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The role of hypoxia on immunomodulation at the colon cancer microenvironment

Ana Rita Santos

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

ANA RITA FIALHO DOS SANTOS

THE ROLE OF HYPOXIA ON IMMUNOMODULATION AT THE COLON CANCER MICROENVIRONMENT

Dissertação de Candidatura ao grau de Mestre em Oncologia – especialização em Oncologia Laboratorial – submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

Orientador – Doutora Ângela Margarida Amorim Costa

Junior Researcher – Tumour and Microenvironment Interactions Group, Instituto de Investigação e Inovação em Saúde/ Instituto Nacional de Engenharia Biomédica (i3S/INEB)

Co-orientador – Doutora Sérgia Maria Aguiar Velho

Associate Researcher – Epithelial Interactions in Cancer Group, Instituto de Investigação e Inovação em Saúde/ Instituto de Patologia e Imunologia Molecular da Universidade do Porto (i3S/IPATIMUP).

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Resumo

O microambiente de tumores sólidos é composto, para além das células malignas, por vários componentes não-malignos como fibroblastos, células endoteliais e células imunes que interagem com as células tumorais modulando as suas funções e influenciando a progressão do tumor e a sua resposta a terapias. Devido à rápida proliferação das células tumorais e vasculatura anormal, o microambiente tumoral de tumores sólidos é frequentemente hipóxico. A hipóxia tem sido associada, em vários tipos de cancro, ao aumento da agressividade tumoral e resistência a terapia. No entanto, no cancro do cólon o efeito da hipóxia ainda não está completamente esclarecido.

Quando se estudou a influência da hipóxia na interação entre células de cancro do cólon e macrófagos, o nosso grupo demonstrou que a hipóxia está associada tanto com a promoção de características associadas à progressão tumoral, como o consumo da glucose e a invasão, como com características associadas com um melhor prognóstico, como a de macrófagos de perfil pro-inflamatório. Mais ainda, quando analisou dados de doentes com cancro do cólon descobriu que tumores *CD68*^{High}*HIF1A*^{High} apresentavam melhor sobrevida que os tumores *CD68*^{High}*HIF1A*^{Low}, um fenómeno particularmente relevante em tumores do cólon direito. Estes resultados levaram o grupo a colocar a hipótese de que estas diferenças poderiam ser devidas a diferentes perfis de expressão de *checkpoints* imunológicos.

Com o intuito de entender quais são os mediadores moleculares envolvidos nas respostas celulares mencionadas, foram estabelecidas triculturas diretas de macrófagos, células T e células de cancro do colon, em condições de normoxia (20% O_2) ou hipóxia (1% O_2), e o RNA foi avaliado através do painel Tumor Signalling 360 da Nanostring. A análise revelou uma dramática alteração no perfil de expressão em hipóxia, e para seleção dos genes mais interessantes para estudos futuros recorreu-se a dados clínicos de doentes de cancro do cólon. Depois de usar uma série de filtros prosseguiu-se com a validação de 5 genes: *CAV1*, *CXCR4*, *FCAR*, *ITGA5* e *PFKFB3.*

O trabalho deste mestrado permitiu validar a nível da proteína a importância da Integrina α5 (*ITGA5*), cuja expressão é aumentada pela hipóxia em todas as linhas celulares avaliadas. Mais ainda, foi avaliado o impacto da expressão desta molécula na viabilidade celular e na ativação das células T.

Em relação ao estudo dos *checkpoints* imunológicos, o trabalho desenvolvido durante este mestrado permitiu concluir que cólon direito e esquerdo apresentam um diferente perfil de expressão, bem como os tipos tumorais que nas análises anteriores

tinham mostrado estar associados com diferentes sobrevidas: *CD68*^{High}*HIF1A*^{High} *versus* *CD68*^{High}*HIF1A*$^{Low.}$

Por último, a expressão de *checkpoints* imunológicos foi avaliada em triculturas de macrófagos, células T e células tumorais (RKO ou SW480) silenciadas ou não para *ITGA5*, sob normóxia ou hipóxia, para avaliar o impacto da Integrina α5 na regulação de *checkpoints* imunológicos. Desta análise pode-se concluir que a Integrina α5 não parece regular a expressão de CD40, CD86 e TIM-3 em hipóxia.

No geral, consideramos que o conhecimento obtido neste trabalho fornece informações relevantes que podem abrir o caminho a mais estudos que culminem no desenvolvimento de novas e mais eficientes abordagens terapêuticas.

Palavras-chave: hipóxia; cancro do cólon; microambiente tumoral; macrófagos; células T; *ITGA5*; *checkpoint* imunológico.

Abstract

The microenvironment of solid tumors is composed, in addition to malignant cells, of various non-malignant components such as fibroblasts, endothelial cells and immune cells that interact with tumor cells modulating their functions and influencing tumor progression and their response to therapies. Due to the rapid proliferation of tumor cells and abnormal vasculature, the tumor microenvironment of solid tumors is often hypoxic. Hypoxia has been associated, in various cancer types, with increased tumor aggressiveness and therapy resistance. However, in colon cancer, the effect of hypoxia is not yet completely understood.

When the influence of hypoxia on crosstalk between colon cancer and macrophages was studied, our group showed that hypoxia is associated with both the promotion of features associated with tumor progression, as glucose consumption and invasion, and with features associated with a better prognosis, as a more pro-inflammatory profile of macrophages. Moreover, when colon cancer patients' data was analyzed, it was found that *CD68*^{High}*HIF1A*^{High} tumors presented better survival than *CD68*^{High}*HIF1A*^{Low} tumors, a particularly relevant phenomenon in right colon tumors. These results led the group to hypothesize that these differences could be due to different immune checkpoints expression profile.

In order to understand which molecular mediators are involved in the mentioned cellular responses, direct tricultures of macrophages, T cells and colon cancer cells were established under normoxia (20% O_2) or hypoxia (1% O_2) conditions, and the RNA was evaluated using the Nanostring Tumor Signaling 360 panel. The analysis revealed a dramatic change in the expression profile under hypoxia, and for selection of the most interesting genes for future studies, clinical data from colon cancer patients were used. After using a series of filters, 5 genes continued for further validation: *CAV1*, *CXCR4*, *FCAR*, *ITGA5* and *PFKFB3.*

This master's degree work allowed to validate at the protein level the importance of Integrin α5 (*ITGA5*), whose expression is increased by hypoxia in all cell lines evaluated. Furthermore, the impact of the expression of this molecule on cell viability and T cell activation was evaluated.

Regarding the study of immune checkpoints, the work developed during this master's degree allowed us to conclude that the right and left colon have a different expression profile, as well as the tumor types that in the previous analyses had shown to be associated with different survival: *CD68*^{High}*HIF1A*^{High} *versus CD68*^{High}*HIF1A*$^{Low.}$

Finally, the expression of immune checkpoints was evaluated in tricultures of macrophages, T cells and tumor cells (RKO or SW480) silenced or not for *ITGA5*, under normoxia or hypoxia, to evaluate the impact of Integrin α5 on the checkpoint's regulation. From this analysis it can be concluded that Integrin α5 does not appear to regulate the expression of CD40, CD86 and TIM-3 in hypoxia.

In general, we consider that the breakthroughs obtained in this work provides relevant information that may pave the way for further studies that culminate in the development of new and more efficient therapeutic approaches.

Keywords: hypoxia; colon cancer; tumor microenvironment; macrophages; T cells; *ITGA5*; immune checkpoints.

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List of Abbreviations

ADM – Adrenomedullin

AJCC – American Joint Committee on Cancer

APC – Adenomatous Polyposis Coli

APCs – Antigen-Presenting Cells

ATCC – American Type Culture Collection

BTLA – B and T Lymphocyte attenuator

CCL – C-C Motif Chemokine Ligand 2

CD – Cluster of Differentiation

CFSE – Carboxyfluorescein Diacetate Succinimidyl Ester

CIMP – CpG Island Methylator Phenotype

CIN – Chromosomal Instability

CMS – Consensus Molecular Subtype

CRC – Colorectal Cancer

CTLA-4 – Cytotoxic T-Lymphocyte-associated Protein 4

CTLs – Cytotoxic T Lymphocytes

CXCL – C-X-C Motif Chemokine Ligand

DCs – Dendritic cells

dMMR–MSI-H – Mismatch Repair deficient and Microsatellite Instability high

ECM – Extracellular Matrix

EGF – Epidermal Growth Factor

EGFR – Epidermal Growth Factor Receptor

EMT - Epithelial-Mesenchymal Transition

FBS - Fetal Bovine Serum

FDA – Food and Drug Administration

HDI – Human Development Index

HIF – Hypoxia-Inducible Factor

HNPCC – Hereditary Non-Polyposis Colorectal Cancer

HRE – Hypoxia Response Elements

Hsc-70 – Heat Shock Cognate protein 70

ICIs – Immune checkpoints inhibitors

IFN-γ – Interferon-gamma

IL – Interleukins

LAG3 – Lymphocyte-Activation Gene 3

LCC – Left-sided Colon Carcinomas

LDHA – Lactate Dehydrogenase A

LOX – Lysyl Oxidase

LPS – Lipopolysaccharides

MAPK – Mitogen-Activated Protein Kinase

M-CSF – Macrophage Colony Stimulating Factor

MFI – Median Fluorescence Intensity

MHC – Major Histocompatibility Complex

MMPs – Matrix Metalloproteinases

MSI – Microsatellite Instability

MSS - Microsatellite Stable

ON - Overnight

PBMCs – Peripheral Blood Mononuclear Cells

PBS – Phosphate-Buffered Saline

PD-1 – Programmed Death 1

PD-L – Programmed-Death Ligand

PFA – Paraformaldehyde

PGE2 – Prostaglandin E2

PHDs – Prolyl Hydroxylases

PI3K – Phosphoinositide 3-Kinase

PMSF – Phenylmethylsulfonyl Fluoride

Pvhl – Von Hippel-Lindau tumor-suppressor protein

RCC – Right-sided Colon Carcinomas

RT – Room Temperature

RTK – Receptor Tyrosine Kinase

SCNA – Somatic Copy Number Alterations

SIRPα – Signal-Regulatory Protein alpha

TAMs – Tumor-Associated Macrophages

TCGA – The Cancer Genome Atlas

TCR – T Cell Receptor

TGF-β – Transforming Growth Factor beta

TME – Tumor Microenvironment

TNF-α – Tumor Necrosis Factor-alpha

TNM – Tumor/Node/Metastases

TP53 – Tumor Protein P53

VEGF – Vascular Endothelial Growth Factor

Introduction

A malignant tumor is an abnormal cellular mass, formed due aberrant proliferation with potential to invade the adjacent tissue, which is originated by mutations or errors that occur in the DNA of normal cells. The accumulation of various mutations promotes a set of characteristics essential to the development of neoplasia, such as excessive cell proliferation, inhibition of cell death, ability to escape control of the immune system, and metastization.

In 2000, Hanahan and Weinberg suggested that the malignant tumor is the manifestation of a set of six physiological changes, and that without these changes there is no development of cancer, the hallmarks of cancer: the acquired capabilities for sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing/accessing vasculature, and activating invasion and metastasis (Hanahan & Weinberg, 2000). Later, taken into account the advances made in understanding the characteristics of the tumor, four new hallmarks were established: reprogramming cellular metabolism, avoiding immune destruction,genome instability and tumor-promoting inflammation (Hanahan & Weinberg, 2011).

Currently are considered four new hallmarks: unlocking phenotypic plasticity, nonmutational epigenetic reprogramming, polymorphic microbiomes, and senescent cells (Figure 1) (Hanahan, 2022).

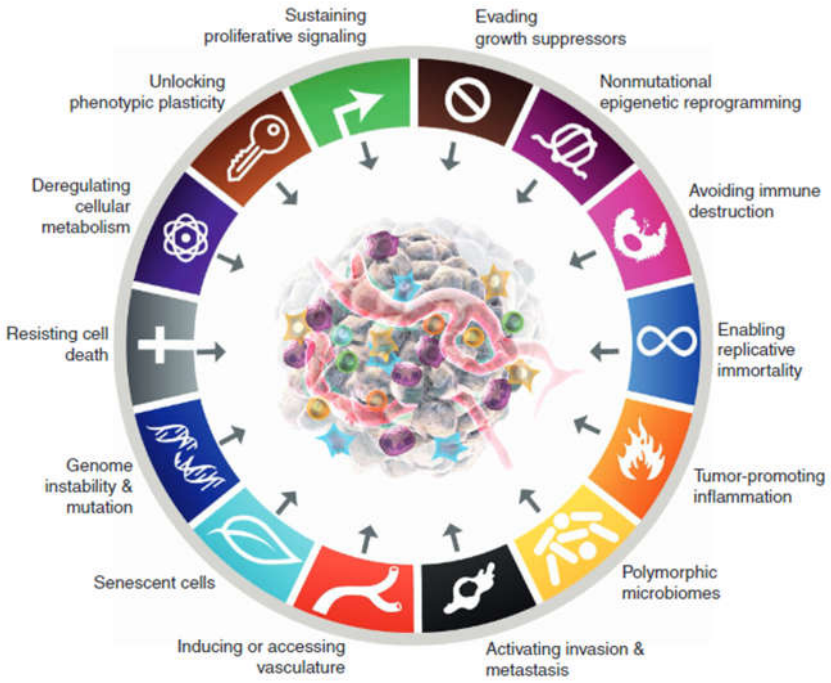


Figure 1. The hallmarks of cancer. This illustration encompasses the most recent version of the hallmark of cancer. Adapted from Hanahan, 2022.

1. Tumor microenvironment

The Tumor Microenvironment (TME) is a complex and heterogenous system composed not only of tumor cells but also of untransformed cells such as immune cells, endothelial cells, and fibroblasts, supported by an Extracellular Matrix (ECM) (Figure 2). Immune cells, such as granulocytes, lymphocytes, and macrophages are involved in various immune responses and activities, such as inflammatory reactions orchestrated by the tumor to promote survival (Joyce & Pollard, 2009). The malignant and the non-malignant cells at the TME interact, indirectly, through the secretion of cytokines, chemokines, growth factors, and matrix remodeling enzymes and, directly, through the surface expression of ligands or of receptors (F. R. Balkwill et al., 2012). These interactions create an environment that provides tumor cells with essential factors for proliferation, immune escape, invasion, and metastasis (Watnick, 2012).

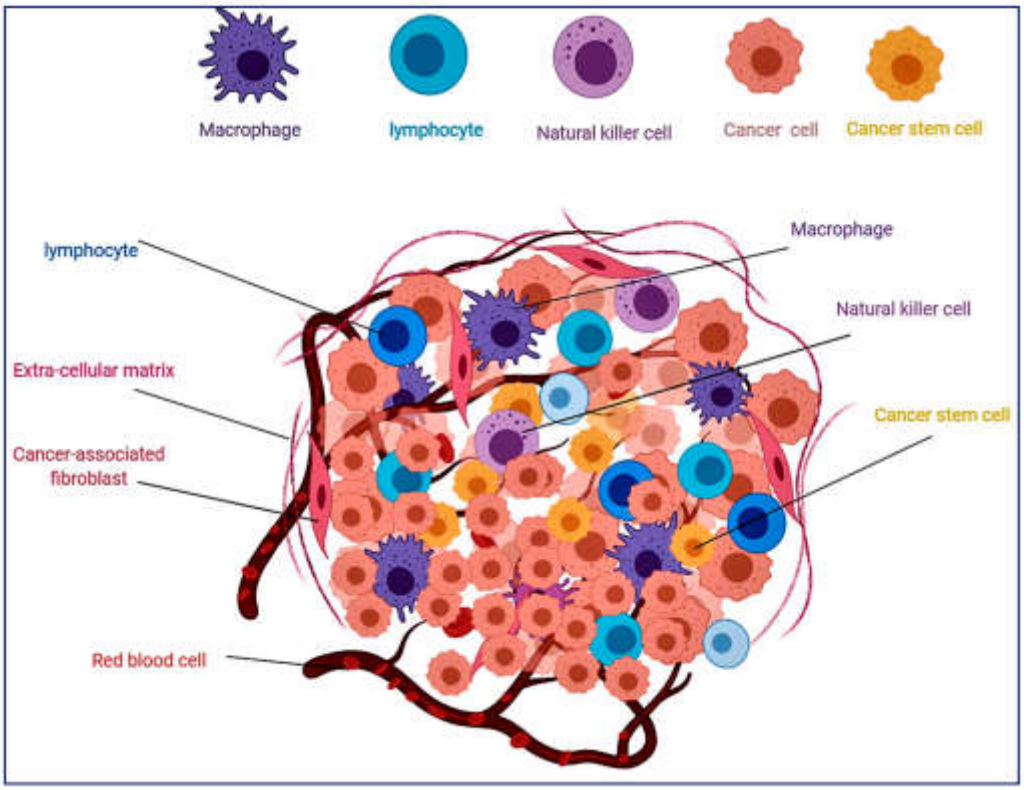


Figure 2. The tumor microenvironment. The complex microenvironment of solid tumors is characterized by the presence of malignant and non-malignant cells, namely, fibroblasts, endothelial cells, and various immune cells supported by an extracellular matrix. Adapted from Hassan & Seno, 2020.

1.1. Immune compartment

One of the main components of the TME are the immune cells, which are responsible for immune surveillance, recognition, and elimination of tumor cells. However, this

elimination is not always successful because, in sites of chronic inflammation, inflammatory cells can be modulated to an immunosuppressive phenotype (Wang et al., 2017).

Immune cells at the TME are both from innate immune system, as macrophages, neutrophils, Dendritic Cells (DCs), and Natural Killer (NK) cells, and from the adapted immune system as T and B cells (M. Wang et al., 2017; Grivennikov et al., 2010). At an early stage, tumor cells release cytokines and chemokines that attract leukocytes to the tumor region (Coussens & Werb, 2002).

The cells from the innate immune system can function as Antigen-Presenting Cells (APCs), which capture, and process antigens released by the tumor cells. Once processed the antigens can be presented to T and B lymphocytes through the Major Histocompatibility Complex (MHC) (D. S. Chen & Mellman, 2013). In turn, activated lymphocytes migrate to the TME, where they recognize and kill tumor cells. When tumor cells die, more pro-inflammatory cytokines are released, enhancing the recruitment of more immune cells to the tumor site (De Visser et al., 2006). Activation of B lymphocytes contributes to memory establishment, allowing the immune system to respond quickly to a second exposure to the same antigen.

The tumor, however, has strategies to avoid an effective anti-tumor immune response leading tumor cells to escape immune surveillance.

1.1.1. Macrophages

Macrophages can be divided, depending on their population of origin, into two: 1) tissue-resident macrophages, which derive from fetal hematopoietic organs present during embryogenesis. The progenitors of monocytes colonize different tissues, differentiating later into resident macrophages, capable of maintaining throughout life; 2) macrophages which differentiate from bone marrow-derived monocytes, and migrate to different tissues depending on the stimuli they are subject to (Davies et al., 2013; Franklin & Li, 2016).

Macrophages, when activated, perform diverse functions, being a hallmark their phagocytic ability. They can phagocyte pathogens, dead cells, or cellular debris. Besides being APCs, produce cytokines and chemokines that increase the recruitment of more immune cells, further enhancing the immune response (Abbas et al., 2021). One of the main characteristics of macrophages is their plasticity. This feature makes them interesting targets for new therapeutic approaches. Depending on the stimuli to which they are subjected, macrophages can polarize into M1 macrophages or classically activated macrophages, which present a pro-inflammatory and anti-tumor phenotype, and M2

macrophages with anti-inflammatory and pro-tumor activities (Cassetta et al., 2011; Leblond et al., 2016; Brundu S, 2015).

When in the presence of Interferon-gamma (IFN-γ), Tumor Necrosis Factor-alpha (TNF-α) and Lipopolysaccharides (LPS) macrophages polarize into M1 macrophages, which are characterized by the release of high levels of pro-inflammatory cytokines such as Interleukins (IL)-1, IL-6, IL-12 and IL-23 (S. Mantovani, 2012). This type of macrophage expresses specific surface receptors, such as Cluster of Differentiation (CD) 80, CD86, MHC class I, and class II molecules, which are essential for antigen presentation (Brundu S, 2015). On the other hand, M2 macrophages are usually polarized by IL-4, IL-13, and IL-10 and produce high levels of IL-2, IL-8, and IL-10 (Brundu S, 2015; Hao et al., 2012; A. Mantovani et al., 2004; Martinez & Gordon, 2014). These macrophages are characterized by exhibiting high levels of the mannose receptor (CD206), which plays an important role in recognition of microorganisms, endocytosis, and phagocytosis and is described as highly expressed in macrophages present in the TME of several types of cancer. In addition to this receptor, M2 macrophages express high levels of the scavenger receptor (CD163), involved in homeostatic functions such as regulation of circulating levels of reproductive hormones, innate immunity, and infections control (A. Mantovani et al., 2004; Cummings, 2022).

M2 macrophages can be subdivided into distinct subpopulations: (1) M2a macrophages, associated with the death and encapsulation of parasites; 2) M2b macrophages, involved in immune regulation; 3) M2c macrophages, associated with immunosuppression, tissue repair and matrix remodeling, and 4) M2d macrophages associated with angiogenesis promotion (Figure 3) (Brundu S, 2015; Curren Smith, 2015; A. Mantovani et al., 2004).

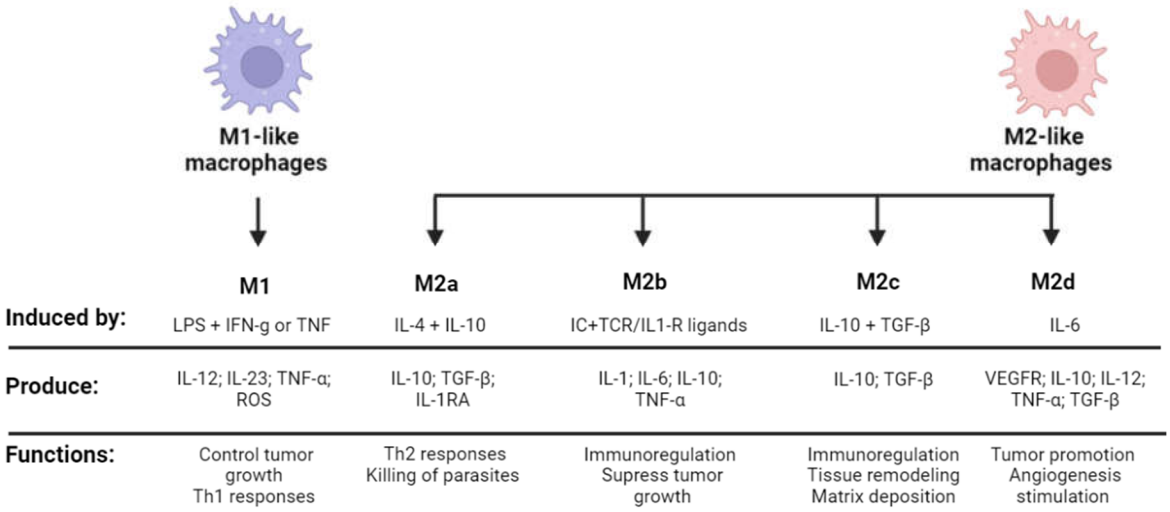


Figure 3. A spectrum of macrophages polarization. Macrophages can be divided into different subpopulations depending on the stimulus to which they are subjected. These cells are defined depending on

the surface receptor they express, the factors secreted, and their functions. There are two main groups of macrophages, M1, and M2, population is divided into four subpopulations. Adapted from Weagel *et al*, 2015.

1.1.2. T cells

T cells are responsible for maintaining immune responses and homeostasis. T lymphocytes originate from bone precursors and are then taken to the thymus where they undergo the maturation process characterized by the development of the T Cell Receptor (TCR), through the recombination of the TCR gene segments. Subsequently, the T cells are subjected to positive and negative selection processes, to eliminate the T cells that react with themselves (Sprent & Surh, 2011). T cells are kept in a quiescent state until they encounter an antigen. In the presence of antigen, the recognition process begins, which consists of presenting an antigenic epitope to the T cell through the TCR-MHC complex binding present in APCs (Pisibon et al., 2021). In addition to this connection, it is essential the connection between the CD28, present on the surface of T cells and the CD80 that is in the APCs, allowing the maximization of the binding between the two cells.

Activated T cells will proliferate giving rise to daughter cells capable of recognizing the antigen exposed on the surface of APCs (Kumar et al., 2018). In turn, these T cells, differentiated into CD8+ T cells and CD4+ T cells will be recruited by chemotaxis to the peripheral tissues.

CD8+ T cells, also called cytotoxic T cells, are responsible for the destruction of antigens (N. Zhang & Bevan, 2011). CD4+ T cells can differentiate into different subtypes (Th1, Th2, Th17, T_{FH}, and Tregs), each expressing a specific repertoire of cytokines, which promotes different responses of the immune system (Figure 4) (Zhu, 2018; Luckheeram et al., 2012).

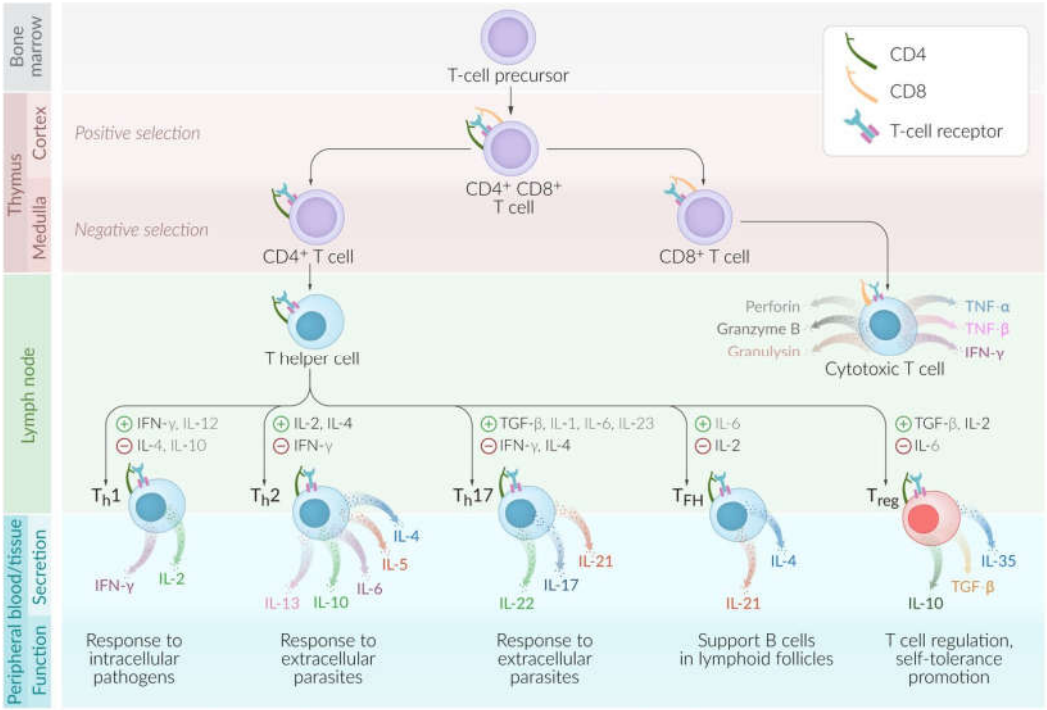


Figure 4. Differentiation of T cells. When activated, T cells divided into two types of cells: CD4+ T cells and CD8+ T cells. CD4+ T cells, depending on the stimuli to which they are subjected, can differentiate into different subtypes. Adapted from https://www.amboss.com/us/knowledge/Adaptive_immune_system.

1.1.3. Immune checkpoints

Immune checkpoints are regulators of the immune system. Under normal physiological conditions the immune checkpoints are essential for maintaining self-tolerance, prevent autoimmunity and control the duration and extent of immune responses, minimizing collateral damage to tissues (Pardoll, 2012).

Immune checkpoints, such as Programmed Death 1 (PD-1), Lymphocyte-Activation Gene 3 (LAG3) and Cytotoxic T Lymphocyte-Associated Protein 4 (CTLA-4) have been shown to modulate T-cell responses to chronic infections, viruses or tumor antigens (Cai et al., 2020; Pardoll, 2012). As surface molecules, their activity can be easily inhibited by blocking antibodies that prevent ligand-receptor engagement and for this reason are considered relevant therapeutic targets (He & Xu, 2020).

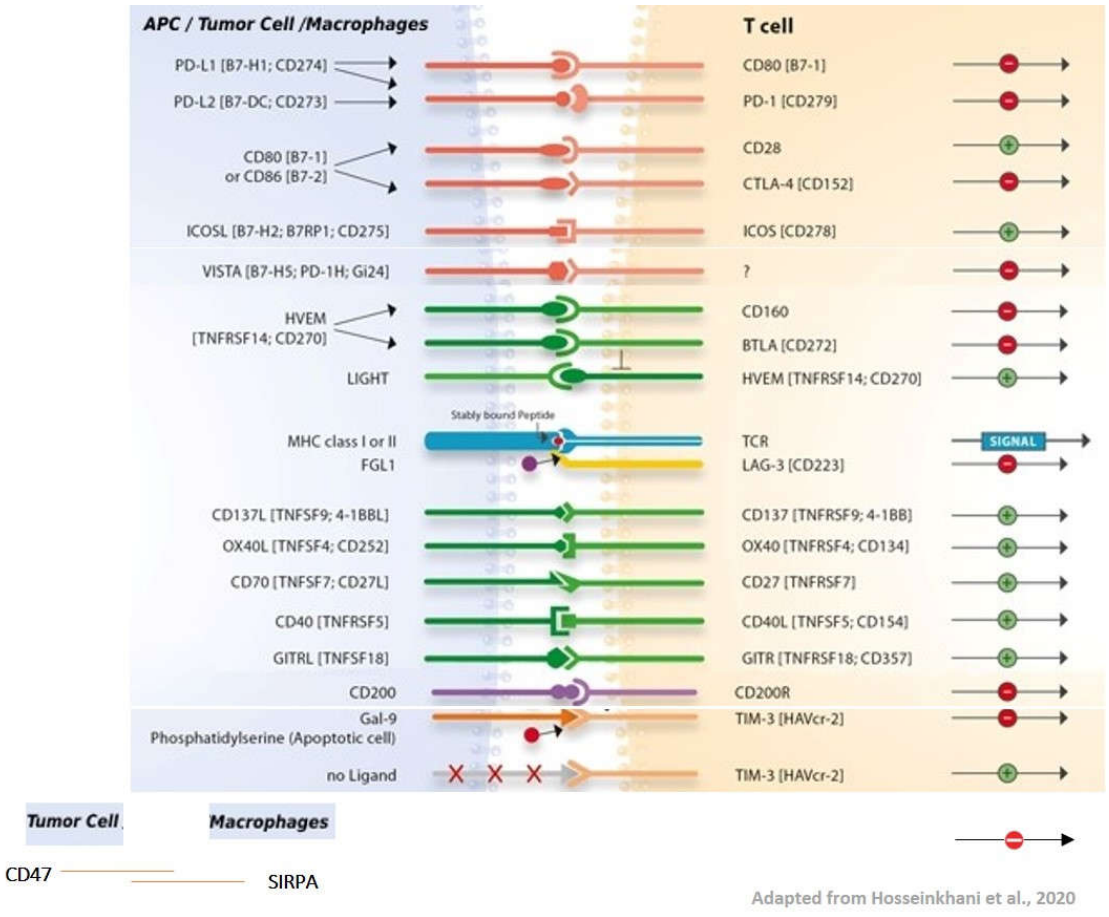


Figure 5. Immune checkpoints. List of immune checkpoints expressed both in tumor cells, APC and T cells. Depending on the link they present, they can activate (green circle) or inhibit (red circle) T cell response, having an anti-or pro-tumor activity. Adapted from Hosseinkhani *et al.,* 2020.

1.1.4. Tumor-associated macrophages and T cells at the tumor microenvironment

Cells present in the TME release cytokines and growth factors such as C-C Motif Chemokine Ligand 2 (CCL)-2, CCL3, CCL4, CCL5, CCL7, CCL8, C-X-C Motif Chemokine Ligand 12 (CXCL)-12, Macrophage Colony Stimulating Factor (M-CSF), Vascular Endothelial Growth Factor (VEGF) and IL-1 promoting the recruitment of monocytes to the tumor region (Hao et al., 2012). Once in the tumor tissue, monocytes differentiate into Tumor-Associated Macrophages (TAMs), which are very similar to M2 macrophages, since they express some typical markers of these macrophages, such as CD163. TME is typically anti-inflammatory, meaning it releases anti-inflammatory cytokines, such as IL-4, IL-13, Transforming Growth Factor beta (TGF-β), and IL-10 (L. Yang & Zhang, 2017). The release of these factors promotes the polarization of macrophages into M2 macrophages, with anti-inflammatory and pro-tumor activities. Studies carried out by our group showed that

macrophages polarized under IL-10 are more efficient in promoting angiogenesis *in vitro* and the invasion of tumor cells contributing to tumor metastasis (A. P. Cardoso et al., 2014).

TAMs play a pro-tumor role by promoting angiogenesis, enhancing cancer cell stemness, promoting their epithelial to mesenchymal transition, immunosuppression activities, migration, and invasion, and contributing to the establishment of the pre-metastatic niche (Aras & Raza Zaidi, 2017; Brundu S, 2015; Ana P. Cardoso et al., 2015; Condeelis & Pollard, 2006). Angiogenesis is induced when TAMs release proangiogenic factors, such as VEGF, Adrenomedullin (ADM), and other factors such as chemokines, TGF-β, and Matrix Metalloproteinases (MMPs). Tumor migration and invasion occur due to the secretion of MMPs, serine proteases and cathepsins, and other factors, which modify the cell–cell adhesion and promote basal membrane disruption. TAMs may also be involved in immunosuppression, by the release of immunosuppressive cytokines, such as IL-10 and TGF-β and by expressing Programmed-Death Ligand (PD-L)-1/PD-L2, ligands of PD-1 in the T cells, inhibiting the immune response (Aras & Raza Zaidi, 2017; Dong et al., 2017; Sun et al., 2018).

TAMs are also described as essential in the preparation of the pre-metastatic niche through the secretion of chemotactic factors, such as CXCL1, and the deposition of ECM components, such as fibronectin. In turn, these components recruit circulating tumor cells to the site where the metastatic niche is forming (Aras & Raza Zaidi, 2017).

Furthermore, the phagocytic activity of macrophages is decreased by the upregulation of the CD47 molecule at the tumor cells, functioning as a “don´t eat me” signal. (Chao et al., 2012; X. Liu et al., 2017; Willingham et al., 2012). CD47 binds to Signal-Regulatory Protein alpha (SIRPα) which is expressed on macrophages and DCs. The CD47-SIRPα binding, prevents the accumulation of myosin-IIA at the phagocytic synapse, which results in inhibition of phagocytosis (Chao et al., 2012; Willingham et al., 2012).

TAMs are preferably found in necrotic and hypoxic regions of the tumor, although they can also be found in highly vascularized regions (Brundu S, 2015; Martins et al., 2020). According to several studies on different types of cancer, high levels of TAMs infiltration are associated with poor prognosis, reduced overall survival, and resistance to therapies (Gordon & Martinez, 2010; Pollard, 2004).

In addition to TAMs, T cells are also an essential component of the tumor microenvironment. Cytotoxic T cells mediate tumor elimination through the secretion of IL-2 and IFN-γ which promotes the recruitment of more cytotoxic T cells to the tumor site (Alspach et al., 2019; Y. Liu et al., 2021). However, the elimination of tumor cells is not

always successful, since tumor cells express immune checkpoints on their surface that “turn off” the immune system and trigger an antitumor immune reaction.

Of the various immune checkpoints discovered so far, CTLA-4, PD-1 and PD-L1 are currently the most studied and the most promising as therapy targets in various types of cancer. However, in recent decades other immune checkpoints have been identified and studied in cancer, such as LAG3, TIM-3 and B and T Lymphocyte attenuator (BTLA) (He & Xu, 2020). Recent findings have revealed that immune checkpoints are subject to specific regulatory mechanisms and have distinct functions depending on the context in which they are inserted (He & Xu, 2020). Thanks to the discovery of immune checkpoints, it was possible to develop one of the most revolutionary therapies against cancer: Immune Checkpoints Inhibitors (ICIs) (Table 1) (Hatic et al., 2021). This therapy aims to boost the immune system to increase its effectiveness in detecting or delaying the growth of tumor cells, thus preventing cancer from spreading.

This immunotherapy is now established as a powerful way to treat cancer and it has been associated with increased survival for many patients with malignancies, however, is still not an option for the majority of solid tumors (Emens et al., 2017).

Table 1.Current immunotherapies for cancer. Adapted from Russel et al., 2021.

Drug (Trade name)	Company	Date of approval	Indication
CTLA-4 inhibitors			
Ipilimumab (Yervoy®)	Bristol-Myers Squibb	2011	Melanoma colorectal cancer Renal cell carcinoma
PD-1 inhibitors			
Nivolumab (Opdivo®)	Bristol-Myers Squibb	2014	Melanoma Hodgkin's lymphoma Diffuse large B-cell lymphoma Urothelial cancer Colorectal cancer Hepatocellular carcinoma Non-small cell lung cancer Small cell lung cancer Renal cell carcinoma Squamous cell carcinoma
Pembrolizumab (Keytruda®)	Merck	2014	Melanoma Cervical cancer Hodgkin's lymphoma Diffuse large B-cell lymphoma Gastric cancer Urothelial cancer Colorectal cancer Hepatocellular carcinoma Non-small cell lung cancer Small cell lung cancer Renal cell carcinoma Squamous cell carcinoma Esophageal cancer Merkel cell carcinoma
Cemiplimab (Libtayo®)	Sanofi	2018	Cutaneous squamous cell carcinoma
PD-L1 inhibitors			
Atezolizumab (Tecentriq®)	Roche, Genentech	2016	Non-small cell lung cancer Triple negative breast cancer
Avelumab (Bavencio®)	Merck, Pfizer	2017	Merkel cell carcinoma Renal cell carcinoma Urothelial cancer
Durvalumab (Imfinzi®)	AstraZeneca	2017	Bladder cancer Non-small cell lung cancer

1.2. Hypoxia: Hallmark of the solid tumor microenvironment

Hypoxia is characterized by low oxygen concentrations (≤2%), and is a feature often found in solid tumors. Hypoxia develops due to uncontrolled proliferation and abnormal vascularization of tumor cells, resulting in a reduced transport of oxygen and nutrients to the tumor (McDonald et al., 2016). Hypoxia is widely associated with cells proliferation, angiogenesis, metabolism and tumor immune response (Y. Li et al., 2021).

1.2.1. Role of hypoxia in tumor progression

Hypoxia is a stress condition that leads to the tumor cells readaptation. In the absence of oxygen and due to aberrant growth, tumor cells resort to aerobic glycolysis for energy production, a phenomenon known as the Warburg effect (Gatenby & Gillies, 2004). This effect is characterized by an upregulation of glucose transporters and glucose intake by cancer cells, by the conversion of glucose into lactate and reflects a metabolic program of

cancer cells driving sustained proliferation and accelerating malignant progression (Vaupel et al., 2019).

Moreover, the hypoxic malignant cells induce alterations in ECM, by the expression of the ECM-matrix remodeling enzymes, such as Lysyl Oxidase (LOX), and MMPs (Gilkes et al., 2014).

Hypoxia is associated with the activation of several signaling pathways, which lead to enhanced angiogenesis, inhibition of apoptosis, regulation of growth factors such as TGF-β and Epidermal Growth Factor (EGF), and promotion of cancer cell migration and invasion.

Interestingly, hypoxia has been also associated with chemotherapy resistance, which occurs because there is a marked distance between hypoxic tumor cells and blood vessels, which makes cells less exposed to some types of anticancer pharmacological agents (Brown & Wilson, 2004). For this reason, hypoxia is associated with a poor prognosis in most solid tumors.

1.2.2. HIFs and mechanism of hypoxia sensing

The molecular response to a hypoxic environment is mainly regulated by the action of the transcription factor Hypoxia-Inducible Factor (HIF). HIF is a heterodimeric complex, composed by an α subunit, whose stability depends on oxygen tension (with three isoforms:1α, 2α and 3α) and a β subunit which is constitutively expressed. The HIF-α isoforms have different gene targets and expression profiles, being the HIF-1α expression ubiquitous and the HIF-2α more restricted (Rosenberger et al., 2002).

Under normoxia conditions, HIF-1α undergoes hydroxylation at proline residues by Prolyl Hydroxylases (PHDs). The interaction between those hydroxylated residues with the Von Hippel-Lindau tumor-suppressor protein (pVHL) leads to the HIF-1α ubiquitination, and consequent targeting to proteasomal degradation. However, in response to hypoxia, PHDs are inactivated and are no longer able to direct HIF-1α for degradation. As a consequence, HIF1α is translocated to the nucleus where it binds to HIF-1β, inducing the activation of promoters of several target genes that contain Hypoxia Response Elements (HRE), resulting in its transcription (Figure 6) (Carnero & Lleonart, 2016).

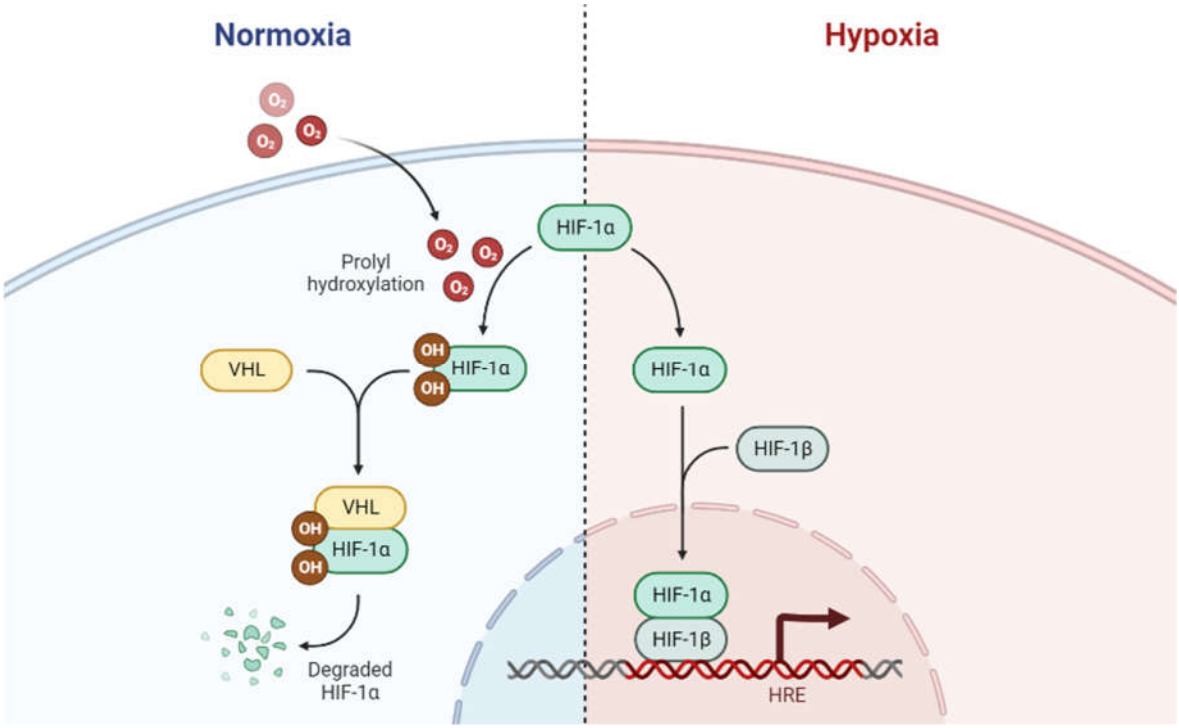


Figure 6. Mechanisms of hypoxia-induced gene expression mediated by the HIF transcription. Under normoxia conditions, PHDs hydroxylate HIF-1α, and when VHL connects to hydroxylated HIF-1α forwards it to degradation. Under hypoxia conditions, HIF-1α is not hydroxylated and is translocated to the nucleus where dimerizes with the HIF-1β subunit and induces the activation of promoters of several target genes, resulting in its transcription. Created with BioRender.com

1.2.3. Hypoxia influence on tumor microenvironment immune compartment: focus on macrophages and T cells

TAMs are the most widely studied immune population in the context of hypoxia (Noy & Pollard, 2014). As a result of the release of chemo-attractants by tumor cells, such as VEGF and endothelins, hypoxic areas of the tumor are generally enriched in macrophages (Murdoch et al., 2004). When TAMs are deprived of oxygen, they stabilize HIF, which results in an increase in VEGF transcription and secretion, thus playing a crucial role in pro-tumoral angiogenesis (Cramer et al., 2003). HIF-1 expression by TAMs can dampen adaptive immunity by suppressing T cells responses (Doedens et al., 2010).

The study of how hypoxia modulates T cells activation function and differentiation is essential to elucidate T cells behavior in the tumor microenvironment. It has been reported that active HIF-1α deficient T lymphocytes showed an increase in proliferation and secretion of pro-inflammatory cytokines, such as INF-γ (Roman et al., 2010). In addition to the direct mechanisms in T cells, HIF-1α and hypoxia play indirect roles, such as the production of cytokines and chemokines by hypoxic tumor cells, TGF-β and CCL28, favoring the entry of

Treg cells into the tumor microenvironment, promoting angiogenesis and tumor tolerance (B. Deng et al., 2013; Facciabene et al., 2011; Hasmim et al., 2013).

2. Colon Cancer

Colorectal Cancer (CRC) is the third most incident cancer type and the second with the highest mortality rate worldwide (Figure 7) (Sung et al., 2021). Despite being commonly addressed as CRC, it is acknowledged that colon and rectum cancers should be viewed as independent entities, due to their differences in presentation, disease outcome, and treatment (Hong et al., 2012). The survival and prognosis of colon cancer is largely dependent on the disease diagnostic capacity.

It is estimated that in 2020 there were more than 1.9 million new cases of CRC and 935,000 deaths. Incidence rates are 4 times higher in developed countries compared to developing countries, largely due to increased access to health units and consequently greater CRC screening. The highest incidence rates of CRC are found in Europe, Australia/New Zealand for both sexes (Sung et al., 2021). Higher intake of foods of animal origin, sedentary lifestyle, smoking and excessive alcohol consumption are factors associated with CRC risk (Siegel et al., 2020). CRC can be considered a socio-economic marker, as incidence rates tend to increase in countries with a high Human Development Index (HDI) (Fidler et al., 2016).

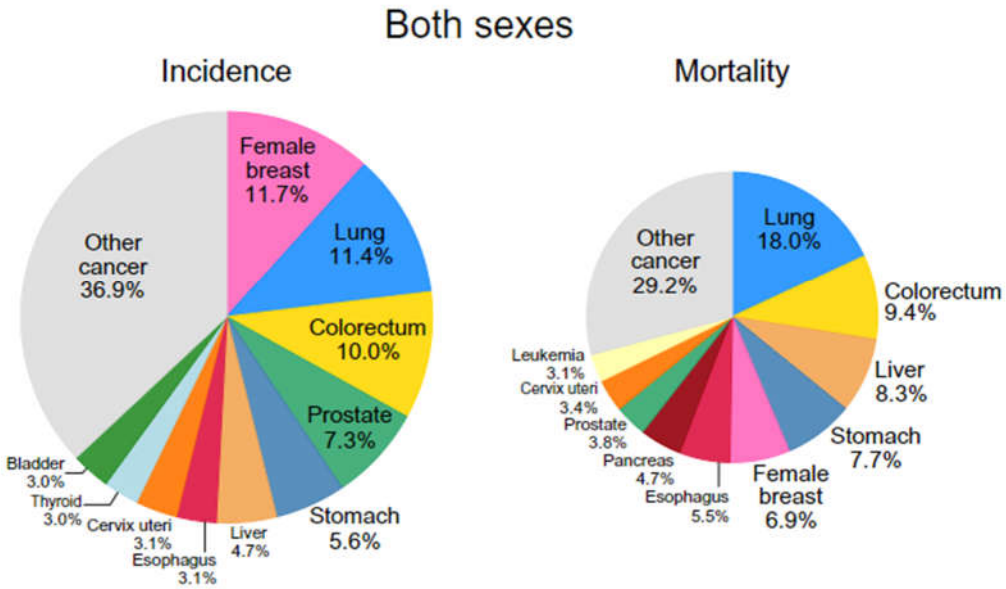


Figure 7. Distribution of cases and deaths taking into account the 10 most common cancers for both sexes. The total number of cases or deaths is reflected according to the graph area. Adapted from Sung et al., 2021.

2.1. Carcinogenesis

The gastrointestinal tract often presents adenomas, which are benign lesions, without invasive capacity, that when not removed can acquire genetic alterations leading to malignant transformation (Tannapfel et al., 2010).

Colon cancer results from the carcinogenesis process which can occur from one or a combination of three different mechanisms, such as Chromosomal Instability (CIN), Microsatellite Instability (MSI), and CpG Island Methylator Phenotype (CIMP). The CIN pathway is characterized by aneuploid tumors, where the total or partial gain or loss of chromosomes is verified. This pathway begins with the accumulation of genomic modifications in the Adenomatous Polyposis Coli (*APC*), a tumor suppressor gene, resulting in an early adenoma. The activation of oncogene *KRAS* is another alteration that may occur in the adenoma-carcinoma sequence. The stage of adenocarcinoma is considered when occurs the functional inactivation of another tumor suppressor gene, the Tumor Protein P53 (*TP53*) (Figure 8) (Armaghany et al., 2012). MSI is characterized by the presence of repetitive DNA sequences that are not present in the corresponding germline DNA, which is the result of dysfunction of proteins the mismatch repair mechanism of DNA, which leads to the accumulation of errors during replication (Nojadeh et al., 2018). On the other hand, colorectal cancer may also originate from CIMP, which is characterized by the hypermethylation of several tumor suppressors and is usually associated with proto-oncogene *BRAF* mutations and with MSI (Tariq & Ghias, 2016).

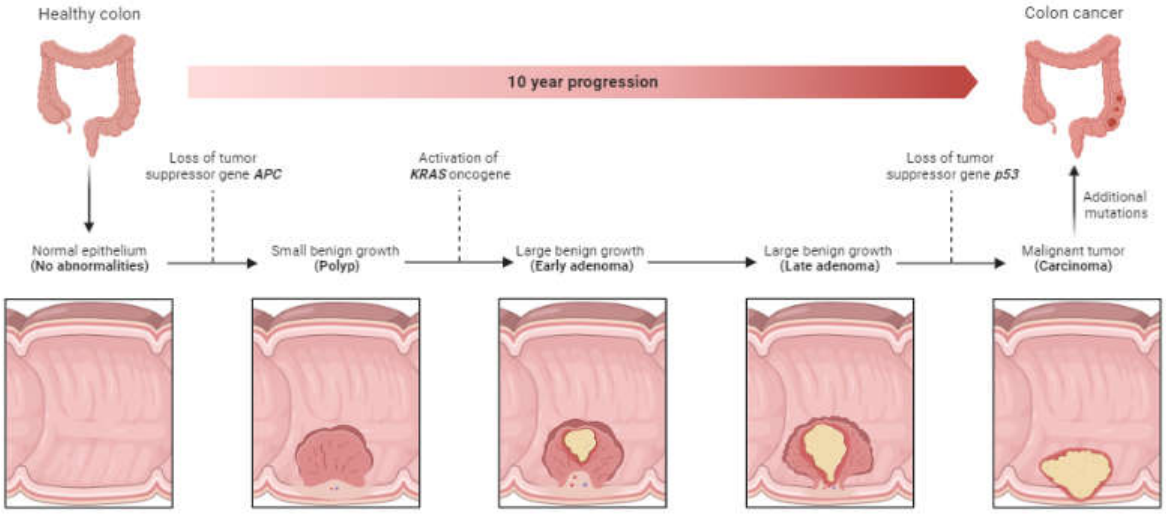


Figure 8. Carcinogenesis of colorectal cancer. Cascade of molecular alterations leading to the establishment of a colon malignant tumor. Adapted from the Campbell, Biology: A Global Approach, 2018.

2.2. Colon cancer subtypes

CRC staging is made according to the Tumor/Node/Metastases (TNM) system specified by The American Joint Committee on Cancer (AJCC). Colon cancer can be divided into different stages depending on whether it is a tumor restricted to the primary site, there is regional lymph node infiltration or distant metastasis. In addition to these three classifications, information on tumor invasion, the number of affected lymph nodes, and the organs affected by metastases can be evaluated. All these data are important for classifying the stage of colon cancer (NCCN Clinical Practice Guidelines in Oncology: Colon Cancer).

More recently, and taking advantage of the data generated from The Cancer Genome Atlas (TCGA) project, a new classification system was developed, Consensus Molecular Subtype (CMS) that allows the categorization of most tumors into one of four robust subtypes: CMS1: MSI-immune, CMS2: Canonical, CMS3: Metabolic, and CMS4: Mesenchymal, based on molecular and physical features, that includes primary tumor location, MSI, CIMP, CIN, Somatic Copy Number Alterations (SCNA), *BRAF, KRAS* and *APC* mutations, WNT/MYC and TGF-β pathway activation, and immune infiltration statues (Figure 9) (Guinney et al., 2015; Inamura, 2018).

The advance in understanding the molecular characteristics of a given CRC subtype can represent new therapeutic targets, allowing the deployment of personalized therapies and better patient management.

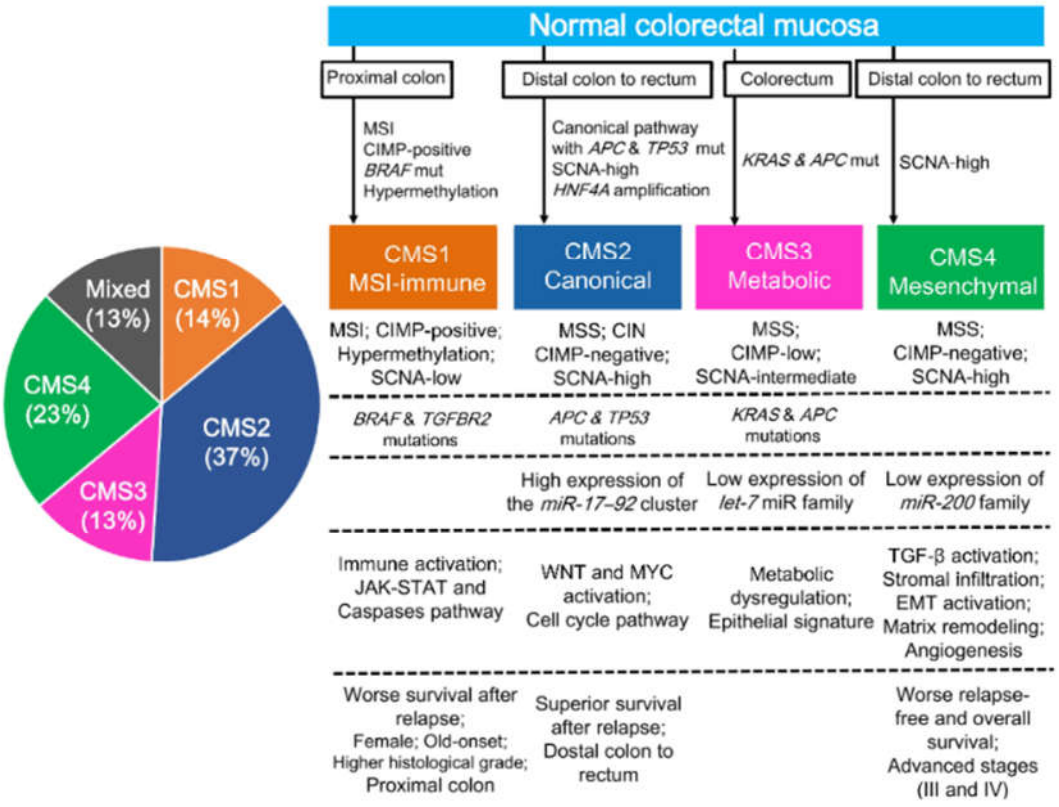


Figure 9. The taxonomy of colorectal cancer according to the Consensus Molecular Subtype. The CMS is a recently developed classification system that allows the categorization of most tumors into four distinct subtypes: CMS1 characterized by being the only group that encompasses MSI; CMS2 exhibits a Microsatellite Stable (MSS) phenotype and the attraction of the WNT-MYC pathway; CMS3 corresponds to approximately 13%, presents epithelial signature and metabolic dysregulation; CMS4 characterized by stromal activation and Epithelial-Mesenchymal Transition (EMT). Adapted from Inamura, 2018.

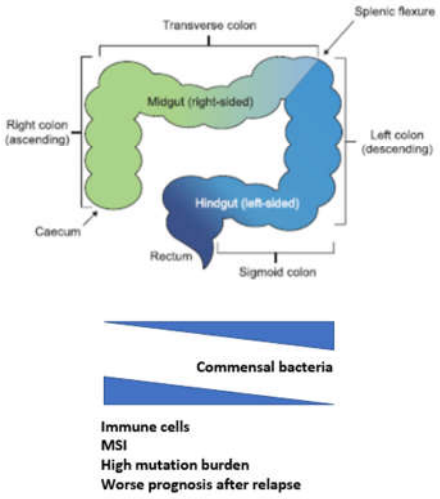
2.3. Left and Right colon tumors

Tumors that arise from different regions of the colon are molecular and clinically distinct, suggesting that carcinomas of the right and left side should be considered as distinct entities. Right Colon is derived from the embryologic midgut, and includes caecum, ascending colon, hepatic flexure, and two-thirds of the transverse colon. Left colon is derived from the embryologic hindgut, and includes the distal third of the transverse colon, splenic flexure, descending colon, and sigmoid colon (Figure 10) (Baek, 2019).

From a molecular point of view, Right-sided Colon Carcinomas (RCC) are associated with Lynch syndrome, also known as Hereditary Non-Polyposis Colorectal Cancer (HNPCC) is a type of inherited cancer syndrome associated with a genetic predisposition to different cancer types, and generally dependent on Mitogen-Activated Protein Kinase (MAPK) and Phosphoinositide 3-Kinase (PI3K) signaling, show a higher prevalence of MSI, CpG island methylation, and mutations of *KRAS* and *BRAF.* Left-sided Colon Carcinomas (LCC) are dependent on Receptor Tyrosine Kinase (RTK) activity, such as Epidermal Growth Factor Receptor (EGFR) signaling. The mutation burden is lower as compared to RCC, with the more frequent gene mutations being those involving *APC*, *TP53* and *NRAS* genes (Figure 10) (Baek, 2019; Shimada et al., 2017).

The microbiome is believed to play an important role in the formation of CRC, and RCCs have a relatively higher abundance of *Prevotella*, *Pyramido*-bacterium, *Selenomonas* and *Peptostreptococcus* than LCC, which have a higher prevalence of *Fusobacterium*, *Escherichia*, *Shigella* and *Leptotrichia*. Additionally, a higher incidence of *Escherichia coli* phylogroup B2 was found in patients with RCC and a higher risk of *Helicobacter pylori* infection in patients with LCC (Figure 10) (Gao et al., 2015; Kohoutova et al., 2014).

The clinical presentation also differs, with iron deficiency anemia from occult blood loss being more prevalent in patients with RCC and, conversely, hematochezia and change in bowel habits being more common as presenting symptoms for LCC. RCC is diagnosed more frequently in women, older ages and usually when diagnosed the tumor is in a more advanced stage, in relation to LCC, which is more frequent in men, with younger ages and when diagnosed the tumors are smaller and are in a less advanced stage (K. Kim et al., 2018).



Microbiome differences between RCC and LCC.

RCC	LCC
• Higher abundance of *Prevotella, Pyramido-bacterium, Selenomonas* and *Peptostreptococcus* • Higher incidence of *Escherichia coli* phylogroup B2	• Higher prevalence of *Fusobacterium, Escherichia-Shigella* and *Leptotrichia* • Higher risk of *Helicobacter pylori* infection

LCC, left-sided colon carcinoma; RCC, right-sided colon carcinoma.

Chromosomal and molecular characteristics of RCC and LCC.

RCC	LCC
• High MSI and CIMP • Hypermutation state • *KRAS* mutations • *BRAF* mutations • *TGFβR2* mutations • *PI3KCA* mutations	• Chromosomal aberrations ◦ Deletion of 8p, 17p (including *TP53*), 18p (including *SMAD4*), gain of chromosome 7, 8q (including *MYC*), 20q, loss of 18q • Aneuploidy • Frequently mutated genes: ◦ *TP53* ◦ *APC* ◦ *KRAS* • Overexpression: ◦ EGFR and HER2 gain ◦ High EGFR ligand expression (EREG and AREG expression) ◦ High VEGF-1 mRNA expression ◦ COX-2
• Distribution of CMS subtypes ◦ CMS1 – 31% ◦ CMS2 – 26% ◦ CMS3 – 19% ◦ CMS4 – 24%	• Distribution of CMS subtypes ◦ CMS1 – 7% ◦ CMS2 – 56% ◦ CMS3 – 10% ◦ CMS4 – 27%

AREG, amphiregulin; CIMP, CpG island methylator phenotype; CMS, consensus molecular subtype; EGFR, epidermal growth factor receptor; EREG, epiregulin; LCC, left-sided colon carcinoma; MSI, microsatellite instability; RCC, right-sided colon carcinoma; VEGF-1, vascular endothelial growth factor 1.

Figure 10. Right and Left-sided colon carcinomas differences. Right and left-sided colon carcinomas present several differences at level of microbiome, chromosomal and molecular characteristics and in the CMS-type. Adapted from Stintzing et al., 2017.

2.4. Diagnosis and therapeutics of Colon cancer

CCR is considered a slowly progressing cancer, taking an average of 7 to 10 years for its clinical appearance, which allows its early detection. There are currently several screening methods available, such as digital rectal examination, colonoscopy, and fecal occult blood testing. However, when diagnosed, about 20% of patients are already in a metastatic state, being considered incurable at this stage. This is the main reason for the extreme importance of early diagnosis and the development of new, more efficient, and less troublesome therapeutic approaches for the detection and treatment of CRC (Simon, 2016).

The choice of treatment for CRC will depend on the patient's health, the size of the tumor, and its location. Usually, surgery is the primary treatment for individuals, however,

other treatments can be applied such as radiofrequency ablation, cryosurgery, targeted therapy, and traditional chemo- and radiation therapy (Yoshihara et al., 2007).

Regarding immunotherapy in colon cancer, therapy targeting PD-1 is only advantageous for the treatment of some tumors that are mismatch repair deficient or MSI high (Stein & Folprecht, 2018). The good response in these patients was associated with a higher tumor mutational rate, a potential source of tumor-specific neoantigens, and increased infiltrating cytotoxic T cells (Castle et al., 2019). However, there is still a high degree of non-responders among MSI colon cancer patients, and immunotherapy is not an option of MSS tumors (Ganesh et al., 2019). This led to the need for new combinatorial targets. Progress led to the approval by the Food and Drug Administration (FDA) of combinatorial therapies for patients with Mismatch Repair deficient and Microsatellite Instability high (dMMR–MSI-H) tumors, such as nivolumab and ipilimumab (Table 1) (Ganesh et al., 2019).

Noteworthy, it was reported that for the promotion of T cell infiltration, neoantigens formation is not enough, and it is also required the activation of specific signaling pathways, emphasizing the need of further assessment of TME molecular players that account for an effective anti-tumor immune response (Lu et al., 2021).

2.5. Hypoxia and colon cancer

It is known that in CRC, hypoxia, through HIF and its ability to bind the HRE promoter regions of several genes, regulates various cellular activities associated with hallmarks of cancer, such as angiogenesis, survival and proliferation (Cao et al., 2009; To et al., 2014b; Wincewicz et al., 2010).

Regarding angiogenesis, VEGF, a major driver of angiogenesis in CRC, is also transcriptionally controlled by HIF-1α under hypoxia, and HIF-1α is positively correlated with VEGF expression in CRC (Cao et al., 2009). Furthermore, in CRC the expression of both HIF-1α and VEGF is significantly associated with tumor stage and metastasis (Cao et al., 2009).

In CRC, hypoxia is also associated with survival, since it is described that β-catenin can interact with HIF-1α, enhancing its transcriptional activity, and promoting the survival of CRC cells under hypoxic conditions (Wincewicz et al., 2010). As β-catenin interacts with HIF-1α, it interacts with the ligand binding domain of Nur77, an orphan receptor that belongs to the NR4A subgroup of the nuclear receptor superfamily (H. Z. Chen et al., 2012). Nur77 drives a positive feedback loop to enhance β-catenin signaling through HIF-1α, a novel

mechanism for survival and aggressive behavior of CRC under hypoxia. Moreover, the overexpression of Nur77 or β-catenin leads to increased expression of the mesenchymal markers N-cadherin and MMP2, which contributes to tumor invasion (To et al., 2014a).

In respect of excessive cell proliferation, it is known that colon cancer cells under hypoxic conditions can promote self-growth through the secretion of exosomes, which shortens the duration of mitosis and activates STAT3, contributing to the proliferation of colon cancer cell lines (Ren et al., 2019).

Recent studies explored the use of an hypoxic signature, based on the behavior of cancer cells of different tumor types under hypoxia, showing that hypoxia is associated with poor prognosis in colorectal cancer (Qi et al., 2020; Q. Zhang et al., 2020). However, none of these reports had in consideration neither the specificities of colon cancer nor its immune compartment, which would be important giving the results of a study that evidence that only immune-cold colorectal tumors are simultaneously associated with poor prognosis and hypoxia (Craig et al., 2020).

3. Aims of this thesis

Currently it is known that macrophages and T cells at the tumor microenvironment impact on various cellular activities associated with cancer. However, most of the studies were established under normoxia conditions, completely discarding one of the main characteristics of the microenvironment of solid tumors: hypoxia.

Previous studies of our group aimed to understand the impact of hypoxia on the crosstalk between macrophages and cancer cells had shown that hypoxia can be both associated with pro-tumor cellular activities, as increased glucose consumption and increased invasion. However, hypoxia also associates with anti-tumor cellular features, as the macrophages presenting a more pro-inflammatory phenotype, with decreased expression of anti-inflammatory cytokines such as IL-10 and TGF-β, and the increase of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α. Moreover, it was also found that when comparing *CD68*^{High}*HIF1A*^{High} and *CD68*^{High}*HIF1A*^{Low} colon cancer tumors, the *CD68*^{High}*HIF1A*^{High} tumors presented better survival, along with increased markers of cytotoxic T cells, and this result was particularly evident in the right colon tumors.

Considering the relevance of hypoxia in the tumor microenvironment, the main aim of this thesis was to understand how macrophages, T cells and cancer cells connect to each other and how they respond to hypoxia, and which is the impact of that interaction on the survival of colon cancer patients. To achieve this aim, three specific tasks were defined: 1)

unravel which are the molecular players involved in the process; 2) evaluate the immune checkpoint expression profile between the groups of cancer patients in which differences in survival were found, and 3) assess whether the molecular players validated in task 1 may function as regulators of the most relevant immune checkpoints found in task 2.

To accomplish this aim, different approaches were used: bioinformatic analysis, and molecular and cellular biology techniques such as monocyte and T cells isolations from blood and establishment of primary cultures, cancer cell culture, western blot and flow cytometry analysis, and siRNA optimization and treatment.

Overall, with this project we aim at understanding how hypoxia affects the survival of colon cancer patients and what are the underlying molecular mechanisms, aiming to contribute to the improvement of therapeutic approaches for colon cancer patients.

Materials and Methods

Bioinformatic Analysis

The Cancer Genome Atlas, (TCGA; https://cancergenome.nih.gov/) dataset of colon carcinomas, COAD, was analyzed. Median values were calculated in normal tissue and used as threshold to define “high” and “low” expression levels in cancer patients. The expression of genes was quantified as FPKM (fragments per kilobase million), which are provided by TCGA consortium. Clinical metadata were also provided by the TCGA consortium at https://gdc.cancer.gov/.

Cancer cell culture

SW48, RKO, HCT-15, Caco-2, HT-29, and SW480 colon cell lines were purchased from the American Type Culture Collection (ATCC). Cells were maintained in a humidified atmosphere with 5% CO_2 at 37°C. RKO, HCT-15, HT-29, and SW480 were maintained in RPMI 1640 medium with GlutaMax (Invitrogen), Caco-2 and SW48 were maintained in DMEM medium with GlutaMax (Invitrogen), all cell lines with medium supplemented with 10% Fetal Bovine Serum (FBS, Biowest) and 100 Penicillin UmL-1 and 100 µgmL-1 streptomycin (Invitrogen) (considered complete medium).

Ethics Statement

The buffy coats used for monocyte and T cell isolation are highly leukocyte-enriched waste-products that results from a whole blood donation of healthy blood donors. The isolation of immune cells from healthy blood donors was approved by the Centro Hospitalar Universitário São João Ethics Committee (protocol 90/19) after collecting each donor’s informed consent.

Human T cells and monocytes isolation and macrophage differentiation

Monocytes and T cells were isolated from buffy coats of healthy blood donors, provided by Centro Hospitalar Universitário São João. The blood was centrifuged at 1200g for 20 minutes at Room Temperature (RT), after which the blood was separated into three distinct layers. To isolate monocytes and T cells, the whitish layer that contains Peripheral Blood Mononuclear Cells (PBMCs) was collected, divided, and incubated for 20 minutes

under rotation with RosetteSep Human Monocyte Enrichment Cocktail (StemCell Technologies) or RosetteSep Human T Cell Enrichment Cocktail (StemCell Technologies) This mixture was then diluted (1:1) with Phosphate-Buffered Saline (PBS) and 2% FBS (Biowest), added cautiously over Ficol-Histopaque 1077 (Sigma) and centrifuged at 1200 g (brake off) for 20 minutes at RT. A new gradient is formed, and the intermediate layer was collected, and washed in PBS three times, by centrifugating at 300g for 10 minutes, and once at 120g for 10 minutes. Both monocytes and T cells were resuspended in complete RPMI medium and counted. After, T cells were placed in freezing medium (90% FBS (Biowest) and 10% dimethyl sulfoxide (DMSO, Sigma), and stored at -80ºC. Monocytes were plated at a density of $0.5x10^6$ cells per well, in 6-well culture plates with glass coverslips, in complete RPMI medium supplemented with M-CSF (ImmunoTools, 50 ngmL-1). Macrophages were differentiated for 7 days. At day 7, medium was replaced by complete medium without M-CSF and tricultures were performed. Cells were maintained at 37 °C and 5% CO_2 humidified atmosphere.

Establishment of macrophages, T cells, and cancer cells triculture

Seven days after monocyte and T cell isolation, and after the replacement of the culture medium by medium without M-CSF, $1x10^6$ cancer cells were added on top of macrophage cultures for 3 hours under normoxia (20% O_2) or hypoxia (1% O_2). After that time, T cells were thawed and centrifuged at 1200 rpm for 5 minutes, counted and added to the culture. $0.5x10^6$ T cells were added to the macrophages and cancer cells, as tricultures. Tricultures were maintained in complete RPMI medium for 24 hours in conditions of normoxia (20% O_2) or hypoxia (1% O_2), after which biological material was collected (Figure 11).

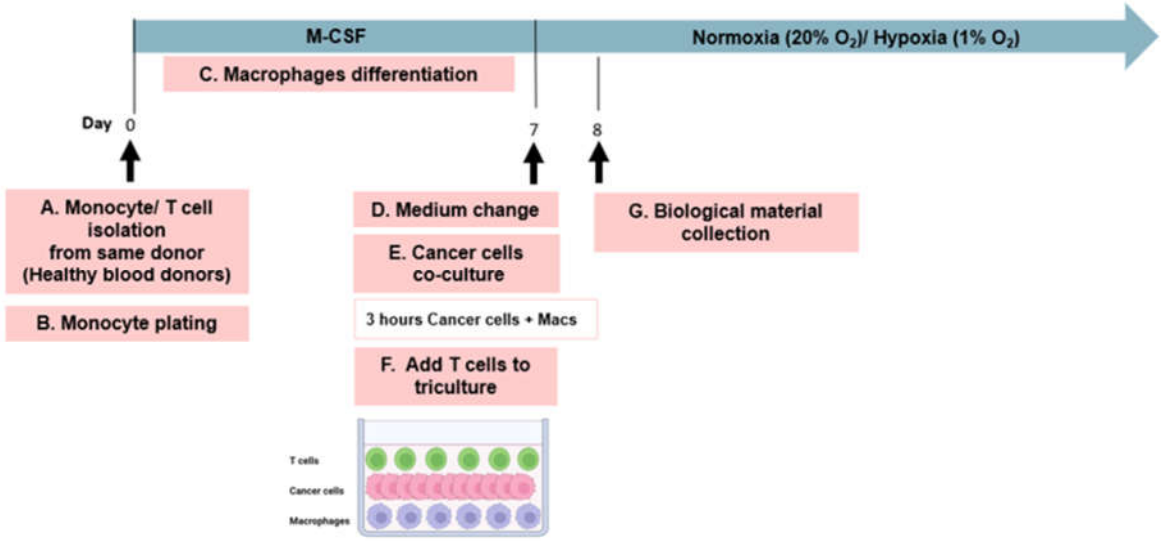


Figure 11. Schematic overview of the methodology used in this work. Monocytes and T cells were isolated from blood from healthy donors provided by Centro Hospitalar Universitário São João. $0.5x10^6$ monocytes were plated with complete RPMI medium and M-CSF and kept for 7 days in a humidified atmosphere with 5% CO_2 at 37°C. After 7 days, the macrophage medium was renewed without M-CSF, and tumor cells ($1x10^6$ cells) were added to the culture. 3 hours later T cells ($0.5x10^6$ cells) were added to the triculture. The tricultures are maintained for 24 hours under normoxia (20% O_2) or hypoxia (1% O_2) conditions, and after biological materials were collected.

Sample preparation for Western Blot

Cell lysates were prepared to perform Western Blot. T cells, in suspension, were collected of the triculture medium by centrifugation at 1200 rpm for 5 minutes, while macrophages and cancer cells were detached from the plates using accutase (BD Bioscience), incubated for 30 minutes in the incubator. Cells were carefully detached with the aid of a scrapper, and added to the T cells previously collected, and centrifuged at 1200 rpm for 5 minutes at RT. After centrifugation, supernatants were discarded, and cold Rippa lysis buffer [50 mM Tris HCl pH=7.5; 1% NonidetP (NP)-40; 150 mM NaCl and 2mM EDTA] with proteases/phosphatases inhibitors [10 mM NaF; 200mM Phenylmethylsulfonyl Fluoride (PMSF); 1 $mgmL^{-1}$ aprotinin and leupeptin; 50mM Na_4VO_3 and 50 $mgmL^{-1}$ $Na_4P_2O_7$] were added to the pellets. The mixture of cells with lysis buffer was placed on ice for 30 minutes and centrifuged again at 13300 rpm for 10 minutes at 4ºC, and supernatants were collected.

Protein concentration was determined using the DCProtein assay kit (BioRad) and 20µg were mixed with Laemmli sample buffer [0.5 M Tris-HCl pH 6.8, 9.2g SDS, 40mL Glycerol, 5% β-mercaptoethanol, 5% bromophenol blue] and boiled for 5 minutes at 95ºC for protein denaturation.

Western Blot

Samples were loaded on a 10% or 15% SDS-polyacrylamide gel and run at 100V. Following electrophoresis, gels were transferred to nitrocellulose membranes (GE Healthcare) for 2 hours at 100V. Membranes were then stained with Ponceau (Sigma) solution until bands of protein were visualized. Subsequently membranes were blocked with 5% non-fat powder milk in TBS + 0.1% Tween-20 (TBS-T 0.1%) for 30 minutes and incubated with primary antibodies (Table 2) at 4ºC Overnight (ON). Membranes were then washed with TBS-T 0.1%, and incubated with the respective secondary antibody (Table 3) for 45 minutes at RT. After final washing, membranes were incubated with Clarity Western ECL Substrate (BioRad) for signal detection.

Table 2. Primary antibody specifications for Western Blot.

Target molecule	Serum	MW (KDa)	Dilutions	Supplier	Incubation conditions
Primary antibodies					
Caveolin-1	Rabbit	21-24	1:1000	Sigma	4ºC ON
CXCR4	Rabbit	45-60	1:500	Abcam	
Hsc-70	Mouse	70	1:1000	Santa Cruz	
Integrin α5	Rabbit	150	1:1000	BD	
PFKFB3	Rabbit	60	1:1000	Cell Signalling	

Table 3. Secondary antibody specifications for Western Blot.

Target molecule	Dilutions	Supplier	Incubation conditions
Secondary antibodies			
Mouse	1:1000	GE Healthcare	45 minutes RT
Rabbit	1:1000	GE Healthcare	45 minutes RT

Transfection with siRNA

For siRNA transfection, RKO and SW480 cells seeded in 6-well plates at 50% confluence were washed with PBS and incubated in serum and antibiotic-free medium. siRNA targeting *ITGA5* and Non-silencing siRNA were obtained from Qiagen and prepared according to the manufacture instructions. Transient transfection was performed using Lipofectamine RNAiMAX (Invitrogen) as transfection reagent. After optimization, siRNA was used at a concentration of 50 nM. The efficiency of siRNA was evaluated by western blot.

Flow cytometry

Tricultures were collected as for the preparation of cell lysates, T cells by centrifugation and macrophages and cancer cells by accutase detachment, but resuspended in flow cytometry buffer (PBS, 2%FBS, 0.01% sodium azide). Staining was performed with antibodies, represented in Table 4, for 40 minutes at 4ºC in the dark.

Unstained cells and single stained with antibodies were used as a control. Then, cells were incubated with Live Dead, for 30 minutes at 4ºC in the dark. This step labels the cells that are dead or the cells with unviable membrane. Next to washing steps with buffer, cells were fixed for 15 minutes in 4% of Paraformaldehyde (PFA) at RT. Samples were filtered and analyzed in a FACS Canto Flow Cytometer (BD Biosciences) using FACS Diva Software. Analysis of the data obtained was performed with FlowJo software.

Table 4. Antibody specifications for Cytometer.

Antibodies				
Target molecule	**Fluorochrome/Visible colors**	**Clone**	**Supplier**	**Dilution**
CD3	FITC	HIT3b	Immunotools	1:25
CD3	PERCP	UCHT-1	Immunotools	1:25
CD4	Pacific Blue	RPA-T4	Biolegend	1:50
CD8	PERCP	HIT8a	Immunotoos	1:25
CD14	Pacific Blue	63D3	Biolegend	1:50
CD14	PERCP	18D11	Immunotools	1:25
CD40	PE	HI40a	Immunotools	1:25
CD69	APC	FN50	Immunotools	1:25
CD80	APC	MEM-233	Immunotools	1:25
CD86	FITC	BU63	Immunotools	1:25
CD89	APC	A59	Biolegend	1:100
Tim-3	PE	F38-2E2	Biolegend	1:100
Live Dead	APC-Cy-7		eBiosciences	1:10000

Statistical analysis

Data were expressed as the mean with Standard Deviation (SD) and collected from at least three independent experiments with immune cells from different donors and cancer cells with three different passages. The normality of the distribution was evaluated using the Kolmogorov–Smirnov test. For comparison between two independent groups, t-test (Wilcoxon, and Mann–Whitney) was used. For comparison among four independent groups, one-way ANOVA (RM one-way ANOVA, Friedman, and Kruskal–Wallis), with Dunn's multiple comparison was used. Correlations were tested with the Spearman test and

considered positive when Spearman $R \geq 0.500$. Survival curves were analyzed with the log-rank (Mantel–Cox) test. Statistical tests were performed in GraphPad Prism 8. Differences were considered significant at $p < 0.05$.

Results

The work that was performed during this thesis was part of an ongoing work of the Tumour and Microenvironment Interactions Group, at i3S, in which it is intended to study the hypoxia role in the modulation of the crosstalk between colon cancer cells and immune cells usually present at the tumor microenvironment. This study is still a work in progress. As such, this section of the results is presented in four different parts in which in the first part are presented the preliminary results of the group that led to the experiments performed during the thesis practical work.

(I) Preliminary results

Hypoxia is associated with pro-tumor cellular features

When studied the hypoxia impact on colon cancer cell-macrophage crosstalk, our group had previously showed that hypoxia is able to positively regulate pro-tumor cellular features. These include increased glucose consumption and expression of the genes that codify to glucose transporter and for Lactate Dehydrogenase A (*LDHA)*, an enzyme involved in lactate production, all associated cellular metabolism favoring glycolysis (Figure 12A). Moreover, it was observed that macrophages and hypoxia alone were sufficient to promote cancer cell invasion and, most importantly, this is potentiated when both factors are combined (Figure 12B) (Martins et al., 2020).

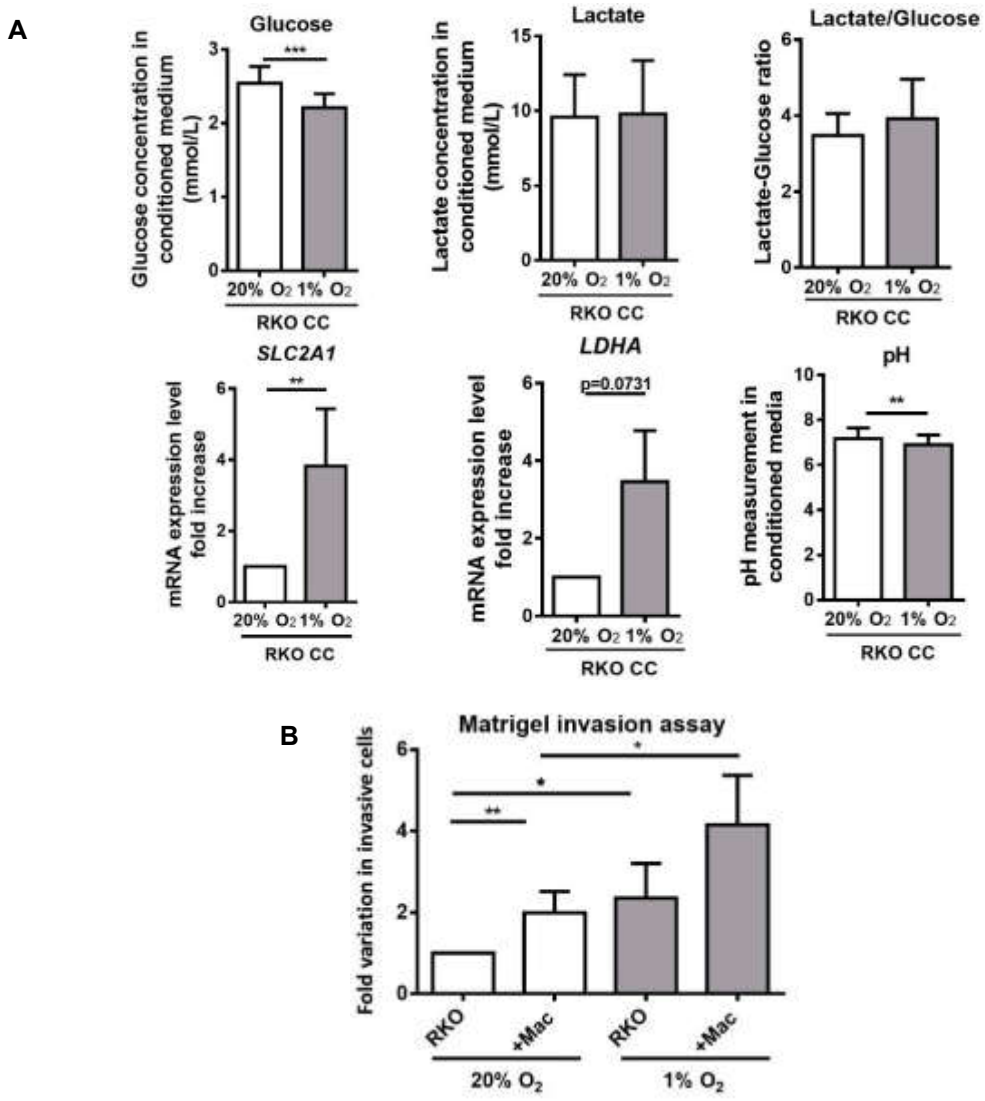


Figure 12. Hypoxia impacts colon cancer cell behavior, increasing invasion and glucose consumption. (A) Indirect co-cultures with macrophages and RKO were established and maintained for 72h at 20% O_2 or 1% O_2 and glucose consumption, lactate production, and pH was analyzed. qRT-PCR was used to measure *SLC2A1* and *LDHA* mRNA levels in tumor cells. (B) Cells maintained at 20% or 1% O_2 and stimulated or not with macrophages previously maintained at 20% or 1% O_2 were cultured for 24 h on Matrigel-coated filters, at 20% or 1% O_2. Data are presented as fold variation in the number of invasive cells relative to RKO cells cultured at 20% O_2 without any stimulation with macrophages. The statistical tests Friedman, RM one-way ANOVA, Wilcox or paired t-test were used *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$. The p-values between 0.05 and 0.1 were presented and considered a tendency. Adapted from F. Martins et al., 2020.

Hypoxia is associated with anti-tumor features

Interestingly, it was observed that hypoxia modulates macrophage-colon cancer interaction in a way that favors anti-tumor cellular activities. These includes increased macrophagic phagocytic activity (Figure 13A), and macrophage polarization into a more pro-inflammatory phenotype (Figure 13B). Moreover, colon cancer patients with high

macrophage infiltration, and high *HIF1A* expression present, as well higher levels of expression of pro-inflammatory cytokines (Figure 13B and 14A), and markers of CD8 cytotoxic T cells (Figure 14B) (Martins et al., 2020).

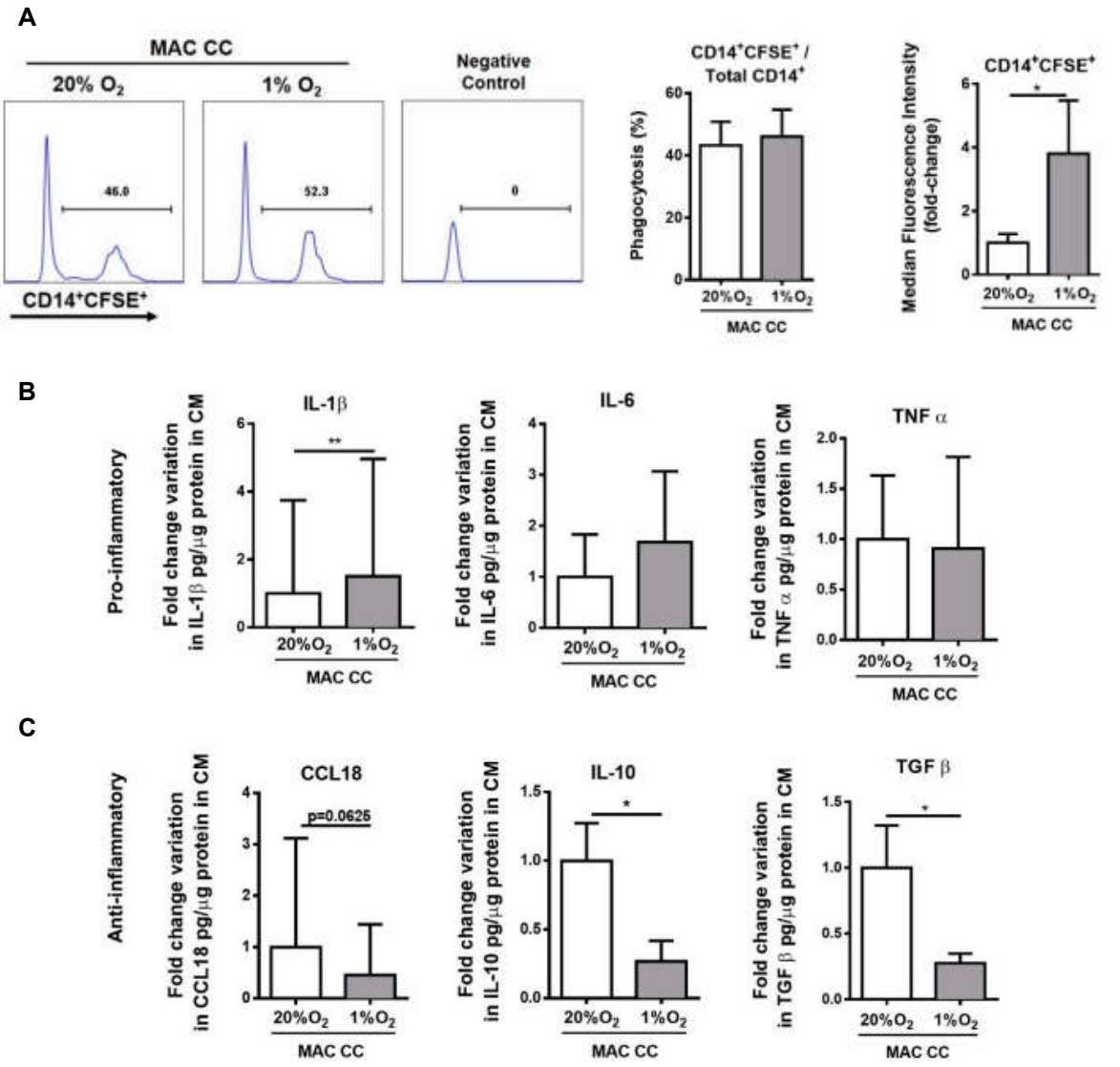


Figure 13. Hypoxia modulates phagocytic activity of macrophages and promotes the decrease of anti-inflammatory cytokines and increases IL-1β secretion. (A) Macrophages were maintained in indirect co-culture with tumor cells (RKO), 20% O_2 and 1%O_2 for 72h, after these times as tumor cells were removed and were added directly to macrophages RKO cells marked with Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE) for 2h. Subsequently, macrophages were analyzed by flow cytometry. Histograms show the gating strategy created with FlowJo and Phagocytic activity was calculated as the ratio of CD14+CFSE+ cells/Total CD14+ cells. (B) Concentration of secreted molecules was measured by ELISA and normalized to conditioned media (CM) protein levels. Graphs represent cytokine concentration of pro- and anti-inflammatory markers. The statistical tests Wilcox or paired t-test were used **** $p < 0.0001$; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$. The p-values between 0.05 and 0.1 were presented and considered a tendency. Adapted from F. Martins *et al.*, 2020.

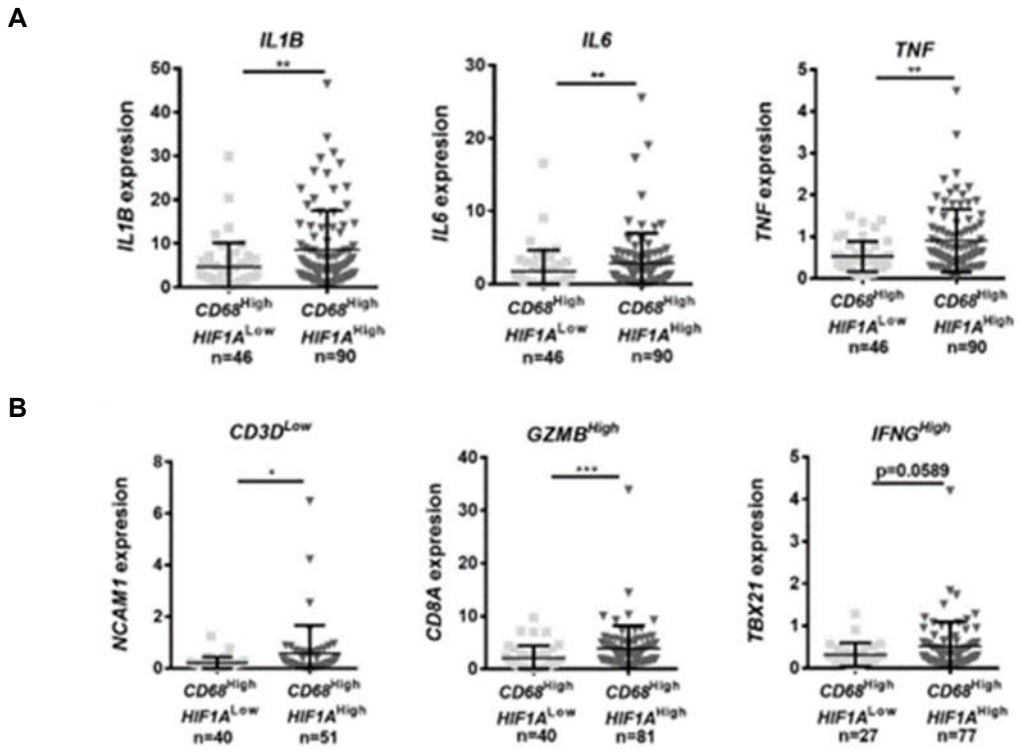


***Figure 14. CD68^{High}HIF1A^{High}* tumors show increased expression of pro-inflammatory cytokines and increased infiltration of cytotoxic T cells.** These data were obtained using TCGA. (A) The expression of *IL1B*, *IL6* and *TNF* was increased in the *CD68*^{High}*HIF1A*^{High} tumors compared to the *CD68*^{High}*HIF1A*^{Low} tumors. (B) The expression of *NCAM1*, *CD8A* and *TBX21* were analyzed in the *CD68*^{High} tumors between the *HIF1A*^{High} and *HIF1A*^{Low} tumors, and there was a significant increase in the expression of *NCAM1 (*within the *CD3D*^{Low}*)* and *CD8A (*within the *GZMB*^{High} population*)*. **** p < 0.0001; *** p < 0.001; ** p < 0.01; * p < 0.05. The p-values between 0.05 and 0.1 were presented and considered a tendency. Adapted from F. Martins et al., 2020.

Colon cancer patients *CD68*^{High}*HIF1A*^{High} presents better prognosis

Interestingly, when the survival curves of *CD68*^{High}*HIF1A*^{Low} and *CD68*^{High}*HIF1A*^{High} colon cancer patients were analyzed, it was found that the tumors *CD68*^{High}*HIF1A*^{High} present better prognosis (Figure 15A). When the patients were separated by the tumor location between left and right colon cancer patients, that tendency was only observed, and in a more evident way, in the case of right colon cancer patients (Figure 15B).

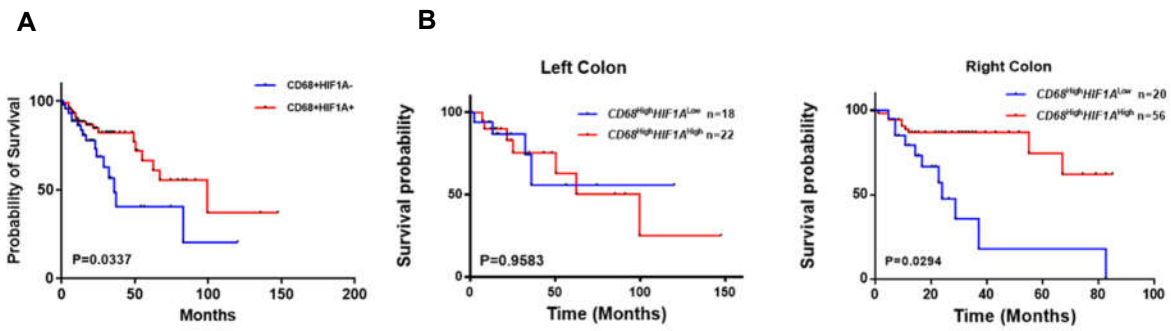


Figure 15. *CD68*^{High}*HIF1A*^{High} tumors presents better prognosis. RNASeq expression data were downloaded from The Cancer Genome Atlas (TCGA). Kaplan-Meier curves were made with patient's survival data, comparing the populations *CD68*^{High}*HIF1A*^{High} and *CD68*^{High}*HIF1A*^{Low} using the total number of tumors (A) or dividing between left and right colon (B). Is considered right colon the cecum, the ascending colon and the hepatic flexure, while it is considered left colon the splenic flexure, descending colon and the sigmoid colon. Transverse colon and rectum were not considered in the analysis. Division High/Low expression was made according to the median expression of the selected genes in normal tissue. Long-rank (Mantel Cox) test was performed in the analysis of the survival curves.

These results showed the importance of considering hypoxia as an important factor in the modulation of the tumor microenvironment. For this reason, it became necessary to understand which molecular players are involved in the response to hypoxia regarding the crosstalk between cancer and immune cells. To shed light on this issue, it was performed triple tricultures of 6 colon cancer cell lines with macrophages, the most abundant immune cells at the colon tumor microenvironment, and T cells. The differences between tricultures under normoxia and hypoxia at the RNA level were addressed using the Nanostring nCounter Tumor Signaling 360 Gene Expression Panel. Analysis revealed substantial gene expression alterations under hypoxia (92 genes up and 34 downregulated) (Figure 16).

Genes presenting the most significant differences between normoxia and hypoxia were chosen for assessment of clinical relevance in colon cancer patients, using TCGA. For further studies the chosen genes presented at the same time, 1) differences in *CD68*^{High}*HIF1A*^{High} and *CD68*^{High}*HIF1A*^{Low} tumors expression; 2) differences in the right colon in *CD68*^{High}*HIF1A*^{High} and *CD68*^{High}*HIF1A*^{Low} tumors expression 3) correlation with *HIF1A* (Spearman R≥0,5). After applying the referred constraints our data revealed 5 upregulated genes: *CAV1*, *CXCR4*, *FCAR*, *ITGA5* and *PFKFB3* (Figure 17).

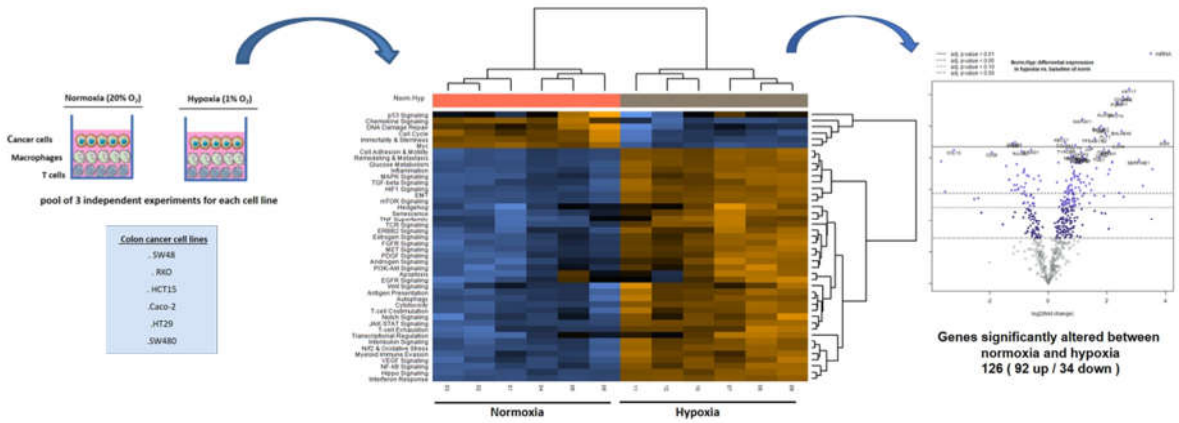


Figure 16. Hypoxia induces a new expression profile in tricultures of colon cancer cells, macrophages and T cells. Macrophages, cancer cells and T cells were directly tricultured at 20% or 1% O_2 for 24h. The differences between tricultures under normoxia and hypoxia at the RNA level were addressed using the Nanostring nCounter Tumor Signaling 360 Gene Expression Panel. Analysis revealed substantial gene expression alterations under hypoxia (92 genes up and 34 downregulated).

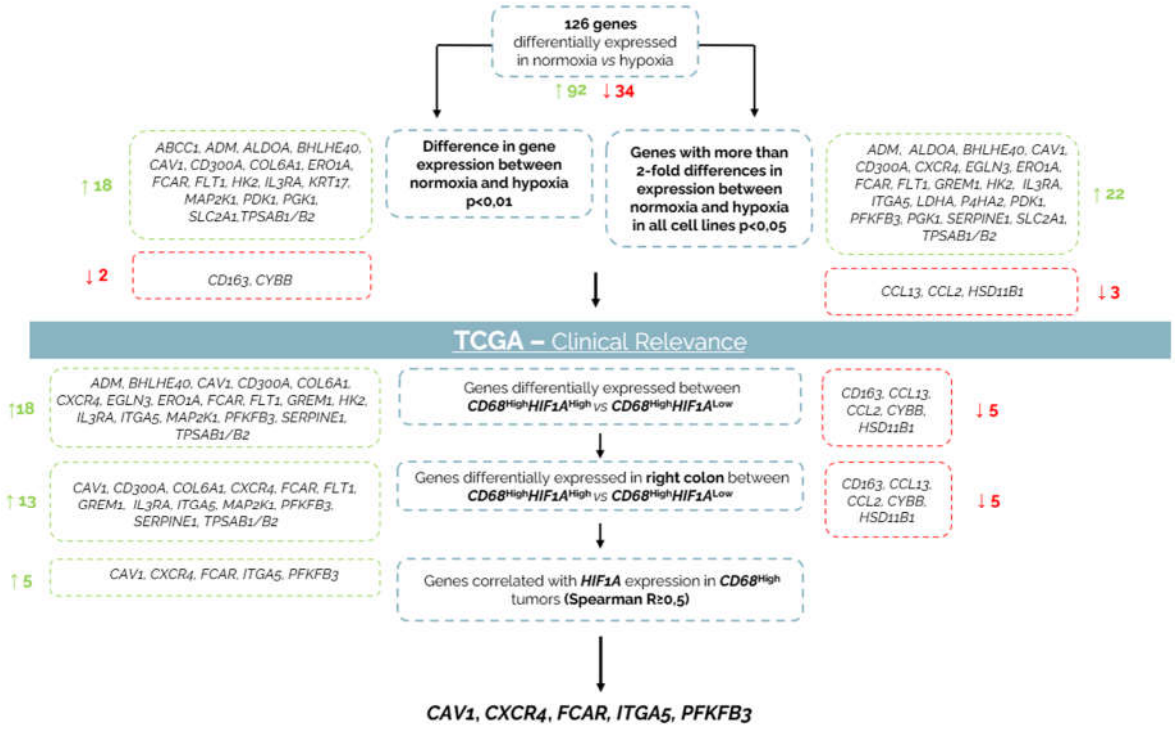


Figure 17. Choice and clinical validation of the most interesting altered genes. Nanostring analysis resulted in 126 differentially expressed genes between normoxia and hypoxia, of which those significantly different in gene expression between normoxia and hypoxia (p-value < 0.01) and genes with more than 2-fold differences in expression between normoxia and hypoxia in all cell lines (p-value < 0.05) were considered relevant for the next phase of the study, which consisted in seeing the clinical relevance of these data. Using data from colon cancer patients present in the TCGA database, genes that respected the following conditions were selected: genes differentially expressed between *CD68*^{High}*HIF1A*^{High} *vs CD68*^{High}*HIF1A*^{Low}; differentially

expressed in right colon between *CD68*^{High}*HIF1A*^{High} *vs* *CD68*^{High}*HIF1A*^{Low}*;* genes correlated with *HIF1A* expression in *CD68*^{High} tumors. After analyzing the results, it was observed that, *CXCR4*, *FCAR*, *ITGA5*, and *PFKFB3* met all conditions.

(II) Hypoxia-induced molecular players

With RNA array data in hand, it became necessary to validate the results obtained at the protein level. For that, macrophage, T cells, and cancer cells tricultures were established under normoxia and hypoxia conditions, and total cell lysates were obtained for western blot, or cells prepared for flow cytometry analysis. The expression of the proteins codified by the genes validated in the array was analyzed (Caveolin-1, CXCR4, Integrin α5, PFKFB3), and Heat Shock Cognate protein 70 (Hsc-70) was used as loading control. CD89 expression, codified by *FCAR*, was analyzed by Flow Cytometry. Our results revealed an increase in Integrin α5 expression in tricultures under hypoxia compared to tricultures under normoxia in all cell lines, but particularly evident in RKO and SW480 (Figure 18A). In addition, no differences were found when comparing normoxia and hypoxia condition in the case of Caveolin-1 (Figure 18B). Regarding PFKFB3 some differences were found but not in all cell lines. In fact, PFKFB3 expression is increased in SW48 tricultures in hypoxia, and reduced in Caco-2 tricultures in hypoxia comparing with the normoxic condition (Figure 18C). We also performed this validation with CXCR4, however, the antibody did not work and for this reason, we could not draw any conclusions about this protein.

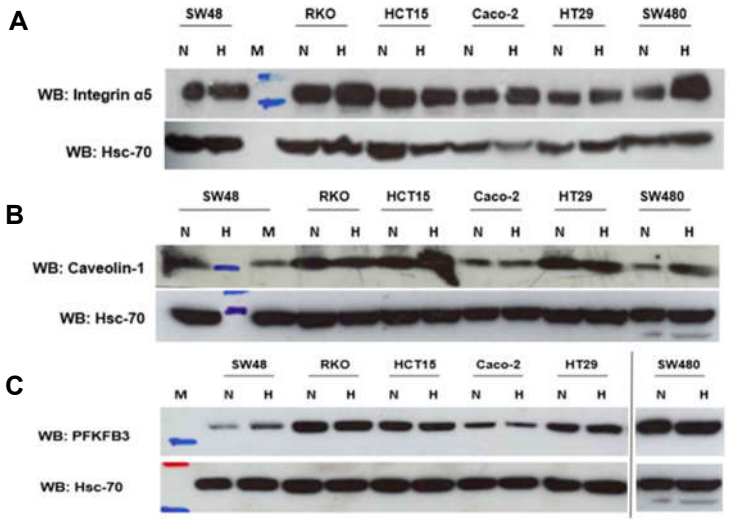
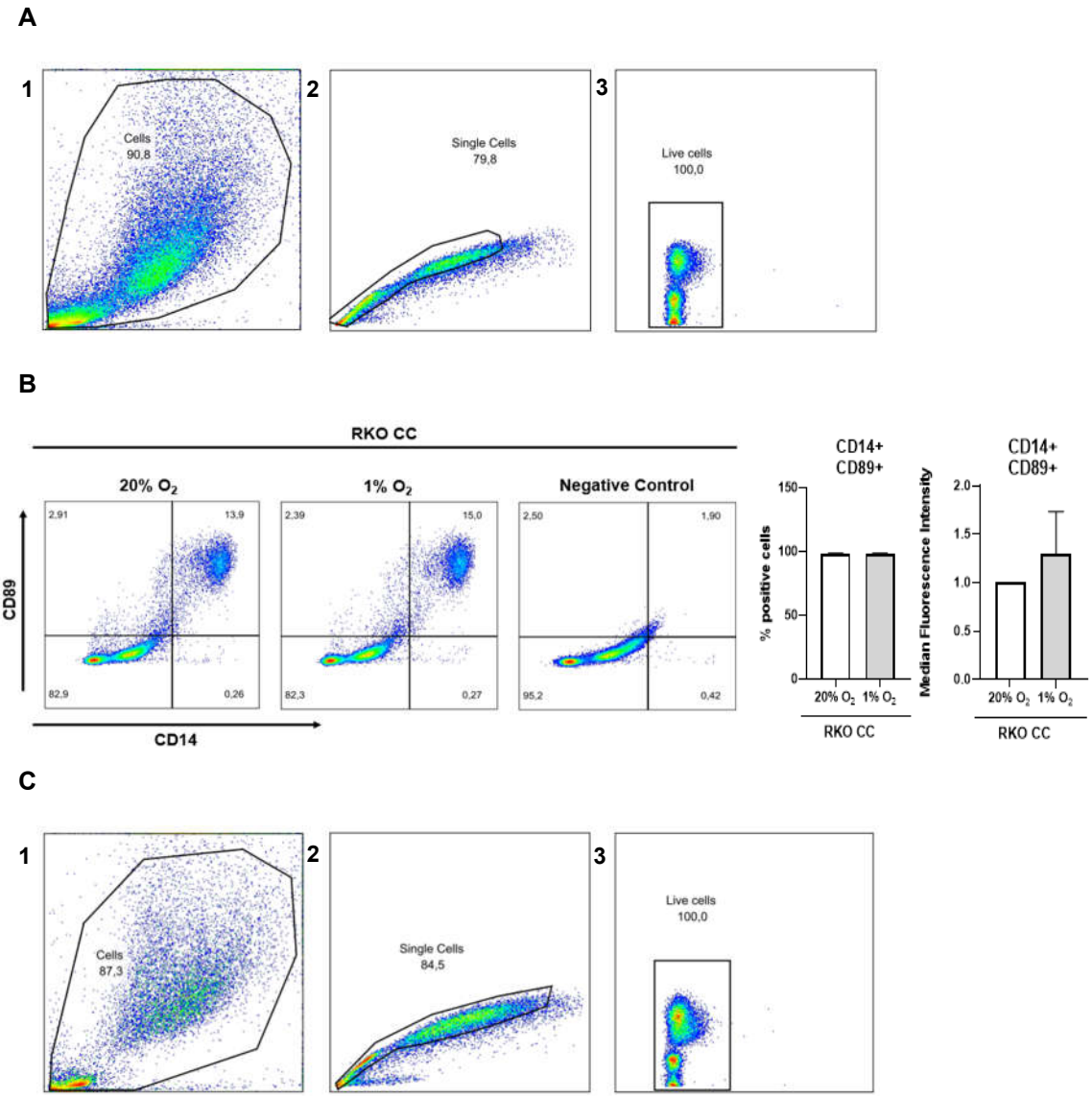


Figure 18. Expression of Integrin α5, Caveolin 1, and PFKFB3 in tricultures under normoxia and hypoxia. (A-C) Total protein levels were evaluated by western blot in tricultures of macrophages, T cells, and cancer cells (SW48, RKO, HCT15, Caco-2, HT29, SW480) under normoxia and hypoxia conditions. Hsc-70 was used as a loading control, and the expression of (A) integrin α5, (B) caveolin-1, and (C) PFKFB3 was evaluated. Images are representative of three independent experiments.

Regarding CD89 validation, for now it was analyzed the expression in tricultures of RKO and SW480, and the analysis in the other cell lines tricultures will be performed in the future. The analysis was done by flow cytometry, using the gating strategy presented in Figure 19A and C. Once it is known that CD89 is expressed in myeloid cells, besides CD89 it was include the CD14 macrophage lineage marker in the analysis. The analysis of the percentage of CD14+CD89+ cells showed that there were no differences between normoxia and hypoxia in both cell lines (Figure 19B and D). However, regarding the Median Fluorescence Intensity (MFI), RKO tricultures showed a tendency to increase in hypoxic condition (Figure 19B). To further clarification the n of the experiments must be increased.



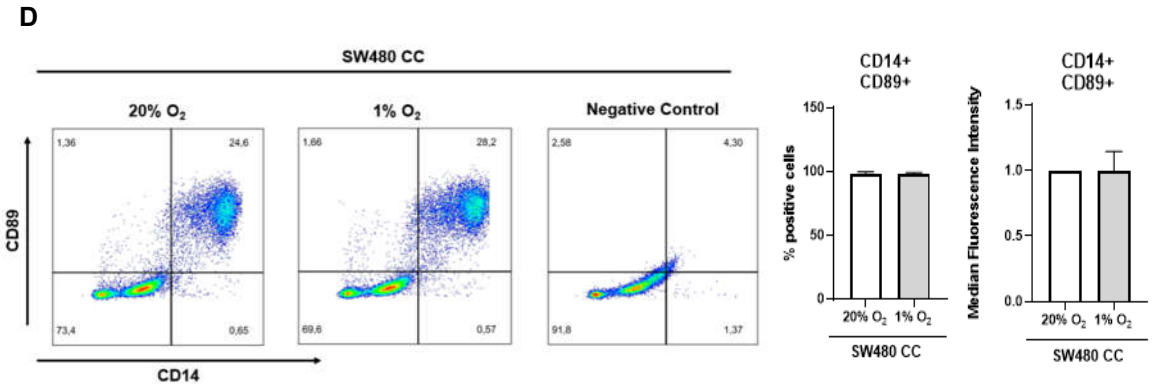


Figure 19. CD89 expression by flow cytometry. *CD89 expression was* determined by flow cytometry in the triculture of macrophages, T cells and cancer cells (RKO or SW480), under normoxia and hypoxia, in three independent experiments. (A) Pseudo color plots display the gating strategy created with FlowJo for flow cytometry. 1: FSC-A/SSC-A - represents the distribution of cells in the light scatter based on cell size and granularity, respectively - to 2: FSC-A/FSC-H – represents single cells and 3: Live dead/FCS-H – represented living cells for tricultures with macrophages, T cells and cancer cells (RKO). (B) Dot plots, graph with representation of double positive cells CD14+ CD89+ and graph representing the intensity of median fluorescence in RKO tricultures in normoxia and hypoxia. (C) Pseudo color plots display the gating strategy created with FlowJo for flow cytometry. 1: FSC-A/SSC-A - represents the distribution of cells in the light scatter based on cell size and granularity, respectively - to 2: FSC-A/FSC-H – represents single cells and 3: Live dead/FCS-H – represented living cells represented living cells for tricultures with macrophages, T cells and SW480. (D) Dot plots, graph with representation of double positive cells CD14+ CD89+ and graph representing the intensity of median fluorescence in SW480 tricultures in normoxia and hypoxia. Paired t test was used, and the differences were considered to be significant when $p < 0.05$.

Since Integrin α5 was the only protein that, as in the array, increased the expression in the tricultures of all cell lines under hypoxia, the study focus was directed to further evaluate its role on the hypoxia modulation during the crosstalk between cancer and immune cells. For that it was decided to silence the Integrin α5 expression by siRNA and assess the impact on cell behavior. RKO and SW480 were chosen to proceed with the studies since were the two cell lines that showed the highest increase on Integrin α5 expression under hypoxia.

For *ITGA5* siRNA optimization in RKO and SW480 monocultures, two concentrations (50nM and 75nM) and two times points for incubation (48h and 72h) were used. From the optimization of the siRNA, we found that in the case of RKO the concentration that worked best was 50nM, and there were no major differences between the different timepoints (Figure 20). For SW480 both concentrations worked very well and the timepoint with the greatest decrease in Integrin α5 expression was 48 hours (Figure 20). For this reason, further experiments will be performed using the concentration of 50nM and 48h of siRNA incubation for both cell lines.

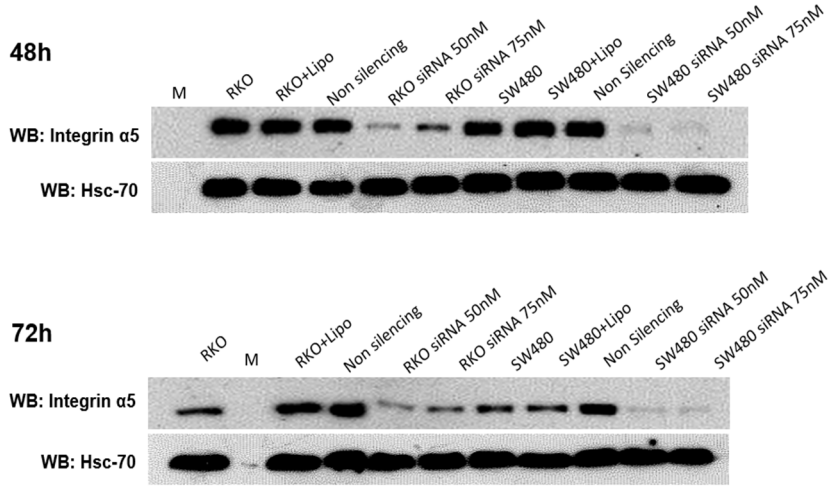


Figure 20. *ITGA5* siRNA optimization. Protein levels were evaluated by western blot in cancer cells (RKO and SW480) under hypoxia conditions. Cells were left untreated or treated with Lipofectamine RNAiMAX (Lipo) or Non-silencing siRNA as control. Hsc-70 was used as loading control.

After siRNA optimization, we started by the evaluation of *ITGA5* impact on the viability of the tricultures of macrophages, T cells and cancer cells under normoxia and hypoxia. The analysis was performed by measuring the percentage of live cells by flow cytometry and showed no major impact on cellular viability by the *ITGA5* silencing (Figure 21A and B).

The impact of Integrin α5 on T cells activation status was also evaluated once these cells can have an anti-tumor activity. CD69 was used as activation marker, and its expression was evaluated both in CD4 and CD8 populations within CD3 cells. The analysis was performed by flow cytometry, and the gating strategy used is presented (Figure 22A and 23A).

It was possible to assume that *ITGA5* had any impact on the CD4/CD8 ratio, for tricultures with RKO (Figure 22B) as well as for tricultures with SW480 (Figure 23B). However, no significant expression of T cells activation marker, CD69, was found in any of the conditions in both cell lines, regarding CD4 (Figure 22C and 23C) and CD8 (Figure 22D and 23D) Since it was not used any positive control condition for T cell activation, one could not conclude whether this is a real result or an artifact. In next experiments, a T cell activation positive control, with PMA and ionomycin treatment, should be included.

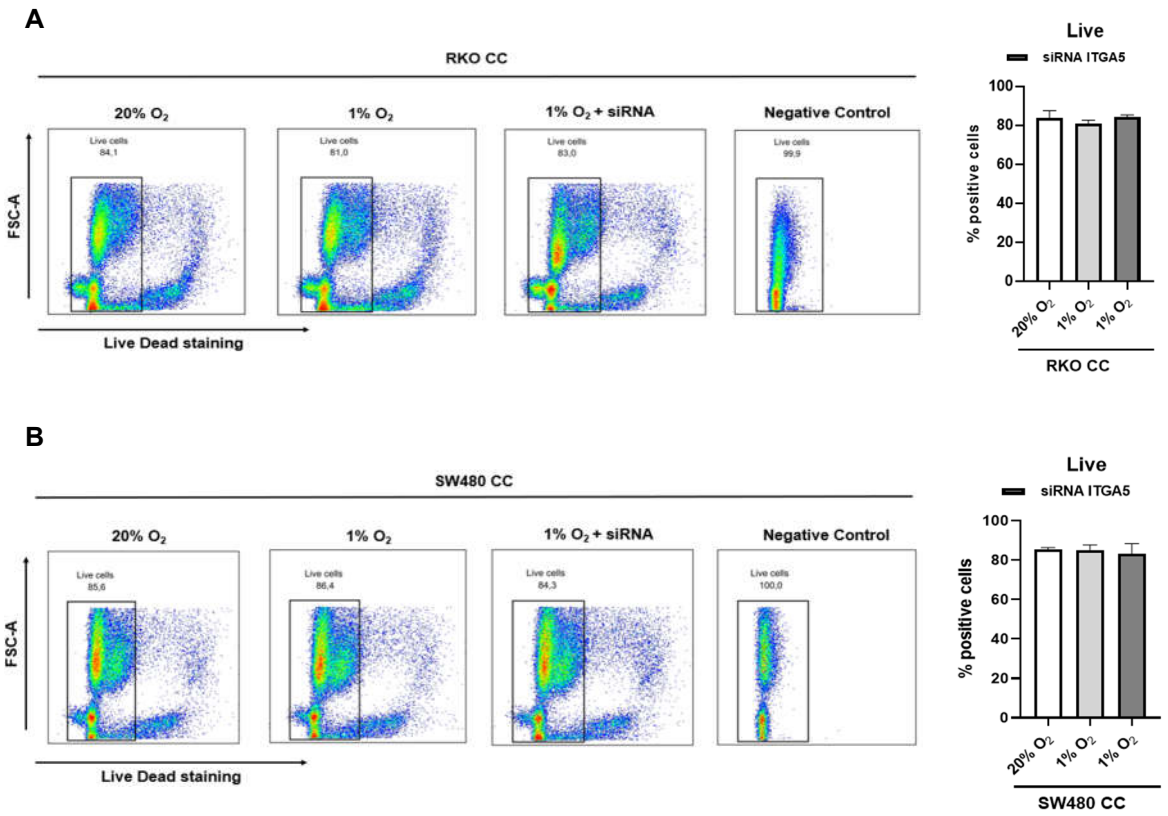
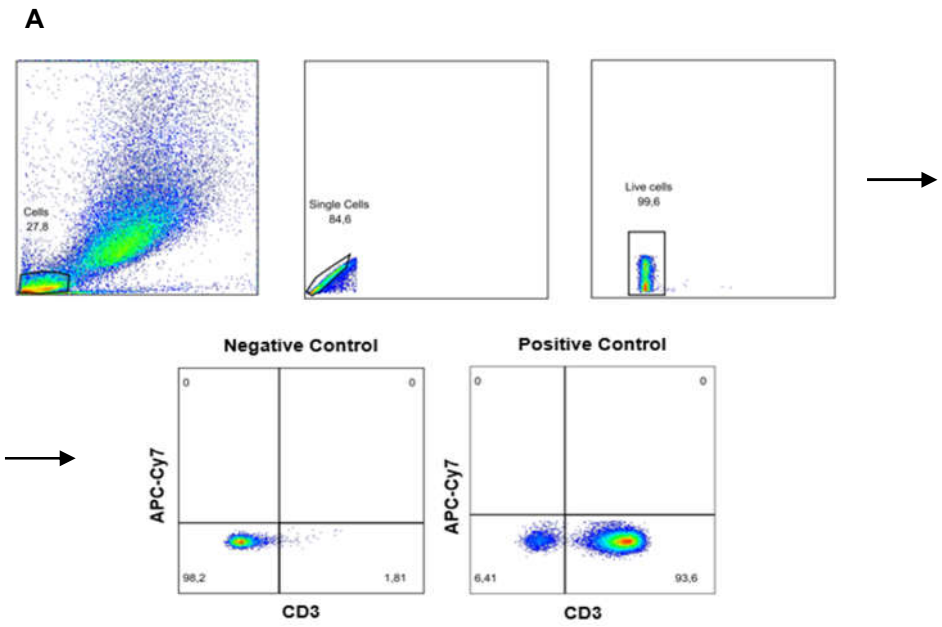
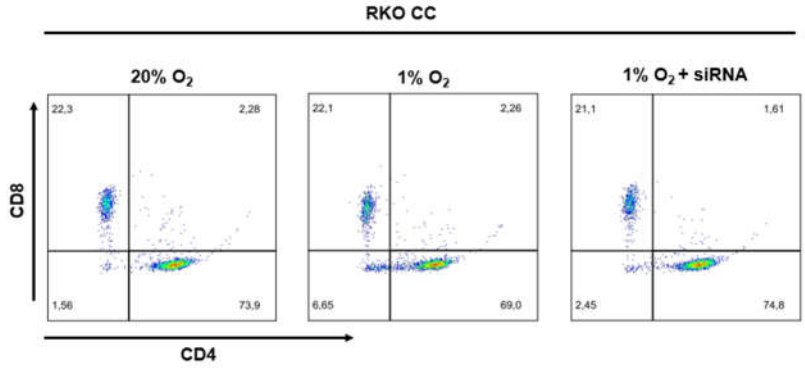


Figure 21. Impact of *ITGA5* in cellular viability. (A) FSC-A versus Live Dead dot plots of tricultures of macrophages, T cells and cancer cells (RKO), at 20% O_2 and 1% O_2, for 24h and graph represent the percentage of live cells in tricultures with macrophages, T cells and cancer cells (RKO). (B) A) FSC-A versus Live Dead dot plots of tricultures of macrophages, T cells and cancer cells (SW480), at 20% O_2 and 1% O_2, for 24h and graph represent the percentage of live cells in tricultures with macrophages, T cells and SW480 cells. The statistical teste RM one-way ANOVA was used.



B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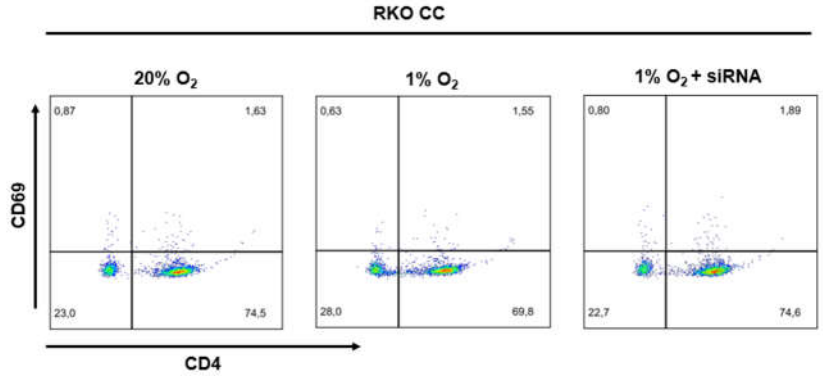
20% O2
1% O2
1% O2 + siRNA
CD8
CD4
22,3
2,28
1,56
73,9
22,1
2,26
6,65
69,0
21,1
1,61
2,45
74,8

C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20% O2
1% O2
1% O2 + siRNA
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CD4
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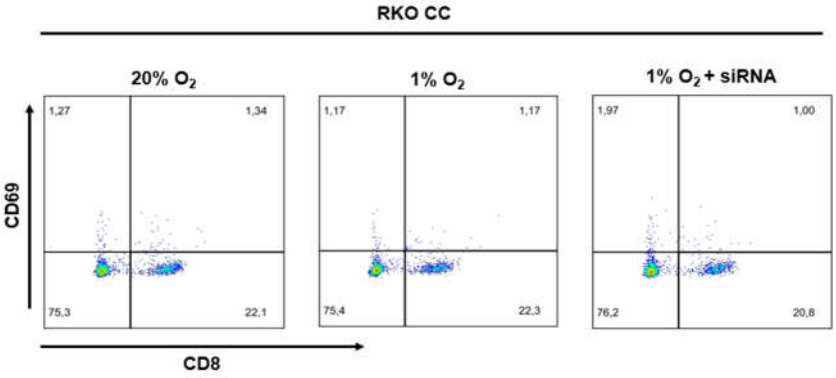
74,5
0,63
1,55
28,0
69,8
0,80
1,89
22,7
74,6

D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20% O2
1% O2
1% O2 + siRNA
CD69
CD8
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1,34
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22,1
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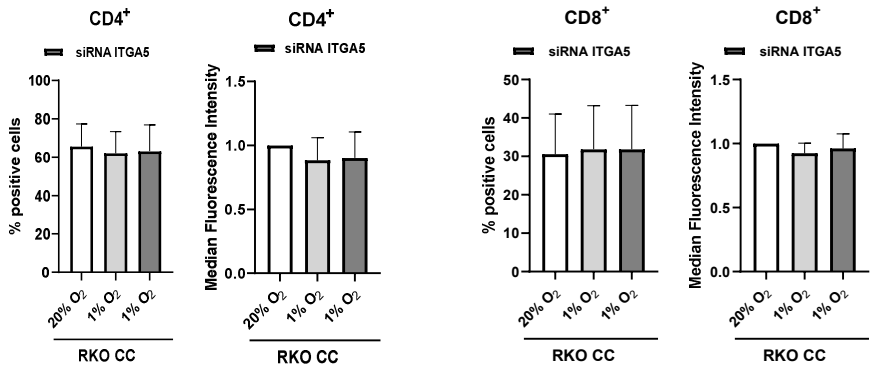
22,3
1,97
1,00
76,2
20,8

E


CD4+
CD8+
siRNA ITGA5
% positive cells
Median Fluorescence Intensity
20% O2
1% O2
1% O2
RKO CC

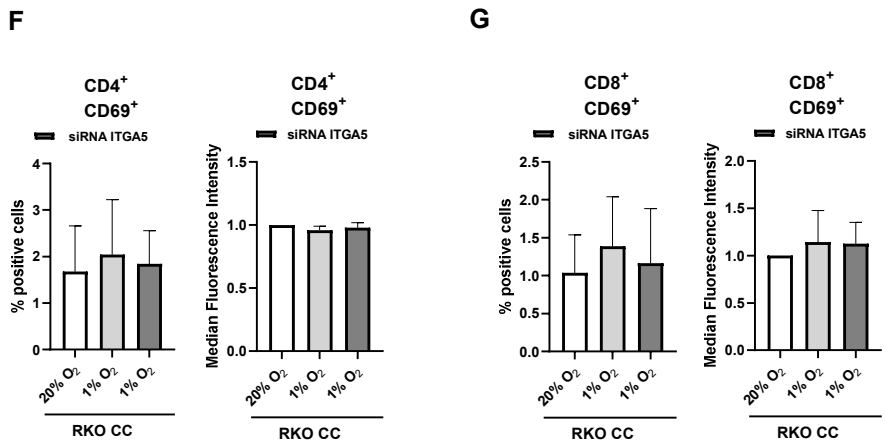
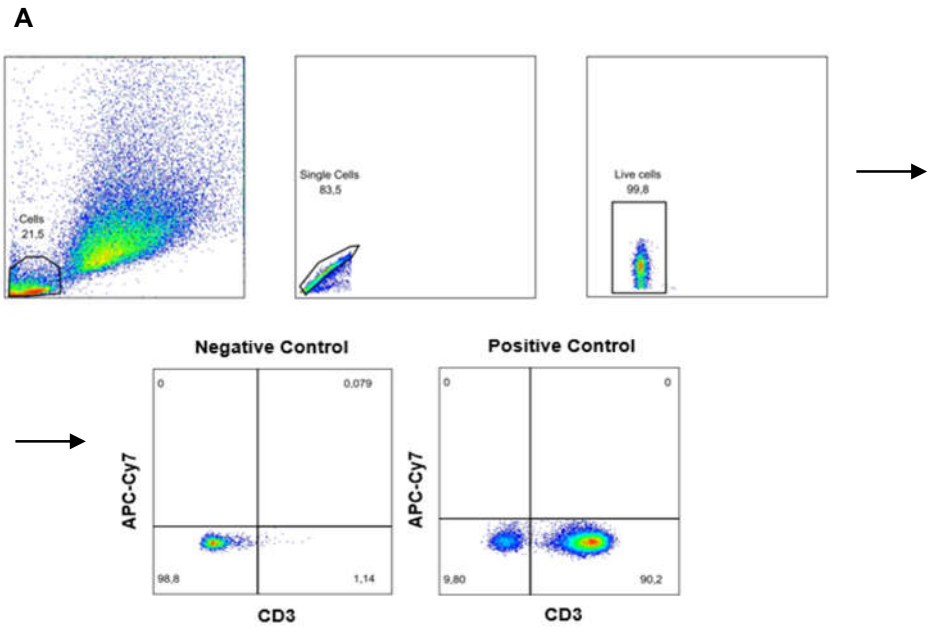


Figure 22. Impact of *ITGA5* in T cells activation. (A) The gating strategy for CD4 and CD8 T cells. (B-D) The expression was determined by flow cytometry in macrophages, T cells cultured with cancer cells (RKO), under normoxia and hypoxia, in three independent experiments. (B) CD4 versus CD8 dot plot. (C) CD4 versus CD69 dot plot, (D) CD8 versus CD69 dot plot. (E) Graph with representation of double positive cells CD4+ and CD8+ graph representing the intensity of median fluorescence in RKO tricultures in normoxia and hypoxia. (F) Graph with representation of double positive cells CD4+CD69+ graph representing the intensity of median fluorescence in RKO tricultures in normoxia and hypoxia. (G) Graph with representation of double positive cells CD8+CD69+ graph representing the intensity of median fluorescence in RKO tricultures in normoxia and hypoxia.



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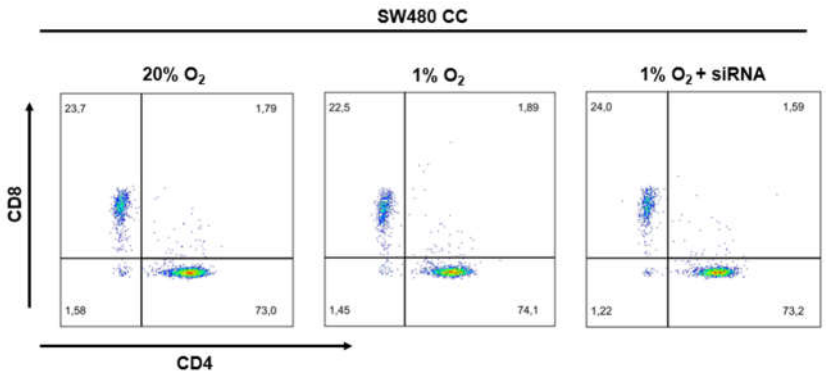
SW480 CC
20% O2
1% O2
1% O2 + siRNA
CD8
CD4
23,7
1,79
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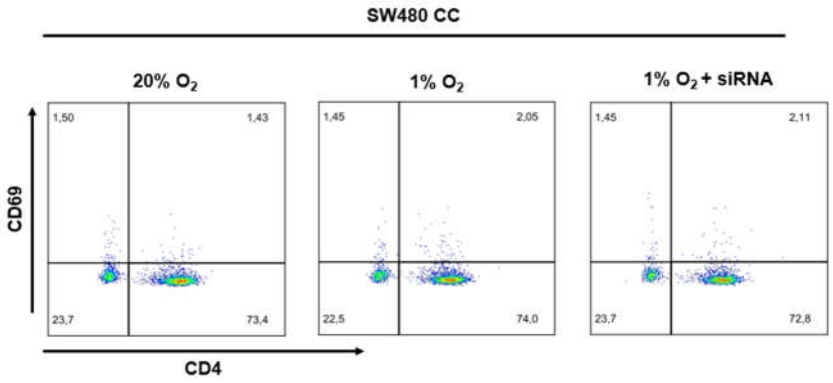
73,2

C


SW480 CC
20% O2
1% O2
1% O2 + siRNA
CD69
CD4
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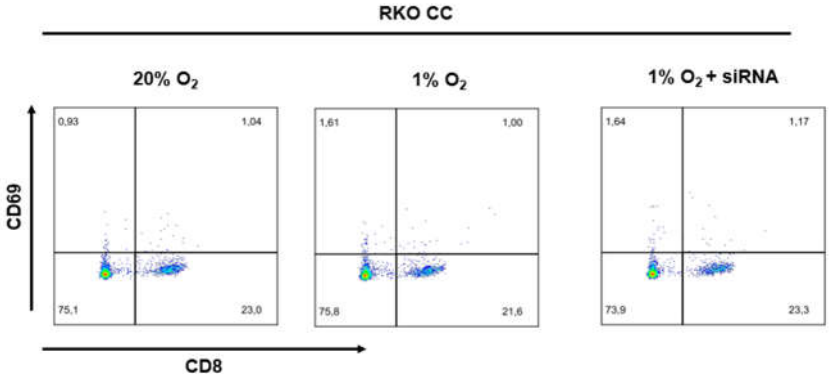
23,7
72,8

D


RKO CC
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1% O2
1% O2 + siRNA
CD69
CD8
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1,61
1,00
75,8
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1,64
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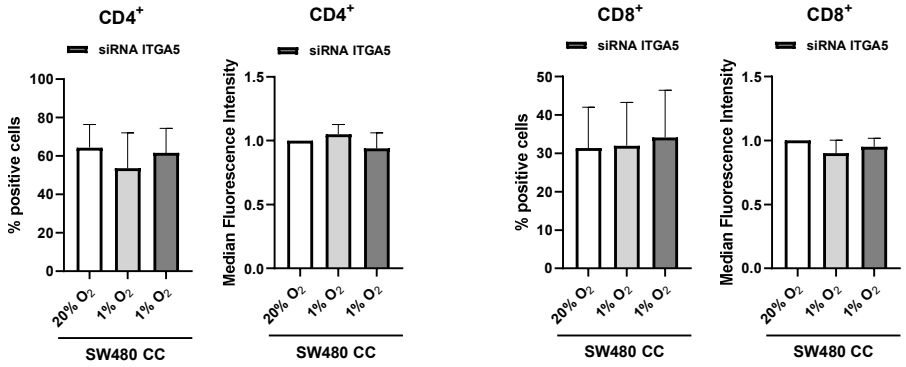
73,9
23,3

E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CD8+
siRNA ITGA5
% positive cells
Median Fluorescence Intensity
20% O2
1% O2
1% O2
SW480 CC

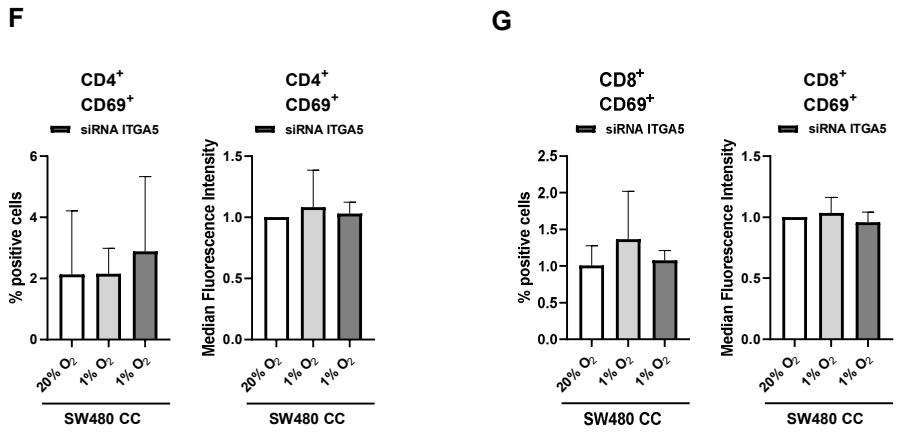


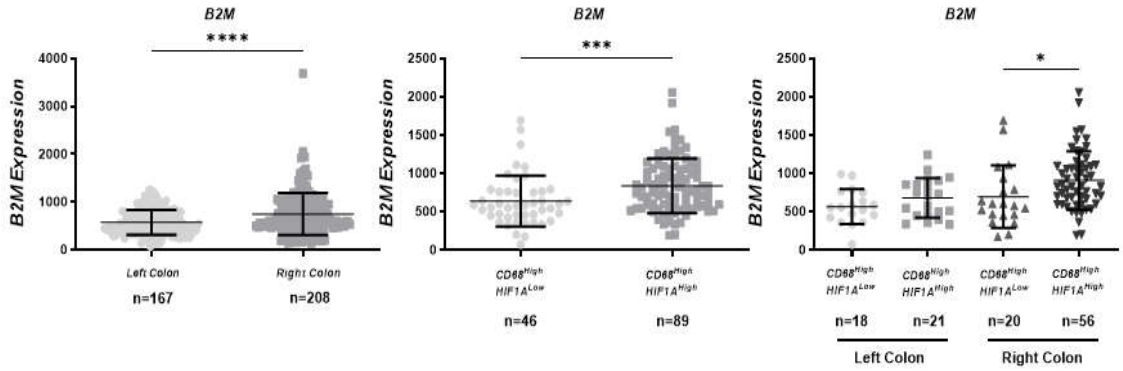
Figure 23. Impact of *ITGA5* in T cells activation. (A) The gating strategy for CD4 and CD8 T cells. (B-D) The expression was determined by flow cytometry in macrophages, T cells cultured with cancer cells (SW480), under normoxia and hypoxia, in three independent experiments. (B) CD4 versus CD8 dot plot. (C) CD4 versus CD69 dot plot, (D) CD8 versus CD69 dot plot. (E) Graph with representation of double positive cells CD4+ and CD8+ graph representing the intensity of median fluorescence in SW480 tricultures in normoxia and hypoxia. (F) Graph with representation of double positive cells CD4+CD69+ graph representing the intensity of median fluorescence in SW480 tricultures in normoxia and hypoxia. (G) Graph with representation of double positive cells CD8+CD69+ graph representing the intensity of median fluorescence in SW480 tricultures in normoxia and hypoxia.

(III) Immune checkpoints expression profile in colon cancer patients

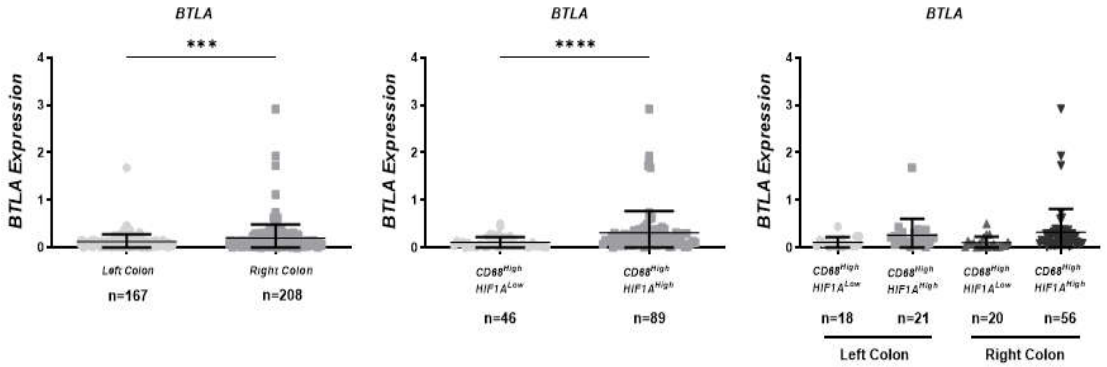
As previously mentioned, the *CD68*^{High}*HIF1A*^{High} colon tumors from the TCGA COAD cohort showed better survival when compared to the *CD68*^{High}*HIF1A*^{Low} tumors (Figure 15A) (Martins et al., 2020), which is also true when we are looking only for the right colon tumors, but not when left colon tumors were analyzed (Figure 15B). Taking this into consideration, we hypothesized that these differences in survival could be related to differences in the expression profile of immune checkpoints in the tumors presenting better prognosis. To test our hypothesis, and using TCGA data it was explored, in colon cancer patients, the expression profile of the immune checkpoints presented in the Figure 5, that can function both as activators or repressors of the anti-tumor immunity.

The immune checkpoints expression was analyzed comparing two group of tumors in four distinct conditions: 1) Left colon *vs* Right colon tumors; 2) *CD68*^{High}*HIF1A*^{Low} *vs* *CD68*^{High}*HIF1A*^{High} tumors; 3) inside the left colon, the tumors *CD68*^{High}*HIF1A*^{Low} *vs* *CD68*^{High}*HIF1A*^{High}; 4) inside the right colon, the tumors *CD68*^{High}*HIF1A*^{Low} *vs* *CD68*^{High}*HIF1A*^{High}.

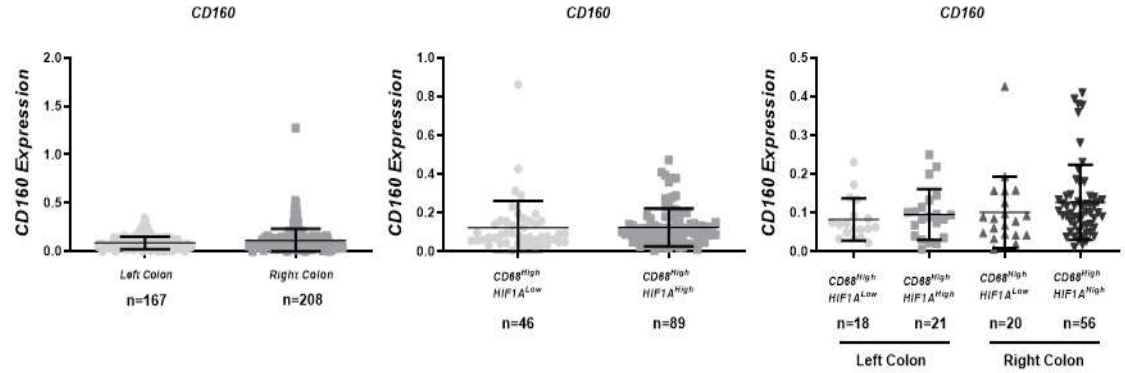
B2M (MHC-I)



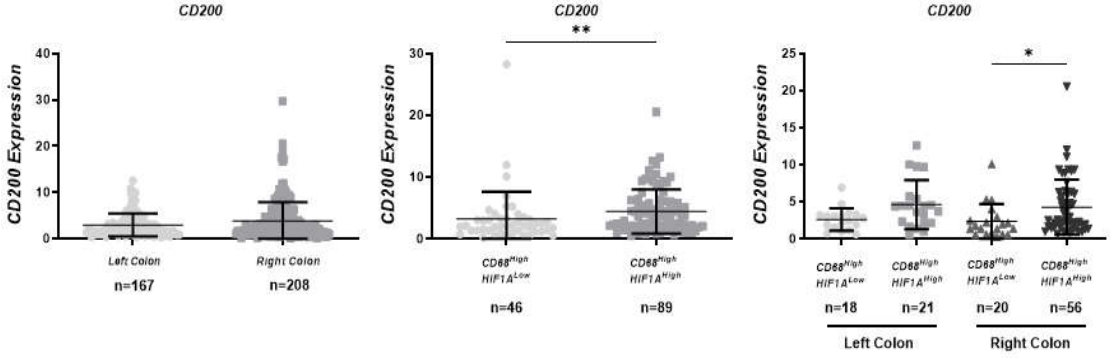
BTLA



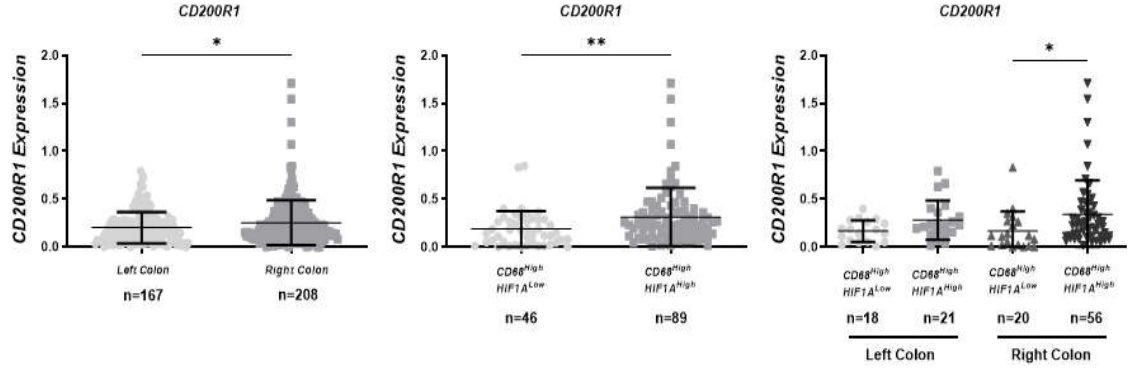
CD160



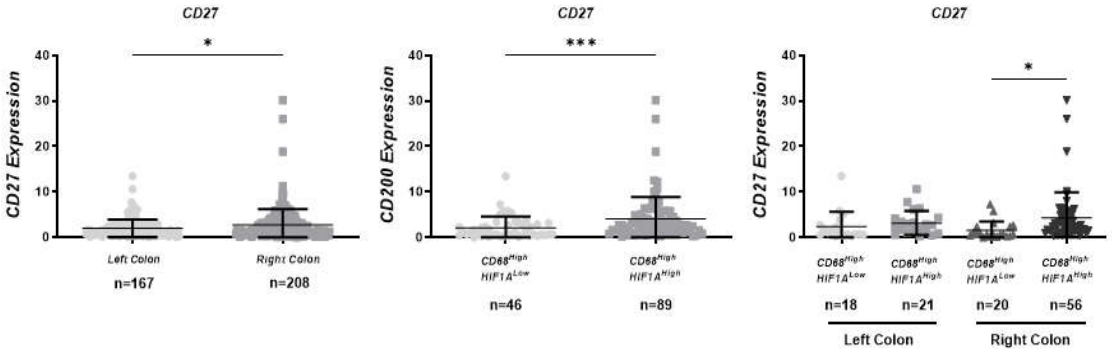
CD200



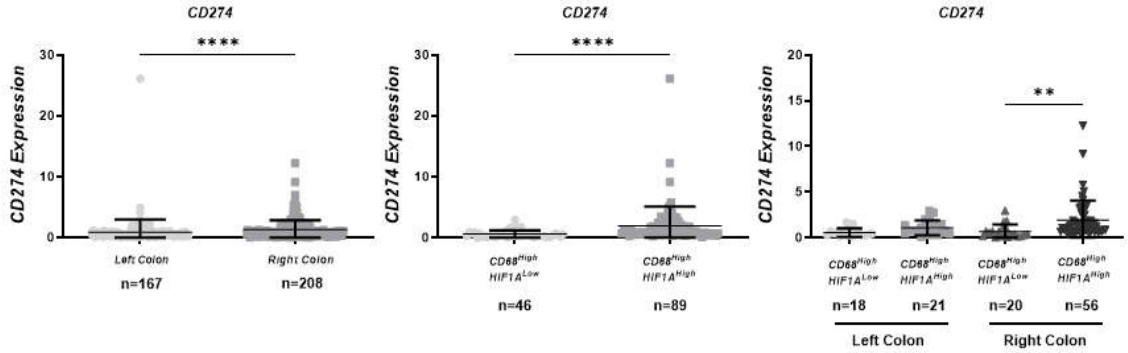
CD200R1 (CD200R)



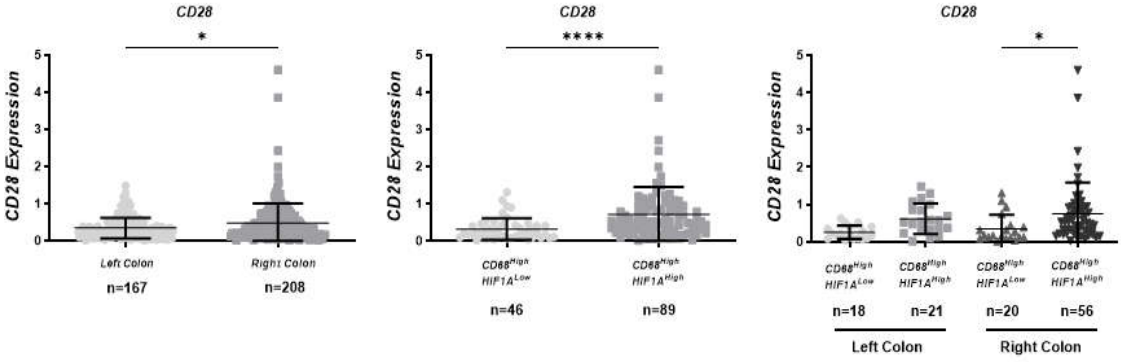
CD27



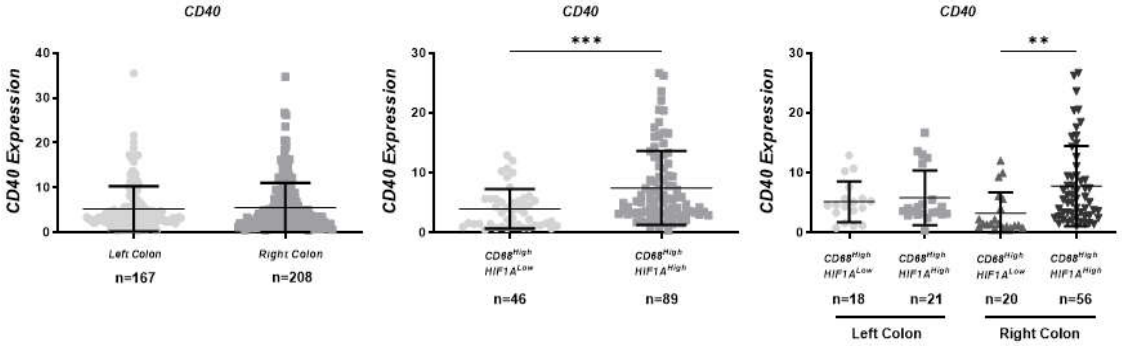
CD274 (PD-L1)



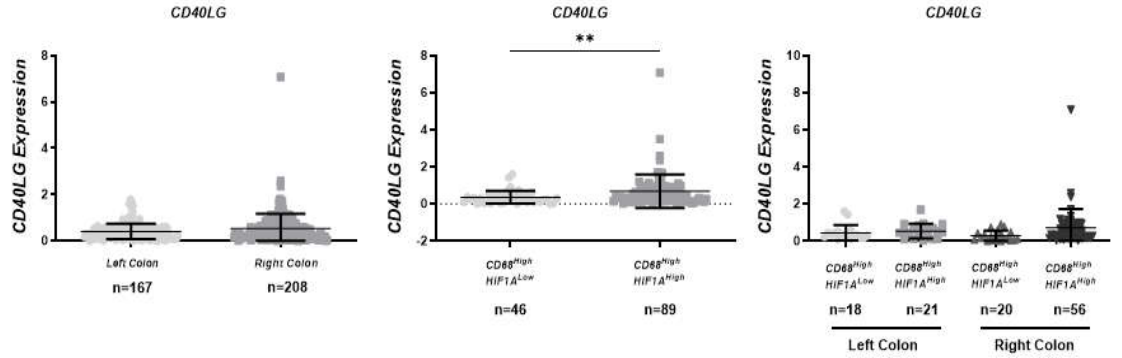
CD28



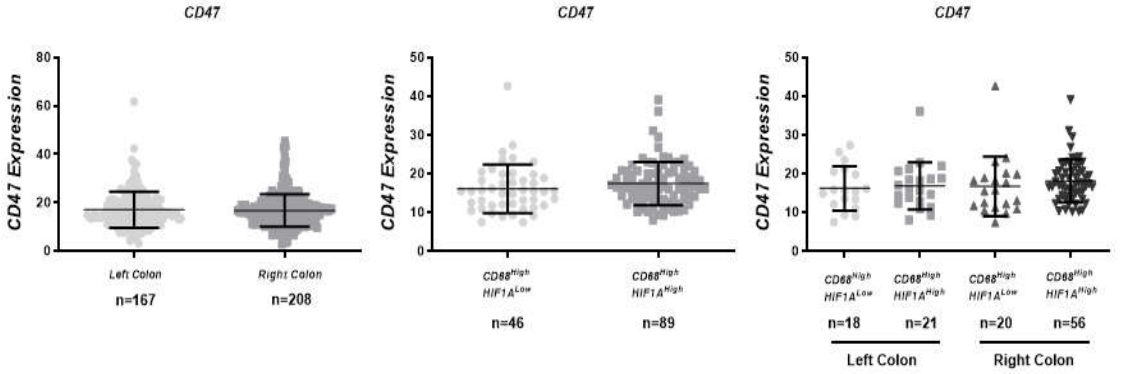
CD40



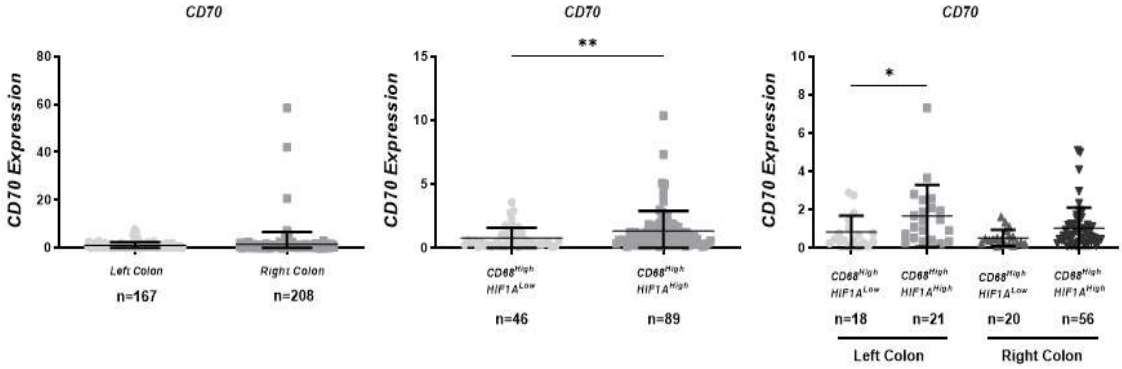
CD40LG (CD40L)



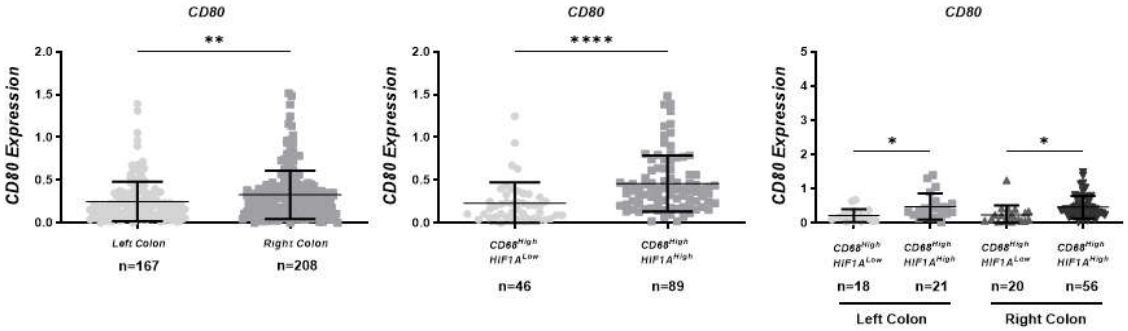
CD47



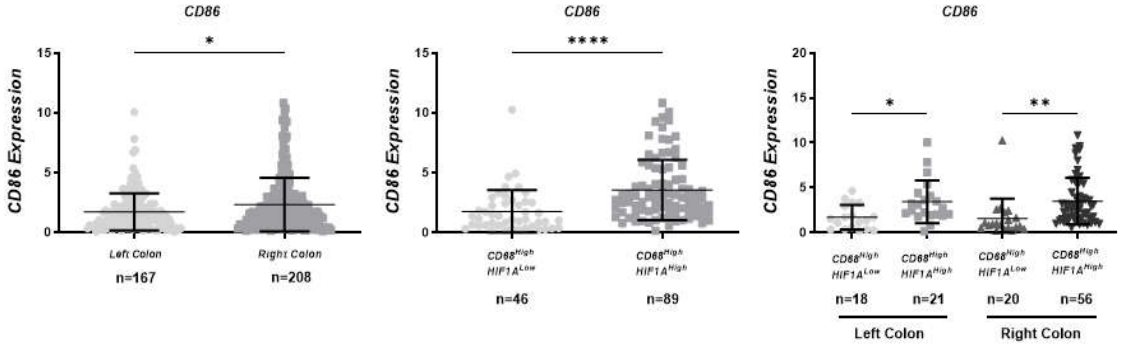
CD70



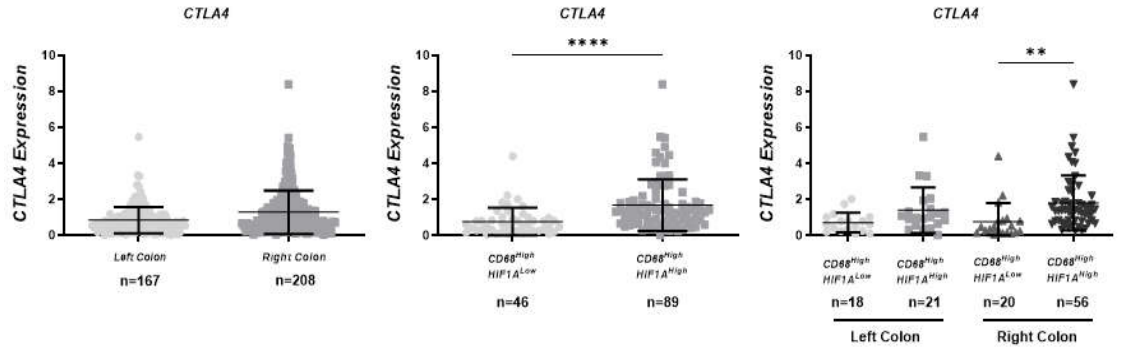
CD80



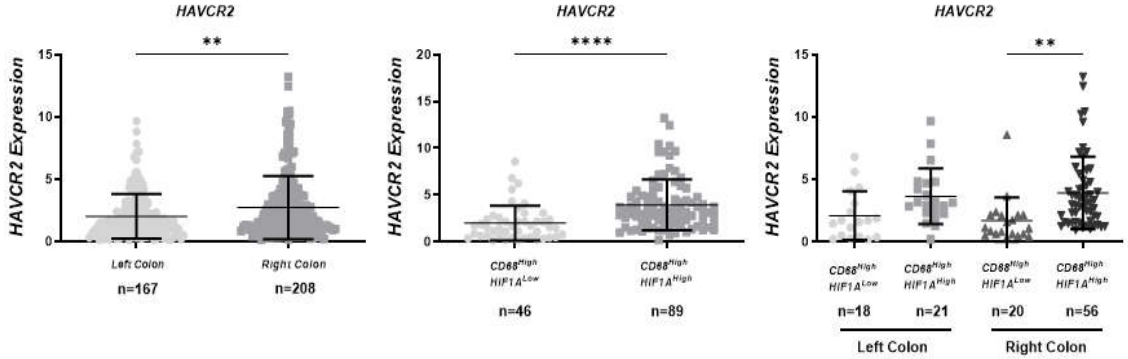
CD86



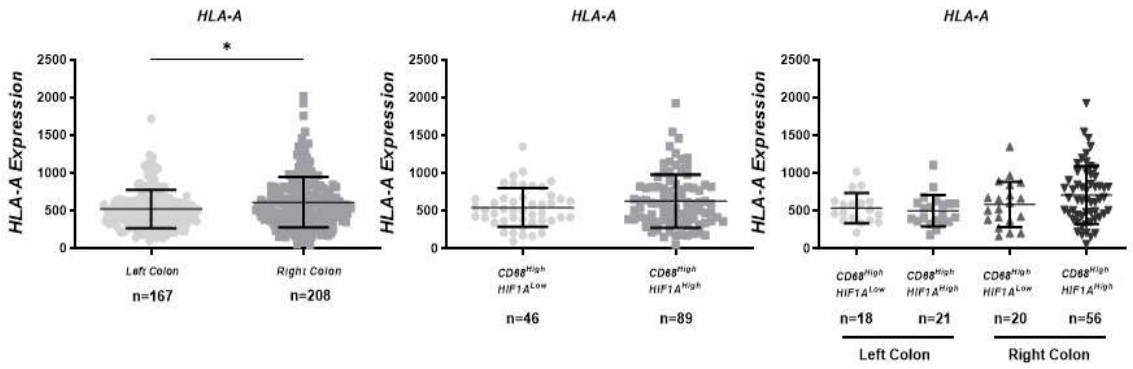
CTLA4



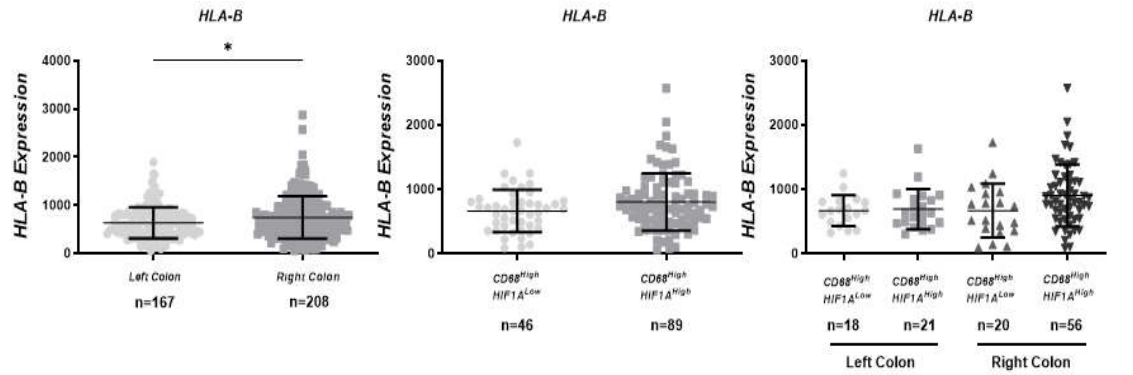
HAVCR2 (TIM-3)



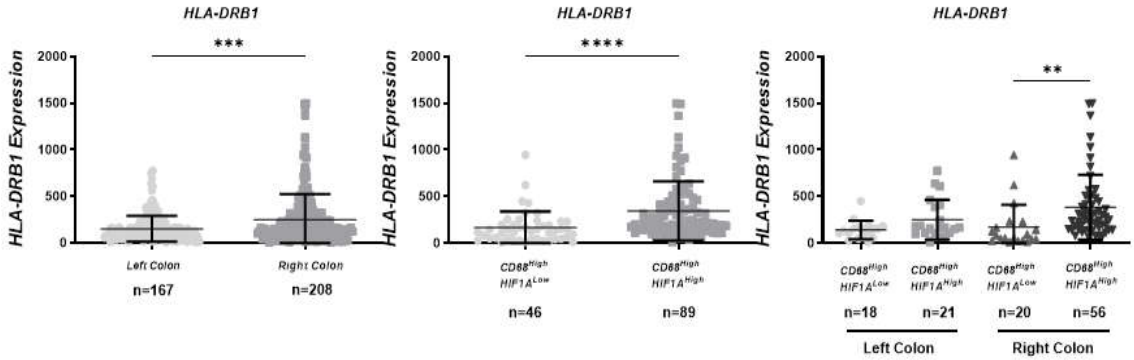
HLA-A (MHC-I)



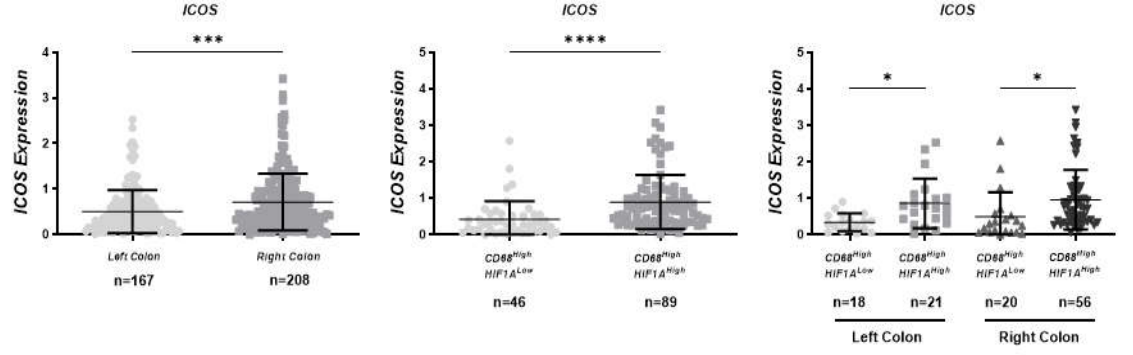
HLA-B (MHC-II)



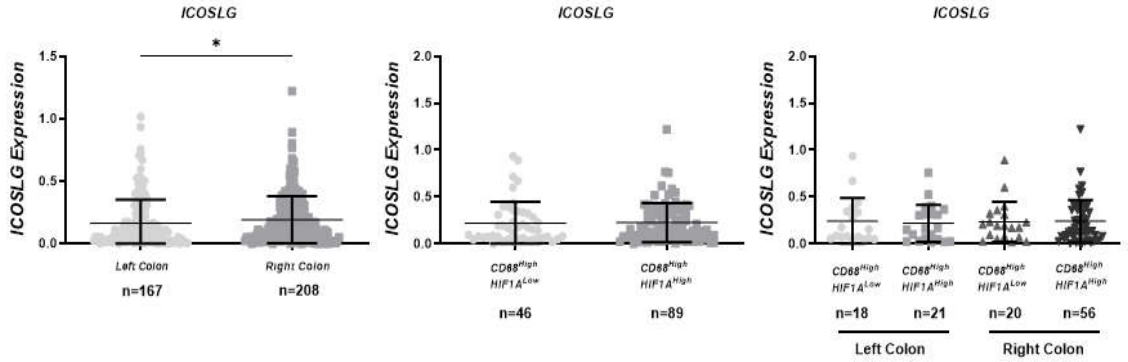
HLA-DRB1 (MHC-II)



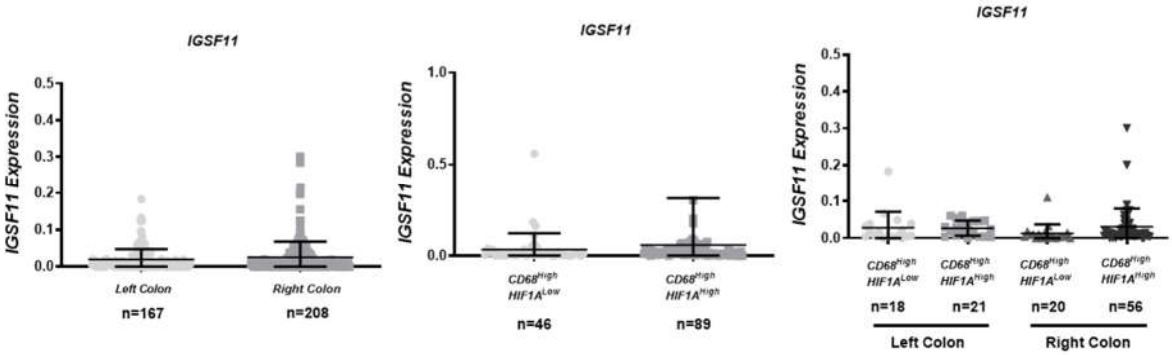
ICOS



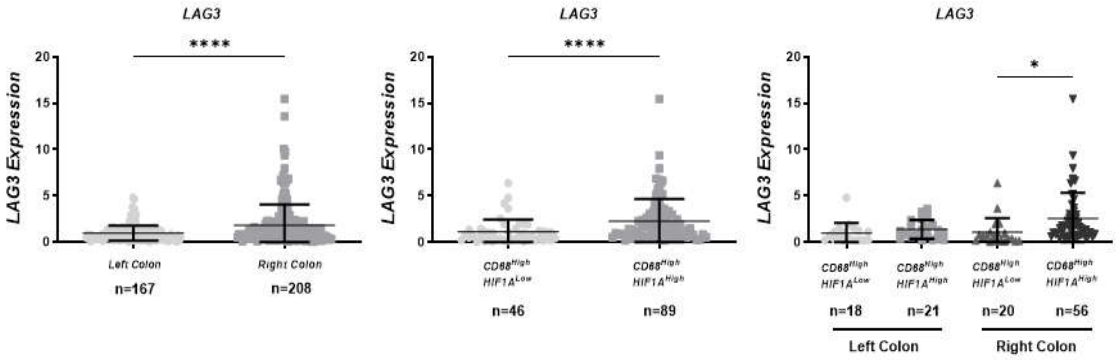
ICOSLG (B7RP1)



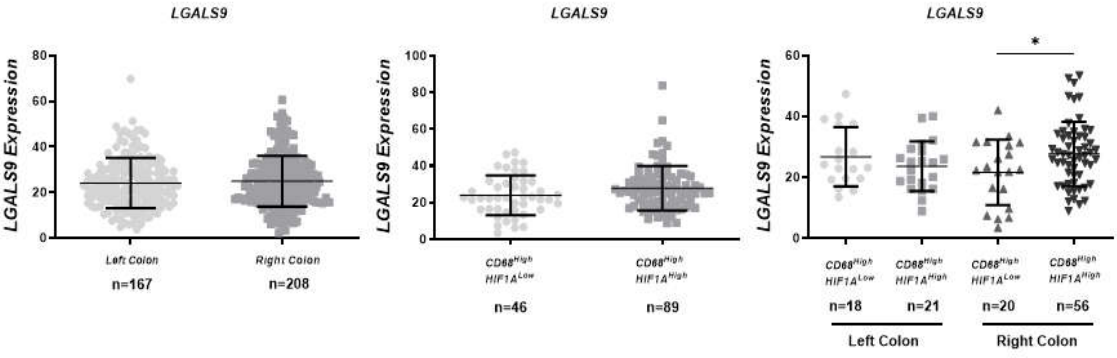
IGSF11



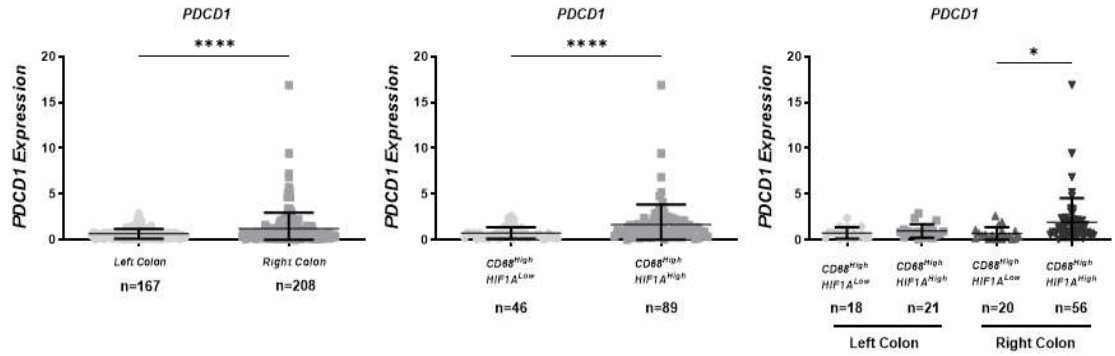
LAG3



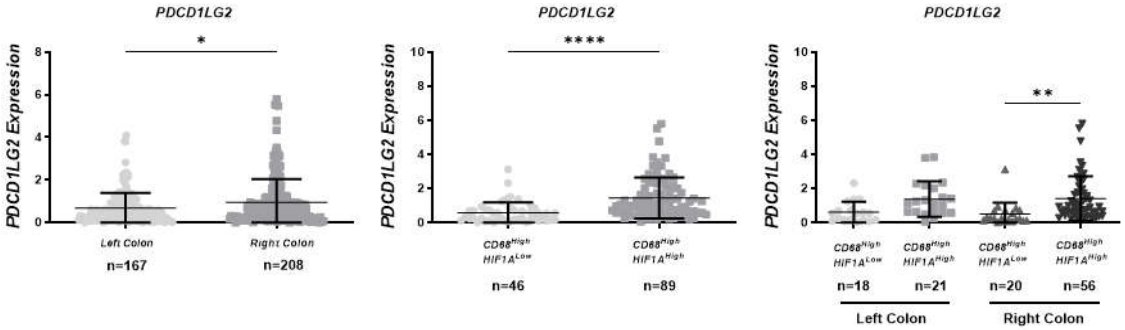
LGALS9 (GAL9)



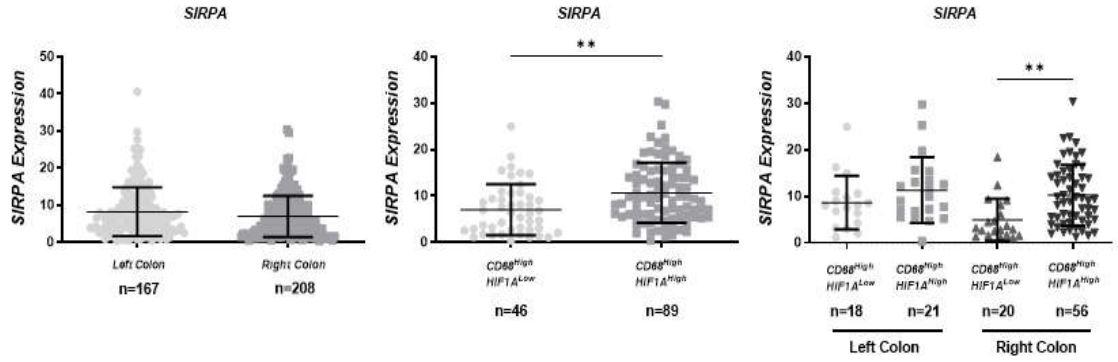
PDCD1 (PD-1)



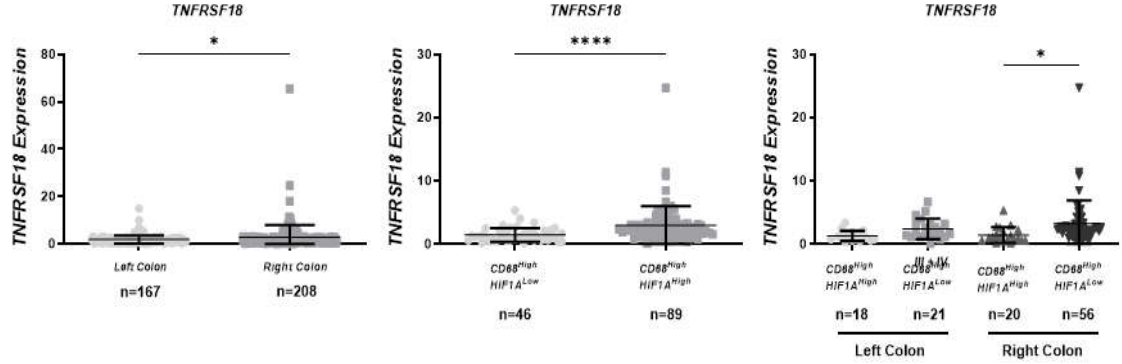
PDCD1LG2 (PD-L2)



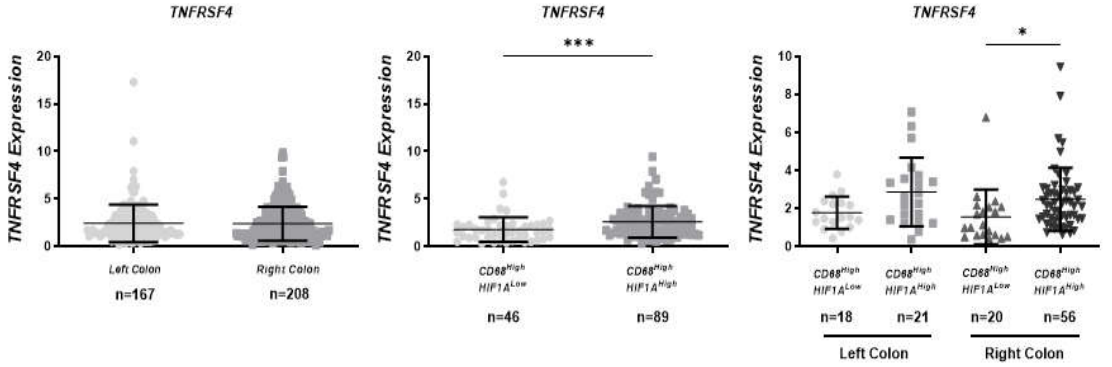
SIRPA (SIRPα)



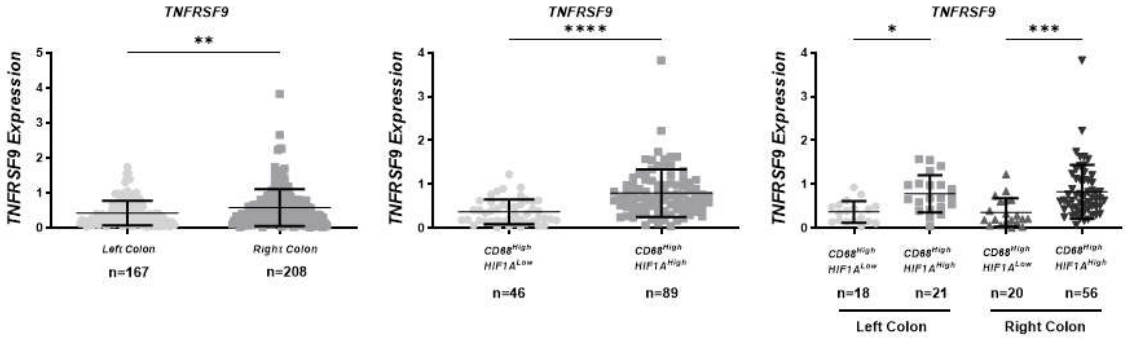
TNFRSF18 (GITR)



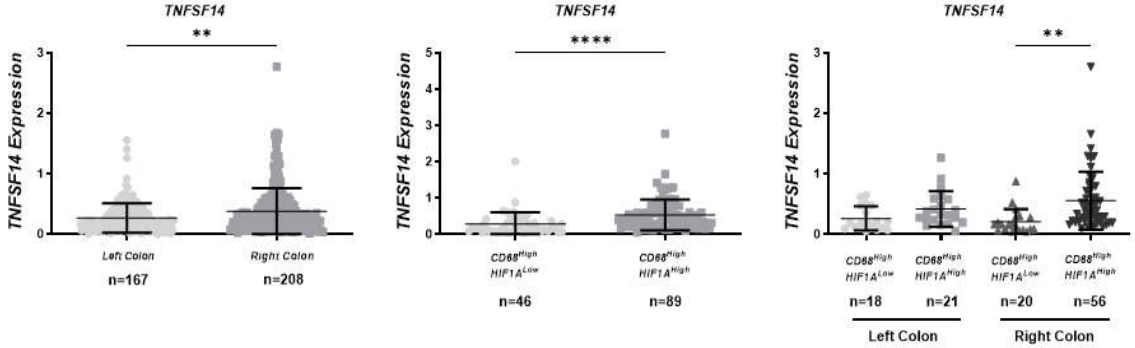
TNFRSF4 (OX40)



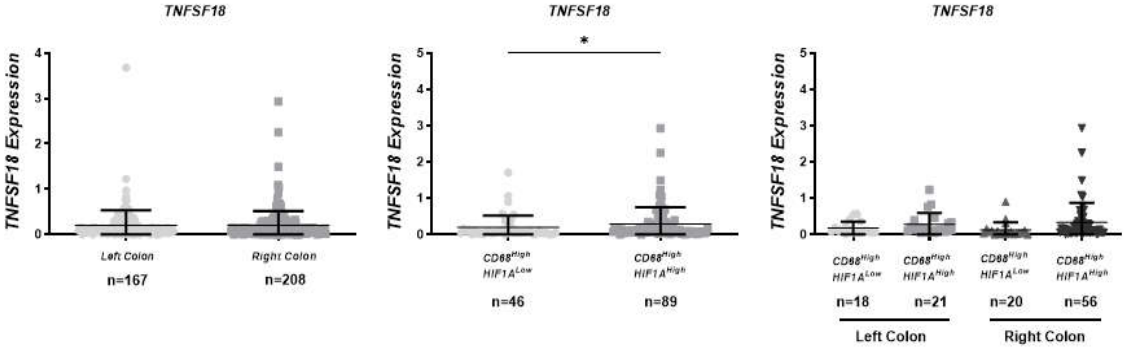
TNFRSF9 (4-1BB)



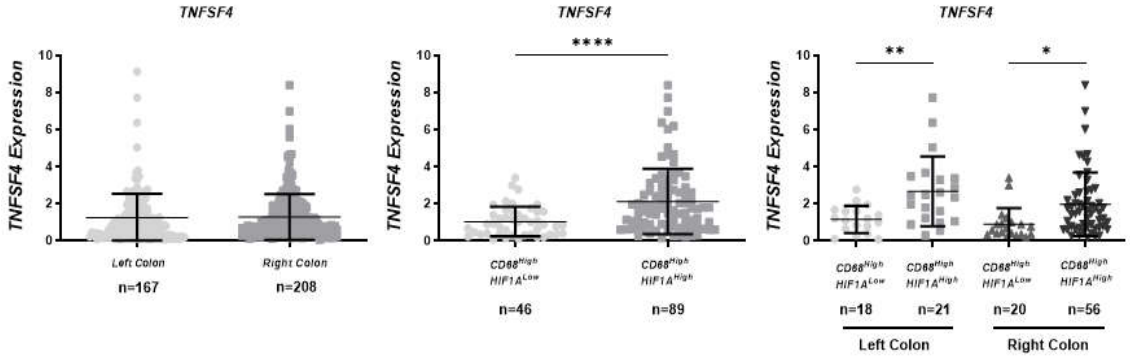
TNFSF14 (HVEM)



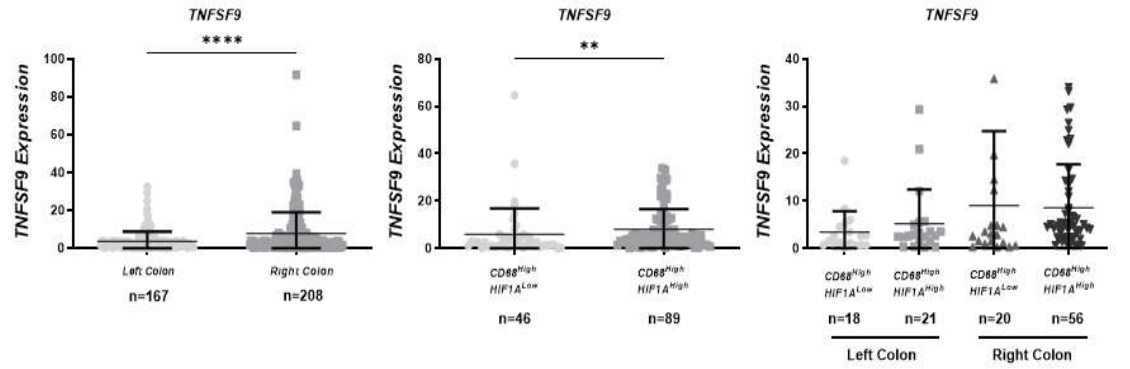
TNFSF18 (GITRL)



***TNFSF4* (OX40L)**



***TNFSF9* (4-1BBL)**



***VSIR* (VISTA)**

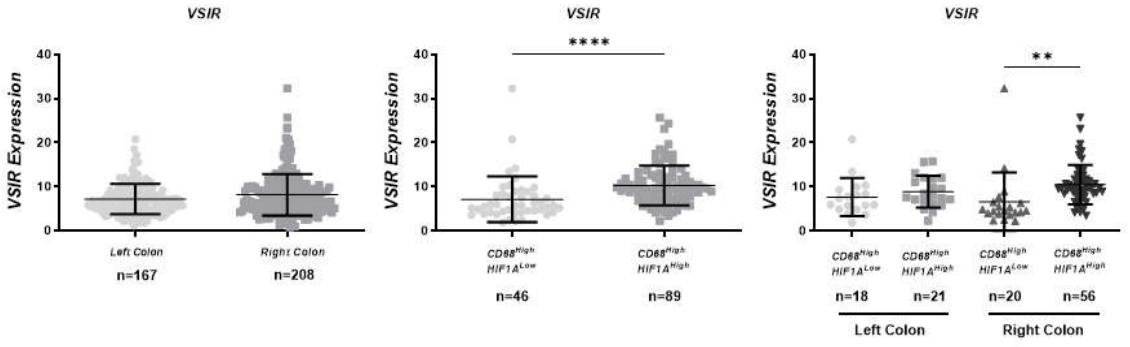


Figure 24. Immune checkpoints expression in colon cancer patients. RNASeq expression data were downloaded from TCGA. Is considered right colon the cecum, the ascending colon and the hepatic flexure, while it is considered left colon the splenic flexure, descending colon and the sigmoid colon. Transverse colon and rectum were not considered in the analysis. Whitin *CD68*^{High} tumors, the expression levels of both genes were compared between *HIF1A*^{Low} and *HIF1A*^{High}, according to the median levels of *HIF1A* expression in normal samples.. Mann–Whitney or one-way ANOVA were used to compare gene expression among groups.. **** $p < 0.0001$; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

A summary of the results found is presented in Table 5. In general, it was found that the right colon presented a higher expression of immune checkpoints when compared with

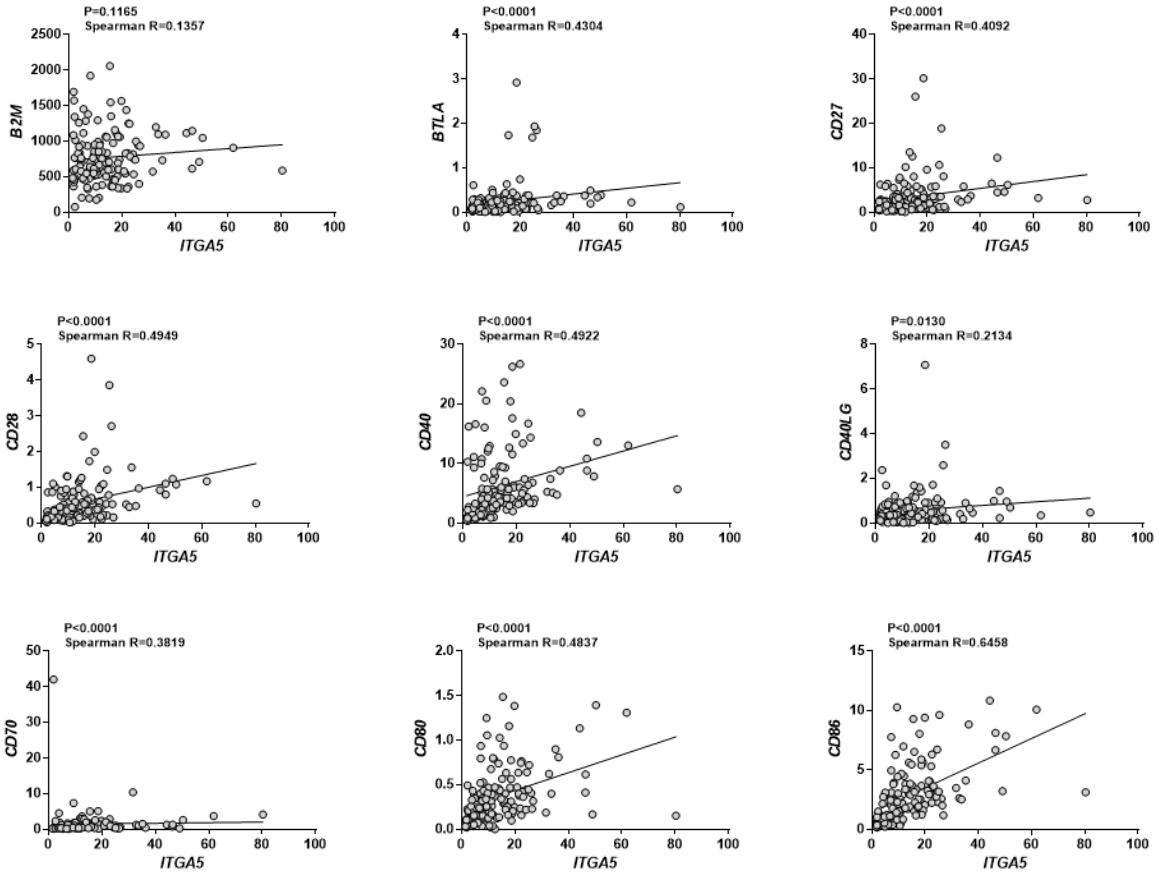
the left colon, both activators and inhibitors of T cells activity. Moreover, and interestingly, the tumors associated with patients better survival, that are the *CD68*^{High}*HIF1A*^{High} tumors when compared to the *CD68*^{High}*HIF1A*^{Low} tumors, showed an increase in the expression of most of the analyzed immune checkpoints, both activators and inhibitors. This tendency is also confirmed when comparing the *CD68*^{High}*HIF1A*^{High} *vs CD68*^{High}*HIF1A*^{Low} population within the right colon. Of note the comparison between *CD68*^{High}*HIF1A*^{High} *vs CD68*^{High}*HIF1A*^{Low} showed a completely different expression profile when compared in right and left colon. For further studies the most interestingly immune checkpoints to explore will be the ones that present distinct results regarding *CD68*^{High}*HIF1A*^{High} *vs CD68*^{High}*HIF1A*^{Low} comparison in right and left colon, and the ones associated with activation of T cells in the *CD68*^{High}*HIF1A*^{High} tumors. Of interest will be also to explore those immune checkpoints differently expressed and that can give both activation and inhibition signals to T cells, depending on the context.

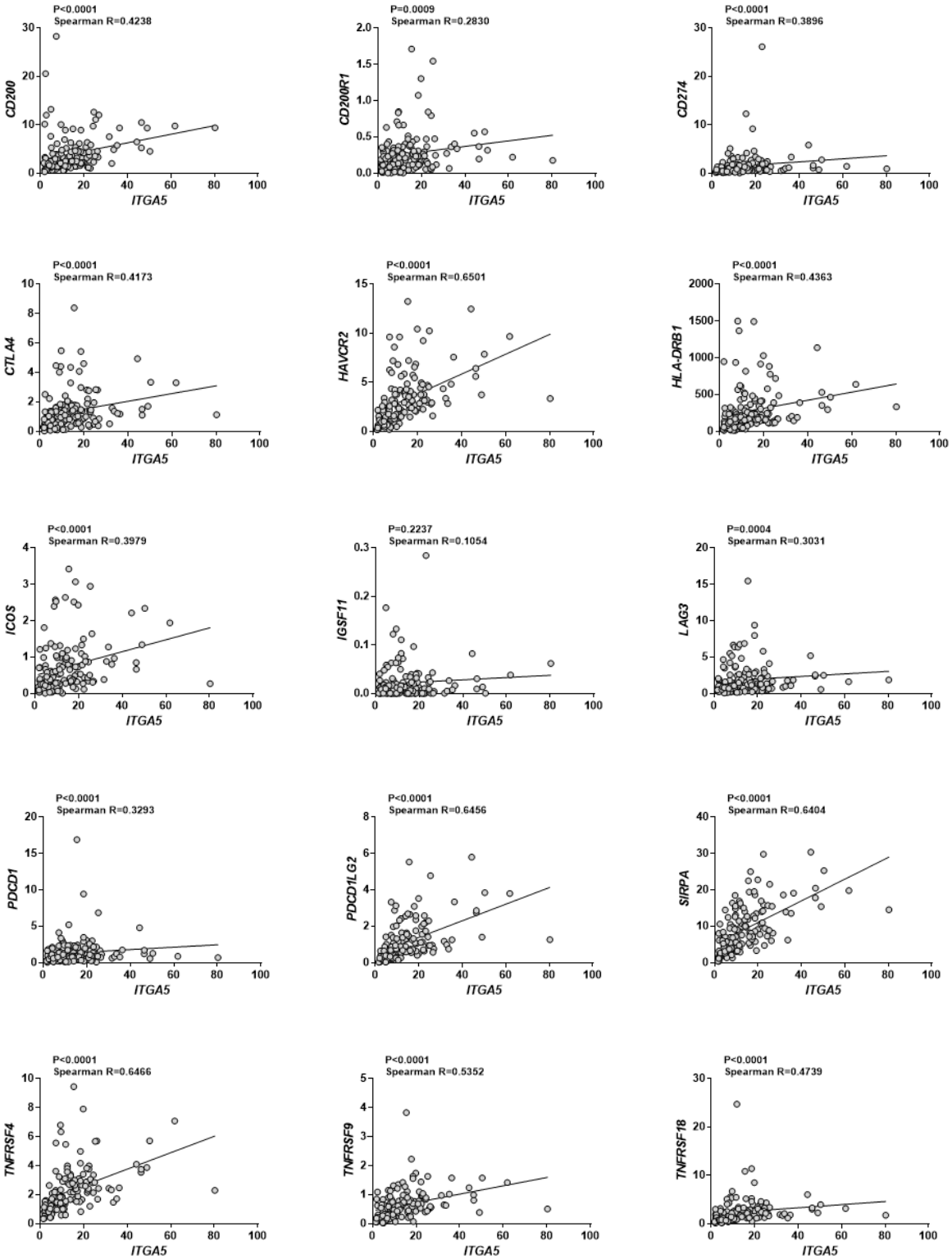
Table 5. Summary of the differences in expression of immune checkpoints in colon cancer patients.

Up regulated at Right Colon in comparison with Left Colon	Up regulated in *CD68*^{High}*HIF1A*^{High} in comparison with *CD68*^{High}*HIF1A*^{Low}	Up regulated in *CD68*^{High}*HIF1A*^{High} in comparison with *CD68*^{High}*HIF1A*^{Low} at the LEFT Colon	Up regulated in *CD68*^{High}*HIF1A*^{High} in comparison with *CD68*^{High}*HIF1A*^{Low} at the RIGHT Colon
B2M	B2M		B2M
BTLA	BTLA		
CD27	CD27		CD27
CD28	CD28		CD28
	CD40		CD40
	CD40LG		
	CD70	CD70	
CD80	CD80	CD80	CD80
CD86	CD86	CD86	CD86
	CD200		CD200
CD200R1	CD200R1		CD200R1
CD274	CD274	CD274	CD274
	CTLA4		CTLA4
HAVCR2	HAVCR2	HAVCR2	HAVCR2
HLA-A			
HLA-B			
HLA-DRB1	HLA-DRB1		HLA-DRB1
ICOS	ICOS	ICOS	ICOS
ICOSLG			
	IGSF11	IGSF11	
LAG3	LAG3		LAG3
			LGALS9
PDCD1	PDCD1		PDCD1
PDCD1LG2	PDCD1LG2		PDCD1LG2
	SIRPA		SIRPA
	TNFRSF4		TNFRSF4
TNFRSF9	TNFRSF9	TNFRSF9	TNFRSF9
TNFRSF18	TNFRSF18	TNFRSF18	TNFRSF18
	TNFSF4	TNFSF4	TNFSF4
TNFSF9	TNFSF9		
TNFSF14	TNFSF14		TNFSF14
	TNFSF18		
	VSIR		VSIR

(IV) *ITGA5* impact on the immune checkpoint's expression

Since it was confirmed that *CD68*^{High}*HIF1A*^{High} tumors present an immune checkpoint expression profile different from the *CD68*^{High}*HIF1A*^{Low} , and that Integrin α5 expression is upregulated under hypoxia, and having into account a previously publication showing that integrins are regulators of immune checkpoint expression (Vannini et al., 2019), we tested whether in our system Integrin α5 is able to modulate the immune checkpoint expression. For that, TCGA was used to assess whether *ITGA5* expression, within the *CD68*^{High} tumors, is correlated with the expression of the immune checkpoints found to be upregulated in the *CD68*^{High}*HIF1A*^{High} tumors compared with *CD68*^{High}*HIF1A*^{Low} tumors, both in total colon cancer cases, and in the right colon cases. *ITGA5* was considered correlated with immune checkpoint expression when Spearman R value was greater or equal to 0.5.




P<0.0001
Spearman R=0.4238
CD200
ITGA5
P=0.0009
Spearman R=0.2830
CD200R1
ITGA5
P<0.0001
Spearman R=0.3896
CD274
ITGA5
P<0.0001
Spearman R=0.4173
CTLA4
ITGA5
P<0.0001
Spearman R=0.6501
HAVCR2
ITGA5
P<0.0001
Spearman R=0.4363
HLA-DRB1
ITGA5
P<0.0001
Spearman R=0.3979
ICOS
ITGA5
P=0.2237
Spearman R=0.1054
IGSF11
ITGA5
P=0.0004
Spearman R=0.3031
LAG3
ITGA5
P<0.0001
Spearman R=0.3293
PDCD1
ITGA5
P<0.0001
Spearman R=0.6456
PDCD1LG2
ITGA5
P<0.0001
Spearman R=0.6404
SIRPA
ITGA5
P<0.0001
Spearman R=0.6466
TNFRSF4
ITGA5
P<0.0001
Spearman R=0.5352
TNFRSF9
ITGA5
P<0.0001
Spearman R=0.4739
TNFRSF18
ITGA5

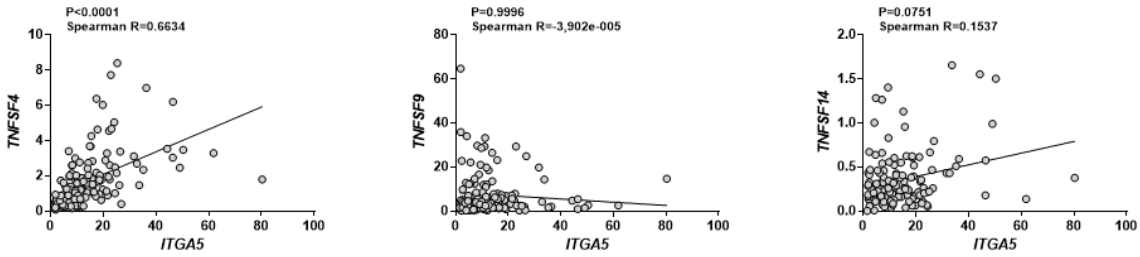


Figure 25. *ITGA5* correlation with immune checkpoints. RNAseq expression data were download from TCGA. The correlation was assessed, within *CD68*^{High} tumors, in relation to immune checkpoint found to be upregulated within the *CD68*^{High}*HIF1A*^{High} tumors compared with *CD68*^{High}*HIF1A*^{Low} tumors, both in total colon cancer cases, and in the right colon cases (Table 5). Spearman statistical test was used to assess correlation, which was considered positive when Spearman R ≥ 0.500.

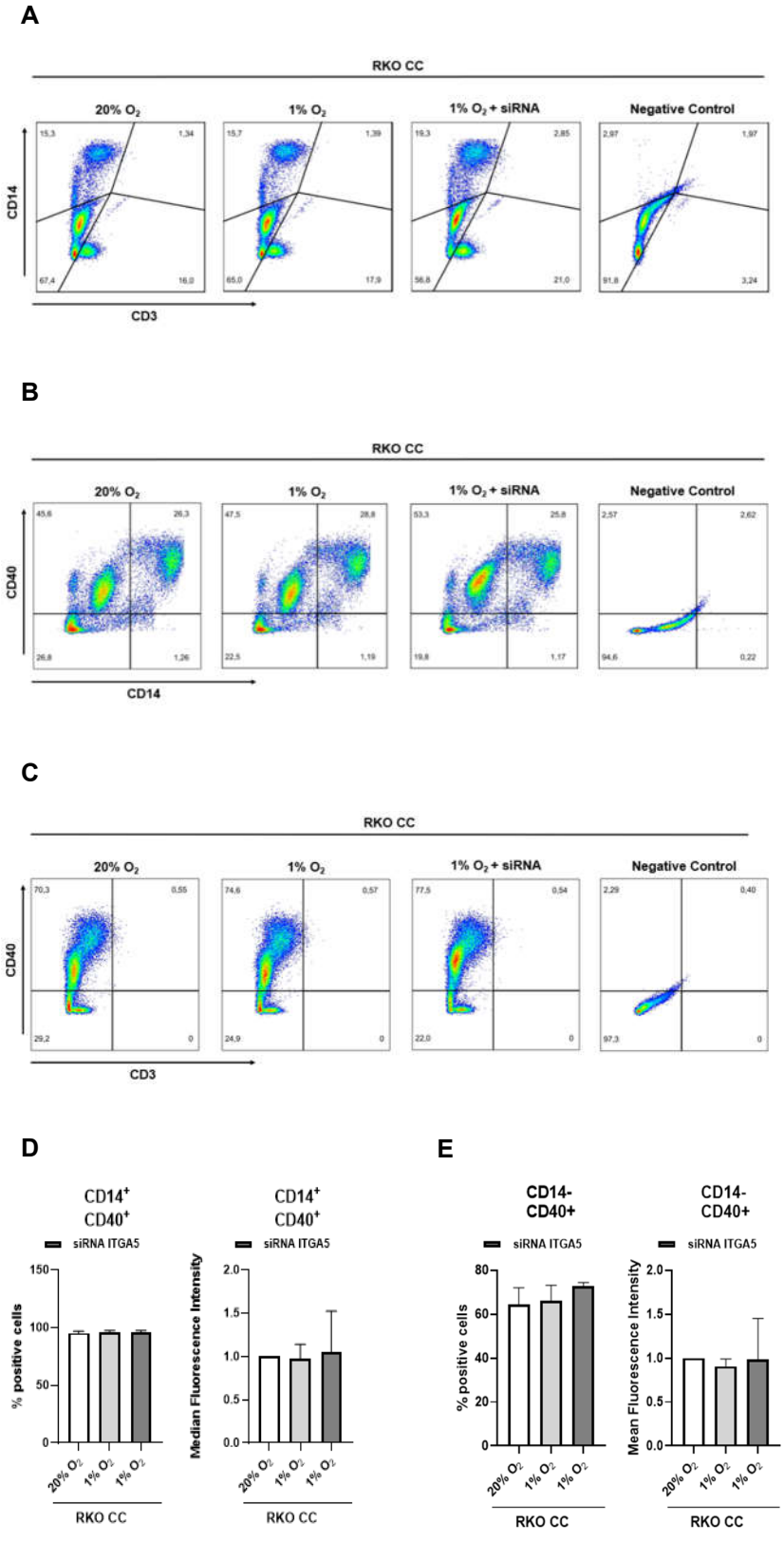
From this analysis it was concluded that twelve of the analyzed immune checkpoints were correlated with *ITGA5*: *CD28*, *CD40*, *CD80*, *CD86*, *HAVCR2*, *PDCD1LG2*, *SIRPA, TNFRSF4, TNFRSF9, TNFRSF18, TNFSF4* and *TNFSF18.*

To test whether Integrin α5 can modulate the expression of these immune checkpoints tricultures of macrophages, T cells and cancer cells RKO or SW480 were established, under normoxia or hypoxia for 24h. In the same experiment tricultures of macrophages, T cells and cancer cells silenced for *ITGA5* were established and maintained under hypoxia for 24h. During the time work of this master, it was only possible to proceed with the experiments regarding four of the immune checkpoints to be tested: CD40, CD80, CD86 and TIM-3 (*HAVCR2*), since the antibodies for cytometry were already available on the lab.

Unfortunately, it was found that the CD80 available for flow cytometry was not working, and the analysis regarding this immune checkpoint was not possible.

CD40 is a member of the tumor necrosis factor receptor family, expressed by B cells, professional antigen-presenting cells, as well as non-immune cells and tumors (Elgueta et al., 2009). The use of macrophage and T cells lineage markers, CD14 and CD3, respectively, allowed to conclude that CD40 is expressed in macrophages and in cancer cells (Figure 26B and G), since there were two distinct CD40 positive populations and none of them was CD3 positive. The expression of CD40 in cancer cells was much higher in the case of RKO than in SW480. Having in consideration that about 60% of the tricultures cells are cancer cells (Figure 26A and F), in the case of RKO almost all population was CD40 positive while in the case of SW480 was a very small percentage.

It was found that there were no significant differences in CD40 expression between the different populations, which happened for tricultures of both cancer cell lines used (Figure 26C and H).



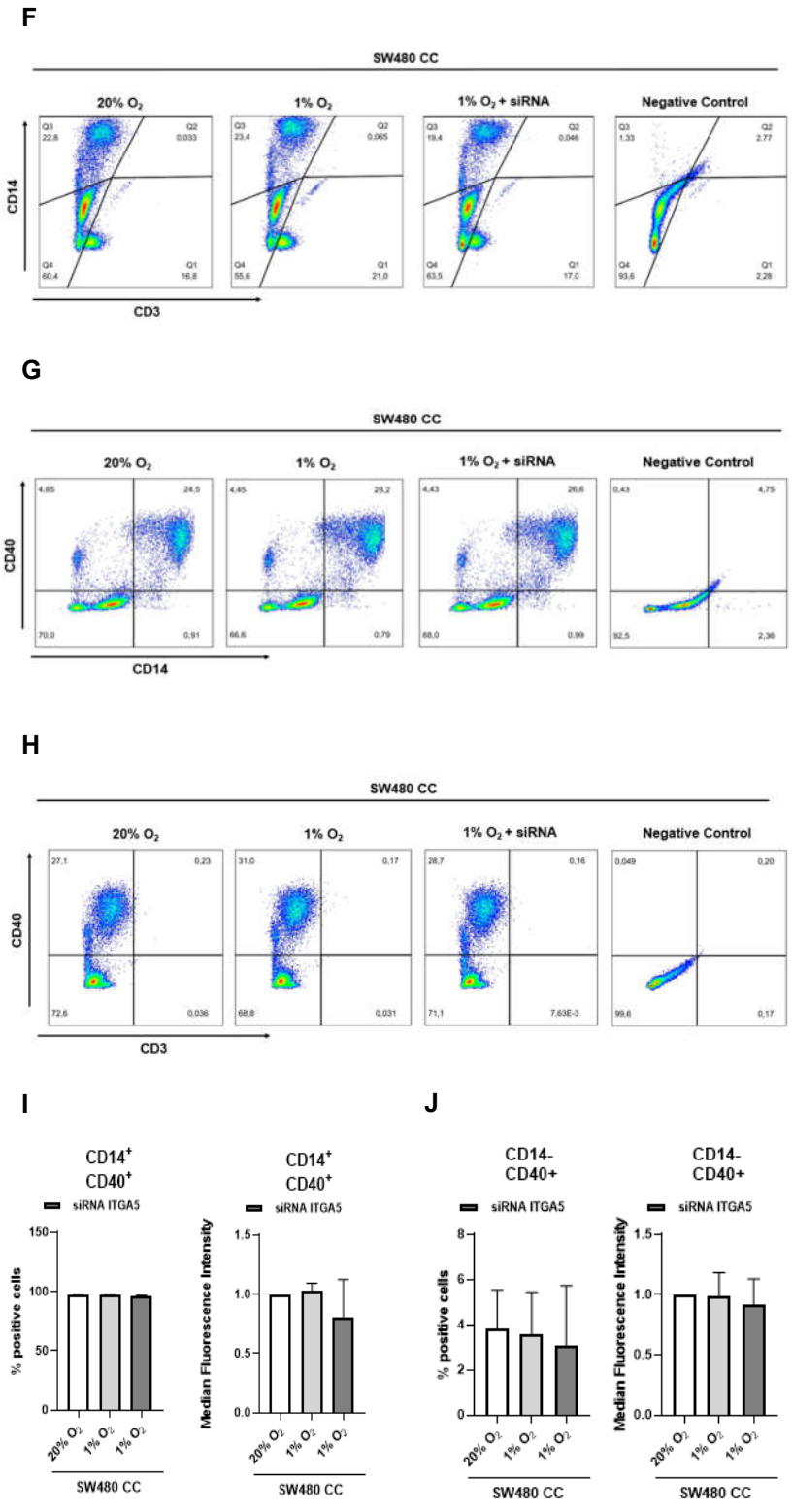


Figure 26. CD40 expression by flow cytometry. CD40 expression was determined by flow cytometry in triculture composed of macrophages, T cells and cancer cells (RKO or SW480), under normoxia and hypoxia, for 24h. Additionally, tricultures of macrophages, T cells and cancer cells silenced for *ITGA5*, exposed to hypoxia for 24h, were used. (A) CD14 versus CD3 dot plots of tricultures with RKO cancer cells. (B) CD40 versus CD14 dot plots of tricultures with RKO cancer cells. (C) CD40 versus CD3 dot plots of tricultures with RKO cancer

cells. (D) Graphs with representation of double positive cells CD14+ CD40+ and graph representing the intensity of median fluorescence in relation to tricultures with macrophages, T cells and RKO under normoxia and hypoxia. (E) Graphs with representation of double positive cells CD14- CD40+ and graph representing the intensity of median fluorescence in relation to tricultures with macrophages, T cells and RKO under normoxia and hypoxia. (F) CD14 versus CD3 dot plots of triculture with SW480 cancer cells. (G) CD40 versus CD14 dot plots of tricultures with SW480 cancer cells. (H) CD40 versus CD3 dot plots of tricultures with SW480 cancer cells. Images are representative of three independent experiments. (I) Graphs with representation of cells CD14+ CD40+ and graph representing the median fluorescence intensity in relation to tricultures with macrophages, T cells and RKO under normoxia and hypoxia. (J) Graphs with representation of cells CD14- CD40+ and graph representing the median fluorescence intensity in relation to tricultures with macrophages, T cells and SW480 under normoxia and hypoxia.

CD86 is constitutively expressed on DCs, Langerhans cells, memory B cells and germinal center B cells. CD86 is also used as a marker for M1 macrophages and together with CD80 plays an important role in T cell activation and survival (Mir, 2015). It was evaluated the expression of CD86 in tricultures with and without cancer cells silenced for *ITGA5*. It was found that CD86 is only expressed in macrophages (Figure 27A and C) and that there were no significant differences in the expression of CD86 between the different populations, which happened for both cancer cell lines used (Figure 27B and D).

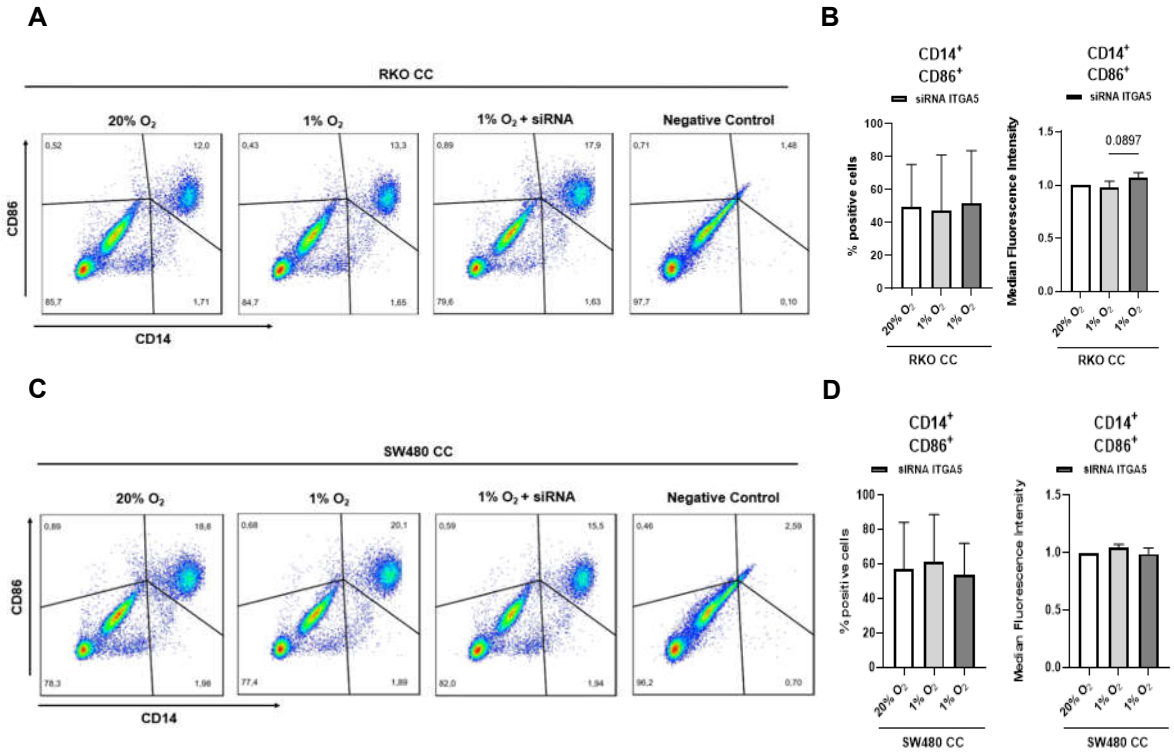


Figure 27. CD86 expression by flow cytometry. (A-D) CD86 expression was determined by flow cytometry in triculture composed of macrophages, T cells and cancer cells (RKO or SW480), under normoxia and hypoxia, for 24h, and tricultures with cancer cells silenced for *ITGA5*, exposed to hypoxia for 24h. (A) CD86 versus CD14 dot plots of tricultures with RKO cancer cells. (B) Graphs with representation of double positive cells CD14+ CD86+ and graph representing the intensity of median fluorescence in relation to tricultures with macrophages, T cells and RKO under normoxia and hypoxia. (C) CD86 versus CD14 dot plots of tricultures with SW480 cancer cells. (D) Graphs with representation of double positive cells CD14+ CD86+ and graph representing the intensity of median fluorescence in relation to tricultures with macrophages, T cells and cancer cells (SW480) under normoxia and hypoxia. Images are representative of three independent experiments.

Finally, it was evaluated the expression of TIM-3, and it was found that there were no statistically significant differences between the analyzed populations (Figure 28B and D).

Experiments presented some variability, expected since primary culture cells were used. As such, the experimental "n" should be increased, for further clarification of the obtained results.

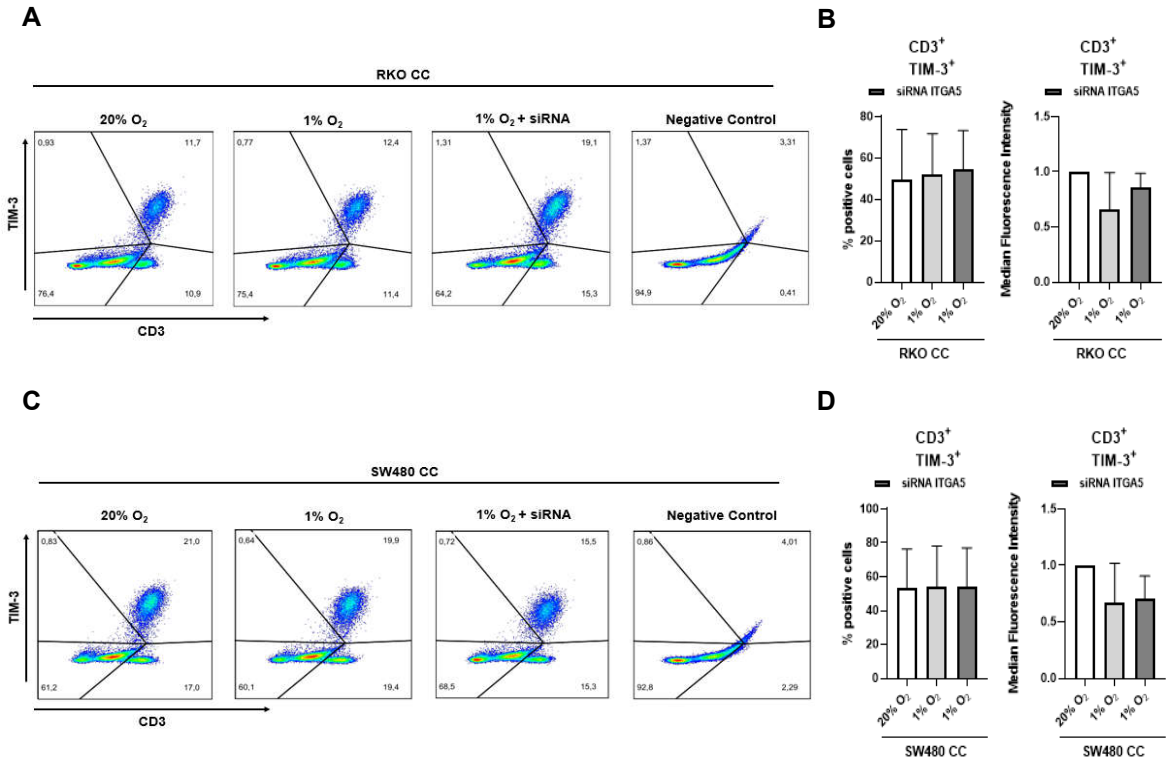


Figure 28. TIM-3 expression by flow cytometry. (A-D) TIM-3 expression was determined by flow cytometry in triculture composed of macrophages, T cells and cancer cells (RKO or SW480), under normoxia and hypoxia, at 24h, and tricultures with cancer cells silenced for ITGA5, exposed to hypoxia for 24h. (A) TIM-3 versus CD3 dot plots of tricultures with RKO cancer cells. (B) Graphs with representation of double positive cells CD3+ TIM-3+ and graph representing the intensity of median fluorescence in relation to tricultures with macrophages, T cells and RKO under normoxia and hypoxia. (C) TIM-3 versus CD3 dot plots of tricultures with SW480 cancer

cells. (D) Graphs with representation of double positive cells CD3+ TIM-3+ and graph representing the intensity of median fluorescence in relation to tricultures with macrophages, T cells and cancer cells (SW480) under normoxia and hypoxia. Images are representative of three independent experiments.

Discussion

Due to excessive proliferation and aberrant vascularization, the solid tumor microenvironment generally presents low oxygen levels (Muz et al., 2015). Despite hypoxia being a common feature in solid tumors, few studies have explored the impact of hypoxia on immune cells in the colon cancer microenvironment. Instead, several studies were carried out in order to understand the interaction between macrophages and cancer cells under normoxia, neglecting a potential role of hypoxia in the modulation of those interactions.

In this study direct triculture systems of colon cancer cells, macrophages and T cells were established.

To analyze the impact of hypoxia on RNA expression profile of the tricultures it was used the Nanostring Tumor Signaling 360 Gene Expression panel, and the analysis confirmed a significant change in the expression profile under hypoxia. For the selection of the most interesting genes to further studies, patients' data (TCGA) was assessed, and it was given priority to genes differently expressed between *CD68*^{High}*HIF1A*^{Low} and *CD68*^{High}*HIF1A*^{High} tumors, in the total number of colon cancer patients and in tumors from the right colon, since those groups of tumors present differences in survival, and genes correlated with *HIF1A*, because is a hypoxia master molecular regulator, and also because of the survival data. From this analysis five genes emerged as the most interesting to further study: *CAV1*, *CXCR4*, *FCAR*, *ITGA5* and *PFKFB3*.

CAV1 codifies for caveolin-1, that exists in high amounts in adipocytes, fibroblasts, endothelial cells, muscle cells and epithelial cells (Williams & Lisanti, 2004). Caveolin-1 is a scaffolding protein that may also regulate endothelial Ca^{2+} influx through structural modulation of caveolae and functional interaction with ion channels (Brazer et al., 2003). Caveolin-1 role in cancer is controversial as is associated with metastasis promotion and described as a tumor suppression gene (Arpaia et al., 2012; Quest et al., 2008). Caveolin-1 has been particularly studied in breast cancer, where in mice is known to play a role in the pathogenesis of breast epithelial cell hyperplasia, tumorigenesis and metastasis (Bouras et al., 2004). Interestingly, the expression of *CAV1* RNAs is reduced in primary breast tumors (Witkiewicz et al., 2009). Regarding colon cancer it was reported that adenocarcinomas present an overexpression of caveolin-1, comparing with adenomas and normal tissue (Fine et al., 2001). Furthermore, the study of chemotherapy sensitive and resistant cell lines showed that *CAV1* is associated with drug resistance (Selga et al., 2008). Also, it was reported that hypoxia induces caveolin-1 expression in HT-29 cell, in a HIF1-α dependent manner, which was associated with increased migration and invasion (Castillo

Bennett et al., 2018). Moreover, and underlining the caveolin-1 dual role in cancer, its expression was associated with reduced transcriptional activity of HIF-1α under hypoxia, which in turn associated with decreased transcription of VEGF, limiting the tumor growth (Sanhueza et al., 2020). Our results, in contrast with the previous reports showed that hypoxia did not alter caveolin-1 protein expression in any of the tricultures analyzed, despite the upregulation at RNA level (Figure 18B). Those differences may be dependent of the presence of the immune cells, once the previous *in vitro* works were performed only with cell lines, or it may be dependent on the timepoint at which the protein was extracted.

CXCR4 is a chemokine receptor of CXCL12, a molecule with potent chemotactic activity for lymphocytes, is not normally expressed in normal tissues, but is expressed in several types of cancer, including breast, ovarian, melanoma, prostate and CRC (F. Balkwill, 2004; Müller et al., 2001; Scotton et al., 2001). CXCR4 plays an important role in the differentiation, mobilization, migration and polarization of hematopoietic cells (Pawig et al., 2015). More recently, CXCR4 and CXCL12 have been shown to control the regeneration of multiple organs and tissues, including lung, heart, liver, and the nervous system (Bianchi & Mezzapelle, 2020). In CRC, CXCR4 expression in tumor cells is associated with recurrence and metastasis (J. Kim et al., 2006). Studies suggested that hypoxia-induced acquisition of cancer stem cell characteristics was associated with CXCR4 activation in lung cancer cells lines (Kang et al., 2019). Furthermore, hypoxia has been shown to be associated with increased metastatic capacity of breast cancer through positive regulation of CXCR4 (Cronin et al., 2010) and with promotion of cell migration in osteosarcoma (Guo et al., 2014). Regarding colon cancer it is reported that hypoxia increased the expression of CXCR4 both at RNA and protein level, in a HIF-1α mediated process, which was associated with increased cancer cell migration (Romain et al., 2014). Given the technical constrains it was not possible to confirm whether the CXCR4 expression increases at protein level under hypoxia in our triculture system, but considering its described role in colon cancer, and also our CXCR4 increase at RNA level, in line with the others studies, we shall invest in a good western blot antibody to address this issue.

FCAR codifies for CD89, a member of the Fc receptor family, and is expressed on neutrophils, monocytes, eosinophils and macrophages and on subsets of DCs. CD89 is associated with several immunologic defense processes, including phagocytosis, antibody-dependent cell-mediated cytotoxicity, and stimulation of the release of inflammatory mediators (Otten et al., 2005; van der Steen et al., 2009). Also, with FcaRI receptor can trigger antibody-dependent cell-mediated cytotoxicity by neutrophils, degranulation of eosinophils and phagocytosis by monocytes and macrophages (Snoeck et al., 2006). For this reason, FcaRI engagement has long been proposed as a way to enhance the well-

recognized anti-tumor ability of neutrophils (Souto et al., 2009). It was previously reported that hypoxia modulate CD89 expression in monocytes (Bosco et al., 2006), however the role of CD89 in the context of colon cancer is not explored. Despite the increased FCAR expression in the tricultures of all cell lines, we only found an increased in MFI in RKO cells between normoxia and hypoxia condition at protein level. However, further validation with the remaining cell lines should be done.

PFKFB3 is a glycolysis critical regulatory enzyme (Navarro-Sabaté et al., 2001). In glioblastoma, reduced expression of PFKFB3 caused by downregulation of HIF-1α promotes cell death (Blum et al., 2005). In breast cancer, increased PFKFB3 expression and glycolysis metabolism in tumor cells occurs due to constitutive loss of HER2 (O'Neal et al., 2016). PFKFB3 is highly expressed in CRC cells, and it is described in the literature that it can be strongly induced under hypoxia (Bando et al., 2005). Interestingly, studies have shown that hypoxia induces the expression of different PFKFBs both *in vitro* and *in vivo* in several mice organs (Minchenko et al., 2003). Our results showed that the observed increased in the *PFKFB3* expression at RNA under hypoxia it is not mirrored at protein level. However, an increased in protein level in hypoxia is observed in SW48 cocultures, while a slightly decreased is observed in Caco-2 cocultures. Of note is the fact that SW48 cell line is MSI, and Caco-2 is MSS (Ahmed et al., 2013) and, even if nothing is reported regarding PFKFB3 with MSI status, could be possible that it may account for the differences in the cell lines behavior.

ITGA5 codifies for Integrin α5, that is a receptor for fibronectin and fibrinogen, which is described as being important for proliferation, migration, invasion and metastasis in several cancer types (Y. Deng et al., 2019; Tani et al., 2003). It is associated with a poor prognosis in triple negative breast, lung and ovarian cancer (Gong et al., 2016; Xiao et al., 2018; Zheng et al., 2016). In colon cancer, upregulation of Integrin α5 increased cell growth, tumorigenesis and decreased cell apoptosis (Yu et al., 2019). *HIF1A* and *ITGA5* are associated with resistance to chemotherapies in triple negative breast cancer (Saatci et al., 2020), and in colorectal cancer, hypoxia is associated with *ITGA5* upregulation, a factor contributing to tumor progression and poor survival (Nersisyan et al., 2021). From the proteins analyzed Integrin α5 was the only one that, as in the array, presented an upregulation under hypoxia in the tricultures of all cell lines studied. In this work it was shown that Integrin α5 expression had no impact neither in cell viability nor on T cell activation under hypoxia. However, giving integrin α5 association with invasion, this protein could also be involved in the macrophage and hypoxia-mediated invasive phenotype reported by the group in a previous work (Martins et al., 2020).

It is described that tumors with high macrophage infiltrate and hypoxic TME are associated with poor prognosis and therapy resistance in breast, gastric and lung cancer (Semenza, 2015; M. Yang et al., 2018). That may not be the case in colon cancer, as previous studies by our group identified a positive correlation between *HIF1A* and *CD68*, and reported that patients with tumors with greater macrophage infiltration and with high *HIF1A* levels were associated with better survival, (Martins et al., 2020) particularly in the right colon tumors. These results led us to hypothesized that the observed survival differences may be due to differences in the expression profile of immune checkpoints. Currently, it is known that hypoxia has been shown to modulate the expression of some of these immune checkpoints, such as CTLA4, PD-1, PD-L1 and Tim-3 (Hu et al., 2021). In order to clarify this question, we evaluated the expression of immune checkpoints in tumors with differences in survival, and it was proved that the tumors with the better survival, *CD68*^{High}*HIF1A*^{High}, show a different immune checkpoint expression profile, when compared with *CD68*^{High}*HIF1A*^{Low} tumors, with marked upregulation of several immune checkpoints. It was also observed, when comparing right colon with left colon, that right colon tumors have an increased expression of most of the immune checkpoints studied, compared with left colon (Table 6). These data can be supported by the fact that tumors that are predominantly located in the proximal region of the colon are associated with immune activation (Inamura, 2018) and upregulation of immune checkpoints molecules, such as PD-1 and PD-L1 (Becht et al., 2016).

Interestingly, immune checkpoints upregulated in *CD68*^{High}*HIF1A*^{High} tumors, considering all tumors analyzed, were also found upregulated in tumors of the *CD68*^{High}*HIF1A*^{High} at the right colon.

The analysis of the tumors with better prognosis showed up-regulation of immune checkpoints associated both with T cell activation and repression, underlined the fact that should be a fine-tuned balance between these two kinds of immune checkpoint that will account for the final outcome.

Finally, and since it was previously reported that integrins can function as regulators of immune checkpoint expression, namely PD-L1 (Vannini et al., 2019), we tried to understand whether *ITGA5* could have the same function. TCGA data showed that *ITGA5* is correlated with twelve of the analyzed immune checkpoints. These data are in line with data published this year showing that *ITGA5* correlates with most of the immune checkpoints present in gliomas (S. Li et al., 2022).

Our results showed that *ITGA5* was not a regulator of none of the three immune checkpoints analyzed (CD40, CD86 and TIM-3), remained to be explored its function in the

other nine immune checkpoint found to be correlated with its expression. Although our studies indicated that *ITGA5* is not involved in the regulation of CD40, it is known that integrin α5β1 binding is required for CD40/CD40L signaling, possibly through CD40-CD40L-integrin complex formation on the cell surface, and that CD40L monomer engages with α5β1 integrin and induces antiapoptotic signals (Takada et al., 2019). However, there are hardly any studies that link integrins with immune checkpoints.

Conclusions and future perspective

This study aimed to understand which molecular mediators were involved in cellular responses in the colon tumor microenvironment under hypoxia, and for this purpose were established direct tricultures of macrophages, T cells and colon cancer cells, under conditions of normoxia (20% O_2) or hypoxia (1% O_2).

It was possible, with this work, to validate the importance of Integrin α5, whose expression is increased by hypoxia in all cell lines evaluated. In parallel, it was possible to conclude that the right and left colon present a different immune checkpoint expression profile that may be related to the colon cancer patient's survival differences previously observed by our group. Finally, the impact of Integrin α5 on the regulation of immune checkpoints was evaluated, and we concluded that Integrin α5 does not seem to regulate the expression of CD40, CD86 or TIM-3 in hypoxia.

Overall, it can be considered that hypoxia influences immunomodulation at the colon cancer microenvironment and that its presence has an impact on the expression profile of immune checkpoints. These data provide relevant information that may pave the way for further studies aiming to unravel new biomarkers and to develop new and more efficient therapies for colon cancer.

However, further work has to be done to consolidate our results. From the Nanostring analysis we only validated the genes that were filtered to meet the criteria established in this master's work, nevertheless, it would be extremely interesting to validate other genes that also show to be significantly altered under hypoxia.

Since it was not possible to validate CXCR4 at protein level because the antibody did not work, it would be relevant to retry to validate the array results with a better antibody in order to understand whether hypoxia affects CXCR4 expression in any of the six cell lines used at protein level. Although there were no changes in all cell lines, it would be important to evaluate the differences observed in the expression of PFKFB3 in SW48 and Caco-2 cell lines.

Another point that would be important to add in this study would be the evaluation of Integrin α5 impact on the regulation of the immune checkpoints with which it is correlated and were not tested: *CD28*, *PDCD1LG2, SIRPA, TNFRSF4, TNFRSF9, TNFRSF18, TNFSF4* and *TNFSF18,* using the same strategy used for the immune checkpoints already analyzed. Moreover, any positive result should be validated in tricultures with all cell lines, and in patients' samples.

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