Escola Superior de Saúde do Porto
Instituto Politécnico do Porto

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Influence of KRAS activation in the colorectal cancer immunosurveillance escape

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"It is not the strongest of the species that survives, nor the most intelligent that survives. It is the one that is most adaptable to change."

Charles Darwin

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Abstract

The immune system as a host defense system watches the cell growth and division, eliminating cells with antigens different from those present in healthy cells. However, some transformed cells have the capacity, through various mechanisms, to escape the immune system. Genomic instability and some mutations are pointed as possible mechanisms supporting the immune surveillance escape, as is the case of oncogenic mutations. KRAS mutation is present in about 40% of cases of colorectal cancer and confers to the tumor a greater potential for malignancy. It is known that KRAS and BRAF mutant cancer cells regulate the recruitment, activation, and differentiation of immune cells, promoting tumor evolution by ensuring leakage to the immune system and increasing the proliferative potential. Few evidences highlight an association between a KRAS mutation and myeloid cells, mainly macrophages and neutrophils infiltration. However, the mechanism which determines this interaction remains unclear.

To investigate how the KRAS and BRAF mutations can influence the immune response, either by regulating the expression of immunomodulatory molecules, leading to altered crosstalk between the tumor and the immune system, or by regulating immune cell infiltration.

In our work, a series of immunomodulatory molecules were analyzed by flow cytometry in a panel of KRAS and BRAF mutant colorectal cancer cells in which *KRAS*/*BRAF* was silenced by small interfering RNA. Additionally, the influence of chemotherapy and IFN-γ administration, which is a non-consensual possible therapy, in the expression of immune checkpoints molecules was also evaluated. It was also performed immunohistochemistry staining of F4/80 to access the *in vivo* infiltration and distribution of macrophages in the mouse colonic epithelium.

Preliminary results suggest that *KRAS* silencing lead to the alteration of some molecules involved in the crosstalk with the immune system cells, such as macrophages and lymphocytes, as long as *BRAF* silencing did not cause any alteration. Additionally, in some cases, chemotherapy and IFN-γ administration showed to have some influence on the upregulation of these immune molecules expression, which was impaired by KRAS silencing. On the other hand, KRAS mutation, was not capable to increase the macrophage

infiltration in the mouse colon epithelium.

KRAS activation seems to be capable of regulating the expression of surface markers, which can regulate and suppress the immune response against cancer cells, and to influence the macrophage infiltration when combined with another frequent mutation.

Key words: Colorectal cancer, tumor microenvironment, immune cells, immunosurveillance, immuneescape, immunotherapy.

Resumo

O sistema imune tendo como função a proteção do organismo está atento, entre outros, ao crescimento e divisão celular, eliminando as células com antigénios diferentes daqueles presentes nas células saudáveis do indivíduo. Contudo, algumas células transformadas conseguem, através de vários mecanismos, escapar ao sistema imune. De entre os mecanismos que suportam esta fuga ao sistema imune encontra-se a instabilidade genómica e algumas mutações, como a do gene *KRAS*. Esta mutação ocorre em cerca de 40% dos casos de cancro coloretal e confere um maior potencial maligno. A capacidade das células KRAS ou BRAF mutadas na regulação, recrutamento, ativação e diferenciação das células imunes é já reconhecido e tem sido associada à promoção da evolução do tumor através do suporte na fuga ao sistema imune e do aumento do potencial proliferativo. Porém, o mecanismo que determina esta interação é ainda desconhecido. Assim, o estudo mais aprofundado da influência da mutação KRAS na resposta imune tanto através da regulação da expressão de moléculas imunossupressoras quer através da regulação da infiltração tumoral por células imunes revela-se bastante pertinente. Para tal, no nosso trabalho, analisamos, por citometria de fluxo, uma série de moléculas imunomodeladoras num painel de células de cancro colorectal com mutação *KRAS* ou *BRAF*, antes e após o silenciamento do oncogene. Consequentemente, verificamos a influência da quimioterapia e da administração de interferão-gama na expressão destas moléculas imunomodeladoras. Por fim, determinamos se a mutação KRAS é capaz de levar à maior ou menor infiltração de macrófagos através de imunohistoquímica.

Resultados preliminares sugerem que apenas mutação *KRAS* e não a mutação *BRAF* aumenta a expressão de moléculas imunossupressoras em determinadas situações, modelando a resposta de células imunes, como macrófagos e linfócitos, sendo que esta resposta é também potenciada, em alguns casos pela quimioterapia e interferão-gama. Por outro lado, a mutação *Kras* não é capaz de aumentar a infiltração macrofagocitária no epitélio do colon de ratinho. Em conclusão, a ativação do KRAS parece ser capaz de regular a expressão de moléculas de superfície, regulando desta forma a supressão do sistema imune contra as células tumorais, e ainda consegue, quando em conjugação com a mutação no gene *Apc*, aumentar a população de macrófagos no tumor.

The work presented in this thesis was presented by myself in the following national and international meetings:

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Abbreviations and acronyms

5-FU	5-Fluoruracil
APC	Adenomatous Polyposis Coli
APC	Allophycocyanin
ATCC	American Type Culture Collection
BSA	Bovine Serum Albumin
B2M	Beta-2-Microglobulin
CAF	Cancer-associated Fibroblast
CAR	Chimeric Antigen Receptor
CD	Cluster of Differentiation
cDNA	Complementary DNA
CFSE	Carboxyfluorescein Diacetate Succinimidyl Ester
CIMP	CpG Island Methylator Phenotype
CIN	Chromossomal Instability
CRC	Colorectal Cancer
CTLA-4	Cytotoxic T lymphocyte–associated antigen 4
DAB	Diaminobenzidine
DC	Dendritic Cell
DMSO	Dimethyl Sulfoxide
ECM	Extracellular Matrix
EGFR	Epidermal Growth Factor Receptor
ERRB3	Erb-B2 Receptor Tyrosine Kinase 3

FAP	Familial Adenomatous Polyposis
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
FITC	Fluorescein Isothiocyanate
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
H&E	Hematoxylin Eosin
HLA	Human Leukocyte Antigen
HNPCC	Hereditary Nonpolyposis Colorectal Cancer
HRP	Horseradish Peroxidase
INF-γ	Interferon–gamma
KRAS	Kirsten Rat Sarcoma Viral Oncogene Homolog
LPS	Lipopolysaccharides
MAF	MAF BZIP Transcription Factor
MAPK	Mitogen-activated Protein Kinases
M-CSF	Mitemphage Colony-Stimulating Factor
MHC	Major Histocompatibility Complex
MMP	Matrix Metalloproteinases
MMR	Mismatch Repair
MSI	Microsatellite Instability
MSS	Microsatellite Stability
NF-κB	Nuclear Factor kappa-B
NIH	National Institutes of Health
NK	Natural Killer
NT	Non-Targeting

PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate-Buffered Saline
PDGF	Platelet-derived Growth Factor
PD-L1	Programmed Death-Ligand 1
PE	Phycoerythrin
PI3KCA	Phosphatidylinositide 3-kinase
qRT-PC	Quantitative Real-Time Polymerase Chain Reaction
RNA	Ribonucleic Acid
RPM	Rotations Per Minute
RT	Room Temperature
SAP2	Stomach cancer-associated protein tyrosine phosphatase-2
SDS	Sodium Dodecyl Sulfate
SEM	Standart Error of the Mean
siRNA	Small Interfering RNA
SIRPα	Signal Regulatory Protein 1 alpha
TAA	Tumor-associated Antigens
TBS	Tris-Buffered Saline
TCR	T Cell Receptors
TERT	Telomerase reverse transcriptase
TGF-β	Transforming Growth Factor-β
TIL	Tumor-infiltrating Lymphocytes
TME	Tumor Microenvironment
TNF-α	Tumor Necrosis Factor α
TP53	Tumor Protein p53

Treg	Regulatory T cells
TTP	Tristetraprolin
VEGF	Vascular Endothelial Growth Factor
WT	Wild Type

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Chapter I

Introduction

1.1 Cancer: hallmarks of a heterogeneous disease

Cancer is a major health problem and the second leading cause of death in the world, currently only surpassed by the heart diseases (Siegel et al., 2015). The risk of developing cancer is associated with the geographic and environmental factors, such as tobacco, alcohol, aging, radiation, among others (Danaei et al., 2005). In a few cases, there is a genetic predisposition to this condition (Knudson, 1985). The majority of cases are characterized by a spontaneous mutation event in a normal cell that leads to the formation of an abnormal cell. Normally, these mutations affect proto-oncogenes, promoting a dominant gain-of-function, or in tumor suppressor genes, resulting in a loss of function (Bishop, 1991). When an abnormality is not checked this cell survives and gradually proliferates increasing the tumor size, disorganization, and malignancy. This progression consists of a multistep process that leads to the accumulation of mutations and constitutes an advantage to the neoplasia by becoming highly heterogeneous (Vogelstein and Kinzler, 1993).

In 2000, Hanahan D and Weinberg RA (Hanahan and Weinberg, 2000) defined the acquired functional capacities of cancer cells that allow them to survive, proliferate and disseminate. These hallmarks of cancer, which were lately revised and completed by the same authors, range from growth suppression evasion, replicative immortality, sustained proliferative signaling and angiogenesis, evading of immune destruction, capability to invade, metastasize and to deregulate the cellular metabolism, genome instability and mutation, resistance to apoptosis and tumour promoting inflammation. The last revision highlighted the fundamental role of the TME in the tumor progression, being the tumor-promoting inflammation proposed as an emergent mechanism underlying the acquisition of certain hallmark traits, Figure 1.1 (Hanahan and Weinberg, 2011).

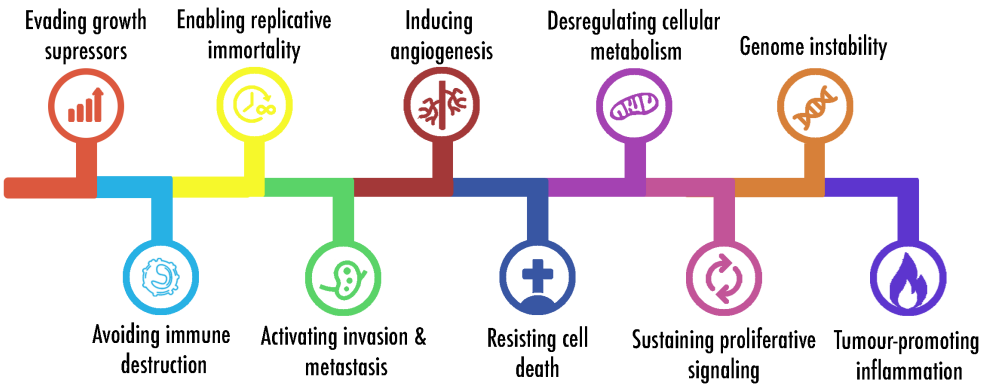

Figure 1.1. The hallmarks of cancer. Cancer cells have fundamental pillars that support its growth, proliferation, immune and growth suppressors evasion. Genomic instability and mutations were pointed out as the main mechanism underlying the acquisition of these hallmark features.

1.2 Tumor Microenvironment

A tumor cannot be seen only as a bulk of cancer cells that overgrowth and proliferate, but it has to take into account the high influence of a constant level of dynamic interactions with what is around these cancer cells, the tumor microenvironment (TME). A tumor consists of cancer cells and tumor-associated host cells. Tumor biology, tumor progression and response to therapy are widely influenced by TME (Quail and Joyce, 2013). TME is constituted by stromal elements, such as fibroblasts, endothelial, adipose and immune-inflammatory cells embedded within an extracellular matrix (ECM), Figure 1.2 (Olumi et al., 1999; Folkman, 2002; Nieman et al., 2013; Murdoch et al., 2008; Nelson and Bissell, 2006). To such degree, neoplastic cancer cells corrupt both the resident and recruited ”normal” cells for their own benefit, while the TME also modulate the cancer features. Indeed, in advanced cancer stages, the TME is considered as a central modulator of cancer cell invasion, intravasation, and extravasation from surrounding blood vessels, and capacity to home and colonize new niches forming metastases (Tauriello and Batlle, 2016). Thus, in the last decade, the interest in the influence of TME on the hallmarks capabilities of the tumor is rising. Although the TMEs are quite heterogeneous among tumors and patients, the common features suggest that targeting the TME cells or their soluble mediators may be an attractive therapeutic strategy (Balkwill et al., 2012; Hanahan and Coussens, 2012).

1.2.1 Cellular components of tumor microenvironment

Apart from the malignant cells, the TME also includes non-cancer cells recruited to tumors, secreted proteins, and blood vessels that surround, support and enhance the tumor

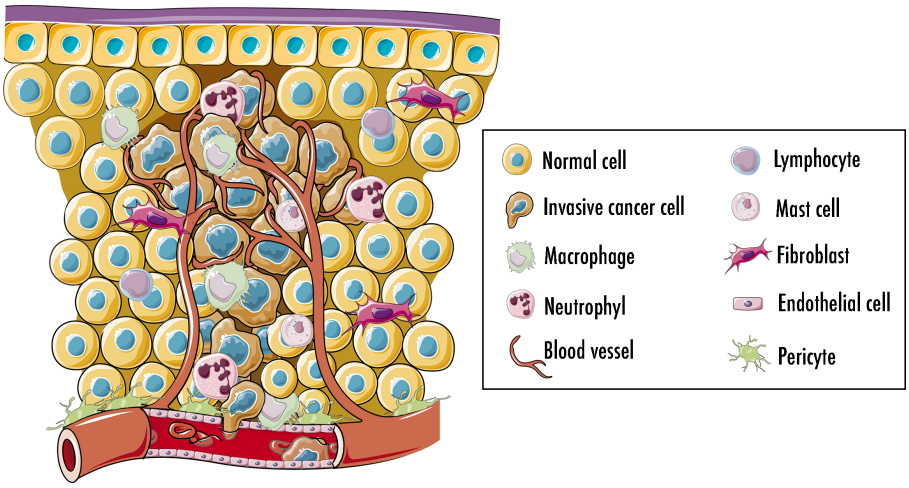

Figure 1.2. The primary tumor microenvironment. Solid tumors are a complex microenvironment composed by neoplastic cancer cells surrounded by numerous cells including endothelial cells from blood and lymphatic circulation, fibroblasts, endothelial cells, pericytes, and a variety of immune cells, namely macrophages, neutrophils, lymphocytes and mast cells.

growth (Hanahan and Coussens, 2012). Interactions of the various components of the TME are significant, as tumor cells can change the behaviour of the other cells present at the surrounding microenvironment and the extracellular matrix architecture, while the TME reciprocally can affect how tumor cells progress and spread (Li et al., 2007; Quail and Joyce, 2013). Initially, the TME can exert inhibitory effects on tumor cells, however, during their progression, they circumvent these inhibitory signals, taking advantage of the surrounding cells to inappropriate growth, invade and ultimately disseminate to distant organs (Joyce and Pollard, 2009). Thus, the tumor behavior is influenced by tumor-associated host cells at both primary and metastatic sites (Mareel et al., 2009).

1.2.1.1 Fibroblasts

Fibroblasts play a critical role in wound healing. Whenever a tissue lesion occurs, fibroblasts are activated in myofibroblasts and promote organ fibrosis (Desmouliere et al., 2004). This tissue remodeling occurs through matrix metalloproteinases (MMP) and other ECM-degrading enzymes produced by fibroblasts that degrade the damaged tissue. This tissue is posteriorly restored also by fibroblasts that synthesize the ECM and collagen. In tumors, they are the most abundant cell type in the stroma (Li et al., 2007). Fibroblasts within tumors acquired the activated phenotype and are so-called cancer-associated fibroblasts (CAFs) (Sugimoto et al., 2006). There are a lot of signals that can mediate the transition of a normal fibroblast to a CAF. Transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF) 2 are key mediators of this transition (Elenbaas and Weinberg, 2001). These mediators are

in the repertoire of the cancer cell signaling, giving to the tumor cells the capacity to activate fibroblasts. On their turn, CAFs have the capacity to promote tumor initiation and progression through specific communications with cancer cells. Additionally, these stromal cells produce ECM-degrading proteases such as the MMP that can help cancer cells to evade the primary tumor site, promoting invasion, metastasis and epithelial-to-mesenchymal transition (Kalluri, 2016; De Wever and Mareel, 2002).

1.2.1.2 Endothelial Cells

During cancer progression, the tumor needs to increase the blood flow to sustain the continuous growth of the neoplastic formation. For this, solid tumors induce the formation of new blood vasculature, creating a complex vascular network, through a process called angiogenesis. During neovascularization there are many soluble factors, present in TME, such as FGFs, vascular endothelial growth factors (VEGFs), PDGFs, and chemokines that in a paracrine manner recruit and stimulate the endothelial cells and the associated pericytes (Carmeliet and Jain, 2011). VEGF is the major tumor angiogenic factor and it is secreted upon genetic or epigenetic induction mainly by cancer cells, but also by fibroblasts, and immune cells in the stroma (Rafii et al., 2002). This genetic induction of VEGF comprises the inactivation of tumor supressors such as TP53 and activation of tumor promoters such as RAS and epidermal growth factor receptor (EGFR) (Wissmann and Detmar, 2006). The reinforced tumor irrigation promotes the high levels of cancer cell proliferation, by the supply of nutrients, oxygen, and growth promoting trophic factors (Bergers et al., 1999). These vessels are also an open way for extravasation of recruited cells, into the tumor, and intravasation of the tumor cells into the circulation, promoting tumor invasion and metastasis (Detmar et al., 2000).

1.2.1.3 Adipocytes

The association between obesity and cancer is not a recent issue, so the role of adipocytes in tumor progression is not surprising. These cells, also known as fat cells, are specialized in the energy excess storage in the form of lipids (Rajala and Scherer, 2003). Additionally, fat cells are also considered an endocrine organ, secreting growth factors, adipokines, and hormones (Kershaw and Flier, 2004). Being a constitutive part of the TME, adipocytes influence not only the cancer cells but also other tumor-associated cells. Adipocyte-produced leptin stimulates angiogenesis and macrophages that in response secret more pro-inflammatory cytokines, like tumor necrosis factor α (TNF-α) (Bouloumié et al., 1998; Suganami et al., 2005). Tumor growth is potentiated by the fatty acids

provided by adipose cells, working as an energy resource that also increases the tumor metastatic potential (Dieudonne et al., 2002).

1.2.1.4 Immune cells

The TME comprises a wide variety of hematopoietic cells and their progeny. There are those intervening in the adaptive immunity, such as T lymphocytes, dendritic cells (DC), and occasionally B cells, and effectors of innate immunity, such as macrophages, polymorphonuclear leukocytes, and natural killer (NK) cells (Li et al., 2007; Whiteside, 2008). Although immune cells are frequent within the tumor, in many cases, they not only fail in the development of an effective antitumoral response but also cooperate with the neoplastic cells promoting oncogenesis (Yu et al., 2007). Additionally, cancer cells can subvert inflammatory cells to have a much more pro- than antitumoral phenotype (Coussens and Werb, 2002). Infiltrating immune cells, as well as other stromal cell types, supply mitogenic growth factors that stimulate and sustain the unchecked proliferation of cancer cells (Balkwill et al., 2005). These cells may also stimulate angiogenesis through VEGF, TNF-α, and other pro-angiogenic chemokines secretion (De Palma and Coussens, 2008). Macrophages, leucocytes, and neutrophils contribute with a wide range of proteases, including MMPs that foster the tumor invasion and metastatic potential by remodeling the ECM components (van Kempen et al., 2006; Kessenbrock et al., 2011).

1.3 Cancer Immunology

Cancer cells, like other aberrant cells in the organism, express a wide and heterogeneous variety of tumor-associated antigens (TAAs). TAAs are often products of mutated cellular genes, those normal genes that became aberrantly expressed or encoding viral proteins, and they differentiate tumor cells from the non-transformed counterparts (Boon et al., 1997; Parmiani et al., 2007). Therefore, the immune system, in its demand to maintain tissue homeostasis, plays an important role in preventing cancer by eliminating neoplastic cells that express not only TAAs, but also innate immune activating receptors (Kawai and Akira, 2010). The principle that the TME exerts a cancer immunosurveillance pressure is widely accepted, but there is another side of the coin that completes the entire scenario of the interrelation between cancer progression and immune infiltrate. The immune system also acts as a tumor-sculpting entity in developing cancers, giving raise to the cancer immunoediting concept (Shankaran et al., 2001; Dunn et al., 2002). This term is more comprehensive considering that it takes into account not only immune system

protection against developing tumors but also their capacity to shape the tumor immunogenicity and tumor development promotion. Previous studies, comparing immunodeficient with immunocompetent mice, demonstrate that those who lacked innate immunity have less immunogenic tumors and, these tumors, also were less immune-edited than similar neoplastic formations derived from immunocompetent mice (Shankaran et al., 2001). The cancer immunoediting process comprises three distinct phases: i) elimination; ii) equilibrium; iii) escape, Figure 1.3. However, some neoplasias enter directly into the equilibrium or escape phases (Dunn et al., 2002).

Elimination

The elimination phase is the hallmark of the original concept of cancer immunosurveillance. In this earlier phase, the immune system orchestrates an organized tumor elimination process through innate and adaptive responses. At this stage, tumors are not clinically detectable, and, if the immune system succeeds, it represents the complete editing process without tumor progression to subsequent phases (Schreiber et al., 2011).

Initially, the innate immune system recognizes the neoplastic transformation and, in a limited way, abrogates it. With the continuous tumor growth, it requires a blood supply and stromal remodeling to support tumor developing. This requirement forces tumor cells and the tumor stroma to release pro-inflammatory cytokines that concomitantly recruit and activate more innate immune cells (Smyth et al., 2001; Dinarello, 2006). NK cells, natural killer T (NKT), $\gamma\delta$ T cells, macrophages, and dendritic cells (DCs) allocate to tumor sites where they also release interferon gamma (IFN-γ) (Smyth et al., 2001). This cytokine in addition to their limited cytotoxicity via anti-proliferative and anti-angiogenic effect also induces apoptosis. Thus, these cumulative anti-tumor effects lead to tumor cell death, and the derived cell debris are ingested by local DCs, that become active (Gollob et al., 2005; Qin et al., 2003; Wall et al., 2003). Mature DCs home into draining lymph nodes where they present TAAs to naive T cells, resulting in the activation of adaptive immune responses (Pardoll, 2002). Activated $CD4^+$ and $CD8^+$ T cells migrate to the primary tumor site and kill the remaining antigen-bearing tumor cells. However, during this whole process, low immunogenic tumor cells are being selected.

Equilibrium

Tumor cell variants that survived the elimination phase enter into a dynamic equilibrium. At this point, the adaptive immune system prevents the tumor outgrowth and continuously sculpts the neoplastic formation. This phase should be the longest part of the cancer immunoediting process, as cancer cells can remain under a dormancy state over a period of many years or decades (Aguirre-Ghiso, 2007). Low immunogenic cells, being

those that are better able to survive in an immunocompetent host, are continuously favored by potent immune selection pressure. Furthermore, although many cancer cells are eliminated, new variants arise in genetically unstable tumors, providing them increased resistance to immune attack. This Darwinian selection, oriented by lymphocytes and IFN-γ, culminates in an immune-insensitive bulk of cancer cells (Whiteside, 2008; Dunn et al., 2004b).

Escape

Surviving tumor variants that acquired insensitivity to immune detection and/or destruction, through genetic and epigenetic changes, come into this last stage of the cancer immunoediting. Progression from equilibrium to escape can occur through: i) disruption of the immune system; ii) in response to cancer-related immunosuppressors; iii) changes in tumor cells, such as antigens loss or increased resistance to cytotoxicity effects of immune cells, driven by immune editings (Dunn et al., 2004b,a). Ultimately, escaping tumors cells give rise to a clinically observable condition that can lead to host death (Dunn et al., 2002).

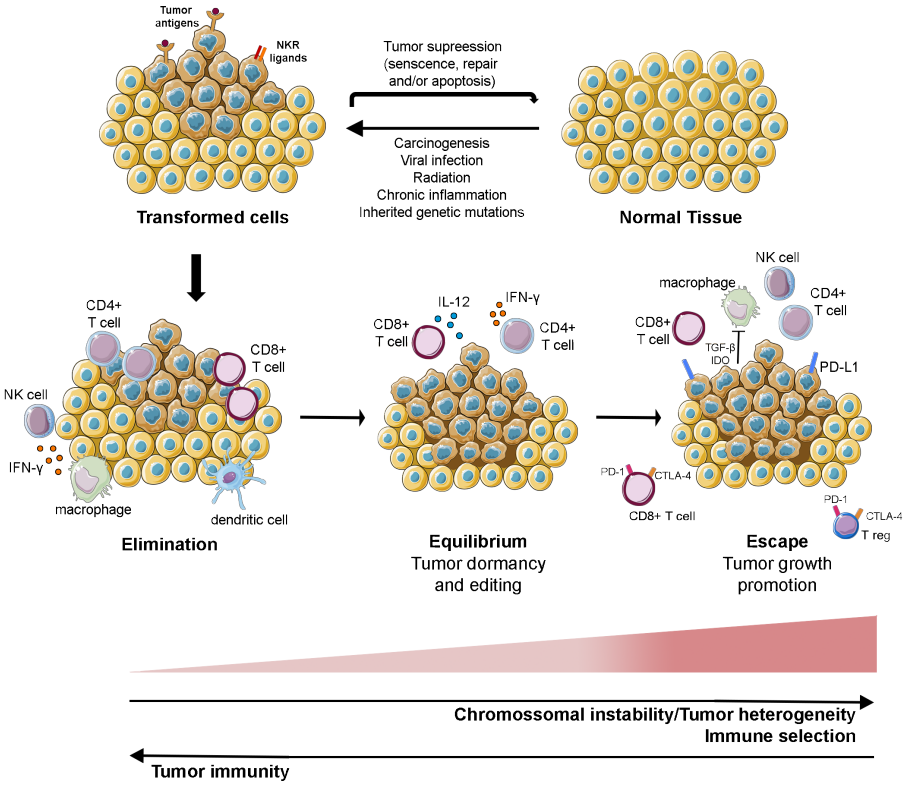

Figure 1.3. The cancer immunoediting concept. Cancer immunoediting encompasses three processes: elimination, equilibrium, and escape. The elimination process corresponds to the cancer immunosurveillance as the immune system tries to eliminate the neoplastic formation. In the equilibrium phase, the tumor is under a dormancy state, since the immune system prevents the tumor outgrowth. In consequence of the constant immune selection, increasing of CIN, and a decrease of tumor immunity the tumor becomes more aggressive and finally escapes from the immune control. In this phase, the outgrowth is no longer controlled and tumors become clinically apparent. Adapted from Schreiber et al. (2011).

1.3.1 Immune cells and cancer

Multiple immune cells invade tumor sites, including T Cells, NK Cells, DCs, Macrophages and B Cells. The extension of the invasion and the frequency with each cell is represented in the immune infiltrate composition largely varies according to the tumor type. However, their anti-tumor functions are frequently repressed being hugely influenced to promote tumor proliferation (Whiteside, 1998). Thus, each immune cell of the TME comprises a certain set of functions. However, in this work we will focus mainly on the regulation of the crosstalk between cancer cells, and macrophages and T cells, since these are the two main types of immune cells present in the TME.

1.3.1.1 Macrophages

Macrophages have fundamental roles in both innate and adaptive immune responses. There are macrophage populations that reside in virtually all tissues, however, when needed the macrophage population increases through monocyte differentiation. They differentiate from circulating peripheral blood mononuclear cells (PBMCs), which migrate into the tissue in response to pro-inflammatory cytokines, growth factors, and microbial stimuli (Tacke and Randolph, 2006). The recruited macrophages contribute to the innate immune responses by the coordination of leukocytic infiltration and by phagocyting foreign particles, the reason they are considered the first line of defense (Martinez-Pomares et al., 2003). Additionally, macrophages also collaborate with T and B cells through both cell-to-cell interactions and by secretion of cytokines, chemokines, enzymes, arachidonic acid, reactive radicals, and metabolites. Beyond this, they are also involved in tissue remodeling, thrombosis, and homeostasis (Sica et al., 2008; Arango Duque and Descoteaux, 2014).

Monocytes recruitment is one of the primary events in tumor development, being the macrophages a major component of leukocyte infiltrates in tumors, where they exhibit complex dual functions (Balkwill and Mantovani, 2001). These immune cells have remarkable functional plasticity due to their capacity to adjust their morphology and function in response to tissue microenvironment. Thus, they can assume a spectrum of roles according to the momentary needs for the tissue homeostasis (Mosser and Edwards, 2008). In general, the two extremes of the possible differentiation states include the classically activated (M1) macrophages and the alternatively activated (M2) macrophages (Mantovani et al., 2002). This nomenclature, M1 and M2 macrophages, is derived from the type of immune response, Th1 or Th2 respectively, that each one supports (Fraternale

et al., 2015).

The classical or M1 macrophages activation occurs in response to lipopolysaccharides (LPS), IFN-γ, microbial products, and TNF and is characterized by enhanced phagocytose of microbes or tumor cells, presentation of antigens to T cells, leading to an adaptive immune response, and production of pro-inflammatory cytokines (Mantovani et al., 2002). M1 macrophages are recognized by their anti-tumor activities that encompass the induction of cytotoxicity and tumor apoptosis which leads to the reduction of tumor size and metastasis (Erreni et al., 2011). Characteristically, macrophages express specific surface receptors, namely cluster of differentiation (CD) 80, CD86, and also major histocompatibility complex (MHC) class I and class II molecules, which are required for the presentation of TAAs (Mantovani et al., 2002, 2004). On the other side, M2 polarized macrophages are induced by IL-10, IL-4 or IL-13, glucocorticoid hormones, and vitamin D3. They exhibit distinct functional phenotypes, and display high levels of the mannose receptor (CD206) and of the scavenger receptor (CD163) (Mantovani et al., 2002, 2004). M2 macrophages population, in contrast with the classic M1 polarization, suppresses Th1 mediated-inflammation through IL-1b and IL-10 secretion and promotes angiogenesis, tissue remodeling, and repair (Mantovani et al., 2004).

In tumors, the cytokine repertoire plays a central role in the orientation and polarization of recruited monocytes (Sica et al., 2006). In the tumor context, TAMs are similar to M2 macrophages and have been shown to influence fundamental aspects of tumor biology. These macrophages have a pro-tumor phenotype secreting IL-10 and TGF-β, along with tumor cells (Mantovani et al., 2002). IL-10 and TGF-β preferentially drive monocyte differentiation into macrophages, instead of DC, and supports the acquisition of the M2 phenotype contributing to a general suppression of antitumor activities. It was proved that IL-10 polarized macrophages, bearing an M2 phenotype, are more efficient in promoting tumor growth, angiogenesis, cancer cell motility and invasion through the secretion of VEGF, EGF (Allavena et al., 2000; Mantovani et al., 2002; Cardoso et al., 2015; Pollard, 2004; Balkwill and Mantovani, 2001; Sica et al., 2008).

High levels of TAM infiltration is frequently associated with poor prognosis in various cancer types, reducing the response to therapy and the overall survival (Mantovani et al., 2006; Zhang et al., 2013). In colorectal cancer, in particular, there are controversial results as some demonstrate poor prognosis associated with TAM infiltration (Pollard, 2004), and others observe a good prognosis when the tumor was more infiltrated by

TAMs (Gordon and Martinez, 2010).

1.3.1.2 T Lymphocytes

T lymphocytes or T cells have a significant role in orchestrating immune responses either through activation of other cells or directly killing foreign pathogens (Raphael et al., 2015). The activation of T cells occurs through the interaction of antigens presented by MHC with a specific variety of T cell receptors (TCR) (Brucklacher-Waldert et al., 2014). There are several different effector T cells, commonly divided into $CD4^+$ and $CD8^+$ T cells. The differentiation pattern of T cells depends on the cytokine milieu during activation (Corthay, 2006). $CD4^+$ T cells are T helper cells that mediate the inflammatory process through the secretion of cytokines that suppress or enhance the immune activity. $CD4^+CD25^+$ T cells, also known as T regulatory (Treg) cells are a subset of $CD4^+$ cells that inhibit the immune response, preventing the action of immune cells from remaining indefinitely active. T regs express high levels of cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4) and programmed cell death protein 1 (PD-1) through which suppress effector T cells (Lohr et al., 2006; Keir et al., 2008). However, other subsets of $CD4^+$ cells also have the capacity to activate and promote the growth of cytotoxic T cells and to maximize the phagocytic activity of phagocytes, such as macrophages (Brucklacher-Waldert et al., 2014). On the other hand, $CD8^+$ T cells the cytotoxic T cells recognize an antigen, bind and kill foreign cells through the secretion of cytolytic granules. These granules contain perforin and granzyme A and B to induce cell apoptosis (Harty et al., 2000; Catalfamo and Henkart, 2003).

Tumor-infiltrating lymphocytes (TIL) can attack tumor cells and eradicate the tumor. The presence of $CD4^+$ T cells has been associated with worse prognosis while the $CD8^+$ T lymphocytes infiltration correlate with better prognosis and overall survival (Mareel et al., 2009). Moreover, the highest expression of cytotoxic T cells markers in tumors is associated with a decrease in tumor recurrence and with the absence of early metastasis (Galon et al., 2006). In colorectal cancer, it was described that approximately 42% of the TIL were $CD4^+$ and 27% were $CD8^+$ (Oliveira and Velho, 2013). Surprisingly, in colorectal cancer, tumor-infiltrating Tregs should be considered allies in the anti-tumor response in that the high density of Tregs infiltration has been associated with improved survival (Ladoire et al., 2011; Salama et al., 2009).

1.3.2 Cell Surface Imune Regulators

The immune system is coordinated by a large number of molecules, some of them positively controlling and enhancing the immune response and others inhibiting it to prevent the immune system from attacking cells indiscriminately. The molecules that compose MHC complex are a good example of immune response activators participating in the antigen presentation to cytotoxic T cells (Townsend et al., 1986). However, immune checkpoints are also very crucial for self-tolerance. CTLA-4 and PD-1/PD-L1 are negative regulators of T cell responses, but others, such as SIRPα/CD47, LAG-3, TIM-3, and BTLA, are also examples of inhibitory immune checkpoints. These molecules through distinct mechanisms and sites of action have been related to the tumor immunosurveillance escape. Positively, inhibitory checkpoint molecules are targetable by monoclonal antibodies thus enabling cancer immunotherapy, which in turn inhibits its suppressive functions in multiple types of cancers (Sabatos et al., 2003; Watanabe et al., 2003; Triebel et al., 1990). This compelling possibility for future new approaches to cancer treatment makes quite interesting the in-depth knowledge of these molecules. Thus, and supported by the recent reports that associate these molecules with the presence of mutations in oncogenes, we proceed to investigate if the presence of KRAS mutations in the tumor cells can affect the number of immune molecules at its cell surface (Casey et al., 2016; Coelho et al., 2017). For this purpose, we focused on the molecules from the immunological domain that are expressed at the surface of tumor cells, such as HLA-ABC, SIRPα-CD47, and PD-L1.

1.3.2.1 HLA-ABC

TAAs *per se* do not guarantee the immune system activation as this process requires antigen-processing machinery used by both normal and tumor cells to present peptides to T cells (Townsend et al., 1986). This process is mediated by the MHC complex that displays peptide fragments of intracellular antigens of expressing-cells, exposing them to cytotoxic ($CD8^+$) T cells recognition. Normal antigens derived from normal cells do not induce a response on $CD8^+$ T cells. However, these immune cells will become activated when exposed to peptide fragments of non-self-proteins such as those present in cancer cells, triggering an immune response directed against the presented TAAs. TAAs presentation to immune cells constitutes a great possibility for tumor recognition by the host immune system, however, as previously mentioned, tumor cells developed strategies to escape the immunosurveillance.

One mechanism through which tumor cells evade T-cell recognition is by the par-

tial or total downregulation of MHC class I genes, *human leukocyte antigen* (*HLA*)-*A*, -*B*, and -*C* (Garrido et al., 1993). The complete loss of HLA class I surface expression has been reported in a large variety of different human tumors, including for example approximately 10% of the colorectal adenocarcinomas (Bodmer et al., 1993). It was defined a variety of altered human leukocyte antigen (HLA) phenotypes in human tumors, including HLA total loss, *HLA-ABC* haplotype loss, *HLA-ABC*-specific locus downregulation, *HLA* allelic losses, and a combination of these phenotypes. The complete loss of *HLA-ABC* genes follows the same principles of inactivation of tumor suppressor genes, being necessary two mutational events during the tumor progression (Garrido and Algarra, 2001; Garrido et al., 1997, 1993; Marincola et al., 1999; Seliger et al., 2002). However, the inactivation by mutation of the *Beta-2-Microglobulin B2M* gene, the subunit of MHC whose expression is needed for the stable surface expression of all HLA class I molecules, has the same effect, leading to deficient antigen presentation (Algarra et al., 2004). Therefore, considering that the downregulation of this class of genes is a remarkable advantage to tumor progression, the immunoselection of MHC class I-negative tumors in an immunocompetent host constitute a reality. Effectively, during the tumor evolution the MHC class I positive tumor cells are preferentially eliminated by cytotoxic T cells (Marincola et al., 1999; Algarra et al., 2004).

1.3.2.2 CD47 and SIRPα synapse

The immune system has a remarkable capacity to control its response, having a set of immune molecules that switch off the immune response. Inhibitory receptor signal regulatory protein alpha (SIRPα) also known as CD172a, SHPS-1 and BIT, is expressed mainly on myeloid cells such as granulocytes and macrophages, and on DCs and neuronal cells (Barclay and Brown, 2006). The SIRP family is constituted by transmembrane glycoproteins, namely SIRPα, SIRPβ, and SIRPγ (van Beek et al., 2005). These proteins have an extracellular immunoglobulin-like domain, that upon ligation generates mostly a negative signal. CD47, known as integrin-associated protein is ubiquitously expressed and serves as a SIRPα ligand. CD47-SIRPα constitutes a cell-cell communication system that plays a role in cell migration, adhesion of B cells and T cell activation (Liu et al., 2002; Motegi et al., 2003; Yoshida et al., 2002; Latour et al., 2001). Additionally, SIRPα phosphorylation induces upon CD47 ligation an inhibitory signal to the phagocytic activity of macrophages. Thus, CD47 is an important marker of ”self” for macrophages and when linked to SIRPα generates a ”don't eat me” signal (Barclay and Brown, 2006; van Beek et al., 2005). In normal conditions, the downregulation of CD47 expression is

necessary to trigger the macrophage phagocytosis of aged or apoptotic cells (Oldenborg, 2004).

Taking advantage of CD47 immunosuppressive functions, various cancer cells types increase CD47 expression (Majeti et al., 2009; Chao et al., 2010). At the cancer cell surface, CD47 binds to SIRPα at the macrophage surface, promoting the inhibition of phagocytosis and tumor cell survival. Additionally, this molecule also mediates tumor dissemination through activation of integrin and chemokine-dependent cell migration (Chao, Tang, Pachynski, Chin, Majeti and Weissman, 2011). Therefore, inhibition of CD47-SIRPα interactions with a CD47 blocking antibody seems to be a promising therapeutic approach (Majeti et al., 2009; Chao et al., 2010; Chao, Tang, Pachynski, Chin, Majeti and Weissman, 2011).

1.3.2.3 PD-1 and PD-L1 immune checkpoint

Programmed cell death protein 1 (PD-1), a member of the CD28/CTLA-4 family, is a membrane protein of activated T cells, NK cells, B cells, and macrophages, and several subsets of DCs (Simon and Labarriere, 2018). This protein has two ligands: programmed death-ligand (PD-L) 1 and PD-L2. PD-L1 is expressed in a wide variety of tissues and its expression is increased by INF-γ (Iwai et al., 2002; Blank et al., 2004). PD-L2 expression is much more restricted and appears to be limited to a subset of bone marrow-derived cells, including DCs and macrophages (Latchman et al., 2001). The interaction between the PD-1 and their ligand (Figure 1.6) alters the activity of T cells in many ways: inhibits human T cells proliferation, survival, cytokine synthesis and other activities such as the increasing of the immunosuppressive regulatory T cells (Tregs) function (Shindo et al., 2015; Butte et al., 2007; Latchman et al., 2004; Ritprajak and Azuma, 2015).

Firstly, when the body detects the presence of neoplastic cells, it activates the innate and adaptive immune system, with the consequent release of IFN-γ to boost the immune response (Zaidi et al., 2011; Rizvi et al., 2015; Ikeda et al., 2002; Bald et al., 2014). However, the presence of this cytokine leads concomitantly to the upregulation of PD-L1 ligand in tumor cells (Syn et al., 2017). PD-L1 upregulation allows the PD-1 signaling axis to become more active, which leads to downregulation of the cytotoxic response. For this reason, immunotherapy approaches based on blockade of PD-1 interaction with its ligands has lend great results when applied to eligible cancer patients (Sunshine and Taube, 2015). Immunotherapy with inhibitors of this immunological checkpoint seems

to shrink tumors and achieve durable responses in melanoma, lung cancer, among others and it is associated with lower levels of toxicity than other immunotherapies (Curran et al., 2010; Reck et al., 2016).

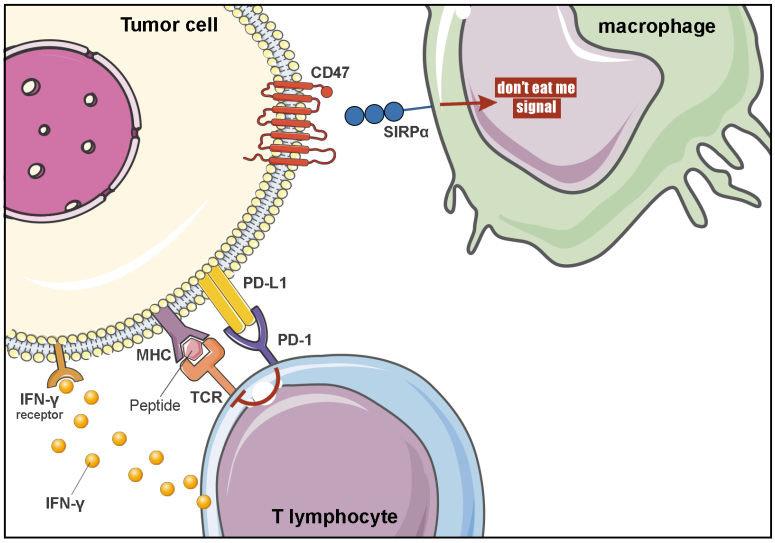

Figure 1.4. PD-1-PD-L1 and CD47-SIRPα immune checkpoints. PD-L1 is a well know T cell inhibitor whose expression is elicited by IFN-γ signaling. CD47 is ubiquitously expressed and interacts with the SIRPα, mostly expressed by macrophages. Upon CD47-SIRPα interaction it is generated a negative signal that suppresses the phagocytosis function of macrophages.

1.3.3 Immunotherapy and cancer

Cancer treatment modalities such as chemotherapy and radiotherapy are nowadays well established, however, the cancer mortality rates worldwide still high. Cancer immunotherapy is seen as an advantageous alternative modality of treatment, and there are two types: active and passive immunotherapy.

Active immunotherapy

The principle of active immunotherapy is based on the possibility to induce an endogenous, long-lasting host immune response that somehow was depleted by the tumor, and consequently, to arrest the tumor growth or in some cases even eradicate an existing solid tumor (Bertolaccini and Olivero, 2001). There are two types of active immunotherapy, the non-specific active immunotherapy, and the specific active immunotherapy (Davis, 2000). Non-specific active immunotherapy does not target specific cells, it stimulates the immune system in a more unspecific way, through administration of immunomodulating agents, such as cytokines, chemokines, interferons, and others. Specific-active im-

munotherapy consists of immune vaccination that uses not only TAAs but also material obtained from tumor biopsies after irradiation. The TAAs inoculation aims to develop an immune response based on the activation of a cytotoxic T cell response directed against that particular TAA. Both, specific and non-specific, techniques are often conjugated so that the two cooperate as adjuvants to boost the immune system, promoting the recognition and elimination of neoplastic cells (Keogh et al., 2001; Rosenberg, 1997; Baxter, 2014).

Passive immunotherapy

Passive immunotherapy is based on the passive use of antibodies to initiate an immune response against the provided antibody. In contrast with active immunotherapy, it does not induce sustained responses, bringing the need of repeated applications (Davis, 2000). Antibody administration uses a high variety of monoclonal antibodies. There are naked antibodies that work by itself and antibodies conjugated to radioactive isotopes or chemotherapeutic drugs which address the conjugated toxic substance directly to cancer cells. There are a large number of TAAs targeted by antibodies in passive immunotherapy, accordingly with the features of each cancer. For example, the chimeric EGFR-specific IgG1 monoclonal antibody, that prevents the binding of activating ligand by which impairs the growth signaling (Weiner et al., 2010). Moreover, therapies using antibodies that directly inhibit immune checkpoints are now among the most promising approaches to activating therapeutic antitumor immunity. The more common immune checkpoints covered by this treatment strategy are PD-1-PD-L1 and CTLA-4 since both acts as a ”switch-off” signal to the immune system (Pardoll, 2012). Another example of passive immunotherapy is the the administration of chimeric antigen receptor (CAR) T cell therapy in which T cells are genetically altered in the lab by adding a specific chimeric antigen receptor to specifically target the cancer cells. Typically, this treatment is conjugated with chemotherapy, some days before CAR T cell administration, to decrease the number of other immune cells and improve the CAR T cell treatment efficacy. When CAR T cells bind to cancer cells they start to increase in number destroying even more cancer cells (Sadelain et al., 2013).

Clearly, there are limitations in the immune attack to the cancer cells, since the immune system does not always recognize cancer cells as foreign. Sometimes it recognizes the cancer cells but is not able to process a consistent, and strong enough immune response to destroy the tumor. To overcome this, the immunotherapy tries to help the immune system in recognizing and effectively destroy the cancer cells. However, this

therapy works better for some types of cancer than others. Now, in different approaches, it is successfully used in particular cases of lung cancer, melanoma, colorectal cancer, breast cancer, leukemia, among others (Brahmer et al., 2015; Hamid et al., 2013; Le et al., 2015; Verma et al., 2012; Grupp et al., 2013)

1.4 Colorectal cancer

Colorectal cancer (CRC) was, in 2018, the third most common cancer both in men and women, and the second deadliest in the United States (medical and editorial content team, 2018). The mortality of this cancer reaches 9% in men and 8% in women, being only surpassed by lung and breast cancer in women (R.L. Siegel, 2017). The risk factors for developing this condition include a personal or family history of adenomas or CRC, inheritance of CRC syndromes, and inflammatory bowel disease. In addition to these factors, a diet rich in red meat and fats, low fruit and vegetable intake, high intake of calories associated and low physical activity, obesity, smoking and high alcohol consumption affect CRC carcinogenesis (Willett et al., 1990; Slattery et al., 1999).

1.4.1 Epidemiology and therapeutics

The incidence rates of CRC vary along distinct regions of the world. Almost 55% of the CRC cases occur in the developed regions, with the high rates occurring in Australia/New Zealand and the lowest in Western Africa (Ferlay et al., 2015). Most cases of CRC, about 70-80%, are sporadic, while 20-30% have an inherited component due to susceptibility syndromes such as Lynch Syndrome (3-4%) and familial adenomatous polyposis (FAP) ($\sim$1%) (Whiffin et al., 2014).

CRC treatment has advanced rapidly in the last decades. The treatment decisions are made in accordance with tumor-specific molecular features. Stage, location, and other tumor characteristics along with risks and benefits associated with each treatment are take in account (Grothey and Sargent, 2016). In most CRC cases, the tumors or the segment of colon containing the tumor are removed by surgical procedures. Adjuvant chemotherapy, most frequently 5-fluorouracil (5-FU), and oxaliplatin, may also be used, mostly at III stage CRC cases, as well as radiation in a smaller number of cases. Recurrence to chemotherapy mainly occurs in metastatic or suspected relapse cases (Sargent et al., 2009; Moertel et al., 1995; Shah et al., 2016). Additionally, a number of immunother-

apeutic strategies have been approved. Some of these drugs inhibit new blood vessels or interfere with tumor growth by targeting the VEGF and the EGFR factors, respectively (Allegra et al., 2015). More recently, PD-1 and PD-L1 inhibitory antibodies have been recommended in a small number of CRC cases, normally conjugated with other therapeutic approaches, such as 5-FU (Birendra et al., 2017).

1.4.2 Biology of CRC

CRC is a biologically heterogeneous disease, sporadic in the major number of cases, with different molecular characteristics. Sporadic tumors arise from aberrant crypt foci, a small lesion, that can progress to polyp and, when not detected or removed, can evolve into CRC with ability to invade and metastasize (Roper and Hung, 2013). Actually, it is widely accepted that CRC can arise through three major pathways, involving combinations of genetic and epigenetic changes: chromosomal instability (CIN) pathway, the microsatellite instability (MSI) pathway, and the CpG island methylator phenotype (CIMP) pathway (Walther et al., 2009; Vilar and Tabernero, 2013).

The CIN pathway is the most common form of genomic instability, occurring in 85% of CRC cases (Grady and Carethers, 2008). This pathway appears early in tumor development and follows the adenoma-carcinoma sequence, Figure 1.5. It is characterized by the accumulation of spontaneous mutations in classical proto-oncogenes and tumor suppressor genes (Shen et al., 2007). The multistep model that underlie the transition from adenoma to carcinoma predicts, first of all, the inactivation of the *Adenomatous Polyposis Coli* (*APC*), a tumor suppressor gene, resulting in an early adenoma. Subsequently, activation of the *Kirsten rat sarcoma viral oncogene homolog* (*KRAS*) gene takes place on a late adenoma. Later, it follows mutations in a number of genes: *phosphatidylinositide 3-kinases* (*PIK3CA*), *tumor protein p53* (*TP53*), and *TGF-*β pathway genes (Roper and Hung, 2013).

Microsatellite unstable CRCs account for approximately 15% of CRCs. MSI and CIN are generally mutually exclusive, each one carrying a distinct panel of gene mutations. However, there is a minimum subset of CRC cases that show both CIN and MSI (Walther et al., 2009). MSI pathway usually involves the inactivation of genes in the DNA mismatch repair (MMR) family leading to the accumulation of mutations. This is the case of Lynch syndrome that has germline mutations in one of the MMR genes, developing almost exclusively MSI CRCs (Grady, 2004). Sporadic MSI CRCs, in con-

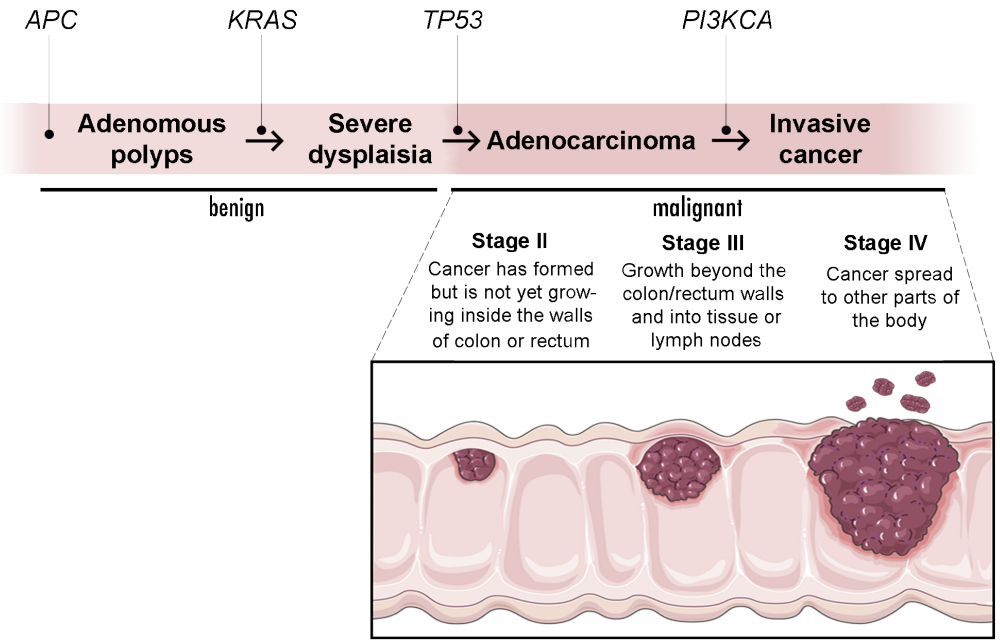

Figure 1.5. The adenoma-carcinoma sequence. The progression of the CRC is characterized by the accumulation of mutations in particular genes. The initial step is the inactivation of the *APC* gene that activates the Wnt pathway. The further progression to adenoma and carcinoma is achieved by mutations in *KRAS* and *TP53*/*PI3KCA*, respectively.

trast, have loss of MMR activity by aberrant methylation that silence *MLH1* gene and are associated with the serrated neoplasia pathway. *MLH1* inactivation is mainly associated with BRAFV600E mutations, and KRAS mutation only occurs in 18% of these cases (Velho et al., 2008; Oliveira et al., 2004). Moreover, MSI cases tend to be associated with local cytotoxic lymphocyte infiltration and with better prognosis than microsatellite stable (MSS) CRCs (Buckowitz et al., 2005). This increase of lymphocyte infiltration is a consequence of the high level of frameshift mutations in MSI tumors, that lead to an enhanced number of abnormal immunogenic peptides (Tougeron et al., 2009). In its turn, inflammation that generates oxidative stress and free radicals has the capacity to further inactivate the DNA MMR system (Chang et al., 2002).

The CpG island methylator phenotype occurs in about 15-20% of CRC cases. These tumors are characterized by the presence of aberrant DNA methylation at the CpG dinucleotide rich regions (CpG islands) frequently found in the promoter region of the tumor suppressor genes. Thereby, methylation at this CpG sites results in the transcriptional silencing of the corresponding genes (Toyota et al., 1999). *APC*, *O-6-methylguanine-DNA methyltransferase* (*MGMT*), and *MHL1* are examples of genes involved in the CRC progression that are frequently epigenetically silenced by hypermethylation (Hiltunen et al.,

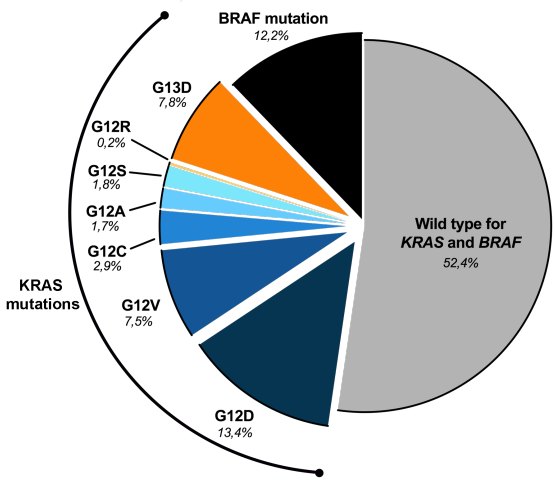

Figure 1.6. KRAS and BRAF mutation frequency in CRC. Adapted from Yoon et al. (2014)

1997; Herman et al., 1998). Thus, there is a substantial overlap of MSI and CIMP in that the majority of MSI tumor also display a CIMP phenotype (Vilar and Tabernero, 2013).

1.4.3 MAPK pathway and CRC

Mitogen-activated protein kinases (MAPK) belong to a family of serine-threonine kinases with three major subfamilies: p38, the c-Jun N-terminal or stress-activated protein kinases (JNK), and finally the extracellular-signal-regulated kinases (ERK/MAPK, Ras/Raf1/MEK/ERK) (Hommes et al., 2003). This pathway is involved in a lot of cellular processes, such as proliferation, cell survival, differentiation, senescence, and apoptosis (Kolch, 2000).

There are large pieces of evidence that support the important role of the ERK/MAPK pathway (Figure 1.7) activation in the pathogenesis and progression on a considerable fraction of CRCs (Wang et al., 2004). *KRAS* and *BRAF* are the proto-oncogenes associated with the activation of the ERK/MAPK pathway in CRC progression, being present in about 50% of CRC tumors, Figure 1.6 (Oikonomou and Pyntzas, 2006). Tumors harbouring these activating mutations are more severe and are associated with worse prognosis when compared with wild-type tumors (Van Cutsem et al., 2011; Oliveira et al., 2007). However, the upregulation of EGF receptor can also activates ERK/MAPK signaling through RAS/RAF signaling and consequently increase mitogenesis (Hsi et al., 2001). Due to these aspects, RAS, RAF, and MEK represent promising strategies for targeted therapies. Effectively, pharmaceutic industries have been, in the last decades, producing new anticancer drugs that specifically target these oncogenes, mainly ERK (Favata et al.,

1998; Sebolt-Leopold et al., 1999; Rice et al., 2003). Although treatment of CRC patients with MEK and ERK inhibitors did not reach the expected clinical benefit, a combinatorial approach with other available therapies may achieve better responses. In accordance with this view, multiple reports have verified that the PD-L1 expression can be regulated by the ERK/MAPK pathway signal in a wide variety of cancers. Accordingly, inhibition of MEK in the HCT116 CRC cell line treated with IL-17 and TNF-α, which stimulate PD-L1 expression, significantly decreased PD-L1 expression (Wang et al., 2017).

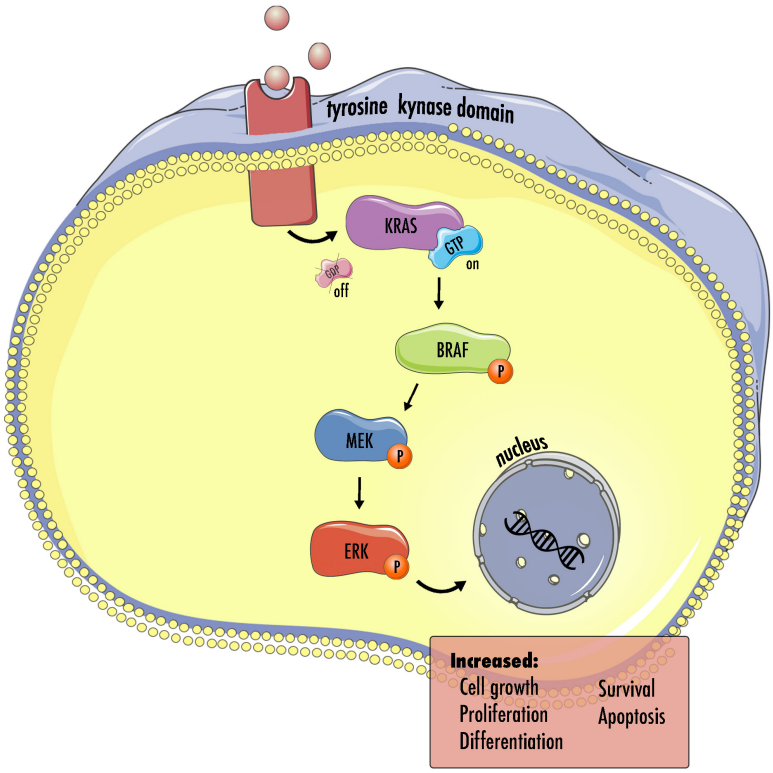

Figure 1.7. An overview of the ERK/MAPK signaling pathway. ERK is the major effector of MAPK pathway. When activated, it translocates to the nucleus and activates transcription factors that lead to cell growth, and increased proliferation, differentiation and survival. ERK can be often activated by increased RTK signaling and genetic alterations in upstream signaling molecules such as RAS, BRAF, and MEK. Mutations on this set of genes normally drive the constitutive activation of this pathway, supporting the continued growth of tumors.

1.4.3.1 KRAS mutation

KRAS is a proto-oncogene that is part of the RAS oncoprotein family (i.e., K-, N-, and H-RAS) that encodes a monomeric GTPase protein (National Center for Biotechnology Information (NCBI), Gene, 2017). This GTPase protein acts as a molecular switch, being activated when bound to GTP and, consequently, inactivated when hydrolyzed from

GTP to GDP, Figure 1.8 (Vetter and Wittinghofer, 2001). When they are bound to GTP they activate other downstream effector proteins, which in turn activate certain signaling pathways that regulate a number of genes. Thus, these proteins are central elements in the control of a wide variety of cellular processes such as cell proliferation, survival, differentiation and metabolism (Lau and Haigis, 2009). KRAS mutations, despite being found in a wide variety of cancers, are more frequent in those with higher mortality, being present in approximately 40% of cases of CRC (Velho and Haigis, 2011). KRAS missense mutations occur in hotspot codons such as G12, G13, Q61, and A146, being G12D and G13D (substitute glycine for aspartate) and G12V (substitute glycine for valine) the most frequent in CRC (Peeters et al., 2013). *KRAS* activating mutations prevents the hydrolysis of GTP in GDP, whereby the protein remains constitutively active, Figure 1.8. Thus, KRAS mutations lead to the continuous activation of the downstream pathways, increasing cell proliferation and promoting primary tumorigenesis in effector cells (Edkins et al., 2006; Lau and Haigis, 2009).

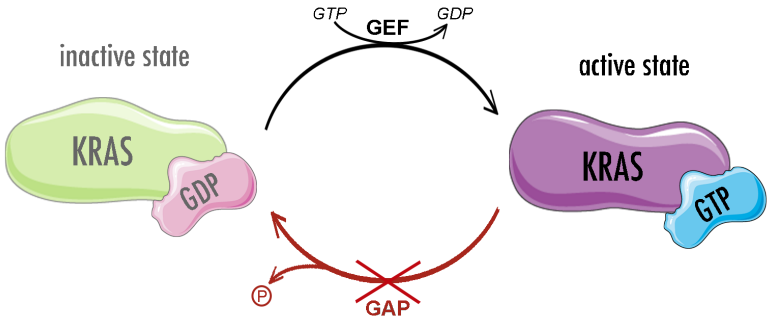

Figure 1.8. KRAS activating mutation. Wild-type KRAS alternate between an active, GTP-bound state, and an inactive, GDP bound-state. In non-dividing cells, it mostly remains in the inactive state, until a proliferating signal appears. Mutant KRAS forces the protein to remain continuously in the active state by blocking the detachment of the GTP molecule, as represented in red at the scheme.

1.4.3.2 BRAF mutation

The *BRAF* gene is a proto-oncogene that encodes the protein BRAF and is a downstream effector of KRAS in the MAPK signaling pathway (Roring and Brummer, 2012). After KRAS activation it associates with the RAS-binding domain of BRAF and recruits the complex to the cell membrane to be activated (Marais et al., 1995). When BRAF is activated it stimulates the downstream signaling cascade that ultimately leads to the regulating of multiple cellular processes controlled by the MAPK signaling transduction pathway. BRAF V600E mutation, consisting on the substitution of a valine for a gluta-

mate in the codon 600, is the most common BRAF mutation found in cancers (Tan et al., 2008). In CRC, this mutation occurs in about 41% of the sporadic MSI cases, and is considered to be mutually exclusive with KRAS mutations (Tan et al., 2008; Benvenuti et al., 2007). The mutation result in a constitutively activated BRAF that phosphorylates MEK independently of KRAS binding (Kyriakis et al., 1992).

Chapter II

Aims

It is currently known that the role of mutant KRAS in tumors is not restricted to the high rates of proliferation that potentiate the tumorigenesis initiation and strongly induce malignancy. This oncogene also influences the surrounding tumor microenvironment components affecting their characteristics and functions (Carvalho et al., 2017). The important intervention of this oncogene is almost transversal to all the immune cells present within a solid tumor. Signaling associated with KRAS mutational activation has been linked to the regulation of tumor-associated immune responses either by promoting the recruitment, activation, and differentiation of immune cells, substantially impacting on their pro-tumorigenic properties or by inducing cancer cell evasion from immune surveillance. In this theme, the specific literature focuses mainly on lung and pancreatic cancers devaluing cancers where there are also a large number of cases with *KRAS* mutations, such as CRC. It is of great importance to understand the role of mutant KRAS in modulating the CRC microenvironment and the mechanisms underlying their actions to improve and adequate the immunotherapeutic approaches in these tumors. As such, the **overall aim** of this thesis is to better characterize and understand the influence of KRAS-mutated signalling in the regulation of the immune response in the context of CRC. For that purpose, **three specific aims** were addressed:

- **Specific aim 1:** To evaluate the capacity of mutant KRAS in regulating the expression of immunomodulatory molecules thus altering tumor cell crosstalk with the immune system.
- **Specific aim 2:** To evaluate whether mutant KRAS is able to up-regulate the expression of immunomodulatory molecules in response to 5-FU and Interferon-gamma treatment.
- **Specific aim 3:** To characterize the level of infiltration and distribution of macrophages in KRAS mutant mouse tumors.

Chapter III

Material and Methods

3.1 Cell lines and culture conditions

Six colon cancer cell lines were included in the present study. HCT-116, HCT-15, SW480, SW620, RKO and HT-29 were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Cell lines were cultured following the supplier's instructions: medium supplemented with 10% fetal bovine serum (FBS, GE Healthcare, Chicago, ILL) and Pen Strep [100 units/mL of penicillin and 100 μg/mL of streptomycin] (Life Technologies, Carlsbad, CO). RPMI medium for HCT-116, HCT-15 and SW480 and DMEM for SW620, RKO and HT-29. Cells were routinely maintained in a humidified incubator at 37°C in an atmosphere consisting of 5% CO_2 in the proper medium.

Table I. Characterization by the molecular pathways MSI, CIMP, CIN and mutation status of *KRAS* and *BRAF*, associated disease and origin of the used CRC cell lines.

Abbreviations: CIN - chromosomal instability pathways; MSI - microsatellite instability; MSS - microsatellite stable; CIMP - CpG island methylator phenotype; WT - wild type. Adapted from Ahmed et al. (2013).

Cell Line	MSI Status	CIMP	CIN	*KRAS*	*BRAF*	Disease	Derived from
HCT116	MSI	+	-	G13D	WT	Colon Carcinoma	Primary tumor
HCT15	MSI	+	-	G13D	WT	Colon Adenocarcinoma	HCT-15/DLD-1 misclassified
SW480	MSS	-	-	G12V	WT	Colon Adenocarcinoma	Primary Tumor
SW620	MSS	+	-	G12V	WT	Colon Adenocarcinoma	Lymph node metastasis
RKO	MSI	+	-	WT	V600E	Colon Carcinoma	Primary Tumor
HT-29	MSS	+	+	WT	V600E	Colorectal Adenocarcinoma	Primary Tumor

3.2 *BRAF* and *KRAS* gene silencing

Gene silencing was performed using four different ON-TARGETplus SMARTpool small interfering RNAs (siRNA), specific for *KRAS* (L-005069-00-0010) and *BRAF* (L-003460-00-0010) from Dharmacon (Carlsbad, CO). A scramble siRNA sequence, with no homology to any gene, referred to as non-targeting (NT) siRNA, was used as negative control at the same concentration as the siRNA targeting the genes of interest. Cells were plated

in a 6-well plates with appropriate completed culture medium ($1,50 \times 10^5$ HCT116 cells, $2,00 \times 10^5$ HCT15 cells, $4,00 \times 10^5$ SW620 cells, $2,50 \times 10^5$ SW480, RKO and HT-29 cells). The gene silencing was performed 12h later using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CO), according to the manufacturer's guidelines. Briefly, the culture medium was replaced with 1,5 ml of Opti-MEM, reduced serum medium 1x (Life Technologies). 10 nM of human *KRAS*, *BRAF* and non-targeting siRNA was added to 250 μl Opti-MEM, also 3 μl of Lipofectamine RNAiMAX was added to another 250 μl of Opti-MEM, then standed for 5 min at room temperature (RT). Individually, the three siRNA and Lipofectamine were mixed together and left to stand for 20 minutes at RT. These complexes were placed in contact with the cells and allowed to incubate for 6 hours at 37°C. After this time, the medium was replaced with fresh complete culture medium. Incubation at 37°C continued until 72h post-transfection, after which the subsequent studies were performed. The *KRAS* expression was assessed by quantitative real-time PCR and *BRAF* expression by Western blotting, to assess the knockdown efficiency.

3.3 Western Blot analysis

Western-blot technique was performed to assess the *BRAF* silencing and α-Tubulin, a constitutively expressed protein in human cells encoded by a housekeeping gene, was used as a loading control.

3.3.1 Samples preparation for Western Blot

Proteins were extracted from cancer cell lysates to perform Western Blot. Cells were detached with cold RIPA Buffer [50 mM Tris HCl; 150 mM NaCl; 2 mM EDTA; 1% IGEPAL CA-630; pH=7.5], supplemented with 1:7 proteases inhibitors cocktail (Roche, Basel, Switzerland) and 1:100 phosphatases inhibitors cocktail (Sigma-Aldrich), scrapped, and centrifuged at 13 300 rpm at 4°C, during 10 min. After centrifugation, supernatants were collected and protein concentration was determined using the Bradford assay with the DCProtein assay kit (BioRad, Hercules, CA). Then, 6 μl of Laemmli sample buffer [0.5 M Tris-HCl pH 6.8, 9.2g sodium dodecyl sulfate (SDS), 40mL Glycerol, 5% 2-mercaptoethanol, 5% bromophenol blue] was added to 50 μg of protein and the mixture was boiled for 5 min at 95°C for protein denaturation.

3.3.2 Western Blotting

50 μg of protein from each sample was loaded on a 7,5% SDS -polyacrylamide gel and run at 90V. A molecular weight ladder (Bio-Rad, Precision Plus protein standard) was also included on each gel. Following electrophoresis, protein was electrophoretically transferred to a Amersham Protran Premium 0,45 μm nitrocellulose blotting membranes (GE Healthcare) for 90 min at 100 V. Following gel transfer, the membranes were incubated with Ponceau (Sigma-Aldrich) solution until protein bands became visible. Subsequently, membranes were blocked with 5% non-fat powder milk in PBS-T [PBS containing 0,1% Tween-20 (OmniPur, Calbiochem)] or 4% bovine serum albumin (NZYTech, Lisbon, Portugal) also in PBS-T for 1 hour. Membranes were incubated overnight at 4°C with primary antibodies according to Table II. Membranes were then washed with PBS-T and incubated with the HRP-conjugated anti-mouse secondary antibody (Table II). After the final wash, the membranes were incubated with Clarity Western ECL Substrate (Bio-Rad) for signal detection. Band density was quantified by densitometric analysis using ImageJ (National Institute of Health, USA) and the quantification of the *BRAF* gene was comparing with the α-tubulin expression.

Table II. Primary and secondary antibody specifications for Western Blot.

Abbreviations: MW - molecular weight.

Target molecule	Serum	MW	Dilutions	Manufacturer	Catalog n^o	Blockade
Primary antibodies						
BRAF	Mouse	87	1:1000	Santa Cruz Biotechnology	sc-5284	4% BSA
α-Tubulin	Mouse	55	1:10000	Sigma-Aldrich	TS168	5% Milk
Secundary antibodies						
Mouse (to BRAF)	—	—	1:2000	GE Healthcare	NA931V	—
Mouse (to α-Tubulin)	—	—	1:20000	GE Healthcare	NA931V	—

3.4 *KRAS* expression quantification

3.4.1 Total RNA Extraction

Cells were lysed directly in the culture dish by adding 500 μl of TripleXtractor (GRiSP Research Solutions, Porto, Portugal) scrapping and passing the lysate several times through a pipette. After being detached cells were incubated for 5 minutes at RT. The lysates were transferred for an RNA-free microtube and then 100 μl of chloroform (Merck Millipore, Burlington, MA) was added. The tubes were shaken vigorously by hand for 15 seconds

and incubate at RT for 3 minutes. After this time the samples were centrifuged at 15.000g for 10 minutes at 4°C. The aqueous phase was transferred to a new tube and the RNA was precipitated by the addition of 500 μl of isopropanol (Fisher Scientific). After 10 minutes of incubation at RT, the tubes were centrifuged at 15.000g at 4°C for 10 minutes. The supernatant was carefully removed and the pellet washed with 1 mL of 70% ethanol and centrifuged at 15.000g at 4°C for 5 minutes. The ethanol was removed by air-drying the pellet and finally, the RNA was resuspended in 20 μl of RNase-free water (GIBCO Invitrogen Life Technologies). RNA concentration and purity were determined using a NanoDrop Spectrophotometer ND-1000 (Thermo Scientific, Waltham, MA). RNA samples were then stored at -80°C until further use.

3.4.2 Complementary DNA synthesis

Complementary DNA (cDNA) was synthesized with 1 μg of total RNA using the qScript™ cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD). After the reagents have been added to a tube 0.2 mL microtubes on ice they were incubated for 5 minutes at 25ºC, 30 minutes at 42ºC, 5 minutes at 85ºC and held at 4ºC in the thermocycler (T100™ Thermal Cycler, BioRad). After completion of cDNA synthesis it was stored at -20ºC until further use.

3.4.3 Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed to verify the *KRAS* and *BRAF* gene silencing. The mix consisted of 0,5 μl of each cDNA sample, 4 μl of RNase Free water, 0,5 μl of the interest probe (Table III) and 5 μl of TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA). Were performed three triplicates for each sample. The qRT-PC program was composed by two holding steps at 50ºC for 2 min and 95ºC for 10 min followed by 40 cycles of 95ºC for 15 s and 60ºC for 1 min. The qRT-PC was run in a 7500 Real-Time PCR System (Applied Biosystems) and each reaction was performed in triplicate. Relative mRNA expression levels of the targeted genes were normalized to expression levels of the housekeeping gene (*GAPDH*) and estimated using the comparative $\Delta\Delta$Ct method.

3.5 Cell treatment

After being *KRAS* silenced, HCT15, HCT116, SW480 and SW620 were treated with 5-FU and IFN-γ. Cells were analyzed by flow cytometry, 48h after 5-FU treatment and 24h

Table III. Taqman Gene Expression Assays used to assess the mRNA levels for the selected genes.

Gene	TaqMan assay reference	Manufacturer
GAPDH	Hs.PT.39a.22214836	Applied Biosystems
KRAS	Hs00270666m1	

after IFN-γ stimulation.

3.5.1 5-Fluorouracil

5-FU (Sigma-Aldrich, St. Louis, MO) was stored at 4ºC and dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich). 24 hours after the *KRAS* silencing, cells were treated with 5 and 10 μg/mL of 5-FU and were left to stand for 48h. After this time, the CD47 and PD-L1 expression levels were evaluated by flow cytometry. RNA was also extracted to assure the *KRAS* silencing. DMSO was used as a control.

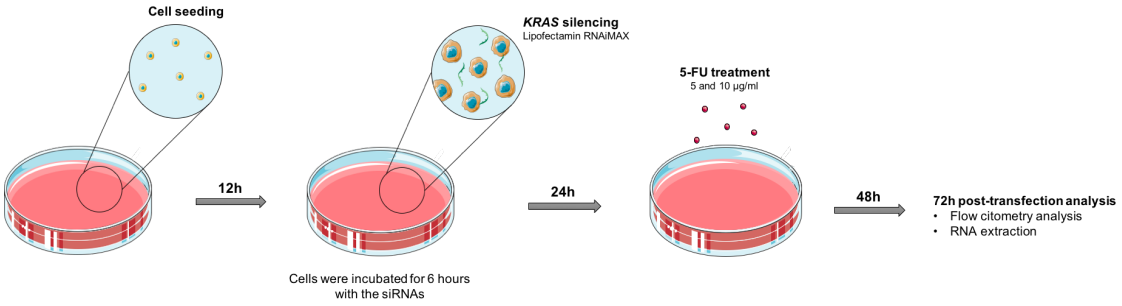

Figure 3.9. 5-FU treatment. After the *KRAS* gene silencing cells were treated with 5 and 10 μg/mL of 5-FU for 48h.

3.5.2 IFN-γ

48h after the *KRAS* gene silencing cells were stimulated with 25 ng/mL of IFN-γ (ImmunoTools, Friesoythe, Germany) for 24h. Such as in the 5-FU treatment, 72h post-transfection cells were analyzed by flow cytometry, for CD47 and PD-L1 expression.

3.6 Flow Cytometry Analysis

Flow cytometry was used to measure the expression of cell surface molecules: CD47, PD-L1, HLA-ABC and SIRPα/β on the studied CRC cell lines. For this analysis cells, after being washed with phosphate-buffered saline (PBS, Fisher Scientiphic, Hampton, NH), were harvested with 0,05% Trypsin-EDTA (GIBCO Invitrogen Life Technologies)

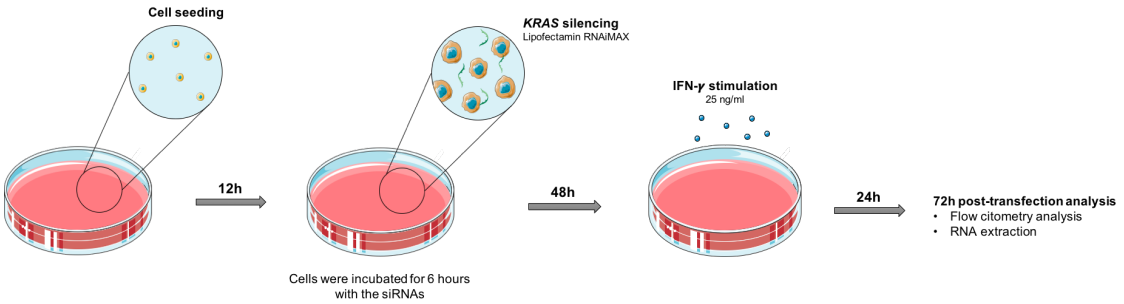

Figure 3.10. IFN-γ stimulation. After the *KRAS* gene silencing cells were stimulated with 25 ng/mL of IFN-γ for 24h.

for long enough to be detached and then complete medium was added to stop the enzyme action. The cells were then left to stand for 30-40 minutes at 37°C to recover the cell surface molecules. Then we proceed with the washing of cells with flow cytometry buffer (PBS and 1%FBS) and centrifuged at 1200 rotations per minute (rpm) for 5 min at 4°C. $2,00 \times 10^5$ cells of each condition were stained with FITC-conjugated CD47, FITC-conjugated PD-L1, PE-conjugated HLA-ABC, APC-conjugated SIRPα/β, Table IV, for 15 minutes at RT. Two additional washing steps were performed with flow cytometry buffer and then the samples were filtered. 30 000 events were acquired with FACS Canto Flow Cytometer (BD Bioscience, Franklin Lakes, NJ) using FACS Diva Software and analyzed using FlowJo software (Treestar, San Carlos, CA). Forward and side scatter gates were set to include all the viable cells.

Table IV. Flow Citometry antibodies used.

Abbreviations: FITC, fluorescein isothiocyanate; PE, phycoerythrin; APC, allophycocyanin.

Antibody	Clone	Conjugated fluorophore	Manufacturer	Catalog nº	$\mu l/10^5$ cells
CD47	MEM-122	FITC	Immunotools	21270473	1
PD-L1	MIH1	FITC	BD Pharmingen, Franklin Lakes, NJ	555748	2
HLA-ABC	W6/32	PE	Immunotools	21159034	1
SIRPα/β	SE5A-ABC	APC	Biolegend, San Diego, CA	323809	2
CD14	18D11	PE	Immunotools	21620144	2
Mouse IgG1	—	FITC	Miltenyi Biotec, Germany	130-098-847	2
Mouse IgG2a	—	PE	Immunotools	21275524	1
Mouse IgG1	—	APC	Miltenyi Biotec	130-098-846	2
Mouse IgG1	—	PE	Immunotools	21279146	2

3.7 Human monocyte isolation and macrophage differentiation

Buffy coats for monocyte isolation was obtained from healthy blood donors, provided by Hospital São João (under a collaboration protocol established by Dr. Maria José Oliveira). The blood from buffy coats was centrifuged at 1200 g for 20 min at RT. The PBMCs which is defined by a whitish layer were collected and incubated for 25 min with RosetteSep Human Monocyte Enrichment Cocktail (StemCell Technologies, Vancouver, Canada) under rotation. This mixture was then diluted (1:1) with PBS and 2% FBS (Biowest, San Marcos, TX) and added cautiously over Ficoll-Histopaque 1077 (Sigma-Aldrich) followed by a 20 min centrifugation at 1200 g (brake off) at RT. The intermediated layer was collected, washed in PBS and centrifuged three times at 700 rpm for 17 min. Cells were then resuspended, in complete macrophage RPMI medium, and $1,2 \times 10^6$ monocytes were plated in 6-well culture plates with glass coverslips. Macrophages were differentiated for 13 days and, at day 7 post-isolation, the medium was renewed. During only 7 of this 13 days, macrophages were in the presence of macrophage colony-stimulating factor (M-CSF, ImmunoTools 50 ng/mL). On the 13th day, the in vitro phagocytic assay was performed.

3.8 *In vitro* phagocytic assay

Fluorescent labeling was performed in 2,4x10^6 wild-type (WT) and *KRAS*-silenced (si*KRAS*) HCT116 and HCT15 cells. For fluorescent labeling was used 2,5 uM green fluorescent dye carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen) which was left to stand for 20 min at RT under rotation. The volume was completed, with macrophage RPMI, until 15 mL and the cells were incubated at 37ºC for 5 minutes. After that, cells were centrifuged at 1200 rpm for 5 minutes. CFSE-labeled cells were resuspended in macrophage RPMI and incubated with macrophages in a ratio of 1 macrophage to 2 CFSE-labeled cells for 2 h at 37ºC. To perform flow citometry, cells were detached by scrapping upon incubation with accutase (BD Biosciences). CD14 and SIRPα/β staining was performed as described previously. The percentage of phagocytosis was obtained by applying the equation:

$$\text{Phagocytosis (\%)} = \frac{\text{CD14}^+\text{CFSE}^+}{\text{CD14}^+} \times 100$$

3.9 Apc, Kras and Apc/Kras mutated mice

Since *Apc* loss and *Kras* activation are two of the earliest genetic alterations found in CRC, we used, previously described (Haigis et al., 2008), *Apc* deficient mice ($Apc^{2lox14/+}$), activated *Kras* ($KRAS^{LSL-G12D/+}$, in which LSL denotes Lox-stop-Lox) and combined mouse mutants all expressing $Fabpl^{-4X@-132}$-Cre^{33} to direct the activation of the mutant genes to the distant small intestine and colonic epithelia. When combined *Apc* deficiency and *Kras* activation lead to tumor development recapitulating the early steps of the disease. When showing disease signs (rectal prolapse), mice were euthanized to dissect the colon. Colonic tissue (with part of the tumors still attached) were then swiss-rolled, flushed with 1x PBS and formalin-fixed for histopathologic and immunohistochemistry analysis.

3.10 Immunohistochemistry

Immunohistochemistry staining of F4/80 (BM8, Biolegend) was performed to visualize macrophages in mouse colon and rectum. The slides were deparaffinized in two changes of xylene for 5 minutes each and then transferred for two changes to 100% alcohol, 3 min each. The slides were rinsed in running tap water for 5 minutes and transfer to deionized water. The antigen retrieval was performed using Proteinase K for 10 min at RT. After two washes in tris-buffered saline (TBS) containing 0,01% of Tween 20 (TBS-T) the endogenous peroxidase activity was blocked by a 10 min incubation with Lab Vision™ Hydrogen Peroxide Block solution (Thermoscientiphic) at RT. The slides were rinsed again for two times in TBS-T and the endogenous biotin was blocked with the Lab Vision™ Avidin-biotin Blocking Solution (Thermoscientiphic). 100 μl of protein blocker was added in order to block unspecific binding sites and without washing, the slides were incubated overnight at 4°C with 100 μl of anti-mouse F4/80 antibody diluted at 1:300. Primary antibody incubation was omitted for negative controls. The slides were maintained at RT for 30 min and then were rinsed in TBS-T two times for 5 minutes. Afterward, followed the incubation with a biotinylated secondary antibody, Goat anti-rat-biotin (ENZO) – 1:300 ALX-211-058-C100 diluted at 1:300 for 30 min at RT and then the two times slides washing in TBS-T. Then was applied 100 μl of Streptavidin, Horseradish Peroxidase, R.T.U. (Vector Laboratories, Burlingame, CA). The color development by DAB Quanto (Thermoscientiphic) was allowed for 5 minutes and after that, the slides were counterstained with Mayer Hematoxylin for 10 min. Finally, the

slides were washed in running tap water for 10 min, dehydrated in alcohol and xylene changes and coverslip using mounting solution.

3.11 Statistical analysis

Data analysis was performed with Prism 7.0a (GraphPad Software, USA). All the data are presented as the mean $\pm$ standart error of the mean (SEM), and statistical significance was determined as $P < 0.05$ by a nonparametric test (Mann-Whitney test) and by Two-way ANOVA followed by Turkey's multiple comparison test. All means were calculated from data of all the independent experiments.

Chapter IV

Results

4.1 Influence of *KRAS* activation in the CRC immunosurveillance escape

4.1.1 Characterization of immunosurveillance molecules expression and KRAS and BRAF mutation influence

In this first part of our work, the main aim was to understand the role of the KRAS in modulating the TME of CRC, focusing mainly on the influence of the KRAS-mutated signaling in CRC immune evasion. For such purpose, we set up a panel of putative molecules that can alter the tumor crosstalk with the immune system and favor tumor immune evasion, invasion, and metastatic potential. The expression levels of HLA-ABC, PD-L1, and CD47-SIRPα/β synapse intervenient, were evaluated by flow cytometry in different CRC cell lines: four of them harboring KRAS mutations (HCT15, HCT116, SW480, and SW620), and two of them BRAFV600E mutated (RKO and HT-29) (Table I from the Chapter III). BRAF mutated cell lines were used to determine whether alterations in KRAS downstream signaling effectors are also able to regulate the expression of immunosuppressive molecules in cancer cells or if it is exclusively dependent on *KRAS* activation. All inhibitions were confirmed through Western Blot for the *BRAF* and qRT-PCR for *KRAS* inhibitions (Supplementary Figures S1 and S2, respectively). Only the experiments where the silencing was more than 60% were considered in the following results.

4.1.1.1 HLA-ABC

The cell surface levels of HLA-ABC molecules were measured by flow cytometry, using the gating strategy shown in Supplementary Figure S3 for each CRC cell line. The results show no alterations in the number of HLA-ABC positive cells upon *KRAS* silencing (Figure 4.11). Nevertheless, in the HCT15, HCT116, and SW620 cell lines there is an increase of the median fluorescence intensity (MFI). HLA-ABC cell surface levels did

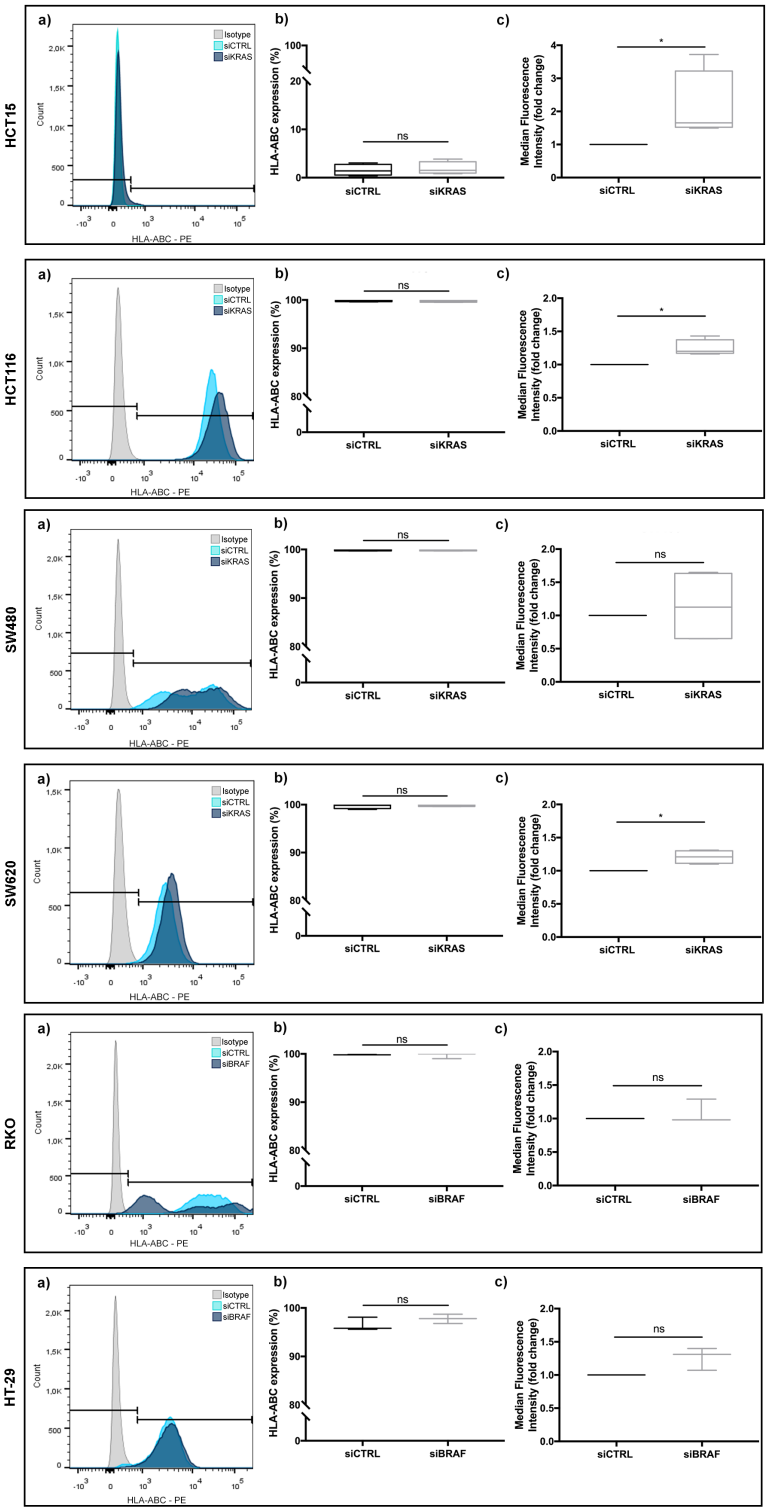

Figure 4.11. HLA-ABC expression on *KRAS* and *BRAF* inhibited CRC cells. HLA-ABC expression levels were assessed by flow cytometry in HCT15 (n= 4), HCT116 (n= 4), SW480 (n= 4), SW620 (n= 4), RKO (n= 3) and HT-29 (n= 3) cell lines; **a)** HLA-ABC expression single parameter histogram of the siCTRL, si*KRAS* or si*BRAF* and isotype (negative control) of the CRC cells analyzed; **b)** Percentage of HLA-ABC-PE positive cells in *KRAS* or *BRAF* inhibited cells; **c)** MFI graph of the HLA-ABC positive cells, values are fold changed relative to siCTRL MFI levels. Mann-Whitney test was performed (* indicates a significantly different result $P<0.05$).

not show alterations upon *BRAF* inhibition, being expressed in almost all RKO and HT29 cells.

4.1.1.2 SIRPα/β

The expression of SIRPα/β was also evaluated in the same panel of CRC cell lines, following the gating strategy showed in Supplementary Figure S3. Considering KRAS mutant cell lines, the number of positive SIRPα/β positive cells was considerably lower in HCT15 in comparison with the others, and only this cell line showed an increase in the number of SIRPα/β positive cells upon *KRAS* inhibition. In HCT116, SW480, and SW620 cell lines, the percentage of positive cells is close to 100%, and no alterations were found, neither in the number of SIRPα/β positive cells nor in MFI values upon *KRAS* inhibition (Figure 4.12). *BRAF* inhibition, following the same tendency as *KRAS*, did not lead to any alteration in SIRPα/β cell surface levels. Additionally, SIRPα/β positive cells population is considerably higher in RKO, while only approximately 8% of HT-29 cells express this molecule (Figure 4.12).

4.1.1.3 CD47

CD47 expression was also accessed for *KRAS* and *BRAF* inhibited cell lines by flow cytometry, using the gating strategy displayed in Supplementary Figure S3. The obtained results are represented in Figure 4.13 and show that *KRAS* inhibition led to a decrease of about 50% of cells expressing CD47 in HCT15 and SW480 cell lines. However, there were no differences, after inhibition of this oncogene, in HCT116 and SW620 cell lines. Nevertheless, *KRAS* inhibition promotes an increase in the MFI of all the mutant KRAS cell lines analyzed. Paradoxically, in the BRAFV600E mutants CRC cell lines, CD47 molecule coats almost all RKO and HT-29 cells and, neither the number of positive cells nor the levels of CD47 expression in each cell, appears to be influenced by the absence of mutant BRAF.

4.1.1.4 PD-L1

PD-L1 expression was also accessed by flow cytometry, following the same gating strategy of the Supplementary Figure S3. KRAS mutant cell lines, HCT15, HCT116, SW480, and SW620 express residual levels of PD-L1 (Figure 4.14) and the same is true for BRAF mutant HT-29 cell line. In contrast, almost all RKO cell population express PD-L1. However, there are no significant differences neither in the percentage of positive cells, nor

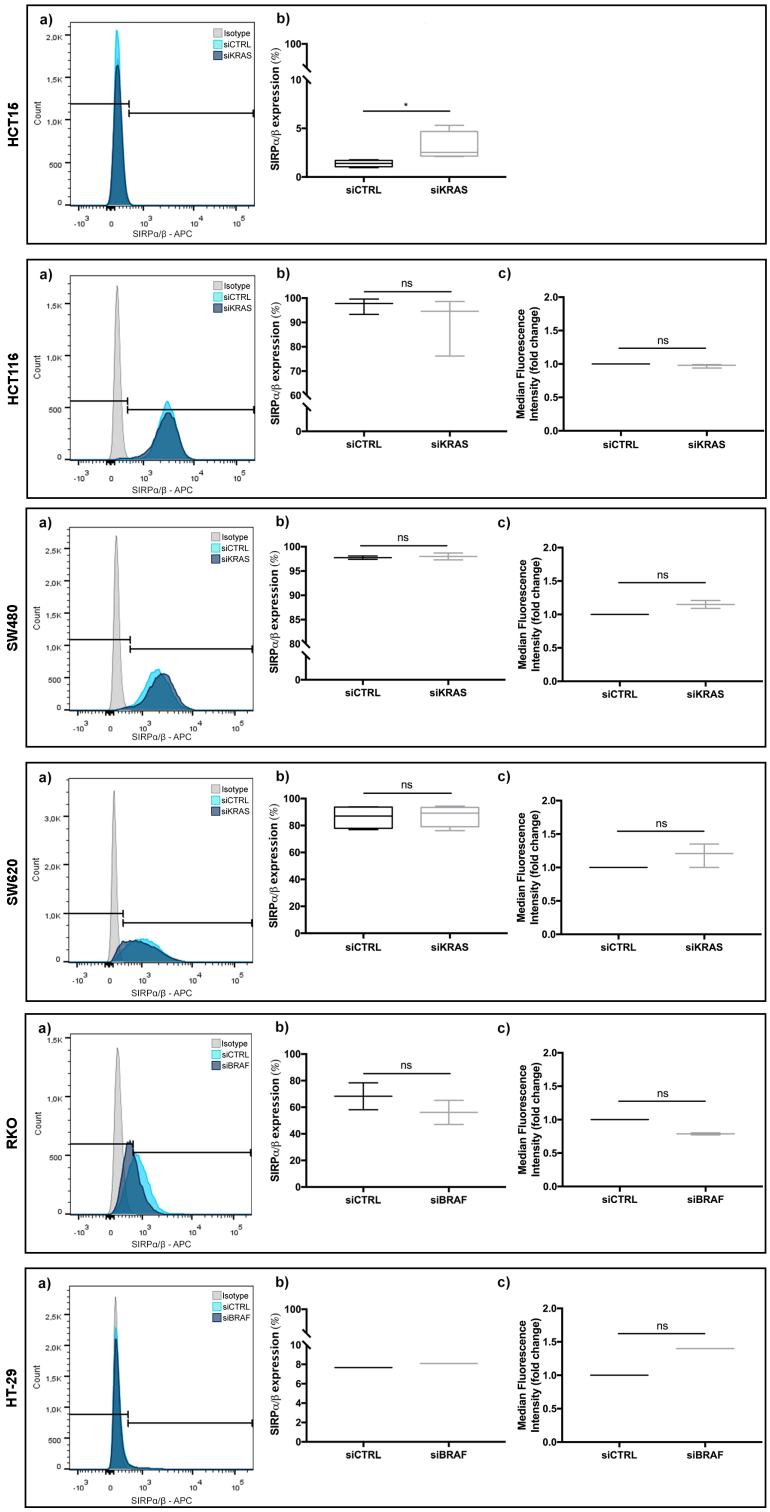

Figure 4.12. SIRPα/β expression in *KRAS* and *BRAF* inhibited cell lines. Flow cytometry analysis of SIRPα/β in HCT15 (n= 4), HCT116 (n= 3), SW480 (n= 2) and SW620 (n= 4), RKO (n= 2) and HT-29 (n= 1) cancer cell lines; **a)** Single parameter histograms of the SIRPα/β (APC) in siCTRL, si*KRAS* or si*BRAF*, and isotype, used as the negative control population; **b)** Representative graph of the percentage of SIRPα/β positive cells in *KRAS* and *BRAF* inhibited cell lines; **c)** MFI of the SIRPα/β positive cells, this measurement could not be assessed in the HCT15 cell line due to the low number of positive events. Mann-Whitney test was performed (* indicates a significantly different result $P<0.05$).

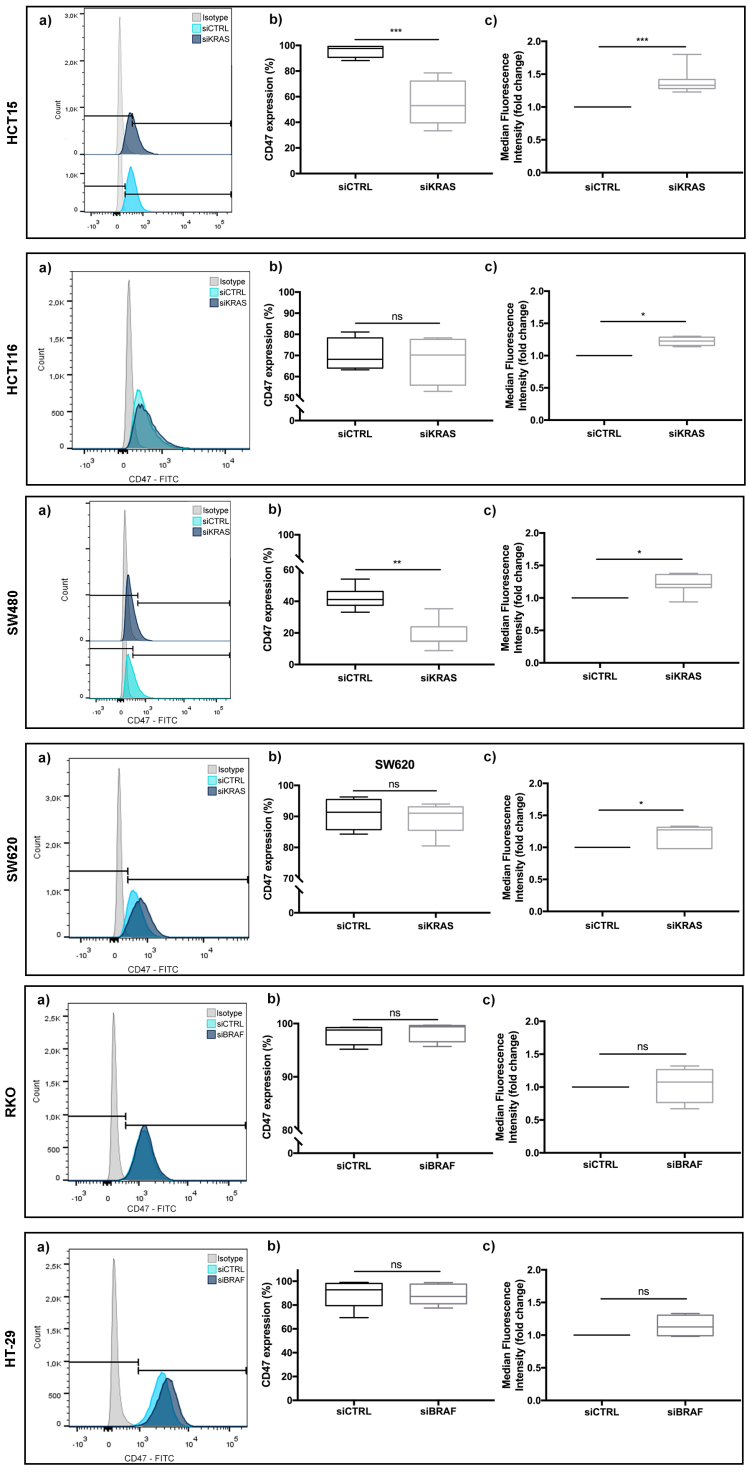

Figure 4.13. CD47 expression in *KRAS* and *BRAF* inhibited CRC cell lines. CD47 expression was assessed by flow cytometry for both *KRAS* and *BRAF* inhibited cell lines, including HCT15 (n=7), HCT116 (n=4), SW480 (n=4), SW620 (n=6), RKO (n= 4) and HT-29 (n= 5); **a)** The single parameter histogram overlay the unstained population, used as the negative population, onto the stained CD47 (FITC) siCTRL and si*KRAS* or si*BRAF* population. For HCT15 and SW480, as the *KRAS* inhibition induces an alteration in the dispersion of the cell population, this histogram is split in two; **b)** The graph represents the percentage of CD47-FITC positive cells in all conditions siCTRL and si*KRAS*/si*BRAF*, for each cell line. **c)** MFI of CD47 positive cells is represented as fold change relative to the MFI of siCTRL. Mann-Whitney test was performed (* indicates a significantly different result $P<0.05$; ** $P<0.01$; *** $P<0.001$).

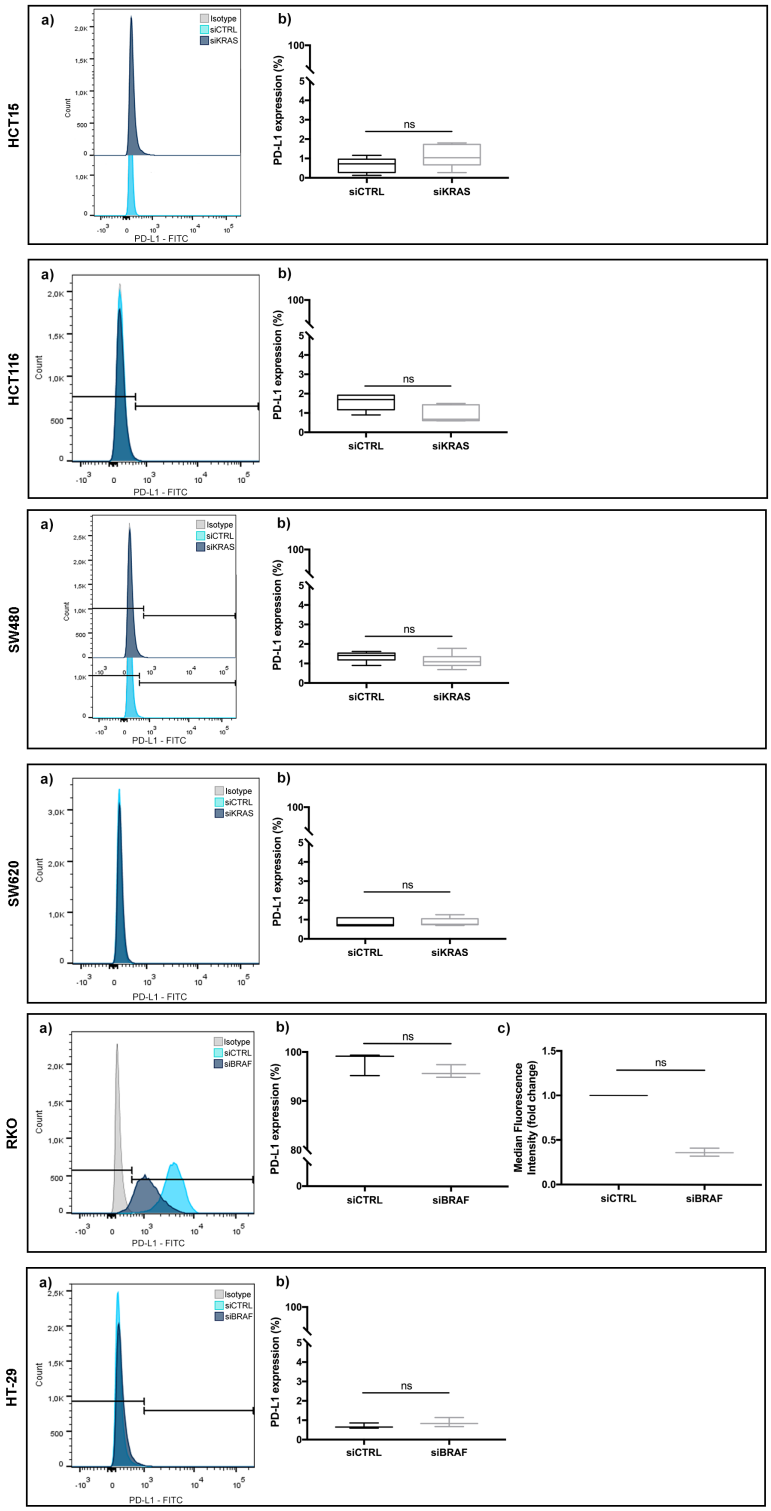

Figure 4.14. PD-L1 expression in *KRAS* and *BRAF* inhibited CRC cell lines. Flow cytometry results of PD-L1 expression in HCT15 (n=8), HCT116 (n=6), SW480 (n=6), SW620 (n=7), RKO (n= 3), and HT-29 (n= 3); **a)** Single parameter histogram of PD-L1 expression of the negative control population (isotype, IgG1-FITC), and the siCTRL and si*KRAS*/si*BRAF* conditions; **b)** Percentage of PD-L1-FITC positive cells in siCTRL and si*KRAS*/*BRAF* cells; **c)** MFI of the PD-L1-FITC positive cells, results are presented as fold change relative to the siCTRL MFI levels. Mann-Whitney test was performed.

in the MFI upon *KRAS* or *BRAF* inhibition (Figure 4.14) despite a slight tendency for a decrease in MFI levels in RKO cells upon *BRAF* inhibition.

4.1.2 IFN-γ influence in the expression of immunosurveillance molecules and its relation with KRAS mutation

IFN-γ is a pro-inflammatory cytokine that activates the immune system and is known to stimulate PD-L1 expression (Blank et al., 2004). Thus, we investigate whether IFN-γ treatment affected the CD47 and PD-L1 expression in CRC cell lines harbouring KRAS mutations and if mutant KRAS mediates IFN-γ effect. KRAS mutated cells used in this study were stimulated for 48h with 25 ng/mL of IFN-γ and the CD47 and PD-L1 levels were determined by flow cytometry. The cell aspect before and after the IFN-γ treatment was not altered and is represented in the Supplementary Figure S4.

In accordance with the previous results, the number of CD47 positive cells decreased to approximately 50% upon *KRAS* inhibition in the HCT15 cell line but remained unaffected in the HCT116 cell line (Figure 4.13). Treatment with IFN-γ, Figure 4.15, did not affect the number of CD47 positive HCT15 cells as this value is already close to 100%. However, CD47 values in HCT15 si*KRAS* cells are, upon IFN-γ stimulation, restored to values close to 100% as seen in the control. A different result was observed for HCT116 as the number of cells expressing CD47 increased in both, siCTRL and si*KRAS*, conditions upon treatment with IFN-γ.

Following the same tendency as HCT15, the number of CD47 positive SW480 cells decrease from 40% in siCTRL to 20% when *KRAS* was inhibited (si*KRAS*). However, in this cell line, IFN-γ stimulation increased the number of CD47 positive cells in the siCTRL but not in the si*KRAS* condition (Figure 4.15). Expression of CD47 on the surface of SW620 cells is found in almost 90% of the cells, both in siCTRL and si*KRAS*. However, with the IFN-γ treatment, was still able to promote an increase in the number of cells expressing CD47 in both conditions.

As already shown, all the KRAS mutant cells used in this work do not express PD-L1. However, IFN-γ treatment stimulates the expression of PD-L1 in all the cell lines (Figure 4.16), allowing the detection, through flow cytometry, of possible differences in PD-L1 levels as a result of *KRAS* silencing. In both HCT15 and HCT116 cells, there is a drastic decrease of IFN-γ-induced PD-L1 expression upon inhibition of *KRAS*. IFN-γ

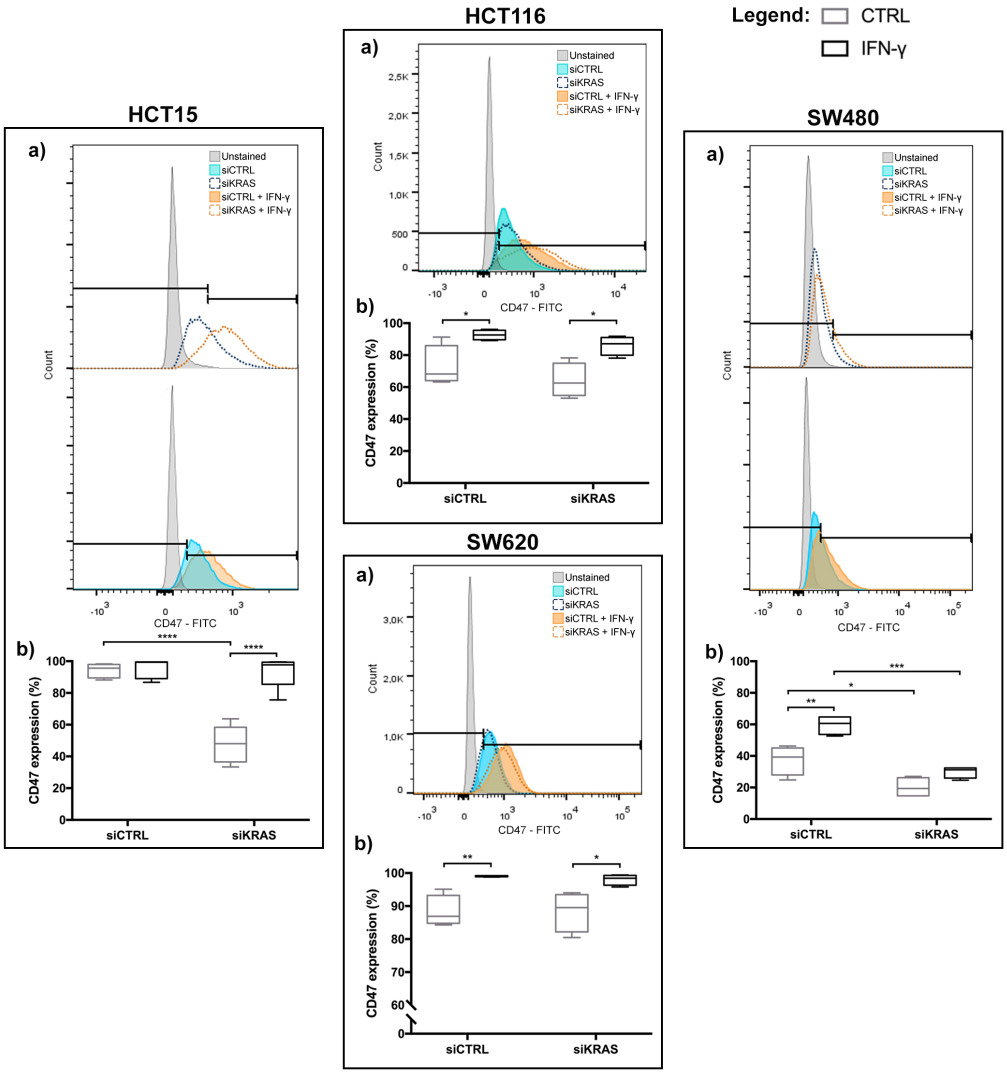

Figure 4.15. CD47 protein expression in *KRAS* inhibited cell lines with and without IFN-γ treatment. Flow cytometry results of CD47 expression in the *KRAS* inhibited HCT15 (n= 5), HCT116 (n= 4), SW480 (n= 4), and SW620 (n= 4) cell lines; **a)** Single parameter histogram of the PD-L1-FITC staining. In HCT15 and SW480 cell lines, the histogram is separated in two, given the difference in the isotype (negative control) behavior which is more extended in the si*KRAS* conditions. This isotype extension influences the negative/positive gate position. In the bellow histogram, the unstained siCTRL, as negative population, was used to define the negative/positive gate for siCTRL conditions. In the other histogram, the negative/positive gate was defined for HCT15 si*KRAS* conditions using the unstained si*KRAS* as the negative population; **b)** Percentage of CD47 positive cells graph, comparing *KRAS* inhibited cell lines with and without IFN-γ treatment (25 ng/mL). Two-way ANOVA followed by Turkey's multiple-comparison tests was performed (* indicates a significantly different result $P<0.05$; ** $P<0.01$; *** $P<0.001$).

treatment is also capable of inducing PD-L1 expression in SW480 and SW620. However, in these cell lines, IFN-γ stimulation of CD47 expression is not so efficient as seen in the previous cell lines, leading to an increase of about 8% in SW480 and 15% in SW620 (Figure 4.16). Additionally, there is no difference in PD-L1 expression between siCTRL cells and si*KRAS* cells, in both cell lines.

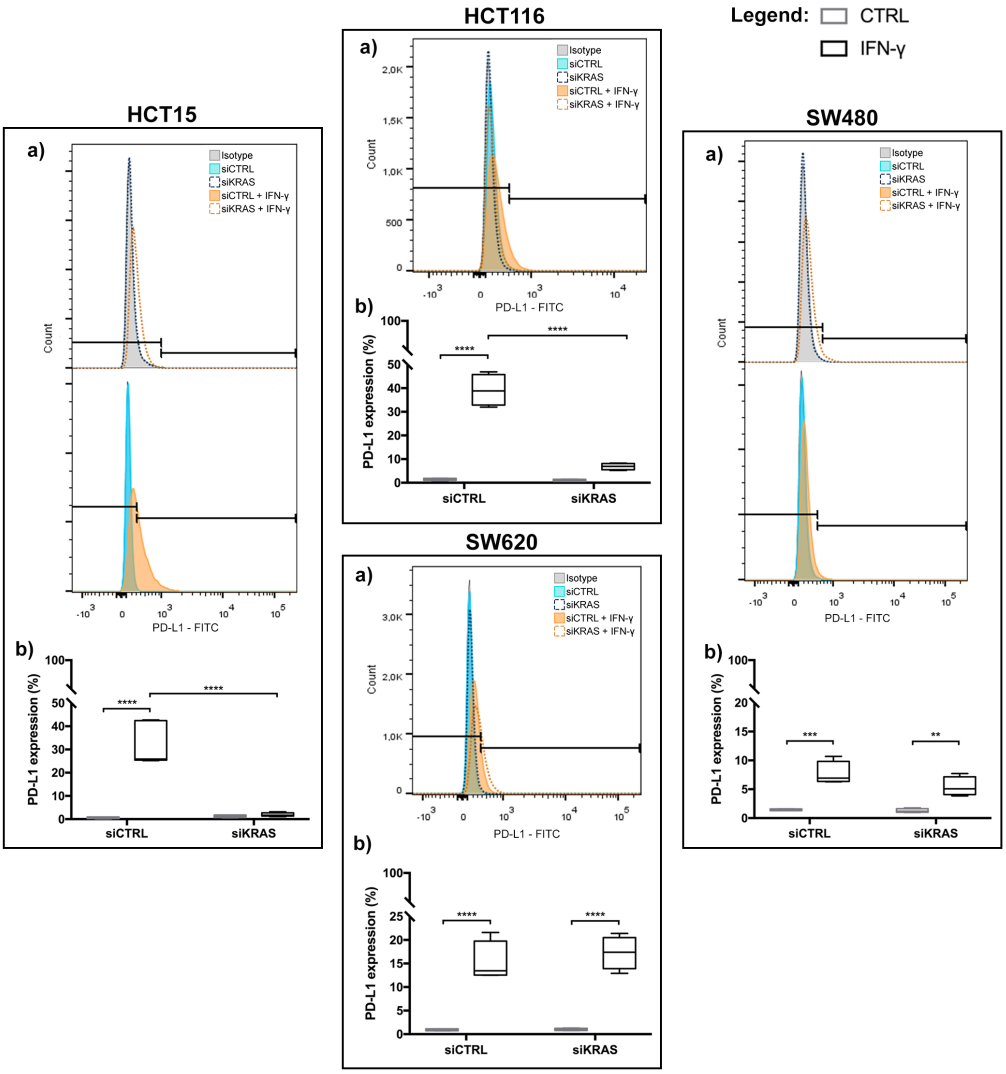

Figure 4.16. PD-L1 protein expression in *KRAS* inhibited cell lines after IFN-γ treatment. PD-L1 expression, flow cytometry results, in the *KRAS* inhibited HCT15 (n= 5), HCT116 (n= 4), SW480 (n= 4) and SW620 (n= 4) cell lines; **a)** Single parameter histogram of the PD-L1 FITC staining with the 4 conditions. The HCT15 and SW480 histograms are separated, given the different extension of the isotype (negative control) which influences the negative/positive gate position; **b)** Percentage of PD-L1 in siCTRL and si*KRAS* with and without IFN-γ stimulation (25ng/mL). Two-way ANOVA followed by Turkey's multiple-comparison tests was performed (** indicates a significantly different result $P<0.01$; *** $P<0.001$; **** $P<0.0001$).

4.1.3 Chemotherapy, *KRAS*, and immunosurveillance escape

5-FU has been pointed out as a possible inducer of cancer immune resistance (Zitvogel et al., 2008). This treatment is a therapeutic alternative in CRC stages II, III and IV, so we tried to understand if this therapeutic approach could have a negative impact on the expression of molecules that control the immune response against cancer cells (Carethers et al., 2004). For this all KRAS mutant CRC cell lines were treated cells with low doses of 5-FU: 5 μg/mL and 10 μg/mL, and then the expression levels of CD47 and PD-L1 were

evaluated before and after *KRAS* silencing. The control used in this part are the cells treated with DMSO, which was used to dissolve 5-FU. 5-FU cell treatment alters cells morphology, resulting in a highly heterogeneous population of larger and star-shaped cells (Supplementary Figure S5). This aspect is also observable in its dispersion in the cytometry results. Thus, as we cannot use the same negative control to all the conditions, due to the morphological alterations prompted by 5-FU treatment, the histograms are presented in separate, the untreated cells below and the treated conditions above. Additionally, the morphological differences caused by *KRAS* silencing in HCT15 and SW480 cells are abolished after 5-FU treatment, so there is no need to separate histograms.

The results show a tendency for a reduction in the number of CD47 positive HCT15 and HCT116 cells upon 5-FU treatment in both the siCTRL and the si*KRAS* (Figure 4.17). Additionally, as we increase the 5-FU dose, there is a decrease on the percentage of CD47 positive cells. However, given the low number of replicates for this experiment, we did not obtain statistically significant differences in CD47 expression with 5-FU treatment. In SW480 cells, the presence of 5-FU at 5 μg/mL tends to increase the number of CD47 positive cells (Figure 4.17). On the other hand, *KRAS* inhibition decreases the number of CD47 positive cells. Additionally, it impairs CD47 induction upon treatment with 5 μg/mL of 5-FU treatment and there is only a slight tendency to increase the number of the CD47 at the highest dose. In this way, the differences in CD47 surface levels previously verified between siCTRL and si*KRAS*, are further intensified with the treatment of 5 μg/mL of 5-FU. In contrast, when the 5-FU dose is increased to 10 μg/mL in the SW480 cell lines, the differences between siCTRL and si*KRAS* almost disappear, as both increase CD47 levels to near values. In SW620 cells, there are no differences in surface CD47 expression at any of the doses included in our study, as can be seen in Figure 4.17.

Both in the HCT15 and HCT116 cell lines (Figure 4.18), there appears to be a greater induction of PD-L1 expression at the lower dose of 5-FU and the PD-L1 expression tends to fall with the increase of 5 to 10 μg/mL of 5-FU. However, whereas in HCT15 cells is only a trend, in HCT116 cells the results are already statistically significant. At a low dose of 5-FU (5 μg/mL), the PD-L1 expression in HCT116 cells, negative in the control, is stimulated to values close to 20%. However, with the dose increment to 10 μg/mL, the values decline to only about 4% of PD-L1 positive cells. Additionally, the stimulus generated by 5-FU (5 μg/mL) treatment for PD-L1 expression is abrogated when the *KRAS* gene is silenced. Contrary to the previous two CRC lines, 5-FU treatment increases the

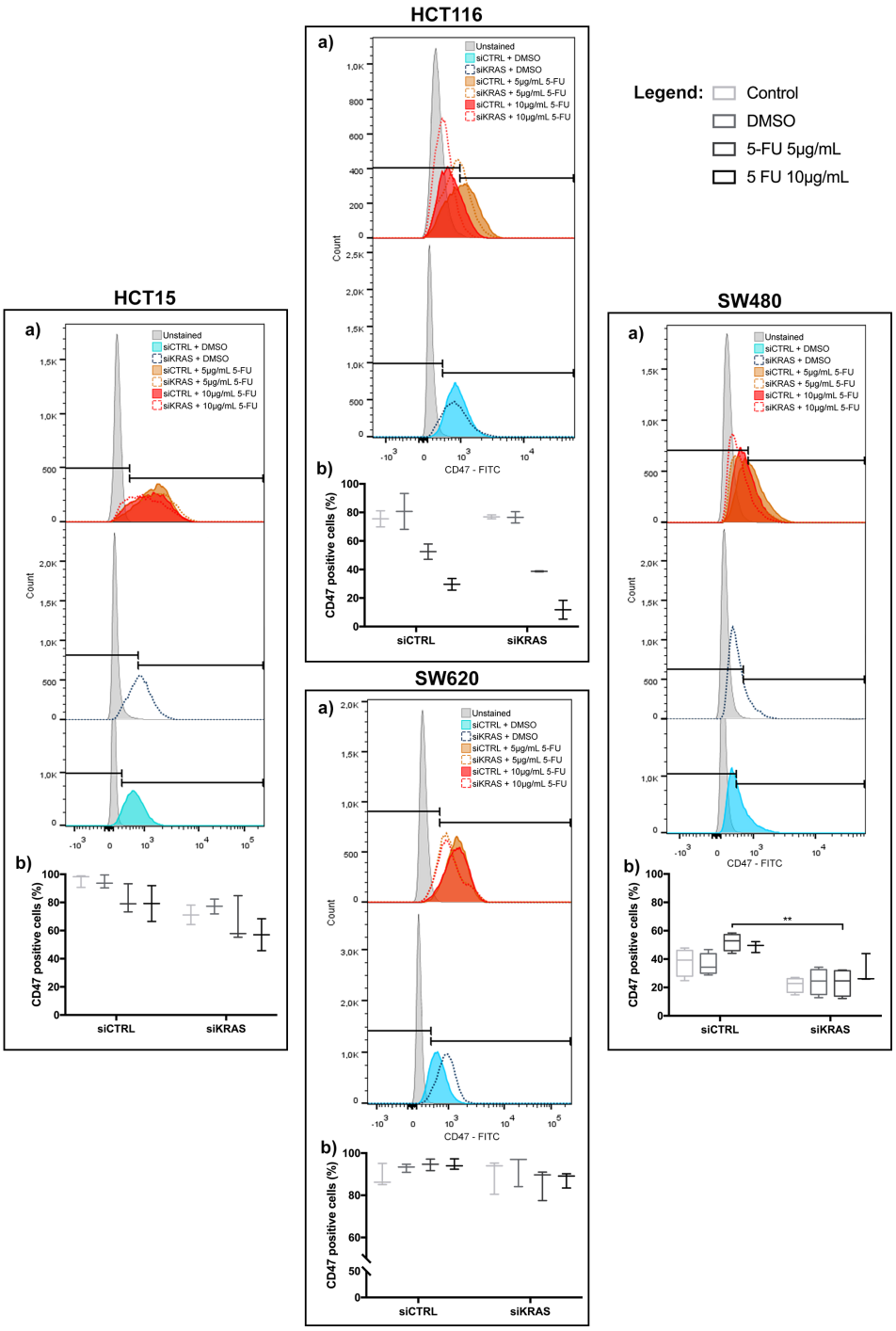

Figure 4.17. CD47 expression in *KRAS* inhibited CRC cell lines after 5-FU treatment. This influence was assessed through the staining of CD47 after cell treatment with 5 μg/mL and 10 μg/mL of 5-FU and analysis by flow cytometry of HCT15 (n= 3 for 5 μg/mL and n= 2 for 10 μg/mL of 5-FU), HCT116 (n= 2 for both concentrations of 5-FU), SW480 (n= 4 and n= 3 for 5 and 10 μg/mL of 5-FU, respectively) and SW620 (n= 3 for both concentrations of 5-FU); **a)** Single parameter histograms of the CD47-FITC staining. The top histogram illustrates the expression levels of the 5-FU treated cells, and the bottom the cells treated with DMSO (control). In HCT15 and SW480 cell lines, the bottom histogram is separated, given the difference in the isotype (negative control) behavior which is more extended in the si*KRAS* conditions. This isotype extension influences the negative/positive gate position. However, when the cells are treated with 5-FU the negative controls (isotypes) of all different conditions overlap surpassing this difference; **b)** Graphs comparing the percentage of positive cells of the different conditions. Two-way ANOVA followed by Turkey's multiple-comparison tests was performed (* indicates a significantly different result $P<0.05$; ** $P<0.01$; *** $P<0.001$).

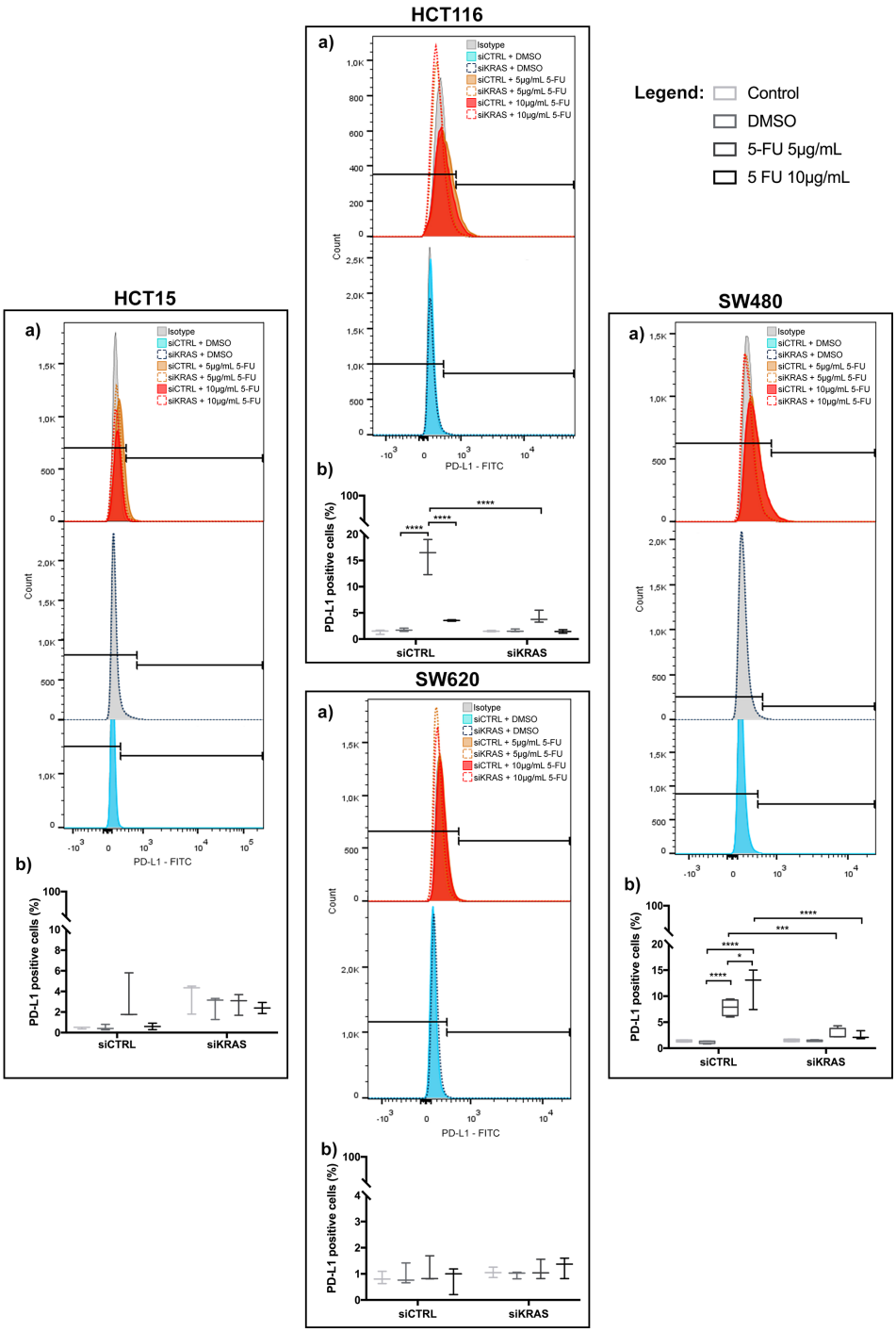

Figure 4.18. Stimulation of PD-L1 expression by 5-FU in *KRAS* inhibited CRC cell lines. Flow cytometry results, after cell treatment with 5 and 10 μg/mL of 5-FU, of HCT15 (n= 3 and n=2 for 5 and 10 μg/mL of 5-FU, respectively), HCT116 (n= 3 and n=2 for 5 and 10 μg/mL of 5-FU, respectively), SW480 (n= 4 and n= 3), SW620 (n= 3 and n= 3 for both concentrations of 5-FU) stained with anti-PD-L1 antibody; **a)** Single parameter histograms of the PD-L1-FITC staining. The top histogram illustrates the expression levels of the 5-FU treated cells, and the bottom the cells treated with DMSO (control). In HCT15 and SW480 cell lines, the bottom histogram is separated, given the difference in the isotype (negative control) behavior which is more extended in the si*KRAS* condition. This isotype extension influences the negative/positive gate position. However, when the cells are treated with 5-FU the negative controls (isotypes) of all different conditions overlap surpassing this difference; **b)** Graphs comparing the percentage of positive cells of the different conditions. Two-way ANOVA followed by Turkey's multiple-comparison tests was performed (* indicates a significantly different result $P<0.05$; ** $P<0.01$; **** $P<0.0001$).

levels of PD-L1 expression in SW480 cells (Figure 4.18) in a dose-dependent manner. However, PD-L1 levels are much lower in *KRAS* inhibited conditions. There are again no statistically significant differences nor a recognizable pattern in the SW620 cell line, Figure 4.18.

In summary, from all the studied immune regulatory molecules frequently altered in tumors, CD47 and PD-L1 are the only ones whose expression is in some particular cases altered with inhibition of the *Kras* gene, possibly promoting cancer immunosurveillance escape. There is also an interesting relationship between these molecules and the ability to be modulated by the *in vitro* treatment with IFN-γ and 5-FU. These molecules, in general, seem to function as a stimulus that increases the expression of these molecules on the surface of the treated cells. Thus, Table V summarizes all the results obtained in this first part of the results. From the analysis of these results, we first observe that only a small percentage of cells analyzed in cytometry, approximately 10%, are CD14 positive. In other words, among the cells analyzed through flow cytometry, only a small percentage of them are macrophages.

4.1.4 Macrophage phagocytosis of CRC cell lines

As an attempt to understand how CD47 expression differences in the HCT15 cells are translated into a biologic effect, we performed phagocytosis assays using macrophages isolated from the PBMC of healthy donors. HCT116 cells were used for comparison, as no changes in CD47 surface expression were observed after *KRAS*-silencing of these cells. Briefly, cells were CFSE-labeled and placed in a ratio of 1 macrophage per 2 cells, in a direct co-culture with the isolated macrophages, for two hours. The efficiency of labeling cells with CFSE-FITC is proven in Figure 4.19a, where all cells that were subsequently placed in contact with macrophages are marked. After two hours of co-culture, the macrophages were labeled with antibodies to CD14 (PE) and SIRPα/β^{+} (APC) and analysed by flow cytometry. CD14 is a macrophage lineage marker, then used to select the macrophagic population.

From the analysis of these results, we first observe that only a small percentage of cells analysed in cytometry, approximately 10%, are CD14 positive as can be seen by the observation of the right quadrants of each plot from Figure 4.19c. In other words, among the cells analysed through flow cytometry, only a small percentage of them are macrophages. As would be expected, by the low ratio of macrophages among the ana-

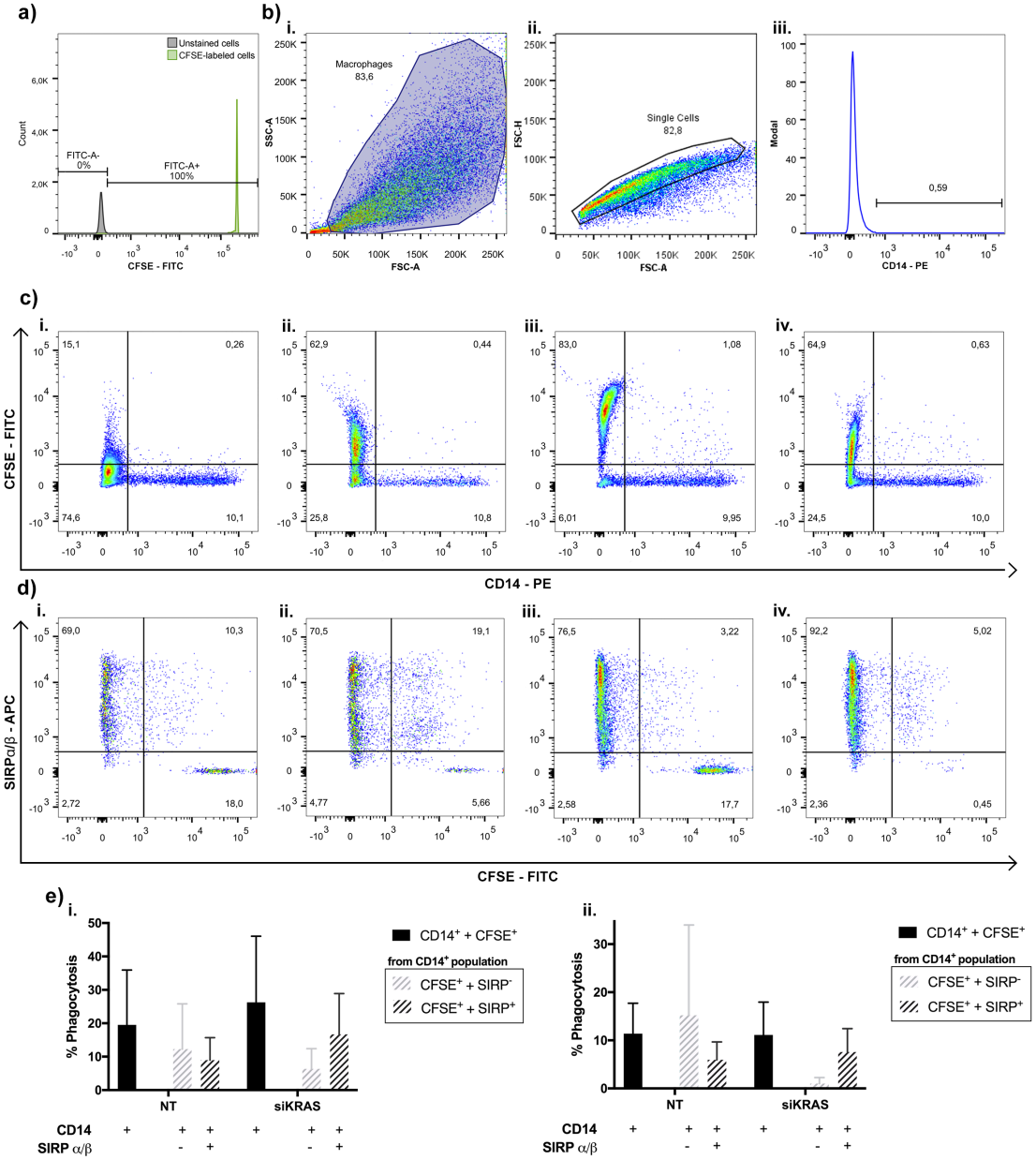

Figure 4.19. Macrophage phagocytosis rate in HCT15 and HCT116 CRC cell lines. a) Representative single parameter histogram of the unstained population in grey and the CFSE-labeled population in green. This histogram refers to HCT15 siCTRL population, however, it is representative of all the other conditions. **b)** Representative pseudo-color plots and histogram of the flow cytometry gating strategy created by FlowJo; **i.** Macrophage population gate, based on forward and side scatter (FSC-A/SSC-A) which represents the cells distribution based on cell size and granularity; **ii.** SSC-A/SSC-H represents the used strategy to determine single cells population; **iii.** The single parameter histogram represents the gating of the CD14 positive cells through the isotype population. **c)** Pseudo-color plots representative of the phagocytosis assay of macrophages (CD14 positive) against CFSE-labeled HCT15 and HCT116 cells; **i.** HCT15 siCTRL; **ii.** HCT15 si*KRAS*; **iii.** HCT116 siCTRL and **iv.** HCT116 si*KRAS* cells. **d)** Pseudo-color plots with the representation of CFSE-FITC/SIRPα/β^+ double positive cells in all conditions (HCT15 and HCT116; siCTRL and si*KRAS*), within the CD14$^+$ cells population. **e)** Graphs display the phagocytosis percentage of four independent experiments for each condition. The last two columns of each condition represent the percentage of macrophages that phagocytized cells that are SIRPα/β^+ or SIRPα/β^-, among the macrophages population (CD14 positive, gate representation in a.iii); **i.** HCT15 and **ii.** HCT116 graph. Two-way ANOVA followed by Turkey's multiple-comparison tests was performed.

lyzed cells population, the phagocytosis values are too weak. Still, no differences in the phagocytosis rate were observed between HCT15 control and *KRAS* inhibited (Figure 4.19), despite the previous results showing a reduction in the number of CD47 positive cells in HCT15 si*KRAS* condition. Similar results were found in the HCT116 cell line. Additionally, there is also a tendency in the si*KRAS* condition of both cell lines to have more SIRPα/β^{+} macrophages phagocyting cells (Figure 4.19d).

4.2 Macrophage distribution within the colon of *Apc*, *Kras* and *Apc*/*Kras* mutated mice

On this second part, we tried to access the *in vivo* infiltration and distribution of macrophages in the colonic epithelium of WT, and *Kras*, *Apc* and *Apc*/*Kras* genetically engineered mouse models of colorectal carcinogenesis, through immunohistochemistry. F4/80 was used as a specific marker of murine macrophages.

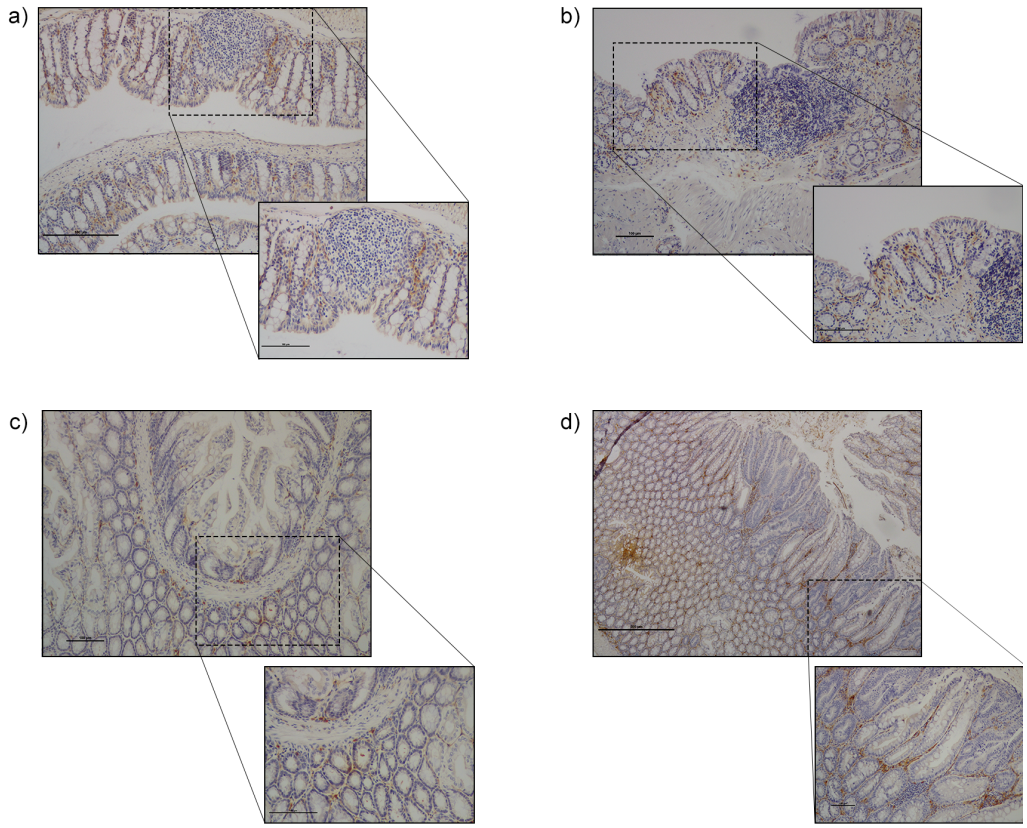

Figure 4.20. Macrophage distribution within the colon of *Apc*, *Kras* and *Apc*/*Kras* mutated mice. F4/80 staining of colon sections **a)** WT, **b)** *Apc* mutated, **c)** *Kras* mutated and **d)** *Apc*/*Kras* mutated mice (100x magnifications in the bigger images and 200x magnifications of boxed sections with the exception of the align d) with 40x magnifications in the bigger image and 100x in the smaller image).

In the normal mucosa of WT mice, macrophages tend to be localized between intestinal crypts (Figure 4.20a). In the *Apc* and *Kras* mutant cases, the macrophagic distribution is very similar to that observed in the WT (Figure 4.20b and 4.20c). However, this increases considerably in the cases where the mice comprise the two mutations, as can be seen in Figure 4.20. At Figure 4.20d, the amount of macrophages is much higher than those found in all the other cases.

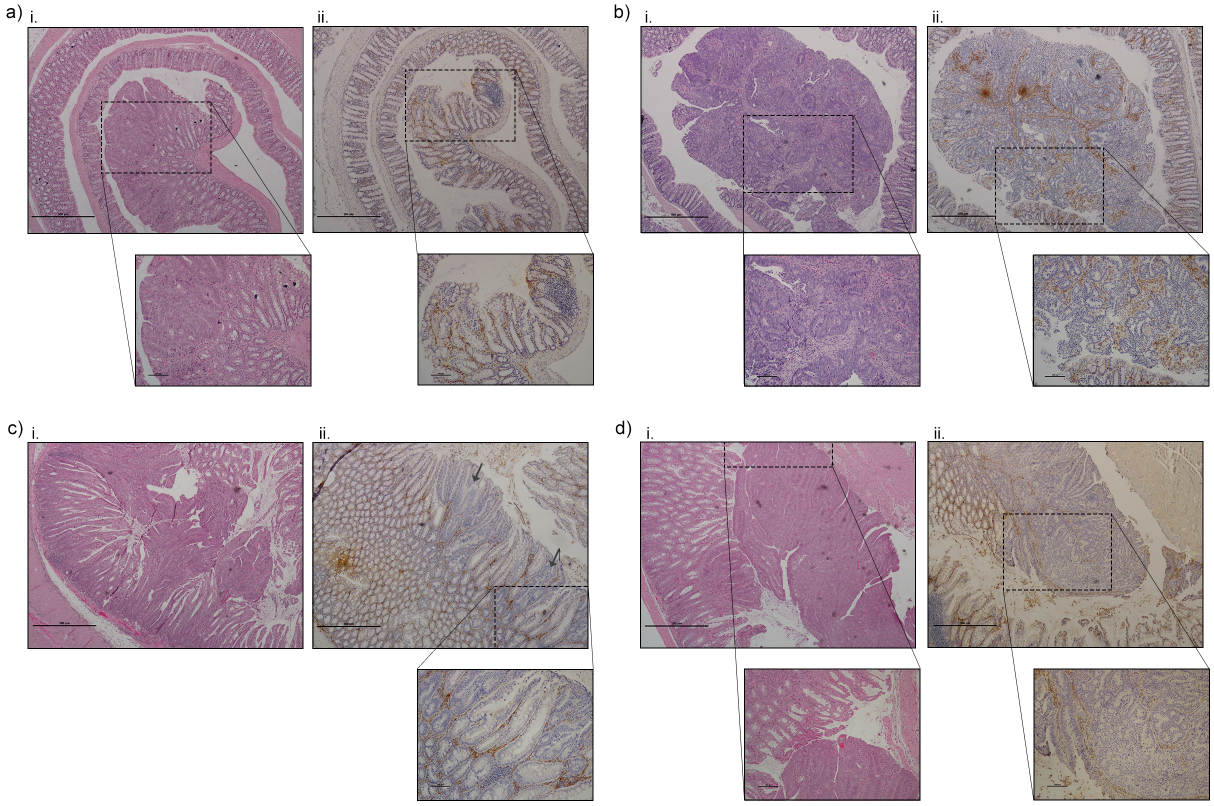

Figure 4.21. Macrophage distribution within tumors at different development stages in *Apc* and *Apc/Kras* mutated mice. a,b) F4/80 staining on *Apc* mutated mice tumors; **a)** Infiltration of less developed and smaller *Apc* tumors; **b)** Macrophage infiltration patterns in more advanced *Apc* tumor. **c,d)** F4/80 staining on *Apc*/*Kras* mutated mice tumors; **c)** Macrophage distribution in a section with different development stages tumors, marked with arrows, in this case the hematoxylin and eosin image does not correspond to the F4/80 staining, it only demonstrates the tissue architecture; **d)** *Apc*/*Kras* more developed tumor macrophage infiltration; **i.** Hematoxylin and eosin (H&E) stain **ii.** F4/80 staining (40x magnifications in the bigger images and 100x magnifications of boxed sections).

In the tumoral context, Apc tumors tend to be more infiltrated when they are smaller, as we can see in Figure 4.21a. However, larger tumors, like that illustrated in Figure 4.21b, tend to be less infiltrated. In addition, infiltration into larger tumors appears to be limited to main stroma branch, whereas in smaller tumors infiltration surrounds the abnormal intestinal crypts. The *Kras* mutation is associated with villous or tubulovillous morphology, considering it has more elongated crypts but, by itself, does not induce

tumorigenesis, unless when conjugated with another mutation (Yuen et al., 2002). Effectively when conjugated to the mutation in the *Apc* gene, tumor development is accelerated and tumors are more advanced when compared to *Apc-driven* tumors. Still, the analysis of macrophage infiltration revealed the same variability found in the tumors developed within the *Apc* loss background. Apc/Kras tumors tend to be more infiltrated when they are still less developed and are therefore smaller, as we can see in Figure 4.21c. As seen in Apc tumors, larger tumors (Figure 4.21d) also appear to be less infiltrated, and this infiltration seems to be more restricted to the main stroma branch supporting the tumor. However, these are very preliminary results that require analysis by a pathologist to assess the degree of tumor development and to enable a correlation between tumor progression and the degree of macrophage infiltration.

Chapter V

Discussion

The emerging role of KRAS mutations as a modulator of the TME and its capacity to shape the immune microenvironment, in particular, is becoming a topic of great interest. Activation of KRAS signaling modulates the nature of immune responses and establish an immunosuppressed state through a dynamical interplay between KRAS-mutant cells and the components of both innate and adaptive immune response (Carvalho et al., 2017). Supported on it we attempt to characterize and understand the influence of KRAS-mutated signalling in the regulation of the immune response in the context of CRC. The work developed in this thesis further supports a role for mutant KRAS in the regulation of the immune response in cancer, highlighting possible mechanisms used by colorectal cancer cells to escape immune surveillance. *KRAS* is, such as *BRAF*, among the most commonly altered genes in CRC (Velho et al., 2008). Both are part of the same signaling pathway, being BRAF a downstream effector of KRAS. Thereby, BRAF was used to assure if the possible obtained alterations are dependent on KRAS mutation.

CD47 upregulation is effectively a common alteration in a large number of cancers and a lot of studies have been done in order to determine the regulatory mechanisms underlying CD47 expression (Jaiswal et al., 2009; Zhang et al., 2015; Lo et al., 2015). For example, nuclear factor (NF)-κB mediates the increase of CD47 in hepatocellular carcinoma, whereas, in breast cancer, CD47 expression was promoted under hypoxic conditions by the hypoxia-inducible factor 1 (HIF-1) (Lo et al., 2015; Zhang et al., 2015). Moreover, MYC-mediated regulation of CD47 is an important hallmark for initiation and development of T cell acute lymphoblastic leukaemia (Casey et al., 2016). Given the capacity of mutant KRAS in regulating these signaling pathways, hereupon, we aimed to understand if mutant KRAS signaling impacts CD47 expression (Bassères et al., 2010; Yeung et al., 2008). In fact, in HCT15 and SW480 cell lines, there is a decrease of CD47 at the cell surface upon *KRAS* inactivation. However, in the other two KRAS mutant cell lines used in this study – HCT116 and SW620 – KRAS downregulation did not affect the expression of CD47. Unexpectedly, *KRAS* inhibition lead to an increase in the number of CD47 molecules expressed at the cell surface of the CD47 positive popula-

tion in all the KRAS mutant cell lines analysed. Although not very pronounced, this increase supports the existence of KRAS-independent mechanisms of CD47 regulation. It is possible that in those cells where *KRAS* downregulation was not enough to inhibit CD47 expression, cells, upon losing the strong pro-tumorigenic signaling derived from *KRAS* activation, up-regulate CD47 as a defense mechanism to avoid being detected and attacked by innate immune cells. We did not proceed with further investigations to understand the molecular mechanism underlying KRAS regulation of CD47 levels. Still, we can already rule out an effect of specific mutations and differences in the MSI status as we could not find a pattern between the two groups of cell lines. As such, we speculate that a combination of factors such as differences in the genetic background of the cell lines and in the capacity of mutant KRAS to activate downstream signaling may be the main reason explaining the observed effects. Regarding the signaling pathways that may be involved in the regulation of CD47 expression, there is some evidence, at the hepatocellular carcinoma field, that activated NF-κB may bind to the promoter region of *CD47* gene and increase their transcription (Lo et al., 2015). Since oncogenic KRAS could induce NF-κB activation, there is a good chance for this to be the mechanism responsible for the influence of KRAS on CD47 expression, Figure 5.22, (Bassères et al., 2010). This speculation is consistent with the IFN-γ ability to stimulate CD47 expression. It was established that IFN-γ is capable of activating NF-κB in a STAT3/PI3K signaling-dependent manner, thereby increasing CD47 levels on the cell surface, as we have seen in all but one KRAS-mutated cell lines studied regardless of the presence or absence of the KRAS mutation (Pfeffer, 2011). Additionally, MYC activation, a KRAS downstream protein, was also related to the upregulation of CD47, so it also can be an alternative mechanism through which KRAS mutation regulates CD47 expression (Yeh et al., 2004; Casey et al., 2016).

SW480 and SW620 cell lines derived from the same patient, established from the primary tumor and from a lymph node metastasis, respectively, and they are a good example of how the genetic background may explain different dependencies of CD47 expression on KRAS activation. A study search for these differences focusing on differential chromosomal alterations found a chromosome segment amplified in SW620 and not in SW480. In this segment were identified four oncogenes: *Telomerase reverse transcriptase* (*TERT*), *Stomach cancer-associated protein tyrosine phosphatase-2* (*SAP2*), *Erb-B2 Receptor Tyrosine Kinase 3* (*ERBB3*), and *MAF BZIP Transcription Factor* (*MAF*) (Melcher et al., 2000). ERBB3, also known as HER3, is a receptor tyrosine-protein kinase that efficiently can activate not only KRAS protein but also PI3K triggering the

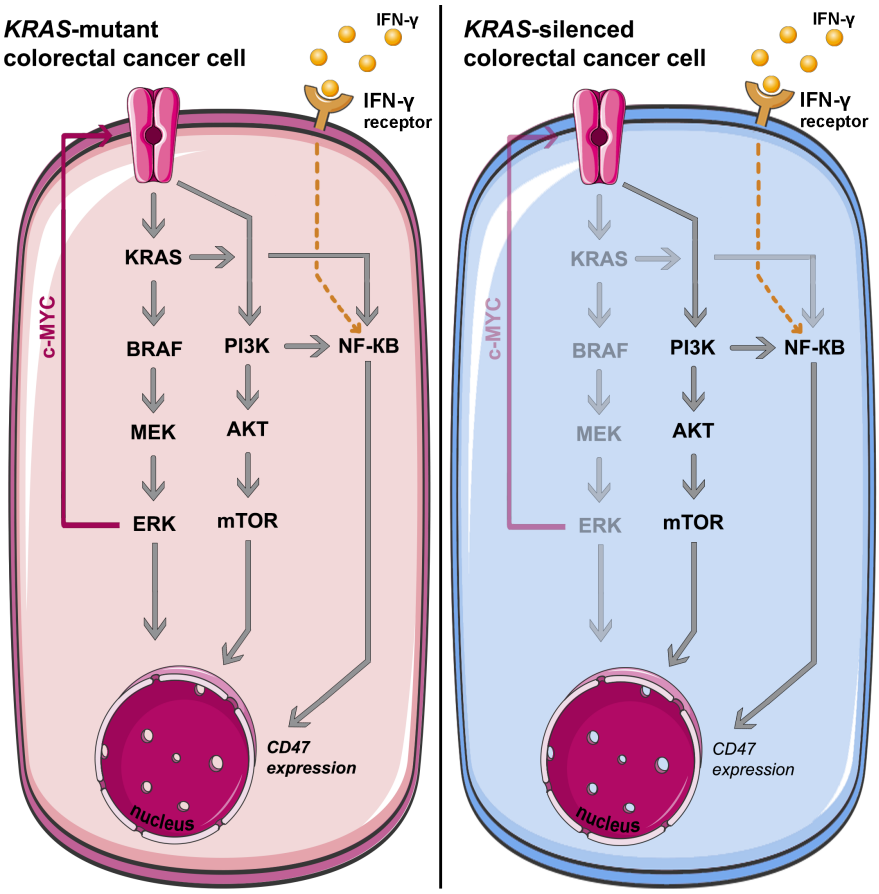

Figure 5.22. Mechanism proposed for KRAS-dependent CD47 expression. KRAS mutation leads to the constitutive activation of the ERK/MAPK pathway, however, this is not the only activated pathway downstream of KRAS protein. This protein is associated with the phosphorylation of various downstream proteins, including PI3K and NF-κB. NF-κB has been associated with the upregulation of CD47 and is also activated via PI3K. So, in a KRAS mutant context, it is expected that the constitutive activation of KRAS proteins brings a continuous activation of NF-κB increasing the CD47 protein at the cell surface. In addition, the pro-inflammatory cytokine IFN-γ can directly increase NF-κB leading to an increase in CD47 expression in a KRAS-independent manner.

MAPK signaling pathway. The differential expression of this receptor can explain the differences obtained, regarding CD47 protein, in SW480 and SW620 cell lines. Effectively, the ERBB3 receptor has been associated with the KRAS-mutated colorectal cancer cells resistance to the MEK inhibitory therapies. MEK-ERK activation enhances MYC stability through phosphorylation of this protein which in turn negatively regulates ERBB2 and ERBB3 gene expression (Sears et al., 1999; Sun et al., 2014). Therefore, the lower activation of MYC prompted by MEK inhibition leads to an increase of ERBB2/3 mRNA. The authors found, in both SW480 and SW620 cell lines, that there was a significant increase of ERBB2 and ERBB3 levels as a consequence of MEK inhibition. However, consistently with the past discoveries, the SW620 levels of ERBB3 were twice bigger than SW480 values (Sun et al., 2014). It suggests that, in SW620, there is a cumulative effect of the baseline higher levels of ERBB3 with the upregulation generated by MEK

inactivation. Thus, SW620 can compensate the *KRAS* inactivation through the higher levels of ERBB3 that activates PI3K, leading to the maintenance of CD47 expression, even after *KRAS* inhibition, promoted by NF-κB activation via PI3K, Figure 5.23. As we have seen, there are several factors that influence and regulate the CD47 expression. Therefore, although HCT15 and HCT116 share the same mutation in the *KRAS* gene, there are a number of differences in the mutational profile that may alter the output, as it occurs with SW480 and SW620. Likely, as we suspect for SW480 and SW620, HCT116 cells can also have a compensatory mechanism for CD47 expression.

Interestingly, in the BRAF-mutated colorectal cancer cell lines that we used, RKO and HT-29, we did not verify significant differences at the CD47 surface levels after *BRAF*-inhibition. Previous studies have already investigated the possible action of BRAF/MEK inhibition in the regulation of CD47 expression. They proved that BRAF/MEK inhibition upregulates CD47 in melanoma. Furthermore, they demonstrate that this upregulation is due to the reactivation of ERK which leads to increased levels of CD47 mRNA. ERK reactivation is a major mechanism whereby BRAF-mutated melanoma cells acquire resistance to BRAF inhibitors. Additionally, CD47 upregulation was associated with an ERK-mediated enhancement of the nuclear respiratory factor 1 (NRF-1), that acts as the terminal effector on this cascade (Liu et al., 2017). Nevertheless, melanoma tumors harboring BRAFV600E are more sensitive to the BRAF inhibitory therapies than CRC tumors with the same mutation and a group from the University of Texas tried to understand the reason why this happens. They found that the mechanism by which CRC tumor "escape" the BRAF inhibition therapies is related to their higher levels of PI3K (Mao et al., 2013). This demonstrates that, although harboring the same mutation, there are different mechanisms and backgrounds within the tumor types, so probably the mechanism by which melanoma cells upregulate CD47 in response to BRAF inhibition does not occur in CRC cases with the same mutation what can explain the differences comparing these tumors. Additionally, in BRAF-mutated analyzed cell lines, almost 100% of cells express CD47 so there is no margin to increase this values and, regarding the median fluorescence intensity, it seems to be increased after BRAF-inhibition but it did not reach statistically significant results. This shows that the differences regarding CD47 expression with oncogenic silencing in CRC is probably exclusive of KRAS mutation, since no differences were obtained with *BRAF* inhibition.

Patients with higher levels of CD47 have a worse prognosis and overall survival, in part, due to the ability of CD47 to prevent phagocytosis by macrophages (Chao, Alizadeh,

Tang, Jan, Weissman-Tsukamoto, Zhao, Park, Weissman and Majeti, 2011). Thus, we attempt to realize *in vitro* how the decrease of CD47, after *KRAS* inhibition, influences the chance of these cells for being phagocytosed. However, we observed that HCT116 control cells are less phagocyted than HCT15 control cells despite having a lower percentage of CD47 positive cells. Additionally, in HCT15 there were no significant differences in the percentage of phagocytose despite the differences in the number of CD47 positive cells observed upon *KRAS* inhibition. Still, we could observe a slight tendency in the si*KRAS* condition of both cell lines to have more SIRPα/β^{+} macrophages phagocyting cells. It is plausible that a different balance of "eat me" and "don't eat me" signals, in each individual cell line, can contribute to this phenomenon. Although there is a lack of consensus on this topic, the most recent studies have unveiled the association of higher levels of SIRPα with tumor-associated macrophages which have an immunosuppressive phenotype like M2 (Qie et al., 2016; Yan Lin and Han, 2018; Zhang et al., 2013). Together with our results, this suggests that M2-polarized macrophages, which are frequently found in the proximity of cancer cells, are less proficient to phagocyte KRAS-mutated cells.

Nevertheless, the results obtained were not consistent with the previous observations regarding regulation of CD47 expression by mutant KRAS. We postulate that the low levels of phagocytosis and the lack of positive results reflect an inefficiency of our experimental approach given that only a small fraction of immune cells were macrophages, and the broad variability of macrophage batches. Moreover, monocyte-derived macrophages which we isolated were not polarized, so they were on an intermediate state between the M1 and M2 polarization, the so-called M0 like phenotype. Thus, our model does not consistently represent the *in vivo* characteristics. Considering that M1 macrophages display a greater phagocytic response than M2 macrophages, it would be interesting to evaluate the influence of macrophage polarization in our model (Zhang et al., 2016). Additionally, the SIRP antibody used targets not only SIRPα but also SIRPβ which may be a confounder variable. Given that SIRPβ is a SIRP family member, similar to SIRPα, but does not bind to CD47, it would be interesting to use a more specific marker disregarding the possible marking of SIRPβ.

There are in course clinical trials regarding anti-CD47 therapies, but a large number of patients have received or still receive chemotherapy as a co-adjuvant therapy (of Health et al., 2012). The effects of chemotherapy on the immune responses is now an emerging issue. In fact, this constitutes one of the most used therapeutic approaches in CRC treatment, and 5-FU prevails as the mainstay of the chemotherapeutic treatment of CRC.

However, some studies have questioned the 5-FU efficacy in some subsets of CRC, such as MSI tumors. In these cases, where there is a high immunogenic component, there is more resistance to this medicine and patients do not benefit from this treatment approach (Carethers et al., 2004). Nevertheless, in CRC, the selection of patients treated with 5-FU has been based only in cancer stage rather than the biology of the tumor (Moertel et al., 1995; Carethers et al., 2004). Our results suggest that 5-FU treatment of colorectal cancer cells decrease the CD47 levels in HCT116. In opposite, the CD47 surface levels are increased in SW480 and are no significantly altered in HCT15 and SW620. However, there is a tendency to a decrease in HCT15, such as in HCT116. IC_{50} of HCT15 (3,93 μg/mL) is higher than in HCT116 cells (2,24 μg/mL) what may explain the higher resistance of HCT15 to 5-FU treatment (Tsuruta et al., 2008). Thus, it is expected that with the continuous increase of the 5-FU dose, the CD47 levels of HCT15 cell line begin to decrease to significant values. It is not surprising that CD47 expression decreases with 5-FU treatment since, during apoptosis, CD47 levels should be removed or inactivated to allow the efficient clearance of debris (Gardai et al., 2005; Lawrence et al., 2009). On the other hand, IC_{50} of HCT15 and HCT116 cell lines is not considerably different from the other cell lines in which the 5-FU effect is different (SW480 - 3,01 μg/mL and SW620 - 3,28 μg/mL) (Tsuruta et al., 2008; González-Vallinas et al., 2013). It proposes that it may be an association between the MSS status and the CD47 response to 5-FU treatment, suggesting that MSI colorectal tumors decrease CD47 levels upon 5-FU treatment whether MSS tumor increase these values, at least one of the studied cell lines. However, interestingly there is evidence that only MSS tumors stage II or III benefit from 5-FU treatment, improving patients survival (Ribic et al., 2003; Sargent et al., 2008). This brings into question the possibility to MSS tumors benefit from the chemotherapy-conjugation with anti-CD47 therapy, since the later can blunt the CD47 increase prompted by 5-FU. The chemotherapy conjugation with anti-CD47 therapies was tested *in vivo* in a murine B cell lymphoma cell line. It was proved that the chemotherapy application before anti-CD47 therapy brings out better results than the inverse employment. Additionally, they also proposed that when chemotherapy was administrated after anti-CD47 therapy it brought adverse consequences on the development of anti-CD47-induced immune memory, promoting relapses after mice re-injection with murine B cell lymphoma cells (Liu et al., 2015). Further tests are required to fully understand the effects of chemotherapy treatment followed by anti-CD47 blockade in MSS colorectal cancers.

SIRPα and β receptors expression is described, in several reports, as restricted to myeloid and neuronal cells (Adams et al., 1998; Brooke et al., 1998). However, in

our research, we found that colorectal cancer cells, except HCT15 and HT-29, express SIRPα/β, in high proportion, at their cell surface. A later study evaluates by immunofluorescent staining the expression levels of SIRPα in a colorectal cancer cell line (SW480) in co-culture with macrophages and contrary there was no expression in these cells (Zhang et al., 2013). Implying that probably, cells are only positive for SIRPβ regarding the duality of the used antibody which targets SIRP α and β. Not disregarding the possibility of SIRP expression being modulated by co-culture with macrophages. Summing-up, more studies are needed in this field considering that less is known about the expression and function of SIRPα/β in tumor or normal cells.

A recent study found out that oncogenic RAS signaling enhances PD-L1 expression in type II pneumocytes harboring $KRAS_{G12V}$ and $KRAS_{G12C}$, and in MSI colorectal cell line harboring $KRAS_{G12C}$. Additionally, in type II pneumocytes it also described that this is possible through RAS signaling via MEK which stabilizes PD-L1 mRNA. PD-L1 mRNA is, in normal conditions, marked by ARE-binding protein tristetraprolin (TTP) which impair the mRNA stability and negatively regulates PD-L1 expression. In lung cancer, oncogenic RAS signaling reduces TTP activity by stabilizing PD-L1 mRNA, thereby increasing PD-L1 expression and immune evasion in cancer (Coelho et al., 2017). However, as colorectal cancer cells were not contemplated in these further studies this regulatory mechanism is not proved for colorectal tumors. PD-L1 is overexpressed in approximately 30% of CRC cases and is associated with poor prognosis and shorter overall survival, being associated with improved cellular proliferation, migration and invasion adding to its immunosuppressive function on T cells (Droeser et al., 2013). Nonetheless, MSI tumors have been pointed out as those who will mostly benefit from immune checkpoint inhibitory therapies, considering the high immunogenicity of this tumor and due to the demonstrated upregulation of several immune-inhibitory ligands and receptors, including the IFN-γ inducible PD-L1 (Llosa et al., 2015). Effectively, our results are in concordance with this whereas HCT15 and HCT116, our MSI representatives, express higher levels of PD-L1 than MSS studied cell lines, after IFN-γ stimulation. However, these higher PD-L1 levels in MSI cell lines are largely dependent on the expression of *KRAS* mutation as PD-L1 expression is decreased upon *KRAS*-mutation silencing, but only in the MSI colorectal cancer cells. Summarily, MSI colorectal tumors show higher levels of PD-L1 expression especially when the KRAS mutations were active, and probably the mechanism underlying this is also the increased PD-L1 mRNA stability. These results provide strong evidence that MSI KRAS-mutated colorectal cancers can hugely benefit from anti-PD-L1 blockade therapy. However, IFN-γ production by T cells oc-

curs very early at the primary tumor stages, and beside its recognition as a positive step for tumor shrink since this cytokine activates the immune system, it also stimulates the PD-L1 upregulation in a lot of different tumors, such as CRC. Thus, although it leads to improved recruitment of immune cells it also influences cancer cells to become more shielded against these cells, through PD-L1 upregulation, which allows the PD-1 signaling axis to become more active, leading to the downregulation of the cytotoxic response. Immunotherapy with inhibitors of this immunological checkpoint (PD-1/PD-L1) seems to shrink tumors in melanoma, lung cancer, among others and it is associated with lower levels of toxicity than other immunotherapies, with durable responses (Curran et al., 2010; Reck et al., 2016). Along with this, the vast majority of ongoing clinical trials for anti-PD-1/PD-L1 immunotherapy in colorectal cancer are aimed at more advanced and mostly metastatic states (of Medicine, n.d.). However, given the celerity with which IFN-γ is secreted at the tumor genesis timeline, our results suggest that induction of PD-L1 expression may be a mechanism used by KRAS-mutant MSI CRC cells to escape immune evasion and progress towards more aggressive stages. Therefore, they raise the hypothesis whether KRAS-mutant MSI CRC patients should undergo anti-PD-1/PD-L1 immunotherapy at the earlier stages of disease development. Indeed, more studies are required to deeply evaluate this hypothesis, to find the exact mechanism behind this KRAS-mediated PD-L1 expression and to understand why this only occurs in MSI CRC cell lines.

The PD-L1 expression has been associated with signatures of the serrated pathway of colorectal carcinogenesis, including BRAF mutation, MSI status, poor differentiation (with medullary morphology), and frequent TIL (Rosenbaum et al., 2016). Immunogenic MSI tumors express PD-L1 under selective pressure factors. Co-occurrence of BRAF mutation and MSI status, in the serrated pathway of colorectal carcinogenesis, is very common and the BRAF mutation was also associated with a higher expression of PD-L1 (Rosenbaum et al., 2016). Accordingly, RKO cells (MSI and BRAF mutant) did not need IFN-γ stimulation to express PD-L1, however, there are no differences after *BRAF* inhibition, suggesting that BRAF activation is not involved in the regulation of PD-L1 expression in this cell line. The other BRAF mutant cell line used in this work, HT29, expressed very low levels of PD-L1 at basal conditions, similarly to what was found in the KRAS-mutated cell lines. We did not performed further studies in this line, but it will be interesting to determine whether HT29 cells increase PD-L1 levels upon stimulation with IFN-γ and whether KRAS or BRAF are involved.

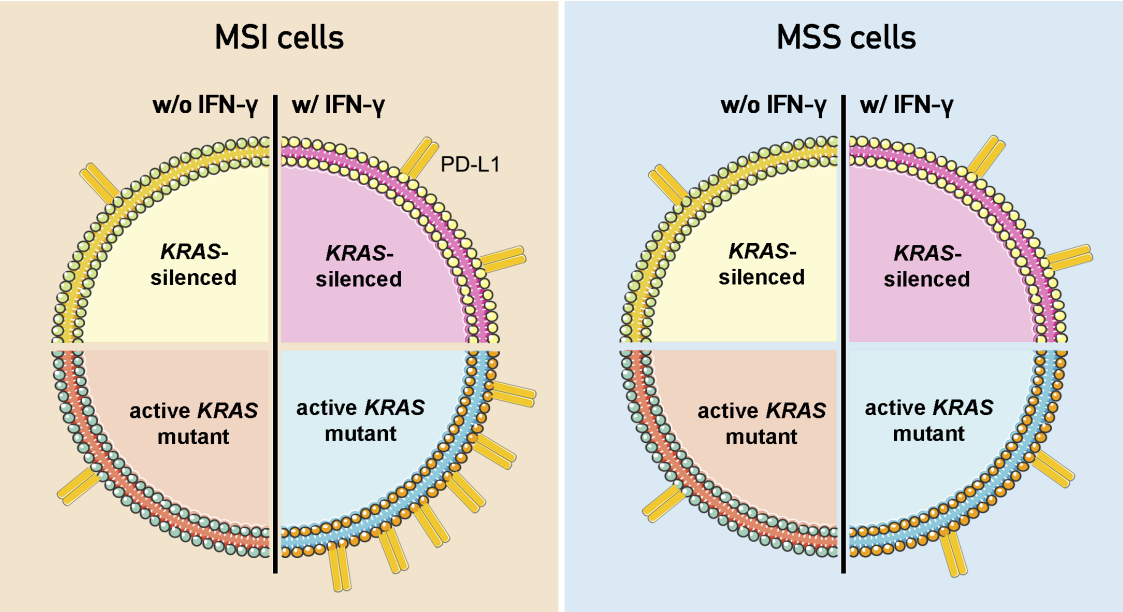

Figure 5.23. The influence of microssatellite instability status on PD-L1 expression. KRAS mutation seems to boost PD-L1 expression in an MSI context after IFN-γ stimulation. On the other hand, this effect is not observed in MSS colorectal cancer cells in which there are lower expression levels of PD-L1 after IFN-γ stimulation comparing with MSI CRC cells and this expression is not affected by the KRAS inhibition. The upregulation of PD-L1 on the cell surface of MSI CRC cells may impair T cell activation and proliferation leading to an immunosuppressive TME.

A previous study demonstrated that chemotherapeutic agents, such as 5-FU, induce PD-L1 expression in breast cancer in an IFN-γ independent manner and, additionally, can potentiate the IFN-γ stimulus (Zhang et al., 2008). Nevertheless, the mechanism behind this association was not described. Years later, in 2016, there was evidence that the same could also be true for colorectal tumors through a study which performed extensive bioinformatics analyses using independent transcriptional profiling datasets. This interesting paper suggests that MSI status effectively correlates with higher PD-L1 expression levels, but it is also regulated through a MSI-independent mechanism since a few MSI-high CRC tumors have lower levels of PD-L1 expression. Importantly, they have aroused that the PD-L1^{high} CRC subgroup have no clinical benefit from chemotherapy and indeed may be damaged by adjuvant 5-FU therapy (Dunne et al., 2016). Interesting, our results suggest that in MSI CRC cell lines, low doses of 5-FU are capable of inducing PD-L1 upregulation. Probably, there is again a higher resistance of HCT15 than HCT116 because HCT15 seems to have the same tendency as HCT116, however, did not result in significant results. Nonetheless, at MSS level, 5-FU induced no response in SW620 and triggered PD-L1 expression in SW480 empowered by the KRAS mutation. However, the higher the concentration of 5-FU, the bigger the expression of PD-L1, contrariously to MSI cell lines results were the lowest concentration of 5-FU generate higher levels of

PD-L1. This suggest that in MSS CRC cases the chemotherapy administration should be accompanied by an anti-PD-L1 immunocheckpoint inhibitory therapy. Further studies, to understand the mechanism by which 5-FU treatment affects differently PD-L1 expression are needed. Nevertheless, it would be quite interesting to evaluate the effect of higher doses of 5-FU and its performance together with IFN-γ, since earlier studies suggested both as having a cumulative action in increasing PD-L1 levels.

There are a lot of studies focused on IFN-γ signaling and its relationship with tumor development that have been elucidating positive and negative effects of this cytokine to the tumor progression. The capacity that IFN-γ has to increase tumor immunogenicity, upregulates MHC class I expression, and inhibits angiogenesis are favor arguments to the potential immunotherapy use of this cytokine (Seliger et al., 1996; Coughlin et al., 1998). However, the growing number of studies reporting adverse cancer immunotherapy effects of this cytokine has put a brake on this possibility. The down-modulation of TAA expression and loss of efficient processing of some TAA, as well as up-regulation of PD-L1 in a lot of different cancers, are some of the negative impacts associated with this cytokine (Beatty and Paterson, 2000; Morel et al., 2000; He et al., 2015; Krönig et al., 2014). These controversy results are the reason why IFN-γ is not an approved treatment possibility in cancer immunotherapy. Additionally, our results suggest that IFN-γ capacity to stimulate CD47 and PD-L1 in colorectal cancer cell lines is a new insight into the negative impacts of this treatment option in CRC cases.

The expression of HLA-ABC in tumors and its downregulation as a mechanism that favors tumor progression, especially at the beginning of tumor development, is a subject long debated by the scientific community (Gutierrez et al., 1987; Menon et al., 2002). Lack of MHC class I complex in tumors has been associated with oncogenic alterations such as the case of *c-Myc* activation in melanoma (Versteeg et al., 1988). Notwithstanding it was observed a connection between *KRAS* gene activation and MHC class II in fibrosarcoma tumor cells, and a downregulation of HLA class I antigen related to *KRAS* codon 12 mutations in non-small cell lung cancer, however, it was not noticed on KRAS-mutated colorectal cancer (Alon et al., 1987; He et al., 2013; Oliva et al., 1990). Our results showed that *KRAS* silencing does not alter the number of HLA-ABC expressing cells, although, it influences the number of molecules each cell has on its surface. In contrast, *BRAF* silencing did not cause any differences neither in the number of HLA-ABC expressing cells nor in the number of molecules expressed at the cell surface. Thus, in the analyzed cell lines, with the exception of the SW480 cell line, the active KRAS appears

to decrease the levels of HLA-ABC per cell. This decrease will probably be translated on a limited targeting and destruction of these cells by cytotoxic T-lymphocytes, given that it is expected that the number of presented antigens diminishes. This is particularly important in tumors with a "mutator phenotype", characterized by defects in the MMR genes which produce altered proteins in a large scale and are correlated with total loss of HLA-ABC (Menon et al., 2002). Interestingly, in CRC, contrasting with other tumors such as breast cancer and melanoma, the lack of HLA-ABC is correlated with a lower tumor stage and a better prognosis (Concha et al., 1991; van Duinen et al., 1988). Probably, this better prognosis associated with loss of HLA-ABC in colorectal cancer may be justified by its frequent occurrence in MSI-high tumors, which, by themselves, already have a better prognosis (Menon et al., 2002). However, this cancer research field has been disregarded with the mass emergence of new targets with greater therapeutic potential than the HLA-ABC system. Nevertheless, these results are somewhat contradictory and very preliminary so further studies are required to realize the actual influence of this molecule in CRC and the way its expression is influenced by the presence of oncogenes.

High infiltration of TAMs correlates with poor prognosis and promote tumor progression in the vast majority of the cancers as the TME benefits macrophage polarization into a pro-tumoral M2 phenotype. In contrast, in CRC high macrophage infiltration has colorectal tumors have been associated with better prognosis and survival rate (Edin et al., 2012). It is supposed that the anti-tumorigenic M1 macrophage phenotype is the most prominent in CRC patients due to the intestinal environment where the functional adaptation of macrophages is needed for the local tissue homeostasis which favors the better immune response against the tumor (Edin et al., 2012). The presence of APC mutation in CRC is thought to increase the signaling between malignant cells and infiltrated macrophages (Smith et al., 1999). However, our results did not suggest any differences in macrophage infiltration in *Apc* mutated cases compared to WT, except when combined with the *Kras* activation. Additionally, tumor-infiltrate macrophages and neutrophils have been pointed out as being also influenced by the KRAS mutation. A detailed analysis of lung cancer postulates the capacity of KRAS mutant tumor cells to secrete high levels of neutrophils chemokines but very low levels of macrophage chemokines. This study also demonstrates that macrophages, in contrast with neutrophils that infiltrate the tumor, preferentially localized on the tumor periphery (Ji et al., 2006). However, later Geou-Yarh Liou group demonstrate that oncogenic KRAS in pancreatic acinar cells upregulates the expression of *ICAM-1* which serves as a chemoattractant for macrophages (Liou et al., 2015). Our results suggest that *Kras*-mutation in CRC does not increase the

macrophage infiltration unless in cooperation with Apc inactivation. *Apc* inactivation and oncogenic activation of the Kras-signaling pathway is very frequent in colorectal cancer and is associated with poor prognosis as both synergize in the promotion of adenoma growth and progression (Janssen et al., 2006). The macrophage infiltration in colon and rectum of *Apc* and *Kras* mutated mice was dramatically higher than the observed in cases with a single mutation in one of these mutations. The higher macrophage infiltration may arise as a result of the chemoattractants soluble factors release by cancer cells or can be a consequence of the higher rates of tumor formation which is a frequent characteristic of this genotype. However, in spite to be an indication of a better prognosis to CRC tumors, these tumors APC- and KRAS-mutated are known to be more aggressive. Therefore, more studies are needed to understand the mechanism through which these mutations cooperate in the promotion of macrophage infiltration and to know which macrophage polarization prevails.

Chapter VI

Conclusion and Future Perspectives

Table V summarizes the results regarding alterations in the expression of immune- modulatory molecules upon *KRAS* and *BRAF* silencing. The results support our initial hypothesis, showing that mutant KRAS is able to regulate the expression of immune stimulatory and inhibitory molecules present at the surface of colorectal cancer cells. In particular, the results obtained award mutant KRAS a role in the development of mechanisms that allow colorectal cancer cells to escape immune surveillance through downregulating the expression of immune-stimulatory molecules such as HLA-ABC, and promoting the expression of immune inhibitory molecules such as CD47 and PD-L1. Moreover, the unexpected increase in the MFI values of CD47 upon *KRAS* inhibition (observed even in cell lines where loss of KRAS reduces the number of CD47 positive population) highlight a possible compensatory mechanism of defense that cancer cells may develop when depleted of a strong oncogenic signal. Additionally, they also suggest that in BRAF mutant CRC cells the expression of immune-modulatory molecules evaluated in this work are likely to be controlled through alternative pathways independent of mutant BRAF. The capacity of mutant KRAS to regulate the expression of immune-modulatory proteins varies amongst the cell lines analyzed. Still, the results allow the construction of two different scenarios considering the MSI status of the cancer cells. In MSI cell lines, PD-L1 expression under IFN-γ stimulation is dependent on mutant KRAS signaling. Given the high degree of lymphocyte infiltration found in MSI tumors, this result pinpoint a KRAS-dependent mechanism used by mutant KRAS MSI CRC cells to evade immune surveillance allowing cancer development and progression. In MSS cell lines, the effect of KRAS on the expression of immune-modulatory proteins was more evident in the cell line derived from the primary tumor. In this cell line, KRAS promotes CD47 expression in basal conditions and upon IFN-γ stimulation. Moreover, it also promotes PD-L1 expression upon 5-FU treatment. Taken together, the results on this MSS cell line suggest that in mutant KRAS MSS primary tumors, lack of response or resistance to 5-FU-based chemotherapies may be caused by the capacity that mutant KRAS confers to cancer cells of inhibiting an anti-cancer immune response triggered by chemotherapy. Unfortunately, the functional *in vitro* and *in vivo* studies developed in this work were very preliminary

and not informative enough to support the implications of the findings. Therefore, additional *in vitro* and *in vivo* experiments are needed to prove the biologic significance of the differences observed and the validity of the two scenarios proposed. As a general conclusion, KRAS seems to have much more functions beyond those accrued from the ERK/MAPK signaling activation to support tumor proliferation, growth, and survival, as previously suggested. It also influences the TME and promotes tolerance to tumor cells by the immune system through the modulation of immune checkpoints-associated proteins. Considering the high resistance of tumor cells to current treatments, and the lack of KRAS-targeted therapies, it is of great importance the broader knowledge on the mechanisms in which the KRAS mutation is involved so that more promising therapeutic approaches can be developed.

Table V. Resume of the principal results relative to the *KRAS* and *BRAF* genes and their putative role in regulating the immunosurveillance molecules expression. In parenthesis are presented the alterations regarding MFI (* indicates a significantly different result $P<0.05$; ** $P<0.01$; *** $P<0.001$;**** $P<0.0001$).

		Cell Line					
		siKRAS				**siBRAF**	
Protein	**Treatment**	**HCT15**	**HCT116**	**SW480**	**SW620**	**RKO**	**HT-29**
CD47	**w/o treatment**	$\downarrow$*** ($\uparrow$*** MFI)	= ($\uparrow$* MFI)	$\downarrow$** ($\uparrow$* MFI)	= ($\uparrow$* MFI)	=	=
	IFN-γ	=	=	$\downarrow$***	=	—	—
	5-FU (5 μg/mL)	=	=	$\downarrow$**	=	—	—
	5-FU (10 μg/mL)	=	=	=	=	—	—
SIRPα/β	**w/o treatment**	$\uparrow$*	=	=	=	=	=
PD-L1	**w/o treatment**	=	=	=	=	=	=
	IFN-γ	$\downarrow$****	$\downarrow$****	=	=	—	—
	5-FU (5 μg/mL)	=	$\downarrow$****	$\downarrow$***	=	—	—
	5-FU (10 μg/mL)	=	=	$\downarrow$****	=	—	—
HLA-ABC	**w/o treatment**	= ($\uparrow$* MFI)	= ($\uparrow$* MFI)	=	= ($\uparrow$* MFI)	=	=

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Supplementary Figures

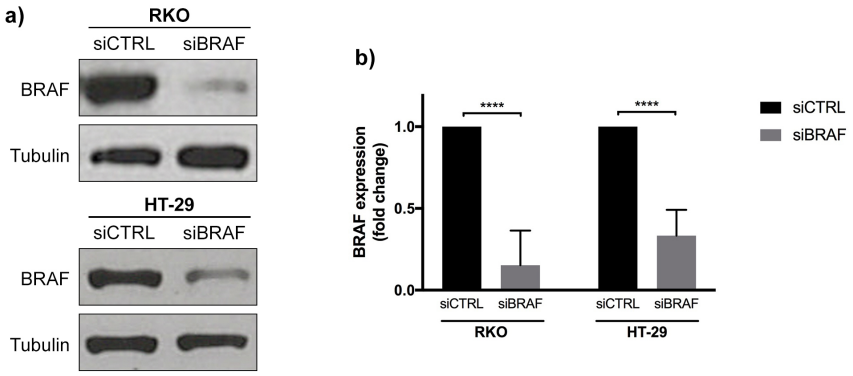

Figure S1. Confirmation of *BRAF* silencing. a) Western blot analysis of both cell lines, RKO and HT-29, revealed a significant decrease of BRAF protein levels after silencing (siBRAF) when compared with the negative control (siCTRL). **b)** Histogram represents the silencing confirmation revealed by the decrease of BRAF protein on the used samples.

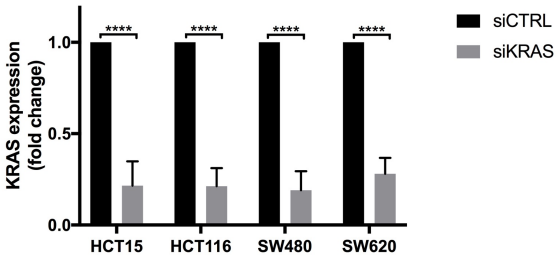

Figure S2. Confirmation of *KRAS* silencing. qRT-PC results after *KRAS* silencing, comparison of *KRAS* expression levels of the negative control (siCTRL) with the si*KRAS* in HCT15, HCT116, SW480 and SW620.

Table SVI. Resources Table.

Product	Manufacturer	Catalog nº
Fetal Bovine Serum	GE Healthcare	SV30160.03
Pen Strep	Life Technologies	15140-122
RPMI medium	Life Technologies	72400-021
DMEM medium	Life Technologies	41965-039
Lipofectamine RNAiMAX	Invitrogen	13778-150
Opti-MEM	Life Technologies	11058-021
Proteases Inhibitors Cocktail	Roche	11836170001
Phosphatase Inhibitors Cocktail	Sigma-Aldrich	P0044-1ML
DCProtein assay kit	BioRad	#500-0114
Molecular weight ladder	BioRad	#161-0374
Nitrocellulose blotting membranes	GE Healthcare	106000002
Ponceau S solution	Sigma-Aldrich	P7170-1L
Tween-20	OmniPur	9480
Bovine Serum Albumin	NZYTech	MB04602
Clarity Western ECL Substrate	BioRad	102030695
TripleXtractor	GRiSP Research Solutions	GB23
Chloroform	Merck Millipore	1024311000
Isopropanol	Fisher Scientific	P/7555/17
RNase-free water	Invitrogen	10977-035
qScript^{TM} cDNA SuperMix	Quanta Bioscience	95161-100
Taqman Universal PCR Master Mix	Applied Biosystems	4324018
Fluorouacil	Sigma-Aldrich	F6627
Dimethyl Sulfoxide	Sigma-Aldrich	D4540
IFN-γ	ImmunoTools	11343536
Trypsin-EDTA	Invitrogen	25300-062
RosetteSep Human Monocyte Enrichment Cocktail	StemCell Technologies	#15028
FBS	Biowest	S181A
Ficoll-Histopaque 1077	Sigma-Aldrich	10771
Macrophage Colony-stimulating factor	ImmunoTools	11343113
Carboxyfluorescein diacetate succinimidyl ester	Invitrogen	C34570
Accutase	BD Biosciences	561527
Formaldehyde solution 37%	Merck Millipore	10400131000
NH_4Cl	Calbiochem	168320
TritonX-100	Sigma-Aldrich	9002-93-1
Vetashield Mounting Medium with DAPI	Vector Laboratories	H-1200
LabVision^{TM} hydrogen peroxide block solution	Thermoscientiphic	TA-060-HP
LabVision^{TM} avidin-biotin blocking solution	Thermoscientiphic	TA-015-BB
Goat anti-rat-biotin	ENZO	ALX-211-058-C100
Horseradish Peroxidase, R.T.U.	Vector Laboratories	SA-5704
DAB Quanto	Thermoscientiphic	TA-060-QHDX

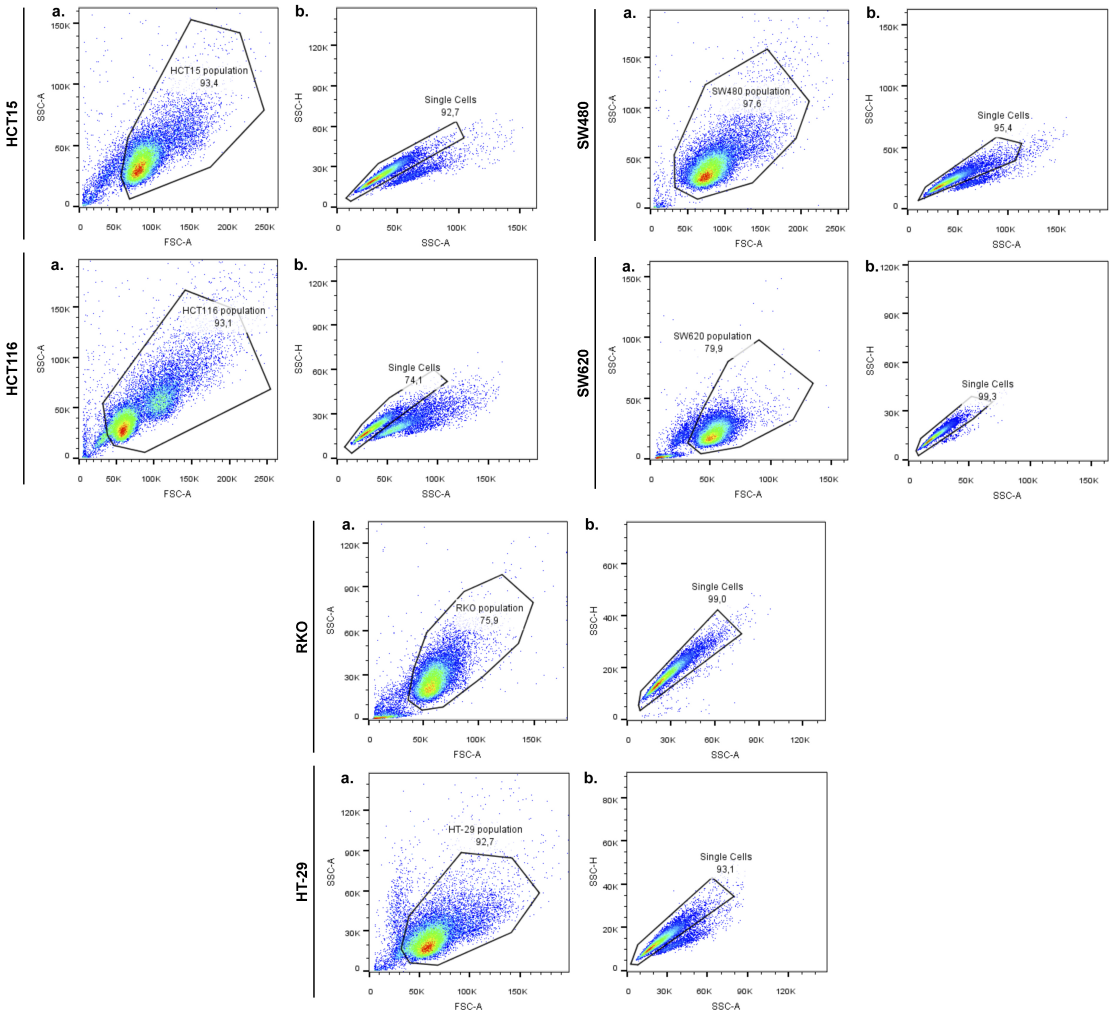

Figure S3. Flow cytometry gating strategy applied to each CRC cell lines. Pseudo-color plots illustrating the flow cytometry gating strategy created by FlowJo. **a.** Gating strategy used to define cells population for each cell line, based on forward and side scatter (FSC-A/SSC-A) which represents the cells distribution based on cell size and granularity; **b.** SSC-A/SSC-H represents the used strategy to determine single cells population for each cell line.

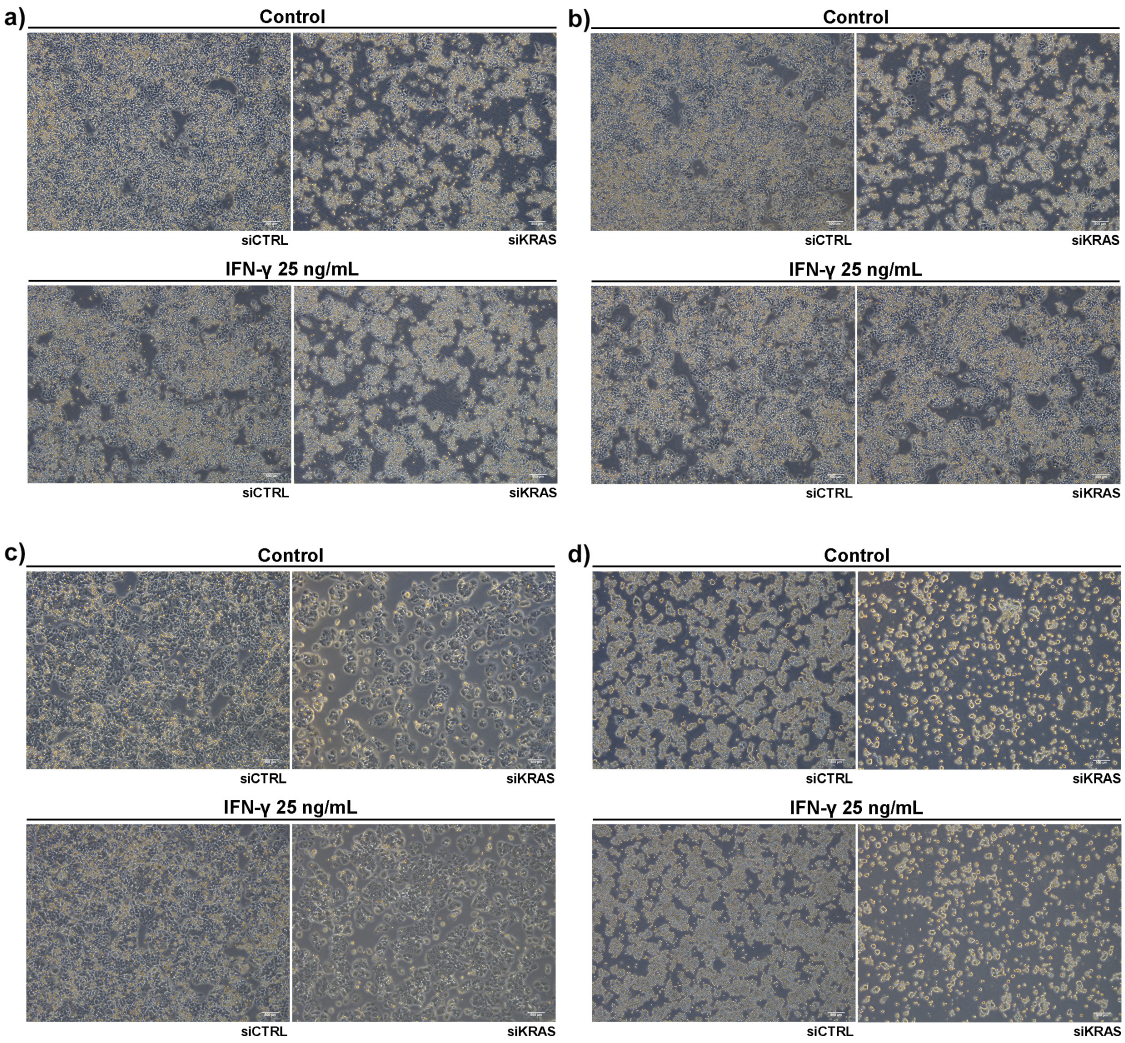

Figure S4. Cell aspect after IFN-γ stimulation. **a)** HCT15; **b)** HCT116; **c)** SW480 and **d)** SW620 control and after stimulation with 25 ng/mL of IFN-γ.

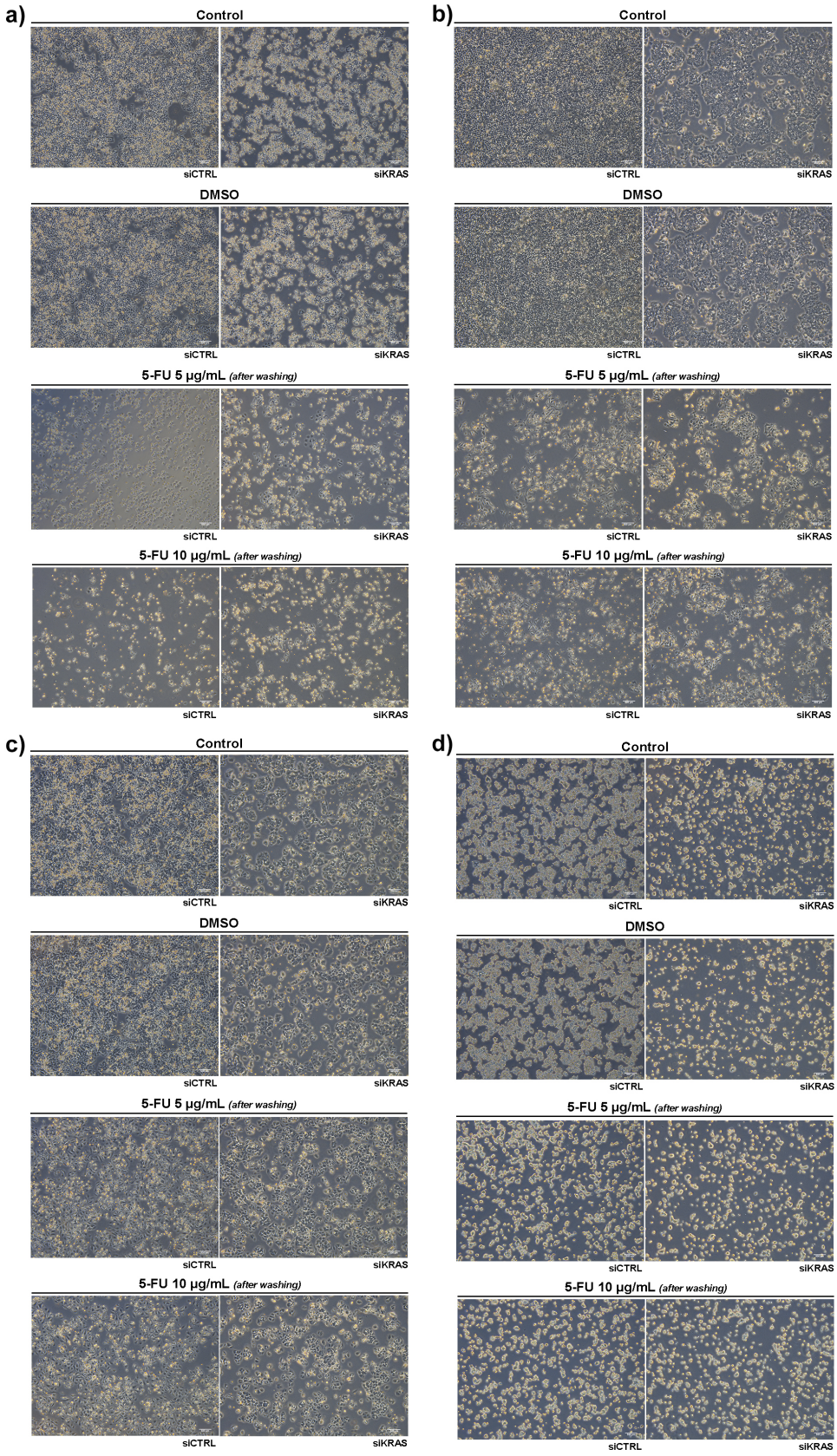

Figure S5. Cell aspect after treatment with different doses of 5-FU. a) HCT15; **b)** HCT116; **c)** SW480 and **d)** SW620 control (DMSO) and after 24h treatment with 5 μg/mL and 10 μg/mL of 5-FU. The 5-FU treated cells picture was taken after a wash with PBS 1x to remove dead cells.