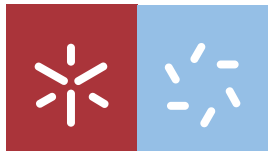
Unravel the role of hypoxia in the tumor microenvironment: a focus on macrophage-cancer cell interplay

Rosa Oliveira

UMinho | 2018



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Doutora Ângela Margarida Amorim Costa
Professora Doutora Ana Paula Fernandes Monteiro Sampaio Carvalho

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DECLARAÇÃO

Nome: Rosa Alexandra Costa Oliveira

Endereço eletrónico: rosa.alexandra_23@hotmail.com Telemóvel: +351 914891851

Cartão do Cidadão: 14389639

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Orientadoras:

Doutora Ângela Margarida Amorim Costa

Professora Doutora Ana Paula Fernandes Monteiro Sampaio Carvalho

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Resumo

"Desvendar o papel da hipoxia no microambiente tumoral com destaque na interação entre macrófagos e células tumorais"

O cancro não resulta apenas de um conjunto de aberrações genéticas, mas também de outros elementos do microambiente tumoral hipóxico, como componentes da matriz extracelular, fibroblastos e células imunes, que afetam as células tumorais, modificando as suas atividades, e influenciando a progressão tumoral e a resposta terapêutica. Apesar de sabermos o impacto que os elementos deste microambiente têm na progressão tumoral, os mecanismos subjacentes que dirigem essas interações são pouco compreendidos, e a sua perceção pode ser traduzida em novas ou mais eficientes terapias. Os macrófagos são uma das células mais abundantes no microambiente tumoral e, dependendo do estímulo a que estão sujeitos, podem ser classificados em pró-inflamatórios (M1) ou anti-inflamatórios (M2). Apesar da hipoxia e dos macrófagos estarem independentemente associados à progressão tumoral, e de os macrófagos serem preferencialmente recrutados para áreas tecidulares com baixos níveis de oxigénio, não há informação suficiente sobre a influência da hipoxia na interação entre macrófagos-células tumorais e na invasão de células tumorais mediada por macrófagos. Portanto, para compreender como a hipoxia pode influenciar a interação entre macrófagos e células tumorais, foi estabelecido um sistema de co-cultura com células tumorais e macrófagos, obtidos através de doadores de sangue saudáveis. Os macrófagos foram mantidos em monocultura e em co-cultura indireta com uma linha celular de cancro colorretal (RKO) em normoxia (20% O_2) ou hipoxia (1% O_2), permitindo a recolha de material biológico para caracterizar ambas as populações. Em primeiro lugar, a nossa experiência foi validada quanto à expressão de marcadores de hipoxia nos macrófagos e nas células tumorais. Os nossos resultados sugerem que a hipoxia desencadeia um fenótipo mais pró-inflamatório nos macrófagos, concomitantemente com alterações na sua função como: fagocitose, capacidade de apresentação de antigénios e regulação da resposta imune. A hipoxia e as co-culturas revelaram um impacto na expressão nos macrófagos de SIRP1α, CD47, MHC-II e PD-L1. Além disso, a caracterização das células tumorais RKO revelou que a hipoxia potencia invasão, expressão de genes envolvidos na transição epitelial para mesenquimal e expressão de genes com atividade proteolítica.

Os nossos resultados demonstram que a hipoxia é uma característica importante a ser considerada no estudo das interações do microambiente tumoral.

Palavras-chave: microambiente tumoral, hipoxia, macrófagos, cancro colorretal, invasão de células tumorais

Abstract

Cancer is a consequence not only of genetic aberrations but also of other elements of the surrounding hypoxic tumor microenvironment such as extracellular matrix components, fibroblasts, and immune cells, which affect cancer cells, modulating their activities and influencing tumor progression and therapy response. Despite this knowledge, underlying mechanisms governing these interactions are poorly understood, and their understanding can be translated into new or more efficient therapies. Macrophages are one of the most abundant cells in the tumor microenvironment and depending of the stimuli that they are subjected to, can either present a pro-inflammatory (M1) or anti-inflammatory (M2) phenotype. Although hypoxia and macrophages are independently associated with tumor progression, and macrophages are preferentially recruited to low oxygen areas, there is insufficient information about the influence of hypoxia on macrophage-cancer cell crosstalk, and on macrophage-mediated cancer cell invasion. Therefore, in order to understand how hypoxia can influence the crosstalk between tumor cells and macrophages a co-culture system with cancer cells and human macrophages, derived from healthy blood donors was established. Macrophages were cultured in monoculture or in indirect co-culture with CRC cell line (RKO) in normoxia (20% O_2) or hypoxia (1% O_2), enabling the collection of biological material to characterize both populations.

Firstly, our experimental setup was validated regarding the response of cancer cells and macrophages to hypoxia. Our results suggest that hypoxia triggers a more pro-inflammatory phenotype on macrophages, along with alterations in macrophages function hallmarks: phagocytosis, antigen presenting capacities, and immune response regulation. Hypoxia and co-cultures modulate macrophage expression of SIRP1α, CD47, MHC-II, and PD-L1. In addition, the characterization of RKO cells was also evaluated and revealed that hypoxia potentiates invasion, expression of EMT genes and expression of proteolytic genes.

Our results demonstrate that hypoxia is a critical feature to have in consideration when studying the tumor microenvironment interactions.

Keywords: tumor microenvironment, hypoxia, macrophages, colorectal cancer, cancer cell invasion

List of contents

List of Figures

List of Tables

List of Abbreviations

ADM – Adrenomedullin

APC – Adenomatous Polyposis Coli

APCs – Antigen-Presenting Cells

ATCC – American Type Culture Collection

BSA – Bovine Serum Albumin

CAFs – Cancer Associated Fibroblasts

CAIX/CA9 – Carbonic Anhydrase IX

CCL – C-C Chemokine Ligand

CCR – C-C Chemokine Receptor

CD – Cluster of Differentiation

CIMP – CpG Island Methylator Phenotype

CIN – Chromosomal Instability

CM – Conditioned Media

CRC – Colorectal Cancer

CTLA-4 – Cytotoxic T Lymphocyte Antigen-4

CXCL – C-X-C Chemokine Ligand

DAPI – 4', 6-Diamidino-2-Phenylindole

DCs – Dendritic Cells

ECM – Extracellular Matrix

EGF – Epidermal Growth Factor

ELISA – Enzyme-linked Immunosorbent Assay

EMT – Epithelial-Mesenchymal Transition

FACS – Fluorescence-Activated Cell Sorting

FBS – Fetal Bovine Serum

FDA – US Food and Drug Administration

FGFs – Fibroblast Growth Factors

FITC – Fluorescein Isothiocyanate

GLUT-1 – Glucose Transporter 1

HIF – Hypoxia-Inducible Factor

HL – Hodgkin Lymphoma

HLA – Human Leukocyte Antigen

HNSCC – Head and Neck Squamous Cell Carcinoma

HRE – Hypoxia Response Elements

IDT – Integrated DNA Technologies

IFN-γ – Interferon - gamma

IL – Interleukin

ITIM – Immunoreceptor Tyrosine-based Inhibitory Motif

ITSM – Immunoreceptor Tyrosine-based Switch Motif

LDHA – Lactate Dehydrogenase A

LOH – Loss of Heterozygosity

LOX – Lysyl Oxidase

LPS – Lipopolysaccharide

M-CSF – Macrophage Colony-Stimulating Factor

MDSCs – Myeloid-Derived Suppressor Cells

MFI – Mean Fluorescence Intensity

MHC – Major Histocompatibility Complex

MMPs – Matrix Metalloproteinases

MSI – Microsatellite Instability

NHL – Non-Hodgkin's lymphoma

NK – Natural Killers

NSCLC – Non-small Cell Lung Cancer

PBS – Phosphate-Buffered Saline

PD-1 – Programmed Cell Death Protein 1

PD-L – Programmed-Death Ligand

PDGFs – Platelet Derived Growth Factors

PFA – Paraformaldehyde

PHDs – Prolyl-4-Hidroxilase Domains

qRT-PCR – Quantitative Real-Time Polymerase Chain Reaction

RPM – Rotations Per Minute

RT – Room Temperature

SEMA3A – Semaphorin 3A

SHP – Tyrosine-protein phosphatase non-receptor type

SIRP1α – Signal Regulatory Protein 1 alpha

SLC2A1 – Solute carrier family 2

TAMs – Tumor Associated Macrophages

TAP – Transporter Associated with Antigen Presentation

TGF-β – Transforming Growth Factor–Beta

TIMPs – Tissue inhibitors of Matrix metalloproteases

TLR – Toll-like Receptor

TNF-α – Tumor Necrosis Factor-alpha

TP – Tumor Protein

Treg – Regulatory T cells

UC – Urothelial Carcinoma

VEGF – Vascular Endothelial Growth Factor

VHL – Von Hippel-Lindau

Introduction

In the last decades, a great effort has been made to find better and novel treatment options for cancer patients. However, due to its complex and dynamic nature, cancer remains in most of the cases an incurable disease. Nevertheless, significant advances have been made in this field with incredible progress to increase life rate and patient's health quality of life.

Cancer is the result of genetic alterations, and cancer cells have specific hallmarks and enable characteristics associated with their transformation and disease: i) resistance to cell death; ii) induction of angiogenesis; iii) inducing and sustaining proliferative signaling; iv) escape to growth suppressors; v) capacity of unlimited replicative potential; vi) induction of tissue invasion and metastasis ; vii) tumor-promoting inflammation; viii) genome instability and mutation; ix) evading immune destruction; x) deregulating cellular energetics (Hanahan & Weinberg, 2011).

1. The tumor microenvironment

The tumor microenvironment is composed by malignant and non-transformed cells such as fibroblasts, adipocytes, endothelial cells, immune cells, supported by an acellular extracellular matrix **(Figure 1).** In the last years, the tumor microenvironment has been the focus of several studies in the cancer research area, due to its critical role in immune evasion, therapy resistance, and metastasis into distant organs, underlying the urgent need for a better knowledge of this field (Allen & Jones, 2011;Chen *et al.*, 2015). The non-tumor cells interact with malignant cells through the secretion of cytokines, chemokines, growth factors, and matrix remodeling enzymes present in the stroma, accounting for malignant progression (Balkwill *et al.*, 2012). The tumor microenvironment provides to cancer cells essential factors for the proliferation, invasion and metastasis, namely oxygen and nutrients, soluble matrix-bound growth factors, and enzymes, via the vasculature existent in this environment (Li *et al.*, 2007; Watnick, 2012). The understanding of the molecular mechanisms underlying this complex interaction is one of the major challenges in cancer research (Kessenbrock *et al.*, 2010; Samples *et al.*, 2013).

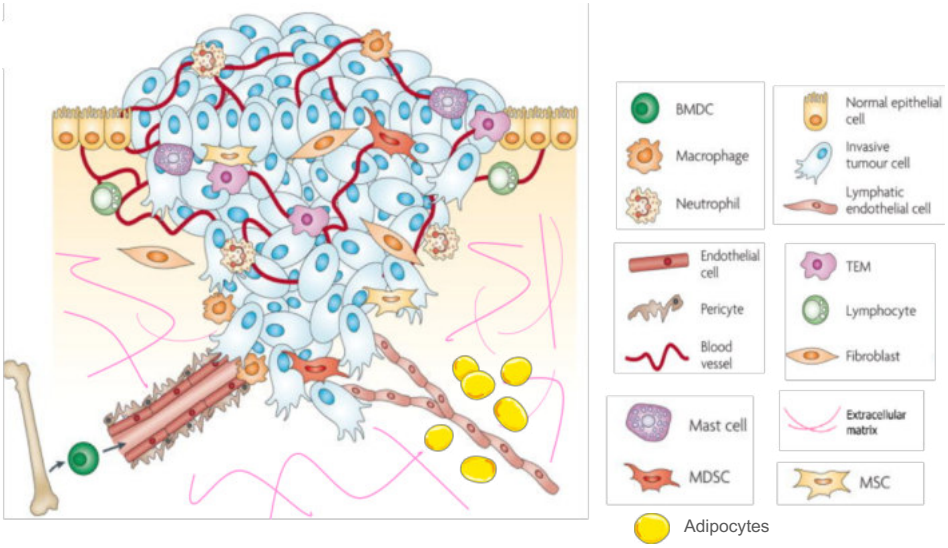


Figure 1: The tumor microenvironment. The tumor microenvironment is characterized by a complex interplay between malignant and non-malignant cells. The cellular components of the tumor microenvironment are fibroblasts, endothelial cells, pericytes, and a diversity of immune cells, including macrophages, and mesenchymal stem cells (MSC). The extracellular matrix, a non-cellular component, is the most abundant element present at the tumor microenvironment. BMDC: Bone marrow-derived cells; MDSC: Myeloid-Derived Suppressor Cells; TEM: TIE-2 expressing monocytes. Adapted from (Balkwill *et al.*, 2012; Joyce & Pollard, 2009; Xu *et al.,* 2014).

1.1 Cellular components and non-cellular components of the tumor microenvironment

The tumor microenvironment is composed by malignant and non-malignant cells that are attracted to this milieu and can be corrupted to become malignant. Each component is distinguished by cell-type-specific markers, often expressed as cell surface molecules and each type of cells has different effects to potentiate cancer progression (Balkwill *et al.,* 2012).

Fibroblasts among other cells constitute the tumor stroma and are mainly responsible for the deposition of extracellular matrix (ECM) components, regulation of epithelial cell differentiation, regulation of inflammation and are involved in wound healing activities. Fibroblasts within the tumor stroma acquire a modified phenotype and become perpetually activated (Kalluri & Zeisberg, 2006). Several growth factors secreted by cancer cells such as transforming growth factor (TGF-β), platelet-derived growth factor (PDGF) and fibroblast growth factor 2 (FGF2) have been described as key mediators of fibroblast activation and tissue fibrosis. The fibroblasts that

are found in association with cancer cells are known as cancer-associated fibroblasts (CAFs) (Kalluri & Zeisberg, 2006). CAFs secrete several molecular mediators as growth factors, chemokines, cytokines and matrix metalloproteinases (MMPs), which altogether promote an enhancement of proliferation, invasion, migration, immune evasion and epithelial-mesenchymal transition (EMT) (Balkwill *et al.*, 2012; Gascard & Tlsty, 2016).

The adipose tissue is also an important element at the tumor microenvironment. Adipocytes stimulate the growth and the survival of tumor cells through the release of different adipokines, which provide fatty acids that can be metabolized and used as an energy source, promoting rapid tumor growth (Corrêa *et al.*, 2017; Nieman *et al.*, 2011). In addition to provide energy to tumor cells, adipocytes are also involved in the promotion of cell proliferation, angiogenesis, survival, migration, invasion and inhibition of immune-mediated destruction (Corrêa *et al.*, 2017; Duong *et al.*, 2017). Adipocytes have also been implicated in the process of recruitment and metastasis to the omentum (organ primarily composed of adipocytes), especially in intra-abdominal tumors, as the case of ovarian cancer (Balkwill *et al.*, 2012; Nieman *et al.*, 2011).

Cancer growth is dependent of the development of a complex vascular network (neovascularization) termed as angiogenesis. Several soluble factors present at the tumor microenvironment, as vascular endothelial growth factors (VEGFs), FGFs, PDGFs and chemokines, are necessary to stimulate endothelial cells and their associated pericytes to contribute for the development of such new vessels. The most predominant angiogenic factor in the tumor microenvironment is VEGF, which is secreted by tumor and immune cells within the tumor stroma. Since VEGF is a pro-angiogenic signal, when quiescent endothelial cells sense this signal, the angiogenesis process takes place. However, in cancer, the vessels resulting from the angiogenesis process are abnormal regarding their structure and function. The abnormal tumor vessels are characterized by an increase of interstitial fluid pressure, heterogenous blood flow, irregular oxygen supply, which promotes hypoxia, leading to tumor invasion and metastasis (Balkwill *et al.*, 2012; Carmeliet & Jain, 2011; Jain, 2005).

Additionally to its supporting role in maintaining tissue structure, the ECM is involved in the regulation of tissue homeostasis, organ development, inflammation and disease (Gilkes *et al.*, 2014; Lu *et al.*, 2012). The ECM is composed by fibrous proteins (such as collagens, elastins, fibronectins and laminins), elastic proteins (such as elastin and laminins), proteoglycans and several soluble factors (namely growth and pro-angiogenic factors, cytokines and chemokines) that are secreted locally (Balkwill *et al.*, 2012; Gilkes *et al.*, 2014).

The ECM controls cell growth, apoptosis, structure, polarity and, in addition, can also promote tumor progression (Bonnans *et al.*, 2014). The ECM remodeling is balanced by the presence of proteolytic enzymes, such as MMPs, or the existence of its inhibitors as Tissue inhibitors of Matrix metalloproteases (TIMPs) (Lu *et al.*, 2012). The abnormal ECM dynamics and the importance and consequences of this dysregulation have been described with a role in cancer initiation and progression (Lu *et al.*, 2011). The density of the ECM and fibrosis, which consists of enhanced ECM fibers deposition, are described as important regulators in breast, liver and pancreas tumor initiation. In addition to initiation, the content, organization and biomechanical properties, such as the presence of hyaluronic acid and the stiffness of the ECM, contribute to tumor progression (Martin *et al.*, 2016).

One of the most important constituents of the tumor microenvironment is the immune system, responsible for immunosurveillance, recognition and elimination of the tumor cells. However, at sites of chronic inflammation, as frequently is the tumor microenvironment, inflammatory cells may be modulated towards an immunosuppressive phenotype, supporting tumor progression (Wang *et al.*, 2017). The adaptive and innate immune systems have been described as contributors in cancer immunoediting, which is comprised in three steps: elimination, equilibrium and escape. The first step concerns the role of the immune system in the removal of the first tumor cells through different inflammatory and signaling molecules. Nevertheless, additionally to protect the host, the immune cells edit the immunogenicity of tumors that may eventually appear, in a process named "cancer immunoediting" (Dunn *et al.*, 2004; Wang *et al.*, 2017). The second step is concerning the equilibrium stage where tumor cells are not completely eliminated but the growth of tumor cells is controlled. The last step include several mechanisms performed by tumor cells to escape immune-mediated destruction (Wang *et al.*, 2017).

The tumor microenvironment is characterized by the presence of innate immune cells, such as macrophages, neutrophils, mast cells, Myeloid-derived Suppressor Cells (MDSCs), Dendritic Cells (DCs), and Natural Killer (NK) cells and it is also characterized by the presence of adaptive immune cells, including T and B lymphocytes (Grivennikov *et al.,* 2010; Wang *et al.,* 2017). During the early stage of neoplasia in the tissue, the cancer-associated antigens existent at the tumor environment are transported to lymphoid organs by DCs which activate the adaptive immune activity, resulting in both tumor-promoting responses, as tissue expansion, malignant transformation, increase of tumor growth, and also in anti-tumor responses. Therefore, in

secondary lymphoid organs, T and B-lymphocytes are activated and due to the constant activation stimulated by the cancer-associated antigens the innate immune system enters in a state of chronic activation in the neoplastic tissues. These constantly activated innate immune cells, such as mast cells, granulocytes and macrophages, stimulate tumor development by the release of potent pro-survival soluble molecules that leads to genomic instability, cell-death inhibition, fibroblast activation, angiogenesis and tissue remodelling with alterations in the matrix metabolism (De Visser *et al.,* 2006). In addition, activation of adaptive immunity also potentiates antitumor responses through T-cell-mediated toxicity, by the induction of perforin, which is a glycoprotein capable of developing pores in the cell membranes of the target cells (Osińska *et al.*, 2014), and/or for the activation and release of cytokines (De Visser *et al.,* 2006).

2. Macrophages: origin and functions

Macrophages are present in essentially all tissues and have an important role as immune effector cells. These cells are very plastic and, depending on the type of stimulator, can acquire distinct phenotypes, and affect the soluble factors released in the milieu (Mosser & Edwards, 2008). There are three main different types of macrophages in the organism: 1) resident macrophages, which derives from fetal hematopoietic organs (precursors in the yolk sac), present during embryogenesis, which differentiate into specialized forms in particular tissues; 2) macrophage produced in the bone marrow and recruited to the inflammatory reactions; 3) macrophages with yolk sac and fetal liver origin that seems to contribute to adult Langerhans cells (Abbas *et al.,* 2014 ;Wynn *et al.*, 2013).

In the bone marrow, hematopoietic stem cells which will give rise to activated macrophages, suffers a maturation process, turning from granulocyte/macrophage colony-forming units, into monoblast, pro-monocytes and finally monocytes, which will be released from the bone marrow into the bloodstream. The monocytes released in the bloodstream will be recruited into different tissues, in response to inflammatory reactions, differentiating into macrophages that will become fully activated (Abbas *et al.*, 2014; Mosser & Edwards, 2008).

Macrophages have different functions, being the phagocytic activity one of their most characteristic features, which allows them to ingest and destroy pathogens. Nevertheless, macrophages can also ingest dead cells or cellular debris, resulting from apoptosis, which is necessary for many physiologic processes, such as development, growth, and renewal of healthy tissues, and works as a cleaning up process to prevent inflammatory responses (Abbas *et al.,*

2014). Additionally, monocytes and macrophages are described as antigen-presenting cells (APCs), being responsible for the degradation of foreign material, and for the presentation of antigenic epitopes to immunocompetent cells, in order to initiate an immune response (Flaherty,2012).

Furthermore, macrophages produce several different cytokines and chemokines that increase the recruitment of monocytes and other leukocytes to the sites of infections, resulting in an upscale of the immune response. Additionally, these cells also indorse the repair of damaged tissues, through the releasing of cytokines that stimulate angiogenesis, and synthesis of a collagen-rich extracellular matrix (fibrosis) (Abbas *et al.,* 2014).

2.1 Macrophages polarization

Macrophages can present different functional phenotypes depending on the local environment signals that they receive, which turns these plastic cells interesting therapeutic targets to explore. Depending on the polarization status, and inflammatory activity, macrophages can be classified as pro-inflammatory or M1-like and anti-inflammatory or M2-like (Wang *et al.*, 2014). This nomenclature (M1/M2) is derived from the immune mediators released by Th1 and Th2 cells that are associated with these macrophage phenotypes (Brundu S, 2015). The M1-like macrophages exhibit bactericidal, tumor suppressive and immunogenic activities, and are characterized by high antigen presentation, high production of pro-inflammatory interleukin (IL)-1, IL-6, IL-12, IL-23, nitric oxide and reactive oxygen intermediates, and low production of anti-inflammatory IL-10. These macrophages are simulated by microbial-derived factors and/or cytokines like Interferon gamma (IFNγ), Toll-like Receptor Ligands (TLR), lipopolysaccharides (LPS), or Tumor Necrosis Factor alfa (TNF-α). In addition, M1-like macrophages express specific surface receptors, namely the co-stimulatory cluster of differentiation (CD) 80, CD86 and also the Major Histocompatibility Complex (MHC)- class I and II (Fraternale *et al.*, 2015; Mantovani *et al.*, 2002). Macrophages can also polarize into the M2 phenotype in response to IL-4, IL-13, IL-10, Transforming Growth Factor β (TGF-β), Macrophage Colony-Stimulating Factor (M-CSF), or glucocorticoids. They also produce anti-inflammatory cytokines such as IL-10, IL-8 and IL-2 and low levels of the pro-inflammatory IL-12, IL-6 and IL-23 (Escribese *et al.*, 2012; Mantovani *et al.*, 2004; Wang *et al.*, 2014). Additionally, they also exhibit high levels of the mannose receptor (CD206), a receptor mainly expressed by macrophages and dendritic cells, with an important role in the endocytosis and phagocytosis and described as highly expressed in cancer, and high

levels of the scavenger receptor (CD163), highly expressed on most of tissue resident macrophages and mainly responsible for homeostatic functions and with also important players in the resolution of inflammation (Azad *et al.*, 2014; Fabriek *et al.*, 2005; Mantovani *et al.*, 2004). These macrophages are described to promote the acquisition of anti-inflammatory, scavenging, tumor-promoting, tissue repair and pro-angiogenic functions, a process called "alternative" or M2 polarization (Escribese *et al.*, 2012; Mantovani *et al.*, 2002). Macrophages are described by a variety of different functions and, therefore, the classification into M1/M2 phenotype has been considered an oversimplification **(Figure 2).** This classification corresponds to the extreme phenotypes of a continuum spectrum of responses (Braster *et al.*, 2015; Weagel *et al.*, 2015). Therefore, M2 macrophages might be subdivided into distinct subpopulations **(Figure 2),** each induced by a different set of molecules and with different activation responses: i) the M2a macrophages, associated with killing and encapsulation of parasites; ii) the M2b, involved in immune regulation; iii) the M2c, associated to immune suppression, tissue repair and matrix remodeling; and finally iv) the M2d which resembles tumor associated macrophages (TAMs) functions, promoting angiogenesis and tumor growth (Mantovani *et al.*, 2004; Weagel *et al.*, 2015; Fraternale *et al.*, 2015).

For a complete and rigorous macrophage characterization, it is essential to evaluate their morphology, gene expression profile, protein surface markers, as well as analyze the secreted inflammatory cytokines and chemokines.

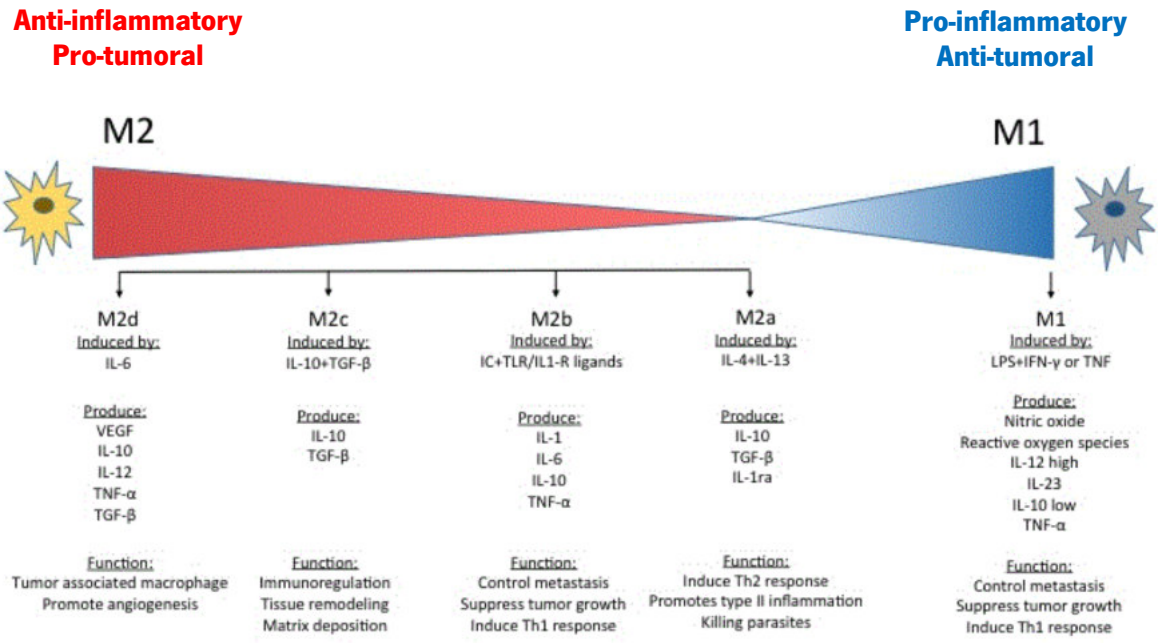


Figure 2: Spectrum of macrophage polarization. Characterization of macrophage polarization spectrum. Macrophages are induced by different stimuli allowing the classification into different subpopulations, defined by the surface receptors they express, the factors they secrete and their function. Adapted from (Weagel *et al.,* 2015).

2.2 Tumor-associated macrophages

Monocytes are recruited to tumor tissues in response to a variety of cytokines, chemokines, and growth factors produced by stromal and tumor cells at the tumor microenvironment (Aras & Raza Zaidi, 2017). Attracted by the presence of chemokine C-C motif ligand (CCL)-2, M-CSF, IL-6, VEGF-A, PDGF, monocytes will differentiate into TAMs (Aras & Raza Zaidi, 2017). In addition, other chemokines and cytokines, such as CCL3, CCL4, CCL5 or Rantes, CCL7, CCL8, C-X-C Chemokine Ligand (CXCL)12, and IL-10, also promote monocyte recruitment to the tumor microenvironment (Hao *et al.*, 2012).

At the beginning of a malignant transformation process, a pro-inflammatory environment could be a trigger, due to the genetic instability caused by the inflammatory process. However, once the tumor is established, an anti-inflammatory environment becomes pro-tumorigenic, contributing to immune evasion. It has been suggested that inflammation can explain the origin of many cancers. Patients with chronic infection or persistent irritation are associated with higher

cancer risk, and as macrophages are major players in the inflammatory process, they have been suggested as having a critical role in cancer induced by inflammation. It has been conjectured that immune cells can create a mutagenic environment since they produce reactive nitrogen and oxygen species during the inflammatory response, which are highly mutagenic in the adjacent epithelial cells, contributing to their transformation. Nevertheless, not all cancers are associated with chronic inflammation and can be derived from another type of spontaneous or hereditary alterations (Qian & Pollard, 2010).

Therefore, the tumor microenvironment possesses macrophages that can either be M1- or M2-like. Nevertheless, it has been evidenced that TAMs resemble generally M2-polarized macrophages, since they express some markers typical of this phenotype such as CD163, Fc fragment of IgG, C-type lectin domains, and heat shock proteins. In addition, some anti-inflammatory chemoattractants, present at the tumor microenvironment such as IL-4, IL-13, TGF-β, and IL-10 lead to the adoption of an M2 phenotype (Hao *et al.*, 2012; Qian & Pollard, 2010; Yang & Zhang, 2017).

Once the tumor is established, cancer cells are able to recruit and benefit from the presence of macrophages, which may promote angiogenesis, enhance cancer cell stemness, promote their epithelial to mesenchymal transition, immunosuppression activities, migration, invasion, and contributing to their establishment at the pre-metastatic niche (seed and soil paradigm), favoring metastasis **(Figure 3).** Angiogenesis is induced when TAMs release pro-angiogenic factors, such as VEGF, adrenomedullin (ADM), PDGF and other factors as chemokines, TGF-β, semaphorin 3A (SEMA3A) and MMPs. Tumor migration and invasion occurs due to the secretion of MMPs, serine proteases and cathepsins and other factors, which modify the cell–cell junctions and promote basal membrane disruption. Some reports described that TAMs are involved in the EMT process, through the involvement of TLR4/IL-10 signaling pathway, and through the expression of TGF-β1, in hepatocellular carcinoma. Through the involvement of EGF, CCL18 and P2Y2 receptor, TAMs are also involved in the process of intravasation and extravasation, which consist in cancer cell entry into and escape from the blood vessels, a step required for metastasis (Aras & Raza Zaidi, 2017). Macrophages have also been appointed as pioneers in preparing the premetastatic niche by the secretion of chemotactic factors, as CXCL1, and the deposition of ECM components, as fibronectin, which then recruit circulating tumor cells to the metastatic site (Aras & Raza Zaidi, 2017; Psaila & Lyden, 2009). Notably, TAMs might be involved in immunosuppression either by direct interaction with T cells,

or by releasing immunosuppressive cytokines, such as IL-10, TGF-β, proteases, Arginase-1 and prostaglandins (Aras & Raza Zaidi, 2017). In addition, programmed-death ligand (PD-L)–1/PD-L2 and CD80/CD86, ligands of PD-1 and cytotoxic T lymphocyte antigen-4 (CTLA-4), respectively, have been also described as expressed by TAMs which lead to the inhibition of immune responses due to the interaction between the ligand and the receptor. The engagement between the ligand and the receptor, leads to T cell dysfunction, due to the high expression of both in the tumor microenvironment, enabling this cell to detect tumor cells and destroyed them (Aras & Raza Zaidi, 2017; Dong *et al.*, 2015; Sun *et al.*, 2018). A report of glioma cancer has described a positive correlation between infiltration of macrophages and induction of initial cancer stem cells. Stimulation of cancer cell stemness has been associated with the presence of TGF-β, IL-10, the lactadherin protein MFG-E8 and IL-6 (Aras & Raza Zaidi, 2017).

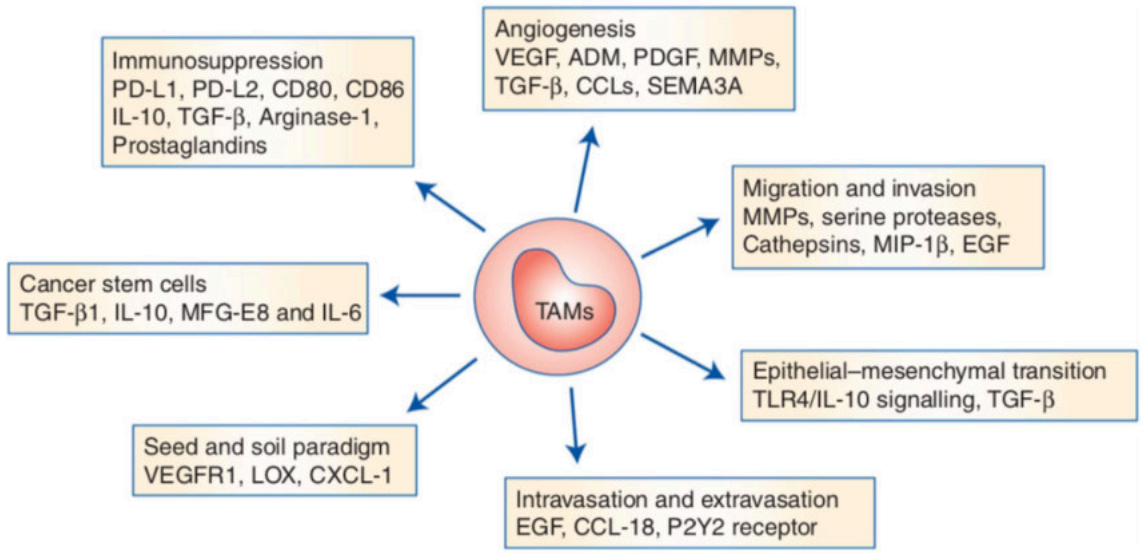


Figure 3: The distinct mechanisms through which TAM may contribute to tumor progression. The impact of tumor microenvironment in manipulating macrophages to acquire a specific phenotype and perform different functions that contributes to tumor progression. VEGF: vascular endothelial growth factor; ADM: adrenomedullin; PDGF: platelet-derived growth factor; MMPs:matrix metalloproteinases; TGF- β: Transforming-Growth factor β; CCLs: Chemokines; IL: Interleukin; PD-L1/PD-L2: Programmed death ligand 1 or 2; MIP-1β: Macrophage-derived protein; EGF: Epidermal Growth Factor; TLR4: Toll-like receptor 4; P2RY2: Purinergic receptor; LOX: Lysyl oxidase; MFG-E8: globule-epidermal growth factor–VIII. Reproduced from (Aras & Raza Zaidi, 2017).

There are evidences in some cancers, as breast, thyroid, lung, and hepatocellular, that increased macrophage density is correlated with poor prognosis and reduced survival. On the other hand, there are exceptions where a high density of macrophages is associated with increased survival like in pancreatic cancer for example (Qian & Pollard, 2010). In the case of colorectal cancer (CRC), the presence of macrophage infiltration is still controversial, since several reports indicate that increase of macrophage density is correlated with poor survival. On the other hand other reports described that the presence of infiltrated macrophages in the tumor microenvironment is associated with an improved survival in CRC patients (Zhong *et al.*, 2018). Notably, recent studies of our group indicate that rather than the density, the immune profile of the existent macrophages, in particular the loss of the pro-inflammatory phenotype, is actually the most relevant feature (Pinto *et al,* not published).

2.3 Mechanisms of immune escape

In the last years, it has been described that additionally to immunosurveillance, the immune system can select for tumors with decreased antigenicity and/or immunogenicity and therefore, promote tumor progression (Beatty & Gladney, 2015). Tumor cells express nonmutated and mutated antigens which trigger the immune-mediated recognition and elimination. However, cancer cells can lose their antigenicity to avoid immune destruction. The immune selection of cancer cells that lack or mutate immunogenic tumor antigens, as well as, an inefficient antigen presentation, due for example to the loss of MHC expression or dysregulation of antigen processing machinery, could lead to a loss of antigenicity. Additionally, the tumor cells that are still recognized by the immune system can escape elimination by decreasing their immunogenicity, through the upregulation of the immunoinhibitory molecules such as PD-L1 on cancer cells, or the inhibition of phagocytosis through the upregulation of the CD47 molecule on cancer cells surface (Beatty & Gladney, 2015; Chao *et al.*, 2012).

2.3.1 CD47-SIRP1α interaction

Tumor cells developed diverse mechanisms to evade immunosurveillance. Despite the recent reports describing the crucial role of macrophages in controlling tumor growth with phagocytic clearance, tumor cells are able to inhibit their phagocytosis, specifically through the CD47-SIRPα pathway (Chao *et al.*, 2012). CD47 is a cell surface molecule of the immunoglobulin superfamily, which function as an anti-phagocytic signal, the “don’t eat me”

signal. Is generally expressed on normal tissues, and is associated with diverse functions as cell migration, T cell and DC activation and axon development (Chao *et al.*, 2012; Liu *et al.*, 2017). Additionally, it has been associated with inhibition of phagocytosis through the ligation to the signal-regulatory protein alpha (SIRPα) expressed on phagocytes. The signaling cascade that results in the inhibition of phagocytosis is due to the phosphorylation of the immunoreceptor tyrosine-based inhibition motifs, present on the cytoplasmic tail of SIRPα, and subsequently binding and activation of Tyrosine-protein phosphatase non-receptor (SHP)—1 and SHP-2 that blocks the engulfment by macrophages and DC, possibly by preventing the accumulation of myosin-IIA at the phagocytic synapse (Chao *et al.*, 2012; Willingham *et al.*, 2012). The expression levels of CD47 have been described as increased in several tumor types such as acute myeloid leukemia, chronic myeloid leukemia, acute lymphoblastic leukemia, non-Hodgkin's lymphoma (NHL), multiple myeloma and in solid tumors, including colon carcinoma (Chao *et al.*, 2012; Willingham *et al.*, 2012). Upregulation of CD47 expression was associated with poor prognosis in some of these malignancies and also appears to influence tumor growth and dissemination (Chao *et al.*, 2012; Murata *et al.*, 2014).

Targeting CD47–SIRPα pathway could be a promising therapeutic strategy for various human cancers, and several therapies have been developed to inhibit the CD47-SIRPα pathway. These therapies are based in the direct block of CD47 through monoclonal antibodies, but also based in a recombinant SIRPα protein that can also bind and block CD47 interaction (Chao *et al.*, 2012), demonstrating that the interaction between "don't eat me signal" on tumor cells and SIRPα on phagocytes impairs a rapid and efficient phagocytosis of multiple tumor cells types, and therefore their elimination (Chan *et al.*, 2009; Chao *et al.*, 2010, 2012).

2.3.2 MHC-I and MHC-II

MHC class I and class II proteins have a crucial role in the immune system, both classes of proteins are responsible for presenting peptides on the cell surface to allow the recognition by T cells and, therefore, initiate the immune-mediated destruction (Wieczorek *et al.*, 2017). MHC class I and II genes in humans, are identified as human leukocyte antigens (*HLAs*) and MHC class I are encoded by three polymorphic genes namely *HLA-A, -B*, and *-C* and MHC class II are also encoded by three polymorphic genes namely *HLA-DR, -DP*, and *-DQ*, and each protein binds to a different range of peptides (Neefjes *et al.*, 2011). MHC class I molecules are expressed by all

nucleated cells and are recognized by cytotoxic $CD8^+$ T cells, due to the protein fragments of cytosolic and nuclear origin expressed at the cell surface by this protein (Neefjes *et al.*, 2011; Wieczorek *et al.*, 2017). The antigens expressed at the cell surface by MHC-I are degraded by cytosolic and nuclear proteasomes, and the resulting peptides are translocated into the endoplasmic reticulum through a transporter associated with antigen presentation (TAP) and loaded on to MHC class I molecules. At first, the MHC class I heterodimer is assembled, the peptides bind to this molecule, and the peptide (antigen)–MHC class I complexes leave the endoplasmic reticulum for presentation at the cell surface (Neefjes *et al.*, 2011). MHC class II molecules are primarily expressed by professional APCs, as DCs, macrophages and B cells, and are recognized by $CD4^+$ T cells. In opposition to MHC class I, the fragments expressed at the cell surface are from exogenous origin (Neefjes *et al.*, 2011; Wieczorek *et al.*, 2017). Loss and defects in the antigen presentation is a common and important mechanism used by tumor cells to avoid immune recognition and destruction (Reeves & James, 2016). Several mechanisms can lead to an inefficient antigen presentation by tumor cells, as lack of expression of tumor antigens; downregulation or mutations of MHC genes which may lead to low or no expression of MHC molecules; modifications in the loading of tumor antigens on MHC molecules. Also downregulation of co-stimulatory molecules can inhibit the MHC signaling to T cells (de Charette *et al.*, 2016). Several tumors have been described with a total loss of MHC class I expression such as melanoma, colorectal carcinoma, prostate adenocarcinoma and breast carcinoma (Bubeník, 2003). The altered expression of MHC class II at cell surface can also be due to defects on the antigen presentation pathway and, in addition, to modifications of endosomes, and down-regulation of cellular processes, such as autophagy, which have a significant impact on the generation and presentation of antigens (Reeves & James, 2016).

Several approaches are under development to re-establish and maximize the antigen presentation of tumor cells, to increase immune–mediated responses. Some of these methods are tumor vaccines, dendritic cell-based vaccines, B cell-based vaccines, ex vivo-expanded and genetically engineered T cells, among others (Thibodeau *et al.,* 2012).

2.3.3 PD-1 and PD-L1

Programmed cell death protein 1 (PD-1) is one of the co-inhibitory receptors that is expressed on the surface of antigen-stimulated T cells. It is described that this receptor interacts with two ligands PD-L1, preferentially expressed on hematopoietic cells including T cells, B cells, macrophages, DCs, mast cells and also with PD-L2, mainly expressed on macrophages, DCs, and mast cells (Sun *et al.*, 2018). Interestingly, tumor cells have been described to express both ligands (Dail *et al.*, 2016). The major role of PD-1 under normal physiological conditions, is to inhibit effector T-cell activity and enhance the function and development of regulatory T cells (Tregs). This mechanism is activated to inhibit T-cell responses and prevent overstimulation of immune responses in peripheral tissues, and therefore protect the host against autoimmunity (Dong *et al.*, 2015). The engagement between PD-1 and its ligand PD-L1 changes the activity of T cells, inhibiting T cell proliferation, survival, and inducing apoptosis of tumor-specific T-cells, and other effector functions (Dong *et al.*, 2015; Sun *et al.*, 2018). The interaction between the extracellular domains of PD-L1 or PD-L2 and the PD-1 induce a receptor conformational change, leading to phosphorylation of the cytoplasmic immunoreceptor tyrosine-based inhibitory motif (ITIM) and of the immunoreceptor tyrosine-based switch motif (ITSM), by Src family kinases. After the phosphorylation of these tyrosine motifs, SHP-2 and SHP-1 protein tyrosine phosphatases are recruited to decrease T cell-activating signals (Dong *et al.*, 2015; Sun *et al.*, 2018). Notably, the components present at the tumor microenvironment, such as cancer cells, inflammatory cells and suppressive cytokines, are crucial for the regulation of T-cell functions, having the ability to drive T cells from differentiated into ‘exhausted' T cells and vice-versa. T-cell exhaustion is frequently associated to T-cell dysfunction in result of constantly high levels of expression of inhibitory receptors such as PD-1 and CTLA-4, among others. This dysfunction is characterized by the stepwise and progressive loss of T-cell functions (Jiang *et al.*, 2015; Yi *et al.*, 2010). In fact, PD-L1 is described as overexpressed in numerous cancers such as, gastric cancer, hepatocellular carcinoma, renal cell carcinoma, esophageal cancer, pancreatic cancer, ovarian cancer, and bladder cancer, and has been associated with poor prognosis. However in lung cancer, colorectal cancer, and melanoma the association of PD-L1 expression with a good or poor prognosis is still controversial, despite their overexpression (X. Wang *et al.*, 2016). Additionally, PD-L2 is also expressed in several tumor types such as, renal cell carcinoma, bladder carcinoma, melanoma, Non–Small Cell Lung Cancer (NSCLC) , Head and Neck Squamous Cell Carcinoma (HNSCC), triple-negative breast cancer and gastric carcinoma, with

expression differing accordantly to the tumor types (Yearley *et al.*, 2017). In addition, several articles in distinct mouse tumor models confirmed that PD-L1 expression, either on tumor or host immune cells, contributes to tumor escape of immune-mediated destruction (F. Tang & Zheng, 2018). Based on the crucial role of this receptor and its ligands and the consequences in T-cell function, blocking the PD-1/PD-L1 pathway, by impairing the binding between these proteins, is currently a therapeutic strategy used in cancer treatment (F. Tang & Zheng, 2018). Three years ago, the first treatments targeting the PD-1 receptor, namely pembrolizumab and nivolumab, were approved by the US Food and Drug Administration Agency (FDA), for treatment of unresectable or advanced melanoma (Gong, Le, *et al.*, 2018). Nowadays, the inhibition of PD-1 and PD-L1 was extended to the treatment of other solid tumors and hematologic malignancies, and five pharmacological agents were already approved for the treatment of different types of cancer. The pembrolizumab (anti-PD-1) was approved for the treatment of NSCLC, HNSCC, Hodgkin Lymphoma (HL), Urothelial Carcinoma (UC), Tissue-agnostic with microsatellite instability-high (MSI-H) and gastric cancer. The Nivolumab (anti-PD-1) was approved for Renal Cell Carcinoma, HL, HNSCC, UC, metastatic CRC with MSI-H and hepatocellular carcinoma. The Atezolizumab (anti-PD-L1) was approved for the treatment of UC and NSCLC. Additionally, Avelumab (Anti-PD-L1) was approved for the treatment of merkel cell carcinoma and UC and Durvalumab, also anti-PD-L1 antibody, was approved for the treatment of UC (Gong, Chehrazi-Raffle, *et al.,* 2018; Gong, Le, *et al.,* 2018).

3. Hypoxia: a critical feature in tumor progression

The solid tumor microenvironment is characterized by an imbalance between oxygen (O_2) supply and consumption, a characteristic known as hypoxia, usually defined as ≤ 2% O_2 levels (Vaupel & Harrison, 2004). This condition results from the abnormal vasculature present, caused by the rapid proliferation of cancer cells, which quickly consume oxygen supplied by the blood vasculature, and accumulate toxic metabolic products (Eales *et al.,* 2016; Henze & Mazzone, 2016).

3.1 Hypoxia sensing mechanisms and HIF regulation

In the tumor microenvironment, it is possible to have different percentages of O_2, with areas of mild-hypoxia and severe hypoxia, leading to necrosis, as well as areas of acute hypoxia and re-oxygenation, being these differences related to tumor cells disorganized vasculature network (Eales *et al.*, 2016). In order to overcome the lack of O_2, several cellular and systemic responses are activated, and one of the key regulators is the hypoxia-inducible factor (HIF-1) (Kuschel *et al.*, 2012). In hypoxia, cells activate HIFs, heterodimeric transcription factors composed of an O_2-regulated HIF-1α, HIF-2α, or HIF-3α subunit, and a constitutively expressed HIF-1β subunit, which regulate the expression of several genes (Semenza, 2013).

In normoxic conditions, HIF is hydroxylated in proline residues by prolyl-4-hydroxylase enzymes (PHDs) and interacts with the Von Hippel-Lindau (VHL) tumor suppressor protein, resulting in its ubiquitination and targeting to proteasomal degradation. When cells are under hypoxic conditions, the activity of PHDs is inhibited, resulting in reduced HIF-1α hydroxylation and suppressed proteasomal degradation. As consequence, HIF-1α subunits accumulate in the cytoplasm and are translocated into the nucleus, where they heterodimerize with the β subunit. The HIF-1 complex binds then to enhancer elements, named hypoxia responsive elements (HRE), present in the target genes, leading to their transcriptional activation (Kuschel *et al.*, 2012). Examples of genes described to be regulated by HIF are cytokines and multiple angiogenic growth factors, such as VEGF, stromal-derived factor 1, angiopoietin 2, metabolism associated molecules, and stem cell factor (Semenza, 2013).

3.2 Impact of hypoxia in tumor progression

Giving the importance of hypoxia in tumor progression, it is critical to study how this factor modulates macrophage-cancer cell crosstalk. It has been evidenced that hypoxia is associated

with increased mutation rates and increased expression of genes associated with angiogenesis, tumor invasion, immune escape, and alterations in the metabolism, promoting tumor progression. One example that evidences how hypoxia promotes this progression is its association with cancer cell metabolic shift, which dysregulates the glycolytic pathway, resulting in an increase in cell survival, tumor growth, and proliferation (Escribese *et al.*, 2012; Liberti & Locasale, 2016). Interestingly, hypoxia has been also associated with radiotherapy and chemotherapy resistance. This occurs due to the significant distance between hypoxic cells and blood vessels, which turns hypoxic cells less exposed to some types of anticancer pharmacological agents; reduction of cellular proliferation related with distance to blood vessels; hypoxia-driven selection of cells that have lost sensitivity to p53-mediated apoptosis, which reduces sensitivity to some anticancer agents; and hypoxia-driven upregulation of genes involved in drug resistance. Due to these factors, a worse tumor prognosis is associated with a hypoxic environment **(Figure 4)** (Brown & Wilson, 2004).

3.3 Influence of hypoxia on macrophages

In the case of breast, endometrium, ovary, bladder, colon, and oral cavity tumor microenvironments, it was described that TAMs are recruited to hypoxic and/or necrotic areas. Once there, macrophages become anti-inflammatory and pro-tumorigenic by alterations on their gene expression profile (Chanmee *et al.*, 2014; Murdoch *et al.*, 2008). Furthermore, it was suggested that TAMs are attracted to hypoxic environments through the release of hypoxia-induced chemoattractants as VEGF, endothelins as Endothelial Monocyte-Activating Polypeptide II, and high mobility group 1 proteins (Murdoch *et al.*, 2008). Once macrophages are entrapped in this environment, tumor cells stimulate angiogenesis, in a process mediated by TAMs, through the upregulation of VEGF, FGF, IL-8 among others, or through upregulation of angiogenic modulators such as cyclooxygenase-2, Nitric Oxide Synthase, and MMP-7. TAMs have also been described as crucial players in cancer cell migration and invasion, through the release of MMPs, which in turn may also facilitate new vessels formation (Henze & Mazzone, 2016) **(Figure 4).**

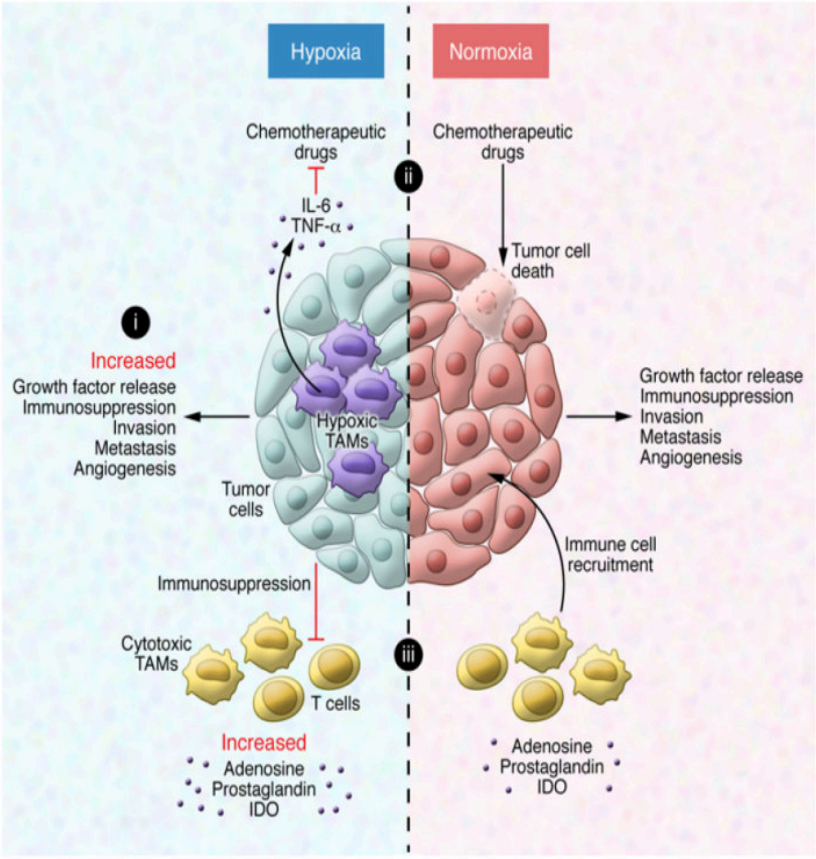


Figure 4: Regulation of TAMs in a hypoxic environment and effects in cancer progression and therapy compared to normoxia conditions. i) Hypoxia environment stimulates the release of chemoattractants that increase macrophage recruitment, which is related to cancer progression-associated mechanisms such as the release of immunosuppressors, invasion, metastasis, and angiogenesis. (ii) TAM release survival factors which can inhibit the action of chemotherapeutics pharmacologic agents. (iii) A hypoxic environment is characterized by its immunosuppression and can suppress the antitumor response which enhances the selectivity of more aggressive tumor cells. Reproduced from (Henze & Mazzone, 2016).

In the last years, it was described that hypoxic macrophages upregulate HIF1 and HIF2, whose activation leads to transcription of many genes, which in turn regulate cell proliferation, metabolism and angiogenesis (Murdoch *et al.*, 2008). It has been described that M1 macrophages alter their metabolism to the anaerobic glycolytic pathway, whereas M2 macrophages proceed with oxidative glucose metabolism and fatty acid oxidation (Escribese *et al.*, 2012). In cancer cells, there are metabolic changes, named 'Warburg Effect', frequently described as present in the tumor microenvironment (Liberti & Locasale, 2016). It is characterized by an increased glucose uptake, associated with an up-regulation of glucose

transporters on tumor cells (Netea-Maier *et al.*, 2018) and macrophages (Burke *et al.*, 2003) and fermentation of glucose to lactate despite the presence of completely functioning mitochondria and oxygen supply (Liberti & Locasale, 2016). Hypoxia is not the main factor responsible for the switch to aerobic glycolysis by cancer cells, however, HIF1α facilitates the production of enzymes necessary for anaerobic glycolysis (Escribese *et al.*, 2012).

4. Colorectal carcinoma

CRC is the third most common cancer worldwide in terms of incidence, but the second in terms of mortality, considering both sexes (Bray *et al.*, 2018). The colon and rectal cancer are often considered to be the same disease since they are anatomically related and have many features in common, however, they represent two separate regions of a contiguous organ and have different local rates of recurrence (Hong *et al.*, 2012). The survival of CRC patients is highly dependent from the stage of the disease at diagnosis and usually, the earlier the better is the prognosis and chance of survival (Haggar & Boushey, 2009).

4.1 Colorectal cancer epidemiology: Incidence and risk factors

This type of cancer exists mainly in developed countries with a Western culture (Haggar & Boushey, 2009). The areas with highest CRC incidence rates are still Europe, Australia/New Zealand, Northern America and Eastern Asia. A tendency of low rates in CRC incidence is observed in most regions of Africa and Southern Asia (Bray *et al.,* 2018).

CRC can be classified as sporadic or as a hereditary disease associated to familial predisposition. Sporadic corresponds to about 70 % of all CRC cases and are related to somatic mutations linked to environmental and lifestyle risk factors. The main hereditary CRC syndromes, which correspond to 7-10 % of all CRC cases, are hereditary nonpolyposis colorectal cancer and familial adenomatous polyposis syndrome (De Rosa *et al.*, 2015). Some examples of factors that have been proved to have a role in CRC development are age, hereditary factors, chronic inflammation of the digestive tract and environmental lifestyle risk factors such as, a diet rich in meat and fat and low in vegetables and fruit, smoking, lack of physical activity, higher consumption of alcohol and obesity (Haggar & Boushey, 2009; Raskov *et al.*, 2014).

4.2 Carcinogenesis

The gastrointestinal tract presents very often adenomas and polyps, which are benign tumors of epithelial tissue which through time can, eventually, turn into adenocarcinomas. If these benign cells are not removed, they can continue to proliferate and progress to malignancy, invading and metastasizing (Tannapfel *et al.*, 2010). The carcinogenesis of colorectal cancer can result from one or from the combination of three different mechanisms, namely chromosomal instability (CIN), CpG island methylator phenotype (CIMP), and MSI. The CIN pathway begins with the accumulation of genomic modifications in the adenomatous polyposis coli (*APC*), a tumor suppressor gene, resulting in an early adenoma. Subsequently, another alteration that might occur in the adenoma-carcinoma sequence is the activation of the oncogene *KRAS*. Another step is the inactivation or loss of tumor suppressor genes, as the Tumor Protein P53 (*TP53*) **(Figure 5).** Additionally to these changes, aneuploidy and Loss of Heterozygosity (LOH) have an important role in tumors, together with this altered pathway. The other two pathways that can contribute for carcinogenesis of CRC are the CIMP pathway, characterized by the hypermethylation of the several tumor suppressors, and usually associated with the Proto-Oncogene *BRAF* mutations and with MSI (Armaghany *et al.*, 2012; Leslie *et al.*, 2002; Tariq & Ghias, 2016).

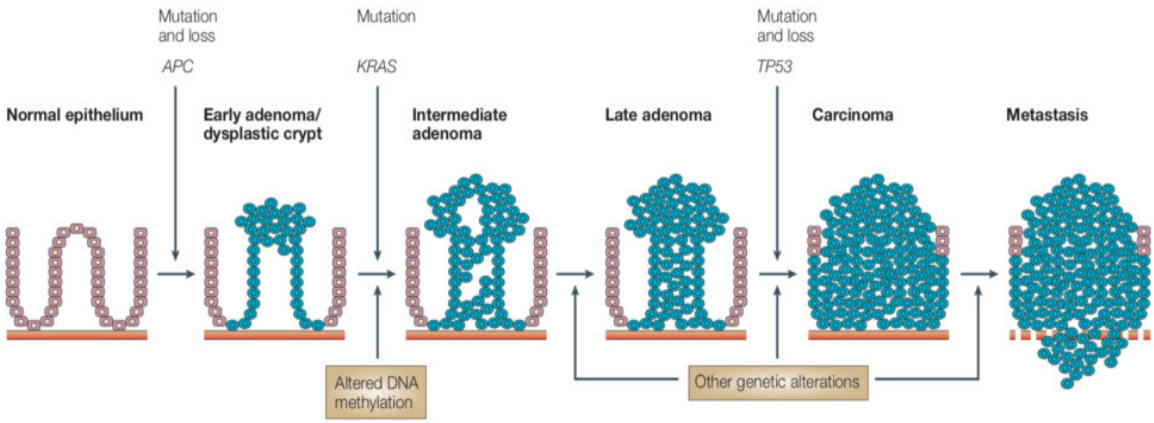


Figure 5: Adenoma-carcinoma sequence of colorectal. The multistep and progression from normal epithelium to carcinoma and ultimately to metastasis is characterized by an accumulation of specific changed genes that lead to transformation. The APC results in an early adenoma and mutation in the oncogene *KRAS* leads to intermediate adenoma. Adenocarcinoma is characterized by mutation and loss of TP53. Adapted from (Davies *et al.,* 2005).

4.3 Diagnostic and therapeutics

To the decrease of CRC mortality rate, diagnosis at early stage is essential. Nevertheless, the diagnostic process is a challenge, especially on CRC, due to vague and the lack of specific symptoms. In consequence, when the symptoms become more serious and require investigation, usually the disease is in a more advanced state (Vega *et al.*, 2015). CRC can be identified by several screening methods such as fecal occult blood testing, rectal exam, double-contrast barium enema and virtual and traditional colonoscopy (Davies *et al.*, 2005; Marley & Nan, 2016; Vega *et al.*, 2015).

The choice of treatment for colorectal cancer will depend of the patient's health, the size of the tumor, and its location. Usually, the surgery is the most common treatment option and different approaches of resection will occur, accordingly to the presence and area of tumor metastasis. Other treatments can be applied such as radiofrequency ablation, characterized by electrodes insertion, cryosurgery, the use of freezing techniques to destroy cancerous tissue, targeted therapy where drugs or other methods, attack the cancer cells or other components of the tumor microenvironment and the traditional chemo- and radiation therapy (Marley & Nan, 2016). Additionally, new immunotherapies have been recently approved for the treatment of CRC with MSI-H (Gong, Chehrazi-Raffle, *et al.*, 2018) . Although many improvements have been made and CRC mortality rate has decreased it is necessary to search for new and effective therapeutic approaches and have a healthy lifestyle.

Aim of the thesis

Our group has previously described that macrophages promote cancer cell invasion and invasion-related activities. Nevertheless, these studies were performed in normoxic conditions without contemplating the effect of hypoxia, characteristic of the tumor microenvironment. Some authors described the role of hypoxia on tumor cells as well on macrophages, but its impact on the macrophage-cancer cell crosstalk is still undetermined. Considering the importance of hypoxia at the tumor microenvironment, the main purpose of this thesis is to understand how it can influence the crosstalk between tumor cells and macrophages. We intend to clarify the functional alterations that occurs in both macrophages and cancer cells, modulated by hypoxia, and explore the underlying molecular mechanisms, especially regarding the invasive phenotype of cancer cells, described as a hallmark of aggressiveness and poor prognosis. For this purpose, monocultures and indirect co-cultures of macrophages and colon cancer cells were established under normoxia or hypoxia conditions. The impact of hypoxia and cancer cells on macrophages was evaluated regarding several features, such as polarization profile, cytoskeleton organization, phagocytic activity and immunological synapse molecules. Moreover, the effect of hypoxia and the presence of macrophages on cancer cells invasion and invasion-related activities, such as migration, proteolytic activity, and EMT, was also assessed.

Material and Methods

Cancer cell culture

RKO cell line derived from a human colon carcinoma were purchased at American Type Culture Collection (ATCC). Cells were cultured in RPMI Medium 1640 with GlutaMax (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Lonza) and 100 UmL^{-1} penicillin and 100 μgmL^{-1} streptomycin (Invitrogen) and maintained at 37° C under a 5% CO_2 in a humidified-atmosphere.

Ethics statement

All experiments regarding monocyte isolation were performed with human monocytes obtained from buffy coats, which are a highly leukocyte-enriched waste-product that results from a whole blood donation of healthy blood donors. A collaboration protocol between Instituto de Investigação e Inovação em Saúde -Universidade do Porto (i3S) and the Hematology Service Team of Centro Hospitalar São João (CHSJ), allows the use of these products for investigation purposes. Informed consent was obtained from all subjects before each blood donation.

Human monocyte isolation and macrophage differentiation

The blood from buffy coats were centrifuged at 1200 *g*, without brake, for 20 min at room temperature (RT). The leukocytes present in the whitish layer were incubated with RosetteSep-Human Monocyte Enrichment Cocktail (StemCell Technologies, Grenoble, France), for 20 min at RT. This mixture was diluted at a 1:1 ratio in phosphate buffered saline (PBS) supplemented with 2% FBS (Biowest) and was then gently added over Ficol-Histopaque 1077 (Sigma-Aldrich), and centrifuged, in the same conditions as previously described. The intermediate layer, enriched in monocytes, was collected, washed and centrifuged at 700 rotations per minute (rpm) for 17 min. The pellet was resuspended in RPMI 1640 medium with GlutaMax (Invitrogen) supplemented with 10% FBS (Biowest) and with 100 UmL^{-1} penicillin and 100 μgmL^{-1} streptomycin (Invitrogen), considered as complete culture RPMI medium, and $1.2x10^6$ monocytes were plated per 6 well culture plates containing glass coverslips. Monocytes differentiated into macrophages for 10 days. In the first 7 days they were maintained in culture in the presence of M-CSF (ImmunoTools 50 $ngmL^{-1}$). At the 7^{th} day, cell culture media were replaced by new medium without M-CSF. At the 10^{th} day, indirect co-cultures with cancer cells were performed.

Macrophage and cancer cell indirect co-culture

To perform indirect co-cultures, $1x10^5$ RKO cells were seeded into a permeable transwell inserts (PET inserts with 1μm pore, Corning) positioned on top of a macrophage culture, 10 days after monocyte isolation. In this system, cancer cells do not contact directly with macrophages, but the inserts allowed the exchange of soluble factors between the two compartments. Co-cultures between macrophages and cancer cells were maintained in complete RPMI medium for 3 days in normoxia (20% O_2) or hypoxia (1% O_2) conditions. As control, macrophage and cancer cell monocultures were also performed and analysed. After 3 days of co-culture, all biological material was collected.

RNA extraction

Total RNA from macrophage and RKO cells were extracted using TRIzol reagent (Invitrogen). Briefly, cells were incubated and detached at RT to dissociate nucleoprotein complexes. Each sample was incubated with chloroform at RT for 15 min. Next, they were centrifuged for 15 min at 12 000 *g* at 4°C. Samples were separated in three phases, and the upper phase was collected to a new eppendorf and incubated with isopropanol for 10 min at RT. Next, were centrifuged at 4°C for 10 min at 12 000 *g.* Pellets were washed with ethanol 75%, and centrifuged at 4°C for 5 min at 7500 *g.* After ethanol removal, the RNA pellet was left drying for 1h, resuspended with RNase/DNase free water (Gibco), and incubated for 1h at 4°C. RNA concentration and purity were determined using a NanoDrop Spectrophotometer ND-1000 (Thermo Scientific). RNA samples were stored at -80 °C until further use.

Complementary DNA (cDNA) synthesis

cDNA was synthetized using 1 μg of RNA previously extracted. The cDNA was synthetized according to manufacturer's instructions (NZY M-MuLV Reverse Transcriptase).

Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed with Kapa Probe Fast qPCR Master Mix (Kapa Biosystems) and using the probes described in **table 1**, according to the following program: 2 annealing steps of 50°C for 20 s and 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Each reaction was executed in triplicate, and the PCR was performed in a 7500 Real Time PCR System (Applied Biosystems). Relative mRNA expression of

the target genes was normalized to the levels of the housekeeping gene, using the comparative ΔΔCt method. The housekeeping gene used for macrophages was *18S* and for cancer cells was *ACTB*.

Table 1: TaqMan gene expression and assay reference accordantly to the different gene used to measure mRNA levels. The supplier of the *18S, ACTB, CCR7, CD80, TGFB1, CD163, CCL18* probes are from Applied Biosystems and the remaining probes are from Integrated DNA Technologies (IDT).

Gene	TaqMan Gene Expresion
	Assay reference
18S	Hs99999901_s1
ACTB	Hs01060665_g1
CA9	Hs00154208_m1
CCR7	Hs01013469_m1
CD80	Hs00175478_m1
CD163	Hs00174705_m1
CCL18	Hs00268113_m1
SLC2A1	Hs.PT.58.25872862
SNAIL 1	Hs.PT.58.2984401
SNAIL 2	Hs.PT.58.1772559
ZEB1	Hs.PT.58.3948500
ZEB2	Hs.PT.58.3785272
FN1	Hs.PT.58.40005963
VM	Hs.PT.58.38906895
CD47	Hs.PT.45656328
SIRP1a	Hs.PT.58.27183318
MMP7	Hs.PT.40068681
MMP1	Hs.PT.38692586

Immunocytochemistry

Morphology of macrophages was assessed through immunocytochemistry. Macrophages from mono and co-cultures in normoxic or hypoxic conditions, and seeded in glass coverslips, were fixed with 4% paraformaldehyde (PFA) for 20 min at RT and quenched with 50mM NH_4Cl for 10 min. After PBS washes, cells were permeabilized with 0.2% Triton X-100 for 5min, rewashed with PBS and blocked for 30 min with 5% bovine serum albumin (BSA, Sigma-Aldrich). Coverslips were incubated for 1h with monoclonal mouse anti human-α-tubulin (Sigma-Aldrich) in a dilution of 1:4000. Cells were washed with PBS and incubated with AlexaFluor 488 goat anti-mouse conjugated-secondary antibody (Invitrogen) in a dilution of 1:500. F-actin was stained for 20 min with Alexa Fluor 568 Phalloidin (Invitrogen, Molecular Probes) in a dilution of 1:40. Coverslips were mounted with Vectashield Mounting Medium with 4',6-Diamidino-2-Phenylindole (DAPI,

Vector Laboratories) and were visualized in a Zeiss Axio Imager Z1 fluorescence microscope (CarlZeiss, Germany).

Measurement of macrophage aspect ratio and area

ImageJ software was used to measure the aspect ratio and area of macrophages from mono and co-cultures with RKO under normoxic or hypoxic conditions, stained with actin and tubulin, as previously described. Aspect ratio was calculated as the ratio between the length of each major and minor cell axes. 100 cells per condition were counted.

Phagocytosis assay with bacteria-derived beads

Evaluation of phagocytic activity of macrophage in mono and co-culture, in normoxic and hypoxic conditions, were evaluated using a Ready-made pHrodo green *Staphylococcus aureus* (*S. aureus*) BioParticles Conjugate (1μm diameter, Invitrogen, Molecular Probes). Phagocytic activity can be measured due to the green fluorescence exhibited by the bioparticles. The green fluorescence occurs in the presence of reduced pH, which is the case of vesicles where the particles are encapsulated after the engulfment by macrophages. pHrodo bioparticles were resuspended in PBS up to 1 mg/mL and gently vortexed and sonicated for homogenous dispersion. Macrophages ($2.4x10^5$ cells) were incubated with $9.6x10^6$ *S. aureus* particles for 1h at 37° C and 5% CO_2. Subsequently, macrophages were washed in PBS and fixed with 4% PFA for 20 min at RT. F-actin was stained with Alexa Fluor 568 Phalloidin (1:40, Invitrogen, Molecular Probes) for 20 min, after previous permeabilization with 0.2% Triton X-100 and NH_4Cl, and blocking with 5% BSA. For nuclei visualization, coverslips were mounted in Vectashield Mounting Medium with DAPI, and visualized with a Zeiss Axio Imager Z1 fluorescence microscope (CarlZeiss). The number of cells able to phagocyte *S. aureus* bioparticles was determined using ImageJ software. 200 cells per condition were counted.

Phagocytosis assay with CFSE

Phagocytosis assay using CellTrace™ CFSE (Invitrogen) was performed to evaluate phagocytosis of RKO cells instead of bioparticles with bacterial origin executed by the previous procedure. Thus, three days before proceeding, RKO cells were seeded and maintained at 37°C under normoxia or hypoxia. Two hours before the experiment, the medium was exchange to RPMI 1640 medium with GlutaMax without serum, considered as incomplete medium. Cancer

cells were then detached and centrifuged at 1200 rpm for 5 min. The pellet was resuspended in PBS and RKO cells ($1x10^7$ cells/mL) were stained with CellTrace™ CFSE (2,5μM), for 20 min under rotation at RT in the dark. Next, complete RPMI medium was added to the suspension and incubated for 5 min at 37°C, and then centrifuged at 1200 rpm for 5 min. Subsequently, the cellular suspension was added to the macrophages previously seeded, and incubated for 2h in the dark at 37°C, in a proportion of 1 macrophage to 2 cancer cells. Following incubation time, cells were processed accordingly to flow cytometry procedure explained next, preferentially without fixation.

Flow cytometry

For cell surface receptor expression analysis, macrophages and cancer cells, both from mono or co-cultures under normoxic or hypoxic conditions, were harvested with accutase for 30 min at 37°C. Cells were gently detached with help of a scraper and washed with cold PBS and kept on ice. Subsequently, were centrifuged for 5 min at 1200 rpm at 4° C, and the pellet was resuspended in buffer (PBS 1x, 2% FBS, 0.01% sodium azide). Staining was performed with antibodies, represented in the **table** 2, for 40 min at 4°C in the dark. Single staining with antibodies or with respectively IGgs was used as control. All IGgs were purchased from ImmunoTools with exception of IgM- FITC which was acquired from Santa Cruz Biotechnology. Next to washing steps with buffer, cells were fixed for 15 min in 4% of PFA at RT. Samples were filtered and analyzed through FACS Canto Flow Cytometer (BD Biosciences) using FACS Diva Software. Analysis of the data obtained was performed with FlowJo software.

Table 2: Antibodies and respectively IGg's used for cell surface receptor expression analysis.

Antibodies	IGg's	Supplier
CD14-APC	IGg1	ImmunoTools
CD14-PE	IGg1	ImmunoTools
CD14- PerCP/Cy 5.5	IGg1	ImmunoTools
CD14- FITC	IGg1	ImmunoTools
CD163-PE	IGg1	BD Biosciences
CCR7-PerCP/Cy 5.5	IGg2aK	Biolegend
PD1-PerCP/Cy 5.5	IGg1	BD Biosciences
PDL1-FITC	IGg1	BD Biosciences
SIRP1α-APC	IGg1	Biolegend
CD47-FITC	IGg M	ImmunoTools
HLA DR-PE	IGg1	ImmunoTools
HLA DR- FITC	IGg1	ImmunoTools
HLA ABC-PE	IGg2a	ImmunoTools

Processing of Conditioned Media

Conditioned Media (CM) collected from monocultures of macrophage or cancer cells and co-cultures of cancer cells in normoxia or hypoxia, were centrifuged at 6000 rpm at 4°C for 3 min. Supernatants were collected and stored at -20 °C until further use.

Enzyme-linked Immunosorbent assay (ELISA)

Cytokines levels in CM of macrophages from mono- and co-cultures in hypoxic or normoxic conditions were determined using ELISA of TGF-β, TNF-α, IL-10, IL-1β, IL-6 (Biolegend) and CCL18 (Abcam) accordantly to manufacturer's instructions. Cytokine levels were determined by plotting values on a standard curve and normalizing them to CM protein concentration. Protein concentration was ascertained using the DCProtein assay kit (BioRad).

In vitro wound healing assay

In vitro wound healing assay was performed in RKO cells to study cell migration. Briefly, RKO cells were seeded on a 24 well plate, in normoxia and hypoxia, until they reach confluency. Successively, wound was performed, and culture medium was carefully removed, to eliminate free-floating cells and debris. Cells were then treated with CM from prior experiments, from mono/co-culture in normoxic and hypoxic conditions, in a proportion of 1/3 with complete RPMI medium. Photos were acquired at the beginning of the experiment and 14h after, using the Leica DM microscope. ImageJ software was used to measure the wound when is created (0h) and 14h later, giving us the percentage of wound closure. The wound closure was calculated through the following formula: [100 % — (Distance measured 14h later *100 % / Distance measured at 0h)].

Matrigel invasion assay

Cancer invasion phenotype was evaluated through Matrigel-coated invasion inserts of 8μm pore-size filters (BD Biosciences) which mimics the basement membrane, using macrophages as invasive stimuli. Prior to experiment, filters were re-hydrated in complete RPMI medium for 1h at 37° C. Subsequently, confluent RKO cells cultured in normoxia were detached and re- suspended in complete RPMI medium. Matrigel-coated invasion inserts were seeded with $2.5x10^4$ cells and placed on top of macrophages, under normoxic and hypoxic conditions, for 24h at 37° C. After this period, the inserts were washed with PBS and fixed in 4% PFA for 20 min at RT, and the non-invading cells in the upper compartment of the inserts were removed. Invasive cells were

mounted in Vectashield Mounting Medium with DAPI (Vectashield, Vector Laboratories) for nuclei staining, and visualized through a Leica DM2000 fluorescence microscope (Leica Microsystems).

Statistical analysis

Data analysis was performed with GraphPad Prism Software v6. Statistical significance was achieved when *P*-value <0.05. The normality of the distribution was tested, and the adequate ANOVA test was performed.

Results

Since hypoxia is a crucial element at the tumor microenvironment, indirect co-cultures between cancer cells and macrophages under normoxic and hypoxic conditions were established, aiming to understand the role of hypoxia on macrophage-cancer cell crosstalk **(Figure 6).** With this approach, we mainly analyzed the differences induced by secreted factors, and we are aware that we could lose information regarding events that need the direct contact between macrophages and cancer cells. However, we know that monocytes/macrophages do not proliferate during the differentiation process, while cancer cells proliferate, which would be a problem with direct co-cultures, particularly in the end-point of the experiment to separate both populations. On the other hand, the required manipulation to separate the two cell populations, such as labelling and sorting, would be performed in normoxia, and since these are time-consuming procedures the hypoxic cellular properties would likely to be lost.

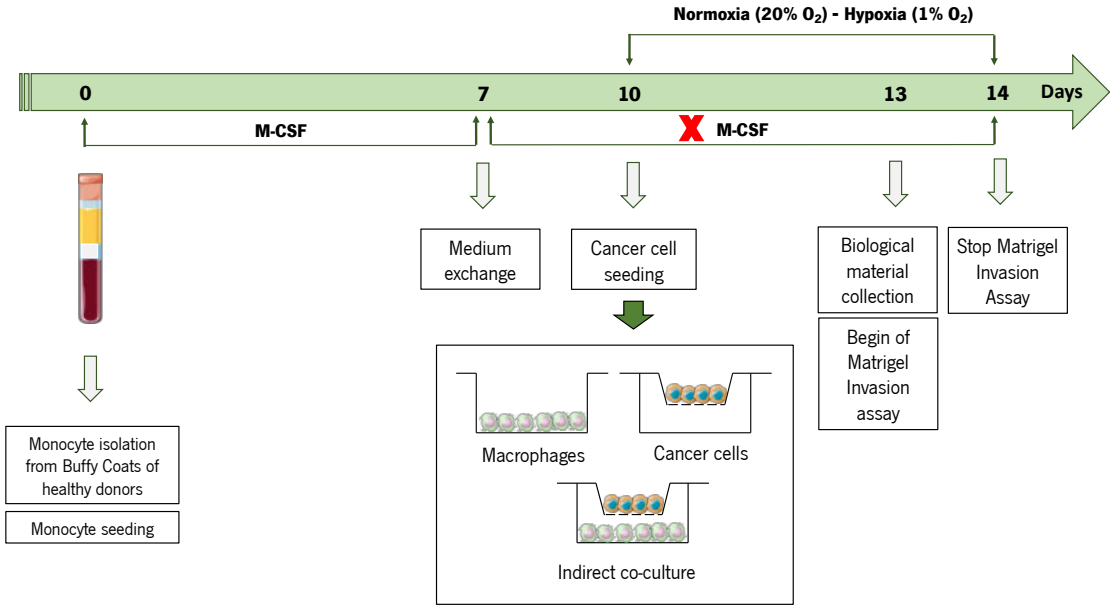


Figure 6: Schematic representation of the experimental setup. Monocytes were plated ($1.2x10^6$) on culture plates with RPMI medium with GlutaMax supplemented with 10% FBS, 1% PenStrep, and M-CSF (50 ngmL-1) for 7 days and differentiated for 10 days. At 7th day of monocyte differentiation, the cell culture medium was renewed without the presence of M-CSF. After 10 days of monocyte isolation indirect co-cultures were established, RKO cancer cells ($1x10^5$ cells/insert) were plated in transwell inserts and allocated on top of macrophage cultures. These co-cultures were kept in complete RPMI medium, under normoxia (20% O_2) or hypoxia (1% O_2) for 3 days. Additionally, monoculture of macrophages and cancer cells were also established as controls. At 13th day of experiment Matrigel invasion assay was initiated and biological material was collected to perform several assays.

Validation of hypoxia in the experimental setup

In order to verify that our experimental setup is working properly, and to evaluate if cancer cells were responding to hypoxia we measured, by qRT-PCR, the mRNA expression levels of carbonic anhydrase IX (*CAIX*), a gene known to be regulated by hypoxia (Li *et al.*, 2015). Our results demonstrated that *CAIX* expression increases significantly in hypoxia, confirming that the cells are responding to the hypoxic stimulation **(Figure 7A).** Since macrophages do not express this gene, the expression levels of solute carrier family 2 (*SLC2A1)* was evaluated. This gene that codifies for the GLUT1 is known to be altered by hypoxia due to alterations in cellular metabolism (Chung *et al.*, 2009) **(Figure 7B).** Our results revealed that in hypoxia, *SLC2A1* mRNA expression levels increased with statistical significance, an indication that macrophages are consuming more glucose, and that metabolic changes are being induced.

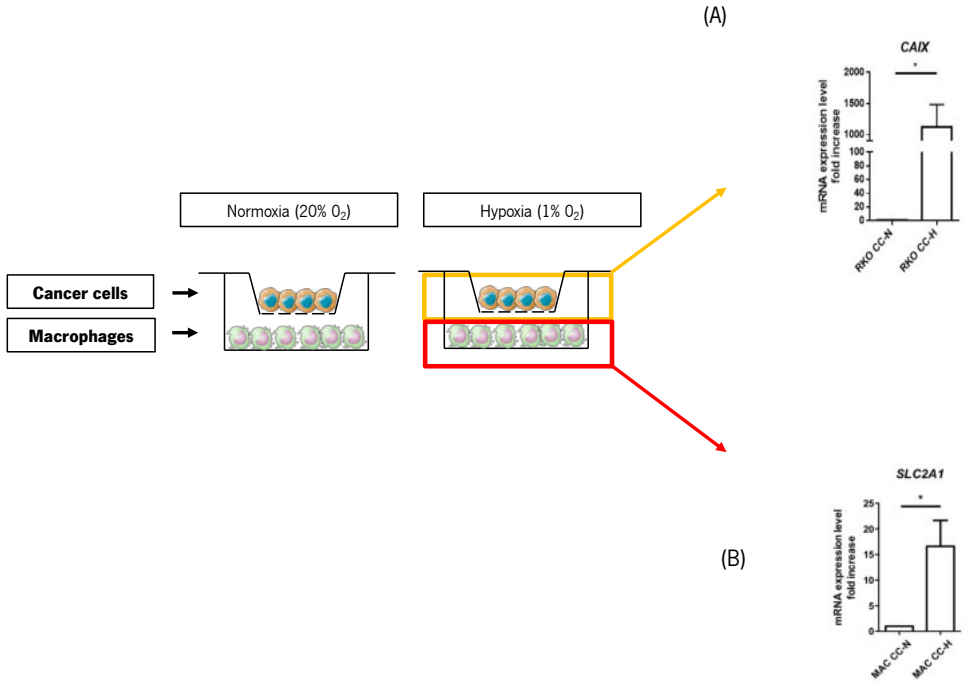


Figure 7: mRNA expression levels of CAIX and *SLC2A1* on cancer cells and macrophages. The expression levels of *CAIX* and *SLC2A1* were assessed on cancer cells and macrophages, respectively, in co-culture both in normoxia and hypoxia. A) mRNA expression levels of *CAIX* were measured on cancer cells co-cultured with macrophages in normoxia (RKO CC-N) and hypoxia (RKO CC-H); B) mRNA expression levels of *SLC2A1* (GLUT1) were quantified on macrophages in co-culture with RKO cells in normoxia (MAC CC-N) and hypoxia (MAC CC-H) conditions. Macrophages mRNA expression levels were normalized to *18S* expression and are represented as fold increase relatively to macrophages in normoxia, while RKO mRNA expression levels were normalized to *ACTB* expression and are represented as fold increase relatively to RKO in normoxia. Graphs represent the mean values and are representative of independent experiments of n=8 for *CAIX* expression and n=5 for *SLC2A1* expression. *$p < 0.05$

Macrophage morphology under normoxic and hypoxic conditions

Alterations on macrophage morphology was visualized after 3 days of co-culture, through brightfield microscopy **(Figure 8).** Our results evidenced that macrophages exhibit a mixed population of rounded-shaped and spindle-shaped cells, independently of the conditions that are subjected to, indicating that, at least at these time-points, hypoxia does not seem to affect their morphology.

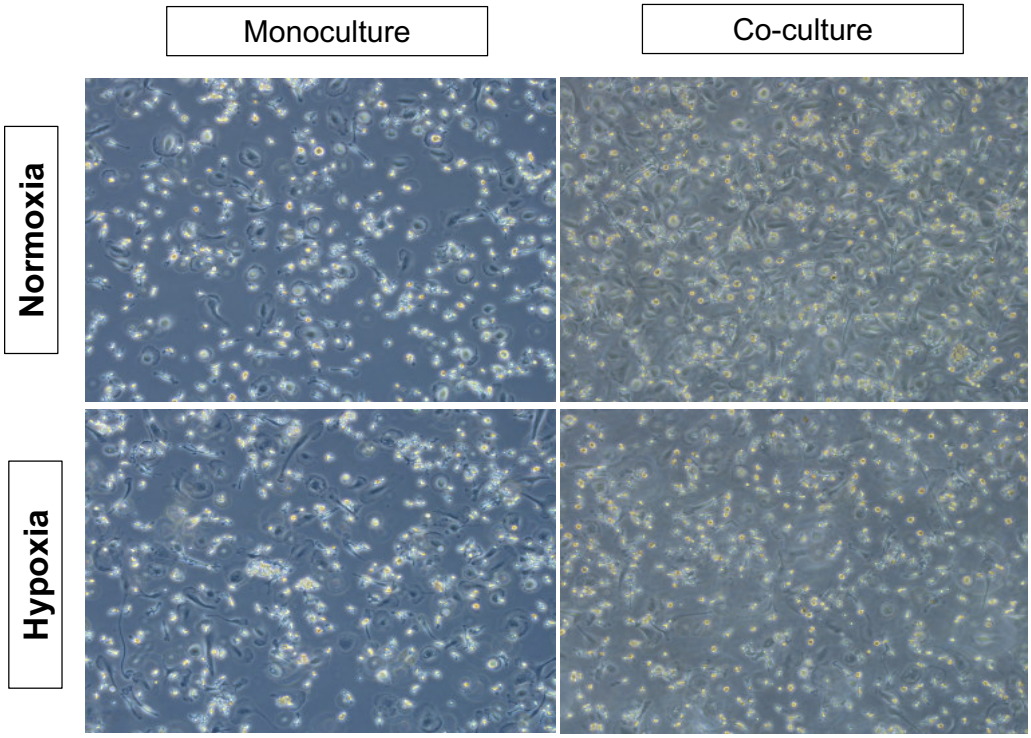
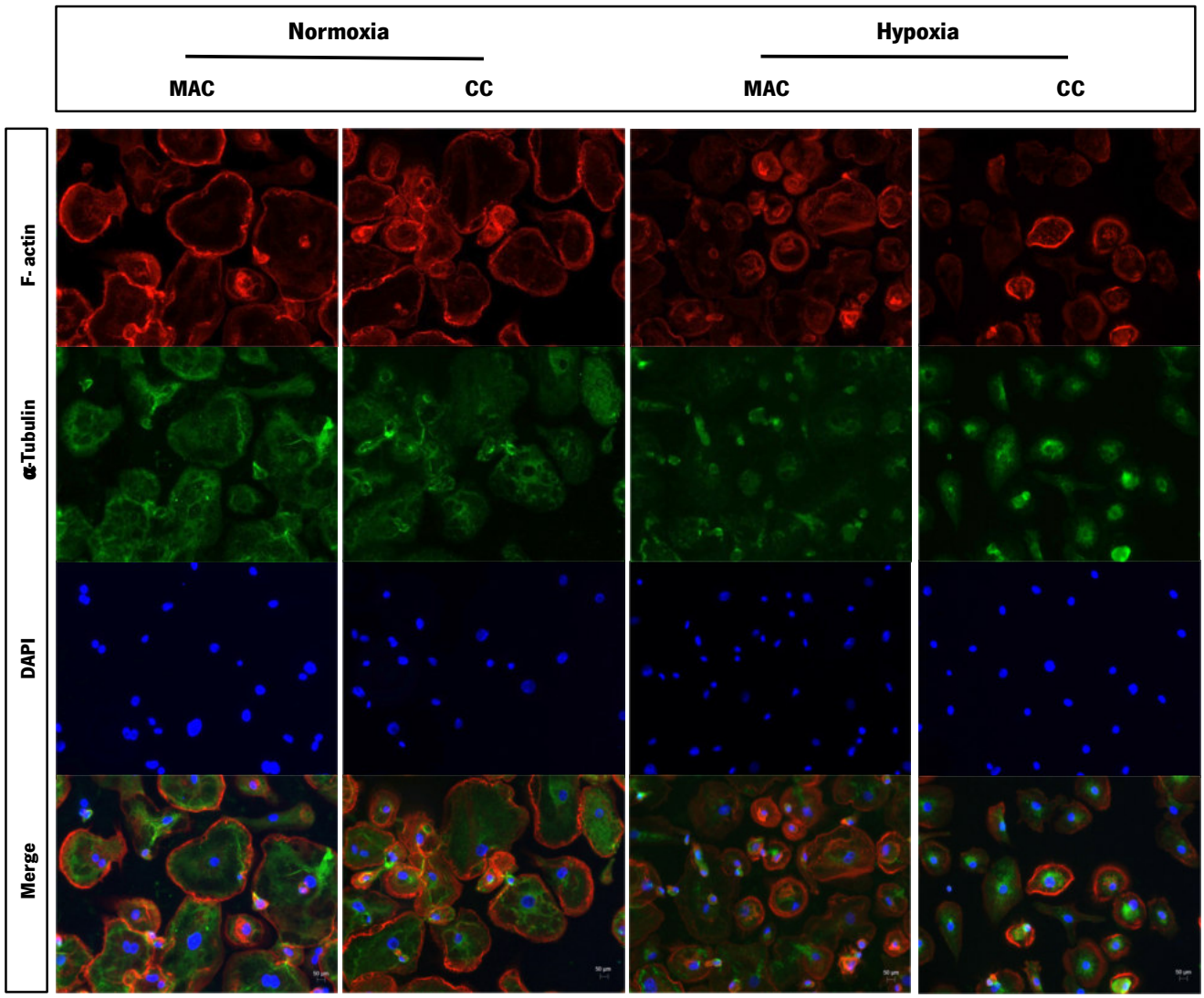


Figure 8: Brightfield observation of macrophages under normoxic and hypoxic conditions. Images of macrophage population in monoculture or in indirect co-culture were acquired at 13th day of the experiment. Images were obtained with 10X amplification.

Macrophages cytoskeleton organization

Nevertheless, the macrophage cytoskeleton organization was evaluated to better analyse their structure among the different conditions. Notably, macrophages cytoskeleton organization have an important role in their structure and function and it is described that could give an indication about their polarization status (Cardoso *et al.,* 2015). The cytoskeleton was evaluated through immunocytochemistry for F-actin and α-tubulin proteins **(Figure 9A).** In the four conditions analysed, hypoxia promoted macrophages morphological alterations when cultured in the absence or in the presence of RKO cells **(Figure 9A).** To better quantify these alterations, the cell aspect ratio, consisting in the ratio between cell major and minor axes length, and the cell area were measured. Co-cultures of macrophages and cancer cells do not exhibit major differences concerning cell area or aspect ratio in normoxia or hypoxia conditions **(Figure 9B-C).** However, a tendency to decrease on cell area was observed in hypoxic conditions, although not significative **(Figure 9C).**

(A)



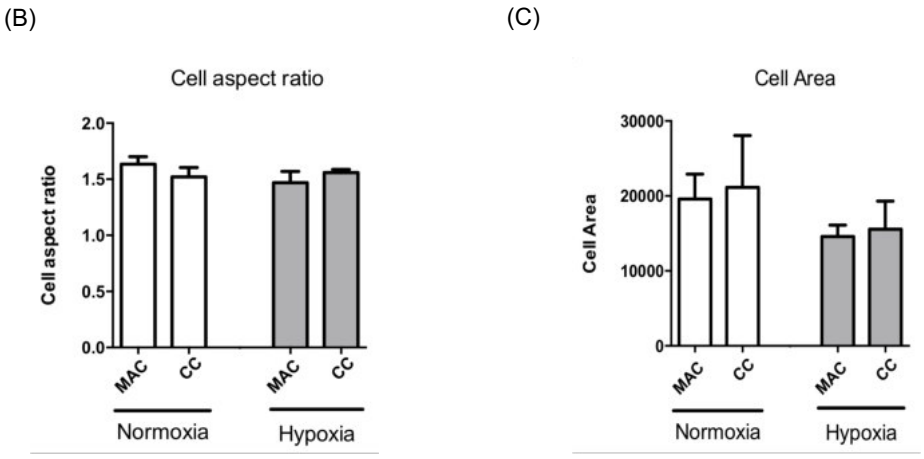


Figure 9: Cytoskeleton organization of macrophages under normoxia and hypoxia conditions. A) Macrophages in monoculture (MAC) and co-culture (CC) with RKO cells were stained for F-actin (red) and for α-tubulin (green) and nuclei were counterstained with DAPI (blue). B) To better characterize the differences in macrophage morphology, cell aspect ratio (ratio between cell major and minor axes length) was calculated in actin/tubulin stained cells. Data represent the mean values of each condition and are representative of nine independent experiments (at least 100 cells/condition). C) Macrophages cell area was quantified in actin/tubulin stained cells (at least 100 cells/ condition). Data represent the mean values and are representative of eight independent experiments (at least 100 cells/condition). Scale bar length: 50 μm.

Macrophage polarization status

Macrophages are a very plastic population, and accordantly to microenvironmental signals they are exposed, can be activated into a more anti- or pro-inflammatory phenotype (Mantovani *et al.*, 2004).

Our aim was to verify the impact of hypoxia and of co-culture with cancer cells on the polarization status of macrophages, which were not exposed to other external stimuli. Therefore, their ability to polarize into M1 or M2-like macrophages, described as a pro- or anti- inflammatory status, and consequently to a more anti- or pro-tumor, respectively, was evaluated. We started to analyze the expression of two pro-inflammatory markers (*CD80* and *CCR7*) and of two anti-inflammatory markers (*CCL18* and *CD163*) by qRT-PCR **(Figure 10A-B).** Our results demonstrated that the presence of cancer cells lead to an increase in the *CD80*, *CCR7* and *CCL18* expression levels. However, when analyzing the effect of hypoxia on macrophage-cancer cell crosstalk, we found that hypoxia led to a decrease in the levels of *CCR7* and *CCL18*, an effect only observed when macrophages are in co-culture with cancer cells. Notably, when comparing monocultures of macrophages in normoxia and hypoxia, an opposite effect was observed, with increased expression of all markers analyzed, and a significant increase on *CCR7* pro-inflammatory marker **(Figure 10A-B).** To confirm whether the significant differences found in hypoxic conditions at RNA corresponded, also, to alterations at the protein level, CCR7 and CD163 receptors expression was measured by flow cytometry. The **Figure 10C** is a representative image of the gating strategy performed for all flow cytometry analysis. The population double positive for the expression of CD14/CCR7 and for CD14/CD163 was subsequently analyzed **(Figure 10D).** Regarding the pro-inflammatory marker, CCR7, and in contrast to what was observed at the RNA level, no differences were detected in normoxia. However, we confirmed a reduction of CCR7 protein levels, when comparing the two co-cultures, a reduction also observed on macrophages monocultures **(Figure 10E).** Concerning the anti-inflammatory marker, CD163, we observed that the presence of cancer cells by itself did not alter the percentage of CD163 positive cells. When comparing the effect of hypoxia on macrophage-cancer cell crosstalk, we found a significant decrease in both the percentage of CD163 positive cells and on its mean fluorescence intensity (MFI), corresponding to the intensity of signal per cell, a process that seems to be mainly influenced by the hypoxia, once the same significant tendency was observed on macrophages monoculture **(Figure 10F).**

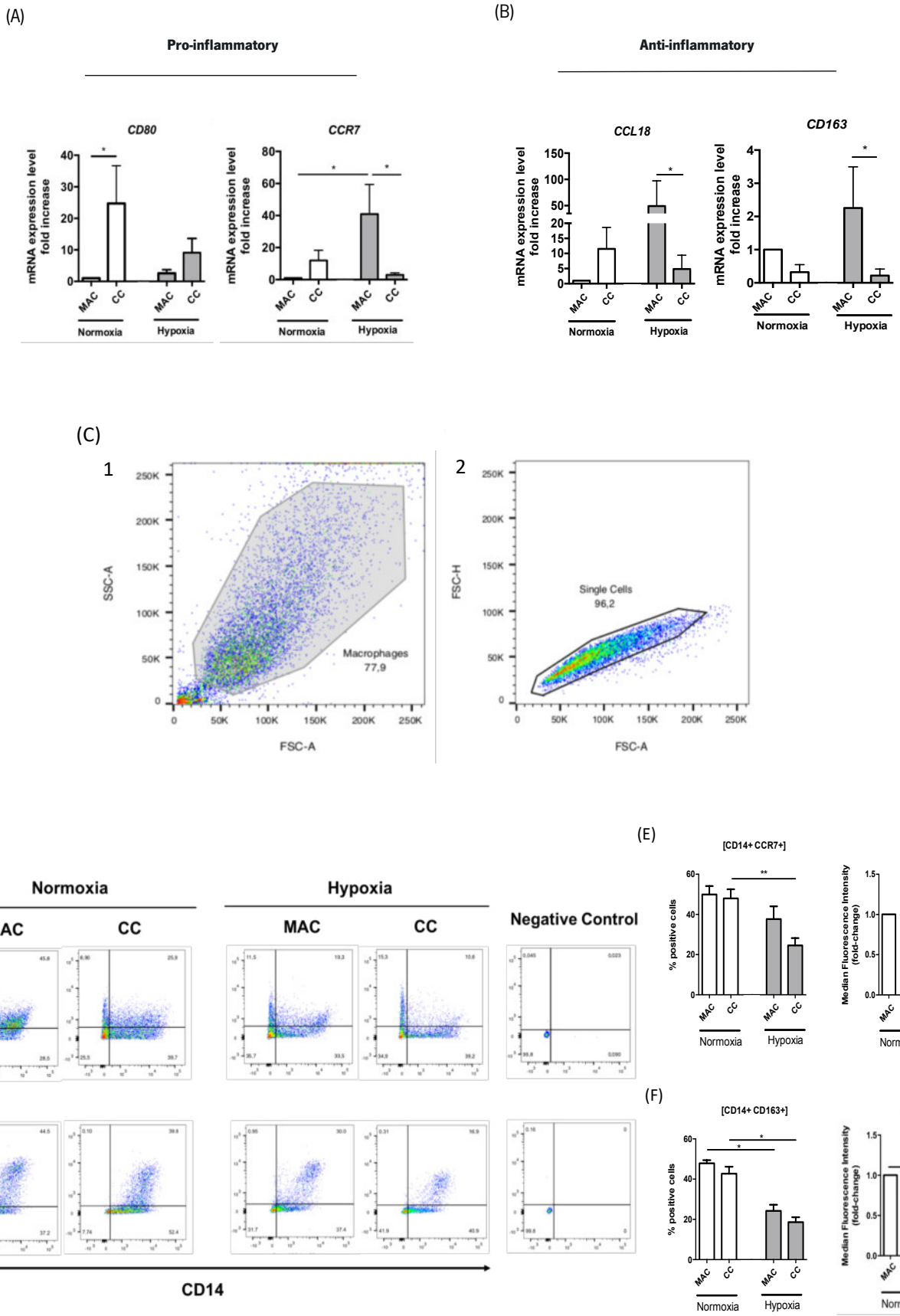

(A)
Pro-inflammatory
CD80
CCR7
mRNA expression level fold increase
MAC
CC
Normoxia
Hypoxia
(B)
Anti-inflammatory
CCL18
CD163
(C)
1
2
SSC-A
FSC-A
FSC-H
Macrophages
77,9
Single Cells
96,2
(D)
Normoxia
Hypoxia
MAC
CC
Negative Control
CCR7
CD163
CD14
(E)
[CD14+ CCR7+]
% positive cells
Median Fluorescence Intensity (fold-change)
(F)
[CD14+ CD163+]

Figure 10: Characterization of macrophages polarization profile. mRNA expression levels were measured on macrophages cultured in mono (MAC) and in co-culture (CC) with RKO cells under normoxia and hypoxia. The expression levels were normalized to housekeeping gene expression (*18S*) and the results are represented as a fold increase relatively to macrophages cultured under normoxia. A) mRNA expression levels of pro-inflammatory markers *CD80* and *CCR7*. B) mRNA expression levels of anti-inflammatory markers *CCL18* and *CD163*. Data represent the mean values and are representative of n= 7 (*CD80*); n=7 (*CCR7*); n=8 (*CCL18*); n=7 (*CD163*) independent experiments. C) The scatter plots exhibit a representative image of the gating strategy created with Flow Jo software for flow cytometry results. 1: FSC-A/SSC-A exemplifies the distribution of cells in the light scatter based on cell size and granularity, respectively; 2: FSC-A/FSC-H represents the single cells of the previous selected population. D) Cell surface expression of CCR7 and CD163 were measured by flow cytometry, on macrophages stained with the macrophage lineage marker CD14. Representation of scatter plots of $CD14^+CCR7^+$ and $CD14^+CD163^+$ in all conditions. Negative control represents the unstained macrophage population. E-F) The left graphs represent the percentage of macrophages with positive expression for E) CCR7 and F) CD163 and the right graphs represents the median fluorescence intensity of this population (MFI). The MFI of both markers are represented as a fold increase relatively to macrophages in normoxia. Data represent the mean values and are representative of n=11 (CCR7/CD14); n=6 (CD163/CD14) independent experiments. $^{**}p < 0.01$; $^{*}p < 0.05$.

Measurement of cytokines levels in conditioned medium

To better characterize our macrophage population, regarding their polarization profile, the levels of the soluble pro-inflammatory molecules IL-1β, IL-6 and TNF-α, and anti-inflammatory molecules CCL18, IL-10 and TGF-β were evaluated by ELISA, in the CM from macrophages cultured in mono and co-culture, under normoxia and hypoxia. We found that the co-culture of cancer cells and macrophages resulted in a significant decrease of CCL18 and IL-10 secretion in normoxia. When we compared the two co-cultures, we found that hypoxia led to a significant increase in IL-1β, and to a significant decrease in TGF-β levels. These effects appear to be exclusive of the co-culture in hypoxia, once the same tendency was not observed in the case of macrophage monocultures **(Figure 11).** In general, the expression of pro-inflammatory markers has the tendency to increase in hypoxia, while there is the tendency to decrease the levels of their anti-inflammatory counterparts **(Figure 11).**

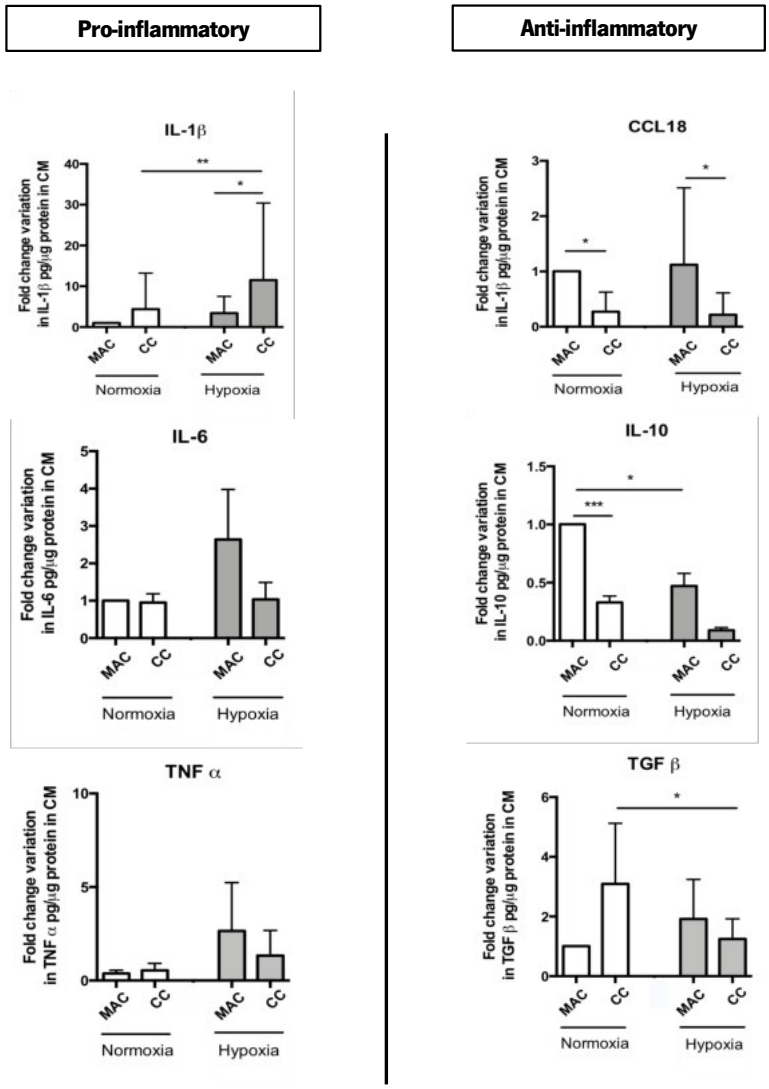
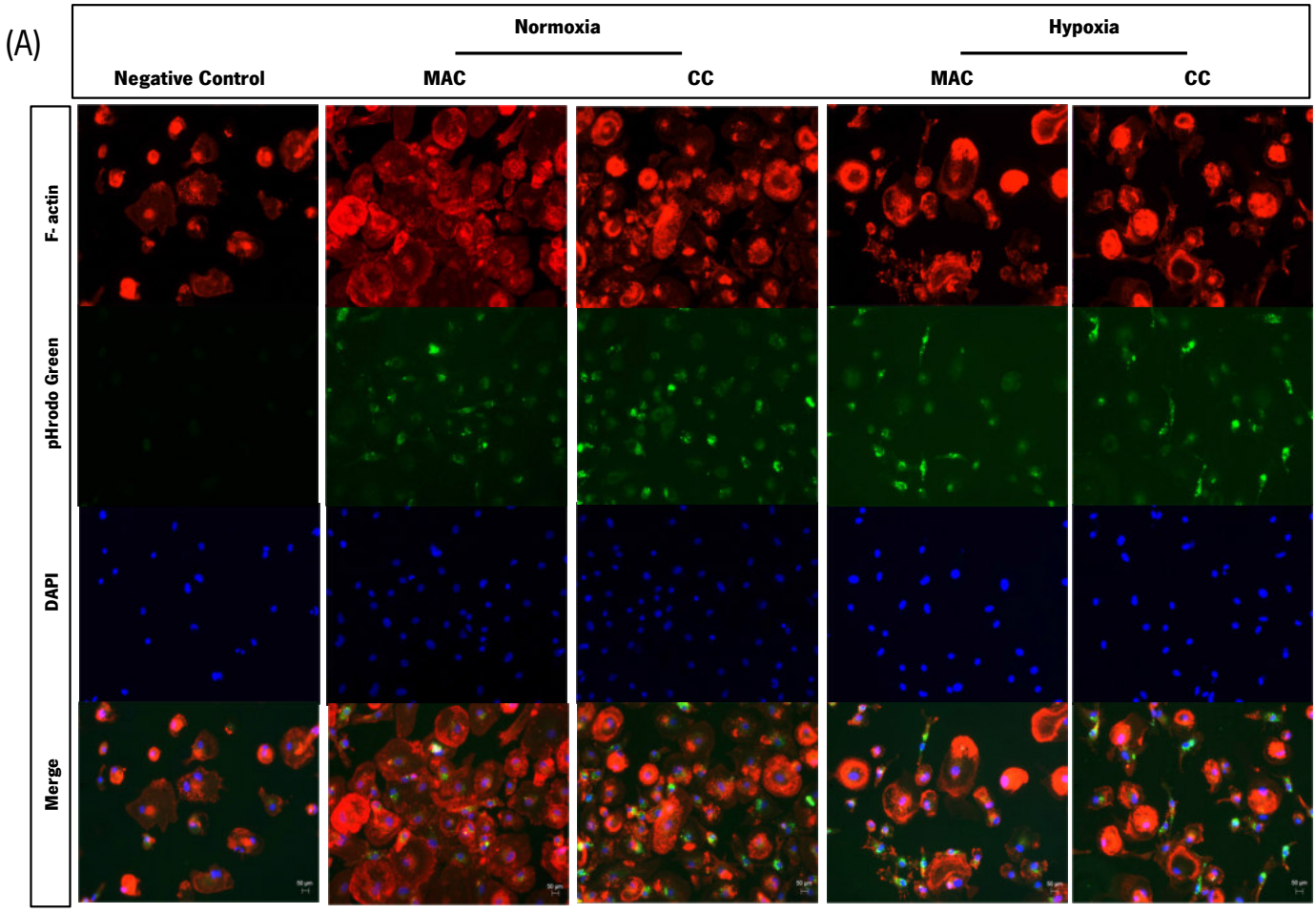


Figure 11: Quantification of soluble polarization markers on macrophage population. The six soluble molecules were evaluated through ELISA in the CM from macrophages grown in mono (MAC) or co-cultures (CC) with RKO cells, under normoxia or hypoxia conditions. The graphs represent cytokine concentration (picograms per micrograms of protein present in CM), of pro and anti-inflammatory polarization markers, as a fold change variation to macrophages cultured in normoxia. Data correspond to mean values and are representative of n=10 (IL-1β); n=9 (IL-6); n= 8 (TNF-α); n=9 (CCL18); n=5 (IL-10); n=8 (TGF-β) independent experiments per marker. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

Evaluation of the phagocytic activity of macrophages under normoxic or hypoxic conditions

One of the macrophages main features is phagocytosis, an activity associated with innate immune response, and a mechanism that is manipulated by the cancer cells in order to escape the immune system (H. Zhang *et al.,* 2015). Therefore, to understand if phagocytosis is affected, macrophages were cultured with or without cancer cells, in normoxia or hypoxia, and stimulated with a pro-phagocytic stimulus: bacteria-derived beads **(Figure 12A).** After the engulfment by macrophages and due to alteration in the pH at the macrophage vesicles, the bacteria-derived particles became green fluorescent, and the phagocytic capacity was then measured under a fluorescence microscope **(Figure 12B).** Our results revealed that hypoxia decreased significantly macrophage phagocytic activity. Interestingly, co-cultures with cancer cells under normoxia enhanced significantly the phagocytic MFI, meaning that each cell is more efficient in engulfing more beads, in contrast to co-cultures established under hypoxia. These results suggest that hypoxia decreases macrophage phagocytic activity MFI, meaning that diminishes the number of macrophages with capacity to phagocytize and, each of those macrophages phagocytize less bioparticles **(Figure 12C-D).**



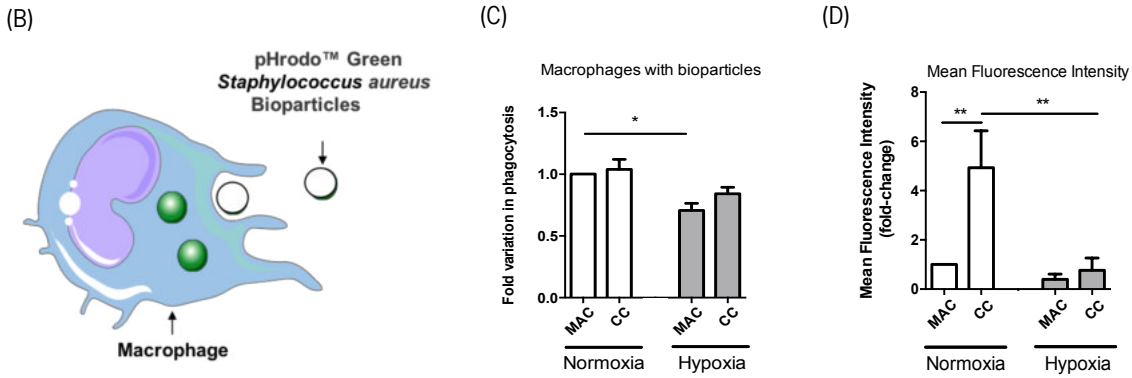


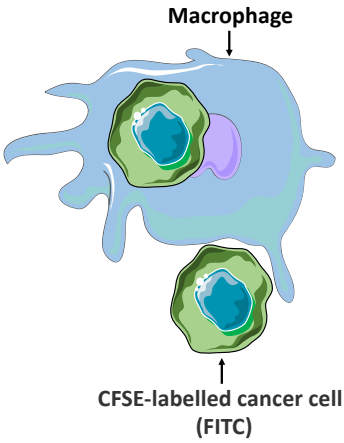
Figure 12: Macrophages phagocytic activity A) Phagocytosis was evaluated on macrophages that were in monoculture (MAC) and co-culture (CC) with RKO cells, in normoxia and hypoxia. Macrophages monocultures that were not in contact with bioparticles were used as negative control. Macrophage cytoskeleton was stained with F-actin (red) and nuclei were counterstained with DAPI (blue). B) Schematic representation of the engulfment of the *Staphylococcus aureus* bioparticles by macrophages. C) The percentage of macrophages able to phagocyte *S.aureus* bioparticles was quantified through Image J software. Fold variation of phagocytosis was evaluated in macrophages cultured alone or with RKO cells, under normoxia or hypoxia, and is presented as a fold-change relatively to monoculture of macrophages in normoxia. D) MFI of macrophages cultured alone or with RKO cells, under normoxia or hypoxia are presented as a fold-change relatively to monoculture of macrophages in normoxia. Data represent the mean values and are representative of six independent experiments (200 cells/ condition). Scale bar length: 50 μm. $^{**}p < 0.01$; $^{*}p < 0.05$.

Phagocytosis of cancer cells by macrophages

Since we obtained differences in the phagocytic behavior when macrophages were stimulated with bacteria-derived beads, we intended to analyze if the same differences were observed when macrophages are phagocytizing cancer cells **(Figure 13A).** Therefore, RKO cancer cells were fluorescently labeled with CellTrace™ CFSE and directly co-cultured with macrophages, previously cultured alone or with cancer cells in normoxic or hypoxic conditions. At the end, the phagocytic activity was measured by flow cytometry. Accordingly, macrophages able to phagocyte cancer cell stained with CellTrace™ CFSE, named as positive cells, corresponded to the $CFSE^{+}CD14^{+}$ population, represented in the **Figure 13 B-C** by the scatter plots on the upper right quadrant. The percentage of phagocytosis corresponds to the quotient of double positive macrophages ($CFSE^{+}CD14^{+}$) able to phagocytize stained cancer cells, per total of $CD14^{+}$ positive cells. Two approaches were performed: i) use as phagocytic stimulus stained cells that were

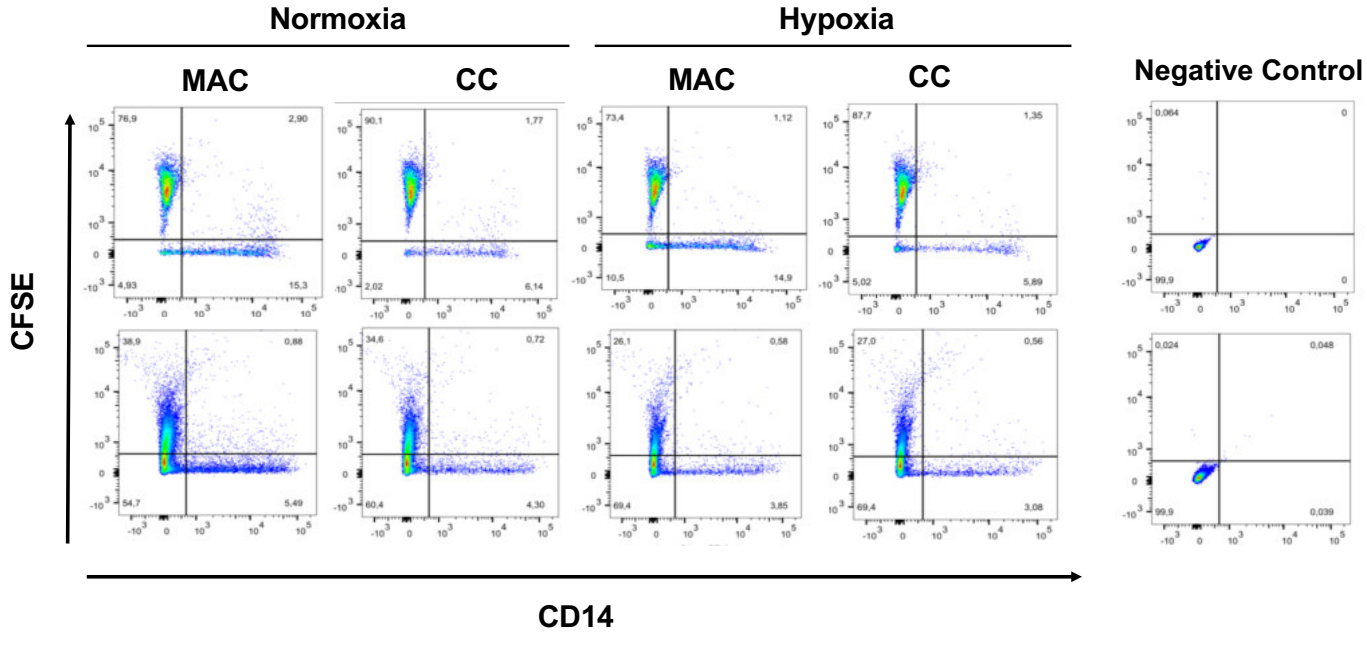
maintained three days in normoxia; ii) or cells that were maintained three days in hypoxia. Our results demonstrate that under normoxia and hypoxia, co-culture with cancer cells does not affect macrophage phagocytosis and analyzing the effect of co-culture in normoxia, no major differences were detected regarding the percentage of phagocytic macrophages, and the same occurred when comparing macrophages that were co-cultured with cancer cells. However, we found that macrophages in hypoxia presented an increase of the MFI, a condition that is significantly potentiated by the presence of cancer cells **(Figure 13D).** Regarding phagocytosis of stained RKO cells maintained in hypoxia no major differences were observed in the percentage of phagocytic cells, but as in the previous case, an increase in the MFI was observed in the hypoxic conditions, more pronounced in the case of co-cultures, but without statically significance **(Figure 13E).**

(A)



(B)

(C)



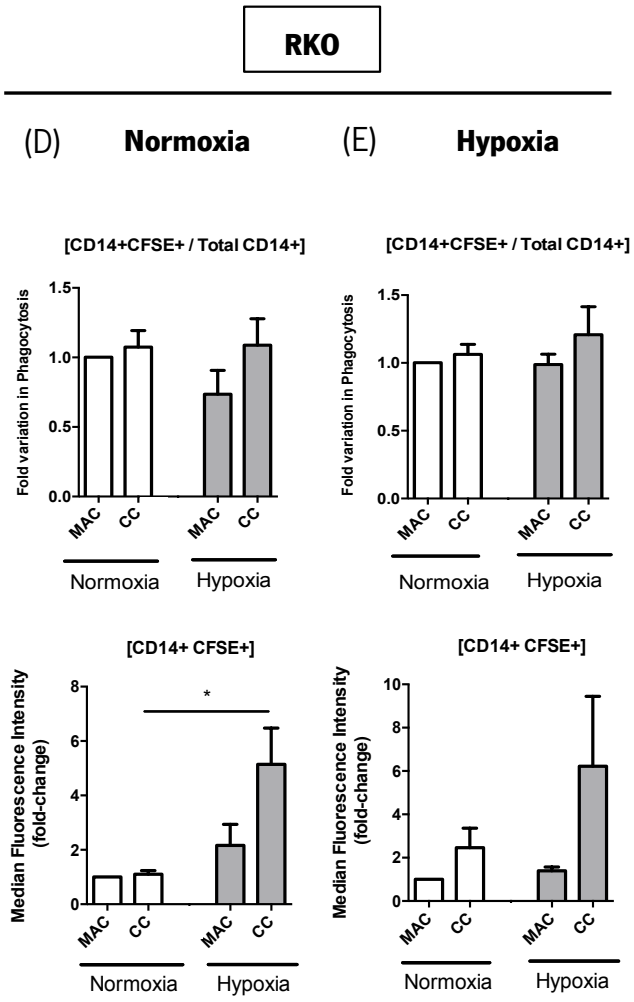


Figure 13: Phagocytosis of cancer cells by macrophages. Macrophages were previously cultured for 13 days in monoculture (MAC) and indirect co-culture (CC) with RKO, under normoxia and hypoxia. CellTrace™ CFSE-labeled cancer cells were used as phagocytic stimulus. A) Schematic representation of the procedure performed consisting on macrophages engulfing CFSE-labelled cancer cells (RKO). B-C) Representation of scatter plots of CD14+CFSE+ that were stimulated with (B) RKO cells maintained in normoxia; (C) RKO cells maintained in hypoxia. The negative controls of each experiment are represented by the unstained macrophage population. D-E) The upper graphs represents the fold variation of phagocytosis, ratio of positive cells for CD14+CFSE+ per total of CD14+ positive cells, represented as fold change relatively to macrophage monoculture in normoxia, when stimulated with stained D) RKO cultured in normoxic or E) RKO cultured in hypoxia. D-E) The lower graphs showed the MFI of the correspondent population. Data represent the mean values and are representative of five independent experiments on both RKO cells. *$p < 0.05$.

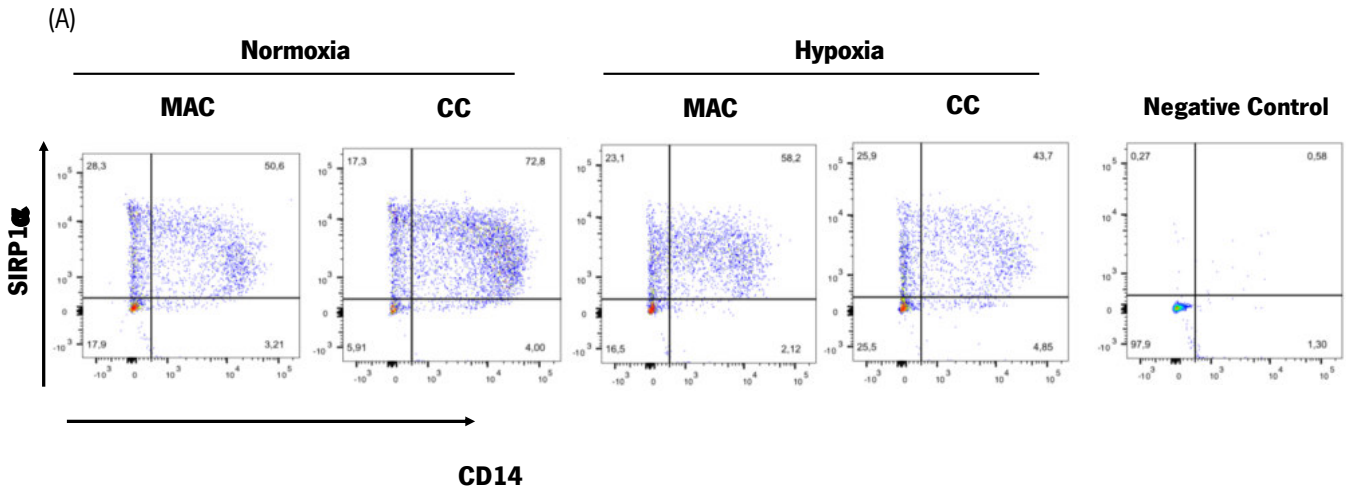
Immunological synapse molecules

"Don´t eat me signalling" - SIRP1α-CD47

Cancer cells have distinct mechanisms to escape the attack of immune cells. One of them, and probably the less exploited, is the enhanced expression of "don't eat me signals". Tumor cells may often increase the expression of CD47 at their surface, which interacts with the signal regulatory protein alpha (SIRP1α) receptor on macrophages, inhibiting phagocytosis (Chao *et al.*, 2012). Once we had found differences regarding macrophage phagocytic activity we decided to explore if this interaction is, in fact, affected by hypoxia.

SIRP1α

We started by evaluating the expression levels of SIRP1α at macrophages surface by flow cytometry, in our four conditions, using CD14 as a macrophage lineage marker, represented by the positive cells, which correspond to the $SIRP1\alpha^{+}CD14^{+}$ represented in the **Figure 14A** by the scatter plots on the upper right quadrant. Our results revealed that co-cultures do not seem to affect macrophage SIRP1α expression. However, hypoxia significantly decreased the MFI levels of this receptor at the surface of macrophages co-cultured with cancer cells **(Figure 14B).** In addition, at the mRNA level, we found that both the co-culture and the hypoxia led to a decrease on *SIRP1α* expression levels **(Figure 14C).**



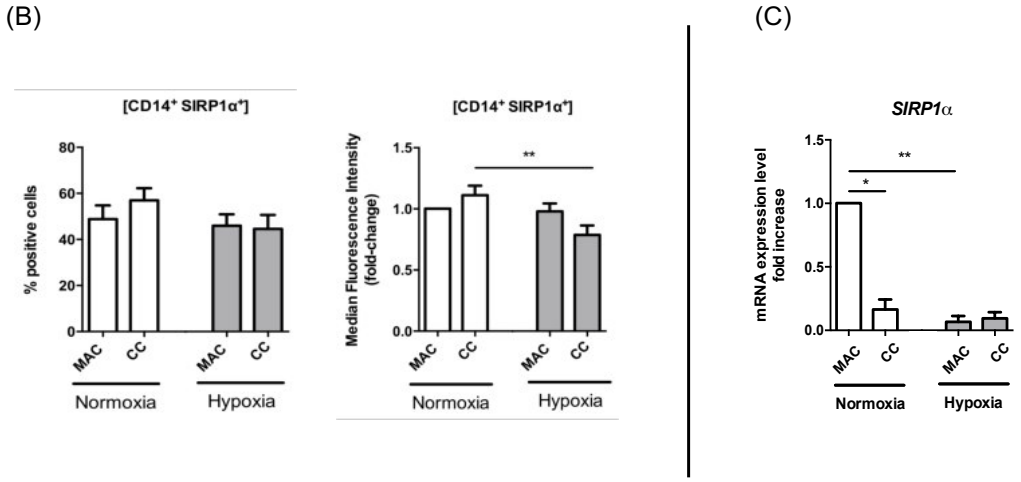


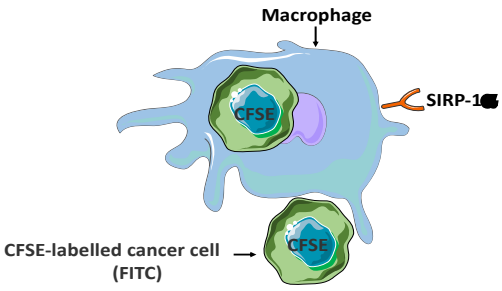
Figure 14: SIRP1α expression. The expression of SIRP1α receptor within the macrophage population, stained with macrophage lineage marker CD14 was determined by flow cytometry in macrophages cultured alone (MAC) or in co-culture (CC) with RKO cells, under normoxia and hypoxia. A) Scatter plots with representation of $CD14^+$ $SIRP1\alpha^+$ in all conditions. Negative control represents the unstained macrophage population. B) The left graph represents the percentage of positive cells for $SIRP1\alpha^+$ within the $CD14^+$ population, while the right graph, the MFI of this population, represented as fold change relatively to macrophages monoculture in normoxia. C) Graph represents the mRNA expression levels of $SIRP1\alpha^+$ on macrophages cultured in the conditions previously mentioned, normalized to the housekeeping gene expression (*18S)*. The results are represented as fold increase relatively to macrophages cultured alone in normoxia. Data represent the mean values and are representative of n=10 for the flow cytometry independent experiments, and n=6 independent experiments for the mRNA expression levels. $^{**}p < 0.01$; $^{*}p< 0.05$.

In parallel, we evaluated the SIRP1α expression on macrophages able to phagocytize CFSE-labelled cancer cells, previously exposed to normoxia or hypoxia conditions **(Figure 15A).** The **Figure 15B** is a representative image of the gating strategy of the steps to select the positive cells for $CFSE^+SIRP1\alpha^+$ within the $CD14^+$ positive population. The phagocytic macrophages that express SIRP1α receptor at their surface corresponded to the double positive population $CFSE^+SIRP1\alpha^+$, represented on the upper right quadrant **figure 15C-D** by the scatter plots. The first finding is that the majority of the macrophages that are phagocytic are also SIRP1α negative, concerning RKO previously cultured in normoxia. When considering the macrophages that are

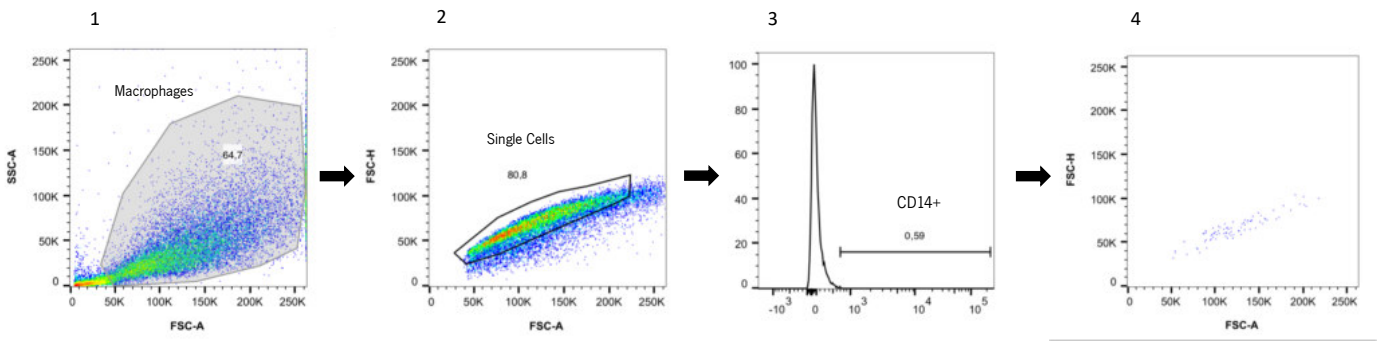
phagocytic and SIRP1α positive, we found that the stimulation with RKO cultured previously in normoxia led to a significant decrease in SIRP1α expression on macrophages that were co-cultured with RKO in hypoxia, compared with the ones co-cultured in normoxia, a condition that seems to be triggered by the co-culture and potentiated by hypoxia. The amount of SIRPα at the cell surface increases with the co-culture and in hypoxia, in a significant manner when comparing mono-cultures of macrophages in normoxia and hypoxia **(Figure 15E).**

Interestingly, in macrophages that phagocyted RKO cultured in hypoxic conditions, we observed no differences on macrophages that were previously in indirect co-culture with RKO in normoxia, but in hypoxic conditions SIRP1α expression increases comparing to normoxia, with a small difference spotted between macrophage that were cultured alone or in indirect co-culture with RKO **(Figure 15F).** The amount of SIRPα at the cell surface on macrophages significantly increased when macrophages were in indirect co-culture with cancer cells in normoxia, but decreases when compared macrophages that were co-cultured with cancer cells in normoxia and hypoxia **(Figure 15F).**

(A)

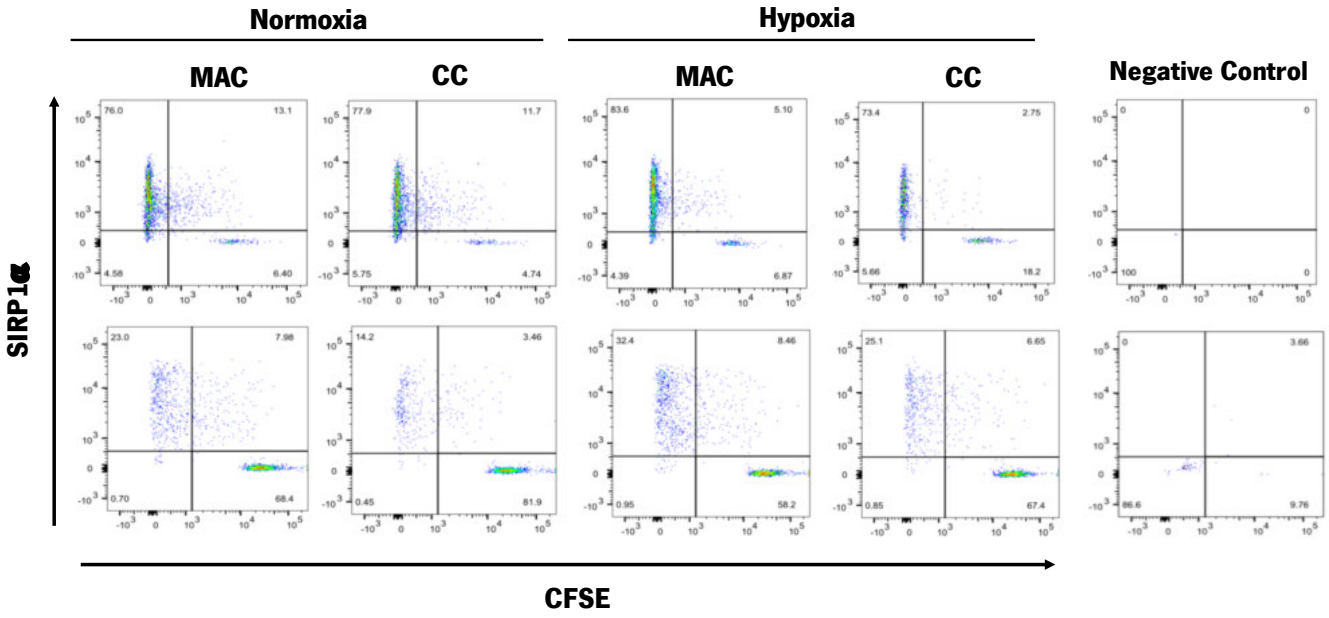


(B)



(C)

(D)


Normoxia
Hypoxia
MAC
CC
MAC
CC
Negative Control
SIRP1α
CFSE

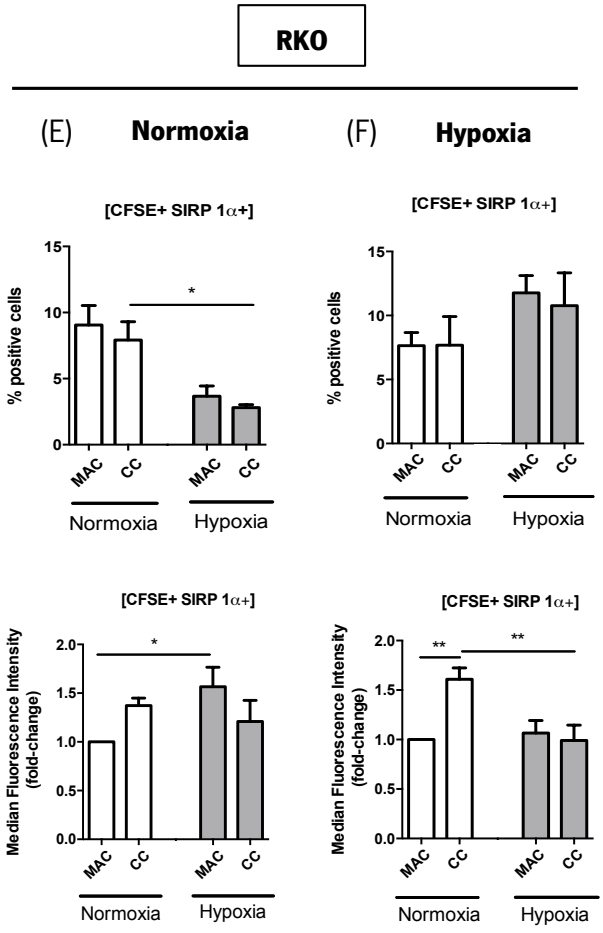

RKO
(E) Normoxia
(F) Hypoxia
[CFSE+ SIRP 1α+]
% positive cells
Median Fluorescence Intensity (fold-change)
MAC
CC
Normoxia
Hypoxia

Figure 15: SIRP1α expression on macrophages able to engulf cancer cells. Macrophages were cultured for 13 days in monoculture (MAC) or indirect co-culture (CC) with RKO cells, under normoxia and hypoxia. After this period, macrophages were stimulated with CellTrace™ CFSE -labelled cancer cells and their phagocytic ability was evaluated ($CD14^+CFSE^+$ cells). In addition, SIRP1α expression was measured on macrophages able to phagocyte stained cancer cells. A) Schematic representation of the procedure performed, with macrophages engulfing CFSE-labelled cancer cells (RKO). B) Sequential representation of the steps performed to select the positive cells for $CFSE^+SIRP1\alpha^+$ within the $CD14^+$ positive population. 1: FSC-A/SSC-A exemplifies the distribution of the unstained population of macrophages in the light scatter based on cell size and granularity, respectively; 2: FSC-A/FSC-H represents the single cells of the previous selected population; 3: Selection of the population of macrophages which is $CD14^+$ (macrophage lineage marker) within the unstained population, by selecting all cells that are in the right side; 4: FSC-A/FSC-H represents single cells of the previous selected population that are $CD14^+$. Representation of scatter plots of $CFSE^+SIRP1\alpha^+$ on macrophages that engulfed C) RKO cells cultured in normoxia or D) RKO cells maintained in hypoxia. Negative control of all experiments represents the unstained macrophage population. E-F) The upper graphs represent the percentage of macrophages that engulfed stained cells with positive expression for SIRP1α, while the lower graphs represent the MFI, when stimulated with E) RKO cells cultured in normoxia or F) RKO cultured in hypoxia. Data represent mean values and are representative of five independent experiments. $^{**}p < 0.01$; $^{*}p < 0.05$.

CD47

After the characterization of SIRP1α receptor on macrophages, the expression of CD47 on cancer cells was evaluated. The protein expression was evaluated by flow cytometry on RKO cells cultured in mono and co-culture with macrophages under normoxia and hypoxia conditions. The **figure 16A** exemplifies the histogram of the population of cancer cells that are positive for CD47, represented as CD47+ on the right side of the histogram. Our results revealed that, regarding RKO cells, hypoxia led to a significant increase on CD47 positive cells and on the MFI when comparing co-cultures in normoxia and hypoxia, and the difference between mono and co-culture in hypoxia is also significant **(Figure 16B).** The *CD47* mRNA levels were also measured to evaluate whether the differences found at protein level are also occurring at RNA level. We observed a significant increase of CD47 expression levels on RKO cells co-cultured with macrophages in normoxia. When the effect of hypoxia was evaluated on macrophage-cancer cell crosstalk, we saw that hypoxia led to a significant decrease of *CD47* levels. Despite not being statically significant we also spotted a tendency of increase on *CD47* expression levels when comparing monocultures in normoxia and hypoxia **(Figure 16C).**

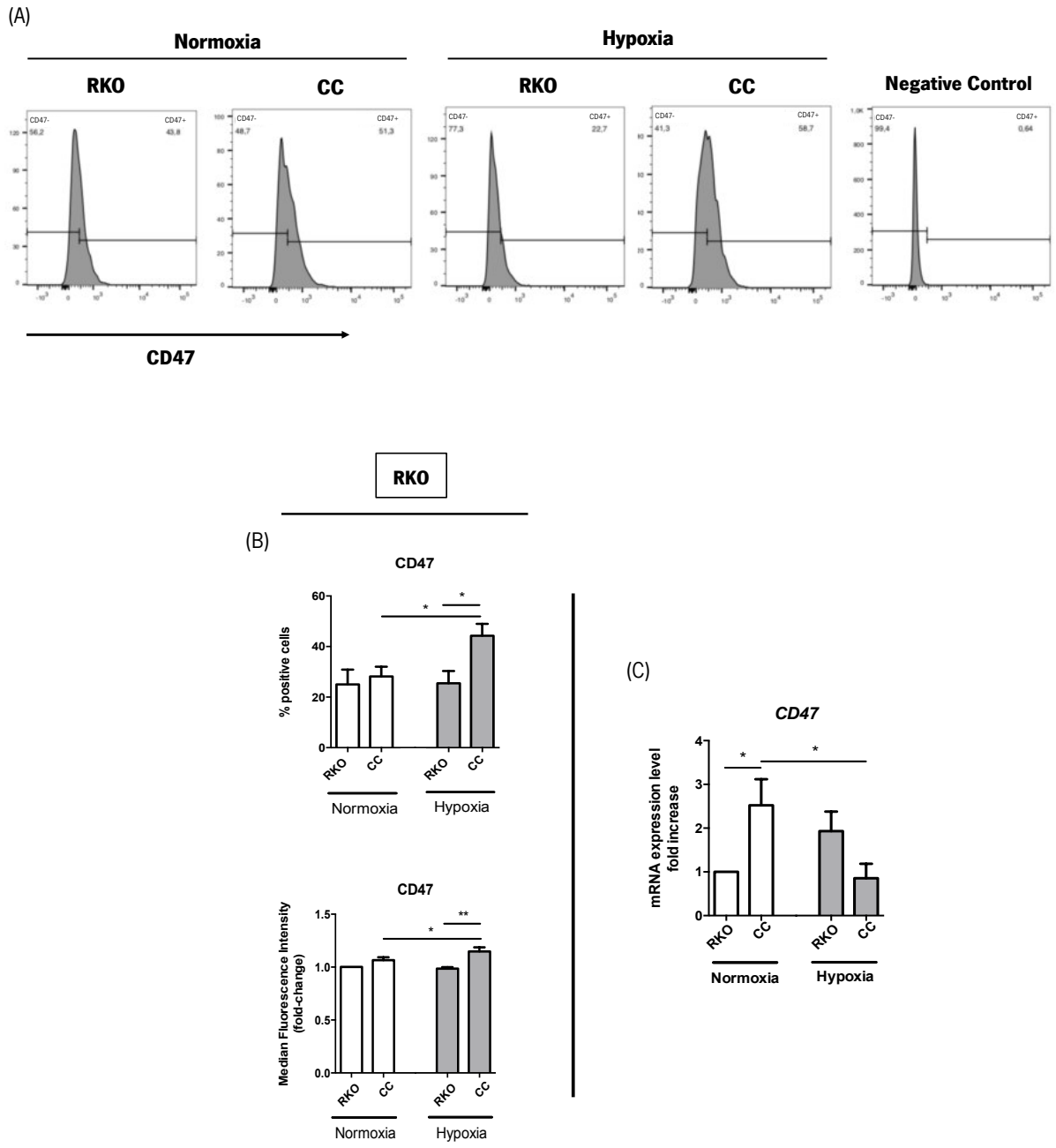
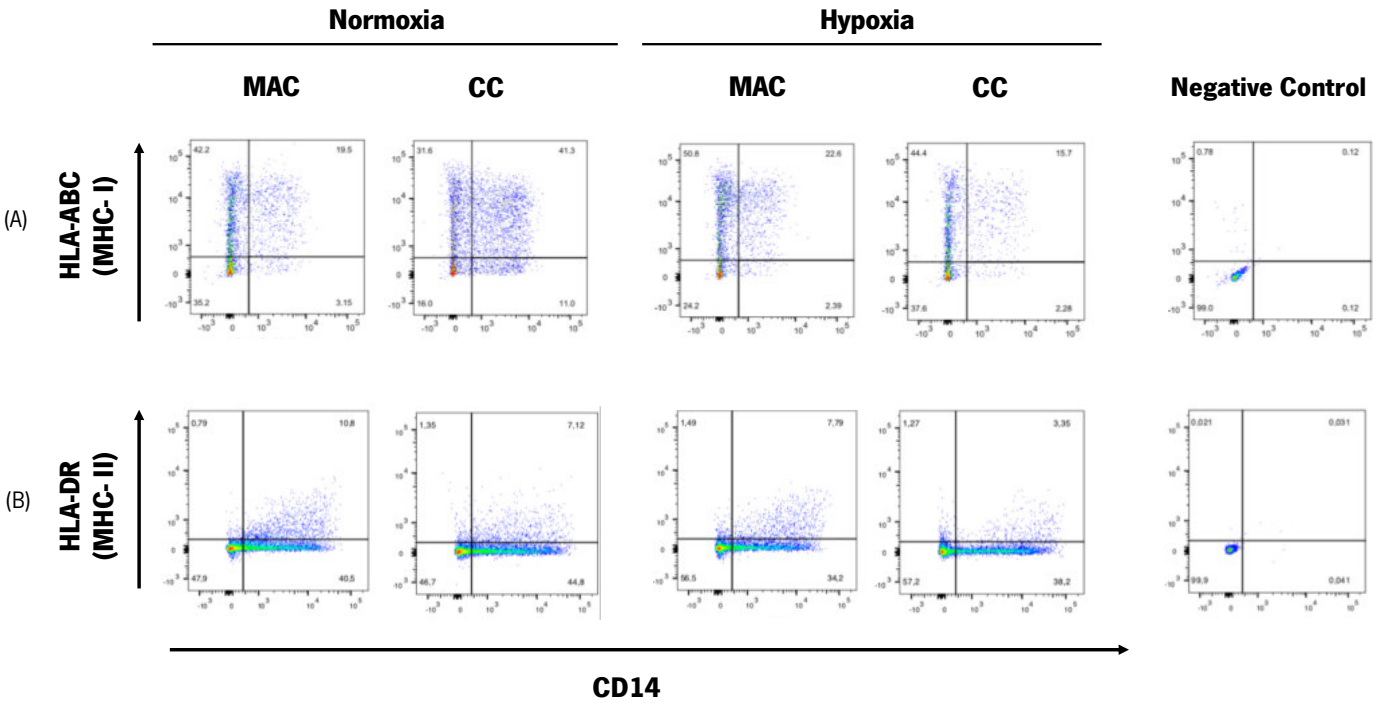


Figure 16: CD47 expression level. The cell surface expression of CD47 was measured by flow cytometry on cancer cells cultured in mono (RKO) and co-culture (CC) with macrophages under normoxia and hypoxia. A) Representation of CD47+ on RKO cells in all conditions. Negative control represents the unstained population. B) The upper graphs represents the percentage of positive cells for CD47 and the lower graphs represents the MFI of this population. The MFI is represented as a fold change relatively to cancer cells cultured in normoxia. C) *CD47* mRNA expression of RKO cells in mono (RKO) or co-culture (CC) with macrophages, under normoxia or hypoxia. The expression levels were normalized to *ACTB* expression and the results are represented as a fold increase relatively to RKO cells cultured under normoxia. Data represent the mean values and are representative of nine independent experiments for both flow cytometry and mRNA expression analysis. **p < 0.01; *p < 0.05

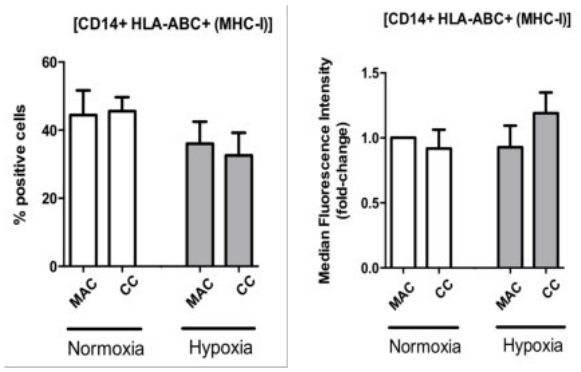
MHC-I and MHC-II

Major histocompatibility complexes (MHC), classified as MHC-I (HLA-ABC) and MHC-II (HLA-DR) are responsible for antigen presentation to T cells, being essential for cell-mediated immunity, and destruction of aberrant cells. However, tumor cells are able to escape to immune recognition and destruction through several mechanisms (de Charette *et al.,* 2016). So, our aim was to evaluate how hypoxia and the co-cultures of macrophages and cancer cells alter the expression of these two molecules. Therefore, their cell surface expression on macrophages, stained with the macrophage lineage marker CD14 was measured by flow cytometry. The **figure 17 A-B** represents the scatter plots of macrophage population which express HLA-ABC and HLA-DR within the macrophage population and are described as positive cells, on the upper right quadrant. Concerning the macrophages, our results evidenced that there is a tendency decrease percentage of MHC-I positive cells with hypoxia. Nevertheless, when the MFI is analysed, we found an increase when comparing co-cultures in normoxia and hypoxia **(Figure 17C).** Regarding MHC-II, we found that the co-culture with cancer cells resulted in a significant decrease on this molecule expression, both in normoxia and hypoxia. However, the MHC-II level of expression are lesser in hypoxia than in normoxia **(Figure 17D)**. The MFI sustain the same pattern with a significant decrease observed in macrophages co-cultured with RKO in normoxia **(Figure 17D).** MHC-I was also evaluated on RKO cancer cells, cultured in mono and in co-culture with macrophages under normoxia and hypoxia and are represented in the **figure 17E** by the histogram of the population of cancer cells that are positive for HLA-ABC represented as HLA-ABC+ on the right side of the histogram.

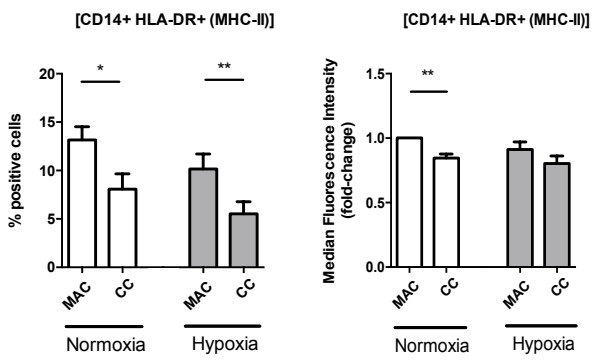
Our results revelled no major differences on percentage of positive RKO cells, but the analysis showed an increase on the MFI with the co-culture **(Figure 17F).**

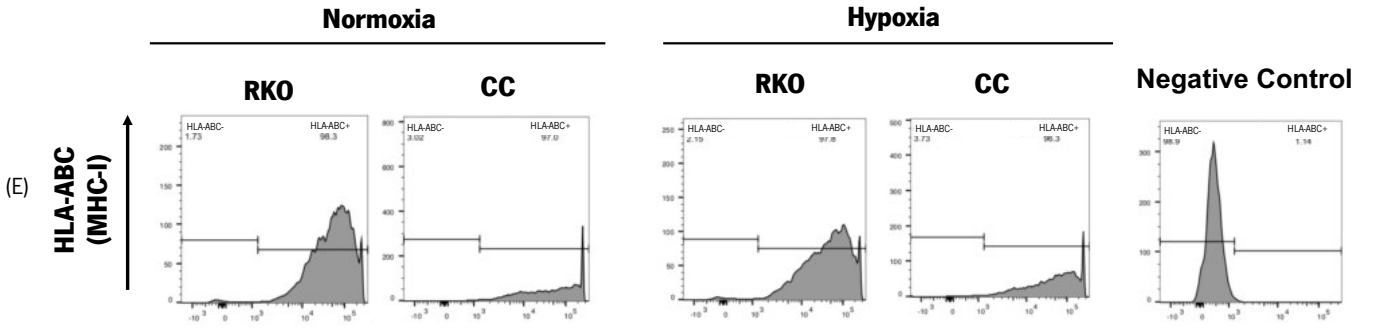

Normoxia
Hypoxia
MAC
CC
MAC
CC
Negative Control
(A)
HLA-ABC (MHC- I)
(B)
HLA-DR (MHC- II)
CD14

(C)


[CD14+ HLA-ABC+ (MHC-I)]
% positive cells
60
40
20
0
MAC
CC
MAC
CC
Normoxia
Hypoxia
[CD14+ HLA-ABC+ (MHC-I)]
Median Fluorescence Intensity (fold-change)
1.5
1.0
0.5
0.0
MAC
CC
MAC
CC
Normoxia
Hypoxia

(D)


[CD14+ HLA-DR+ (MHC-II)]
% positive cells
20
15
10
5
0
*
**
MAC
CC
MAC
CC
Normoxia
Hypoxia
[CD14+ HLA-DR+ (MHC-II)]
Median Fluorescence Intensity (fold-change)
1.5
1.0
0.5
0.0
**
MAC
CC
MAC
CC
Normoxia
Hypoxia


Normoxia
Hypoxia
RKO
CC
RKO
CC
Negative Control
(E)
HLA-ABC (MHC-I)

(F)

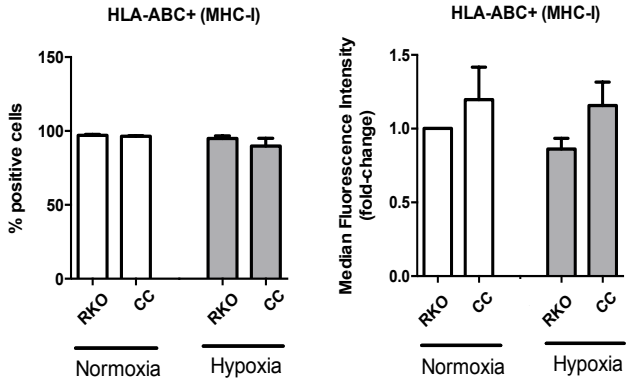
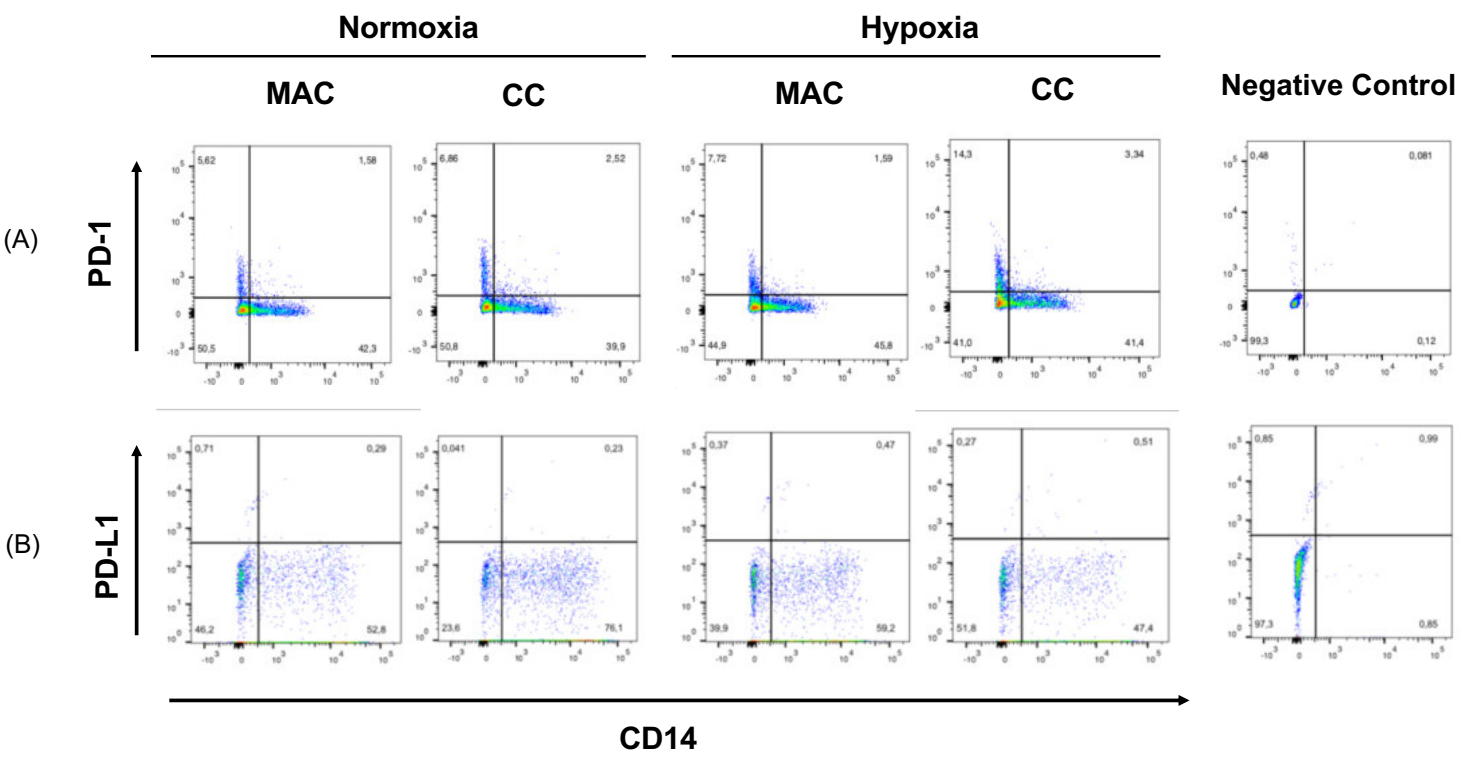


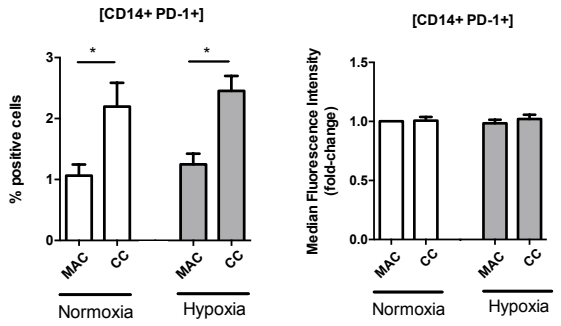
Figure 17: Cell surface expression of MHC-I and MHC-II. The MHC-I and MHC-II expression was measured by flow cytometry on macrophages cultured in mono (MAC) and in co-culture (CC) with RKO cells under normoxia and hypoxia. A) Scatter plot representation of macrophages stained with the macrophage lineage marker CD14 and positive for HLA-ABC in all conditions. B) Representation of scatter plots of macrophages $CD14^{+}$ $HLA\text{-}DR^{+}$ in all conditions. Negative control represents the unstained population of macrophages. C-D) The left graphs represent the percentage of macrophages positive for C) MHC-I and D) MHC-II and the right graphs represent the MFI of this population. The MFI in all experiments are represented as a fold change relatively to macrophages cultured in normoxia. Data represent the mean values and are representative of n= 7 (MHC-I); n=8 (MHC-II) independent experiments. The MHC-I levels were also measured by flow cytometry on RKO cells cultured in mono (RKO) and in co-culture with macrophages (CC) under normoxia and hypoxia. E) Representation of MHC-I+ cells within the RKO population in all conditions. F) The left graphs represent the percentage of RKO that are positive for MHC-I and the right graphs the MFI of this population. The MFI is represented as a fold change relatively to RKO cultured in normoxia. Data represent the mean values and are representative of eight independent experiments for RKO measurements. *$p < 0.05$; **$p < 0.01$.

PD-1 and PDL-1

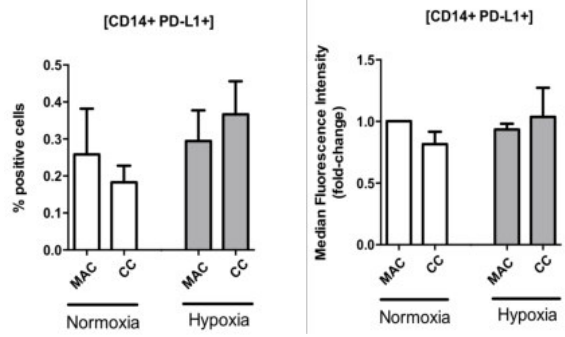
PD-1 and PD-L1 are described as being an axis altered by cancer cells to allow their immune evasion (Valentini *et al.,* 2018). We wanted to understand how hypoxia and the presence of cancer cells or macrophages affects the expression of these two proteins. To do so, we measured the cell surface expression of PD-1 and PD-L1 on macrophages, stained with the lineage marker CD14, in mono and co-culture with RKO under normoxia and hypoxia, by flow cytometry. The **figure 18 A-B** represents the scatter plots of macrophages that express PD-1 and PD-L1 within the macrophage population, named as positive cells in the upper right quadrant. Regarding PD-1 expression on macrophages we found a very low percentage of positive cells. However, we found that the presence of cancer cells in normoxia and in hypoxia triggered a significant increase of PD-1 positive cells **(Figure 18C).** Concerning PD-L1 expression, we found a very low percentage of positive cells, and observed that hypoxia led to an increase on its expression, slightly higher on macrophages co-culture with RKO cells **(Figure 18D).** The same receptor and ligand were evaluated on RKO cancer cells cultured in mono and co-culture with macrophages in normoxia and hypoxia and the **figure 18 E-F** exemplifies the histogram of the population of cancer cells that are positive for PD-1 and PD-L1, respectively, represented as PD1+ and PD-L1+ on the right side of the histogram. Regarding PD-1 and PD-L1 expression on cancer cells we found that the expression of PD-1 receptor decreased whenever cancer cells were in presence of the macrophages, an effect that is not triggered by hypoxia. However, the monocultures of RKO showed increased levels when exposed to hypoxic conditions **(Figure 18G).** Additionally, the presence of macrophages increases PD-L1 expression in normoxia, but the presence of hypoxia in the co-culture led to a significantly opposite effect, which seems to be specific, as we observed that in monocultures of cancer cells, the hypoxia also led to an increase of PD-L1 levels **(Figure 18H).**


Normoxia
Hypoxia
MAC
CC
MAC
CC
Negative Control
(A)
PD-1
5,62
1,58
50,5
42,3
6,86
2,52
50,8
39,9
7,72
1,59
44,9
45,8
14,3
3,34
41,0
41,4
0,48
0,081
99,3
0,12
(B)
PD-L1
0,71
0,29
46,2
52,8
0,041
0,23
23,6
76,1
0,37
0,47
39,9
59,2
0,27
0,51
51,8
47,4
0,85
0,99
97,3
0,85
CD14

(C)


[CD14+ PD-1+]
% positive cells
MAC
CC
MAC
CC
Normoxia
Hypoxia
[CD14+ PD-1+]
Median Fluorescence Intensity (fold-change)
MAC
CC
MAC
CC
Normoxia
Hypoxia

(D)


[CD14+ PD-L1+]
% positive cells
MAC
CC
MAC
CC
Normoxia
Hypoxia
[CD14+ PD-L1+]
Median Fluorescence Intensity (fold-change)
MAC
CC
MAC
CC
Normoxia
Hypoxia

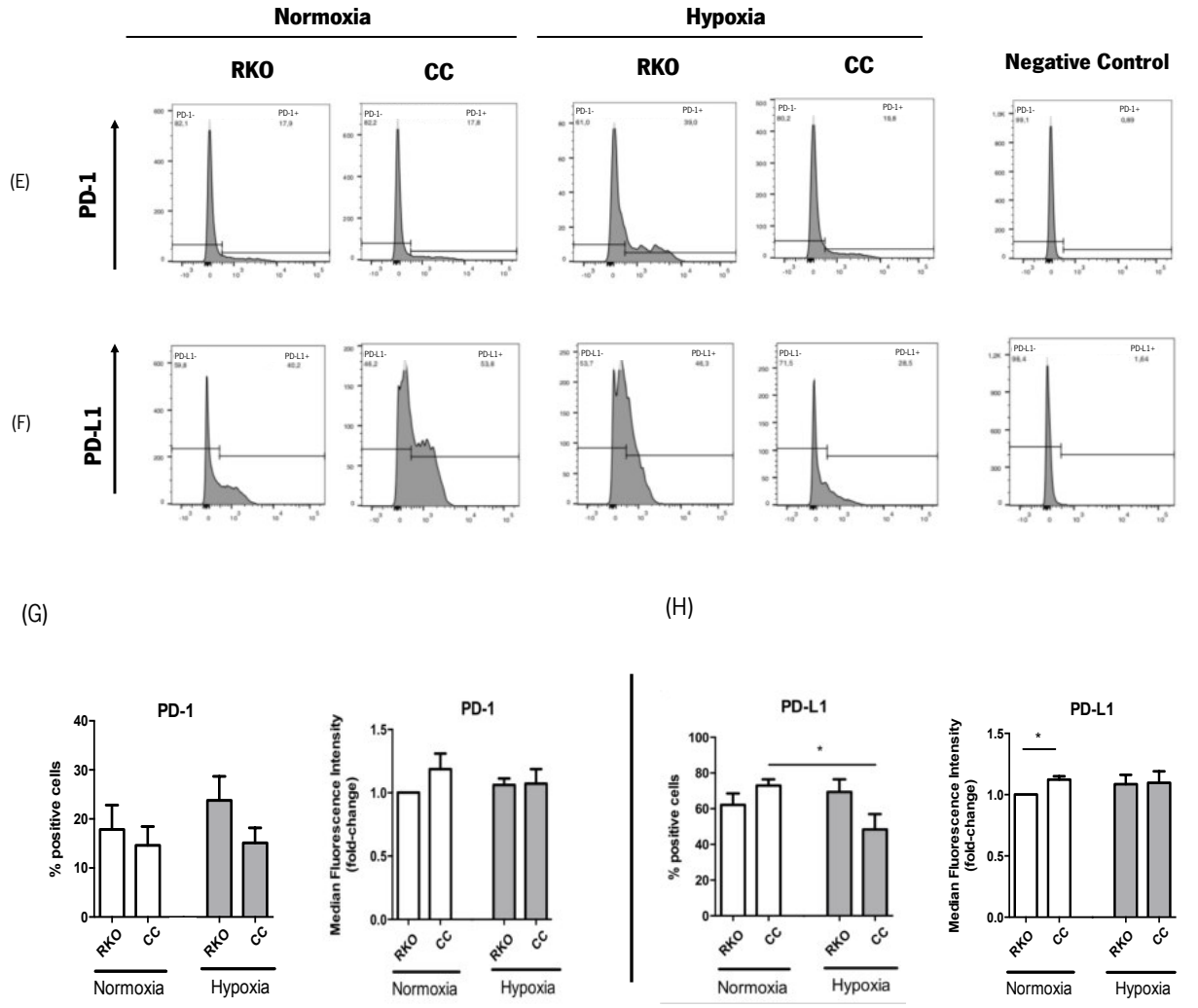


Figure 18: Cell surface expression of PD-1 and PD-L1 measured on macrophages and RKO cells.

The PD-1 and PD-L1 expression was measured by flow cytometry on macrophages cultured in mono (MAC) and co-culture (CC) with RKO cells under normoxia and hypoxia. A) Representation of scatter plots of macrophages stained with macrophage lineage marker CD14 and positive for PD-1 in all conditions. B) Representation of scatter plots of macrophages CD14+ PD-L1 + in all conditions. Negative control represents the unstained population of macrophages. C-D) The left graphs represent the percentage of positive macrophages and the right graphs represent the MFI of C) PD-1 and D) PD-L1. The MFI in all experiments are represented as a fold change relatively to macrophages cultured in normoxia. Data represent the mean values and are representative of n= 13 (PD-1); n=7 (PD-L1) independent experiments. The same ligand and receptor were measured by flow cytometry on RKO cells cultured in mono (RKO) and in co-culture (CC) with macrophages under normoxia and hypoxia. E) Representation of PD-1+ cells within the RKO population in all conditions. F) Representation of PD-L1+ cells within the RKO population in all conditions. Negative control characterizes the unstained population. G-H) The left graphs represent the percentage of RKO cells that are positive for G) PD-1 and H) PD-L1 and the right graphs represents the MFI of the correspondent populations. The MFI in all experiments are represented as a fold change relatively to RKO cultured in normoxia. Data represent the mean values and are representative of seven independent experiments for both PD-1 and PD-L1 measurements *$p < 0.05$;

Evaluation of hypoxia impact on macrophage-driven cancer cell invasive phenotype

Cancer invasion is one of the hallmarks of cancer, defined as the cancer cell capacity to invade adjacent normal tissue, and is the first step for the switch from a locally growing tumor into a systemic, metastatic disease (Friedl & Alexander, 2011). This mechanism is of great interest to us since it was previously described by our group that macrophages promote gastric and colorectal cancer cell invasion (Cardoso *et al.,* 2014). However, this characterization was performed under normoxic conditions and our aim now it is to understand if in hypoxic conditions and in the presence of macrophages, cancer cells still promote cell invasion. In order to evaluate invasion of cancer cells, a Matrigel invasion assay was performed in normoxia and hypoxia, allowing the quantification of cancer cells able to infiltrate through the Matrigel-coated insert, using macrophages as invasive stimulus. Our results revealed that in the presence of macrophages, RKO invasive capacity increased significantly, confirming the previous results described by our group. Interestingly we observed that hypoxia without macrophages is sufficient to induce an increase in invasion, with statically significance, and the presence of macrophages and hypoxia potentiate even more the cancer cell invasive potential **(Figure 19).**

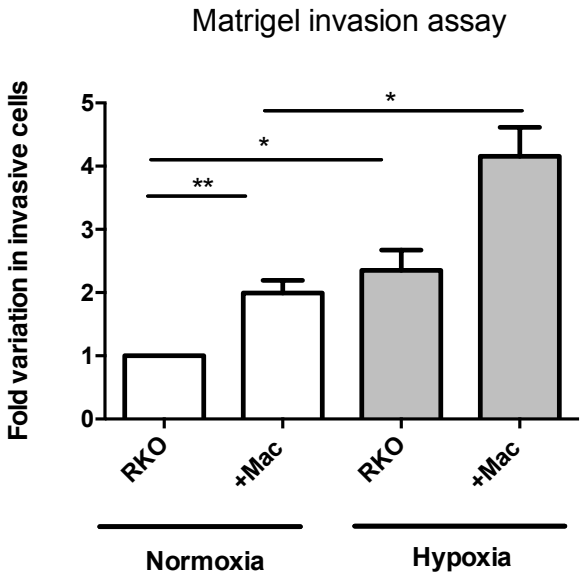
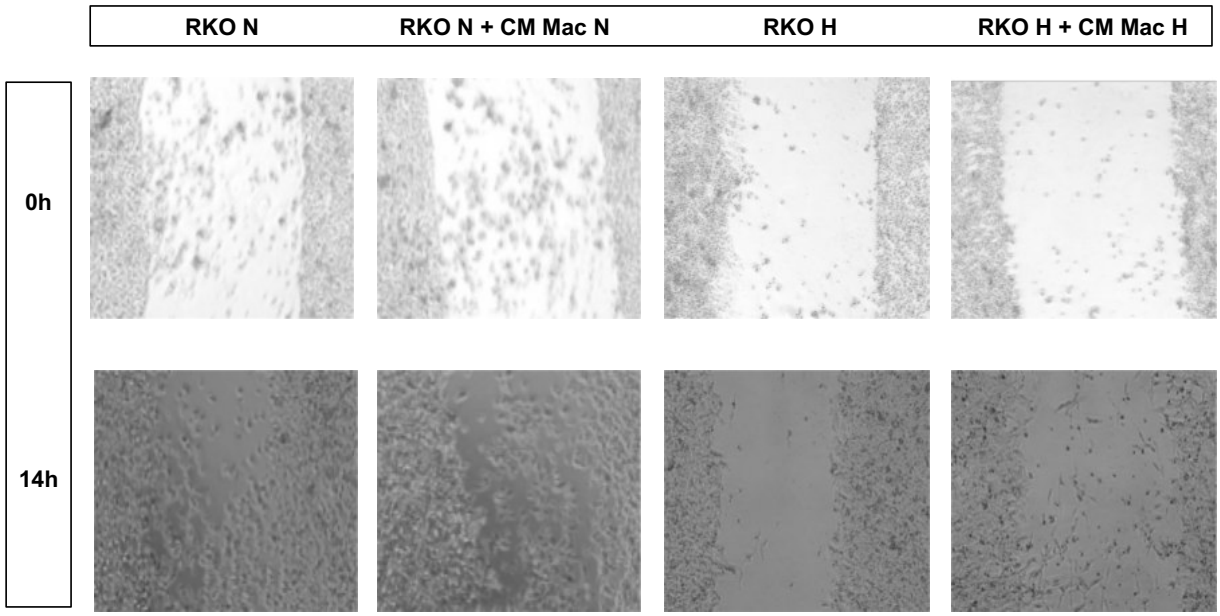


Figure 19: Influence of macrophages and hypoxia on cancer cell invasion. The experiments were performed using RKO cancer cells seeded alone (RKO) or in co-culture with macrophages (+Mac) in a Matrigel coated transwell filters, under normoxic or hypoxic conditions. The invasion capacity of cancer cells is represented as a fold change variation relatively to RKO cultured in normoxia. Data represent the mean values and are representative of seven independent experiments. $^{**}p < 0.01$; $^{*}p < 0.05$.

Impact of hypoxia and macrophages on cancer cell migration

Several cellular alterations underlie the cancer invasive process, namely the increase in migration, in proteolytic activity, and at the level of gene reprogramming and signalling. Once differences were observed when cancer cells were stimulated both by macrophages and by hypoxia regarding invasion, we decide to explore the impact of these factors on invasion-associated mechanisms, starting by the cancer cell migratory capacity. Therefore, we analysed migration of RKO cells through a wound healing assay, performed in a confluent cell monolayer cultured in normoxia and hypoxia and consecutively exposed, or not, to CM of macrophages from prior experiments grown under both conditions **(Figure 20A)**. Cancer cell migration was evaluated through the quantification of wound closure measured in each condition. Our results demonstrated that hypoxia by itself alters the migratory potential of the cells. The addition of soluble factors of the conditioned medium from macrophages cultured in normoxia has the same effect. However, the conjugation of these two factors did not show any cumulative effect **(Figure 20B).**

(A)



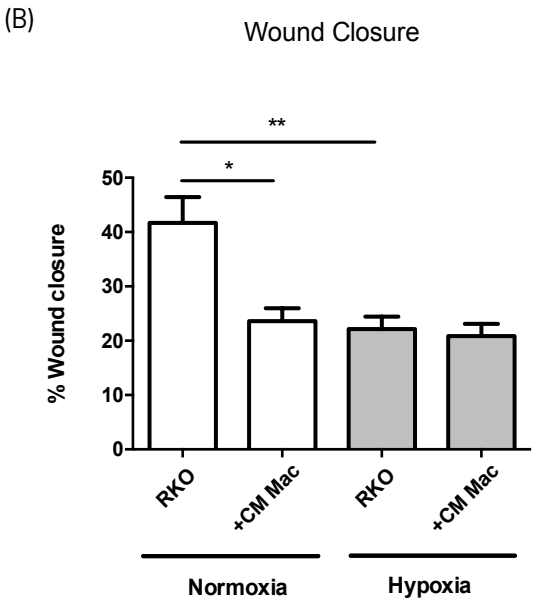


Figure 20: Migration of RKO cells. Cancer cell migration was evaluated by a wound healing assay, performed on RKO monolayer cultured alone in normoxia or hypoxia or treated with CM of macrophages (+CM Mac), cultured in normoxic or hypoxic conditions. (A) Images represent the wound performed in each condition and were acquired with 10X amplification. B) Graph represents the percentage of wound closure measured 14h after the wound. Data represent the mean values and are representative of eleven independent experiments. **$p < 0.01$; *$p<0.05$.

Impact of hypoxia and macrophages on proteolytic activity

Cancer cells become more invasive due the upregulation of extracellular matrix-degrading enzymes such as MMPs (Friedl & Wolf, 2003). Once it was previously described that cancer cells and macrophages can produce the invasion-associated enzymes MMP-1 (Cierna *et al.,* 2014) and MMP-7 (Burke *et al.,* 2003), we analysed their expression by qRT-PCR on RKO cells and macrophages, in mono and co-culture in normoxia and hypoxia.

Noteworthy, we verified with the same procedure, no significant levels of *MMP1* expression on macrophages, and of *MMP7* on cancer cells. We observed a significant increase of *MMP1* levels when cancer cells were co-cultured with macrophages in normoxia, and a significant decrease when comparing the two co-cultures **(Figure 21)**. Regarding the mRNA expression levels of *MMP7*, we found that hypoxia significantly increase its expression, and that even in normoxia the presence of cancer cells is sufficient to trigger an increase on this MMP **(Figure 21).**

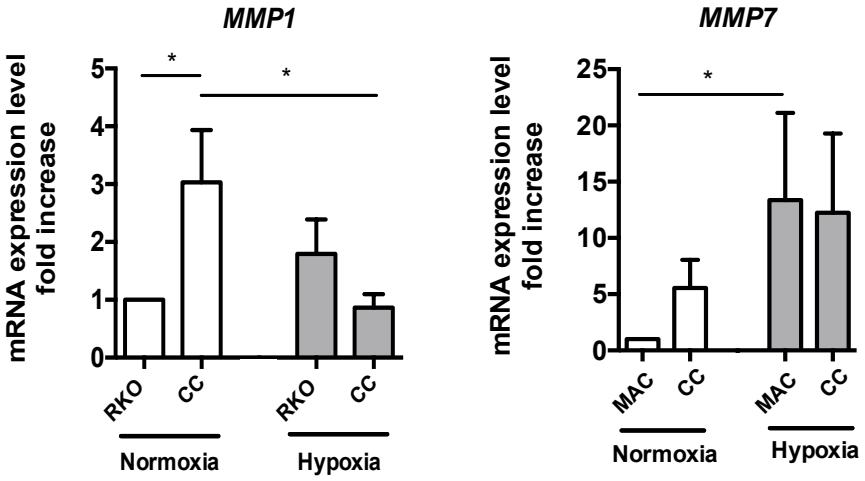


Figure 21: mRNA expression levels of MMPs measured in RKO cells and macrophages. The mRNA expression levels of *MMP1* were measured on RKO in mono-culture (RKO) and in co-culture (CC) with macrophages in normoxia and hypoxia conditions. RKO mRNA expression levels were normalized to *ACTB* expression and are represented as fold increase relatively to RKO in normoxia. The mRNA expression levels of *MMP7* were measured on macrophages in mono (MAC) and in co-cuture (CC) with RKO cells in normoxia and hypoxia. mRNA expression levels of macrophages were normalized to the housekeeping gene *18S* and are represented as a fold increase relatively to macrophages in normoxia. Graphs represent the mean values and are representative of n=8 (*MMP1*); n= 7 (*MMP7*) independent experiments *$p<0.05$.

Epithelial-mesenchymal transition (EMT) related genes

The increase of tumor invasion is associated to a process denominated EMT which is characterized by the transition of epithelial-like cancer cells to a more mesenchymal phenotype, allowing the dissemination and migration of cancer cells. (Friedl & Wolf, 2003).

Accordantly to EMT gene profile, cancer cells can become more migratory, invasive and aggressive. So, we intended to understand how hypoxia and the presence of macrophages controls the acquisition of the mesenchymal-like characteristics. Therefore, the expression profile of the EMT associated genes *Snail1, Snail2, Zeb1, Zeb2, VIM* and *FN1* were measured by qRT-PCR in RKO cancer cells, cultured alone or in co-culture with macrophages, under normoxia and hypoxia. We found no significant expression levels on *Snail2*, *Zeb2* and *FN1* genes.

In general, the conditions in which the expression was higher was in hypoxia. Our results revealed that there is a tendency to *Snail1* decrease expression when RKO cells are in the presence of macrophages in normoxia. However, a significant opposite effect is seen when comparing co-cultures in normoxia and hypoxia, an effect that seems to be exclusive of the combination of these two factors, co-culture and macrophages, once no differences are observed when monoculture of cancer cells are compared **(Figure 22).** Regarding *Zeb1* expression no differences were observed in normoxia, but an increase in RKO monoculture was detected in hypoxic conditions. Due to that alteration, when we compared the *Zeb1* levels in mono-cultures and co-cultures in hypoxia, a significant decrease is observed **(Figure 22).** Concerning *VIM* mRNA expression levels, we observed a significant increase when mono-cultures are compared. A significant decrease is observed when mono and co-cultures are compared in hypoxia **(Figure 22).**

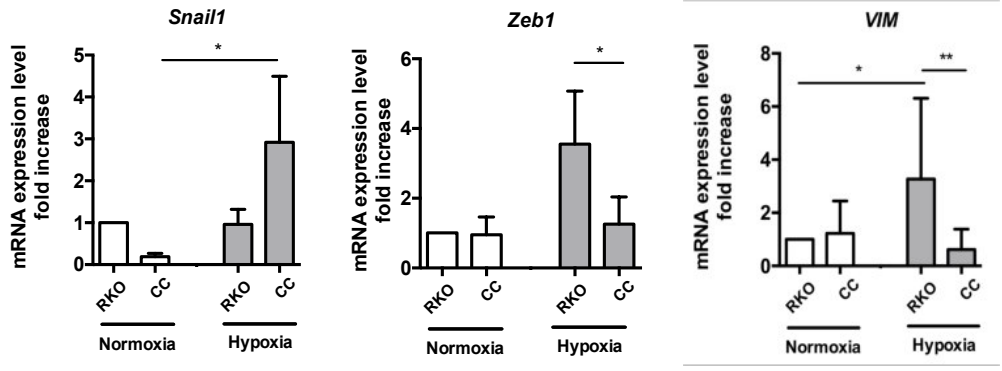


Figure 22: mRNA expression levels of EMT-associated genes. The expression levels were assessed on RKO cells cultured in mono (RKO) or in co-culture (CC) with macrophages under normoxia and hypoxia. All mRNA expression levels were normalized to the housekeeping gene *ACTB* and the results are represented as fold increase relatively to RKO monoculture in normoxia. Data represents the mean values and are representative of n=7 (*Snail1*); n= 7 (*Zeb1*); n=10 (*VIM*) independent experiments. **p<0.01; *p < 0.05.

Overall characterization of macrophage-cancer cell crosstalk in normoxia and hypoxia

Several experiments allowed us to better characterize in normoxia and hypoxia the macrophage-cancer cell crosstalk that could occurs at the tumor microenvironment. Bellow it is represented a schematic overview of the differences that were found **(Figure 23).**

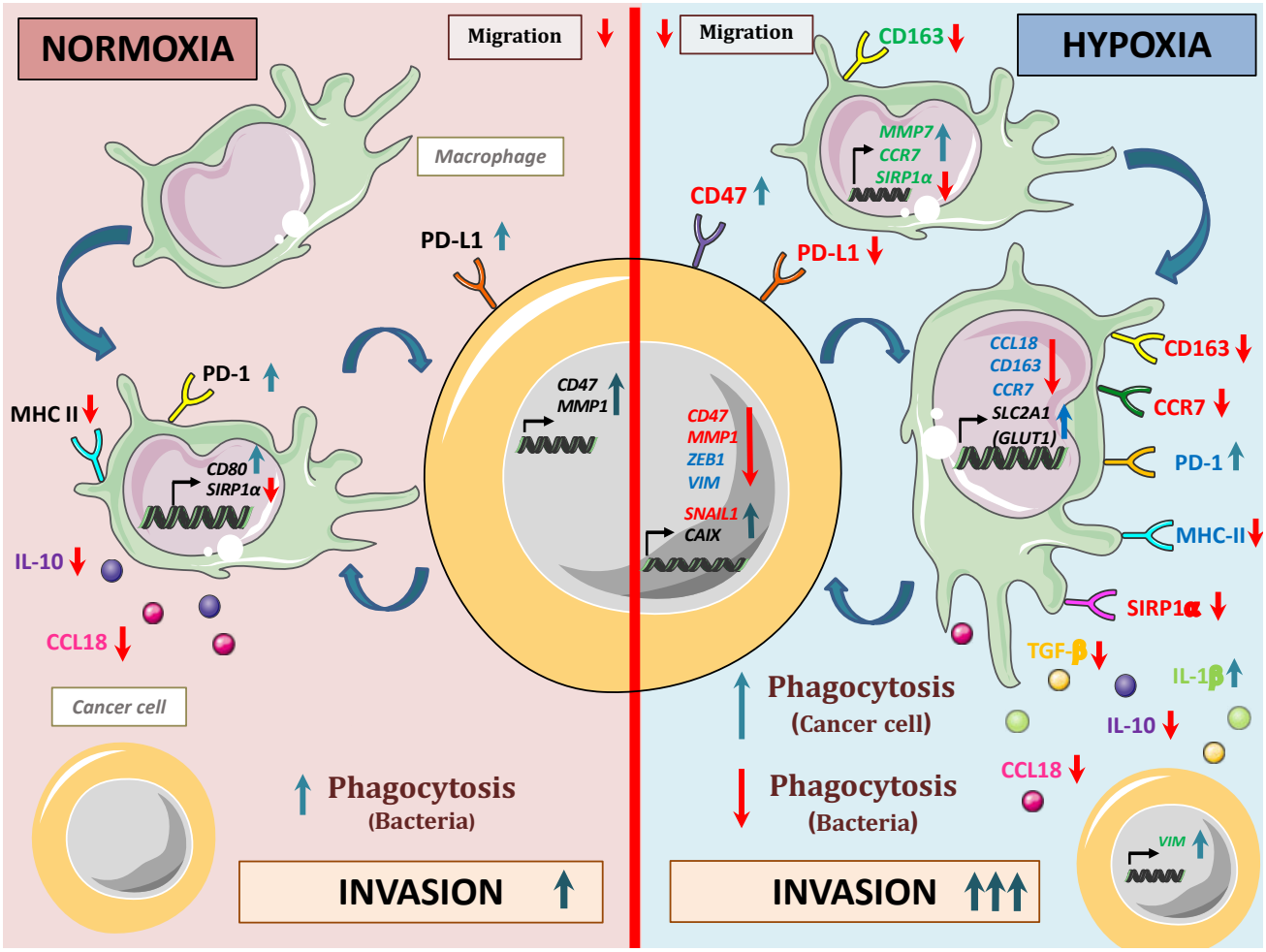


Figure 23: Schematic representation of macrophage-cancer cell crosstalk in normoxia and hypoxia. Illustration of all results obtained with statistical significance, through the different assays performed in macrophages (represented by the green cells) and cancer cells (represented by the yellow cells). The blue arrows indicating upwards direction represent an increase and the red arrows pointing downwards direction indicate a decrease. Differences in green are exclusive of the comparison between monocultures in normoxia and hypoxia. Differences in blue are exclusive of the comparison between mono and co-culture in hypoxia. Differences in red are exclusive of the comparison between co-cultures in normoxia and hypoxia.

Discussion

The solid tumor microenvironment is characterized by reduced oxygen levels, known as hypoxia, which occurs due to aberrant cellular proliferation and deficient vascularization, and has been associated with tumor progression (Muz *et al.*, 2015). Previous research had shown that both hypoxia and macrophages are associated with tumor progression, and that macrophages are preferentially recruited to hypoxic areas (Henze & Mazzone, 2016; Murdoch *et al.*, 2004). However, there is little information regarding the role of hypoxia on macrophage-cancer cell crosstalk. In the majority of the cases, neither the experiments studying the interplay tumor cell-macrophage were performed in hypoxia, nor had the studies about hypoxia focused on macrophage-cancer cell interplay. As a result, there are critical gaps in the tumor microenvironment understanding.

This work aimed to understand the impact of hypoxia on macrophage-cancer cell crosstalk, underlying the need of having in consideration this tumor microenvironment feature and the differences that one can obtain when are only looking to macrophages or cancer cells. Our experimental setup tried to overcome previous studies limitations. As an example, instead of focusing on the widely used human monocytic cell line THP-1, derived from an acute monocyte leukemia patient (Auwerx, 1991; Marcuello *et al.*, 2018), we decided to use primary cultures of macrophages, which will add the variability factor to our equation, and give us more confident results.

Since differences on the polarization status of macrophages are associated with their anti- and pro-tumor effect, we decided to study whether the presence of cancer cells and hypoxia can function as a polarization stimulus. The morphology of macrophages could give us some hints regarding this issue, as McWhorter *et al.*, 2013 group also described that macrophages exhibit different morphologies, accordingly to the cytokines that lead to an M1- or M2-like state (McWhorter *et al.*, 2013), however no significant differences were found in our results (Figure 10-11). Regarding the impact of hypoxia on cancer cell-macrophage crosstalk, when specific pro- and anti-inflammatory markers were analyzed at RNA and protein levels, we found that it induced a mixed phenotype, more balanced to pro-inflammatory, with a significant decrease of CD163 and TGF-β protein levels, a tendency to decrease CCL18 and IL-10, and a significant increase on IL-1β (Figure 10-11). Since we also observed a significant decrease on CCR7, and a tendency to decrease on CD80 (Figure 10A), it seems that hypoxia function is more related with the downregulation of anti-inflammatory markers than with the up-regulation of pro-inflammatory markers. Nevertheless, the validation of this statement would need a more broad analysis of

polarization-associated markers, expanding the screening for other pro- and anti-inflammatory receptors. While in the case of CD163 and TGF-β the effect occurred both in mono-cultures and in co-cultures, showing that the effect of hypoxia is stronger than the presence of cancer cells, in the case of IL-1β, the effect only occurred when hypoxia and co-culture occur at the same time (Figure 10-11). The results at mRNA levels were not concordant with the protein analysis of CCR7, CCL18 and CD163. The case of CCR7 is particularly interesting, once we verified that the presence of hypoxia, but not co-cultures, clearly enhanced the expression of this macrophage receptor at the mRNA level while, the combination of co-cultures with cancer cells and hypoxia was required to decrease CCR7 levels, both at mRNA and protein levels (Figure 10A and 10E). Similar to CCR7, the CCL18 and CD163 RNA levels increased when macrophages mono-cultures were exposed to hypoxia, a behavior that did not have parallel at protein level (Figure 10-11). Once protein and RNA were extracted at the same time point of the experiment, this might be explained to a distinct temporal regulation of these genes. The results comparing macrophages in mono and co-cultures with RKO in normoxia, were in line with the previously data published by our group regarding CD80, CCR7 and CCL18 mRNA levels (Pinto *et al.*, 2016). In contrast, others have reported that co-culture of macrophages and gastric cancer cells decreased CD80 levels (X.-Y. Liu *et al.*, 2013).

Regarding macrophage mono-cultures, it was also described that hypoxia triggered a decreased on CD80 expression (Lahat *et al.*, 2003) both on M1 and M2 macrophages (Raggi *et al.*, 2017). To our knowledge, we are the first to report the impact of hypoxia and macrophage-cancer cell crosstalk on CD80 expression and on the CCR7 regulation. However, regarding the influence of hypoxia, others have reported that CCR7 expression decrease in human primary macrophages mono-cultures (Raggi *et al.*, 2017), and on T cells (Sun *et al.*, 2010), and increase on dendritic cells (Köhler *et al.*, 2012). Curiously, it is also described that hypoxia is able to increase the expression of this receptor in lung (Li *et al.*, 2009), ovarian (Cheng *et al.*, 2014) and prostate cancer cells (Huang *et al.*, 2013), which was associated with an increase in migration, invasion and EMT activities. The decrease on CD163 mRNA levels on primary monocytes exposed to hypoxia was also described by other group, in concordance with our protein levels, but opposite to our mRNA expression results (Figure 10B and 10F) (Bosco *et al.,* 2006). Additionally, other groups described a decrease of CD163 RNA levels in a U937 macrophage cell line, in co-culture with pancreatic cancer cells, and similarly, they observed that macrophages also increased cancer cell invasion activities (Meng *et al.*, 2014). In opposition to our results, it

was described that macrophages co-cultured with ovarian (Ning *et al.*, 2018) and gastric cancer cells (Yamaguchi *et al.*, 2016) resulted in an increase on CD163 expression. In contrast to our CCL18 mRNA results, others reported that hypoxia did not induce alterations on this chemokine levels (Dehne & Brüne, 2016). Others had also reported that hypoxia decreased CCL18 secretion in M1 macrophages (Raggi *et al.*, 2017) and in dendritic cells in monoculture (Ricciardi *et al.*, 2008), a different tendency of our results. The IL-1β secretion was shown to increase on M1 macrophages (Raggi *et al.*, 2017) and THP-1 cell line (Zhang *et al.*, 2017) when stimulated by hypoxia, a condition only observed in our system when cancer cells are also present. Curiously, it was reported that co-cultures of macrophages with colon (Honda *et al.*, 2013) or breast cancer cells (Burnett *et al.*, 2008) induced and increase on IL-1β mRNA levels. In our results, the increased in IL-1β expression could be also due to increased expression by the cancer cells themselves. To address this issue, we should prompt analyze the expression levels of each population separately. Regarding TNF-α, a tendency to hypoxia increase its secretion was also previously observed by others (Cramer *et al.*, 2003; Murdoch *et al.*, 2005). On its turn, IL-10 decrease in hypoxia was also described in dendritic cells (Köhler *et al.*, 2012) and when macrophages were co-cultured with pancreatic cancer cells (Meng *et al.*, 2014). In opposition to our results, it was reported that co-culture of breast (Joimel *et al.*, 2010), ovarian (Ning *et al.*, 2018), and gastric cancer cells (Shen *et al.*, 2012) with macrophages led to a significant IL-10 increase. Also, in contrast with our results other groups reported that THP-1 co-cultured with ovarian (M. Tang *et al.*, 2018) increased TGF-β mRNA expression levels and TGF-β secretion into the culture medium . Additionally, it was also observed that THP-1 co-cultured with colon cancer cells (Honda *et al.*, 2011) enhanced TGF-β mRNA expression levels.

This apparent discrepancy in some results may be due to the use of macrophage cell lines instead of primary cultures, and of specificities of the tissue of origin of the cell lines used. It cannot be discarded the duration of the hypoxic stimulus may also have an influence on cell behavior.

As described by other researchers, human monocytes in the presence of colorectal primary cancer cell supernatant (Caras *et al.*, 2011) or, after direct contact with human colorectal cell lines, present a mixed phenotype (Wu *et al.*, 2014). Although, the current information suggests that macrophages which infiltrated tumors acquire the properties of a M2-like macrophage (Mantovani *et al.*, 2002), obtained accordantly to different environmental stimuli such as hypoxia (Leblond *et al.*, 2016), presence of cancer cells (Chen *et al.*, 2018), or of tumor cell

supernatants (Wu *et al.*, 2014), our macrophages are still in the "middle" of the polarization spectrum. This observation is in accordance with a report that analyzed the macrophagic population of mouse lung carcinoma, a mixed population, in which was found that hypoxia fine-tuned the M2 population (Laoui *et al.*, 2014), where reduced tumor hypoxia did not alter the abundance of TAMs nor the M2 expression markers, but lowered the hypoxia-associated gene expression and angiogenesis.

After analyzing the impact of hypoxia on macrophage polarization, we intended to analyze whether such alterations have impact on macrophage main functions phagocytosis, antigen presentation, and cell behavior. Phagocytosis is a crucial feature of macrophage function in host defense and tissue homeostasis (Kapellos *et al.*, 2016), and has been described as one of the mechanisms target by tumor cells in order to escape of tumor destruction, by the immune system (H. Zhang *et al.*, 2015)

When we were just looking to how macrophage behavior was modulated by the presence of cancer cells in normoxia, we saw that this stimulation was not sufficient to alter the percentage of cells that are able to phagocytize bacteria derived bioparticles, but significantly increase the amount that each cell is able to phagocytize (Figure 12C-12D). The phagocytic activity of macrophages in normoxia was not compromised and we suggest that their ability to engulf bacterial bioparticles is potentiated by factors released by the cancer cells present in the co-culture system. This effect of cancer cells on macrophage phagocytic ability was already observed by Wu *et al.* 2014, who described an enhancement of the phagocytic activity of THP-1 cells, when cultured with supernatants from three colon cancer cell lines (Wu *et al.*, 2014). When the phagocytic stimulus was cancer cells grown under normoxia, no alteration on macrophage phagocytic behavior was observed. However, when the stimulus was cancer cells grown under hypoxia, the behavior was very similar to the one with bacteria derived bioparticles, with no alterations on the number of phagocytic cells, but with a tendency to increase the amount of phagocytized material by macrophages (Figure 12-13). These results are in accordance with the report by Romano *et al.* 2018, who demonstrated that melanoma cells exposed to hypoxia are more phagocytized than normoxic cells (Romano *et al.*, 2018). The influence of cancer cells on macrophage phagocytic activity is still controversial, with some authors claiming that the presence of breast cancer cells leads to a decrease on macrophages phagocytic activity (Arsenijević *et al.*, 2005; H. Zhang *et al.*, 2015) . Nevertheless, other claimed the opposite using THP-1 macrophages cultured with conditioned medium from colorectal cancer cell lines (Wu *et*

al., 2014), indicating that alterations on phagocytosis may be dependent on cancer type and macrophage origin.

When we looked to the hypoxia effect on the macrophage phagocytic potential, and considering bacteria-derived bioparticles stimulation, we observed that hypoxia triggers a tendency to decrease the number of phagocytic cells, and a significant decrease on the amount of bioparticles that each cell is able to phagocytize. This effect is due to the hypoxia and not to the cancer cells, once the same effect is observed in macrophages that were previously on monocultures or in co-cultures (Figure 12C-12D). Interestingly, when the stimulus was cancer cells (RKO), in hypoxic conditions the macrophages have a tendency to increase the percentage of phagocytic cells and the amount each cell can phagocytize, independently of the stimulus (Figure 13D-13E). Since the macrophages were identically stimulated before the assay, our results show that macrophages phagocytic activity is also modulated depending on the material that have to phagocytize. Previous works evidenced that macrophages conditioned by hypoxia became more phagocytic, when the phagocytic stimulus are beads (Anand *et al.*, 2007), bacteria (Guan *et al.*, 2017), or dead cells (Ortiz-Masià *et al.*, 2012) while other showed that there is no alteration caused by oxygen tension (Nickel *et al.*, 2012), or that hypoxia decrease the phagocytic activity on pulmonary alveolar macrophages (Leeper-Woodford & Mills, 1992). These differences may be due to the different origin of macrophages, and different oxygen tensions.

Giving that activated macrophages induce formation and repair of tissues by secretion of factors such as TGF-β, and our results evidenced decrease levels of this factor, and that when stimulated with cancer cells macrophages, conditioned by hypoxia and cancer cells, increased phagocytosis, we conclude that macrophages modulated both by hypoxia and cancer cells present an increase of the scavenging function, but a decrease of repair capacity function. In addition, an increase on phagocytic activity could also indicate an increase of antigen presentation capacity.

Many cancer cells have developed immune evasion mechanisms such as the inhibition of phagocytosis through the CD47-SIRPα pathway (Chao *et al.*, 2012). CD47 is highly expressed on tumor cells and it is defined as an inhibitor of phagocytosis, due to its interaction with SIRP1α receptor expressed on macrophages (Chao *et al.*, 2012). We found that, independently of giving any extra phagocytic stimulus, the presence of hypoxia and cancer cells led to a significant decrease on the amount of SIRP1α per macrophage (Figure 14B). The conjugation of these two factors was essential to the observed reduction, once the same effect was not observed in mono-

cultures. In opposition, the hypoxic environment and the presence of cancer cells was sufficient to enhance the expression of CD47 on cancer cells (Figure 16B). Curiously, the differences found at protein level were not seen at mRNA level (Figure 16C). Concerning SIRP1α expression, the presence of cancer cells, as well as the hypoxic stimulus per se were sufficient to induce a significant decrease on RNA levels, which seems not to be cumulative on the condition where macrophages were double stimulated with cancer cells and hypoxia (Figure 14C). Regarding CD47 levels, the stimulation only with cancer cells resulted in a significant increase on RNA levels, and when hypoxia is added to the equation, it resulted in the opposite effect (Figure 16C). These differences on mRNA/protein levels, as in the case of the polarization markers, may be due to temporal-dependent regulation mechanisms. In contrast to our results, others have previously demonstrated that SIRP1α expression is reduced when macrophages are co-cultured with cancer cells, showing, however, similarly to us, that this alteration is partly due to soluble factors (Pan *et al.*, 2013). Interestingly, these alterations on SIRP1α protein expression occurred in the same conditions in which an increase on phagocytic activity was seen, when the cancer cells were used as phagocytic stimulus (Figure 13D and 13E).

In addition, to explore whether a reduction of SIRP1α expression was associated with increased levels of phagocytosis, we looked at SIRP1α levels on macrophages that phagocytize cancer cells (Figure 15). Our results confirmed that the great majority of the phagocytic cells do not express SIRP1α, and there was even a significant decrease on the number of SIRP1α positive cells when macrophages, modulated by hypoxia and the presence of cancer cells, were stimulated with normoxic cancer cells, despite the tendency to increase in the amount of SIRP1α per cell (Figure 15E-15F). Curiously, it was reported that LPS-induced down regulation of SIRP1α which contribute to innate immune activation in macrophages and that SIRP1α downregulation increased the production of pro-inflammatory cytokines, TNFα and IL-6 (Kong *et al.,* 2007), IL-1β and the anti-inflammatory IL-10 (Pan *et al.,* 2013). In our case SIRP1α downregulation was associated with an increase in IL-1β, known as a pro-inflammatory cytokine. Previous reports described a downregulation of cell surface expression of SIRP1α on THP-1 macrophage cell line when treated with conditioned media of oral cancer cells. In the same study, the inhibition of SIRPα resulted in a decreased phagocytic activity of macrophages. Importantly, the low expression of SIRPα was associated with increased cancer cell invasion and migration (Ye *et al.*, 2016). In another study, the silencing of SIRP1α in RAW264.7 macrophage cell line had an opposite effect, with the promotion of the phagocytic activity of osteosarcoma cancer cells (Ray *et*

al., 2018). So, we suggest that the levels of SIRP1α at macrophage surface are a limiting factor to trigger the "don´t eat me" signal by the cancer cells CD47. However, even if the cancer cells express high levels of CD47, if the protein cannot bind to the ligand on the macrophages, the phagocytosis is not repressed. The increase in phagocytosis triggered by the hypoxia is according to the fact that in hypoxic areas exists an increase in necrosis, and consequently in cellular debris, and the macrophages could be reprogrammed to increase their activity to clear this debris. However, "anticipating" the potential increase in phagocytosis, cancer cells could increase the CD47 expression.

In the case of CD47, it was previously report that hypoxia, through activation of HIF-1α, induces an increase on its expression on breast cancer cells. In this case there were no differences on phagocytosis comparing normoxia and hypoxia, but phagocytic activity increased when HIF-α was silenced or the CD47 was silenced (H. Zhang *et al.*, 2015). To our knowledge, in colon cancer, there are no studies addressing the role of hypoxia and macrophages on CD47 expression on cancer cells, but it was described that treatment with conditioned medium from THP-1 macrophage cell line polarized into M2-like macrophages is sufficient to induce an increase on CD47 expression on colorectal cancer cell lines, and to enhance cancer cell migration (Zhang *et al.*, 2013). Curiously, in this work was also reported that an inhibitory antibody directly to SIRPα implied less CD47-mediated cancer cell migration on SW480 cells. (Zhang *et al.*, 2013). Interestingly, in glioblastomas EGFR inhibition resulted in downregulation of SRC and significantly up-regulation of SIRP1α promoter activity (Kapoor *et al.*, 2004). Once our group had previously demonstrated that macrophages induced cancer cell invasion through EGFR (Cardoso *et al.*, 2014), it will be interesting to explore whether EGFR is also associated with down-regulation of SIRP1α mediated by hypoxia.

Besides phagocytic activity another hallmark of macrophage function is their antigen presentation. When we analysed the expression of MHC-II on macrophages, and also the MHC-I on macrophages and cancer cells we found that co-cultures induced a decrease on MHC-II on macrophages (Figure 17C and 17D), meaning that the cancer cells are able to secrete factors that will reduce the ability of macrophages to present antigens, but that hypoxia had no major role on this process. These results are in line with others showing that co-culture of THP-1 macrophages with a gastric cell line also resulted in MHC-II decrease (X.-Y. Liu *et al.,* 2013) and that in RAW 264.7 mouse macrophage cell line in hypoxia did not induce any alteration (Lahat *et al.,* 2003). In contrast, other group described that in polarized mono-cultures of macrophages,

both M1 and M2, the hypoxia condition led to a decrease on MHC-II (Raggi *et al.,* 2017). Curiously it is known that IL-10 leads to the inhibition of MHC-II expression (Chadban *et al.,* 1998), but in our system both IL-10 and MHC-II are reduced when macrophages are in the presence of cancer cells are under normoxia. Several authors have reported the loss or the downregulation of MHC-I expression in several tumors, including colorectal carcinoma (Garrido *et al.,* 1993; Watson *et al.,* 2006) and hypoxia has been described as responsible for the downregulation of expression of MHC-I in vivo and in 3D culture of mice tumors (Sethumadhavan *et al.,* 2017). However, in our system, we found that neither the presence of macrophages nor hypoxia account for MHC-I downregulation in RKO cells.

Certain immune-checkpoints pathways such as the PD-1-PD-L1 have been described as one of the mechanisms modulated by cancer cells and responsible for immune evasion (Alsaab *et al.,* 2017). Our results revealed that co-culture and hypoxia induce alterations on the expression of these markers, since a significant increase of PD-1 on macrophages was observed when they are in the presence of cancer cells, independently of hypoxia, suggesting that the interaction with PD-L1 expressing cells could be potentiated (Figure 18C). We observed that the percentage of positive cells for these markers is very low on macrophages comparing with the levels on cancer cells. Nevertheless, Rodriguez-Garcia *et al.* 2011, reported that the basal expression of PD-L1 on human monocyte derived macrophages (MDMs) was only enhanced upon the stimulation with LPS (Rodriguez-Garcia *et al.*, 2011). Despite most studies only report an increase of PD-L1 expression on tumor cells, we proved that our macrophages also express this ligand, as previous studies reported the expression of PD-L1 on tumor biopsy specimens from patients with colorectal tumors (Herbst *et al.*, 2014). Moreover, our results revealed a higher expression of PD-L1 per cancer cell when co-cultured with macrophages in normoxia, which is in line with the increased described by other authors on colorectal carcinoma cell line (Shi *et al.*, 2013) and on colorectal cancer specimens (Valentini *et al.*, 2018).

Since we observed a decrease in hypoxia, it would be interesting to confirm, using patient´s specimens, whether the positive PD-L1 cells are located. In contrast to our results, it was described that hypoxia increase PD-L1 expression on macrophages and tumor cells, in a tumor-bearing mice in a HIF1α dependent way (Noman *et al.*, 2014). Most studies regarding PD-1 expression have been mainly described on immune-competent cells of the hematopoietic lineage however, Kleffel *et al.* 2015 described that murine and human melanomas contain cancer subpopulations expressing PD-1 (Kleffel *et al.*, 2015). In concordance with this study, we also

observed a PD-1 expression on colon cancer cells, with a tendency to increase in hypoxic conditions, but independent from macrophages presence.

Subsequently, we explored the effect of hypoxia and the presence of macrophages on cancer cells invasion-related activities. Our results revealed that RKO cell invasion in normoxic conditions increases in the presence of macrophages, confirming the results that our group previously reported (Cardoso *et al.*, 2014) . Interestingly, hypoxia by itself was sufficient to induce an invasive phenotype, which is potentiated by the presence of macrophages. In 2013, Shen and collaborators (Shen *et al.* 2013) described identical results regarding gastric cancer cell invasion, evaluated in the presence of macrophages and of hypoxia (Shen *et al.*, 2013) and Wang *et al.* 2016 also described that both hypoxia and macrophage supernatants promoted glioblastoma cancer cell invasion (Y. Wang *et al.*, 2016). Despite the more pro-inflammatory behaviour of macrophages under hypoxia, their regulation of key cancer cell behaviour associated with the hallmarks of cancer prevail.

Curiously, we found that the impact on invasion was not due to the impact on migration, once we found that both the presence of macrophage and hypoxia, not in a cumulative way decrease the migratory capacities of the cells. This was more in line with a more pro-inflammatory phenotype of macrophages, once the wound healing is more favored by an anti-inflammatory profile. In another way the hypoxic environment is favoring both the MMP secretion and the expression of EMT-associated genes. Our results regarding migration are, however, the opposite to studies described by Green *et al.* 2009, who demonstrated that a mouse macrophage cell line (RAW 264.7) induce the migration of a mouse colon cancer line (CT26) (Green *et al.*, 2009). More recently, Tátrai *et al.* 2017 proved that migration of the colon cancer cell lines (HT25 and HT29) remain unchanged in hypoxia comparing to normoxic conditions (Tátrai *et al.*, 2017).

Regarding MMP-1, the increase of its expression levels on RKO in monoculture in hypoxia is in agreement with other authors that described the same pattern with a highly metastatic cell line from a human bladder (Shin *et al.*, 2015). Additionally, MMP7 expression, also described as associated with decreased survival in advanced CRC (Said *et al.*, 2014) was also described to be up-regulated by hypoxia by Burke *et al.* 2003, on macrophages in hypoxic conditions or in hypoxic areas of human breast tumors (Burke *et al.*, 2003).

The results obtained regarding EMT-associated genes are in line with previous results from Zhang *et al.* 2015, who described that HIF-1α expression was positively associated with *Zeb 1*

and *VIM* expression in primary and metastatic CRC specimens (W. Zhang *et al.*, 2015), and that hypoxia induces an increase of *Snail* and *VIM* on hepatocellular carcinomas (Liu *et al.*, 2016) . A previous study from Liu *et al.* 2013 described an increase of *Snail 1* and *VIM* expression in pancreatic cancer cells co-cultured with M2 polarized macrophages (C. Y. Liu *et al.*, 2013), and in bladder cancer it was also reported that an increase in *VIM* was only observed when cancer cells are co-cultures with M2 macrophages (Zhou *et al.*, 2018). Our results were the opposite of these studies and this could be due to the mixed phenotype of our macrophagic population, which can have both M1 or M2-like characteristics, and therefore affect differently the expression of EMT genes.

Overall, our results reveal that hypoxia had an impact on the complex crosstalk between macrophages and cancer cells. In general, more than the structural alterations, the differences discovered are more related with functional alterations. This study allowed us to better understand this interplay that occurs in a tumor microenvironment.

Final remarks and future perspectives

Our study demonstrated that the establishment of the indirect co-culture with macrophages and colorectal cancer cells in normoxic and hypoxic conditions indorsed a better characterization of the crosstalk between these two populations.

The differences obtained when analyzing the distinct conditions underlined the importance of consider the different elements of the tumor microenvironment, namely hypoxia, when trying to understand the disease outcome.

In the future, our studies should be expanded to other cancer cell lines, and explore the impact of common genetic alterations associated with CRC on cellular behavior. It would be also important to explore signaling pathways that have been described as involved in tumor progression and associated with the invasive phenotype. It would be interesting to do a more broad characterization of the underlined molecular alterations both in cancer cells and macrophages, using RNA-Seq, Multiplex, proteomic and metabolomic approaches.

The understanding of how macrophage and hypoxia impacts on cancer cells would be of critical relevance concerning the development of new and more efficient cancer therapies.

References

Abbas, A. K., Lichtman, A. H., Pillai, S. (2014). Cells and tissues of the immune system (Chapter 2). *Cellular and molecular immunology* (8th ed.). Philadelphia, PA: Elsevier-Saunders. ISBN: 9780323222754

Allen, M., & Jones, J. L. (2011). Jekyll and Hyde: The role of the microenvironment on the progression of cancer. *Journal of Pathology*. https://doi.org/10.1002/path.2803

Alsaab, H. O., Sau, S., Alzhrani, R., Tatiparti, K., Bhise, K., Kashaw, S. K., & Iyer, A. K. (2017). PD-1 and PD-L1 checkpoint signaling inhibition for cancer immunotherapy: mechanism, combinations, and clinical outcome. *Frontiers in Pharmacology*. https://doi.org/10.3389/fphar.2017.00561

Anand, R. J., Gribar, S. C., Li, J., Kohler, J. W., Branca, M. F., Dubowski, T., ... Hackam, D. J. (2007). Hypoxia causes an increase in phagocytosis by macrophages in a HIF-1α-dependent manner. *Journal of Leukocyte Biology*. https://doi.org/10.1189/jlb.0307195

Aras, S., & Raza Zaidi, M. (2017). TAMeless traitors: Macrophages in cancer progression and metastasis. *British Journal of Cancer*. https://doi.org/10.1038/bjc.2017.356

Armaghany, T., Wilson, J. D., Chu, Q., & Mills, G. (2012). Genetic alterations in colorectal cancer. *Gastrointestinal Cancer Research : GCR*, *5*(1), 19–27. http://www.ncbi.nlm.nih.gov/pubmed/22574233%5Cn

Arsenijević, N., Baskić, D., Popović, S., Ristić, P., & Aćimović, L. (2005). Preliminary study of mononuclear phagocytosis during breast cancer therapy. *Journal of B.U.ON.* https://www.ncbi.nlm.nih.gov/pubmed/17335140

Auwerx, J. (1991). The human leukemia cell line, THP-1: A multifacetted model for the study of monocyte-macrophage differentiation. *Experientia*. https://doi.org/10.1007/BF02041244

Azad, A. K., Rajaram, M. V. S., & Schlesinger, L. S. (2014). Exploitation of the Macrophage Mannose Receptor (CD206) in Infectious Disease Diagnostics and Therapeutics. *Journal of Cytology & Molecular Biology*, *1*(1), 1000003. https://doi.org/10.13188/2325-4653.1000003

Balkwill, F. R., Capasso, M., & Hagemann, T. (2012). The tumor microenvironment at a glance. *Journal of Cell Science*, *125*(23), 5591 LP-5596.

https://doi.org/10.1242/jcs.116392

Beatty, G. L., & Gladney, W. L. (2015). Immune escape mechanisms as a guide for cancer immunotherapy. *Clinical Cancer Research*. https://doi.org/10.1158/1078-0432.CCR-14-1860

Bonnans, C., Chou, J., & Werb, Z. (2014). Remodelling the extracellular matrix in development and disease. *Nature Reviews Molecular Cell Biology*. https://doi.org/10.1038/nrm3904

Bosco, M. C., Puppo, M., Santangelo, C., Anfosso, L., Pfeffer, U., Fardin, P., … Varesio, L. (2006). Hypoxia Modifies the Transcriptome of Primary Human Monocytes: Modulation of Novel Immune-Related Genes and Identification Of CC-Chemokine Ligand 20 as a New Hypoxia-Inducible Gene. *The Journal of Immunology*, *177*(3), 1941 LP-1955. https://doi.org/10.4049/jimmunol.177.3.1941

Braster, R., Bögels, M., Beelen, R. H. J., & Van Egmond, M. (2015). The delicate balance of macrophages in colorectal cancer; their role in tumour development and therapeutic potential. *Immunobiology*. https://doi.org/10.1016/j.imbio.2015.08.011

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., & Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians*, *0*(0). https://doi.org/10.3322/caac.21492

Brown, J. M., & Wilson, W. R. (2004). Exploiting tumour hypoxia in cancer treatment. *Nat Rev Cancer*, *4*(6), 437–447. https://dx.doi.org/10.1038/nrc1367

Brundu S, F. A. (2015). Polarization and Repolarization of Macrophages. *Journal of Clinical and Cellular Immunology*, *06*(02), 1–10. https://doi.org/10.4172/2155-9899.1000319

Bubeník, J. (2003). Tumour MHC class I downregulation and immunotherapy (Review). *Oncology Reports*. https://doi.org/10.3892/or.10.6.2005

Burke, B., Giannoudis, A., Corke, K. P., Gill, D., Wells, M., Ziegler-Heitbrock, L., & Lewis, C. E. (2003). Hypoxia-Induced Gene Expression in Human Macrophages. *The American Journal of Pathology*. https://doi.org/10.1016/S0002-9440(10)63483-9

Burnett, G. T., Weathersby, D. C., Taylor, T. E., & Bremner, T. a. (2008). Regulation of inflammation- and angiogenesis-related gene expression in breast cancer cells and co-cultured macrophages. *Anticancer Research*. https://www.ncbi.nlm.nih.gov/pubmed/18751381

Caras, I., Tucureanu, C., Lerescu, L., Pitica, R., Melinceanu, L., Neagu, S., & Salageanu, A. (2011). Influence of tumor cell culture supernatants on macrophage functional polarization: In vitro models of macrophage-tumor environment interaction. *Tumori*. https://doi.org/10.1700/989.10726

Cardoso, A. P., Pinto, M. L., Pinto, A. T., Oliveira, M. I., Pinto, M. T., Gonçalves, R., … Oliveira, M. J. (2014). Macrophages stimulate gastric and colorectal cancer invasion through EGFR Y 1086, c-Src, Erk1/2 and Akt phosphorylation and smallGTPase activity. *Oncogene*. https://doi.org/10.1038/onc.2013.154

Cardoso, A. P., Pinto, M. L., Pinto, A. T., Pinto, M. T., Monteiro, C., Oliveira, M. I., … Oliveira, M. J. (2015). Matrix metalloproteases as maestros for the dual role of LPS- and IL-10-stimulated macrophages in cancer cell behaviour. *BMC Cancer*. https://doi.org/10.1186/s12885-015-1466-8

Carmeliet, P., & Jain, R. K. (2011). Molecular mechanisms and clinical applications of angiogenesis. *Nature*. https://doi.org/10.1038/nature10144

Chadban, S. J., Tesch, G. H., Foti, R., Lan, H. Y., Atkins, R. C., & Nikolic-Paterson, D. J. (1998). Interleukin-10 differentially modulates MHC class II expression by mesangial cells and macrophages in vitro and in vivo. *Immunology*, *94*(1), 72–78. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1364333/

Chan, K. S., Espinosa, I., Chao, M., Wong, D., Ailles, L., Diehn, M., … Weissman, I. L. (2009). Identification, molecular characterization, clinical prognosis, and therapeutic targeting of human bladder tumor-initiating cells. *Proceedings of the National Academy of Sciences of the United States of America*. https://doi.org/10.1073/pnas.0906549106

Chanmee, T., Ontong, P., Konno, K., & Itano, N. (2014). Tumor-associated macrophages as major players in the tumor microenvironment. *Cancers*. https://doi.org/10.3390/cancers6031670

Chao, M. P., Alizadeh, A. A., Tang, C., Myklebust, J. H., Varghese, B., Gill, S., … Majeti, R. (2010). Anti-CD47 Antibody Synergizes with Rituximab to Promote

Phagocytosis and Eradicate Non-Hodgkin Lymphoma. *Cell*. https://doi.org/10.1016/j.cell.2010.07.044

Chao, M. P., Weissman, I. L., & Majeti, R. (2012). The CD47-SIRPα pathway in cancer immune evasion and potential therapeutic implications. *Current Opinion in Immunology*. https://doi.org/10.1016/j.coi.2012.01.010

Chen, F., Zhuang, X., Lin, L., Yu, P., Wang, Y., Shi, Y., ... Sun, Y. (2015). New horizons in tumor microenvironment biology: challenges and opportunities. *BMC Medicine*, *13*(1), 45. https://doi.org/10.1186/s12916-015-0278-7

Chen, L., Wang, S., Wang, Y., Zhang, W., Ma, K., Hu, C., ... Xu, N. (2018). IL-6 influences the polarization of macrophages and the formation and growth of colorectal tumor. *Oncotarget*, *9*(25), 17443–17454. https://doi.org/10.18632/oncotarget.24734

Cheng, S., Han, L., Guo, J., Yang, Q., Zhou, J., & Yang, X. (2014). The essential roles of CCR7 in epithelial-to-mesenchymal transition induced by hypoxia in epithelial ovarian carcinomas. *Tumor Biology*, *35*(12), 12293–12298. https://doi.org/10.1007/s13277-014-2540-6

Chung, F.-Y., Huang, M.-Y., Yeh, C.-S., Chang, H.-J., Cheng, T.-L., Yen, L.-C., ... Lin, S.-R. (2009). GLUT1 gene is a potential hypoxic marker in colorectal cancer patients. *BMC Cancer*, *9*(1), 241. https://doi.org/10.1186/1471-2407-9-241

Cierna, Z., Mego, M., Janega, P., Karaba, M., Minarik, G., Benca, J., ... Mardiak, J. (2014). Matrix metalloproteinase 1 and circulating tumor cells in early breast cancer. *BMC Cancer*. https://doi.org/10.1186/1471-2407-14-472

Corrêa, L. H., Corrêa, R., Farinasso, C. M., de Sant'Ana Dourado, L. P., & Magalhães, K. G. (2017). Adipocytes and macrophages interplay in the orchestration of tumor microenvironment: New implications in cancer progression. *Frontiers in Immunology*. https://doi.org/10.3389/fimmu.2017.01129

Cramer, T., Yamanishi, Y., Clausen, B. E., Förster, I., Pawlinski, R., Mackman, N., ... Johnson, R. S. (2003). HIF-1α is essential for myeloid cell-mediated inflammation. *Cell*. https://doi.org/10.1016/S0092-8674(03)00154-5

Dail, M., Yang, L., Green, C., Ma, C., Robert, A., Kadel, E. E., ... Venstrom, J. M. (2016). Distinct Patterns of PD-L1 and PD-L2 Expression By Tumor and Non-Tumor Cells in Patients with MM, MDS and AML. *Blood*, *128*(22), 1340 LP-1340.

http://www.bloodjournal.org/content/128/22/1340.abstract

Davies, R. J., Miller, R., & Coleman, N. (2005). Colorectal cancer screening: Prospects for molecular stool analysis. *Nature Reviews Cancer*. https://doi.org/10.1038/nrc1569

de Charette, M., Marabelle, A., & Houot, R. (2016). Turning tumour cells into antigen presenting cells: The next step to improve cancer immunotherapy? *European Journal of Cancer*. https://doi.org/10.1016/j.ejca.2016.09.010

De Rosa, M., Pace, U., Rega, D., Costabile, V., Duraturo, F., Izzo, P., & Delrio, P. (2015). Genetics, diagnosis and management of colorectal cancer (Review). *Oncology Reports*, *34*(3), 1087–1096. https://doi.org/10.3892/or.2015.4108

De Visser, K. E., Eichten, A., & Coussens, L. M. (2006). Paradoxical roles of the immune system during cancer development. *Nature Reviews Cancer*. https://doi.org/10.1038/nrc1782

Dehne, N., & Brüne, B. (2016). Hypoxic inhibition of JMJD3 reduces H3K27me3 demethylation and induction of the STAT6 target gene CCL18. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms*, *1859*(12), 1490–1501. https://doi.org/https://doi.org/10.1016/j.bbagrm.2016.10.004

Dong, Y., Sun, Q., & Zhang, X. (2015). PD-1 and its ligands are important immune checkpoints in cancer. *Oncotarget*. https://doi.org/10.18632/oncotarget.13895

Dunn, G. P., Old, L. J., & Schreiber, R. D. (2004). The Three Es of Cancer Immunoediting. *Annual Review of Immunology*. https://doi.org/10.1146/annurev.immunol.22.012703.104803

Duong, M. N., Geneste, A., Fallone, F., Li, X., Dumontet, C., & Muller, C. (2017). The fat and the bad: Mature adipocytes, key actors in tumor progression and resistance. *Oncotarget*. https://doi.org/10.18632/oncotarget.18038

Eales, K. L., Hollinshead, K. E. R., & Tennant, D. A. (2016). Hypoxia and metabolic adaptation of cancer cells. *Oncogenesis*, *5*(1), e190. https://doi.org/10.1038/oncsis.2015.50

Escribese, M. M., Casas, M., & Corbi, A. L. (2012). Influence of low oxygen tensions on macrophage polarization. *Immunobiology*, *217*(12), 1233–1240. https://doi.org/10.1016/j.imbio.2012.07.002

Fabriek, B. O., Dijkstra, C. D., & van den Berg, T. K. (2005). The macrophage scavenger receptor CD163. *Immunobiology*, *210*(2), 153–160. https://doi.org/https://doi.org/10.1016/j.imbio.2005.05.010

Flaherty, D. K., (2012). Antigen-Presenting Cells (Chapter 5). Immunology for Pharmacy. St. Lois, Missouri 63043, Elsevier. ISBN: 9780323069472

Fraternale, A., Brundu, S. & Magnani, M., 2015. Polarization and Repolarization of Macrophages. *Journal of Clinical and Cellular Immunology*, 6(2), pp.1–10. https://doi.org/10.4172/2155-9899.1000319

Friedl, P., & Alexander, S. (2011). Cancer invasion and the microenvironment: Plasticity and reciprocity. *Cell*, *147*(5), 992–1009. https://doi.org/10.1016/j.cell.2011.11.016

Friedl, P., & Wolf, K. (2003). Tumour-cell invasion and migration: Diversity and escape mechanisms. *Nature Reviews Cancer*. https://doi.org/10.1038/nrc1075

Garrido, F., Cabrera, T., Concha, A., Glew, S., Ruiz-Cabello, F., & Stern, P. L. (1993). Natural history of HLA expression during tumour development. *Immunology Today*. https://doi.org/10.1016/0167-5699(93)90264-L

Gascard, P., & Tlsty, T. D. (2016). Carcinoma-associated fibroblasts: Orchestrating the composition of malignancy. *Genes and Development*. https://doi.org/10.1101/gad.279737.116

Gilkes, D. M., Semenza, G. L., & Wirtz, D. (2014). Hypoxia and the extracellular matrix: Drivers of tumour metastasis. *Nature Reviews Cancer*. https://doi.org/10.1038/nrc3726

Gong, J., Chehrazi-Raffle, A., Reddi, S., & Salgia, R. (2018). Development of PD-1 and PD-L1 inhibitors as a form of cancer immunotherapy: a comprehensive review of registration trials and future considerations. *Journal for Immunotherapy of Cancer*, *6*, 8. https://doi.org/10.1186/s40425-018-0316-z

Gong, J., Le, T. Q., Massarelli, E., Hendifar, A. E., & Tuli, R. (2018). Radiation therapy and PD-1/PD-L1 blockade: the clinical development of an evolving anticancer combination. *Journal for ImmunoTherapy of Cancer*, *6*(1), 46. https://doi.org/10.1186/s40425-018-0361-7

Green, C. E., Liu, T., Montel, V., Hsiao, G., Lester, R. D., Subramaniam, S., ... Klemke, R. L. (2009). Chemoattractant signaling between tumor cells and macrophages

regulates cancer cell migration, metastasis and neovascularization. *PLoS ONE*. https://doi.org/10.1371/journal.pone.0006713

Grivennikov, S. I., Greten, F. R., & Karin, M. (2010). Immunity, Inflammation, and Cancer. *Cell*. https://doi.org/10.1016/j.cell.2010.01.025

Guan, F., Lu, X.-J., Li, C.-H., & Chen, J. (2017). Molecular characterization of mudskipper (*Boleophthalmus pectinirostris*) hypoxia-inducible factor-1α (HIF-1α) and analysis of its function in monocytes/macrophages. *PLoS ONE*, *12*(5), e0177960. https://doi.org/10.1371/journal.pone.0177960

Haggar, F. A., & Boushey, R. P. (2009). Colorectal cancer epidemiology: Incidence, mortality, survival, and risk factors. *Clinics in Colon and Rectal Surgery*, *22*(4), 191–197. https://doi.org/10.1055/s-0029-1242458

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: The next generation. *Cell*. https://doi.org/10.1016/j.cell.2011.02.013

Hao, N. B., Lü, M. H., Fan, Y. H., Cao, Y. L., Zhang, Z. R., & Yang, S. M. (2012). Macrophages in tumor microenvironments and the progression of tumors. *Clinical and Developmental Immunology*. https://doi.org/10.1155/2012/948098

Henze, A. T., & Mazzone, M. (2016). The impact of hypoxia on tumor-associated macrophages. *Journal of Clinical Investigation*. https://doi.org/10.1172/JCI84427

Herbst, R. S., Soria, J. C., Kowanetz, M., Fine, G. D., Hamid, O., Gordon, M. S., ... Hodi, F. S. (2014). Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. *Nature*. https://doi.org/10.1038/nature14011

Honda, T., Inagawa, H., & Yamamoto, I. (2011). Differential expression of mRNA in human monocytes following interaction with human colon cancer cells. *Anticancer Research*. https://www.ncbi.nlm.nih.gov/pubmed/21873165

Honda, T., Yamamoto, I., & Inagawa, H. (2013). Angiogenesis-, metastasis- and signaling pathway-related factor dynamics in human colon cancer cells following interaction with monocytes. In *Anticancer Research*. https://www.ncbi.nlm.nih.gov/pubmed/23780976

Hong, T. S., Clark, J. W., & Haigis, K. M. (2012). Cancers of the colon and rectum:

Identical or Fraternal Twins? *Cancer Discovery*. https://doi.org/10.1158/2159-8290.CD-11-0315

Huang, X., Su, K., Zhou, L., Shen, G., Dong, Q., Lou, Y., & Zheng, S. (2013). Hypoxia preconditioning of mesenchymal stromal cells enhances PC3 cell lymphatic metastasis accompanied by VEGFR-3/CCR7 activation. *Journal of Cellular Biochemistry*, *114*(12), 2834–2841. https://doi.org/10.1002/jcb.24629

Jain, R. K. (2005). Normalization of tumor vasculature: An emerging concept in antiangiogenic therapy. *Science*. https://doi.org/10.1126/science.1104819

Jiang, Y., Li, Y., & Zhu, B. (2015). T-cell exhaustion in the tumor microenvironment. *Cell Death and Disease*. https://doi.org/10.1038/cddis.2015.162

Joimel, U., Gest, C., Soria, J., Pritchard, L.-L., Alexandre, J., Laurent, M., … Soria, C. (2010). Stimulation of angiogenesis resulting from cooperation between macrophages and MDA-MB-231 breast cancer cells: proposed molecular mechanism and effect of tetrathiomolybdate. *BMC Cancer*, *10*, 375. https://doi.org/10.1186/1471-2407-10-375

Joyce, J. A., & Pollard, J. W. (2009). Microenvironmental regulation of metastasis. *Nature Reviews Cancer*, *9*(4), 239–252. https://doi.org/10.1038/nrc2618

Kalluri, R., & Zeisberg, M. (2006). Fibroblasts in cancer. *Nature Reviews Cancer*. https://doi.org/10.1038/nrc1877

Kapellos, T. S., Taylor, L., Lee, H., Cowley, S. A., James, W. S., Iqbal, A. J., & Greaves, D. R. (2016). A novel real time imaging platform to quantify macrophage phagocytosis. *Biochemical Pharmacology*. https://doi.org/10.1016/j.bcp.2016.07.011

Kapoor, G. S., Kapitonov, D., & O'Rourke, D. M. (2004). Transcriptional Regulation of Signal Regulatory Protein α1 Inhibitory Receptors by Epidermal Growth Factor Receptor Signaling. *Cancer Research*, *64*(18), 6444 LP-6452. http://cancerres.aacrjournals.org/content/64/18/6444.abstract

Kessenbrock, K., Plaks, V., & Werb, Z. (2010). Matrix Metalloproteinases: Regulators of the Tumor Microenvironment. *Cell*, *141*(1), 52–67. https://doi.org/10.1016/j.cell.2010.03.015

Kleffel, S., Posch, C., Barthel, S. R., Mueller, H., Schlapbach, C., Guenova, E., … Schatton, T. (2015). Melanoma Cell-Intrinsic PD-1 Receptor Functions Promote

Tumor Growth. *Cell*. https://doi.org/10.1016/j.cell.2015.08.052

Köhler, T., Reizis, B., Johnson, R. S., Weighardt, H., & Förster, I. (2012). Influence of hypoxia-inducible factor 1α on dendritic cell differentiation and migration. *European Journal of Immunology*, *42*(5), 1226–1236. https://doi.org/10.1002/eji.201142053

Kong, X.-N., Yan, H.-X., Chen, L., Dong, L.-W., Yang, W., Liu, Q., ... Wang, H.-Y. (2007). LPS-induced down-regulation of signal regulatory protein α contributes to innate immune activation in macrophages. *The Journal of Experimental Medicine*, *204*(11), 2719–2731. https://doi.org/10.1084/jem.20062611

Kuschel, A., Simon, P., & Tug, S. (2012). Functional regulation of HIF-1a under normoxia-is there more than post-translational regulation? *Journal of Cellular Physiology*, *227*(2), 514–524. https://doi.org/10.1002/jcp.22798

Lahat, N., Rahat, M. A., Ballan, M., Weiss-Cerem, L., Engelmayer, M., & Bitterman, H. (2003). Hypoxia reduces CD80 expression on monocytes but enhances their LPS-stimulated TNF-α secretion. *Journal of Leukocyte Biology*, *74*(2), 197–205. https://doi.org/10.1189/jlb.0303105

Laoui, D., Van Overmeire, E., Di Conza, G., Aldeni, C., Keirsse, J., Morias, Y., ... Van Ginderachter, J. A. (2014). Tumor Hypoxia Does Not Drive Differentiation of Tumor-Associated Macrophages but Rather Fine-Tunes the M2-like Macrophage Population. *Cancer Research*, *74*(1), 24 LP-30. https://doi.org/10.1158/0008-5472.CAN-13-1196

Leblond, M. M., Gérault, A. N., Corroyer-Dulmont, A., MacKenzie, E. T., Petit, E., Bernaudin, M., & Valable, S. (2016). Hypoxia induces macrophage polarization and re-education toward an M2 phenotype in U87 and U251 glioblastoma models. *OncoImmunology*. https://doi.org/10.1080/2162402X.2015.1056442

Leeper-Woodford, S. K., & Mills, J. W. (1992). Phagocytosis and ATP levels in alveolar macrophages during acute hypoxia. *American Journal of Respiratory Cell and Molecular Biology*. https://doi.org/10.1165/ajrcmb/6.3.326

Leslie, A., Carey, F. A., Pratt, N. R., & Steele, R. J. C. (2002). The colorectal adenoma-carcinoma sequence. *British Journal of Surgery*. https://doi.org/10.1046/j.1365-2168.2002.02120.x

Li, H., Fan, X., & Houghton, J. (2007). Tumor microenvironment: The role of the tumor stroma in cancer. *Journal of Cellular Biochemistry*. https://doi.org/10.1002/jcb.21159

Li, J., Zhang, G., Wang, X., & Li, X.-F. (2015). Is carbonic anhydrase IX a validated target for molecular imaging of cancer and hypoxia? *Future Oncology (London, England)*, *11*(10), 1531–1541. https://doi.org/10.2217/fon.15.11

Li, Y., Qiu, X., Zhang, S., Zhang, Q., & Wang, E. (2009). Hypoxia induced CCR7 expression via HIF-1alpha and HIF-2alpha correlates with migration and invasion in lung cancer cells. *Cancer Biology & Therapy*. https://doi.org/10.4161/cbt.8.4.7332

Liberti, M. V., & Locasale, J. W. (2016). The Warburg Effect: How Does it Benefit Cancer Cells? *Trends in Biochemical Sciences*, *41*(3), 211–218. https://doi.org/10.1016/j.tibs.2015.12.001

Liu, C. Y., Xu, J. Y., Shi, X. Y., Huang, W., Ruan, T. Y., Xie, P., & Ding, J. L. (2013). M2-polarized tumor-associated macrophages promoted epithelial-mesenchymal transition in pancreatic cancer cells, partially through TLR4/IL-10 signaling pathway. *Laboratory Investigation*. https://doi.org/10.1038/labinvest.2013.69

Liu, W.-T., Jing, Y.-Y., Yu, G., Chen, H., Han, Z., Yu, D., … Wei, L.-X. (2016). Hepatic stellate cell promoted hepatoma cell invasion via the HGF/c-Met signaling pathway regulated by p53. *Cell Cycle*, *15*(7), 886–894. https://doi.org/10.1080/15384101.2016.1152428

Liu, X.-Y., Miao, Z.-F., Zhao, T.-T., Wang, Z.-N., Xu, Y.-Y., Gao, J., … Xu, H.-M. (2013). Milky spot macrophages remodeled by gastric cancer cells promote peritoneal mesothelial cell injury. *Biochemical and Biophysical Research Communications*, *439*(3), 378–383. https://doi.org/10.1016/j.bbrc.2013.08.073

Liu, X., Kwon, H., Li, Z., & Fu, Y. xin. (2017). Is CD47 an innate immune checkpoint for tumor evasion? *Journal of Hematology and Oncology*. https://doi.org/10.1186/s13045-016-0381-z

Lu, P., Takai, K., Weaver, V. M., & Werb, Z. (2011). Extracellular Matrix Degradation and Remodeling in Development and Disease. *Cold Spring Harbor Perspectives in Biology*, *3*(12), 10.1101/cshperspect.a005058 a005058.

https://doi.org/10.1101/cshperspect.a005058

Lu, P., Weaver, V. M., & Werb, Z. (2012). The extracellular matrix: A dynamic niche in cancer progression. *Journal of Cell Biology*. https://doi.org/10.1083/jcb.201102147

Mantovani, A., Sica, A., Sozzani, S., Allavena, P., Vecchi, A., & Locati, M. (2004). The chemokine system in diverse forms of macrophage activation and polarization. *Trends in Immunology*. https://doi.org/10.1016/j.it.2004.09.015

Mantovani, A., Sozzani, S., Locati, M., Allavena, P., & Sica, A. (2002). Macrophage polarization: Tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends in Immunology*. https://doi.org/10.1016/S1471-4906(02)02302-5

Marcuello, M., Mayol, X., Felipe-Fumero, E., Costa, J., López-Hierro, L., Salvans, S., … Pera, M. (2018). Modulation of the colon cancer cell phenotype by pro-inflammatory macrophages: A preclinical model of surgery-associated inflammation and tumor recurrence. *PLoS ONE*. https://doi.org/10.1371/journal.pone.0192958

Marley, A. R., & Nan, H. (2016). Epidemiology of colorectal cancer. *International Journal of Molecular Epidemiology and Genetics*. https://doi.org/10.1093/bmb/64.1.1

Martin, J. D., Fukumura, D., Duda, D. G., Boucher, Y., & Jain, R. K. (2016). Reengineering the Tumor Microenvironment to Alleviate Hypoxia and Overcome Cancer Heterogeneity. *Cold Spring Harbor Perspectives in Medicine*, *6*(12), a027094. https://doi.org/10.1101/cshperspect.a027094

McWhorter, F. Y., Wang, T., Nguyen, P., Chung, T., & Liu, W. F. (2013). Modulation of macrophage phenotype by cell shape. *Proceedings of the National Academy of Sciences*. https://doi.org/10.1073/pnas.1308887110

Meng, F., Li, C., Li, W., Gao, Z., Guo, K., & Song, S. (2014). Interaction between pancreatic cancer cells and tumor-associated macrophages promotes the invasion of pancreatic cancer cells and the differentiation and migration of macrophages. *IUBMB Life*, *66*(12), 835–846. https://doi.org/10.1002/iub.1336

Mosser, D. M., & Edwards, J. P. (2008). Exploring the full spectrum of macrophage

activation. *Nature Reviews Immunology*. https://doi.org/10.1038/nri2448

Murata, Y., Kotani, T., Ohnishi, H., & Matozaki, T. (2014). The CD47-SIRPα signalling system: Its physiological roles and therapeutic application. *Journal of Biochemistry*. https://doi.org/10.1093/jb/mvu017

Murdoch, C., Giannoudis, A., & Lewis, C. E. (2004). Mechanisms regulating the recruitment of macrophages into hypoxic areas of tumors and other ischemic tissues. *Blood*, *104*(8), 2224 LP-2234. https://doi.org/10.1182/blood-2004-03-1109

Murdoch, C., Muthana, M., Coffelt, S. B., & Lewis, C. E. (2008). The role of myeloid cells in the promotion of tumour angiogenesis. *Nature Reviews Cancer*, *8*(8), 618–631. https://doi.org/10.1038/nrc2444

Murdoch, C., Muthana, M., & Lewis, C. E. (2005). Hypoxia Regulates Macrophage Functions in Inflammation. *The Journal of Immunology*, *175*(10), 6257 LP-6263. https://doi.org/10.4049/jimmunol.175.10.6257

Muz, B., de la Puente, P., Azab, F., & Azab, A. K. (2015). The role of hypoxia in cancer progression, angiogenesis, metastasis, and resistance to therapy. *Hypoxia*. https://doi.org/10.2147/HP.S93413

Neefjes, J., Jongsma, M. L. M., Paul, P., & Bakke, O. (2011). Towards a systems understanding of MHC class I and MHC class II antigen presentation. *Nature Reviews Immunology*. https://doi.org/10.1038/nri3084

Netea-Maier, R. T., Smit, J. W. A., & Netea, M. G. (2018). Metabolic changes in tumor cells and tumor-associated macrophages: A mutual relationship. *Cancer Letters*. https://doi.org/10.1016/j.canlet.2017.10.037

Nickel, D., Busch, M., Mayer, D., Hagemann, B., Knoll, V., & Stenger, S. (2012). Hypoxia triggers the expression of human β defensin 2 and antimicrobial activity against Mycobacterium tuberculosis in human macrophages. *Journal of Immunology (Baltimore, Md. : 1950)*. https://doi.org/10.4049/jimmunol.1100976

Nieman, K. M., Kenny, H. A., Penicka, C. V., Ladanyi, A., Buell-Gutbrod, R., Zillhardt, M. R., ... Lengyel, E. (2011). Adipocytes promote ovarian cancer metastasis and provide energy for rapid tumor growth. *Nature Medicine*. https://doi.org/10.1038/nm.2492

Ning, Y., Cui, Y., Li, X., Cao, X., Chen, A., Xu, C., ... Luo, X. (2018). Co-culture of ovarian

cancer stem-like cells with macrophages induced SKOV3 cells stemness via IL-8/STAT3 signaling. *Biomedicine & Pharmacotherapy*, *103*, 262–271. https://doi.org/10.1016/j.biopha.2018.04.022

Noman, M. Z., Desantis, G., Janji, B., Hasmim, M., Karray, S., Dessen, P., … Chouaib, S. (2014). PD-L1 is a novel direct target of HIF-1α, and its blockade under hypoxia enhanced MDSC-mediated T cell activation. *The Journal of Experimental Medicine*. https://doi.org/10.1084/jem.20131916

Ortiz-Masià, D., Díez, I., Calatayud, S., Hernández, C., Cosín-Roger, J., Hinojosa, J., … Barrachina, M. D. (2012). Induction of CD36 and Thrombospondin-1 in Macrophages by Hypoxia-Inducible Factor 1 and Its Relevance in the Inflammatory Process. *PLoS ONE*, *7*(10), e48535. https://doi.org/10.1371/journal.pone.0048535

Osińska, I., Popko, K., & Demkow, U. (2014). Perforin: an important player in immune response. *Central-European Journal of Immunology*, *39*(1), 109–115. https://doi.org/10.5114/ceji.2014.42135

Pan, Y., Tan, Y., Wang, M., Zhang, J., Zhang, B., Yang, C., … Wang, H. (2013). Signal regulatory protein α is associated with tumor-polarized macrophages phenotype switch and plays a pivotal role in tumor progression. *Hepatology*, *58*(2), 680–691. https://doi.org/10.1002/hep.26391

Pinto, A. T., Pinto, M. L., Velho, S., Pinto, M. T., Cardoso, A. P., Figueira, R., … Rocha, S. (2016). Intricate Macrophage-Colorectal Cancer Cell Communication in Response to Radiation. *PLoS ONE*, *11*(8), e0160891. https://doi.org/10.1371/journal.pone.0160891

Psaila, B., & Lyden, D. (2009). The metastatic niche: adapting the foreign soil. *Nature Reviews. Cancer*, *9*(4), 285–293. https://doi.org/10.1038/nrc2621

Qian, B., & Pollard, J. (2010). Macrophage diversity enhances tumor progression and metastasis. *Cell*, *141*(1), 39–51. https://doi.org/10.1016/j.cell.2010.03.014

Raggi, F., Pelassa, S., Pierobon, D., Penco, F., Gattorno, M., Novelli, F., … Bosco, M. C. (2017). Regulation of human Macrophage M1-M2 Polarization Balance by hypoxia and the Triggering receptor expressed on Myeloid cells-1. *Frontiers in Immunology*. https://doi.org/10.3389/fimmu.2017.01097

Raskov, H., Pommergaard, H. C., Burcharth, J., & Rosenberg, J. (2014). Colorectal

carcinogenesis-update and perspectives. *World Journal of Gastroenterology*, *20*(48), 18151–18164. https://doi.org/10.3748/wjg.v20.i48.18151

Ray, M., Lee, Y.-W., Hardie, J., Mout, R., Yeşilbag Tonga, G., Farkas, M. E., & Rotello, V. M. (2018). CRISPRed Macrophages for Cell-Based Cancer Immunotherapy. *Bioconjugate Chemistry*, *29*(2), 445–450. https://doi.org/10.1021/acs.bioconjchem.7b00768

Reeves, E., & James, E. (2016). Antigen processing and immune regulation in the response to tumours. *Immunology*. https://doi.org/10.1111/imm.12675

Ricciardi, A., Elia, A. R., Cappello, P., Puppo, M., Vanni, C., Fardin, P., … Varesio, L. (2008). Transcriptome of Hypoxic Immature Dendritic Cells: Modulation of Chemokine/Receptor Expression. *Molecular Cancer Research*, *6*(2), 175 LP-185. https://doi.org/10.1158/1541-7786.MCR-07-0391

Rodriguez-Garcia, M., Porichis, F., de Jong, O. G., Levi, K., Diefenbach, T. J., Lifson, J. D., … Kavanagh, D. G. (2011). Expression of PD-L1 and PD-L2 on human macrophages is up-regulated by HIV-1 and differentially modulated by IL-10. *Journal of Leukocyte Biology*. https://doi.org/10.1189/jlb.0610327

Romano, E., Rufo, N., Korf, H., Mathieu, C., Garg, A. D., & Agostinis, P. (2018). BNIP3 modulates the interface between B16-F10 melanoma cells and immune cells. *Oncotarget*. https://doi.org/10.18632/oncotarget.24815

Said, A., Raufman, J.-P., & Xie, G. (2014). The Role of Matrix Metalloproteinases in Colorectal Cancer. *Cancers*. https://doi.org/10.3390/cancers6010366

Samples, J., Willis, M., & Klauber-DeMore, N. (2013). Targeting Angiogenesis and the Tumor Microenvironment. *Surgical Oncology Clinics of North America*, *22*(4), 629–639. https://doi.org/10.1016/j.soc.2013.06.002

Semenza, G. L. (2013). Cancer–stromal cell interactions mediated by hypoxia-inducible factors promote angiogenesis, lymphangiogenesis, and metastasis. *Oncogene*, *32*(35), 4057–4063. https://doi.org/10.1038/onc.2012.578

Sethumadhavan, S., Silva, M., Philbrook, P., Nguyen, T., Hatfield, S. M., Ohta, A., & Sitkovsky, M. V. (2017). Hypoxia and hypoxia-inducible factor (HIF) downregulate antigen-presenting MHC class I molecules limiting tumor cell recognition by T cells. *PLoS ONE*. https://doi.org/10.1371/journal.pone.0187314

Shen, Z., Kauttu, T., Seppanen, H., Vainionpaa, S., Ye, Y., Wang, S., ... Puolakkainen, P. (2013). Both macrophages and hypoxia play critical role in regulating invasion of gastric cancer in vitro. *Acta Oncologica*. https://doi.org/10.3109/0284186X.2012.718444

Shen, Z., Seppänen, H., Vainionpää, S., Ye, Y., Wang, S., Mustonen, H., & Puolakkainen, P. (2012). IL10, IL11, IL18 are differently expressed in CD14+ TAMs and play different role in regulating the invasion of gastric cancer cells under hypoxia. *Cytokine*, *59*(2), 352–357. https://doi.org/10.1016/j.cyto.2012.04.033

Shi, S.-J., Wang, L.-J., Wang, G.-D., Guo, Z.-Y., Wei, M., Meng, Y.-L., ... Wen, W.-H. (2013). B7-H1 Expression Is Associated with Poor Prognosis in Colorectal Carcinoma and Regulates the Proliferation and Invasion of HCT116 Colorectal Cancer Cells. *PLoS ONE*. https://doi.org/10.1371/journal.pone.0076012

Shin, D. H., Dier, U., Melendez, J. A., & Hempel, N. (2015). Regulation of MMP-1 expression in response to hypoxia is dependent on the intracellular redox status of metastatic bladder cancer cells. *Biochimica et Biophysica Acta - Molecular Basis of Disease*. https://doi.org/10.1016/j.bbadis.2015.09.001

Sun, C., Mezzadra, R., & Schumacher, T. N. (2018). Regulation and Function of the PD-L1 Checkpoint. *Immunity*. https://doi.org/10.1016/j.immuni.2018.03.014

Sun, J., Zhang, Y., Yang, M., Zhang, Y., Xie, Q., Li, Z., ... Qu, X. (2010). Hypoxia induces T-cell apoptosis by inhibiting chemokine C receptor 7 expression: the role of adenosine receptor A(2). *Cellular and Molecular Immunology*, *7*(1), 77–82. https://doi.org/10.1038/cmi.2009.105

Tang, F., & Zheng, P. (2018). Tumor cells versus host immune cells: Whose PD-L1 contributes to PD-1/PD-L1 blockade mediated cancer immunotherapy? *Cell and Bioscience*. https://doi.org/10.1186/s13578-018-0232-4

Tang, M., Liu, B., Bu, X., & Zhao, P. (2018). Cross-talk between ovarian cancer cells and macrophages through periostin promotes macrophage recruitment. *Cancer Science*, *109*(5), 1309–1318. https://doi.org/10.1111/cas.13567

Tannapfel, A., Neid, M., Aust, D., & Baretton, G. (2010). The Origins of Colorectal Carcinoma. *Dtsch Arztebl International*, *107*(43), 760–766. https://doi.org/10.3238/arztebl.2010.0760

Tariq, K., & Ghias, K. (2016). Colorectal cancer carcinogenesis: a review of mechanisms.

Cancer Biology & Medicine. https://doi.org/10.28092/j.issn.2095-3941.2015.0103

Tátrai, E., Bartal, A., Gacs, A., Paku, S., Kenessey, I., Garay, T., ... Tóvári, J. (2017). Cell type-dependent HIF1 α-mediated effects of hypoxia on proliferation, migration and metastatic potential of human tumor cells. *Oncotarget*. https://doi.org/10.18632/oncotarget.17806

Thibodeau, J., Bourgeois-Daigneault, M.-C., & Lapointe, R. (2012). Targeting the MHC Class II antigen presentation pathway in cancer immunotherapy. *OncoImmunology*. https://doi.org/10.4161/onci.21205

Valentini, A. M., Di Pinto, F., Cariola, F., Guerra, V., Giannelli, G., Caruso, M. L., & Pirrelli, M. (2018). PD-L1 expression in colorectal cancer defines three subsets of tumor immune microenvironments. *Oncotarget*. https://doi.org/10.18632/oncotarget.24196

Varin, A., Mukhopadhyay, S., Herbein, G., & Gordon, S. (2010). Alternative activation of macrophages by IL-4 impairs phagocytosis of pathogens but potentiates microbial-induced signalling and cytokine secretion. *Blood*, *115*(2), 353–362. https://doi.org/10.1182/blood-2009-08-236711

Vaupel, P., & Harrison, L. (2004). Tumor hypoxia: causative factors, compensatory mechanisms, and cellular response. *The Oncologist*, *9 Suppl 5*(Supplement 5), 4–9. https://doi.org/10.1634/theoncologist.9-90005-4

Vega, P., Valentín, F., & Cubiella, J. (2015). Colorectal cancer diagnosis: Pitfalls and opportunities. *World Journal of Gastrointestinal Oncology*. https://doi.org/10.4251/wjgo.v7.i12.422

Wang, M., Zhao, J., Zhang, L., Wei, F., Lian, Y., Wu, Y., ... Guo, C. (2017). Role of tumor microenvironment in tumorigenesis. *Journal of Cancer*. https://doi.org/10.7150/jca.17648

Wang, N., Liang, H., & Zen, K. (2014). Molecular mechanisms that influence the macrophage M1-M2 polarization balance. *Frontiers in Immunology*, *5*(NOV), 1–9. https://doi.org/10.3389/fimmu.2014.00614

Wang, X., Teng, F., Kong, L., & Yu, J. (2016). PD-L1 expression in human cancers and its association with clinical outcomes. *OncoTargets and Therapy*. https://doi.org/10.2147/OTT.S105862

Wang, Y., Liu, T., Yang, N., Xu, S., Li, X., & Wang, D. (2016). Hypoxia and macrophages promote glioblastoma invasion by the CCL4-CCR5 axis. *Oncology Reports*. https://doi.org/10.3892/or.2016.5171

Watnick, R. S. (2012). The role of the tumor microenvironment in regulating angiogenesis. *Cold Spring Harbor Perspectives in Medicine*, *2*(12). https://doi.org/10.1101/cshperspect.a006676

Watson, N. F. S., Ramage, J. M., Madjd, Z., Spendlove, I., Ellis, I. O., Scholefield, J. H., & Durrant, L. G. (2006). Immunosurveillance is active in colorectal cancer as downregulation but not complete loss of MHC class I expression correlates with a poor prognosis. *International Journal of Cancer*. https://doi.org/10.1002/ijc.21303

Weagel, E., Smith, C., Liu, P. G., Robinson, R., & O'Neill, K. (2015). Macrophage Polarization and Its Role in Cancer. *Journal of Clinical & Cellular Immunology*. https://doi.org/10.4172/2155-9899.1000338

Wieczorek, M., Abualrous, E. T., Sticht, J., Álvaro-Benito, M., Stolzenberg, S., Noé, F., & Freund, C. (2017). Major histocompatibility complex (MHC) class I and MHC class II proteins: Conformational plasticity in antigen presentation. *Frontiers in Immunology*. https://doi.org/10.3389/fimmu.2017.00292

Willingham, S. B., Volkmer, J.-P., Gentles, A. J., Sahoo, D., Dalerba, P., Mitra, S. S., ... Weissman, I. L. (2012). The CD47-signal regulatory protein alpha (SIRPa) interaction is a therapeutic target for human solid tumors. *Proceedings of the National Academy of Sciences*. https://doi.org/10.1073/pnas.1121623109

Wu, T. H., Li, Y. Y., Wu, T. L., Chang, J. W. C., Chou, W. C., Hsieh, L. L., ... Yeh, K. Y. (2014). Culture supernatants of different colon cancer cell lines induce specific phenotype switching and functional alteration of THP-1 cells. *Cellular Immunology*. https://doi.org/10.1016/j.cellimm.2014.05.015

Wynn, T. A., Chawla, A., & Pollard, J. W. (2013). Macrophage biology in development, homeostasis and disease. *Nature*, *496*(7446), 445–455. https://doi.org/10.1038/nature12034

Xu, X., Farach-Carson, M. C., & Jia, X. (2014). Three-dimensional in vitro tumor models for cancer research and drug evaluation. *Biotechnology Advances*. https://doi.org/10.1016/j.biotechadv.2014.07.009

Yamaguchi, T., Fushida, S., Yamamoto, Y., Tsukada, T., Kinoshita, J., Oyama, K., ... Ohta, T. (2016). Tumor-associated macrophages of the M2 phenotype contribute to progression in gastric cancer with peritoneal dissemination. *Gastric Cancer*, *19*(4), 1052–1065. https://doi.org/10.1007/s10120-015-0579-8

Yang, L., & Zhang, Y. (2017). Tumor-associated macrophages: from basic research to clinical application. *Journal of Hematology & Oncology*. https://doi.org/10.1186/s13045-017-0430-2

Ye, X., Zhang, J., Lu, R., & Zhou, G. (2016). Signal regulatory protein α associated with the progression of oral leukoplakia and oral squamous cell carcinoma regulates phenotype switch of macrophages. *Oncotarget*, *7*(49), 81305–81321. https://doi.org/10.18632/oncotarget.12874

Yearley, J. H., Gibson, C., Yu, N., Moon, C., Murphy, E., Juco, J., ... McClanahan, T. (2017). PD-L2 expression in human tumors: Relevance to anti-PD-1 therapy in cancer. *Clinical Cancer Research*. https://doi.org/10.1158/1078-0432.CCR-16-1761

Yi, J. S., Cox, M. A., & Zajac, A. J. (2010). T-cell exhaustion: Characteristics, causes and conversion. *Immunology*. https://doi.org/10.1111/j.1365-2567.2010.03255.x

Zhang, H., Lu, H., Xiang, L., Bullen, J. W., Zhang, C., Samanta, D., ... Semenza, G. L. (2015). HIF-1 regulates CD47 expression in breast cancer cells to promote evasion of phagocytosis and maintenance of cancer stem cells. *Proceedings of the National Academy of Sciences*. https://doi.org/10.1073/pnas.1520032112

Zhang, J., Zhang, Q., Lou, Y., Fu, Q., Chen, Q., Wei, T., ... Liang, T. (2017). Hypoxia-inducible factor-1α/interleukin-1β signaling enhances hepatoma epithelial–mesenchymal transition through macrophages in a hypoxic-inflammatory microenvironment. *Hepatology*, *67*(5), 1872–1889. https://doi.org/10.1002/hep.29681

Zhang, W., Shi, X., Peng, Y., Wu, M., Zhang, P., Xie, R., ... Wang, J. (2015). HIF-1α promotes epithelial-mesenchymal transition and metastasis through direct regulation of ZEB1 in colorectal cancer. *PLoS ONE*.

https://doi.org/10.1371/journal.pone.0129603

Zhang, Y., Sime, W., Juhas, M., & Sjölander, A. (2013). Crosstalk between colon cancer cells and macrophages via inflammatory mediators and CD47 promotes tumour cell migration. *European Journal of Cancer*. https://doi.org/10.1016/j.ejca.2013.06.005

Zhong, X., Chen, B., & Yang, Z. (2018). The Role of Tumor-Associated Macrophages in Colorectal Carcinoma Progression. *Cellular Physiology and Biochemistry*, *45*(1), 356–365. https://doi.org/10.1159/000486816

Zhou, Z., Peng, Y., Wu, X., Meng, S., Yu, W., Zhao, J., … Li, W. (2018). CCL18 secreted from M2 macrophages promotes migration and invasion via the PI3K/Akt pathway in gallbladder cancer. *Cellular Oncology*. https://doi.org/10.1007/s13402-018-0410-8