the body's antitumor immune functions, has been shown to be an effective treatment for multiple types of tumors[4]. Some of the most common immunotherapies make use of monoclonal antibodies (mAb) targeting immune checkpoint inhibitors or rely on cell-based approaches. Although immunotherapy is a rapidly expanding field in cancer research, cancer patients often respond differently to the same treatment. So, in 2016, Blank *et al*. described a framework – *"cancer immunogram"* –, whose purpose is to assist in the decision of the most effective cancer immunotherapy for each patient[5]. This framework is composed of seven classes of parameters: tumor foreignness, general immune status, immune cell infiltration, absence of checkpoints, absence of soluble inhibitors, absence of inhibitory tumor metabolism and tumor sensitivity to immune effectors[5]. In each one of these classes, multiple parameters can be analyzed, MDSC being one of them. MDSC are a group of cells characterized by the ability to suppress the immune system and have been subject of research in recent years[6].

The aim of this work was to identity and quantify MDSC present in the blood of cancer patients, as well as to study the function of these cells, with a view to integrate MDSC assessment in the cancer immunogram under development in the Immunology Service of the IPO-Porto. So, in the following sections, a brief description of cancer immunobiology, including the concept of *"cancer immunoediting"* and the tumor immunoescape/immunosuppressive mechanisms will be discussed (subchapter 1.1.). Then, some antitumoral immunotherapy approaches, such as immune checkpoint inhibitors, dendritic and natural killer cells vaccines, CAR T cells and tumor-infiltrating cells will be explained (subchapter 1.2.). Finally, the use of the cancer immunogram as a tool to help in

the selection of the potentially most effective immunotherapy will be discussed, along with the role of MDSC in the cancer immunogram (subchapter 1.3.). In section 2, the materials and methods used in this work are presented, namely regarding the identification and quantification of MDSC, performed by flow cytometry, and the study of the immunosuppressive characteristics of these cells, such as the expression of various molecules (*TGF-β* and *NOS2*) and the reduction in the expression of the zeta chain in cells of the immune system (T- and NK-cells). The results and their discussion, as well as some conclusions of this work, are presented in the remaining sections (section 3, 4 and 5, respectively).

Chapter 1

Introduction

Cancer is a general term used to designate diseases characterized by uncontrolled growth and spread of abnormal cells[1]. It is a global public health problem being one of the leading causes of morbidity and mortality, with an estimated 18,1 million new cases and 9,6 million deaths just in 2018[7]. According to the recent report from the International Agency for Cancer Investigation (IARC), a quarter of the Portuguese population is at risk of developing cancer until the age of 75, while 10% is at risk of dying of cancer. In 2018, the number of new cases in Portugal was about 58.000 and the deaths from oncologic disease reached 29.000[7]. Population growth and ageing, as well as environmental factors and the changing prevalence of certain causes of cancer linked to social and economic development (e.g., diet, nutrition and physical activity), are critical determinants of the increasing cancer burden[8].

Since cancer is the second leading cause of death worldwide, multiple efforts have been made over the years to develop effective and robust treatments. Currently, the standard strategies used for treatment of cancer are surgery, radiotherapy and chemotherapy[9]. Surgery was the first approach for cancer treatment and has been the cornerstone for patients with solid tumors, consisting in the removal of cancerous tissue[10]. But, its applicability is dependent on several factors such as the location of the solid mass and on the physical status of the patient[11]. Radiotherapy was discovered years later, in the XIX century, and consists in the use of high-energy radiation to kill cancer cells[12]. This strategy of treatment is used in the treatment of localized tumors, but it can also be used in patients with hematological malignancies, like leukemia, multiple myeloma and lymphoma, by doing total body irradiation. It is also used to control symptoms – symptom relief in locally advanced or disseminated cancers –, to shrink a tumor before surgery and to destroy remaining tumor cells after surgery[13]. However, the use of this strategy causes side effects, which can be divided into acute and late side effects, based on when they occur. Typically, acute radiation side effects are first observed during radiotherapy and include, for example, erythema, alopecia, dry or moist desquamation, among others. Yet, late radiation side effects are caused

by damage to the supporting stroma or vasculature that supplies an area or tissue and can occur in any tissue[14]. Finally, chemotherapy, the most common therapeutic strategy, consisting in the use of a combination of drugs to kill cancer cells[15]. This type of treatment is used in multiple malignancies, such as Hodgkin's lymphoma and acute leukemia in children, palliative treatment for many types of advanced cancers and adjuvant treatment before, during and/or after local treatment, in order to eradicate remaining micrometastasis[12]. The first documented clinical use of chemotherapy was in 1942, in which Goodman and Wintrobe used an alkylating agent, nitrogen mustard, to obtain a brief clinical remission in a patient with lymphoma[16]. Later Sidney Farber demonstrated that aminopterin produced remissions in children with acute leukemia[17]. However, just like radiotherapy, chemotherapy can cause severe side effects, because it doesn't have a selective destructive effect against cancer cells and can produce toxicity. Both radiotherapy and chemotherapy destroy uncontrolled dividing tumor cells but also cells that physiologically rapidly divide in tissues, such as the mucosa[18]. Also, cancer cells tend to develop drug resistance rapidly during treatment, due to mutation or overexpression of the drug target, inactivation of the drug or elimination of the drug from the cell[15].

Therefore, great efforts have been made towards the development of other therapies. Immunotherapy has been emerging as one of the most promising approaches. This type of treatment aims to stimulate, enhance or restore the immune system capacity to identify and destroy malignant cells. The concept dates back decades, but it was only translated to clinical application recently, proving to be a powerful strategy to treat cancer[4,19].

1.1. Immunobiology of Cancer

The role of the immune system in the prevention or repression of tumor development has been object of numerous studies for many years[2]. In 1909, Paul Ehrlich proposed that the immune system may eliminate neoplastic cells before they develop into tumors[20]. Later, in 1970, Burnet proposed that tumor cell neo-antigens induce an immunological reaction against cancer, formulating the immune surveillance theory[21].

Over the last decades, extensive work revealed that this theory was incomplete, showing only part of the story. The concept of *"cancer immunoediting"* was defined, whereby the many facets of the interactions between tumor cells and the immune system are described[3]. This dynamic process, by which the immune system protects against tumor development and

also sculpts their immunogenicity, is composed of three phases, termed as the "three Es of cancer immunoediting" – elimination, equilibrium and escape – (Figure 1), and both innate and adaptive arms of the immune systems take part on it[3].

The elimination phase consists in an updated version of the cancer immunosurveillance theory, in which the immune system detects the presence of transformed cells and destroys them[22]. There are four proposed stages that describe the elimination phase: in the first stage tumor cells proliferate, leading to increased tumor size [22]. When the tumor reaches a certain size, it begins to invade surrounding tissue, leading to the recruitment of innate immune cells such as macrophages, dendritic cells (DC) and natural killer (NK) cells[23]. Once recruited, these cells recognize the structures on the transformed tumor cells, leading to the production of interferon-γ (IFN-γ); in the second stage, IFN-γ induces a limited amount of tumor death via proliferative and anti-angiogenic effects[3]. Besides that, the chemokines derived from tumor and surrounding normal tissue have potent angiostatic capacities and block the formation of new blood vessels, which contributes to the induction of tumor death[24]. Necrotic tumor cells are then ingested by local dendritic cells, which than migrate to draining lymph nodes; in the third stage, the chemokines produced during the escalating inflammatory process recruit more NK cells and macrophages that produce more IFN-γ, which kills more tumor cells by activating cytotoxic mechanisms such as perforin and reactive oxygen intermediates[25]. In the lymph nodes the newly migrated DC present the tumor-antigens to naïve $CD4^+$ T lymphocytes that differentiate into Th1 cells, which, in turn, facilitate the polarization of $CD8^+$ cytotoxic T lymphocytes (CTL)[26]; finally, in the fourth stage, the homing of tumor-specific Th1 cells and CTL to tumor site promote the destruction of the remaining tumor cells[27].

In the equilibrium phase, any tumor cell variant that has survived the elimination phase enters into a dynamic equilibrium, where tumor cells continue to produce cells resistant to immune destruction and the immune system continues to destroy the original escape variants of the tumor cells[22]. Basically, in this phase, immune responses are still active, regulating and controlling tumor growth and metastasis, while keeping the tumor in a dormancy state. This is the longest of the three phases and may occur over a period of many years, being estimated a 20-year interval between initial carcinogen exposure and clinical detection of the tumor[28].

Lastly, in the escape phase, the heterogeneity and genetic variations in tumor cells enable them to acquire insensitivity to immunological detection and/or elimination and to become immune-evasive[29]. Tumor cells have evolved to suppress the immune system by several mechanisms. Some of these mechanisms, which will be further discussed in this work, are: overexpression of CD47, decreased expression of MHC-I, which results in the failure of CTL to recognize cancer cells, increased expression of immunosuppressive molecules, such as PD-1, PD-L1, PD-L2, CTLA-4 and STn. Also, the presence of myeloid-derived suppressor cells (MDSC) and regulatory T (T_{reg}) cells and the lack of tumor-infiltrating lymphocytes (TIL) contribute to an immunosuppressive microenvironment. Altogether, this allows tumor cells to evade the host immune system, resulting in a clinical observable malignant disease[22].

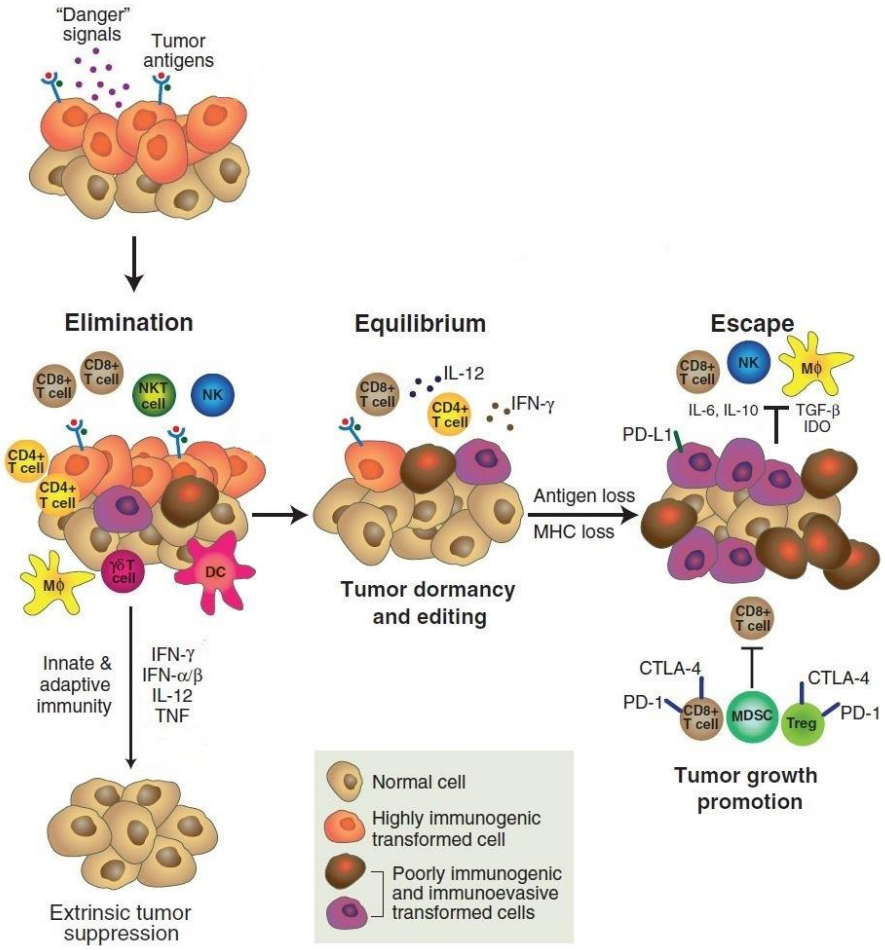

Figure 1. The three phases of cancer immunoediting – elimination, equilibrium and escape. The elimination phase corresponds to the cancer immunosurveillance concept. In the equilibrium phase is obtained an equilibrium between progression of cancer and tumor cells elimination by the host's immune system. In the last phase, the escape phase, the genetically unstable tumor cells held in equilibrium may emerge and evade the immune system of the host, causing a malignant disease. Adapted from [28].

1.1.1. Tumor Immunoscape/Immunosuppressive Mechanisms

CD47, also known as integrin-associated protein (IAP), is a 50-kDa glycoprotein that possesses an extracellular amino-terminal immunoglobulin domain, five transmembrane domains and a short C-terminal cytoplasmic tail[30]. CD47 is expressed in the cell-surface of transfused red blood cells, platelets and lymphocytes to protect them from elimination by macrophages, as it acts as a *"don't eat me"* signal[31]. CD47 functions as a ligand for signal regulatory protein-α (SIRP-α) and as a receptor of thrombospondin-1 (TSP-1)[30]. SIRP-α is a regulatory plasma membrane protein that is mainly expressed in phagocytic cells, such as macrophages and DC, and the CD47/SIRP-α triggers the phosphorylation of immunoreceptor tyrosine-based inhibition motifs (ITIM), that subsequently leads to the binding and activation of SHP-1 and SHP-2 (Src homology 2 (SH2) domains), resulting in the inhibition of phagocytosis through inhibition of myosin accumulation at the cell surface[32]. TSP-1 is a homotrimeric adhesive glycoprotein that is secreted by platelets, monocytes, macrophages and DC and whose expression is elevated during wound healing[33]. The TSP-1/CD47 pathway inhibits nitric oxide (NO)/cyclic guanosine monophosphate (cGMP) signaling in vascular cells, leading to suppression of angiogenic responses[34]. Also, the ligation of CD47 by TSP-1 acts as a negative regulator for DC activation, mediating the arrest of cytokine production and suppressing their differentiation into functional DC[35]. CD47 is overexpressed in many different cancer cell types and is linked to tumor evasion and metastasis, being connected to lower rates of progression-free and overall patient survival[36,37].

Once tumor cells display genetic instability, they can have altered major histocompatibility complex I (MHC-I) expression, leading to low or no expression of MHC-I molecules on their cell surface[38]. MHC-I loss or downregulation has been reported in up to 90% of certain types of human cancers and is a major tumor escape mechanism from the host T lymphocytes[39]. Reduced levels of MHC-I antigens result in decreased sensitivity to cytotoxic T lymphocyte-mediated lysis but imposes another response – the NK cell-mediated immune response, that recognizes the missing MHC-I molecules and lyses the MHC-I-negative or -deficient tumor cells. Basically, high levels of MHC-I molecules favor CTL as anti-tumor effectors and low levels of MHC-I molecules favor NK cells as tumor defense effectors[40]. The problem is that under physiologically conditions, NK cells are almost absent

or only found in low concentrations in tumors, being unlikely to make a major contribution to tumor cell elimination[41].

The programmed cell death 1 (PD-1) protein, also known as CD279, is an immune inhibitory receptor that is expressed on activated immune cells, such as NK cells, B and T cells, macrophages, T_{regs}, among others[42]. This receptor was first described in the 1990s and its major role is to limit the activity of cytotoxic T cells in peripheral tissues, when an inflammatory response to infection occurs, as well as to limit autoimmunity[43]. The two ligands of PD-1 belong to the B7 family and are: PD-L1, also known as CD274, that is expressed in DC, macrophages, activated monocytes, NK cells, T and B cells, among others, and PD-L2, also known as CD273, which is predominantly expressed in DC and some macrophages[44]. Tumor cells can express high levels of PD-L1 that, once binding to PD-1 expressed by T cells, triggers their apoptosis (Figure 2)[45]. Thus, the activation of PD-1/PD-L1 signaling serves as a main mechanism by which tumor cells evade antigen-specific T cell immune responses.

Just like PD-1, the cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), also known as CD152, is an immune-checkpoint receptor that downregulates the amplitude of T cell activation[46]. CTLA-4 is a membrane glycoprotein that is exclusively expressed on T cells, including $CD4^+$ T cells and T_{regs}. This immune-checkpoint receptor is a homolog of CD28, a T cell co-stimulatory receptor, sharing up to 30% of identity at the amino acid level[47]. CTLA-4 and CD28 share two identical ligands: CD80, also known as B7-1, and CD86, also known as B7-2, but CTLA-4 binds with much higher affinity to these ligands. The relative amount of CD28/B7-1/2 binding and the relative amount of CTLA-4/B7-1/2 binding determines whether a T cell will undergo activation or anergy (Figure 2)[48]. CTLA-4/B7-1/2 binding results in reduced T cell proliferation, repression of cell cycle progression and reduced production of cytokine such as IL-2, which stops potentially autoreactive T cells at the initial stage of naïve T cell activation[48]. Also, CTLA-4 may remove CD80 and CD86 from the cell surface of antigen-presenting cells (APC), resulting in a reduced availability of these receptors to stimulate CD28-expressing T cells[49].

Glycosylation, a process that allows the sequential covalent addition of specific glycan structures to the backbone of lipids and proteins, is commonly observed in cancer and can lead to the expression of aberrant tumor-associated carbohydrate antigens (TACA)[50]. TACA can result from incomplete *O*-glycosylation, for example, the sialyated version of Thomsen-

nouveau (Tn, GalNAcα1-*O*-Ser/Thr) antigen, the sialyl-Tn (STn) antigen, also known as NeuAcα2-6GalNAcα-*O*-Ser/Thr or CD175s. STn results from an early sialylation of the Tn antigen and it contains a sialic acid residue α2,6-linked to GalNAcα1-*O*-Ser/Th[51]. Its biosynthesis is mediated by a specific sialyltransferase, the STn synthase, also known as ST6GalNAc-I. This enzyme competes with *O*-glycans elongating glycotransferases and its overexpression leads to an increase expression of STn[52]. STn is rarely expressed in normal tissues but is expressed by more than 80% of human carcinomas, being associated with an adverse outcome and decreased overall survival of cancer patients. The poor prognosis is mainly due to the fact that these STn antigens confer resistance to chemotherapy and are involved in the induction of a tolerogenic phenotype on immune cells[50].

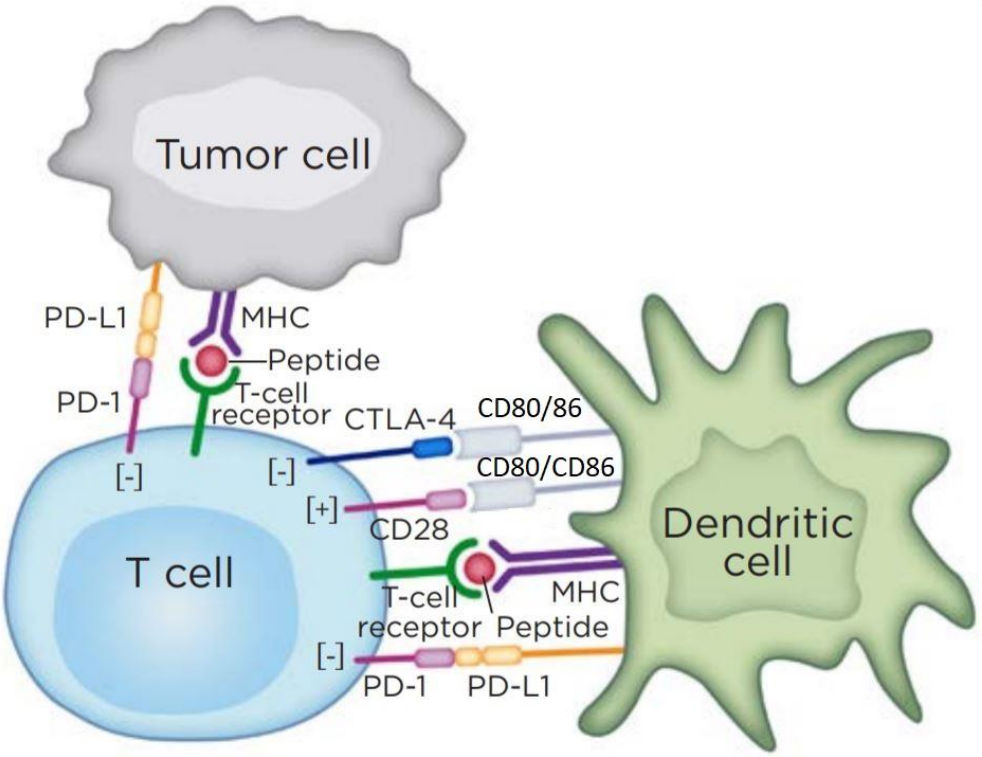

Figure 2. PD-1 and CTLA-4 pathways. PD-1: Tumor cells can express PD-L1 (and PD-L2, not shown) as a consequence of inflammatory cytokines and/or oncogenic signalling pathways. PD-1/PD-L1 binding inhibits TCR-mediated positive signalling, leading to the apoptosis of T cells. **CTLA-4:** T cells activation occurs when the antigen displayed in the MHC on APC binds to TCR, in concert with CD28:CD80/CD86-mediated co-stimulation. Cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) is a homolog of the receptor CD28 and has a much higher affinity for both ligands (CD80 and CD86) of CD28. CTLA-4:CD80/CD86 binding results in a net negative signal that blocks T cell activation. Adapted from [107].

Tumor microenvironment is often prone to induce an intermediate maturation status of DC which results in an increased polarization of regulatory T (T_{reg}) cells. T_{reg} are a small immunosuppressive subpopulation of $CD4^+$ T cells (about 5%), characterized by the expression of the transcription factor Forkhead box protein 3 (FOXP3), also known as scurfin[53]. They can be divided into two different groups: the naturally occurring T_{reg} (nT_{reg}) and the inducible or adaptive T_{reg} (iT_{reg}), and they play an important role in maintaining immune homeostasis and mediating peripheral tolerance[54]. FOXP3-expressing T_{reg} can suppress most immune cells, including $CD8^+$ T cells, B cells, NK cells, DC and macrophages. They express several molecules, such as interleukin (IL)-2, IL-10, transforming growth factor β (TGF-β), CTLA-4 and PD-1, that may directly suppress or induce cell death. Moreover T_{reg} also exhibit their suppressive activity via inhibition of proliferation and cytokine production of $CD8^+$ T cells and through attenuation of co-stimulation and antigen presentation by APC[55]. Accumulating studies have demonstrated that T_{reg} cells infiltrate into various types of tumors and decreased ratios of tumor-infiltrating CTL to T_{reg} cells are correlated with poor clinical prognosis[54].

Other important cellular players contributing to tumor immunoescape are MDSC. MDSC are a heterogenous population of immature myeloid cells that have potent immune suppressive activity[56]. These cells will be discussed in more detail in section 1.3.1.

1.2. Antitumoral Immunotherapy Approaches

Cancer immunotherapy is revolutionizing cancer treatment through the discovery and development of new approaches that enhance the body's antitumor immune functions[4]. Some of the most effective and widely used antitumoral immunotherapy approaches are the use of monoclonal antibodies (mAb) targeting immune checkpoints and the use of immune cell-based therapies[19].

1.2.1. Immune Checkpoint Inhibitors

Immune checkpoints are stimulatory or inhibitory signals that are crucial for the maintenance of self-tolerance and assistance with immune response[46]. The two immune checkpoint receptors that have been most actively studied in the context of cancer immunotherapy are CTLA-4 and PD-1, which are both inhibitory checkpoint receptors. They are selectively upregulated in various types of cancer and are major targets for blockade[46].

The CTLA-4 targeting antibodies ipilimumab, also known as MDX-010, and tremelimumab, also known as CP-675, were the first immune checkpoint inhibitors to be tested and approved by the US Food and Drug Administration (FDA) for the treatment of advanced melanoma[57]. The mechanism is primarily through direct blockade of CTLA-4 competition for CD80 and CD86 ligands, which allows the positive CD28-mediated co-stimulation. Basically, the binding of antibodies to CTLA-4 blocks its interaction with CD80 and CD86, which leads to the augmentation of T cell activation and proliferation, activation and proliferation of tumor infiltrating $CD8^+$ T cells and reduction of T_{reg} cells. These mechanisms lead to a general increase in T cell responsiveness and, consequently, to tumor regression[57].

The first phase III clinical trial in patients with advanced metastatic melanoma to be completed was for tremelimumab and showed survival benefit relative to the standard chemotherapy, without reaching, however, statistical significance[58]. Better results were further obtained with ipilimumab, although with increased incidence of immune-related adverse events (irAE). This is because CTLA-4 blockade removes CTLA-4-mediated protection to autoimmune responses. The most common irAE affects the gastrointestinal tract and the skin, diarrhea and rash being the most frequently reported adverse effects. These adverse effects are typically low grade and manageable with steroids and other supportive cares, but can also be serious and life threatening[59]. Several phase III and phase II trials are being performed in other types of cancer, namely lung, kidney and prostate cancer (phase III) and cervical, colorectal, gastric, pancreatic, ovarian and urothelial cancer (phase II)[48].

Pembrolizumab, a fully human IgG4 antibody, was the first immune checkpoint inhibitor targeting PD-1 approved by the FDA, being used for the treatment of patients with advanced or metastatic melanoma. It's mechanism of action consists in the blockade of the PD-1-mediated signaling, which disrupts the negative signal that downregulates T cell activation and proliferation, restoring antitumor immunity[60]. In a phase III study conducted in patients with advanced melanoma, pembrolizumab showed prolonged progression-free survival and overall survival with fewer high-grade toxicity compared to ipilimumab[61]. Pembrolizumab was later approved to treat patients with metastatic, refractory non-small cell lung carcinoma (NSCLC), head and neck squamous cell carcinoma, Hodgkin lymphoma, urothelial carcinoma and gastric and gastroesophageal carcinoma. Additionally,

multiple phase II and phase III studies are running in order to evaluate pembrolizumab efficacy to treat other cancers[62].

In following years, nivolumab, another immune checkpoint inhibitor for PD-1, was also approved for the treatment of unresectable or metastatic melanoma. In a phase III study in patients with metastatic melanoma, nivolumab alone or combined with ipilimumab, resulted in significantly longer progression-free survival than ipilimumab alone. However, in patients with PD-L1-negative tumors, the combination of nivolumab and ipilimumab was shown to be more effective than nivolumab alone[63]. This PD-1 inhibitor antibody was also approved for treating patients with metastatic NSCLC, renal carcinoma, Hodgkin lymphoma, head and neck squamous cell carcinoma, urothelial carcinoma and hepatocellular carcinoma[62].

Recently, atezolizumab, an immune checkpoint inhibitor for PD-L1, was approved for treating patients with urothelial carcinoma. Atezolizumab is an engineered humanized monoclonal G1 immunoglobulin that binds selectively to PD-L1 and prevents its interaction with PD-1, sparing the interaction between PD-L2 and PD-1[64]. This anti PD-L1 antibody showed a clinically relevant improvement of overall survival compared with docetaxel (standard of care for urothelial carcinoma) and a favorable safety profile[65]. The FDA further approved atezolizumab for treating NSCLC.

Despite the remarkable progress achieved with immune checkpoint inhibitors in monotherapy, there is a tremendous need to improve efficacy across tumor types and it is important to understand which aspects of the tumor microenvironment functionally limit responses to immune checkpoint blockade. For instance, increased expression of PD-L1 in some patients may explain, in part, why monotherapy with anti-CTLA-4 was not effective. So, it is expected that simultaneous blockade of PD-1 and CTLA-4, may improve therapeutic efficacy compared to monotherapy[62].

1.2.2. Cell-Based Therapies

Another emergent immunotherapeutic strategy in the oncology field is the use of cell-based approaches. These therapies explore the use of autologous or heterologous *ex vivo* expanded and manipulated immune cell effectors, such as TIL, CAR T cells or NK cells, or the use of tumor antigen-loaded DC[66]. In the following sections we will briefly discuss each one of these approaches.

1.2.2.1. Dendritic Cells Vaccines

DC are the most powerful APC being highly efficient at generating and modulating immune responses. These cells are therefore viewed as a promising arm to activate the immune system in immunotherapeutic strategies against cancer[67].

The main goal of DC vaccines is the activation of an immune response able to eliminate cancer cells and produce long-lasting immunity. They mostly consist of *ex vivo* expanded DC matured and loaded with tumor antigens, that are then administrated via subcutaneous or intravenous routes into patients[67]. DC can be generated through the differentiation from peripheral blood monocytes or from $CD34^+$ hematopoietic stem cells. The tumor antigens loading step normally involves the exposure of DC to peptides, proteins or apoptotic non-dividing tumor cells, or the transfection with tumor mRNA or DNA coding for antigens such as mucin 1, p53, tyrosinase, melan-A, and gp100. Finally, the maturation of DC is a particularly relevant process to be done prior vaccination, as immature DC are prone to induce tolerogenic rather than immunogenic responses. This is, therefore, a key step in DC vaccine production. However, there is no consensus on an appropriate maturation stimulus, which makes it difficult to compare different studies[68].

DC vaccines are generally well tolerated and induce minimal toxicity, as shown in numerous phase I and II trials. The most common manifestations are local reactions at the injection site, such as rash and pruritus, and occasionally systemic effects can occur, like fever and malaise[67]. Another limitation of DC-vaccines is that tumors can express negative costimulatory molecules, therefore suppressing the immune response. Hence, it is important to combine DC-based vaccines with approaches that overcome the inhibitory signals, such as the immune checkpoint inhibitors for CTLA-4 and PD-1, allowing a more robust immune response against cancer[67].

Sipuleucel-T was the first DC-based vaccine to be approved by the FDA, for use against asymptomatic or minimally symptomatic castration-resistant prostate cancer. This vaccine comprises an enriched preparation of white cells containing a significant fraction of APC, especially DC. These cells are pulsed with prostatic acid phosphatase (PAP) fused *ex vivo* with GM-CSF – PA2024 –, and then reintroduced intravenously in the patient[69]. However, correlation with an immune response against the PA2024 antigen was not significantly strong. Sipuleucel-T is now being reassessed as a combination therapy, for example, plus ipilimumab and atezolizumab, and is also being tested in earlier-stage disease[70].

1.2.2.2. NK cells

NK cells are granular lymphocytes of the innate immune system that have the inherited ability to kill malignant cells in a non-MHC and non-tumor antigen-restricted manner. Due to their key role in first-line immune responses, their rapid effects and the absence of pre-immunization, there has been an increasing interest in the development of NK cell-based immunotherapies[71]. There are many different approaches used for NK cell-based immunotherapy, some of them involve the use of stimulatory cytokines, antibodies and the adoptive transfer of *ex vivo* activated NK cells (allogenic or autologous). The main goal is always to improve the persistency, activation, numbers and tumor cell-targeting of NK cells[71].

The use of NK cell-based immunotherapy shows potential, but the long-term antitumor efficiency remains modest. In order to benefit patients, a combination with other approaches such as chemotherapy or radiotherapy, could help create space for NK cell expansion and integration[72].

1.2.2.3. Tumor-Infiltrating Lymphocytes

Tumor-infiltrating lymphocytes (TIL) are frequently found in tumors and can be characterized by three histologically distinct phenotypes: the immune inflamed phenotype, where there is a robust immune infiltrate; the immune-excluded phenotype, where T cells are particularly present in the stroma; and the immune desert phenotype, which is characterized by the absence of infiltrating lymphocytes[73].

Several studies have reported a survival benefit associated with the presence of TIL, being possible to correlate the quantity, the quality and the spatial distribution of these cells with patient survival[74,28]. In a study with melanoma patients, Clemente and collaborators, indicated that TIL are associated with a favorable patient prognosis and Naito *et al.*, suggested that, in colon cancer, the accumulation of a particular type of TIL – the CTL – within the tumor had a particularly important influence on patient survival, while the accumulation of the same cells at the tumor margin had no effect[75,76]. Also, in a study with patients with colorectal cancer (CRC), Galon *et al.* concluded that the type, density and location of lymphocytes in CRC had a better prognostic value than the previous pathological criteria for tumor staging[77].

TIL can be used in the treatment of diverse types of cancer, for example advanced melanoma. For this, TIL residing within the tumor material are isolated and expanded *ex vivo* to ~ 1 billion cells, and then are reintroduced back in the patient. The treatment with TIL appears to be a promising treatment option for advanced melanoma, but has not yet been adopted widely, due to the lack of robust clinical evidence[78].

1.2.2.4. CAR T cells

Chimeric antigen receptor (CAR) T cell therapy emerged in the 1990s and consists in the genetic modification of patient's autologous T cells[79]. It is a patient-specific therapy, in which a person's T cells are harvested and genetically modified *ex vivo* using viral or non-viral transfection methods. The genetic modification consists in the introduction of a hybrid receptor with an extracellular moiety that recognizes specific tumor antigens and an intracellular signaling domain that commands T cell activation. These genetically modified T cells are expanded *ex vivo* and then infused back in the patient[80]. CARs recognize unprocessed antigens without the requirement of antigen presentation through the MHC pathway, which enables both $CD8^+$ and $CD4^+$ subsets to be recruited for recognition and destruction of tumor cells. But it is important that the targets are expressed only in tumor cells to avoid various consequences associated with autoimmune disease[79].

This type of therapy has shown more robust results in blood malignancies rather than in solid tumors. This is due to greater genetic instability of solid tumors, which renders them more difficult to eliminate, as they can stop expressing targeted antigens[79]. The combination strategies of CAR T cell therapy and immune checkpoint inhibitors have shown a great potential. For example, the blockade of the PD-1 pathway can potentially enhance CAR T cell therapy[81].

However, CAR T cell therapy may elicit severe side effects, the most common being the cytokine release syndrome (CRS), a process characterized by a storm of inflammatory molecules. This can cause hypotension, hypoxia, high-grade fever and is normally accompanied by neurological disturbance, including seizure, delirium and hallucinations. Some of these adverse effects can be mitigated by using tocilizumab, a monoclonal antibody that blocks IL-6 cytokine receptor[82].

1.3. The Cancer Immunogram

Immunotherapy is a rapidly expanding field in cancer research. However, cancer patients do not always respond equally to treatment. In 2016, Blank *et al*. described a framework – the *cancer immunogram* – that illustrates the state of multiple parameters influencing cancer-immune system interactions, in order to help deciding which is the most effective cancer therapy in individual cases[5].

The cancer immunogram is composed of seven parameter classes: **(1)** tumor foreignness; **(2)** general immune status; **(3)** immune cell infiltration; **(4)** absence of checkpoints; **(5)** absence of soluble inhibitors; **(6)** absence of inhibitory tumor metabolism; **(7)** tumor sensitivity to immune effectors[5]. **(1)** Tumor foreignness can be determined by the expression of neoantigens derived from cancer, but the mutational load can also serve as a surrogate marker. High tumor neoantigen load and high tumor mutational load are associated with a higher likelihood of an immunotherapy response[83]. **(2)** The general immune status can be determined by simple blood analyses (lymphocyte and eosinophil counts and neutrophil/lymphocyte ratio), where a decrease in lymphocyte counts and an increase in absolute neutrophil levels have been correlated with a poor patient outcome and elevated eosinophil counts with an improved outcome. Also, elevated circulating MDSC counts have been seen as a negative predictor of patient outcome[84]. **(3)** The immune cell infiltration, such as the presence of tumor-infiltrating $CD8^+$ T cells in the tumor-immune microenvironment has been associated with longer survival in several malignancies. The infiltration of tumor-reactive T cells into the tumor is required for T cell-mediated tumor control and the absence of these cells can be due to a defect at the level of T cell priming, a mechanical barrier by cancer-associated fibrosis, an impermeable tumor-associated vasculature, or the absence of T cell attracting chemokines, such as the chemokine C-X-C motif ligand 9 (CXCL9) and the chemokine C-X-C motif ligand 10 (CXCL10). Differentiation between these possibilities may be of value for guidance in therapy choice. Also, the presence of other immune cells subpopulations, such as T_{reg} cells, that inhibit $CD8^+$ T cell function, may facilitate cancer progression[5,85,86]. **(4)** Regarding the immune checkpoint inhibitors, their expression functions as a valuable biomarker, because it reports the presence of specific therapeutic targets, as mentioned in section 1.2.1.. Measurement of the expression of PD-1, PD-L1 and CTLA-4 on T cells, tumor cells and DC help to choose the best therapeutic strategy[5]. **(5)** Soluble immunosuppressive factors, such as vascular endothelial growth factor (VEGF),

growth factor A, interleukins IL-1, IL-6 and IL-17, can create a hostile and immunosuppressive tumor microenvironment and promote tumor progression. These factors are often released by tumor cells, T_{reg} and MDSC. Also, the C-reactive protein (CRP), which is induced by IL-1 and IL-6 can be used as a clinical marker for tumor-associated inflammation[5,87]. **(6)** In relation to tumor metabolism, in healthy cells, in the presence of oxygen, the pyruvate resulting from glycolysis enters the Krebs cycle in the mitochondria. Under conditions of hypoxia, pyruvate is converted to lactate by lactate dehydrogenase (LDH). However, in cancer cells, even in the presence of sufficient oxygen, pyruvate is preferably converted into lactate[88]. Crucial T cell functions, such as cytokine production, proliferation and lytic activity, can be impaired by lactic acid and low pH. Therefore, high serum LDH concentrations correlate strongly with poor outcome. Besides that, an enzyme highly expressed by tumor cells and MDSC, the indolamine 2,3-dioxygenase-1 (IDO1), that converts tryptophan into kynurenine, also exhausts antitumor T cells. The enzymes glutaminase and arginase-1 (ARG1) are also overexpressed in tumor cells, which leads to: glutamine deprivation, that promotes T_{reg} polarization; and arginine depletion that inhibits T cell and NK cell functions and promotes generation of MDSC[5,86]. Hence, interfering with metabolic pathways might facilitate direct tumor elimination or enhance $CD8^+$ T cell cytolytic functions. **(7)** Finally, the last parameter – tumor sensitivity to immune effectors – is measured by the visibility for the immune system, i.e., the antigen presentation and recognition, which is performed through the binding of T cell receptor (TCR) to MHC. The loss or inactivation of components of the antigen presentation machinery has been observed in human cancers and by the analysis of this parameter it will be possible to identify patients who are less likely to respond to T cell-activating therapies[89]. However, $CD8^+$ T cell effector function can be impaired despite successful binding to tumor cells, due to the loss of IFN-γ signaling, which is associated with resistance to anti-CTLA-4 immunotherapy[5].

Figure 3 represents three examples of hypothetical patient cases with different cancer immunogram parameters. **Case 1** corresponds to a patient bearing a tumor with a low mutational load and, potentially because of this, with significant low $CD8^+$ T cell infiltrate. Hence, a possible effective immunotherapy would be the administration of CAR T cells, which can enable the recruitment of $CD8^+$ T cells for recognition of tumor cells. **Case 2** represents a patient with a high mutational load tumor, who scores well to all parameters, except for the absence of checkpoints, displaying a strong expression of PD-L1 at the tumor

site. For this patient, theoretically an effective immunotherapy would be the administration of an immune checkpoint inhibitor, more specifically, a PD-1 inhibitor. Finally, the patient in **Case 3** is similar to that in case 2, scoring well to all parameters, except for the strong expression of PD-L1 at the tumor site **(A)**. The patient was submitted to anti-PD-1 antibody treatment and answered well to treatment, which led to restoring of the parameter **(B)**. However, the patient suffered a relapse, as the tumor was insensitive to T cell effector mechanisms **(C)**. A first treatment of choice could be the administration of CAR T cells or NK cells, which are independent of MHC expression[5].

Overall, the cancer immunogram provides a framework that incorporates several multidimensional biomarkers that help in the clinical decision. The individualized data may be obtained from the combination of tumor genomics, immunohistochemistry and standard assays on the peripheral blood and can be monitored during the course of the disease, to adjust treatment accordingly[90].

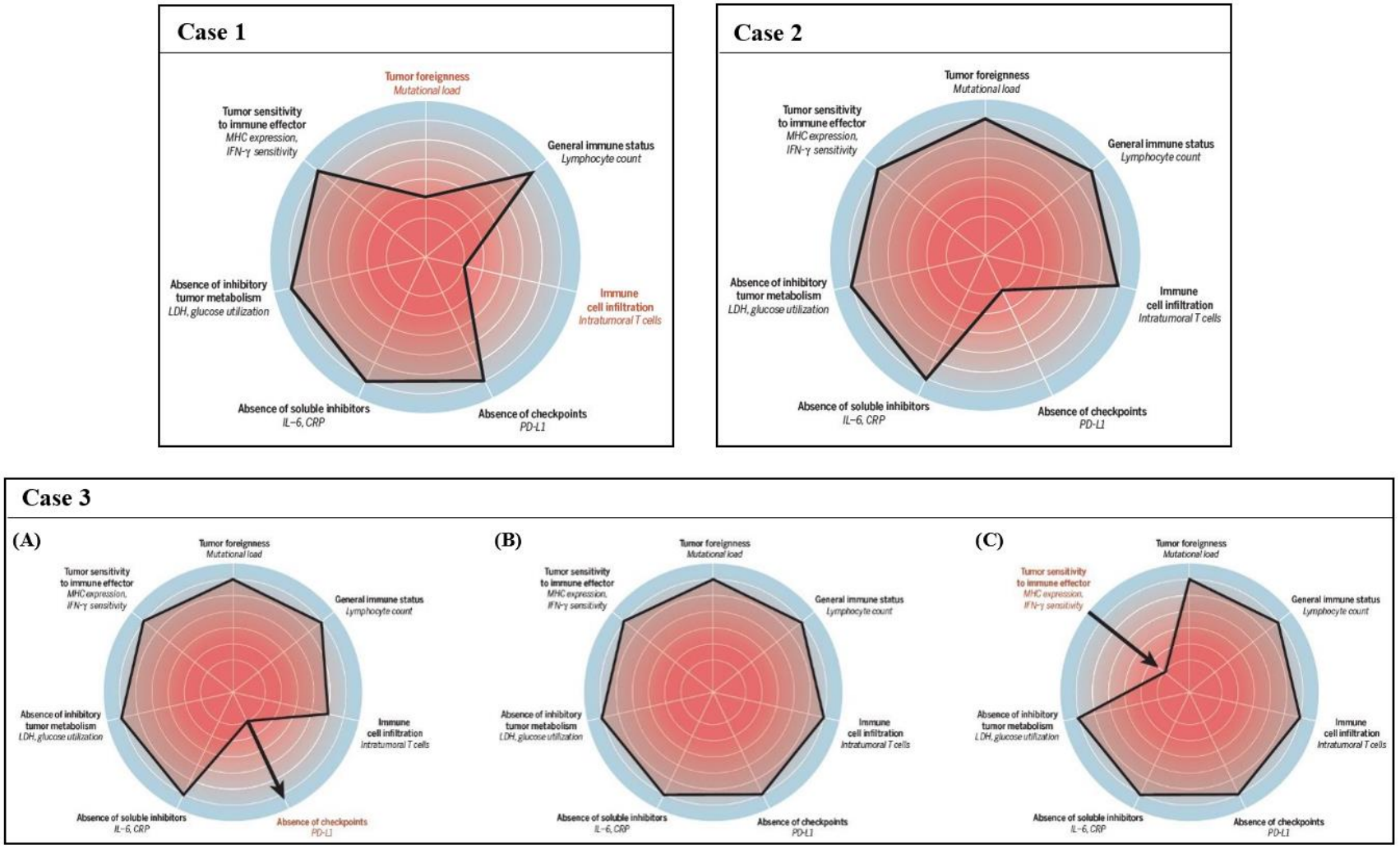

Figure 3. Hypothetical patient cases with different cancer immunogram parameters. Adapted from [5].

1.3.1. MDSC relevance in Cancer Immunogram

MDSC are one of the multiple parameters analyzed in the cancer immunogram and, as mentioned before, they are a heterogeneous population of myeloid lineage defined by an immature state and the capacity to suppress the immune system[56]. In normal conditions, hematopoietic stem cells (HSC), located in the bone marrow, give rise to immature myeloid cells (IMC), that later differentiate into mature macrophages, DC or neutrophils. However, under pathological conditions, such as chronic inflammation, infection and mainly cancer, abnormal myelopoiesis is promoted through persistent stimulation by a range of growth factors, cytokines and chemokines, which eventually results in the accumulation of immature myeloid cells. These accumulated cells can acquire profound immunosuppressive properties through a complex network of signals in the peripheral microenvironment. The immature myeloid cells with immunosuppressive activity are collectively named as MDSC[91] (Figure 4).

In humans, there are three different subsets of MDSC: the polymorphonuclear MDSC (PMN-MDSC), which are phenotypically similar to neutrophils, the monocytic MDSC (M-MDSC), which are phenotypically similar to monocytes, and finally, the immature or early-stage MDSC (e-MDSC), recently discovered, which consists in a small population that includes a mixed group of MDSC that comprises a more immature phenotype[92,93].

In healthy individuals, MDSC are present in low numbers in the circulation, with PMN-MDSC corresponding to less than 1% of total of neutrophils. However, in cancer patients these numbers can rise up to 4-15% of total neutrophils and up to 40% of neutrophils infiltrating tumor tissue[94]. In solid tumors, M-MDSC can rapidly differentiate into tumor-associated macrophages (TAM), which have a long-established role as inhibitors of immune responses and promoters of tumor progression[6]. The amount of MDSC in the peripheral blood is positively correlated with cancer stage and tumor burden, as high numbers of MDSC were associated with shorter progression-free interval, overall survival and were also correlated with negative response to chemotherapy[6].

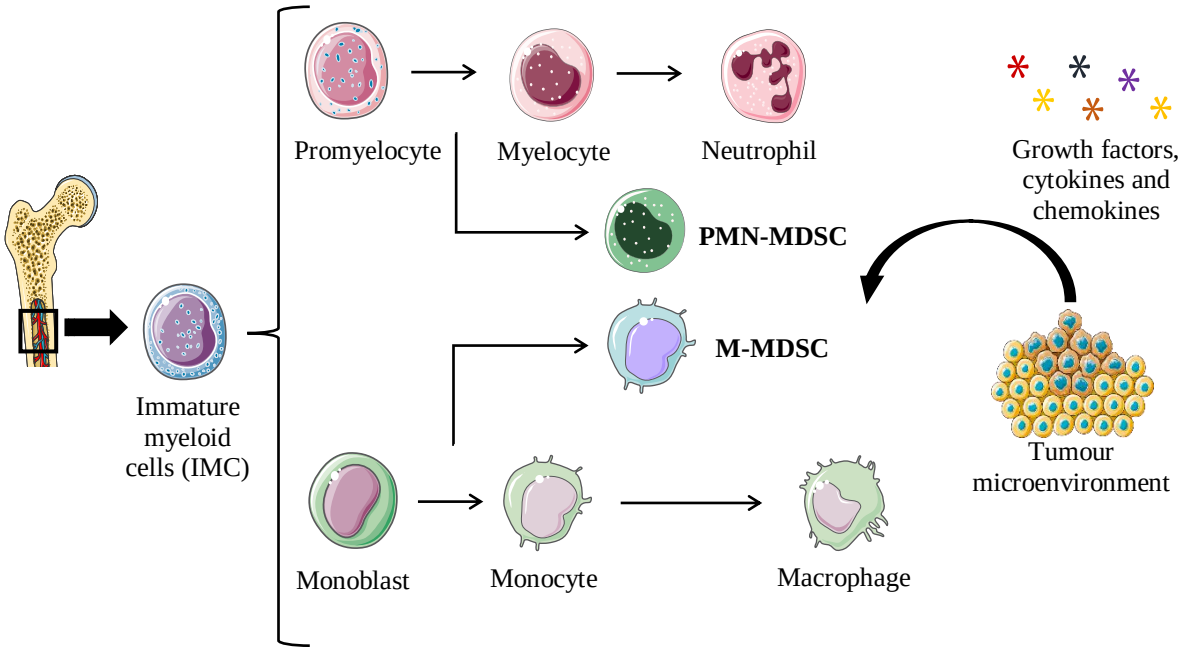

Figure 4. MDSC formation and accumulation. Neutrophils and monocytes are differentiated in bone marrow from hematopoietic progenitor cells, via common myeloid progenitors. Neutrophil differentiation progresses through several progenitor and precursor stages (promyelocyte, myelocyte, metamyelocyte (not shown) and, finally, neutrophil). Monocytes derive from monoblasts and can later differentiate to macrophages. Normally, IMC migrate to different peripheral organs where they differentiate into granulocytes, macrophages or DC. However, under pathological conditions, such as cancer, due to the altering of myelopoiesis by sustained production of inflammatory mediators, such as growth factors, cytokines and chemokines, there is a promotion of immature myeloid cells accumulation, prevention of their differentiation to fully mature cells and induction of their activation. These cells exhibit immunosuppressive functions and are known as myeloid-derived suppressor cells (MDSC), and the pathological activated immature neutrophils and monocytes are known as PMN-MDSC and M-MDSC, respectively.

Several soluble mediators, such as the interleukins IL-1β, IL-6, as well as tumor necrosis factor α (TNF-α), granulocyte/monocyte colony stimulating factor (GM-CSF) and VEGF stimulate the development of MDSC in the bone marrow. Cyclooxygenase-2 (COX-2), prostaglandins, stem-cell factor (SCF), macrophage colony-stimulating factor (M-CSF), IL-6, GM-CSF and VEGF enhance the expansion of MDSC, and finally IFN-γ, IL-3, IL-4 and TGF-β promote MDSC activation[95]. The mechanisms by which MDSC display its immunosuppressive activity include the direct suppression of NK and B cells, as well as their primary target, the T cells. Some of the mechanisms are: **1)** increased expression of the

enzyme ARG1, that converts L-arginine into urea and L-ornithine, which leads to the depletion of L-arginine, an essential amino acid needed for the proper expression of the TCR zeta (ζ) chain, also known as CD247, and coupling of TCR-mediated antigen recognition to diverse signal transduction pathways[96]; **2)** upregulation of inducible nitric oxide synthase (iNOS or NOS2), that under limiting amounts of L-arginine leads to the generation of NO, which reacts with superoxide and generates peroxinitrite (PNT), a reactive nitrogen specie (RNS). The production of PNT causes the nitration and nitrosylation of TCR, leading to T cell tolerance. Also, PNT causes the nitration of T cell-specific chemokines, such as C-C motif chemokine ligand 2 (CCL2), which causes the reduction of the binding of antigenic peptides to MHC molecules on tumor cells and blockade of T cell migration[97]; **3)** induction of oxidative stress via production of reactive oxygen species (ROS), which leads to decrease of TCR functionality, by loss of the zeta chain[95]; **4)** overexpression of the enzyme IDO1, an enzyme involved in tryptophan catabolism, which causes the depletion of this amino acid, leading to the induction of cell cycle arrest and apoptosis in T cells, inhibition of T cell function within the tumor microenvironment or skewing T cell differentiation towards T_{reg}[98]; **5)** stimulation of T_{reg} differentiation and expansion[95]; **6)** upregulation of PD-L1, which inhibits T cell-mediated reactivity, by interacting with PD-1 receptor expressed on T cells[90]. There are several other factors involved in the immunosuppressive activity of the MDSC, such as TGF-β, a cytokine involved in several cellular events, including proliferation, survival and migration, and whose increased secretion leads to the suppression of T cell proliferation, IL-10 and COX-2 and most of the mechanisms do not act simultaneously, being dependent on the type of tumor, type of MDSC and location of the cells[95].

The ratio of PMN-MDSC to M-MDSC is important, once these cells use different mechanisms of suppression of T cell responses. For example, M-MDSC have the ability to suppress T cell activation in an antigen-specific and nonspecific manner, being associated with the increased expression of NOS2, production of NO and secretion of inhibitory cytokines, such as IL-10. In turn, PMN-MDSC suppress immune responses primarily in an antigen-specific manner, which induces $CD8^+$ T cell tolerance, being associated with the increased expression of ARG1 along with high levels of ROS[96].

Although the field of MDSC research is still recent their phenotype has already been established. In humans, PMN-MDSC can be defined as $CD11b^+$ $CD14^-$ $CD15^+$ $CD33^+$ HLA-$DR^{-/low}$, M-MDSC as $CD11b^+$ $CD14^+$ $CD15^-$ $CD33^+$ HLA-$DR^{-/low}$ and e-MDSC as Lineage$^-$

(CD3, CD14, CD15, CD19, CD56) $CD33^+$ $HLA\text{-}DR^-$ [93]. However, these surface markers are not specific to MDSC, which makes difficult their distinction from neutrophils and monocytes, and consequently their identification and quantification. Nevertheless, a recent study indicated that the lectin-type oxidized LDL receptor-1 (LOX-1) can be used as a distinct surface marker for human MDSC, more specifically for PMN-MDSC found in blood and tumor tissue, because PMN-MDSC are LOX-1 positive and neutrophils are LOX-1 negative[94].

1.3.1.1. Therapeutic Strategies targeting MDSC

The main strategies to target MDSC involve their **(1)** elimination, **(2)** inactivation or **(3)** blocking their accumulation. Several of these strategies have been developed and are currently being tested in the clinic[99]. **(1)** Preclinical and clinical studies have shown that MDSC can be eliminated with low doses of chemotherapeutic drugs, resulting in MDSC cell death. Some chemotherapy agents capable of eliminating MDSC in tumor models are gemcitabine, 5-fluorouracil, cisplatin, doxorubicin, among others[100]; **(2)** MDSC use several mechanisms to suppress antitumor immune responses. Thus, targeting the suppressive machinery of MDSC also acts as an anti-MDSC therapy. Inhibitors for the production of ROS using ROS scavengers, such as nuclear factor erythroid 2-related factor 2 (Nrf2), a transcription factor involved in the activation of the antioxidant response and in protecting cells against damage caused by ROS, has been shown to be able to neutralize human MDSC activity by reducing the production of ROS and dampening their suppressive function *ex vivo*[101]. Also, the use of scavengers of NO, such as carboxy-PTIO (C-PTIO) decreased the function of MDSC and improved the efficacy of adoptive T cell transfer in tumor-bearing mice. Another approach consists in the inhibition of the catabolic enzymes upregulated by MDSC, including ARG1 and NOS2[102]. A specific inhibitor for ARG1, N-hydroxy-nor-L-arginine (nor-NOHA) could inhibit tumor growth and decrease ARG1 levels in MDSC. NG-methyl-L-arginine (L-NMMA), an ARG1 and NOS2 inhibitor, had shown to reduce MDSC function and improve T cell proliferation *in vitro*. Nitroaspirin, a NO-releasing aspirin, has been shown to induce downregulation of ARG1, NOS2 and PNT in MDSC[100]; **(3)** A third anti-MDSC therapeutic strategy consists in blocking their expansion and inducing differentiation. The differentiation of MDSC into mature myeloid cells can be achieved by treatment with all-trans retinoic acid (ATRA), a natural oxidative metabolite of vitamin A.

Cancer patients treated with ATRA shown an improvement in their myeloid/lymphoid dendritic cell ratio and immune responses[103].

1.4. Objectives of this Master Thesis

Immunotherapy has emerged as one of the most promising approaches to treat cancer. However, cancer patients do not always respond equally to treatment. The cancer immunogram was recently designed to help solving this problem, and it is currently seen as an important tool to assist clinicians in choosing a personalized immunotherapy.

MDSC are one of the multiple parameters analyzed in the cancer immunogram, as they can suppress the immune system, and consequently lead to resistance to treatments. For this reason, the main objective of the work carried out within the scope of this master thesis was to optimize and validate a method for identification and quantification of MDSC, to be further implemented at the Immunology Service of IPO-Porto. It is of interest that this method will subsequently be included in the patient's immunogram, also to be developed in this Service.

To achieve the above-mentioned purpose, samples of peripheral whole blood from patients with hematologic and non-hematologic neoplasias, such as multiple myeloma (MM), diffuse large B-cell lymphoma (DLBCL), Waldenström's macroglobulinemia, colon carcinoma (CC) and prostate cancer were collected, labeled with specific antibodies and analyzed by flow cytometry (FC). Two different subsets of MDSC (PMN-MDSC and M-MDSC) were analyzed. As a proof of concept, the immunosuppressive phenotype of these cells was evaluated, more specifically, the transcriptional levels of *NOS2* and *TGF-β*, by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). The expression of the zeta chain in T lymphocytes and NK cells, a consequence of the interaction with immunosuppressive MDSC, was also analyzed by FC.

Chapter 2

Materials and Methods

2.1. Identification and quantification of MDSC by Flow Cytometry

The studies were performed using peripheral whole blood samples from 31 patients with solid or hematologic neoplasias (22 with multiple myeloma (MM), 1 with MM and prostate cancer, 1 with diffuse large B-cell lymphoma (DLBCL), 1 with Waldenström's macroglobulinemia and 7 with colon carcinoma (CC)). For control, 12 samples from healthy donors were used. All samples were processed within 2 hours after blood withdrawal.

For the processing of 4 mL of peripheral whole blood, a specific lysing solution – BulkLysis™ (Cytognos, Catalog Code CYT-BL) – was used. The use of this solution requires a prior step of dilution (1:10) in distilled water (dH_2O) and no more than 2 mL of sample per 50 mL of lysing solution should be used. To process 4 mL of peripheral whole blood, two 50 mL Falcon tubes were used, and 2 mL of sample were transferred to each tube. The tube was filled up to reach 50 mL volume with pre-diluted BulkLysis™ solution and mixed well by inverting and incubated for 15 min in a roller device. The samples were centrifuged at 2200 rpm for 10 min and the cell pellet was vigorously resuspended in 2 mL of washing solution (1x Becton Dickinson (BD) Biosciences FACSFlow™ (Catalog No. 342003), 2-5% (v/v) bovine serum albumin (BSA), 2 mM ethylenediamine tetraacetic acid (EDTA) and 2 mM sodium azide (NaN_3)). The volume of the tube was then completed up to 50 mL with washing solution. Another centrifugation step (2200 rpm, 5 min) was performed and the cell pellet was resuspended in 2 mL of washing solution and transferred to a 5 mL polystyrene round-bottom Falcon tube. To recover cells that might have been left in the Falcon tubes, they were washed with 2 mL of washing solution and this volume was then added to the 5 mL polystyrene Falcon tubes mentioned previously. The tubes were centrifuged at 1800 rpm for 5 min and the cell suspensions from the same samples combined. After this, each sample was stained with an eight color panel, using the appropriate amount of the following fluorescently-labeled antibodies: CD15-V450 (clone MMA, BD

Biosciences), CD45-PO (clone MHCD4530, Life Technologies), Lineage Cocktail 2-FITC (CD3, CD14, CD19, CD20 and CD56, BD Biosciences), LOX-1-PE (BioLegend), CD33-PerCP-Cy^{TM} 5.5 (clone P67.6, BD Biosciences), HLA-DR-PC7 (Beckman Coulter), CD11b-APC (clone D12 BD Biosciences) and CD14-APC-H7 (clone MψP9, BD Biosciences). An incubation step of 30 min at room temperature (RT) protected from the light was performed and then 2 mL of 1X FACS^{TM} Lysing Solution (10X FACS^{TM} Lysing Solution diluted 1/10 vol/vol in dH_2O; 10X FACS^{TM} Lysing Solution from BD Bioscience, Catalog No. 349202) were added. Another incubation of 10 min at RT protected from the light, a centrifugation at 1800 rpm for 5 min and a washing step with 2 mL of washing solution was performed. The supernatant was removed, and the cell pellet resuspended in 500 µL of acquisition buffer (FACSFlow^{TM}), for further data acquisition in the BD FACSCanto^{TM} II (at least 5 million cells were acquired). If the cells were not immediately acquired, they were stored at 4°C. The data analysis was performed using Infinicyt^{TM} 1.7 software (Cytognos). PMN-MDSC were defined as CD11b^{+} CD14^{-} CD15^{+} CD33^{+} HLA-DR^{-} LOX-1^{+}, while M-MDSC were defined as CD11b^{+} CD14^{+} CD15^{-} CD33^{+} HLA-DR$^{-/low}$.

2.2. Study of MDSC immunosuppressive activity

2.2.1. Analysis of the mRNA levels of *ARG1*, *IDO1*, *NOS2* and *TGF-β*

In order to study the immunosuppressive activity of MDSC, the expression of the enzymes, such as ARG1, IDO1, NOS2 and of the cytokine TGF-β was assessed. For that, it was necessary to perform the isolation of the two subsets in study – the PMN-MDSC and M-MDSC.

For this, we used the fluorescence-activated cell sorting (FACS) technique, which is a specialized type of flow cytometry that consists in the separation of a heterogeneous mixture of cells into sub-populations, based on the specific light scattering and fluorescent characteristics of each cell. For this, samples of peripheral whole blood from 3 patients were used and 6 mL of peripheral whole blood from each patient were stained using the previously mentioned protocol. The fluorescently-labeled antibodies used were the following: CD15-FITC (clone MMA, BD Biosciences), LOX-1-PE (BioLegend), CD33-PerCP-Cy^{TM} 5.5 (clone P67.6, BD Biosciences), HLA-DR-PC7 (Beckman Coulter), CD11b-APC (clone D12 BD Biosciences) and CD14-APC-H7 (clone MψP9, BD Biosciences). For control, a

peripheral whole blood sample from 1 healthy donor was collected and the T cells separated by FACS. For this, the sample was stained with CD45-PC7 (clone HI30, BD Biosciences) and CD3-APC (clone UCHT, Beckman Coulter) and all the $CD3^+$ cells were selected. This technique was performed at *Instituto de Investigação e Inovação em Saúde* (i3S), using a BD FACSAria™ II, and according to the phenotype previously mentioned.

The sorted cells were preserved in TripleXtractor (Reagent for RNA isolation, GRiSP, Reference GB23.0050), at -80ºC, for further RNA extraction. The RNA extraction and purification was performed using the GRS Total RNA Kit – Blood and Cultured Cells (GRiSP, Catalog No. GK08.0100). After the extraction, the RNA was quantified using a NanoDrop™ Lite Spectrophotometer (Thermo Fisher Scientific) and reversely transcribed using the High-Capacity cDNA Reverse Transcription Kit (Thermofisher, Catalog No. 4368814) according to the manufacturer's protocol.

Real-time quantitative PCR (qPCR) reactions were performed using SYBR Green as probe on a Bio-Rad CFX Connect device. *GAPDH* was used as reference gene and the results presented as the ratio of mRNA molecules of the studied genes/mRNA molecules of *GAPDH*. T cells from the healthy donor was used as negative control. Primer sequences were designed using Beacon Designer software version 8 (Premier Biosoft International, Palo Alto, CA, USA) and thoroughly tested (Table 1).

Table 1. The qPCR primers for detecting mRNA expression of interested genes.

Gene Name	Primer Sequences	Reference
NOS2	Forward: TCAGTATCACAACCTCAG Reverse: TTCTGGAGACTTCTTTCC	ID NM_000625
IDO1	Forward: CCTGACTTATGAGAACAT Reverse: ATTGCCTTGAATACAGTA	ID NM_002164
ARG1	Forward: AAGAGAAGTGTCAGAGCATGAG Reverse: CTCGTGGCTGTCCCTTTG	ID NM_000045
TGF-B	Forward: GGAAACCCACAACGAAATC Reverse: GCTCTGATGTGTTGAAGAAC	ID NM_000660
GAPDH	Forward: ACAGTCAGCCGCATCTTC Reverse: GCCCAATACGACCAAATCC	ID NM_002046

2.2.2. Evaluation of the zeta chain expression in T lymphocytes and NK cells

This study implied the optimization of the mAb CD247-Alexa Fluor 647 (clone 6B10.2, BD Biosciences). For the mAb to work in its best conditions, it was necessary to isolate the peripheral blood mononuclear cells (PBMC) from the peripheral whole blood samples. For this, 1 mL of each of the 10 peripheral blood samples from cancer patients and of the 7 peripheral blood samples from healthy donors were diluted in 1 mL of PBS Buffer and then, using 15 mL Falcon tubes, layered on top of 1 mL of Lymphoprep™ (STEMCELL Technologies, Catalog No. 07801). The Falcon tubes were centrifuged at 2100 rpm, for 20 min, and the mononuclear cells layer was removed and retained. Two washing steps with washing solution were performed and the cells were then stained with the following fluorescently-labeled antibodies: CD27-BV421 (clone M-T271, BD Horizon), CD45-PO (clone MHCD4530, Life Technologies), CD45RA-FITC (clone ALB11, Beckman Coulter), CD8-PE (clone B9.11, Beckman Coulter), CD4-PerCP (clone SK3, BD Biosciences), CD56-PC7 (clone N901 (NKH-1), Beckman Coulter) and CD3-APC-H7 (clone SK7, BD Biosciences). An incubation step of 30 min at RT protected from light was performed and then the cells were washed with 2 mL of washing solution (centrifugation at 1800 rpm, 5 min). The supernatant was removed and then the staining of the zeta chain using the cytoplasmic antibody – CD247 – was performed using the BD Cytofix/Cytoperm Kit, which contains a Fixation/Permeabilization solution and a BD Perm/Wash Buffer, according to the manufacturer's protocol. Before acquisition, the cells were resuspended in 500 µL of acquisition buffer (FACSFlow™) and data acquisition was done in the BD FACSCanto™ II. If the cells were not immediately acquired, they were stored at 4°C. The data analysis was performed using Infinicyt™ 1.7 software (Cytognos).

2.3. Statistical Analysis

A statistical analysis of data was performed using GraphPad Prism 6 for Windows (version 6.01, GraphPad Software). Differences between the groups were determined by Mann-Whitney U test and were considered statistically significant with $P < 0.05$.

Chapter 3

Results

3.1. Identification and quantification of MDSC by Flow Cytometry

An 8-color, 8-parameter flow cytometric assay was developed, using whole blood as starting sample. For PMN-MDSC, the gating strategy began with the elimination of doublets and cell debris using FSC and SSC-A (Figure 5). The $CD45^-$ cells were excluded (not shown) and then the $CD15^+$ cells were selected using SSC-A on the y-axis. Eosinophils were excluded using their typical location on the CD45 vs SSC-A plot. The cells co-expressing CD33 and CD11b were selected and then CD14 and HLA-DR positive cells were excluded using a tight gate on the CD14 or HLA-DR negative population on a CD14 vs SSC-A plot or HLA-DR vs SSC-A plot, respectively. Finally, PMN-MDSC were identified as the $CD15^+$ cells that express LOX-1.

For the identification of M-MDSC, the gating strategy also began with the elimination of doublets and cells debris using FSC and SSC-A (Figure 6). The $CD45^-$ cells were excluded (not shown) and after this, the $CD14^+$ cells were selected using a CD14 vs SSC-A plot. The cells co-expressing CD33 and CD11b were selected and the $CD15^+$ cells were excluded using a gate on a CD15 vs SSC-A plot. Finally, M-MDSC were identified as the $CD14^+$ cells that don't express HLA-DR.

Two characteristic plots are represented in Figure 7, one from a healthy donor (A) and another one from a cancer patient with MM in an advanced state (ISS-III) (B), showing the difference between the two samples in terms of PMN-MDSC content. In the healthy donor PMN-MDSC represents 0.07% of total of neutrophils, while in the cancer patient the value rises to 1.04%, a percentage fourteen times higher. Similarly, in Figure 8 are represented the characteristic plots for the same healthy donor (A) and the same cancer patient (B) relatively to M-MDSC content. In the healthy donor M-MDSC represents 0.44% of total of monocytes versus of 1.07% (two times higher) in the cancer patient.

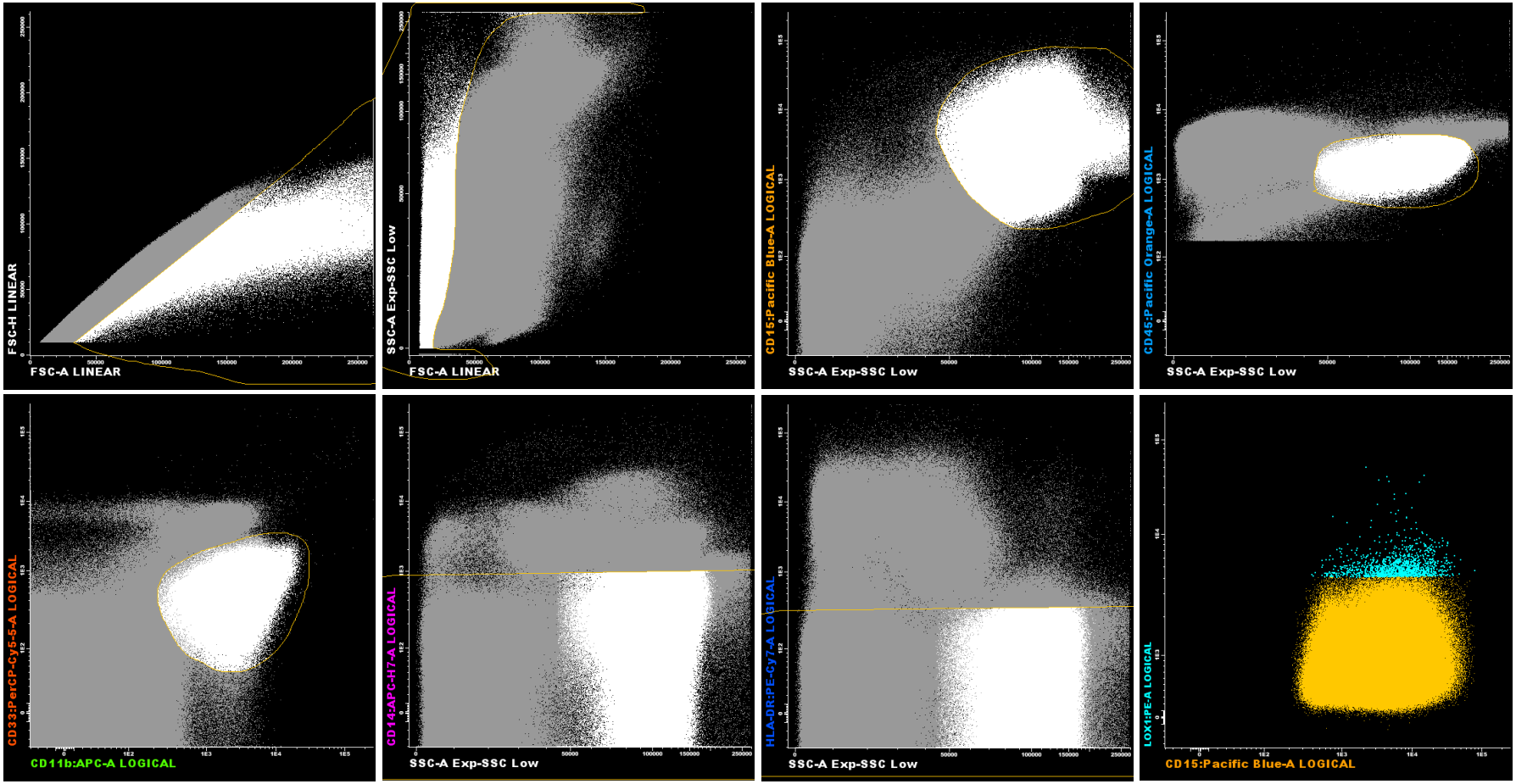

Figure 5. Gating strategy used for the identification of human PMN-MDSC in peripheral blood samples. After the exclusion of doublets and cell debris, $CD15^+$ cells were selected, and the eosinophils excluded using their typical location on the CD45 vs SSC-A plot. Then, the cells $CD33^+$ $CD11b^+$ $CD14^-$ $HLA\text{-}DR^-$ were selected, and finally, only the $CD15^+$ cells that express LOX-1 were selected, which corresponds to the PMN-MDSC population.

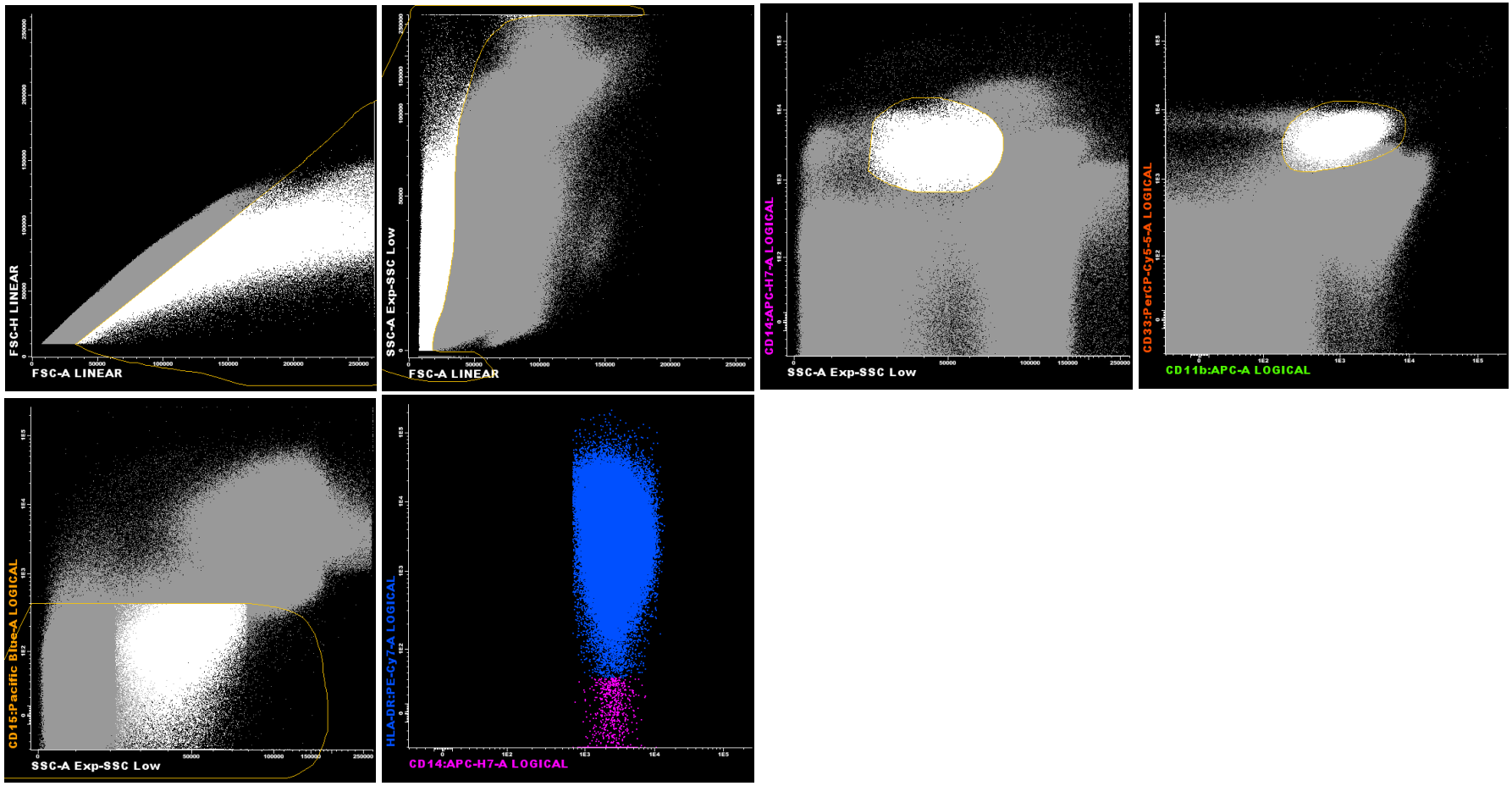

Figure 6. Gating strategy used for the identification of human M-MDSC in peripheral blood samples. After the exclusion of doublets and cell debris, $CD14^+$ cells were selected and then only the cells co-expressing CD33 and CD11b were included. After this, $CD15^+$ were excluded using a gate on CD15 vs SSC-A plot. Finally, only $CD14^+$ cells that don't express HLA-DR were selected, which corresponds to the M-MDSC population.

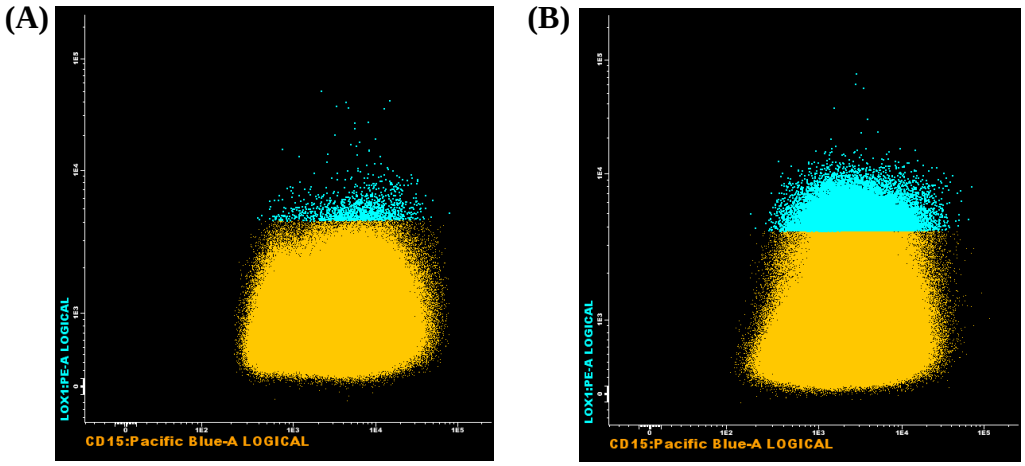

Figure 7. LOX-1 vs CD15 plot for identification and quantification of PMN-MDSC representative healthy donor (A) and cancer patient (B). For the healthy donor it was obtained a percentage of PMN-MDSC of 0.07%, while for the cancer patient it was obtained a percentage of PMN-MDSC fourteen times higher (1.04%). The percentages of PMN-MDSC were obtained in relation to the total neutrophils' population.

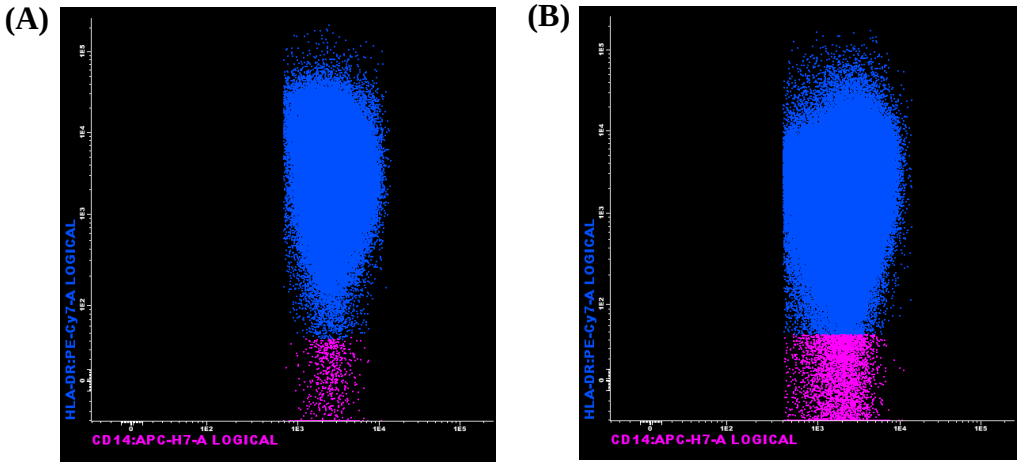

Figure 8. HLA-DR vs CD14 plot for identification and quantification of M-MDSC for representative healthy donor (A) and cancer patient (B). For the healthy donor it was obtained a percentage of M-MDSC of 0.44%, while for the cancer patient it was obtained a percentage of M-MDSC two times higher (1.17%). The percentages of M-MDSC were obtained in relation to the total monocytes' population.

The results concerning the quantification of the MDSC subsets for the 31 cancer patients and 11 healthy donors are represented in Figure 9 (A – PMN-MDSC and B – M-MDSC). In the cancer patients' group, the percentages of PMN-MDSC ranged from 0.134% to 3.136%, with a median value of 0.531, and were found to be about 12-fold higher relatively to the healthy donors, where percentages ranged from 0.004% to 0.151%, with a median value of 0.045. In relation to M-MDSC, in the cancer patients' group the percentages ranged from

0.144% to 8.495%, with a median value of 0.931, and were about 1.1-fold higher relatively to the healthy donors, where percentages ranged from 0.197% to 2.185%, with a median value of 0.845. The differences between cancer patients and healthy donors for the PMN-MDSC cell populations were statistically significant (P < 0.0001), while for the M-MDSC the results obtained were not statistically different (P = 0.5124).

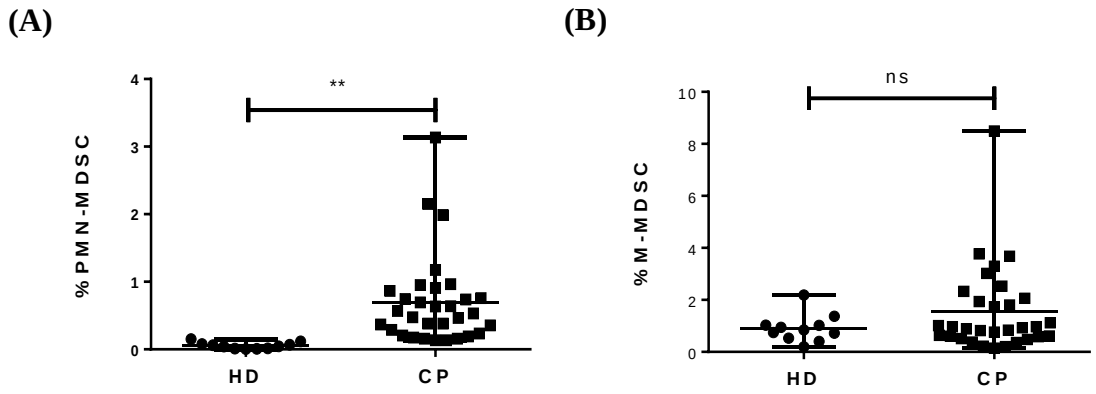

Figure 9. Percentages of PMN-MDSC (A) and of M-MDSC (B) in both cancer patients (CP) and healthy donors (HD). Percentages of PMN-MDSC about 12 times higher in the group of cancer patients comparatively to the group of healthy donors were obtained, being the difference statistically significant (P < 0.0001). Relatively to the M-MDSC, it were obtained percentages about 1.1 times higher in the group of cancer patients comparatively to the group of healthy donors. However, this last result is not statistically different (P = 0.5124).

3.2. Study of MDSC immunosuppressive mechanisms

3.2.1. Analysis of the mRNA levels of *ARG1, IDO1, NOS2 and TGF-β*

MDSC express several factors that contribute to their immunosuppressive activity, including ARG1, IDO1, NOS2 and TGF-β, which can suppress T cell function. To evaluate the production of these factors by PMN-MDSC and M-MDSC, the mRNA level was measured by qPCR. Figure 10 shows that it was possible to detect *NOS2* and *TGF-β* in these cells, which means that they have immunosuppressive activity. More specifically, *NOS2* was mainly produced by PMN-MDSC (Figure 10A) and *TGF-β* was mainly produced by M-MDSC (Figure 10B). Although it was also expected that MDSC expressed *ARG1* and *IDO1*, these were not detected.

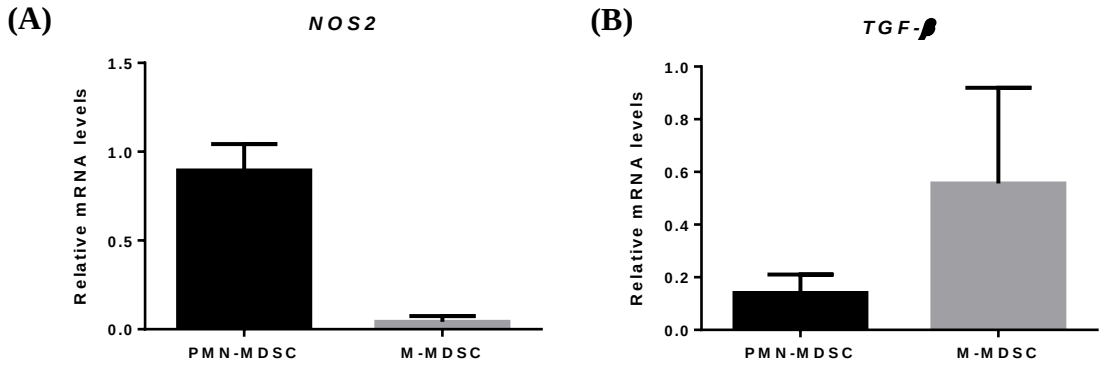

Figure 10. Relative mRNA levels of *NOS2* and *TGF-β*. PMN-MDSC and M-MDSC were sorted from 3 patients and used for RNA isolation. It was possible to detect ***NOS2*** mRNA, being its levels about 21 times lower in M-MDSC compared to PMN-MDSC (A), and ***TGF-β***, being the mRNA levels of this growth factor about 4 times lower in PMN-MDSC compared to M-MDSC (B).

3.2.2. Evaluation of the zeta chain expression in T lymphocytes and NK cells

For the zeta chain, the results were presented in terms of mean fluorescence intensity (MFI) ratios of CD247 expression between T and B cells and between NK and B cells. The B cells were used as a negative control, as they do not express CD247. As it can be seen in Figure 11, the mean fluorescence intensity ratios of CD247 expression between T and B cells and between NK and B cells are significantly lower in the group of cancer patients, comparatively to the group of healthy donors ($P = 0.0004$ and $P = 0.0046$, respectively). This means that there is a reduction in the expression of the zeta chain in both T lymphocytes and NK cells from cancer patients.

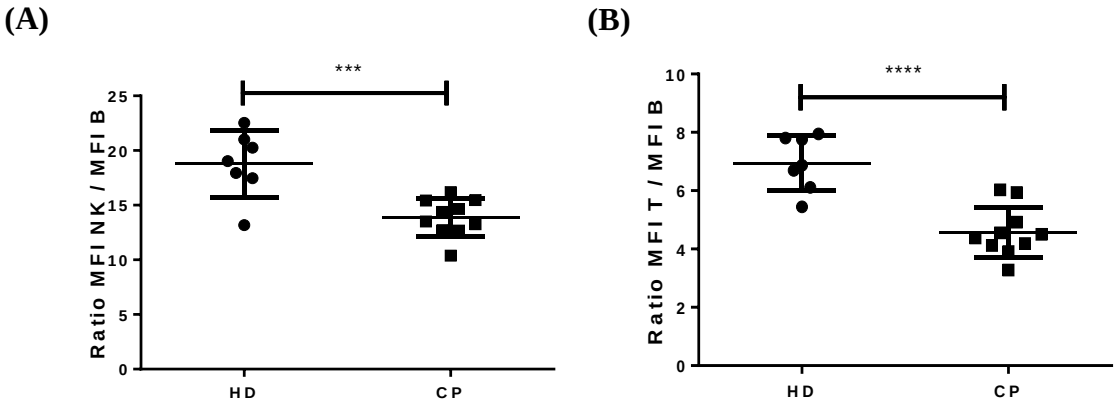

Figure 11. Comparison of the mean fluorescence intensity (MFI) ratios of CD247 expression between NK and B cells (A) and between T and B cells (B) in both healthy donors and cancer patients. Lower ratios for both T lymphocytes and NK cells were obtained in the group of cancer patients, being the differences statistically significant (P = 0.0004 and P = 0.0046 for T- and NK-cells, respectively).

Chapter 4

Discussion

Myeloid-derived suppressor cells are known to be significant contributors to the immunosuppressive tumor microenvironment and are increased in most cancer patients. They are responsible for the negative regulation of immune responses, being correlated with poor clinical outcome and metastatic propensity[6].

In the present work, the expression of LOX-1 receptor was used as a marker that defines PMN-MDSC population, once Condamine and collaborators had previously described the possible usage of this marker for the distinction of human neutrophils and PMN-MDSC without the use of a gradient. That's because PMN-MDSC express LOX-1, while normal neutrophils don't[94]. Additionally, as monocytes are HLA-DR$^+$, its low expression or its absence in CD14 positive cells allowed the discrimination of M-MDSC from monocytes, enabling their identification and quantification. However, it still doesn't exist a marker similar to LOX-1 for the distinction of M-MDSC from monocytes and the cut-off value in the HLA-DR vs CD14 plot it's hard to define, once it is not possible to perform a fluorescence minus one (FMO) control. So, for both populations of MDSC there is still the need to expand the already existing panel of markers to allow the easy phenotypic distinction.

In terms of quantification, although the levels of both subsets of MDSC obtained in the group of cancer patients were significantly higher than the ones obtained in the group of healthy donors, the values were lower than those referred in literature. The percentage of PMN-MDSC in healthy donors had a median value of 0.045% of total of neutrophils, which agreed with the expected low percentage (< 1%). However, in the peripheral blood of cancer patients, these numbers were expected to rise up to 4-15% of total neutrophils[6]. In our group of cancer patients, the median value was of 0.531%, which stills corresponds to less than 1% of the total of neutrophils. This could be due to the gating strategy that leans toward a tighter definition of MDSC. Nevertheless, the percentages obtained for the cancer patients were about twelve times higher than those obtained for the healthy donors. As for M-MDSC,

higher percentages in both cancer patients and healthy donors were obtained relatively to the PMN-MDSC subset. This can be due to the lack of specific markers for the identification of this subset.

It was also expected that the amount of MDSC in the peripheral blood was positively correlated with cancer stage, i.e., high numbers of MDSC were expected in advanced cancer patients or in patients with aggressive cancers. In the present work, at least three groups of patients were analyzed: one composed of colon carcinoma patients, which are patients who already developed metastases and that have not yet initiated chemotherapy; another one composed of MM patients stage ISS-III, the last and most aggressive stage of the disease, and MM patients in disease relapse; and lastly, a group composed of random cancer patients with lower degrees of severity. Comparing the percentages of MDSC between these three groups, it was possible to observe that the groups of advanced or relapsed cancer had higher percentages of both MDSC subsets, which confirms the information that MDSC levels correlate with cancer stage.

MDSC are not only defined by the expression of certain surface markers, but also by some functional characteristics, as Bronte and co-workers described, such as the ability to suppress immune cells, being the inhibition of T cells the gold standard for the evaluation of MDSC function[93]. In the present work, in order to study the immunosuppressive activity of MDSC, the transcription of several molecules implicated in the suppression mechanisms was evaluated by qPCR, on FACS-sorted PMN-MDSC and M-MDSC populations. It was possible to detect the expression of *NOS2* and *TGF-β*, which means that these cells, indeed, possess immunosuppressive activity. According to the literature, the immunosuppression by M-MDSC is more associated with the increased expression of *NOS2* and production of NO, while the immunosuppression by PMN-MDSC is more associated with the increased expression of *ARG1* along with high levels of ROS and PNT. However, in this study, the mRNA levels of *NOS2* were found to be higher in PMN-MDSC than in M-MDSC. As for *TGF-β*, it was verified that in the samples analyzed it was mainly produced by M-MDSC and there was no detection of *ARG1* and *IDO1*. These discrepancies can be due to the low number of biological samples analyzed, since just two of each subset of MDSC were processed. Moreover, the very low quantity of RNA used for the reactions hindered the detection of low abundance transcripts, such as *ARG1* and *IDO1*. For obtaining better results, the ideal would be to perform the cell sorting technique using a bigger volume of peripheral

blood sample, in order to obtain a higher quantity of cells and, consequently, a larger quantity of RNA. Other alternative would be the use of Cells-to-CT kits, that allow the measure of relative gene expression by qPCR, without having to purify RNA prior to amplification. Also, relatively to the *ARG1* detection, some studies refer that it is only expressed by MDSC after the exposure to CD3/CD28 activated T cells.

Another immunosuppressive mechanism of MDSC is related to their capacity to modulate the expression of the zeta chain. CD247 is present in T lymphocytes as a subunit of the T-cell antigen receptor, being required in the TCR complex to guarantee its surface expression and function. However, NK cells also express the zeta chain as a heterodimer co-associated with CD16[104]. Reduced zeta chain levels are functionally relevant because the phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAM) on the intracytoplasmic component of the zeta chain is an important early event in T cells and NK cell activation. For this reason, reduced levels of zeta chain can lead to impaired T and NK cell activation, proliferation and cytokine production, due to the relative lack of tyrosine residues for phosphorylation and, consequently, the reduced recruitment and phosphorylation of downstream signal-transducing molecules, such as ZAP-70[105]. In this work, the zeta chain expression in T lymphocytes and NK cells was evaluated by flow cytometry. The results showed that there was a statistically significant reduction in the expression of the zeta chain in both T lymphocytes and NK cells in the group of cancer patients. Therefore, this could possibly be attributed to the immunosuppressive activity of MDSC, since as we showed in this work, they are in high number in cancer patients and are known to reduce the expression of the zeta chain in T and NK lymphocytes.

Overall, the results obtained in the present work showed that cancer patients present higher levels of MDSC and that these cells are possibly exerting immunosuppressive effects over T lymphocytes and NK cells, as reflected in their decreased expression of zeta chain.

Chapter 5

Conclusions

MDSC are a heterogeneous population with multifaceted phenotypic characteristics and they are recognized as one of the major negative regulators of immune responses in many pathologic conditions, such as cancer[91]. However, there are still challenges related to accurate and reproducible measurements of MDSC levels, since these cells share immunophenotypic markers with other myeloid cells, such as monocytes and neutrophils. In accordance to what has been proposed by Condamine and collaborators, LOX-1 is a candidate marker to distinguish human immunosuppressive PMN-MDSC from normal neutrophils in peripheral blood samples from cancer patients[94]. Also, the expression of CD14 on HLA-DR negative cells allowed the identification of the M-MDSC subset. However, there is still the need to expand the already existing panel of markers to allow for an easier phenotypic distinction. Hence, improved identification and quantification of these cells remains a major priority in this field.

The ability to suppress immune cells, by multiple mechanisms, is a key feature of MDSC. In this work, the immunosuppressive activity of these cells was assessed by the increased transcription of some molecules, namely *NOS2* and *TGF-β*, and by the reduced expression of the zeta chain in both T- and NK-cells. However, these functional assays need to be further optimized and expanded to a higher number of biological samples to confirm the observed tendencies.

Overall, despite the limitations and experimental difficulties encountered, we have confirmed that circulating MDSC were increased in cancer patients compared to healthy subjects, which supports the relevance of their inclusion in the cancer immunogram, currently under development. Also, this work has contributed to highlight a few potentially useful MDSC phenotypic and functional markers, to be validated in larger sample sets. Finally, the fact that MDSC were almost absent in healthy/homeostatic conditions represents a unique opportunity to target these cells without possible side effects. In fact, in recent years, the combination of MDSC targeting with immune checkpoint inhibitors treatment has

already proven valuable to overtake the therapeutic resistance that occurs in the majority of cancer patients[106].

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