Faculdade de Engenharia da Universidade do Porto,
Instituto de Ciências Biomédicas Abel Salazar

Cancer-associated fibroblasts and tumor-associated macrophages: exploring their mutual regulation.

Carlos Ferreira Guimarães

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Supervisor: Sérgia Velho (PhD)
Co-supervisor: Maria José Oliveira (PhD)

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Abstract

Cancer is one of the main causes of death in modern days. Genetic changes – mutations – are the path from which a normal cell transits into a tumorigenic one, but the evolution to metastasis is extremely dependent on the tumor microenvironment and infiltrating cells. Tumor associated macrophages are normally activated into an M1-like phenotype, having the ability to promote an inflammatory response, lethal to cancer. However, cancer-derived signals can shift these cells to an alternative phenotype, M2-like, causing macrophages to promote the development of tumors. Cancer-associated fibroblasts (CAFs) represent the main modulators of tumor stroma. In the proximity of cancer cells, fibroblasts remain perpetually activated, possessing a fibrotic phenotype, which is significant enough to change the physicochemical properties of tumor tissue, creating a favorable niche for tumor cell growth, invasion and migration. In this work, the interaction of cancer-associated fibroblasts with macrophages was evaluated in order to determine whether these two microenvironment cell populations could regulate each other's tumor-promoting properties.

The initial hypothesis was that CAF-like fibroblasts would promote a pro-tumoral, M2-like macrophage phenotype, favoring cancer cells. To verify this, cancer-associated fibroblasts were extracted from tumor tissue and fibroblasts from a colorectal cell line were activated *in vitro* with TGF-β. These fibroblasts were co-cultured with macrophages in a transwell system. When analyzed, macrophages that were co-cultured with fibroblasts were morphologically different and further investigation revealed not an increase of M2 markers such as CD163 but a decrease, with levels of the M1-like CD86 marker increasing, resulting in a significant reduction of anti-inflammatory ratios upon communication with activated fibroblasts. Macrophages previously altered by fibroblasts led to diminished invasion ratios with half the number of invading cells when compared to naive M0 macrophages. Surprisingly, when cancer cells were under the influence of fibroblasts, their invasion numbers increased, especially when fibroblasts were previously near macrophages. These events revealed a very interesting dual behavior with different direct and indirect effects from the same cell type. A molecular analysis was done in order to identify relevant modulator cytokines that could explain the observed effects. TGF-β levels showed very little variation, with a slight decrease when fibroblasts and macrophages were co-cultured. Increased levels of macrophage TGF-β mRNA on the same condition pointed at TGF-β losses at the fibroblast level (inhibition or degradation) or increased consumption by the two cell types, out ruling TGF-β as the main macrophage modulator in this scenario. HGF levels showed an increase in media from fibroblasts co-cultured with macrophages, but the small increase to levels of

activated conditions that showed less invasion also suggest this is not the molecule behind fibroblast-induced cancer cell invasion that is being affected by macrophage signals.

This knowledge paves way for pinpointing and targeting the pathways through which fibroblasts and macrophages are subverted into a pro-tumoral phenotype, which might allow a reversion of these cell types into a tumor-destructive mode - hampering cancer cell invasion, migration and ultimately blocking metastasis.

Resumo

O cancro é uma das principais causas de morte dos dias modernos. As alterações genéticas – mutações – são o caminho através do qual uma célula normal se transforma numa célula cancerígena, mas a evolução para metástase é extremamente dependente do microambiente tumoral e das células infiltradas. Os macrófagos associados ao tumor são normalmente ativados num fenótipo tipo-M1, tendo a capacidade de promover uma resposta inflamatória, letal para o cancro. No entanto, os sinais derivados das células tumorais podem deslocar estas células para um fenótipo alternativo, tipo-M2, levando os macrófagos a promover o desenvolvimento dos tumores. Os fibroblastos associados ao cancro (CAFs) representam os principais moduladores do estroma tumoral. Na proximidade de células cancerosas, os fibroblastos permanecem perpetuamente ativados, possuindo um fenótipo fibrótico que é significante o suficiente para mudar as propriedades físico-químicas do tecido tumoral, criando um nicho favorável ao crescimento das células do tumor, à invasão e à migração das mesmas. Neste trabalho, a interação entre os fibroblastos associados ao cancro e os macrófagos foi avaliada de maneira a determinar se estas duas populações de células do microambiente tumoral conseguem regular as suas propriedades pro-tumorais.

A hipótese inicial era que os fibroblastos tipo-CAF promoveriam um fenótipo pro-tumoral, tipo-M2 dos macrófagos, favorecendo as células do cancro. Para verificar isto, fibroblastos associados a tumores foram extraídos de tecido tumoral e fibroblastos de uma linha celular colorectal foram ativados *in vitro* com TGF-β. Estes fibroblastos foram co-cultivados com macrofagos num sistema transwell. Quando analisados, os macrófagos que tinham sido co-cultivados com fibroblasts eram morfologicamente diferentes e investigação adicional revelou não um aumento dos marcadores M2 como o CD163 mas uma redução, com aumento dos níveis do marcador tipo-M1 CD86, resultando numa diminuição significativa dos rácios anti-inflamatórios após comunicação com fibroblastos ativados. Os macrófagos previamente alterados por fibroblastos levaram a rácios de invasão de células tumorais diminuídos com apenas metade do número de células invasoras em relação aos macrófagos naive, tipo-M0. Surpreendentemente, quando as células tumorais estavam sob a influência de fibroblastos, os seus números de invasão aumentaram, especialmente quando os fibroblastos tinham estado previamente em cultura com macrófagos. Estes eventos revelaram um papel dual interessante com diferentes efeitos diretos e indiretos do mesmo tipo celular. Uma análise molecular foi feita de forma a identificar citocinas moduladoras relevantes que pudessem explicar os efeitos observados. Os níveis de TGF-β mosraram muito pouca variação, com uma ligeira diminuição quando fibroblastos e macrófagos estavam em co-cultura. Simultaneamente, níveis aumentados do

mRNA do TGF-β dos macrófagos nas mesmas condições apontam para perdas desta molécula ao nível do fibroblasto (inibição ou degradação) ou aumento do consumo pelas duas células, desconsiderando o TGF- β como principal modulador dos macrófagos neste contexto. Os níveis de HGF mostraram um aumento no meio de fibroblastos co-cultivados com macrófagos, mas o ligeiro aumento que iguala os valores das condições com ativação que resultaram em menor invasão indicam que não será o HGF o sinal afetado pela comunicação com macrófagos e responsável pelo aumento da invasão tumoral.

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“That is not dead which can eternal lie,
And with strange aeons even death may die.”

Howard Phillips Lovecraft

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List of Abbreviations

ANOVA – Analysis Of Variance
APC (fluorescence) – Allophycocyanin
APC (gene) – Adenomatous Polyposis Coli
bFGF – Basic Fibroblast Growth Factor
BM – Basement Membrane
BSA – Bovine Serum Albumin
CAF – Cancer Associated Fibroblast
CC – Co-culture
CCD-18co – Colorectal Fibroblast Cell Line
CD – Cluster of Differentiation
cDNA – Complementary DNA
CSF1 – Colony Stimulating factor 1 (CSF1)
CXCL12 – C-X-C Motif Chemokine Ligand 12
DAPI – 4', 6-Diamidino-2Phenylindole, Dihydrochloride
DC – Dendritic Cell
DMEM – Dulbecco's Modified Eagle Medium
DNA – Deoxyribonucleic Acid
ECL – Enhanced Chemiluminescence
ECM – Extracellular Matrix
EGF – Epidermal Growth Factor
ELISA – Enzyme-Linked Immunosorbent Assay
EMT – Epithelial-to-Mesenchymal Transition
EPC – Endothelial Progenitor Cell
EpCam – Epithelial Cell Adhesion Molecule
FACS – Fluorescence-activated cell sorting[1]
FAP – Fibroblast Activation Protein
FBM – Fibroblast Basal Medium
FBS – Fetal Bovine Serum
FGF2 – Fibroblast Growth Factor 2
FGM-2 – Fibroblast Growth Medium 2
Fib. – Fibroblast
FITC – Fluorescein isothiocyanate
FSP1 – Fibroblast Specific Protein 1

[1] Sometimes used as a synonym for Flow Cytometry

GAPDH – Gyceraldehyde 3-Phosphate Dehydrogenase
HGF – Hepatocyte Growth Factor
HIF-1 – Hypoxia Inducible Factor 1
HRP – Horseradish Peroxidase
HSF1 – Heat Shock Factor 1
IFN-Y – Interferon Gamma
IL (4,6, 10, 13) – Interleukin (4, 6, 10, 13)
KRAS – Kristen Rat Sarcoma viral oncogene homolog
LPS – Lipopolysaccharide
M-CSF – Macrophage Colony Stimulating Factor
Mac. – Macrophage
MDSC – Myeloid-Derived Suppressor Cell
MET – Mesenchymal-to-Epithelial Transition
MMP – Matrix Metalloproteinase
mRNA – Messenger RNA
MSC – Mesenchymal Stem Cell
NK – Natural Killer
PAGE – Polyacrylamide Gel Electrophoresis
PBS – Phosphate Buffer Saline
PBS-T – PBS – Tween
PCT – Polymerase Chain Reaction
PDGF – Platelet-Derived Growth Factor
PDGFR – PDGF Receptor
PE – Phycoerythrin
Pen – Penicillin
PET – Polyethylene Terephthalate
PI3K – Phosphoinositide 3-kinase
qRT-PCR – Quantitative(Real Time) Reverse Transcriptase Polymerase Chain Reaction
RIPA – Radioimmunoprecipitation Assay
RKO, HCT116 – Colorectal cancer cell lines
RNA – Ribonucleic Acid
RPMI – Roswell Park Memorial Institute Medium
RT – Room Temperature
SDF-1 – Stromal cell-Derived Factor 1
SDS – Sodium Dodecyl Sulfate
SPARC – Secreted Protein Acidic and Cysteine Rich
Strep – Streptomycin
TAM – Tumor-associated Macrophage
TCP – Tissue Culture Polystyrene
TGF-β – Transforming Growth Factor Beta

TGFBR2 – TGF-β receptor 2

TMEM – Tumor Microenvironment of Metastasis

TNF-α – Tumor Necrosis Factor alpha

TP53 – Tumor protein p53

VEGF – Vascular Endothelial Growth Factor

YAP1 – Yes-Associated Protein 1

ZEB – Zinc finger E-box-Binding homeobox 1

α-SMA – Alpha Smooth Muscle Actin

ΔCt – Difference in PCR cycles in comparison to housekeeping

Chapter 1

Introduction

The soil needs the seed and the seed needs the soil. The one only has meaning with the other. Paulo Coelho, Brida.

This quote from Paulo Coelho's novel mirrors the current state of tumor microenvironment understanding about its role in cancer progression and the subversion of the first by the latter.

Cancer is a major health concern and usually tops the charts of leading causes of deaths surpassed only by heart diseases, order which is expected to flip in the nearby future [1]. Human tissues are extremely dynamic structures, from the simplest biological molecules to cells - everything comes with an expiration date nowhere close to a human's life expectancy. In order to circumvent this issue and assure permanent homeostasis, nature developed the process of turnover: the continuous process of renewing cells too old to maintain their function [2]. Turnover takes place at different extents in different tissues. The human skin, hematopoietic system and intestine epithelium are the three tissues with greatest levels of self-renewal (turnover) [3]. In a very elegant and classic work with nuclear marking of cells [4], it was shown that colon cells lining the epithelium had a turnover of one day. However, more recent and accurate analysis revealed that it can take from 5 to 7 days for the colon epithelium turnover to take place [5]. While conceptually simple, the process of turnover is yet not completely understood. For example, the hematopoietic system has different turnovers for the different cellular components. These are mainly replaced through hematopoietic stem cells, which have the ability to repopulate a whole organism. However, their proliferation *ex-vivo* remains to be achieved [6], demonstrating how complex the process of turnover and all the signaling involved can be. When cells go through division, errors can happen and accumulate in their genetic material – mutations. These mutations can be silent, with no impact whatsoever, which is quite frequent: reactive oxygen species alone can cause up to 10.000 DNA lesions per day [7]. The

reason why most of these lesions have no practical effect is due to natural repair mechanisms that range from DNA repair to last-resource apoptosis of cells no longer fit to survive and proliferate [8]. However, the whole system can be compromised when the mutation affects genes related to these same controlling mechanisms. If a certain cell becomes insensible to DNA damage, proliferates instead of dying and its mutations are perpetuated, daughter cells become neoplastic and eventually malignant [9], giving rise to cancer. This rise is amplified by tumor-promoting inflammation and genomic instability. The first leads to an increase of cellular turnover to compensate inflammatory damage, and both together increase the chance of perpetuating mutations [10].

The hallmarks of cancer were defined by Weinberg [11] back in 2000 as 6 fundamental characteristics: evasion of apoptosis, self-sufficiency of growth signals, loss of anti-growth sensitivity, limitless proliferation, sustained angiogenesis (development of new vasculature) and eventually tissue invasion and metastasis. Eleven years later, these hallmarks were redefined at the light of the scientific progress, and two new hallmarks were added to the list: metabolic reprogramming and immune evasion – with the addition of a new entity external to cancer cells – the tumor microenvironment [12], discussed further ahead.

In the practical aspect, the aggressiveness of cancers varies considerably but is extremely aggravated when metastasis occurs. In the case of colorectal cancer, the fourth most common form of cancer-related deaths, 90% of patients with metastasis die within 5 years [13]. This holds true to most cancers, where the main threat is the moment when cells can move onto new niches and compromise the performance of vital organs. Therefore, it is fundamental to understand the processes that empower tumor cells with metastatic potential in order to develop new and more efficient metastasis-preventive treatments.

1.1 - Metastasis

The progression of tumors towards metastasis is usually described as a series of complex steps [14]: Initially, cancer cells may acquire the ability to proteolytic degrade and migrate through the underlying basement membrane through a process termed invasion. Invasive cancer cells reach then the adjacent conjunctive tissue being attracted to neighbor blood vessels and enter the blood stream through a process named of intravasation. Afterwards, cancer cells must survive in the circulation for long enough to reach other tissues and move onto them and out of circulation – extravasation. After reaching a new environment, these cells must colonize it in order to establish a niche that allows them to survive and proliferate, originating a new focus of cancer metastases. All these biological events have been extensively reviewed [15] and the presence and accumulation of oncogenic mutations have been associated with the metastatic path along its different phases.

It is also very relevant to consider that the kinetics of this process can be very different between tissues where the primary focus is established and the destination tissue, host of

tumor cells. In some cases, e.g. breast or prostate cancer, metastasis only manifests itself years after patients remove early and small malignancies, even when they are considered initially as low-risk in prognostics[16], [17]. The fact that there is latency in the establishment of tumor cells in new tissues suggests that either cells acquire further malignancy as time pasts or modulate and subvert the microenvironment into a cancer-permissive niche, or both, in a time-dependent fashion. On the other side of the spectrum, as with lung [18] or pancreatic cancer [19], there is a frustratingly fast evolution from the diagnosis of cancer to the appearance of disseminated metastasis, frequently present as patients are firstly diagnosed, with very small margins for prevention and treatment. This accelerated evolution implies that, most likely, cells acquire a very potent malignant phenotype, probably more related to the microenvironment of origin.

It all begins with the process of carcinogenesis, through which a normal cell evolves to a malignant one. This process as a whole has been extensively associated with mutations in specific genes. In the case of colorectal cancer, the whole event begins with the activation of the Wnt pathway, through inactivation of the tumor suppressor APC (adenomatous polyposis coli) or activation of β-catenin, a Wnt pathway co-activator [20]. The resulting colorectal tumor transits into a carcinoma with the mutational activation of KRAS [21], followed by the activation of the PI3K pathway [22], inactivation of TP53 [23] and loss of the TGFβ (transforming growth factor-β) pathway [24], which is a tumor suppressor pathway. After cancer cells acquire these genetic alterations, they need only to invade the wall of the colon and metastasis is then able to happen. Therefore, the progression from *in situ* to invasive carcinomas is slow, but the transition to the metastatic scenario happens quickly, resulting in the spreading to the liver in 80% of recurrent disease cases [25]. The evolution of colorectal cancer is evaluated based on how far the primary tumor has grown into the wall of the intestine and the presence or absence of metastasis or lymph node spreading.

From the pattern of evolution of this cancer type, one can induce that the primary site microenvironment is the main accountant for the evolution to an aggressive invasive carcinoma. This is due to the fact that the relevant mutations accumulate *in situ* and the colonic wall invasion (adjacent tissue) is the decisive step for metastasis to occur.

It is this metastatic cascade that leads to most colorectal cancer related deaths [26]. However, the road that leads neoplastic cells from the acquisition of malignant traits to invade and colonize new tissues is yet poorly understood. Even though certain gene mutations can be linked with the metastatic outcome, with some of them having a direct impact on the metastatic behavior of cells, as happens with breast cancer mutations and metastasis to the lung [27], mutational profiles fall short from being accountable for the whole set of cellular events that must take place for cells to disseminate [28].

1.2 - The Tumor Microenvironment

In contemplation of the issue on metastatic transition, the scientific community started looking not at the cancer cell but turning its attention to the tumor microenvironment.

A tumor is a combination of several cells with different phenotypes, in different cellular stages with different genetic mutations acquired, resulting into an heterogeneous mix of cells from different populations with different, complex and still fairly unknown contributions to the overall cancer progression [29]. However, these cancer cells do not exist on their own, since they are embedded and surrounded by a very complex niche: the tumor microenvironment.

In fact, cells are intimately surrounded by other cells from adjacent tissues or those that infiltrate into the tumor tissue, such as immune cells like tissue-associated macrophages, or other stromal cells as adipocytes, endothelial cells or fibroblasts. In fact, the process of inflammation and the inflammatory mediators are seen as major contributors to the tumor microenvironment. This, essentially since inflammation was identified as the seventh hallmark of cancer integrating the list of the six hallmarks defined by Weinberg.[10].

The microenvironment of tumor cells is essentially composed of tumor-infiltrating cells, vascular tissue, extracellular matrix (ECM), and some other soluble and matrix-associated molecules.

The ECM is a three-dimensional structure that embeds the cellular components of tissues. Once considered a static network of fibers and other molecules, the ECM is now seen as a highly dynamic structure, with clear roles in the physiology and pathology of tissues. In fact, this cellular niche is crucial for cancer evolution and cancer cell proliferation, invasion and metastasis [30]. The ECM is also a reservoir of cytokines and growth factors other than a regulator of homeostatic events such as migration, differentiation and proliferation. Collagen, laminins, fibronectin, proteoglycans and hyaluronan are the main classes of macromolecules comprising the ECM [31]. Despite the common composition, ECMs from different tissues have different ratios of these molecules and these are present in different isoforms resulting into stiffer or more fluid matrixes. Similarly, ECM composition and stiffness also differs between normal and tumor tissue – being tumors usually related to increased ECM stiffness [32] that serves as an indication for diagnosis on its own. The ECM can be separated into two groups: Basement membrane (BM) and stromal matrix. The stromal matrix is a composition of polysaccharide gels, proteoglycans and fibrous proteins and is usually the more gel-like portion of the ECM. However, in a cancer microenvironment context, this stromal component can be heavily modified by cancer-associated fibroblasts (CAFs), that remodel the ECM into a cancer-permissive environment [33]. BM, on its hand, is a thin and dense sheet of specialized ECM that surrounds epithelial or endothelial cells, muscle and nerve cells, separating these cells from the stromal matrix [34]. This so-called membrane is composed of dense networks of fibers such as collagen type IV. Functioning as a surrounding barrier, the BM is usually the first level of defense against invasive cancer cells as invasion requires cells to degrade and cross the BM

to access the underlying stromal tissue. This process can be very complex but usually bases itself on the expression of matrix metalloproteases (MMPs) by cancer and neighbor cells. These MMPs are proteases with the ability to degrade the ECM, creating room between this dense network permitting cells to move through [35], [36].

A tumor, being a local outburst of cells, requires an increased supply of nutrients and oxygen and therefore one of the first carcinogenesis steps, essential for tumor survival and growth is the process of angiogenesis [37]. In fact, the density of vascularity within a tumor has direct influence in colorectal cancer patient's prognostics and survival [38]. The whole process begins with the rapid growth of cancer cells and the local depletion of oxygen leading to a hypoxic environment. This state of low oxygen levels is translated by cells into the activation of the transcriptional hypoxia inducible factor-1 (HIF-1) leading to the expression of angiogenic factors – mainly VEGF, bFGF and PDGF by tumor cells [39]. Other than these, microenvironment cells from the tumor-surrounding stroma, like tissue-associated macrophages (TAMs) and cancer-associated fibroblasts (CAFs), can also have a direct contribution to the pro-angiogenic response, as discussed later in this paper. Tumor vasculature is of such importance that the current therapeutic picture is evolving from indiscriminate cell targeting to the prevention of angiogenesis in order to fight back tumor growth [40].

Last but (definitely) not least; the microenvironment of tumor cells is composed by distinct cell types. As coined by Dvorak [41], tumors are wounds that do not heal. This situation results into local inflammation at the tumor site that persists with time and results in the gathering of several cell types. Colon carcinomas, as several other solid tumors, are infiltrated by tumor-associated macrophages (TAMs), cancer-associated fibroblasts (CAFs), myeloid-derived suppressor cells (MDSCs), mast cells, monocytes, neutrophils, T-lymphocytes (CD8 and CD4), dendritic cells (DCs), NK cells, endothelial cells and progenitors (EPCs), platelets and even mesenchymal stem cells (MSCs) [42] (fig. 1). The presence of all of these cells, especially the immune ones, works in a quite paradoxical [43] way: at first, they are recruited to the tumor site and exert a pro-inflammatory and anti-tumor effect. However, as time goes by and tumor cells communicate with the infiltrated types, there is a subversion of immune cells, which transit into a tumor-permissive environment, promoting tumor cell proliferation, survival and eventually metastasis [44].

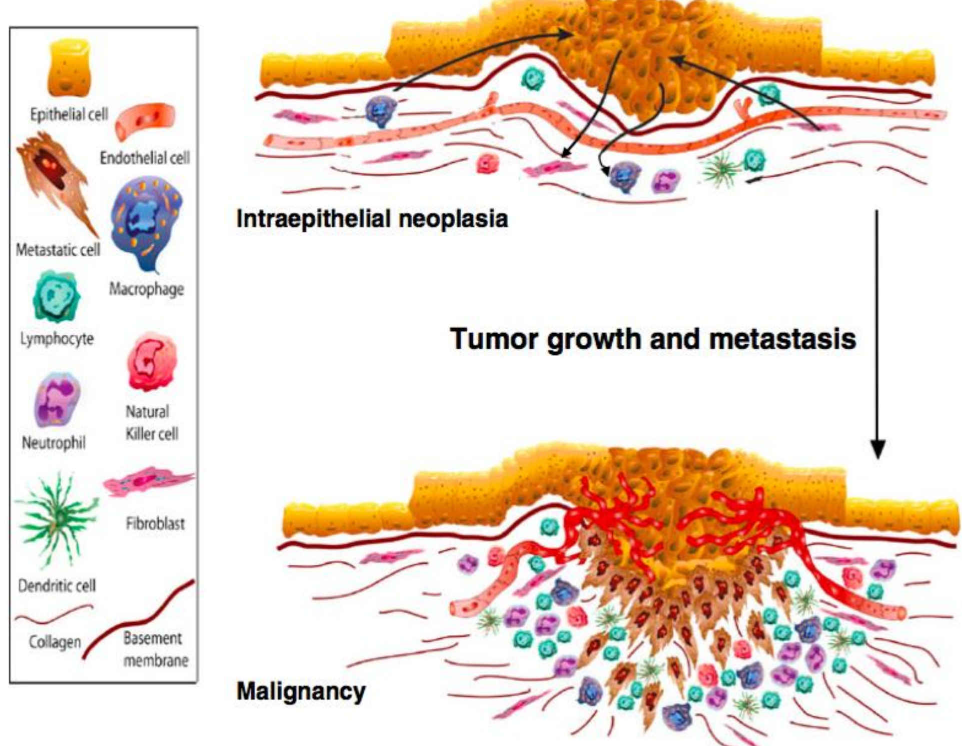

Figure 1 - Tumor infiltrating Cells [42] **-** Model representing the tumor microenvironment and associated cell types. Tumor infiltrating cells include mainly Epithelial and Endothelial Cells, Macrophages (TAMs), Lymphocytes, NK cells, Neutrophils, Fibroblasts (CAFs) and Dendritic Cells. Cells infiltrate the tumor with the natural inflammation that comes with the neoplasia growth. The crosstalk with tumor cells results into new blood vessels formation (angiogenesis), ECM remodeling (depicted by collagen changes) and eventually ends with the proteolytic dissolution of the basement membrane and invasion of the stroma.

This dependency cancer cells have on the stromal interaction has led to the understanding that eventually most cancers converge to this point where their stroma has been subverted into a suitable niche for cancer development. The understanding and ability to target this modulation of adjacent tissue by cancer cells might prove valuable as a way to prevent tumor invasion and metastasis.

1.3 - Cancer-associated Fibroblasts

One very common microenvironment cell type that is present in several cancers, including colorectal, is the fibroblast. Fibroblasts are abundant stromal cells that can be very diverse within and across tissues.

Fibroblasts were first described in the 19th century and are established as non-vascular, non-epithelial and non-inflammatory cells that represent most of the cellular part of the connective tissue of several organs [45]. Fibroblasts can be very diverse within and across tissues, but they have essentially 3 well established roles: Extracellular matrix (ECM) deposition, regulation of epithelial differentiation and an important role on inflammation and wound healing [46], [47].

Concerning fibroblasts roles on the ECM, their action is centered on the deposition and ECM synthesis, by producing collagen (I, II and V), fibronectin and also collagen IV, which together with laminin are needed for basement membrane structuring [48], [49]. However, they can play quite the opposite role, since they can produce matrix metalloproteinases (MMPs), enzymes able to degrade the ECM. In a homeostasis context, this is very important since it allows fibroblasts to regulate the ECM turnover and remodel it when necessary [50].

Fibroblasts are also able to regulate the surrounding epithelia. This is achieved mainly through the secretion of growth factors but also through direct cell-cell interactions [51]. With all these capabilities, fibroblasts are major players in wound healing events. They are able to move into injury sites and produce more ECM components which will recruit and support other cells. Furthermore, they can promote wound contraction through their cytoskeletal capabilities [52]. On a parallel to wound healing, fibroblasts, as ECM producers, are also the main responsible for tissue fibrosis. Fibrosis takes place when there is an increase of ECM secretion and proliferation by fibroblasts, leading to unorganized fiber deposition and the appearance of scar tissue. This increase of fibroblastic activity is usually called activation (fig 2) [47], and can be caused by specific growth factors, such as the transforming growth factor (TGF-β). In normal wound healing events, the number of activated fibroblasts decreases as the wound resolves, possibly through phenotype reversion or senescence and apoptosis [53].

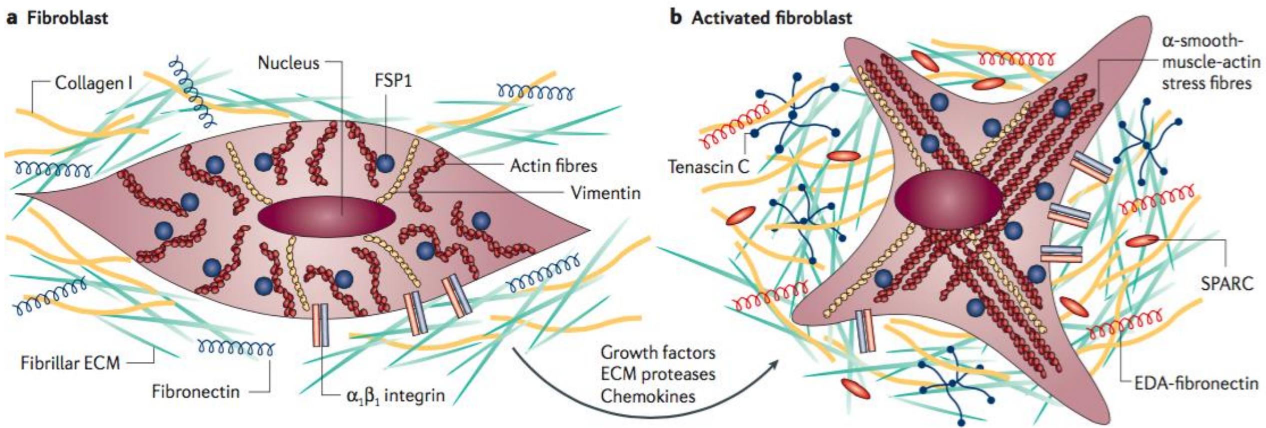

Figure 2 - Fibroblast activation [54] - Normal (a) fibroblasts have a fusiform shape and are placed in a type I collagen and fibronectin ECM with which they interact through integrins (namely $\alpha_1\beta_1$). They express the fibroblast specific protein 1 (FSP1) that allows for their detection in the normal state. Upon changes in the fibroblast environment such as chemokines, growth factors, e.g. transforming growth factor β (TGF-β), or ECM changes, they can transit into an activated (b) phenotype. These activated fibroblasts have an increased proliferation rate and deposit ECM to a higher extent (namely tenascin C, fibronectin and collagen I). Typically, they are identified by the expression of α-smooth muscle actin (αSMA) and are subsequently named myofibroblasts.

Fibroblasts are present in the stroma of tumors in abundance (just like most tissues). Once there, these cells can be activated and subverted into this fibrotic phenotype, perpetually, unlikely what happens temporarily in the fibrotic response. These cancer-associated fibroblasts

(CAFs) are usually defined by the expression of α-smooth-muscle actin (α-SMA), which gives them the myofibroblast classification (fig 2.). The Fibroblast Activation Protein (FAP) is a surface protein expressed mainly by fibroblasts in pathologic situations and is also a way of identifying the presence of this activated phenotype [55].

Since most of the tumor mass consists of stroma, which is, to the highest extent, controlled by fibroblasts, fibroblast changes translate in significant tumor changes. These tend to happen as a response to several signals, namely pro-fibrotic growth factors released by the cancer cells. The key mediators of this process include tumor growth factor β (TGF-β), fibroblast growth factor 2 (FGF2) and platelet-derived growth factor (PDGF) [56]. TGF-β is one of the main modulators of CAF roles in the outcome of cancer. While this growth factor promotes the activation of fibroblasts, as discussed, its action seems to be dual: some works have shown that increased expression of TGF-β boosts disease progression while others observed an increase in cancer initiation and metastasis of mammary tumors when manipulated CAFs lacking the expression of TGBR2 (the receptor of TGF-β) were present [57]. These variations suggest that there might be a dependence of the tumor-stroma interaction on the fibroblast phenotype.

Another relevant growth factor is the PDGF, which is also secreted by cancer cells resulting in cancer progression. PDGF promotes fibroblast proliferation despite its inability to induce the activation of such cells [58]. Also, the fact that cancer cells have no receptor for this growth factor suggests that its relevance is confined to a paracrine effect on microenvironment cells.

FGF2 is a growth factor that is produced and mostly associated with fibroblasts (hence its name) and fibrotic events, playing a significant role at the tumor microenvironment. In fact, recent studies with colorectal cancer cells and fibroblasts have shown a direct relation of FGF2 signaling and the promotion of cancer cell migration and invasion [59].

The complex signaling that might take place through the action of different growth factors within the tumor stroma hints at changes on the microenvironment as responsible for different tumor-CAF crosstalk outcomes. Therefore, there is a very relevant need of characterizing tumor microenvironment subtypes and of looking at these cells in different stages of tumor evolution. This information might shine a light on whether or not CAFs evolve and change their phenotype and action as tumors develop, opening ground for new CAF-targeting therapies upon specific stages of the disease.

CAFs are able to promote tumor initiation and progression once stimulated by cancer cells. The overexpression of, e.g., TGF-β or hepatocyte growth factor (HGF) by fibroblasts has been linked to the initiation of breast cancer [60]. Also, grafting normal fibroblasts versus CAFs with cancer cells demonstrated that the emergence of prostatic neoplastic lesions happens only in the presence of CAFs [61]. Such results have been replicated in different cancer types [62], showing that fibroblasts partially promote cancer growth through the induction of angiogenesis and recruitment of bone-marrow derived endothelial cells. These results show that normal

fibroblast function is important for epithelial homeostasis as opposite to cancer initiation but that activated fibroblasts can lead to the promotion of tumor initiation and growth.

Fibroblasts can also influence tumor progression after it is initiated by communicating with cancer cells [59]. Recently, events such as the upregulation of heat shock factor 1 (HSF1) in CAFs have been identified as promoters of changes in HSF-1 driven cancer cells [63]. Furthermore, the Yes-associated protein 1 (YAP1) in CAFS enhances ECM stiffness and promotes cancer cell invasion [64].

Another mechanism through which CAFs influence tumor progression is by the promotion of EMT. Epithelial-to-mesenchymal transition (EMT) is an event through which epithelial cells lose contact with adjacent cells, gaining new properties similar to those of mesenchymal cells. EMT depends on the expression of specific transcription factors such as Snail and ZEB, leading to the changes in cell behavior and cell differentiation [65]. This reprogramming is initiated by extracellular cues, namely TGF-β signaling and likely modulated by fibroblasts present at the tumor microenvironment. This transition is highly relevant in the field of cancer since cancer cells usually gain the ability to invade adjacent tissues and migrate through the acquisition of an EMT phenotype. Once cells reach new tissues, the reverse process occurs, MET (mesenchymal-to-epithelial transition), and so these cells can once again establish themselves in an epithelial fashion in the metastatic foci [66]. EMT can also play a role in the recruitment of CAFs. In tissue fibrosis, it is well established that epithelial cells undergoing EMT due to microenvironment cues lead to the accumulation of fibroblasts [67]. This suggests that the EMT cells go through in cancer might also be related with the recruitment of fibroblasts. Of note, this action can be performed by the cancer cells but also by healthy cells from the epithelia that might undergo EMT changes due to microenvironment signaling [54].

Furthermore, the immune response in the vicinity of tumors is also modulated by CAFs. These fibroblasts have immunosuppressive activity through the secretion of chemokines. In the case of pancreatic cancer, FAP^+ CAFs secrete the chemokine CXCL12, which hinders the function of T cells. When CAFs are depleted, the immune response is restored leading to cancer cell death [68], [69]. This occurs mainly through the action of cytotoxic T cells, which are the main executors of cancer cells.

Importantly, fibroblasts have also the ability of producing MMPs, proteases able to degrade the ECM. These proteases are also very relevant to promote cancer cell invasion. In particular, MMP3 (also known as stromelysin 1) is produced by activated fibroblasts and is able to cleave the invasion-suppressor cell-cell adhesion molecule E-cadherin. This cleavage is capable of inducing EMT and promote the invasion of cancer cells [70]. Activated fibroblasts also produce MMP1 that gives rise to a similar outcome of invasiveness [71]. Moreover, fibroblast signaling is also responsible for causing cancer cells to produce MMP7 promoting tumor progression [72]. Additionally, fibroblasts' exosomes have been gaining importance in promoting cancer progression and remodeling of the stroma. In fact, fibroblast-derived exosomes have been

identified as inductors of cancer stem cell features and more recently shown to contribute to the chemoresistance of colorectal cancer through this induction of cancer stemness [73]. Fig.3 summarizes the main roles of CAFs in tumor development.

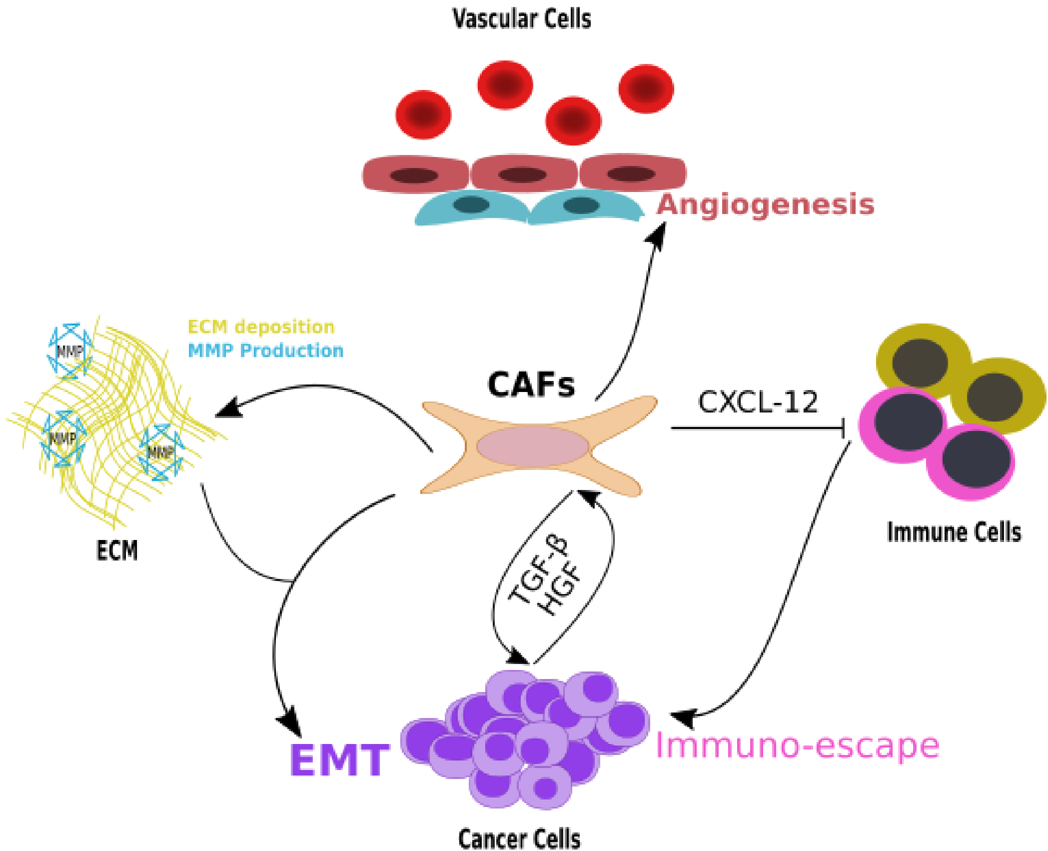

Figure 3 - Cancer-associated Fibroblasts and their pro-tumorigenic roles - CAFs contribute to the development and progression of cancer through several other microenvironment components, altering the ECM, promoting Angiogenesis and inhibiting the immune response.

Altogether, these properties make CAFs perfect partners in tumor development and progression. However, their role doesn't end once a tumor is established. In fact, CAFs have been shown to interfere with the therapeutic efficiency of molecular, cytotoxic and hormonal therapies. This effect is not always in the direction of increased resistance as in some cases CAFs increase the sensibility of cancer cells to conventional therapies [74]. The instability of cancer cells, associated with high mutation rates is one of the main reasons why most therapies have failed. In comparison, CAFs are genetically stable cells from the microenvironment, which places them in a privileged place as targets for anti-cancer therapies. Nevertheless, the most significant challenge to face is CAFs heterogeneity within different and similar cancer microenvironments, with properties that can make them tumor-suppressive or tumor-promoting, rendering cells drug-resistant or drug-sensitive - hence the fundamental need to understand

CAF profiles and their interaction not only with tumor cells but with other microenvironment components.

Knowing this, the topic of CAF heterogeneity has to be addressed. As reviewed above, several markers can identify activated fibroblasts. These include fibroblast-specific protein (FSP1), FAP, vimentin, α-SMA and PDGFR. Moreover, the fibroblast-myofibroblast spectrum is also quite complex and plastic, with some markers being more specific for certain stages of activation than others (fig. 4).

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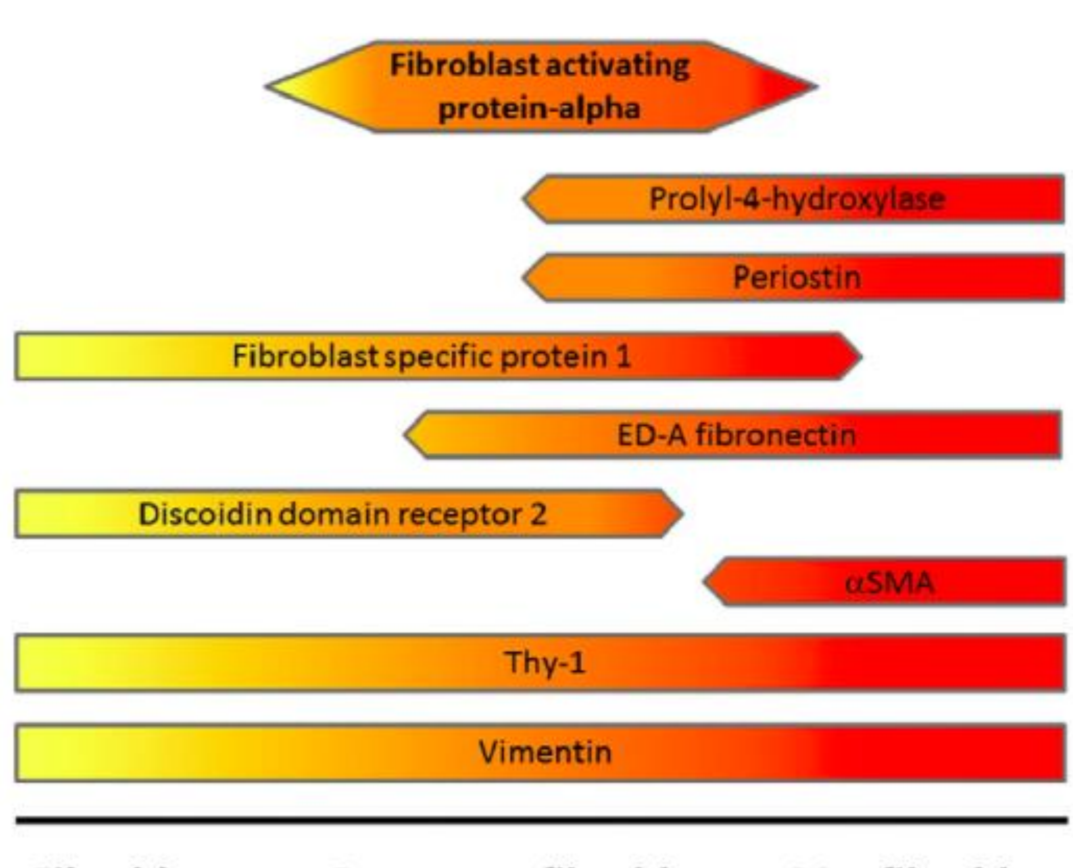

Figure 4 - Schematic representation of fibroblast markers according to the state of activation [75]**-** Several markers are related to the more and less activated fibroblast phenotypes. Also, some markers are only present in specific stages of the fibroblast-myofibroblast spectrum.

It is also important to notice that these markers are not specific for activated fibroblasts and are also present in other cell types. Therefore, the identification of resting or activated fibroblasts should take into account other factors such as context, morphology and spatial distribution. Furthermore, not all activated fibroblasts are expected to express these markers at the same time or amount [76]. It is yet unknown if different fibroblasts subsets might have different overlapping markers and perform distinct functions. A very recent review [77] hypothesized that fibroblasts, similarly to T-cells, can be activated into distinct subsets with

diverse activities, proposing a subtype classification. The fact that these possible branches of fibroblast activation is still very little explored, makes it extremely relevant to look at CAFs not as a single type of cells but as composed of different subtypes. CAFs can have a tumor-restraining role or the interconvertible tumor-promoting action, secrete growth factors that mediate tumor evolution or specialize in ECM-remodeling roles. All of these actions might contribute differently to the outcome of different tumors.

On the other hand, as a tumor evolves, there are changes that occur at the microenvironment level, which might result into different fibroblast subtypes being present in distinct stages of tumor development. Understanding such changes might shine light on the precise effect of CAFs during tumorigenesis, invasion and metastasis and open way for new microenvironment-targeting therapies. This knowledge will tell if different stages of cancer are associated with functionally different fibroblasts that might contribute differently to the outcome of tumors (through growth gactor secretion, matrix remodeling or other mechanisms of invasion promotion) or if, opposed to this, CAFs remain functionally similar along tumor progression, something yet unknown.

1.4 - Tumor-associated Macrophages

Another cell type that is frequently present near tumors is the macrophage. Macrophages are cells from the immune system that differentiate from monocytes according to the needs of different tissues. Therefore, they are inherently heterogenic, i.e., different tissues having different cellular microenvironments lead to different macrophage profiles [78], [79]. Extended research has been conducted as an attempt to identify these profiles, leading to the popular division between pro-inflammatory, classically activated, M1 macrophages versus the pro-healing, alternatively activated, M2 macrophages [80]. This elegant distinction of different activation profiles of macrophages was initially proposed by Mantovani *et al.* [81] and was based on some specific markers that were thought to be responsible for differences in macrophage response to stimuli. The M1 classic profile is related to the inflammatory phenotype, with IFN-γ being the main M1-promoter cytokine. Combined with lipopolysaccharides (LPS), these two molecules are paradigmatic in the M1, pro-inflammatory activation of macrophages [82], [83]. Regarding the alternatively activated marcophages, it has always been seen as a more complex profile, with a subdivision into three subtypes, already proposed in the initial model by Mantovani. While all three subtypes share an anti-inflammatory profile, M2a was defined as the main alternative (opposite to the classic M1) profile, associated with the cytokines IL-4 and IL-13. M2b and M2c were characterized as having a more significant role in immunoregulation. M2c macrophages, also considered as deactivated ones, have IL-10 as the main modulator cytokine [81].

In order to evaluate macrophage polarization, several markers have been studied along the years. The most common, but not unique, are CD86, CD16 and CD32 for M1 profiling; arginase

receptor and mannose receptor (CD206) for M2 identification [84], [85]. CD163, a scavenger receptor protein, has also been linked to the M2 phenotype, namely the IL-10 induced M2c-like [86], [87]. All of these markers are usually combined with CD14 as a monocyte/macrophage lineage marker [88] differing however the selected markers between mice and human samples.

Functionally, macrophages are continually screening different tissues - eliminating pathogens, damaged cells and promoting the repair of different matrices. M1-like macrophages are crucial to eliminate pathogens, infected or cancer cells, through the promotion of an inflammatory response. On the other hand, the M2 profile (alternatively activated) represents the pro-healing phenotype, encompassing the promotion of ECM remodeling, inhibition of the inflammatory response and eventual promotion of tumor growth [89].

In spite of being widely adopted, this dual, almost binary division between macrophage phenotypes has been losing strength with time. This is mainly due to the general belief that macrophage populations can be anywhere on the M1-M2 spectrum and not only on one side or the other. This has led to several attempts of macrophage polarization reassessment [83], that now culminate on M1-like and on M2-like classifications representing shifts on macrophage phenotypes as a response to the whole conjugation of stimuli in a given tissue at a given time.

With such heterogeneity and different roles according to tissues' microenvironments, it is not surprising that macrophages modulate and are modulated by the microenvironment of tumors. In fact, the notion of tumor-associated Macrophages (TAMs) is not recent [90]. These tumor microenvironment cells have been associated with pleiotropic functions that can result into different tumor growth and progression outcomes. The direction of tumor inhibition or opposite to it, tumor promotion, depends on the actions of macrophages, which are a consequence of their differential activation.

At the tumor site, TAMs promote malignancy by inducing tumor cell invasion. The M2-like macrophages, induced by anti-inflammatory stimuli, traced back to other cell types such as B cells and CAFs [91], are able to promote cell invasion [92], [93]. This occurs via a paracrine loop of tumor-originating CSF1 and macrophage-derived EGF that directs tumor cells together with macrophages along collagen fibers in the direction of the vasculature [94]. This is accompanied by the TGF-β signaling that drives EMT in tumor cells promoting migration. This pre-metastatic event is further amplified by the production of VEGF by TAMs, which stimulates angiogenesis, increasing the targets for intravasation (fig.5). The latter occurs through a complex named the Tumor Microenvironment of Metastasis (TMEN) [94].

As a result of this interaction, the presence of TAMs in the microenvironment of certain tumors has direct implications in the prognostics of patients as seen, for example, with bladder [95] and colorectal [96] cancer. Furthermore, there are different interactions between anti-cancer therapies [97], [98] and tumor associated macrophages, with some tumors becoming

resistant due to macrophage influence and others relying upon these same cells for effective healing [99].

This duality exists because macrophages in early neoplastic microenvironments are, in fact, more pro-inflammatory and tumoricidal (through cytotoxic T-cell stimulation), being capable of suppressing tumor growth. However, as the time of exposure to tumor cells increases, macrophages are subverted into a permissive state which then directs this process in an opposite fashion: suppressing the immune system and promoting tumor evolution [100]. This inherent duality hints at the possibility of molding macrophages' plasticity back to restore their antitumor properties allowing them to escape the grasp of tumor cells. In order to achieve this, it is necessary to fully understand the interaction of macrophages not only with tumor cells but also with other cancer microenvironment cells, such as fibroblasts.

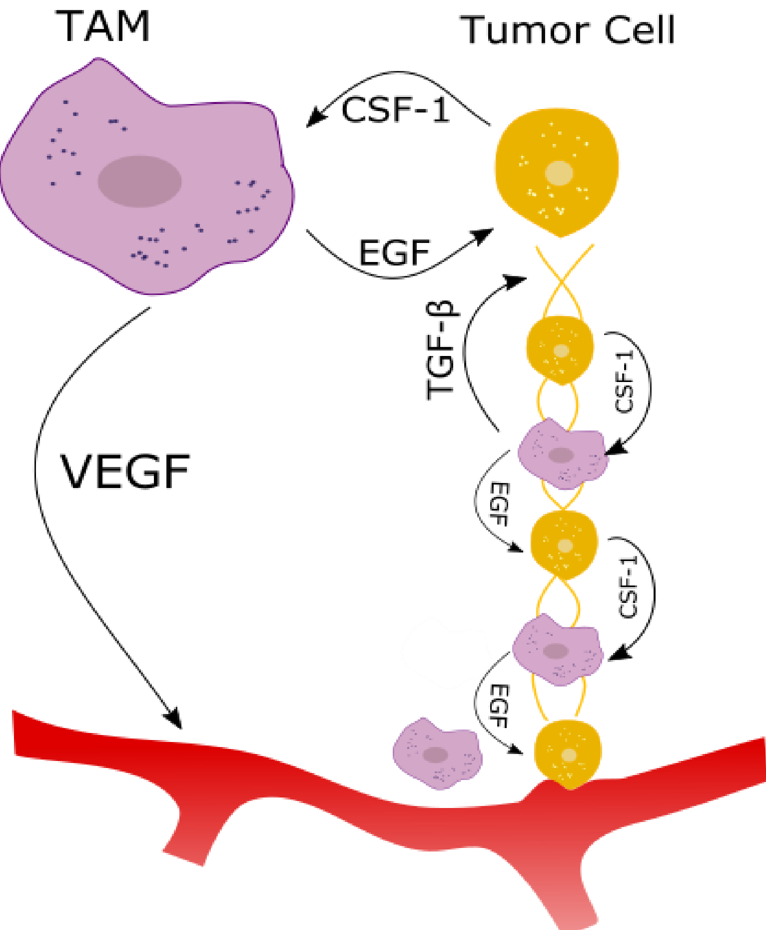

Figure 5 - Macrophage-directed cancer cell Invasion - The tumor associated macrophage (TAM) is stimulated by CSF-1 from cancer cells and promotes the invasion of the cancer cell by paracrine EGF signaling. This loop signaling directs cancer cells and associated macrophages to the vicinity of blood vessels after TAM-originated TGF-β influences tumor cells to go through EM transition, bolstering their ECM-migrating abilities. VEGF is also produced by tumor macrophages resulting into an increase of target-vessels for the intravasation of tumor cells to take place.

1.5 - The CAFs-TAMs crosstalk

As a matter of fact, medical data has shown that CAFs and M2 macrophages markers are co-related with the prognostics of patients with colorectal cancer [101], providing some evidence that some relation between both cell types might exist.

In wound healing and fibrosis situations, the fibroblast-macrophage interaction has been shown to be relevant in extracellular matrix remodeling processes and similar events [102], [103]. Since tumors can be seen as wounds that do not heal, this evidence suggests indeed that interactions between fibroblasts and macrophages take place at the tumor microenvironment level.

At least in some specific settings, this interaction within cancer microenvironments has been observed. In aggressive prostate carcinomas, CAFs were shown to promote the recruitment of monocytes as well as their differentiation into M2-like macrophages with the secretion of stromal-derived factor 1 (SDF1) [104]. An active collaboration between CAFs and M2 macrophages in the activation of endothelial cells, leading to *de novo* angiogenesis has also been reported, and this cooperation bolsters the escape of cancer cells and ultimately facilitates metastasis [104].

A recent work on the fibroblast-macrophage interaction has led to the development of 3D co-cultures as a way to model lung cancer and better understand the impact of these microenvironment cells [105]. In this experiment, the researchers found that the presence of both fibroblasts and macrophages had a clear impact on the expression of MMP1 and VEGF, molecules that play clear roles in matrix degradation leading to cell migration, and angiogenesis, respectively.

Another work with breast cancer cells and co-culture systems aimed at identifying functional changes of cancer cells [106]. Researchers concluded that fibroblasts model the surrounding microenvironment, promoting tumor cell spreading and motility while restricting their 3D-growth capacity. The presence of macrophages, on the other hand, showed a disruptive effect on the influence of fibroblasts and enhanced the formation of spheroids by the tumor cells.

Furthermore, CAF-TAM cooperation has also been revealed in the development of bladder cancer [107] and neuroblastoma [108].

All these evidences combined suggests distinct interactions between tumor associated macrophages and fibroblasts, with a clear impact in the progression of cancer. However, it is not yet clear how both cells interact in such conditions and what results from that interaction, i.e., what changes can fibroblasts induce in macrophages, in polarization and downstream cancer cell invasion and migration, and vice-versa. Unraveling the results of fibroblast-macrophage interaction in phenotypical and functional changes related to the behavior of cancer cells is crucial to understand where the immune subversion arises from and what pathways should be targeted in metastasis-preventive therapies.

1.6 - Objectives and Chapter Description

The main goal of this project was to understand the effects of normal and activated fibroblasts (including carcinoma-derived fibroblasts) on macrophage polarization and the downstream consequences on cancer cell behavior. This was achieved through phenotypical and functional analysis of fibroblasts (activated and non-activated) and macrophages, when alone or cultured in the presence of the other cell type.

The second chapter of this document provides a detailed description of materials and methods used throughout the work. The obtained results are presented and discussed in chapter 3:

Firstly, the growth of fibroblasts from the CCD-18co line (colorectal fibroblasts) for activation with a TGF-β protocol as well as the isolation of cancer-associated fibroblasts from colorectal cancer biopsies was attempted. The achievement of fibroblast cultures as well as the confirmation of fibroblast cell line activation is discussed in the first section of results.

After establishing fibroblasts , monocytes were isolated and differentiated into macrophages. Upon co-culturing macrophages with more and less activated fibroblasts (even without stimuli, fibroblasts showed some degree of activation), we peered into morphological changes of macrophages as well as M1/M2 surface markers expression and polarization shifts. The analysis of macrophage polarization and discussion can be seen in section 3.2.

Section 3.3 shows the functional confirmation of these phenotypical results: the outcomes of fibroblasts and fibroblast-polarized macrophages on cancer-cell invasion (decisive step for metastasis to occur), using a matrigel system and colorectal cancer cell lines.

Finally, section 3.4 deals with the possible molecular mechanisms behind these changes. Since TGF-β is a very important modulator cytokine for fibroblast and macrophage markers, it was expected that its levels on different single and co-cultures could change accordingly, and so it was analyzed through ELISA and qRT-PCR.

HGF, another very important cytokine previously known to be highly secreted by the CCD18-co cell line was also evaluated. Since macrophages showed some promotion of fibroblast-induced invasion, the levels of HGF in the different co-cultures were analyzed and discussed in section 3.4.

Altogether, this knowledge will be crucial to the development of stromal-disruptive therapies by exploiting possible tumoricidal actions between both macrophages and fibroblasts, crucial microenvironment cells in a synergistically anti-metastatic way, promoting inflammatory phenotypes, inhibiting cancer cell invasion and blocking pro-metastatic events, i.e., and suppressing pro-tumoral changes

Chapter 4 is composed of a summary of the main points of discussion as well as the concluding remarks that can be extracted from the integration of all the obtained results. This leads to chapter 5 where the main limitations of the work as well as the future directions in the context of the macrophage-fibroblast studies are pointed out.

Chapter 2

Materials and Methods

2.1 Cancer-associated Fibroblasts Isolation

CAFs were isolated from colorectal cancer patient biopsies as previously described [109]. Briefly, colonic samples of patients were collected and preserved in falcon tubes with 10 mL DMEM (Gibco) supplemented with a high concentration of antibiotics and kept at 4ºC after extraction. From here samples were taken to the flow hood and washed with PBS with antibiotics. Considering these samples are highly populated with bacteria from patient's intestine, the inclusion of antibiotics in the PBS used for washing was an addition to the referred protocol.

Following 30 minutes of incubation with PBS with antibiotics, samples were cut into $2mm^3$ pieces and placed in T-25 flasks with FBS carrying a high concentration of antibiotics. It is very important that pieces do not exceed this size, since it allows a higher surface/volume ratio which translates into more area of contact with the TCP from tissue culture flasks and promotes the outgrowth of fibroblasts.

This medium was replaced by FBS with lower concentration of antibiotics once samples were free of contamination. This process can take more or less time depending on the sample and the patient, but usually samples are contamination-free within 2 or 3 days. After 10 days in culture, fibroblasts start to outgrow from the tissue and onto the flask surface. At this point, FBS was removed and replaced by a more selective medium FBM (fibroblast basal medium) with FGM-2 (fibroblast growth medium) bulletkit (Lonza). This specific medium was developed to promote the growth of primary fibroblasts, favoring these cells and not others that are present in colorectal samples (such as endothelial cells). At all points, cells were kept in the incubator at the standard 37ºC, 5% CO2.

After around 40 days of fibroblast growth, fibroblasts were grown out of the tissue and confluent all around the flasks. At this point, primary cultures of CAFs that were well established were selected for further characterization.

2.2 Fibroblast Characterization

CAFs and fibroblasts characterization was carried out resorting to different techniques. To quantify fibroblast activation, protein was extracted in order to carry western blot analysis of the expression of α-SMA (myofibroblast marker of activation) and vimentin (mesenchymal marker). The expression of e-cadherin (an epithelial marker) was also assessed in order to evaluate the purity of the primary cultures and to assure that cells were not epithelial. Further information regarding the material and methods used for western blot analysis can be found below. The purity of primary cultures was also evaluated with flow cytometry, through a quantification of the Epithelial Cell marker EpCam. Flow cytometry analysis is also described in an individual section.

2.3 Cell Lines

The fibroblast cell line that was used was CCD-18Co which is composed of fibroblasts derived from human normal colon. In order to assay cancer cell invasion, RKO and HCT116 cells derived from human colon carcinoma were used. All these cell lines were purchased from the American Type Culture Collection (ATCC). Cells were maintained at 37ºC, 5% CO2 in DMEM (Gibco) for CCD-18Co and RPMI (Gibco) for tumor cell lines, supplemented with 10% FBS, 100U/mL penicillin (Pen) and 100µg/mL streptomycin (Strep) (Invitrogen). The addition of FBS and Pen/Strep leads to complete medium, most normally used in cell cultures.

2.4 Monocyte Isolation and Macrophage Differentiation

Human monocytes were isolated from healthy blood donors as previously described [110]. Briefly, buffy coats (leukocyte-enriched byproducts of whole blood donations) provided by the Portuguese Blood Institute (Porto, Portugal) were centrifuged at 1200g for 20 min. at room temperature. This centrifugation separates blood components in 3 liquid portions: the upper volume which is plasma, the lower volume which are red blood cells and a white ring of peripheral blood mononuclear cells in between. This ring was collected and incubated with RossetteSep Monocyte Enrichment Cocktail (StemCell Technologies) for 20 min. under rotation. This cocktail is designed to isolate monocytes by negative selection. Cells which are not monocytes are targeted with tetrameric antibody complexes, which in this case recognize unwanted cells (non-monocytes, including red blood cells). Afterwards, this mixture was diluted in PBS+2%FBS, added over Ficol-Histopaque (Sigma) and centrifuged. This centrifugation causes unwanted cells to pellet together with red blood cells, allowing the formation of a highly monocyte-enriched interface between plasma and the higher density medium. This new ring, rich in monocytes, was collected, washed with PBS and centrifuged for 17 min. at 700 rpm, to

remove most of the unwanted material that comes with the aspiration of the ring. The resulting cells (monocytes) were resuspended in complete RPMI (Gibco), ready to be cultured.

In order to allow monocyte differentiation into macrophages, cells were seeded at a density of $1.2x10^6$ cells/well (6-well plates) on top of glass coverslips in complete RPMI medium (described above) with the addition of 50ng/mL of macrophage colony-stimulating factor (M-CSF) (ImmunoTools). The use of glass coverslips is important since glass leads to better monocyte adhesion than normal TCP. M-CSF is a cytokine which facilitates monocyte-macrophage differentiation yielding higher differentiated macrophage numbers, with a more macrophage-like morphology.

After seeding, monocytes were kept in the incubator, at 37ºC 5% CO2 for 7 days, allowing differentiation without any intervention. At this point, the culture medium (1.2mL/well) was replaced by fresh RPMI without M-CSF. At this stage macrophages were set up for co-culture.

2.5 CCD-18Co Activation

Fibroblasts were kept in culture and the medium was renewed twice a week until confluence was around 80%. At this point, cells were subcultured and the same number of cells was divided onto two T75 flasks. This is an important step to make sure that cells can be activated at the same time in the same confluence and that no differences arise from fluctuations of cell number. Before reaching confluence, cells were washed twice with PBS1x to remove all the FBS contained in the medium. FBS removal is very important since it contains several growth factors that might influence fibroblast activation, namely TGF-β [111]. Afterwards, one flask was filled with 10 mL of DMEM supplemented only with 1% Pen/Strep (for non-activated fibroblasts) and the other with 10 mL of DMEM supplemented with 1%Pen/Strep plus 10ng/mL rhTGF-β1 (ImmunoTools), in order to activate fibroblasts, in a controlled, TGF-β dependent manner. In parallel, the same conditions were used in flasks with no cells as controls for the conditioned medium. This step is important if the need to test for specific molecules' concentration arises.

After 4 days, the media from all conditions were retrieved, centrifuged at 1200rpm for 5 minutes, filtered through a 0,2µm filter and stored at -20ºC. Fibroblasts were used for co-cultures with macrophages and the level of activation was confirmed through the expression of alpha smooth muscle actin (α-SMA) by western blot, extracting the protein of remaining fibroblasts not used for co-cultures, as described ahead in the western blot section.

2.6 Fibroblast-Macrophage co-cultures

After 7 days of macrophage differentiation, CAFs, activated and non-activated fibroblasts were seeded at a density of $1x10^5$ cells/insert on 1µm-pore PET inserts (Corning) and were co-cultured with the macrophages in this transwell system allowing free flow of media and soluble

factors. The flow is extremely important in order to allow cells to communicate with each other through soluble factors and not physical interaction. The density of $1x10^5$ was calculated considering that fibroblasts divide and proliferate whereas macrophages don't. So, after 3 days of fibroblasts doubling their numbers, the final density is expected to be around $8x10^5$ cells/insert, which is a number close to the estimated $1x10^6$ macrophages/well, yielding a ratio close to 1:1. As controls for polarization, macrophages were also cultured alone (M0), with LPS (Sigma-Aldrich), which is a modulator of classically activated, inflammatory macrophages (M1-lilke) and IL-10 (Immunotools) which polarizes macrophages towards an alternative, anti-inflammatory phenotype (M2-like), both at 10ng/mL [112]. These polarization controls are extremely important since they act as normalizers for the levels of M1/M2 markers, creating a comparison point between observed outcomes and true M1/M2 levels.

All co-cultures were done using RPMI complete medium, more suitable for macrophages, due to the fact that these cells do not proliferate and are much more sensible than fibroblasts, which can perfectly thrive in different culture media.

After 72h of co-culture, macrophages were detached using 400mL accutase for 30 min. at 37ºC. Accutase is an alternative to the more widely used Trypsin, mimicking its action plus that of collagenase. Accutase is usually formulated at lower concentrations and it is less toxic and gentler and does not need to be neutralized. Slight scrapping was also applied after accutase incubation to ensure maximum detachment. Wells were washed with cold PBS after scrapping and removal to collect additional macrophages left on the well. Afterwards, cells were stored on 5mL PBS (1x) on ice.

Fibroblasts were lysed from the transwell inserts for protein extraction as described on the western blot section.

Some macrophages were also used for RNA extraction using TriPure Isolation Reagent (Roche), according to manufacturer's instructions, for qRT-PCR analysis. Further RNA purification procedure is described below.

All conditioned media was retrieved, centrifuged and stored as previously described, to assess the levels of soluble factors in the different co-culture conditions.

2.7 Macrophage Morphology Screening

In order to evaluate the effect of fibroblast co-culturing in macrophage morphology, bright-field pictures were taken prior to and after the 72h of co-culture using a ZOE Fluorescent Cell Imager (Bio-Rad). Since these revealed some differences, further assessment was done through immunofluorescence staining. At the end of the co-cultures, one glass coverslip with macrophages from each condition was retrieved to a 24-well plate, washed with PBS and fixed with paraformaldehyde for 15 min. at RT. After this, cells were kept in sterile PBS until staining. For the staining process, fixed cells were incubated in NH4Cl 50mM for 10 minutes, followed by a triple wash with PBS for 5 minutes. The ammonium chloride solution blocks formaldehyde

groups that might not have reacted therefore avoiding the resulting background fluorescence. Afterwards, cells were incubated with TritonX-100 0,2% for 5 min. and washed 3 times with PBS at the end of this time. TrixonX-100 is used here to increase the permeabilization of cell membranes, therefore allowing antibody and reagent penetration into the cells. After these steps, samples were blocked in 5% BSA for 30 minutes to block nonspecific antibody ligation. After blocking, the primary mouse antibody against α-tubulin (Sigma) was incubated in BSA 5% for 1h, according to the manufacturer's suggested dilution. Afterwards, samples were washed 3 times for 5 min. and the secondary anti-mouse alexa488 labeled antibody (sigma) was incubated in BSA 5% for 1h. 20 minutes before ending, 5µL of Rhodamine Phalloidin (Termo Fischer) was added to each sample, for actin staining. At the end of the 1-hour of secondary antibody incubation, samples were washed and mounted in glass slides with 3µL of vectashield with DAPI for nuclei staining. Slides were sealed and stored at -20ºC until measurement. Immunofluorescence imaging was done with the Zeiss Axio Imager Z1 Apotome, using the 63x immersion objective. Images were obtained using the apotome and through fusing of 10 different vertical planes.

2.8 Macrophage Polarization Assessment – Flow Cytometry

After retrieval from the co-culture, Macrophages were stained for flow cytometry quantification of polarization levels. Briefly, cells were centrifuged at 1200rpm for 5 minutes, 4ºC to remove remaining accutase and medium and resuspended in 400µL of FACS buffer (PBS with 2% FBS and 0.01% sodium Azide). This PBS/FBS buffer is widely used in cell suspensions for cytometry since it allows cells to stay stable and live. The inclusion of sodium Azide prevents heterophilic antibody interference which is known to cause false positives and false negatives. It also prevents antigens from being internalized during the staining process, so it should be used when cell function recovery is not necessary.

Afterwards, cells were stained for CD14-APC (clone MEM-18) as lineage marker, CD86-FITC (clone BU63) as M1-like marker (Immunotools) and CD163-PE (clone GHI/61) (R&D systems) as M2-like marker. The staining was done in 50µL of FACS buffer with the following dilutions: CD14 and CD86 2:50, CD163 5:50. Unstained, single stained and isotype-matched antibodies were also used as flow cytometry controls. Unstained controls are essential to set the difference between a negative and positive response. The isotypes are antibodies that target a non-existing protein on the cell surface, with the same heavy and light chains of the antibody of interest. If positive results arise with the use of the isotype, it means that the antibody is binding non-specifically to low affinity targets. The isotype control is then important to assure that the measurement is related only to specific binding to the antigen of interest.

The staining was done by incubating cells with the antibody mix for 40 minutes at 4ºC in the dark. Afterwards, cells were washed 3 times with FACS buffer with centrifugations at 1200rpm, 5 min, to remove antibodies that failed to bind to cells.

At the end of the washes, cells were fixed by adding 200μL of 4% Formaldehyde for 15 minutes, time after which it was removed with an additional centrifugation in the same conditions. Stained and fixed, cells were stored in FACS buffer at 4ºC until analysis.

Flow cytometry was conducted using a FACSCanto device (BD biosciences), reading 1×10^4 cells per condition. Analysis for quantification of surface marker expression was performed with FACSDiva and FlowJo software.

Anti-inflammatory ratios of macrophage polarization were calculated based on equation (1).

$$Anti - inflammatory\ ratio = \frac{\%\ M2\ marker\ CD163}{\%\ M1\ Marker\ CD86} \qquad (1)$$

2.9 Western Blots

Whole cell protein extraction were performed after washing cells with PBS1x to remove proteins from the media. The protein extracts were obtained using RIPA lysis buffer, which includes SDS, sodium deoxycholate and Triton X-100 as active components able to disrupt cell membranes. This buffer was supplemented with a cocktail of proteases and phosphatases inhibitors, as previously described [113], to avoid degradation of cell proteins. This lysis was further amplified by scrapping cells on wells and inserts, to ensure maximum release of intracellular proteins.

Extracts with protein alone were obtained after centrifugation at 14000 rpm for 10 minutes at 4ºC, in order to pellet cellular debris and collect the protein mix in the supernatant.

Protein quantification was done using the Biorad protein quantification kit according to the manufacturer’s instructions. Calibration curves were obtained with standards of BSA ranging from 0.25 to 3 mg/ml. After quantification, the volume corresponding to 15μg of protein was mixed with laemmli buffer (1x), which contains mercaptoethanol, SDS, bromophenol blue and glycerol. Mercaptoethanol reduces natural protein bonds, SDS denaturates the protein and gives them negative charge so they can be separated according to their size. The bromophenol blue dyes the sample and runs ahead of the protein in the gel. The glycerol is able to increase sample density so that it layers in the sample well. After mixing the protein solution with this buffer well, samples were placed in the heating plaque for 5 min. at 95ºC for further denaturation.

Once ready, samples were loaded into the PAGE gel at the volume of 15μL together with the protein marker for weight. Gels ran at 80mV until the running front reached the bottom.

Afterwards, SDS-PAGE gels were transferred onto nitrocellulose membranes for 90 minutes at 100mV. These membranes were incubated with primary antibodies overnight at 4ºC.

For fibroblast activation screening and characterization, antibodies against the following proteins were used: α-SMA, Vimentin and E-cadherin (Santa Cruz). GAPDH (Santa Cruz) was used as the housekeeping gene and membranes were also incubated with the antibody against it.

After overnight primary incubation, membranes were washed with PBS + 0.05% Tween (PBS-T) and anti-rabbit or anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies were used on membranes for 1h at RT. After this secondary incubation, membranes were washed again with PBS-T and the detection of antibody signal was done using ECL (BioRad).

The quantification of western blot images was done using Image-J software (FIJI).

2.10 Matrigel Invasion Assays

To evaluate the effects of macrophages and fibroblasts on cancer cell invasion, two cell lines were used. RKO cells were used to assess the effects of macrophages and HCT116 cells were used in the case of fibroblast. This choice of using two cell lines was taken as they proved to respond differently to different cell signaling (S.Velho unpublished results) and so in order to maximize the cancer cell response to macrophages and fibroblasts, the respective responsive cell line was used. At the end of the macrophage-fibroblast co-cultures, where both cells had mutual influence on each other, these cancer cells were seeded on the upper compartment of Matrigel-coated inserts of 8-μm pore size (BD Biosciences) at a density of $5x10^4$ cells/insert. Matrigel is a preparation of solubilized basement membrane extracted from mouse sarcoma, a tumor rich in ECM components such as laminins, collagen, proteoglycans, among other ECM molecules, and was therefore used to mimic the *in vivo* environment cancer cells face and must invade through.

Beneath these cells, one glass coverslip with macrophages from different co-culture conditions was placed on the lower compartment as well as half the number of fibroblasts trypsinized from the 6-well inserts of the co-cultures. By doing so, the transwell system allows free circulation of soluble molecules between macrophages, fibroblasts and cancer cells, which can then invade more or less through the matrigel layer. After 24h at 37ºC and 5% CO2, the invasion assay was stopped, all conditioned media was retrieved and the wells were washed with PBS 1x, fixed for 15 mins with 4% paraformaldehyde and kept in sterile PBS until analysis.

To evaluate the level of invasion on the different conditions, the inserts were washed with PBS and the top surface of the membrane was scrapped gently with a swab to remove non-invasive cells, leaving only a small portion with these cells to allow the distinction of the two planes of focus on the microscope.

Once the porous membranes were clean, they were cut out of the insert with a scalpel and mounted on a glass slide in Vectashield mounting medium with DAPI (Vector Laboratories) for nuclei staining and fluorescent identification of cells. The slides were kept at 4ºC until analysis and -20ºC for storage.

The number of invasive cells was counted under fluorescent light on a microscope (Leica).

The schematics of co-cultures cell transference to invasion assays can be seen in fig 6.

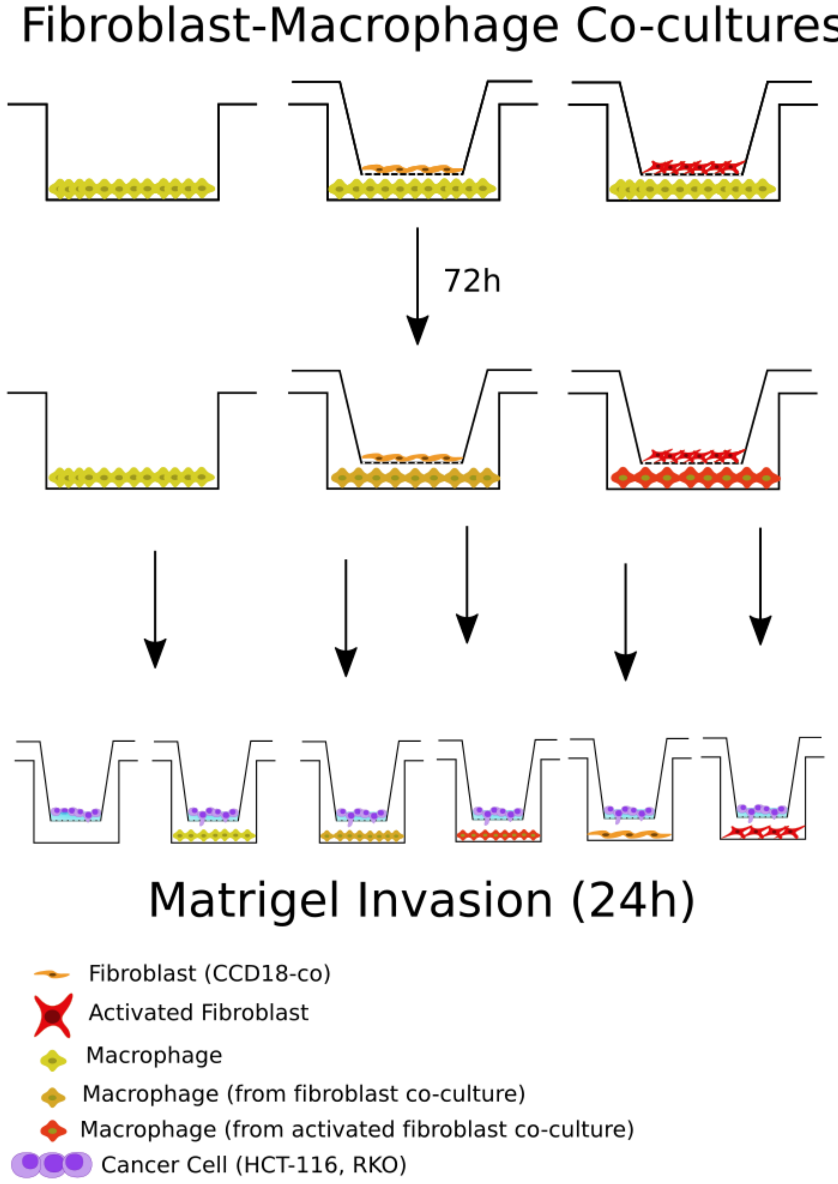

Figure 6 - Co-culture schematics - Macrophages were co-cultured with fibroblasts and activated fibroblasts, as well as alone in single culture. After 72h, macrophages and fibroblasts from all different conditions were transferred to new wells on top of which cancer cells were seeded for the invasion analysis, during 24h.

2.11 Enzyme-linked immunosorbent assay

To measure the levels of TGF-β in the conditioned medium of co-cultures and invasion assays, an ELISA kit with pre-coated plates (Legend Max, BioLegend) was used according to the manufacturer's instructions. Briefly, conditioned media samples were first treated with the acidification and neutralization solutions and then diluted 40x. After this, the plate was washed four times, blotting the plate against absorbent paper to remove the maximum amount of excipient. After, the TGF-β standards and treated samples were incubated in the wells for 2 hours, at room temperature (RT), with shaking at 200 rpm. Following another series of washes, the detection antibody solution was added and incubated for 1 hour, RT, shaking. After this, Avidin-HRP solution was added and incubated for 30 min., RT, shaking. Following another wash series, the substrate was added for 10 minutes, RT, in the dark and after this the reaction was stopped and the blue solution became yellow. The absorbance was read at 450nm and 570nm, the latter subtracted to the first. The standards' values were used to obtain a calibration curve and were plotted on a log-log scale allowing the creation of a linear trend curve and quantification of TGF-β concentration on cultures' conditioned medium.

For HGF measurements, the RayBio Human HGF ELISA kit with pre-coated plates was used according to manufacturer's instructions. Briefly, HFG standards and samples were placed directly into each well and incubated overnight at 4ºC with gentle shaking. The day after, solutions were discarded and the wells washed 4 times, always blotting against absorbent paper. Afterwards, the HGF biotin antibody was added to each well and incubated for 1h at RT, shaking. Following another series of washes, the Streptavidin solution was added and incubated for 45 minutes at room temperature. Wells were then washed for the last time and incubated for 30 minutes with the TMB One-Step substrate reagent. After this time, the solutions turned blue and the reaction was stopped. The absorbance was read at 450nm immediately. The standard curve was built in resemblance with that of TGF-β ELISA.

Both kits are based upon the same interactions, in what is called a sandwich ELISA. The pre-coated wells have an antibody which is able to bind to the desired molecule, trapping it in the bottom of the well. Afterwards, a secondary antibody is able to bind to this trapped molecule. This secondary antibody possesses biotinylated residues on its FC extremity. Through biotin-avidin interaction, the Streptavidin/Avidin-HRP binds to the biotinylated residue. HRP (horseradish peroxidase) is an enzyme linked to avidin, able to degrade the specific substrate producing a colored compound, which is at the end proportional to the level of the molecule present in the assayed solution, increasing the absorbance when more substrate is degraded, meaning more desired molecule was initial present.

All the absorbance readings were performed using a BioRad model 680 microplate reader with 3 seconds of medium-speed shake before measurement to uniformly mix the solutions in the wells.

2.12 Quantitative reverse transcriptase PCR (qRT-PCR)

As previously reported, total macrophage RNA extraction was carried out after fibroblast co-cultures, using the TriPure Isolation Reagent (Roche), in accordance with manufacturer's instructions. Extracted RNA was quantified using a Nanodrop 1000 micro volume system.

After quantification, RNA was converted to cDNA using the qScript XLT cDNA SuperMix (Quantabio). This mix was used at the proper dilution (manufacturer's instructions) with 1μg of RNA in DNase/RNase free water (Gibco). Once properly mixed, the solution was incubated in the thermocycler in the following series: 5 minutes at 25ºC, 1 hour at 42ºC and 5 minutes at 85ºC, maintained at 4ºC until pick up.

The evaluation of TGF-β expression at the mRNA level was done through quantitative reverse transcriptase PCR (qRT-PCR), using TaqMan probes for TGF-β1 and GAPDH as the housekeeping gene, both diluted on the TaqMan Universal PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions.

TaqMan probes are built with a fluorochrome and quencher nearby each other, so that when normally together, the quencher prevents fluorescence to be released. When there is amplification of the desired sequence, the probe is inserted in the cDNA strand and the fluorochrome is released from the 5' portion due to the 5' nuclease activity of the proper polymerase. As the fluorochrome is released from the probe, the quencher action is lost and fluorescence is detected. The quantification arises from the amount of amplification, which leads to higher levels of fluorescence, allowing for the quantification of a certain gene expression (directly related to the amplification).

The housekeeping gene serves the same role as it does in western blotting: normalization of the expression levels. GAPDH is the glyceraldehyde 3-phosphate dehydrogenase, which has the essential function of breaking down glucose for the cell to obtain energy and carbon molecules. This enzyme is expressed throughout all kinds of human tissues and not affected by most genetic and phenotypic modifications of cells, being very reliable as an housekeeping gene [114].

The qRT-PCR was performed using an iQ5 Real-Time PCR Detection System. Samples were run in triplicate with blanks to confirm no self-amplification of probes was taking place. On analysis, the most deviant value of the triplicates was excluded (if present), considering only the two less variable number of cycles for the average calculation. To obtain the fold variation of TGF-β expression, ΔCt (difference in cycles compared to the housekeeping) was converted to fold variation as $2^{-\Delta Ct}$ and normalized against the fold value for macrophages alone.

2.13 Statistical Analysis

Statistical Analysis and Graphs were elaborated using GraphPad Prism (v6). One-way ANOVA tests were performed to test for statistically significant differences between different

conditions. Multiple comparisons were made using Turkey's multiple comparisons test for comparison between all conditions. Dunnett's multiple comparisons test was used for comparisons in relation to a control condition (e.g. Macrophages alone). Differences were assigned statistical significance when $P < 0.05$.

Chapter 3

Results and Discussion

3.1 Establishment of Fibroblast Cultures

One of the goals of this work was to isolate, culture and characterize cancer-associated fibroblasts, specifically colorectal cancer-associated fibroblasts. By establishing a collaboration with the Portuguese Institute of Oncology, it was possible to obtain samples from biopsies of colorectal cancer patients in order to derive fibroblasts from the tumor tissue. As referred in the methods section, colorectal samples of tumor, adjacent as well as distant normal mucosa tissues were extracted from voluntary patients and taken to the laboratory. By using an already developed method [109], with some alterations for contamination control, and resorting to selective cell growth medium, patient's tissues were cultured for several time-points and their development was followed as seen in fig. 7.

Throughout this work, colorectal samples were obtained from 4 different patients, numbered from 1 to 4. In all of these patients, only patient 4 showed some moderate fibroblast outgrowth prior to day 10 in the mucosal tissues. However, just like every other sample, mucosal fibroblasts would degenerate before allowing the establishment of a primary culture. In the case of the two last patients, colorectal contamination was not efficiently controlled by routine antibiotics, leading to re-emergence of contamination around day 25 and requiring samples to be discarded.

Patient 1 and 2, however, yielded fibroblasts on the tumor tissues. These two cases showed very different behavior in the respective fibroblasts. Patient 1 tissues allowed the establishment of primary cultures within 30-40 days, and these fibroblasts were subcultured for successive passages for characterization and always showed a normal growth rate, similar to cell lines, as visualized in fig. 7P1. The morphology of these fibroblasts was also very similar to the one exhibited by established fibroblasts cell lines (comparison with fig. 7P2).

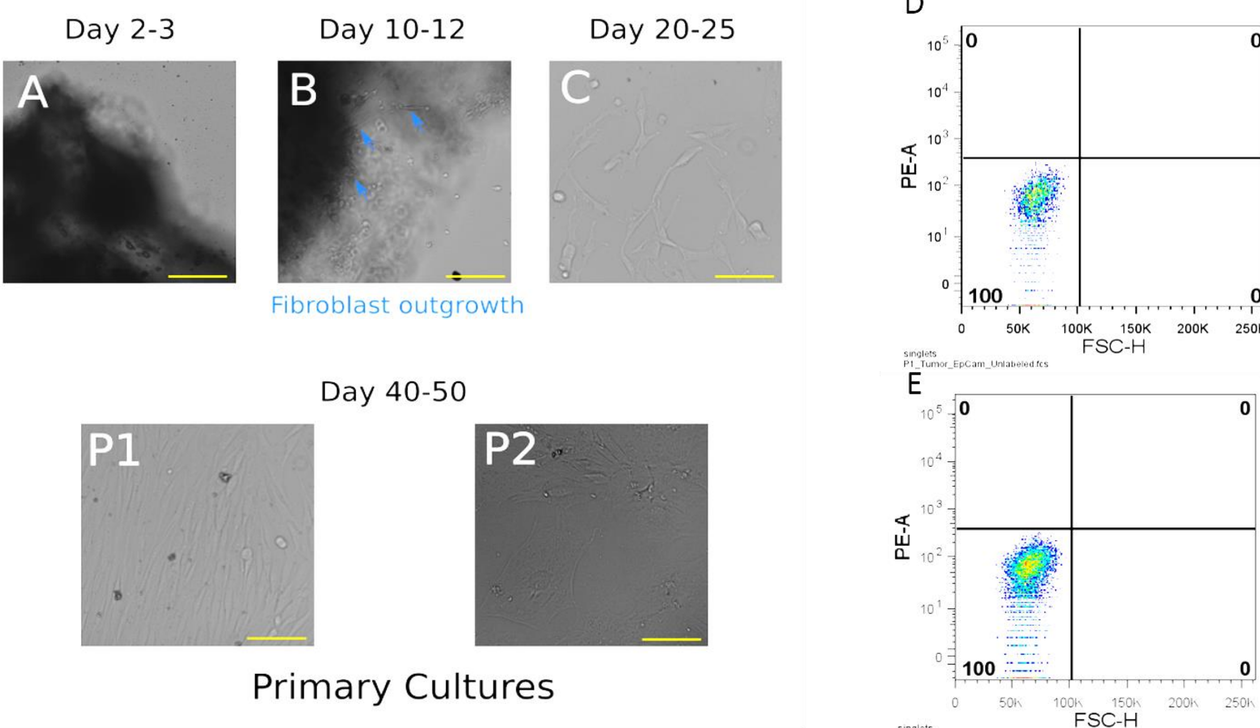

Figure 7 - Primary CAF Culture establishment - Biopsies from Colorectal patient's tissue were cultured and the daily evolution was accompanied. At the first 2-3 days (A), only tissue mass can be seen. Around day 10-12, some fibroblast outgrowth is evident, as indicated by the blue arrows (B). Around 20 days after culture, fibroblasts start to proliferate through the culture dish (C). Fibroblasts obtained from different patients had distinct morphologies and growth rates (P1 vs P2). Scale bars: 100 µm. Flow cytometry characterization showed negative presence of the epithelial marker EpCam-PE (E), similarly to the unstained sample (D) on fibroblasts derived from Patient 1.

Patient 2 samples, however, behaved entirely different. Firstly, these fibroblasts had a much lower growth rate, and took more than 50 days to occupy a T-25 culture flask to a moderate confluence. Not only the growth rate was different, but the morphology of patient 2 fibroblasts was also completely distinct. Some fibroblasts showed elongation while others were round and some of them resembled large cellular complexes, spread in an egg-like shape, as the one seen in fig. 7P2.

The fibroblasts from patient 1 had a very functional behavior and so their characterization was possible. First of all, the purity of the primary culture was assayed, and these fibroblasts revealed the absence of epithelial markers (fig 7 D and E) and expression of other non-fibroblast molecules such as E-cadherin, with no western blot traces of such proteins. In that same analysis, patient 1 fibroblasts showed reduced levels of the fibroblast activation marker α-SMA. This result is however being compared with a cell line that might be extremely activated and the confirmation on whether or not these fibroblasts are significantly activated would require a comparison with normal tissue from the same patients, which failed to yield fibroblasts.

It could also be possible that some of the activation was lost due to the culturing process. However, when we look at the literature, cancer-associated fibroblasts are described as

activated, α-SMA expressing, myofibroblast-like cells. Moreover, this activation, usually screened through the expression of α-SMA, is described as perpetual and irreversible [115]. Being so, the culturing and passage of fibroblasts should not, theoretically, influence their level of activation, which translates into their α-SMA expression.

In fact, most results that identify this myofibroblast profile in tumor tissues are focused on breast cancer, where immuno-histochemistry results clearly show that stromal cells are positive for α-SMA in tumor tissue sections [116]. However, there are no solid reports on the quantification of this protein expression, only about its presence. This holds especially true for the case of colorectal cancer and its different stages.

The fact that patient 1 and patient 2 fibroblasts showed such distinct morphology and growth behavior, suggested that we were dealing with very different profiles. The characterization of patient 2 fibroblasts was unfortunately not possible due to the appearance of contamination in a very late stage, until which their low proliferation impeded efficient passage and growth to have enough cell numbers for analysis. Further characterization would have been essential to confirm if these cells were indeed fibroblasts and if so, whether or not the phenotypical and cell growth differences would reflect on the expression of α-SMA or other CAF markers.

Fibroblasts from patient 1 were used as a low activation population and named CAFs due to their origin for the sake of identification. Their efficient establishment and manipulation allowed for the inclusion in macrophage co-cultures, together with the fibroblast cell line. Some of these fibroblasts were also frozen according to the regularly used cell-freezing protocol. Unfortunately, upon defrosting and culturing, no fibroblasts adhered, remaining in suspension in a very rounded morphology even for several days. This shows that this type of primary cells do not easily endure freezing, at least with the standard protocol, and the fact that, as a primary culture, they cannot be passed on forever, shows that CAF primary cultures are extremely unstable and should always be very well planned in order to maximize the cellular uptime for required experiments, avoiding excessive passages and freezing of cells.

In order to have an additional, more reliable model of fibroblasts for testing, the CCD18-co cell line was used (fig. 8). These cells allowed more manipulation, staying stable until around passage 10 and tolerating freezing and thawing procedures, allowing more testing and reproducibility in comparison to primary cultures.

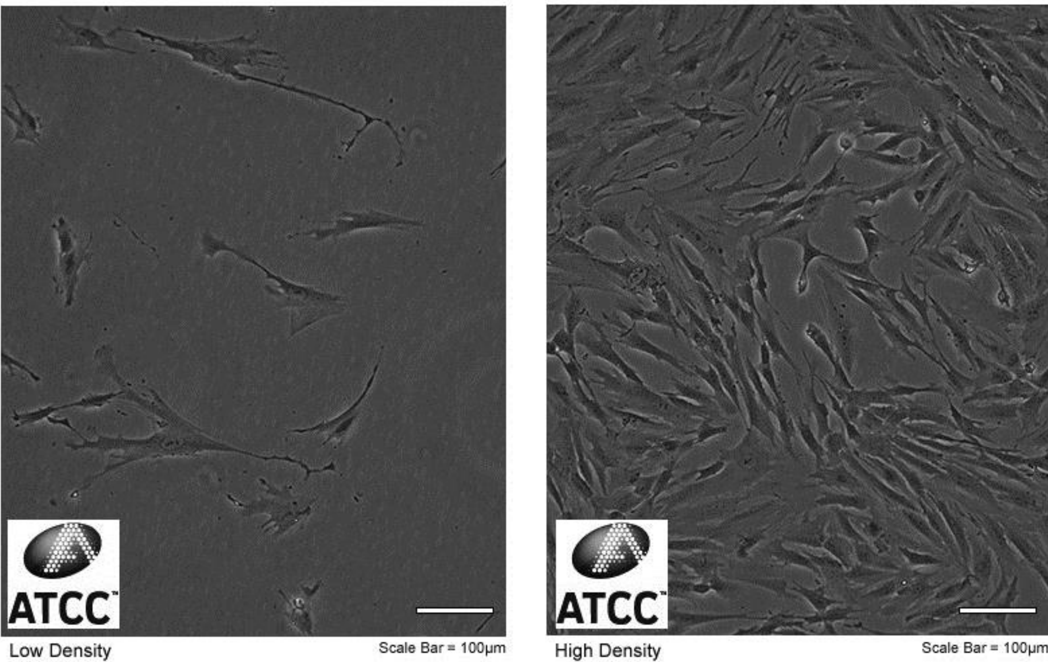

Figure 8 - CCD-18co fibroblast morphology and growth behavior.

Nevertheless, the CCD18-co cell line is composed of colorectal fibroblasts immortalized after derivation from healthy patients. This means that these are actually not CAFs but normal fibroblasts. In order to induce CAF-like properties in this cell model, an activation protocol was used. Therefore, TGF-β, a well-known modulator of fibroblast activation [117] was added to the culture medium of CCD18-co fibroblasts, and the exposure to this cytokine for 4 days allowed a stable and durable activation, as measured through western blot for α-SMA expression levels (fig. 13A). The only pitfall of using this protocol was that non-activated fibroblasts still showed a significant degree of activation, even if always lower than their activated counterparts. This is probably due to the fact that the CCD18-co line has already a certain basal degree of activation. This protocol was attempted with different degrees of confluence but this basal activation level was always similar among non-stimulated fibroblasts, regardless of confluency. This led to the use of more and less activated fibroblasts rather than activated and non-activated fibroblasts. However, the use of the CAF primary culture (with very low levels of α-SMA expression) in some experiments, even if without replicates, allowed some validation of the effects of activation.

3.2 Fibroblast – Macrophage Interaction

Monocytes were isolated from different healthy blood donors and differentiated to a certain extent *in vitro*, point at which fibroblasts in different activation profiles were co-cultured in a transwell system, allowing the flow of media and soluble molecules, i.e., allowing paracrine communication, while avoiding cell-cell physical interaction. This model is widely used and fully described in the methods section.

Using bright field microscopy, the morphology of macrophages before and after fibroblast co-cultures as well as after their full differentiation were followed, as detailed in fig. 9.

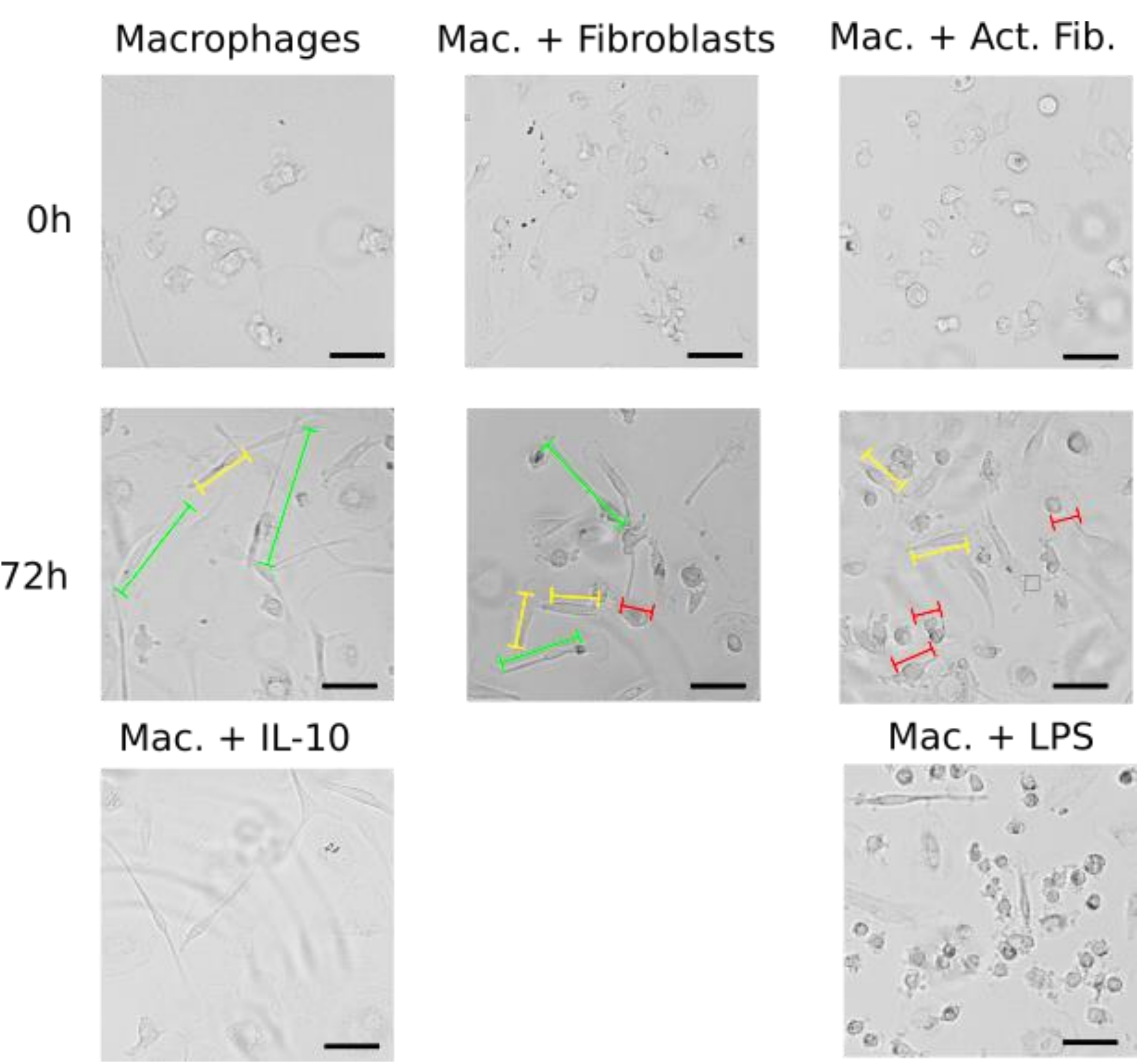

Figure 9 - Bright-field pictures of macrophages prior to and after 3 days of fibroblast co-culture. Images from macrophages alone, macrophages co-cultured with fibroblasts (mac. + fibroblasts) and macrophages co-cultured with activated fibroblasts (mac. + act. Fib.) as well as the macrophages with polarization controls IL-10 (Mac. + IL-10) and LPS (Mac. + LPS). Green, Yellow and Red bars illustrate the varying elongation of different cells. Scale bar: 50µm.

At 0h, which corresponds to 7 days of differentiation with M-CSF, prior to the interaction with any other cell types, all macrophages have a similar, round-shaped morphology. Some differences between patients are expected since they consist of primary cultures originated from the blood of distinct individuals. At this point, accompanied by M-CSF removal, fibroblasts were added on the transwell inserts.

Macrophages that remain alone maintain a more elongated shape (fig. 9). Instead, macrophages that were co-cultured with fibroblasts were a varying population of elongated and

round-shaped cells, and when activated fibroblasts were present, macrophages tended to acquire a more circular shape. Even though some donors presented several elongated macrophages when these were co-cultured with activated fibroblasts, the elongation seemed reduced in comparison to that of macrophages cultured alone. Also, by looking at the macrophages exposed to polarization controls during the 72h, we can see that IL-10 stimulated macrophages seem to be the ones with the highest elongation and the LPS-stimulated population appears rich in small, rounded cells. Since IL-10 and LPS are known inducers of M2-like and M1-like phenotypes [118], respectively, these changes in morphology were the first cue indicating that fibroblasts could be interfering with macrophage polarization to some extent, especially when activated (CAF-like). Nevertheless, these changes in morphology are based only on the observation of cells. The solidification of these claims will only be possible upon precise quantification of cell elongation and evaluation of macrophage cell surface receptors expression. .

Be that as it may, the changes in morphology were further explored through actin/tubulin immunostaining (fig. 10). Macrophages that differentiated without the interference of fibroblasts presented an overall more elongated shape, with protuberances and outgrowths rich in tubulin. With the inclusion of fibroblasts, this largely elongated cells were much less visible, and most macrophages were more round, even though some protuberances an elongated portions of these cells could still be detected, as visible in the merge picture. This change was even more evident when activated fibroblasts were co-cultured with macrophages. In this condition, most macrophages exhibited a round shape, the presence of elongated cells seemed to be reduced and the size of elongations appeared to be overall small, much resembling the LPS-stimulated macrophages of fig. 9. All together, these results pointed at a shift on macrophage morphology through the communication with fibroblast that seems to be related to the polarization of these cells, if we compare their morphology with that of LPS- and IL-10-stimulated control cells.

Knowing this, it must also be noticed that the communication between different cells is a very complex process [119], which can be translated into two essential forms: contact-dependent and paracrine (contact-independent). On the first type of cellular communication, a membrane-bound molecule from one cell is recognized by a receptor on the other cell's membrane. On the second, one cell signals the other by secreting specific molecules, which then travel to other cells' receptors. Both of these types of communication can be very relevant in any context, but paracrine signaling has been successively identified as critical on the microenvironment of tumors, the cancer cell-microenvironment interaction [120] and ultimately the behavior of cancer cells and macrophages [121]. In this case, the changes in macrophage morphology are associated only with paracrine signaling, since this is the only one allowed by the physics of the transwell system.

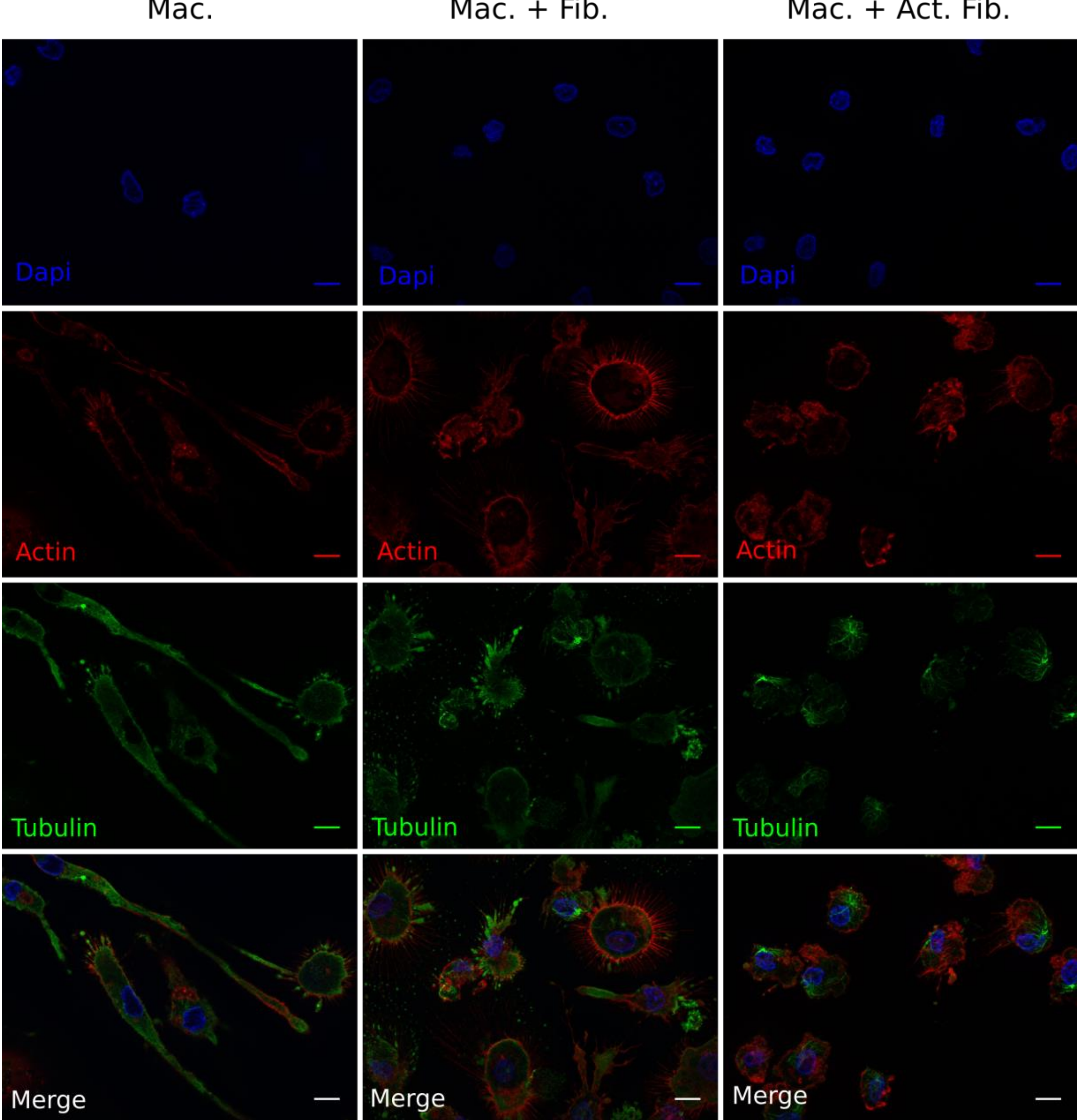

Figure 10 - Immunofluorescence images of macrophages - Macrophages from single cultures (Mac.) and co-cultures with fibroblasts (Mac. + fib.) and activated fibroblasts (Mac. + Act. Fib.) were stained and pictures represent these cells' Nuclei (Dapi), Actin, Tubulin and the Merge of all three, under visualization at 63x magnification. Scale bar: 15μm.

Be that as it may, if we look at the literature, we can see that macrophage morphology is intimately related to macrophage phenotype. McWhorter *et al.* reported that M2-like macrophages exhibited a more elongated shape in comparison to M1-like cells [122]. This higher elongation is not representative of increased elongated cell number or changes in cell area but increased elongation factor, i.e., increased long/short axis ratio. Furthermore, this paper showed that not only is elongation a consequence but also a cause of macrophage polarization by demonstrating that surface patterning controlling macrophage shape can increase elongation and expression of M2 markers. This shows that cell morphology is extremely important and together with the obtained results it seems in fact that fibroblast signals

have some weight in macrophage phenotypes. To further explore these effects of fibroblast-derived signals, the evaluation of macrophage polarization markers was carried.

Macrophages exhibit different surface markers in different contexts and when going through different polarization events. These markers have been extensively studied in order to try and establish a molecular signature of M1 and M2 cells. However, this dual division has evolved to a more complex scenario where macrophages can be placed anywhere in the M1-M2 spectrum, being identified as more M1- or M2-like depending on their expression of surface markers. To identify to which point the changes induced by fibroblasts and observed on macrophage morphology would relate to M1/M2 profiles, macrophages were retrieved after co-culture and stained with a cocktail of antibodies targeting specific surface markers, namely CD14 for macrophage lineage identification (other cellular components can be present as a residual defect of the isolation procedure), CD86 as an M1-like marker and CD163 as an M2-like marker.

After proper staining, macrophages from different donors and different conditions were analyzed through flow cytometry in order to quantify the level of the referred surface markers (fig. 11). This technique combines single-cell measurement with the fact that cell surface markers are quantified in live or similarly fixed cells and so these are analyzed in a very realistic fashion.

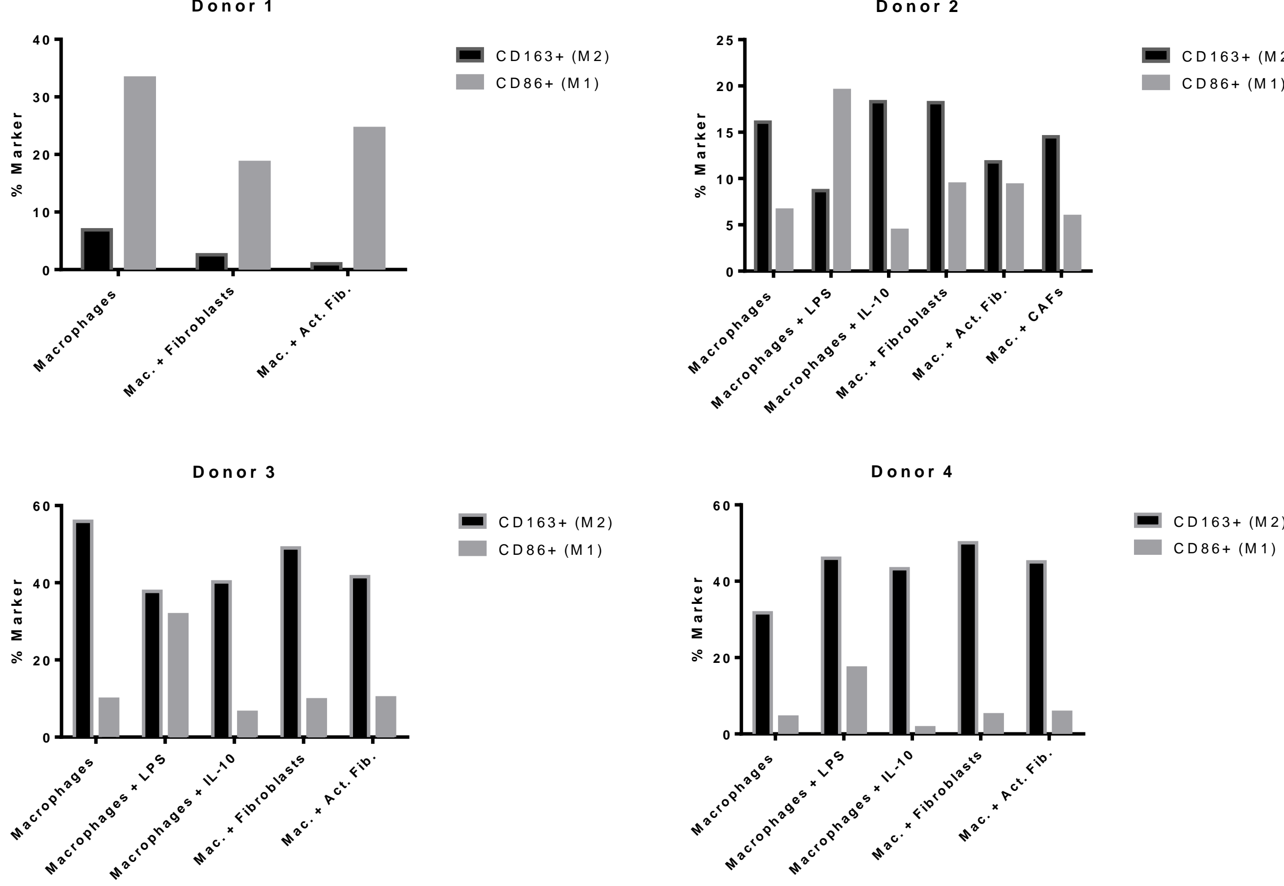

Figure 11 - Macrophage polarization assessment of 4 different donors. The percentage of cells expressing the polarization markers CD163 (black bars) and CD86 (gray bars) were quantified using a flow cytometer. The expression of each marker is represented as %Marker and macrophages from all conditions are present: Macrophages, Macrophages exposed to LPS (Macrophages + LPS), Macrophages exposed to IL-10 (Macrophages + IL-10), Macrophages previously co-cultured with fibroblasts (Mac. + Fibroblasts), Macrophages previously co-cultured with activated fibroblasts (Mac + Act. Fib.) and Macrophages co-cultured with the colorectal biopsy fibroblasts (Mac + CAFs).

On the first, more exploratory experiment with the first donor (fig. 11 donor 1), only three basic conditions were used in co-cultures, comparing macrophages that had developed alone and macrophages that were in co-culture for 3 days with fibroblasts and activated, CAF-like fibroblasts. This experiment was carried out to explore the initial hypothesis that activated fibroblasts would induce an M2 shift on macrophages, related to pro-tumoral events. However, we could see that the presence of fibroblasts caused a relevant decrease on the M2 marker CD163, from 7% when macrophages were alone to 2.6% when fibroblasts were present and 1% when activated fibroblasts were co-cultured. Also here, the effect of fibroblast activation appeared to have some effect in the expression of the M1 marker CD86, as it increased from 18.6% to 24.5% when fibroblasts were activated. This initial results pointed at a shift on macrophage polarization, with activated fibroblasts leading to a decrease of the anti-inflammatory potential of naïve macrophages. With donor 1 as a starting point, this experiment

was repeated with 3 more donors. On these, macrophages cultured in the presence of LPS and IL-10 were included in order to have polarization controls of M1 and M2, respectively, and allow the validation of data. On the macrophages from donor 2, the polarization shift was even more obvious. First we can see that IL-10 and LPS have a reverse effect on the expression of markers. In the presence of IL-10, the highest level of CD14+CD163+ cells is obtained with 18.3% of cells positively expressing this marker and only 4.4% expressing the M1 marker CD86. Reversely, when stimulated by LPS, only 8.7% of the cell population expresses CD163 and 19.5% are CD86 positive. This is very important since it confirms that these markers are very appropriate to evaluate polarization. In this same donor, the presence of activated fibroblasts leads to a decrease of the M2 marker to 11.8% and an increase of the M1 marker to 9.3%, in comparison to naïve macrophages. These more inflammatory levels are second only to LPS-stimulated, M1-like cells, which reinforces activated fibroblasts as responsible for an inflammatory shift. In this assay with macrophages from donor 2, the population of CAFs that was established as a primary culture was also used to see to what extent these fibroblasts could affect macrophage polarization. As seen in fig.11, these CAFs had very little effect on macrophage marker's levels, representing the condition that most similarly resembles that of naïve macrophages. Since these fibroblasts extracted from colorectal cancer patient's biopsies showed very little degree of activation, as represented ahead on western blot results, suggesting that less-activated fibroblasts have no effect on macrophage polarization. This also indicates that the activation of fibroblasts is a decisive event in what comes to macrophage modulation. However, the introduction of CAFs was only possible in one of these experiments with a single monocyte donor and so it lacks some repetition and solidification in order to fully confirm these statements.

After donor 2, the experiment was repeated with two more donors and overall the same could be observed with activated fibroblasts promoting an increase of CD86 expression and diminishing the levels of CD163. Throughout all donors, some more or less unexpected events were observed, such as the highest expression of the M2 marker being in naïve macrophages on donor 3 and the lowest expression of the same marker being too in naïve macrophages on donor 4. Also, the relative percentages of each marker vary greatly from donor to donor. These variations are, however, expected. Each donor is a completely different individual with distinct age, sex and life and so each set of cells are inherently different from the others. Because of this, the compilation of all the donor's information would yield a very deviating set of results and its true significance would be lost. In order to overcome this, the information was compiled in a different fashion. First, the anti-inflammatory potential was defined as the ratio between the levels of expression of M2 and M1 markers. Higher ratios mean higher expression of CD163 in relation to CD86 hence a more anti-inflammatory, tumor favoring potential. Secondly, since each donor has different macrophages with different marker's expression levels, each condition's values were normalized against the simplest condition: naïve macrophages. By

doing so, observed differences are a result of the effect of fibroblasts on the anti-inflammatory profile of macrophages. This allows for a much more clear way of interpreting the information (fig. 12).

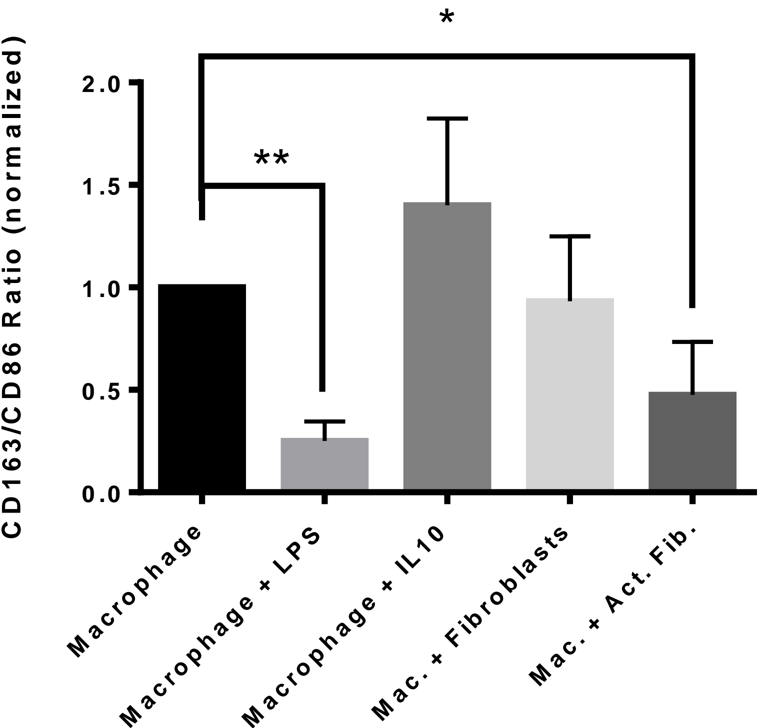

Figure 12 - Macrophage anti-inflammatory ratios: The ratio of CD163(M2)/CD86(M1) markers' expression are represented and normalized against the level for naïve macrophages of each donor. Statistics: ANOVA, *P<0,05; **P<0,01; n=3.

The anti-inflammatory potential of naïve macrophages (fig 12) is significantly reduced by the presence of LPS, a known modulator of the M1-like profile, and increased when IL-10 is added to the culture medium. Also, combining the data from all donors we can see that the presence of activated fibroblasts significantly reduces the anti-inflammatory potential of macrophages, giving rise to a CD163/CD86 ratio which, on average, halves that of naïve macrophages. These results are in agreement with the observed responses on macrophage morphology. First, activated fibroblasts' signals led to a decrease on macrophage elongation, with round cells more similar to those stimulated by LPS, and related with more inflammatory profiles. Now, activated fibroblasts also showed a significant decrease on the anti-inflammatory, pro-tumoral ratio of macrophages markers.

In spite of the changes observed in these markers, which are related to different polarizations, it is very important to mention that these surface proteins are not able to fully

define the M1 or M2 state since the signature of both these polarization states is more complex and relies not only on cell surface but also on the secretion of very important cytokines. So, in order to effectively conclude that macrophages are indeed in an M1- or M2-like state, the production of cytokines such as IL-10 (anti-inflammatory), IL-6 and TNF-α (pro-inflammatory) [110] must also be evaluated in the future. This will allow to solidify how M1 or M2 are fibroblast-modulated macrophage phenotypes. At this point we can only see that there is a trend of increasing expression of inflammatory markers in comparison to decreasing anti-inflammatory markers suggesting that the evolution is to the M1 side of the spectrum.

Furthermore, in order to ensure that these changes were indeed related to fibroblasts in different activation states, it was necessary to confirm and quantify fibroblast activation. As the co-cultures terminated and macrophages were retrieved, fibroblasts were also lysed and whole-cell protein extracted for quantification.

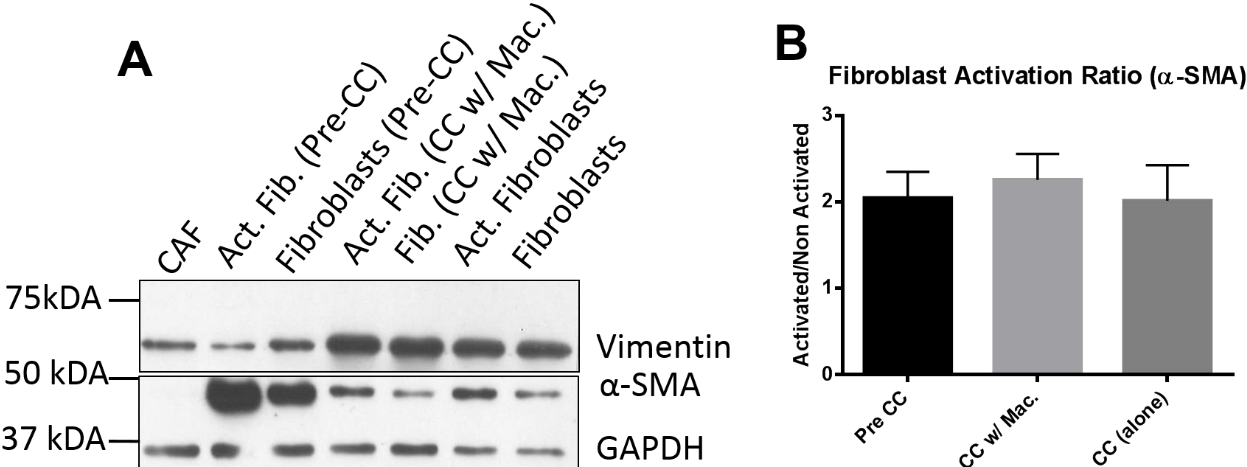

Figure 13 - Fibroblast Activation Results - (A) The activation of fibroblasts was measured through the expression of α-SMA. Several fibroblasts were analyzed: CAF – fibroblasts extracted from colorectal tissue, Act.Fib. (Pre-CC) – Activated fibroblasts prior to the co-cultures, Fibroblasts (Pre-CC)) – Fibroblasts prior to the co-cultures (not activated with TGF-β), Act. Fib. (CC w/ Mac.) – Activated fibroblasts extracted from the macrophage co-culture, Fib. (CC w/ Mac.) – Fibroblasts extracted from the macrophage co-culture, Act. Fibroblasts – Activated fibroblasts seeded alone at the same time of co-culture, Fibroblasts – Fibroblasts seeded alone at the same time of co-culture. (B) α-SMA ratios between activated and non-activated fibroblasts after quantification from before the co-culture (Pre CC), after the co-culture with macrophages (CC w/ Mac.) and after co-culture times but seeded alone (CC (alone)).

Through western blotting, it was possible to simultaneously characterize and evaluate the degree of activation of fibroblasts by looking at the expression of certain proteins. First of all, α-SMA as a marker of fibroblast activation was looked at (fig. 13A). Fibroblasts that were activated with the inclusion of TGF-β in growth media for 4 days showed increased levels of α-SMA expression in comparison to those of non-activated fibroblasts. This difference was equivalent to activated fibroblasts expressing double the amount of α-SMA than non-activated ones. However, even without the addition of TGF-β, fibroblasts still expressed a quite significant amount of α-SMA. This is a relevant limitation since this procedure with the CCD-18co cell line

does not yield activated and inactivated fibroblasts but rather more and less activated fibroblasts. Part of this problem is compensated through the inclusion of fibroblasts from the primary cultures, which revealed low levels of activation, with α-SMA western bands being only visible after exposures of 10 minutes or longer. These fibroblasts, which show almost no activation, serve then as a non-activated control. As referred, these cells were named CAFs in the context of this work but they are probably fibroblasts that still are not cancer-associated ones in the true meaning of the word.

Another problem that had to be resolved through western blot analysis is the maintenance of activation. As described on the methods sections, fibroblasts were activated for 4 days prior to the co-cultures. Once these cells are washed and transported to transwell inserts with macrophages beneath, one problem arises: how to maintain the activation. The most obvious choice would be to include TGF-β in the co-culture medium. However, this molecule is not responsible for activating fibroblasts alone and is a powerful modulator of macrophage phenotypes, as already explored. So, in order to include TGF-β, several controls would need to be added since the validation of results and the logical induction of the effect of fibroblasts alone and not medium TGF-β would complicate tremendously. As an attempt to remove as many interference as possible and more directly approach the objective, activated and non-activated fibroblasts were co-cultured without the inclusion of any additional cytokine, to see if the levels of activation would somewhat be maintained.

In fact, fibroblasts that were analyzed after the 72h of co-cultures showed a decrease in the absolute levels of α-SMA expression (fig. 13A). However, the difference between activated and non-activated fibroblasts regarding α-SMA, i.e., the ratio of activated cells α-SMA in comparison to non-activated cells kept stable and similar before and after co-culture, with activated cells showing always around twice the levels of α-SMA (fig. 13B). This result was very positive since the fact that α-SMA ratios kept similar allowed observing the effects of the activation level since despite the fact that the absolute activation could be lower and results not so powerful, observed differences between more and less activated cells would be explained by the activation to some extent. This is an essential result to validate the effect of fibroblast activation on macrophages. Conversely, macrophages do not appear to significantly affect fibroblast α-SMA expression - even though the levels of expression can appear to be slightly lower in macrophage co-cultures, this difference is far from being significant. This means that fibroblasts are most likely activated through pathways that are independent from macrophage signaling while fibroblast-derived communication might play a role on macrophage polarization.

Another marker that was screened was vimentin (fig. 13A). Vimentin is expressed in fibroblasts from different origins and is not a differentiator marker of activation but rather a mesenchymal marker of more or less activated fibroblasts, being useful as a protein that characterizes this cell type, whose expression can moderately change with the progression towards a myofibroblast phenotype, mainly through mechanical cues [123]. Curiously, the

expression of vimentin increases once fibroblasts are passed from the culture flask to the transwell inserts. This effect appears to be the same in the presence and absence of macrophages so it should not be a result of both cells' interaction. The most promising hypothesis that explains this similar increase of vimentin expression is the fact that fibroblasts are moved onto a different surface, and are no longer cultured on a TCP flat surface but a PET grid with pores. As a matter of fact, fibroblast's expression of vimentin has been shown to vary according to the topography of the surface they are cultured on [124]. Even though the referred work deals with topographies on the nanometer scale, and transwell inserts have 1μm pores, which are in a greatly higher order of magnitude, the increased vimentin expression is most likely explained by cytoskeletal rearrangements that take place once cells are cultured on the pored inserts.

The fact that the CCD-18co cell line is high in α-SMA expression regardless of activation might indicate that these fibroblasts are closer to the proto- or myofibroblast classification and further away from the non-activated fibroblast. So, this marker was used to test for different levels of activation since it is one of the main myofibroblast markers and fits this cell line very well. Nonetheless, the classification through other markers as well as the use of less activated cell types is still something extremely relevant to look at.

Notwithstanding this, since the goal of this work is to approach cancer-associated, perpetually activated, myofibroblast-like cells, the use of this cell line and activation screening through the expression of α-SMA appears as extremely proper indicating that the effect on macrophages approaches that of deeply subverted CAFs.

At this point we see that activated fibroblasts are able to modulate macrophage polarization and shift it towards a less anti-inflammatory profile, and, at the same time, macrophages do not seem capable of interfering with the degree of fibroblast activation. It remains unclear the extent to which these modifications and the interaction between these two cell types is translated in different functional outcomes, especially in changes of cancer cell behavior. Therefore, in order to further explore the mutual regulation of macrophages and fibroblasts, the consequences of such process in cancer cell behavior were explored.

3.3 Outcomes in Cancer Cell Invasion

So far we know that activated fibroblasts are able to significantly reduce the anti-inflammatory M2-like profile of macrophages, identified through higher CD163 levels. Since this profile is the one that most resembles TAMs [125], [126], it is reasonable to hypothesize that by shifting macrophages towards a less anti-inflammatory phenotype, fibroblasts would have an impact in macrophage-mediated cancer cell invasion. This, however, requires functional on top of phenotypical confirmation.

So, in order to confirm if the previous results could be translated into functional changes on macrophage behavior, the interaction with cancer cells and responses in terms of invasion were assessed.

At the end of 72h of co-cultures, one coverslip with macrophages from each condition was transferred to a new well, as well as fibroblasts retrieved from different conditions, on top of which cancer cells were seeded on the matrigel inserts. With this setup, cancer cells must be able to degrade the matrigel coating, which mimics *in vivo* ECM, in order to reach the bottom side of the insert. The RKO cell line was chosen to evaluate macrophage-mediated invasion since these cells were previously shown to properly respond to macrophage stimuli [113]. In the case of fibroblasts, the cell line that was used was HCT116 since this one is responsive to fibroblast signals (S. Velho unpublished results). This test served the purpose of evaluating not only the fibroblast invasive potential but also the effect macrophages could have on fibroblast-mediated invasion.

Macrophages were already known to promote RKO cell invasion in their naïve, M0 state [113]. In fact, when in the presence of macrophages that did not go through co-culture with any other cell type, the invasive ratio of RKO cells increased on average to double the value of these cancer cells alone (fig. R14A). However, when macrophages were co-cultured with fibroblasts, the invasive cell ratio lowered and when activated fibroblasts communicated with macrophages it significantly decreased to half the ratio obtained with naïve macrophages, similarly approaching the numbers of invasion of unstimulated RKO. This reduction in the capacity to stimulate cancer-cell invasion reinforces the data obtained on macrophage phenotype changes – the inflammatory, M1-like shift caused by activated fibroblasts greatly diminished the pro-invasive behavior of macrophages.

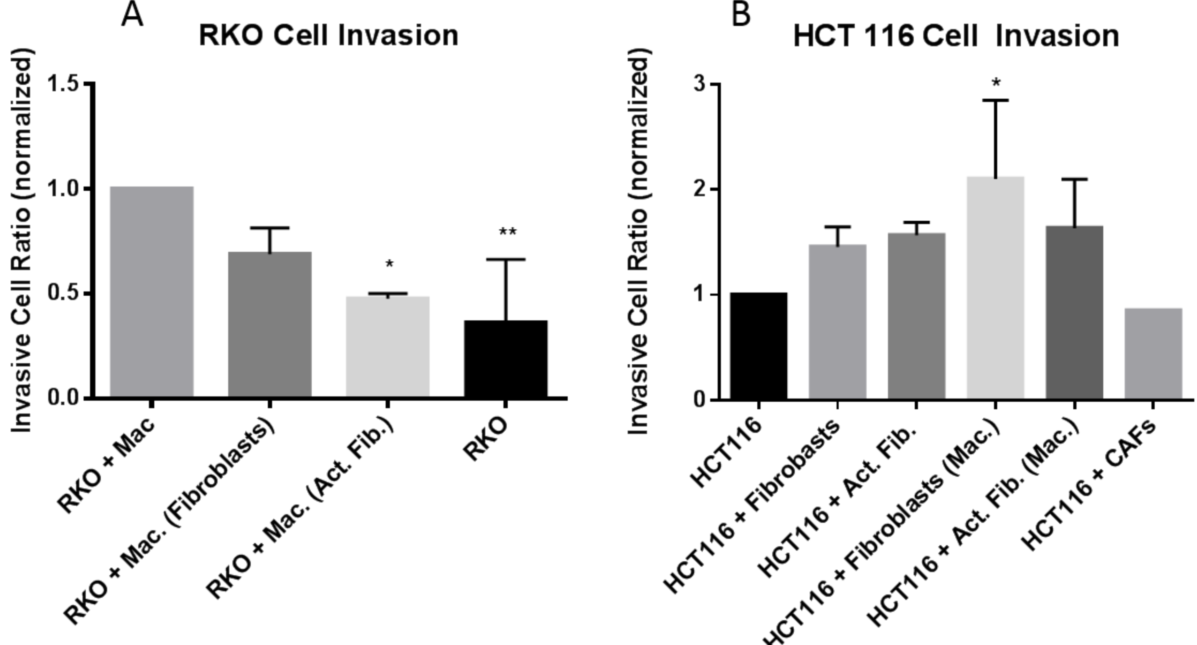

Figure 14 - Cancer cell invasion results - RKO invasion (A) in response to naïve macrophages (RKO + Mac), macrophages previously cultured with fibroblasts (Mac. (Fibroblasts)), macrophages previously cultured with activated fibroblasts (Mac. + (Act. Fib.) and RKO invasion levels alone, normalized against naïve macrophage-derived invasion. HCT116 invasion (B) in response to fibroblasts (HCT116+Fibroblasts), activated fibroblasts (Act. Fib.), fibroblasts previously cultured with macrophages (Fibroblasts (Mac.)), activated fibroblasts previously cultured with macrophages (Act. Fib. (Mac.) and the colorectal biopsy fibroblasts (CAFs). Statistics: ANOVA, *P<0,05; **P<0,01; n=3.

At the same time, fibroblasts increase the invasive potential of cancer cells. The HCT116 cell line is a less invasive cell line, requiring stimuli to reach higher invasive numbers. By comparing the invasive ratios of these cells in the absence or presence of fibroblasts, we can see that both more and less activated fibroblasts appear to bolster HCT116 invasion (fig. 14B). More curiously, the interaction with macrophages appears to increase the pro-invasive potential of fibroblasts, with non-activated fibroblasts leading to a significant increase of HCT116 invasion when previously co-cultured.

Regarding the effect of activation, the results are not so clear. On one hand, fibroblasts and activated fibroblasts appear to lead to similar invasive ratios and the test with the CAF population, which were the least activated showed no effect on HCT116 invasion, with an invasive ratio almost equal to HCT116 alone. This points at increased activation leading to increased invasion. On the other hand, when co-cultured with macrophages, normal fibroblasts lead to an average invasive ratio more than 2 fold that of HCT116 alone while activated fibroblasts do so to a slightly lower extent.

These results show us an interesting and mutual macrophage-fibroblast modulation, with fibroblasts playing a curious dual role: on one side, these cells inhibit the pro-invasive response of anti-inflammatory macrophages, reducing cancer cell invasion to half. On the other side, fibroblasts on their own promote the invasion of cancer cells, especially when stimulated by

macrophages. This result clearly shows how complex the tumor microenvironment and the signals and regulatory pathways between all cell types can be. It also tells us that it is extremely important to understand not only the effects of each cell type on the behavior of cancer cells but also how this behavior is influenced by the interaction between the referred cell types. The integration of all this knowledge is essential in the development of future microenvironment-disrupting therapies.

Notwithstanding this, knowing the outcomes of the mutual interaction between these two cell types in terms of phenotype and function is still not enough to pave way for efficient therapeutic targets. It is necessary to know the mechanisms through which these cells significantly impact cancer cell behavior, i.e., the molecular cues that promote tumor-favorable outcomes and are behind the modulation of the microenvironment, which might be blocked to effectively disrupt the pro-tumoral progression. This necessity is addressed on the following section.

3.4 Molecular Signals at the Macrophage-Fibroblast level

The transforming growth factor-beta – TGF-β is a long-known cytokine [127] that has been extensively studied in the most varied of contexts, biological tissues and cellular events including, evidently, cancer [128]. Moreover, TGF-β is a very powerful modulator of fibroblasts and macrophages. In the case of fibroblasts, it is the cytokine used for activation and so it self-proves its importance in this case since, as shown at the beginning of this work, it clearly modulates fibroblast activation. On the other hand, TGF-β is also able to modulate the polarization of macrophages. Being a naturally anti-inflammatory cytokine, it plays an important role in the alternative, anti-inflammatory polarization of macrophages [129]. Additionally, both fibroblasts [130] and macrophages [131] are able to produce and secrete TGF-β, making it very important to consider what can be happening downstream or upstream from the TGF-β signals in this case.

When in culture, fibroblasts and macrophages secrete factors to the culture medium, which then becomes conditioned culture media. This conditioned media can be retrieved and analyzed for the presence of different soluble molecules that concentrate with time, through ELISA. Knowing this, at the end of the 72h of macrophage and fibroblast co-cultures, the media were retrieved for analysis of TGF-β levels.

When macrophages, fibroblasts and activated fibroblasts are cultured alone, the concentration of TGF-β is very similar, slightly above 1.5 ng/ml (Fig. 15A). Most likely, a relevant portion of this is TGF-β from the FBS present in the culture medium, which contains significant amounts of this cytokine. Be that as it may, once fibroblasts and macrophages are co-cultured, a curious decrease in the concentration of TGF-β can be observed. This slight decrease is quite unexpected since when these cells are alone they lead to similar levels of the

cytokine and, theoretically, when co-cultured they should both contribute with TGF-β secretion leading to higher, not lower values. This would hold true, of course, if this growth factor was produced in the same rates in both situations. However, since co-cultures revealed lower concentrations, we suspected that some inhibition or degradation could be the reason behind this decrease in TGF-β levels. However, the use of co-cultures' conditioned media does not allow deducing how the secretion of any molecule is dependent on a specific cell type of that same co-culture. To overcome this caveat, TGF-β mRNA expression levels were quantified using macrophage RNA extracted from blood donor 1.

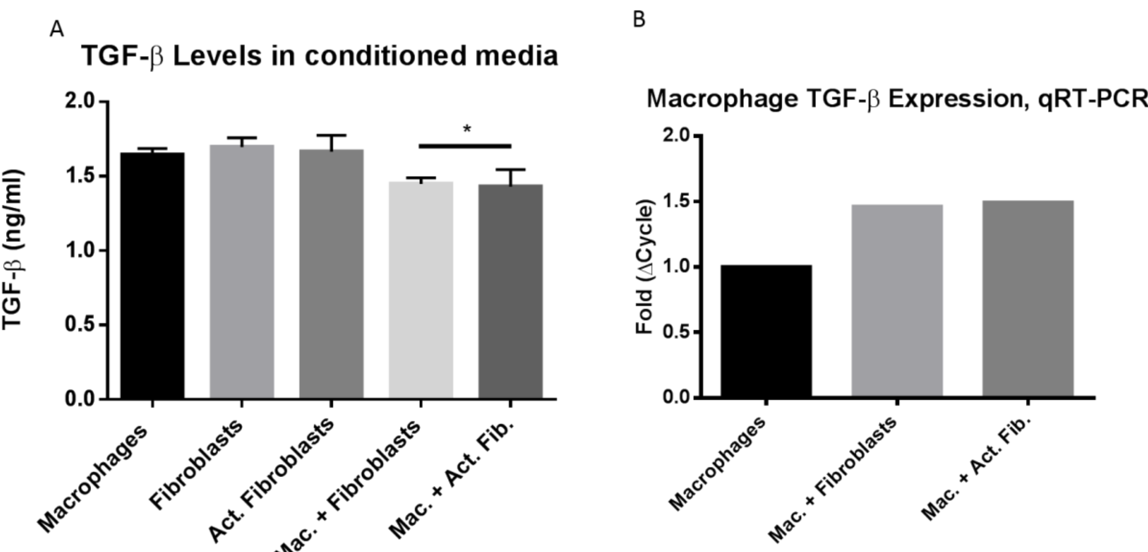

Figure 15 - TGF-β Analysis - TGF-β concentration (ng/ml) as measured through ELISA (A) on the cultures and co-cultures of Macrophages, Fibroblasts, Activated (Act.) Fibroblasts, Macrophages with Fibroblasts (Mac. + Fibroblasts) and Macrophages with activated fibroblasts (Mac. + Act. Fib.). qRT-PCR levels of mRNA in macrophages, macrophages co-cultured with fibroblasts (Mac. + Fibroblasts) and activated fibroblasts (Mac. + Act. Fib.) The values are represented as fold expression. Statistics: ANOVA, $^*P<0.05$. N=4 for ELISA, N=1 for qRT-PCR.

In fact, the expression of TGF-β at the mRNA level seems to increase to 1.5 fold when macrophages are co-cultured with either fibroblasts or activated fibroblasts (fig. 15B). Even though this result is only obtained from one donor and would require additional replicates in order to solidify this claim, it appears that macrophage-wise the TGF-β expression increases when fibroblasts are in the vicinity. However, mRNA levels might not translate into fully secreted proteins, and this increase in mRNA does not necessarily imply that macrophages are releasing more TGF-β to the culture medium. Nevertheless, this increase on mRNA levels of the cytokine is very indicative of the fact that, at least, the production of TGF-β at the macrophage level does not diminish by fibroblast interaction.

Considering both these approaches, once TGF-β concentration is lower in co-cultures despite its mRNA synthesis being higher on macrophages in the same condition, we can consider that TGF-β is subjected to possible inhibition/degradation effects, plausibly at the

fibroblast level. However, it can also be due to increased consumption of this cytokine since more cells are present.

So, as far as TGF-β goes, it seems that there might be some decrease at the fibroblast level or simple increase in consumption, but overall it does not seem to be the main cytokine behind the modulation of macrophage phenotype because although it slightly decreases in co-cultures, no difference between activated and normal fibroblast co-culture can be seen whereas in macrophage phenotype and invasion induction there was a much more clear difference between these two cases. Further work should address other types of modulator cytokines that can be at play in the change of macrophage phenotype, e.g. IL-10, which is used as a polarization control due to its high capacity to induce M2-like changes [132].

Since the invasion of cancer cells was not only affected by changes in macrophages but also in fibroblasts, it too makes sense to explore which pathway might be behind the increased fibroblast-induced cancer cell invasion when these are previously in communication with macrophages. This fibroblast line was previously shown to predominantly produce hepathocyte growth factor (HGF). Additionally, several works have shown that HGF is intimately related to fibroblasts and the induction of cancer cell invasion in several contexts [133]–[135]. This makes HGF a candidate as the fibroblast-secreted cytokine promoting cell invasion, which could be upregulated upon interaction with macrophages. To verify this possibility, an ELISA was performed to quantify the levels of HGF in the conditioned media from co-cultures.

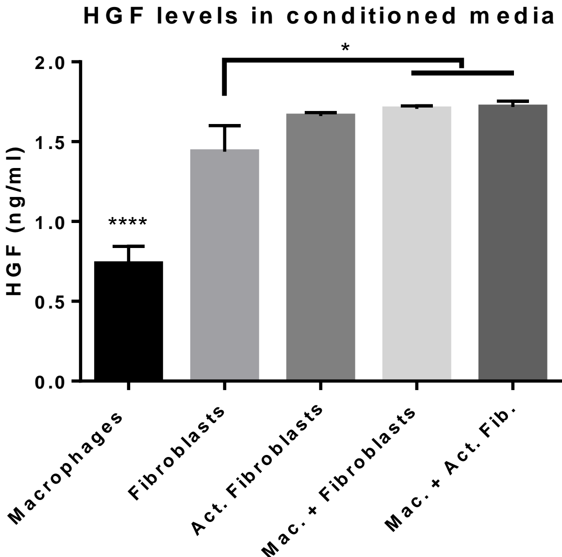

Figure 16 - HGF Analysis HGF Analysis – The concentration of HGF in conditioned media from macrophages, fibroblasts, activated (act.) fibroblasts cultures, macrophages with fibroblasts (Mac. + Fibroblasts) and macrophages with activated fibroblasts (Mac. + Act. Fib.) co-cultures. Evaluated through ELISA, HGF levels are represented in ng/ml. Statistical analysis: ANOVA, * P<0,05; **** P<0,001; n=3.

As initially expected, the production of HGF is very much increased in scenarios where fibroblasts are present (fig. 16), since these cells are the most likely to produce it. However, despite the fact that the level of HGF significantly increases when we compare normal fibroblasts with the ones co-cultured with macrophages, this does not hold true if we look at activated fibroblasts, since their HGF production appears to be similar with or without macrophages. Furthermore, the increase that is seen in non-activated fibroblasts with macrophages is not superior to that of activated fibroblasts single cultures, whereas in the invasion values there is a much more clear difference between these two scenarios (fig. 14B) This evidence shows that HGF is probably not the mechanism through which fibroblasts promote higher levels of invasion when co-cultured with macrophages. The answer to this might rely on other fibroblast-derived molecules that might be promoting cancer cell invasion, namely one of several MMPs these cells produce[136]. Moreover, the fibroblast-macrophage interaction has already been implied on the MMP production, even if the other way around [137].

Through another point of view, one can also argue that the increase in HGF can have a contribution of macrophages since these show some HGF level and the fact that it increases in co-culture can be due to any of the two cell types. Notwithstanding this, there are two main reasons on why this should is not very important: first, Sakaguchi *et al.* showed that the expression of HGF is most likely related to the anti-inflammatory, M2-like phenotype of these cells [138]. Since we can see that co-cultures with fibroblasts are, in principle, leading to increasingly inflammatory macrophages the odds of the HGF increase being responsibility of the macrophages are relatively little. Second, these macrophages are not present in fibroblast invasion tests and so the HGF they produce is of little relevance when it comes to the final outcome.

Chapter 4

Discussion Summary and Concluding Remarks

During this work we have isolated and established primary cultures of cancer-associated fibroblasts, which allowed to see that these cells behave very differently between different patients. However, most biopsy samples failed to lead to primary cultures due to cell degeneration or contamination rising along the way. The primary culture of fibroblasts that fully worked revealed cells with relatively low activation levels. These cells served as an important contrast in comparison to the CCD-18co cell line, which revealed some baseline activation regardless of the activation with TGF-β. Hence, we were able to test the action of more and less activated fibroblasts from this cell line and also these CAFs with low α-SMA expression. All things considered, this primary culture population revealed itself very useful but future work should focus on improving the isolation and establishment of primary CAF cultures, in its true meaning.

Through the co-culture of macrophages and fibroblasts, it was possible to screen the effects of paracrine communication between these cells. By analyzing the levels of α-SMA expression, it was possible to see that the activation ratio of fibroblasts endured the 72h of co-cultures, validating the possibility of performing such a test without the inclusion of TGF-β in the medium, and all the additional controls it would require. By looking at macrophages, it was shown that when fibroblasts, especially activated, were present, there was a change in morphology with apparent reduction of macrophage elongation. Since macrophage elongation has been linked with the anti-inflammatory phenotype of these cells, the change in morphology hinted at a shift on macrophage polarization. By using flow cytometry to look at two important markers of macrophage polarization: CD163 as an anti-inflammatory, M2-like marker and CD86 as the opposite inflammatory, M1-like marker, we could see that when co-cultured with activated, CAF-like fibroblasts, the expression of CD163 decreased in comparison to increasing CD86,

evidencing a pro-inflammatory shift to the M1-like side of the spectrum, which should be further confirmed through analysis of cytokine's profiles.

Since fibroblasts were clearly influencing the profile of macrophages, moving them from the TAM-like anti-inflammatory phenotype to a more inflammatory, anti-tumoral profile, it was necessary to assess if this change would effectively impact the behavior of cancer cells. By using a matrigel system and cancer cell lines, it was possible to see that activated fibroblasts, which shifted macrophages towards a more inflammatory phenotype, led to significantly diminished macrophage-induced cancer cell invasion, as compared with naïve, non-stimulated macrophages, with only half the invasion numbers once activated fibroblasts influenced these cells. This result proves to be somewhat unexpected since these CAF-like fibroblasts should in principle promote a TAM-like phenotype, and the opposite can be observed here. Simultaneously, fibroblasts on their own promoted the invasion of cancer cells, substantially more when previously co-cultured with macrophages. This effect revealed a dual action of fibroblasts – anti-invasion modulation of macrophages while pro-invasive on their own. This dual behavior is a perfect example of how complex the microenvironment of tumors and the signaling involved can be. It is extremely necessary to understand not only how each constituent plays a role in the behavior of cancer cells but also to integrate all the microenvironment components and understand the crosstalks between them. Failing to do so and prematurely developing microenvironment therapies might lead to unpredicted negative outcomes. A good example of this is anti-angiogenic therapy. Angiogenesis is a very important hallmark of tumor development, as previously discussed, and without proper blood supply tumors are not able to grow. This knowledge led to the development of the now popular anti-angiogenic therapies, which aim at preventing tumor growth by blocking angiogenesis through, e.g., inhibiting pro-angiogenic factors [40] or by directly targeting new endothelial cells [139], which makes sense since these cells are not so able to develop resistance, unlike tumors. However, what happens at the microenvironment level is extremely more complex. Once blood supply is diminished, oxygen drops to lower levels. While conceptually positive, since cancer cells can't survive without proper oxygen supply, what happens is that these cells respond and adapt by invading the adjacent tissue, in order to reach more oxygenated areas [140]. In other words, a therapy that was designed to prevent tumor growth comes with the secondary side effect of accelerating cancer cell invasion and, most dangerously, metastasis.

To further explore the mechanisms through which fibroblasts and macrophages interact as well as how the responses of cancer cells might be modulated, it is necessary to study molecular pathways through which these cells might communicate. The concentration of TGF-β in conditioned media was very similar between all the single cultures of macrophages and fibroblasts. However, when co-cultured, TGF-β levels were slightly decreased, which suggested that inhibition/degradation, or even increased consumption was taking place. Through qRT-PCR it was possible to see that macrophages had higher levels of TGF-β mRNA when co-

cultured with fibroblasts, with a 1.5 fold increase. Even though mRNA does not necessarily translate into secreted protein, it appears the inhibitory effect on TGF-β happens plausibly at the level of the fibroblast or simply overall consumption. Either way, TGF-β levels between co-cultures with activated and non-activated fibroblasts showed no difference whereas the macrophage phenotypes were visibly different between these two conditions, which indicates that this cytokine is not the main modulator behind the observed macrophage polarization shifts.

Regarding the action of fibroblasts, we can see that the levels of HGF in the conditioned media of fibroblasts cultures and co-cultures overcomes that of macrophages, and a slight increase of HGF concentration can be seen when non-activated fibroblasts are in the presence of macrophages compared to when alone. However, this small increase leads to HGF levels similar to those of activated fibroblast conditions, which had lower cancer cell invasion, so HGF doesn't seem to be the macrophage-regulated signal of fibroblast-induced cancer cell invasion.

Therefore, we can see the macrophage-fibroblast interaction as pictured in fig. 17: Fibroblasts modulate macrophages towards an increase of inflammatory marker's ratio, significantly leading to a decrease of macrophage-induced cancer cell invasion. At the same time, the pro-invasive action of fibroblasts is reinforced through macrophage signals. Further analysis is needed in order to understand which pathways are regulating the behavior of cancer cells and, most importantly, which signaling pathway(s) allow(s) these cells to modulate each other's behavior.

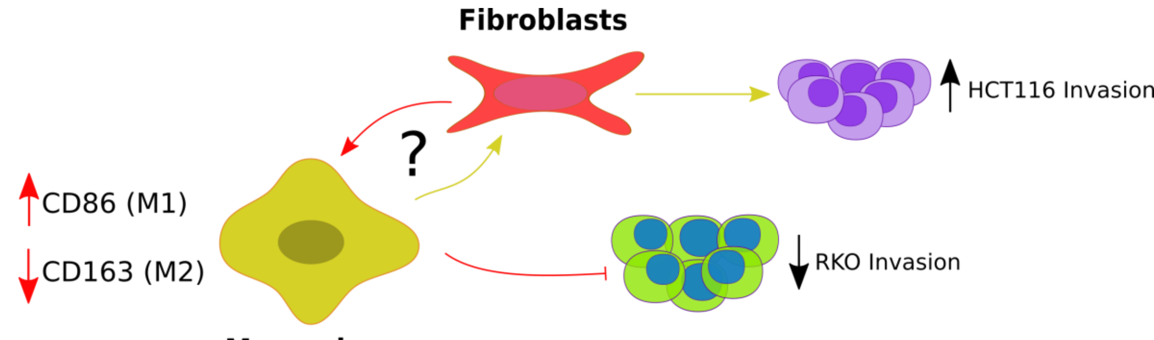

Figure 17 - Fibroblast-Macrophage Mutual Regulation - Fibroblasts, especially when activated, are able to increase CD86 and decrease CD163 expression on macrophages leading to lower cancer cell invasion. At the same time, macrophages can stimulate fibroblast-derived cancer cell invasion. Further assessment is needed to understand the interaction between both cell types (?) as well as the signals that direct the invasion of cancer cells.

Finally, it is very important to state that, in the scope of this work, we are only looking at the interaction and outcomes of two isolated components of the tumor microenvironment. We can already see that this simple view is already quite complex, with this knowledge requiring further complementation. However, we must consider that, in the context of cancer microenvironment, macrophages and fibroblasts are never alone and in exclusive communication. For one, it is

necessary to consider the lack of cancer cell signals, since these are thought to be the origin of all cellular subversion that takes place in the microenvironment. Also, very importantly, tumors are far from being composed of cells alone and as previously explained there is another layer of extreme complexity – the ECM. All the cells of the tumor mass are embedded in this matrix and it is not only complex and dynamic but essential for cells to behave in a certain way. Aguilera *et al.* showed, in a recent work, that the signals from ECM molecules are capable of leading to entirely different cancer outcomes [141]. Here, this group used a model of pancreatic ductal adenocarcinoma to reveal that VEGF inhibition induced hypoxia leading to a mesenchymal transition of cancer cells, higher levels of TGF-β expression, collagen deposition and an overall increased tumorigenesis. They also showed that collagen signaling is a critical factor for the pro-tumoral action of TGF-β (a very common cytokine whose tumorigenic effect here is relying on ECM signals). Furthermore when collagen signaling was increased in mice lacking SPARC, a protein that reduces collagen binding to cell surface, tumor progression was accentuated, revealing how important and significant signals from the ECM are and the need to consider them in microenvironment research. In this work, the absence of ECM and cancer cell signals, might be the reason why CAF-like fibroblasts do not correlate with TAM-like macrophages. This is not negative, at all - a step like this is essential to isolate the component responsible for the CAF and TAM phenotypes – by looking at two components and increasingly adding others, it is possible to know where the subversion derives from, even if it only exists once the whole system is integrated.

Chapter 5

Future Directions

In the future, the CAF isolation, being the process of this work with the most limitations, must be addressed. To try and obtain primary cultures with a better yield, larger tissue samples might be more prone to release higher CAF numbers, and these might be acquired with a partnership with colorectal surgery. If this does not work, the protocol might need to be refined or changed to another one that promotes CAF separation through more advanced procedures such as the use of beads that might allow positive selection of these cells, through specific markers such as FAP or FSP1. It will also be very important to characterize CAFs from different patients and different stages of colorectal cancer by looking at differences in activation (looking at markers other than α-SMA), proliferation and cancer cell invasion, among other responses. This will allow to pinpoint when fibroblast activation starts taking place and how it evolves in time, knowing the consequences of such event.

Regarding the fibroblast-macrophage effects, the interaction must be further studied in order to identify which pathways regulate both cell types and which signals might be important to downstream effects on cancer cell behavior. The characterization of macrophage polarization should also be complemented by assessing the cytokine profile of these cells, in order to be able to strongly conclude about their M1-like or M2-like polarization. The analysis of cytokines such as IL-10, IL-6 and TNF-α will be essential. Still in the macrophage-fibroblast studies, these can be extended to see macrophage effects on fibroblasts by polarizing macrophages towards M1 and M2 profiles and then seeing the consequences of these profiles on fibroblast behavior. Furthermore, in the co-cultures and invasion assays, an analysis of the production of MMPs will also be very important since these are molecules that significantly impact the metastatic advance of cancer cells, and are extremely associated with macrophages and even more with fibroblasts. Differences in MMP production and secretion might be behind some of the observed results. Furthermore, other important cytokines that might be behind macrophage/fibroblast

modulation and downstream cancer cell invasion must also be pursued, some of which are also important for the macrophage characterization, such as IL-10.

Macrophage-wise, the effects of fibroblasts on macrophage differentiation and not only polarization might also be looked at by including fibroblasts on early points after monocyte isolation. Obviously, this will require the inclusion of TGF-β in culture to sustain fibroblast activation for several days, which we opted not to pursue in this work in order to have a more clean and direct approach, minimizing the interference that derives from the inclusion of such a modulator cytokine.

Finally, the inclusion of other components from the microenvironment will be extremely important. Including cancer cells or cancer cell signals through the use of conditioned media will be one way of complementing this work. Additionally, ECM signals will also be extremely important in this context. This can be obtained by introducing ECM molecules such as collagen, performing similar experiments with fibroblasts or macrophages embedded in, e.g., a 2D, or even more realistic 3D collagen matrix. By following these steps, a more complex characterization and knowledge on CAF-TAM mutual regulation can be achieved with results that will increasingly approach the real *in vivo* cancer microenvironment situation. This will hold true especially with the integration of all the main microenvironment components and observation of changes in cell phenotypes and ultimately of the metastatic behavior of cancer cells. Having this knowledge and pinpointing the related molecular pathways will shine a light on how to tackle and prevent the microenvironment subversion as the cancer cell niche, envisioning new ways to deal with and treat cancer and, especially, metastasis.

References

[1] R. L. Siegel, K. D. Miller, and A. Jemal, “Cancer statistics, 2015.,” *CA. Cancer J. Clin.*, vol. 65, no. 1, pp. 5–29, Jan. .

[2] J. Pellettieri and A. Sánchez Alvarado, “Cell turnover and adult tissue homeostasis: from humans to planarians.,” *Annu. Rev. Genet.*, vol. 41, pp. 83–105, 2007.

[3] Leblond, “Classification of cell populations on the basis of their proliferative behavior,” *Natl. Cancer Inst.*, vol. 14, pp. 119–150, 1964.

[4] B. Creamer, R. G. Shorter, and J. Bamforth, “The turnover and shedding of epithelial cells,” *Gut*, vol. 2, no. 2, pp. 110–116, 1961.

[5] N. Barker, “Adult intestinal stem cells: critical drivers of epithelial homeostasis and regeneration.,” *Nat. Rev. Mol. Cell Biol.*, vol. 15, no. 1, pp. 19–33, 2014.

[6] H. Glimm, I. H. Oh, and C. J. Eaves, “Human hematopoietic stem cells stimulated to proliferate in vitro lose engraftment potential during their S/G(2)/M transit and do not reenter G(0).,” *Blood*, vol. 96, no. 13, pp. 4185–4193, 2000.

[7] L. J. Martin, “DNA damage and repair: relevance to mechanisms of neurodegeneration.,” *J. Neuropathol. Exp. Neurol.*, vol. 67, no. 5, pp. 377–387, 2008.

[8] C. J. Norbury and B. Zhivotovsky, “DNA damage-induced apoptosis,” *Oncogene*, vol. 23, no. 16, pp. 2797–2808, 2004.

[9] P. Valent, D. Bonnet, S. Wöhrer, M. Andreeff, M. Copland, C. Chomienne, and C. Eaves, “Heterogeneity of neoplastic stem cells: Theoretical, functional, and clinical implications,” *Cancer Research*, vol. 73, no. 3. pp. 1037–1045, 2013.

[10] F. Colotta, P. Allavena, A. Sica, C. Garlanda, and A. Mantovani, “Cancer-related inflammation, the seventh hallmark of cancer: Links to genetic instability,” *Carcinogenesis*, vol. 30, no. 7. pp. 1073–1081, 2009.

[11] D. Hanahan and R. A. Weinberg, “The hallmarks of cancer.,” *Cell*, vol. 100, no. 1, pp. 57–70, 2000.

[12] D. Hanahan and R. A. Weinberg, “Hallmarks of cancer: The next generation,” *Cell*, vol. 144, no. 5. pp. 646–674, 2011.

[13] B. Mlecnik, G. Bindea, A. Kirilovsky, H. K. Angell, A. C. Obenauf, M. Tosolini, S. E. Church, P. Maby, A. Vasaturo, M. Angelova, T. Fredriksen, S. Mauger, M. Waldner, A. Berger, M. R. Speicher, F. Pagès, V. Valge-Archer, and J. Galon, “The tumor microenvironment and Immunoscore are critical determinants of dissemination to distant metastasis.,” *Sci. Transl. Med.*, vol. 8, no. 327, p. 327ra26, 2016.

[14] D. X. Nguyen, P. D. Bos, and J. Massagué, “Metastasis: from dissemination to organ-specific colonization.,” *Nat. Rev. Cancer*, vol. 9, no. 4, pp. 274–84, 2009.

[15] A. F. Chambers, A. C. Groom, and I. C. MacDonald, “Metastasis: Dissemination and growth of cancer cells in metastatic sites,” *Nat Rev Cancer*, vol. 2, no. 8, pp. 563–572, Aug. 2002.

[16] Y. T. Margaret Lee, “Patterns of metastasis and natural courses of breast carcinoma,” *Cancer Metastasis Rev.*, vol. 4, no. 2, pp. 153–172, 1985.

[17] M. Popiolek, J. R. Rider, O. Andrén, S.-O. Andersson, L. Holmberg, H.-O. Adami, and

J.-E. Johansson, “Natural History of Early, Localized Prostate Cancer: A Final Report from Three Decades of Follow-up,” *Eur. Urol.*, vol. 63, pp. 428–435, 2012.
[18] R. Feld, L. V Rubinstein, and T. H. Weisenberger, “Sites of recurrence in resected stage I non-small-cell lung cancer: a guide for future studies,” *J Clin Oncol*, vol. 2\rhttp://, no. 12, pp. 1352–1358, 1984.
[19] J. Nieto, M. L. Grossbard, and P. Kozuch, “Metastatic pancreatic cancer 2008: is the glass less empty?,” *Oncologist*, vol. 13, no. 5, pp. 562–576, 2008.
[20] K. W. Kinzler and B. Vogelstein, “Lessons from hereditary colorectal cancer,” *Cell*, vol. 87, no. 2. pp. 159–170, 1996.
[21] B. Vogelstein, E. R. Fearon, S. R. Hamilton, S. E. Kern, A. C. Preisinger, M. Leppert, Y. Nakamura, R. White, A. M. M. Smits, and J. L. Bos, “Genetic Alterations during Colorectal-Tumor Development,” *N. Engl. J. Med.*, vol. 319, no. 9, pp. 525–532, 1988.
[22] Y. Samuels, Z. Wang, A. Bardelli, N. Silliman, J. Ptak, S. Szabo, H. Yan, A. Gazdar, S. Powel, G. Riggins, J. Willson, S. Markowitz, K. Kinzler, B. Vogelstein, and V. Velculescu, “Brevia: high frequency of mutations of the PIK3Ca gene in human cancers,” *Science (80-.).*, vol. 304, no. 5670, p. 554, 2004.
[23] S. J. Baker, E. R. Fearon, J. M. Nigro, S. R. Hamilton, A. C. Preisinger, J. M. Jessup, P. VanTuinen, D. H. Ledbetter, D. F. Barker, Y. Nakamura, R. White, and B. Vogelstein, “Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas.,” *Science*, vol. 244, no. 4901, pp. 217–221, 1989.
[24] S. Markowitz, J. Wang, L. Myeroff, R. Parsons, L. Sun, J. Lutterbaugh, R. S. Fan, E. Zborowska, K. W. Kinzler, and B. Vogelstein, “Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability.,” *Science*, vol. 268, no. 5215, pp. 1336–1338, 1995.
[25] K. R. Hess, G. R. Varadhachary, S. H. Taylor, W. Wei, M. N. Raber, R. Lenzi, and J. L. Abbruzzese, “Metastatic patterns in adenocarcinoma,” *Cancer*, vol. 106, no. 7, pp. 1624–1633, 2006.
[26] C. L. Chaffer and R. a Weinberg, “A Perspective on Cancer Cell Metastasis,” *Science (80-.).*, vol. 331, no. 6024, pp. 1559–1564, 2011.
[27] A. J. Minn, G. P. Gupta, P. M. Siegel, P. D. Bos, W. Shu, D. D. Giri, A. Viale, A. B. Olshen, W. L. Gerald, and J. Massagué, “Genes that mediate breast cancer metastasis to lung,” *Nature*, vol. 436, no. 7050, pp. 518–524, 2005.
[28] S. Gout and J. Huot, “Role of cancer microenvironment in metastasis: Focus on colon cancer,” *Cancer Microenvironment*, vol. 1, no. 1. pp. 69–83, 2008.
[29] D. P. Tabassum and K. Polyak, “Tumorigenesis: it takes a village,” *Nat. Rev. Cancer*, vol. 15, no. 8, pp. 473–483, 2015.
[30] P. Lu, V. M. Weaver, and Z. Werb, “The extracellular matrix: A dynamic niche in cancer progression,” *Journal of Cell Biology*, vol. 196, no. 4. pp. 395–406, 2012.
[31] F. T. Bosman and I. Stamenkovic, “Functional structure and composition of the extracellular matrix.,” *J. Pathol.*, vol. 200, no. 4, pp. 423–8, 2003.
[32] O. Chaudhuri, S. T. Koshy, C. Branco da Cunha, J.-W. Shin, C. S. Verbeke, K. H. Allison, and D. J. Mooney, “Extracellular matrix stiffness and composition jointly regulate the induction of malignant phenotypes in mammary epithelium,” *Nat. Mater.*, vol. 13, no. June, pp. 1–35, 2014.
[33] J. Alexander and E. Cukierman, “Stromal dynamic reciprocity in cancer: Intricacies of fibroblastic-ECM interactions,” *Current Opinion in Cell Biology*, vol. 42. pp. 80–93, 2016.
[34] R. G. Rowe and S. J. Weiss, “Breaching the basement membrane: who, when and how?,” *Trends in Cell Biology*, vol. 18, no. 11. pp. 560–574, 2008.
[35] K. Kessenbrock, V. Plaks, and Z. Werb, “Matrix Metalloproteinases: Regulators of the Tumor Microenvironment,” *Cell*, vol. 141, no. 1. pp. 52–67, 2010.
[36] K. Hotary, X. Y. Li, E. Allen, S. L. Stevens, and S. J. Weiss, “A cancer cell metalloprotease triad regulates the basement membrane transmigration program,” *Genes Dev.*, vol. 20, no. 19, pp. 2673–2686, 2006.
[37] R. S. Kerbel, “Tumor angiogenesis.,” *N. Engl. J. Med.*, vol. 358, no. 19, pp. 2039–49, 2008.
[38] H. J. Choi, M. S. Hyun, G. J. Jung, S. S. Kim, and S. H. Hong, “Tumor angiogenesis as a prognostic predictor in colorectal carcinoma with special reference to mode of metastasis and recurrence,” *Oncology*, vol. 55, no. 6, pp. 575–581, 1998.

[39] D. Liao and R. S. Johnson, “Hypoxia: A key regulator of angiogenesis in cancer,” *Cancer and Metastasis Reviews*, vol. 26, no. 2. pp. 281–290, 2007.

[40] Y. Kubota, “Tumor angiogenesis and anti-angiogenic therapy,” *Keio J.Med.*, vol. 61, pp. 47–56, 2012.

[41] H. F. Dvorak, “Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing.,” *N. Engl. J. Med.*, vol. 315, no. 26, pp. 1650–1659, 1986.

[42] V. G. Peddareddigari, D. Wang, and R. N. Dubois, “The tumor microenvironment in colorectal carcinogenesis.,” *Cancer Microenviron.*, vol. 3, no. 1, pp. 149–66, 2010.

[43] K. E. De Visser, A. Eichten, and L. M. Coussens, “Paradoxical roles of the immune system during cancer development,” *Nat. Rev. Cancer*, vol. 6, no. January, pp. 24–37, 2006.

[44] B. F. Zamarron and W. Chen, “Dual roles of immune cells and their factors in cancer development and progression,” *Int. J. Biol. Sci.*, vol. 7, no. 5, pp. 651–658, 2011.

[45] C. B. Croft and D. Tarin, “Ultrastructural studies of wound healing in mouse skin. I. Epithelial behaviour.(ANALISE DA PELE),” *J. Anat.*, vol. 106, no. Pt 1, pp. 63–77, 1970.

[46] P. Bainbridge, “Wound healing and the role of fibroblasts,” *J. Wound Care*, vol. 22, no. 8, pp. 407–412, 2013.

[47] J. J. Tomasek, G. Gabbiani, B. Hinz, C. Chaponnier, and R. a Brown, “Myofibroblasts and mechano-regulation of connective tissue remodelling.,” *Nat. Rev. Mol. Cell Biol.*, vol. 3, no. 5, pp. 349–63, 2002.

[48] A. El Ghalbzouri, M. F. Jonkman, R. Dijkman, and M. Ponec, “Basement membrane reconstruction in human skin equivalents is regulated by fibroblasts and/or exogenously activated keratinocytes,” *J. Invest. Dermatol.*, vol. 124, no. 1, pp. 79–86, 2005.

[49] M. Varkey, J. Ding, and E. E. Tredget, “Superficial dermal fibroblasts enhance basement membrane and epidermal barrier formation in tissue-engineered skin: implications for treatment of skin basement membrane disorders.,” *Tissue Eng. Part A*, vol. 20, no. 3–4, pp. 540–52, 2014.

[50] C. Bonnans, J. Chou, and Z. Werb, “Remodelling the extracellular matrix in development and disease,” *Nat. Rev. Mol. Cell Biol.*, vol. 15, no. 12, pp. 786–801, 2014.

[51] H. Steiling and S. Werner, “Fibroblast growth factors: Key players in epithelial morphogenesis, repair and cytoprotection,” *Current Opinion in Biotechnology*, vol. 14, no. 5. pp. 533–537, 2003.

[52] H. P. Ehrlich and T. K. Hunt, “Collagen Organization Critical Role in Wound Contraction.,” *Adv. wound care*, vol. 1, no. 1, pp. 3–9, 2012.

[53] J.-I. Jun and L. F. Lau, “The matricellular protein CCN1 induces fibroblast senescence and restricts fibrosis in cutaneous wound healing.,” *Nat. Cell Biol.*, vol. 12, no. 7, pp. 676–85, 2010.

[54] R. Kalluri and M. Zeisberg, “Fibroblasts in cancer,” *Nat. Rev. Cancer*, vol. 6, no. 5, pp. 392–401, 2006.

[55] T. Kelly, Y. Huang, A. E. Simms, and A. Mazur, “Fibroblast Activation Protein-??. A Key Modulator of the Microenvironment in Multiple Pathologies,” *Int. Rev. Cell Mol. Biol.*, vol. 297, pp. 83–116, 2012.

[56] B. Elenbaas and R. Weinberg, “Heterotypic signaling between epithelial tumor cells and fibroblasts in carcinoma formation.,” *Exp. Cell Res.*, vol. 264, no. 1, pp. 169–84, 2001.

[57] S. Busch, a Acar, Y. Magnusson, P. Gregersson, L. Rydén, and G. Landberg, “TGF-beta receptor type-2 expression in cancer-associated fibroblasts regulates breast cancer cell growth and survival and is a prognostic marker in pre-menopausal breast cancer.,” *Oncogene*, no. November 2012, pp. 1–12, 2013.

[58] Z. M. Shao, M. Nguyen, and S. H. Barsky, “Human breast carcinoma desmoplasia is PDGF initiated.,” *Oncogene*, vol. 19, no. 38, pp. 4337–4345, 2000.

[59] S. Knuchel, P. Anderle, P. Werfelli, E. Diamantis, and C. Rüegg, “Fibroblast surface-associated FGF-2 promotes contact-dependent colorectal cancer cell migration and invasion through FGFR-SRC signaling and integrin αvβ5-mediated adhesion.,” *Oncotarget*, vol. 6, no. 16, pp. 14300–17, 2015.

[60] C. Kuperwasser, T. Chavarria, M. Wu, G. Magrane, J. W. Gray, L. Carey, A. Richardson, and R. A. Weinberg, “Reconstruction of functionally normal and malignant human breast

tissues in mice.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 101, no. 14, pp. 4966–71, 2004.

[61] A. F. Olumi, G. D. Grossfeld, S. W. Hayward, P. R. Carroll, T. D. Tlsty, and G. R. Cunha, "Carcinoma-associated Fibroblasts Direct Tumor Progression of Initiated Human Prostatic Epithelium," pp. 5002–5011, 1999.

[62] A. Orimo, P. B. Gupta, D. C. Sgroi, F. Arenzana-Seisdedos, T. Delaunay, R. Naeem, V. J. Carey, A. L. Richardson, and R. A. Weinberg, "Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion," *Cell*, vol. 121, no. 3, pp. 335–348, 2005.

[63] R. Scherz-Shouval, S. Santagata, M. L. Mendillo, L. M. Sholl, I. Ben-Aharon, A. H. Beck, D. Dias-Santagata, M. Koeva, S. M. Stemmer, L. Whitesell, and S. Lindquist, "The reprogramming of tumor stroma by HSF1 is a potent enabler of malignancy," *Cell*, vol. 158, no. 3, pp. 564–578, 2014.

[64] F. Calvo, N. Ege, A. Grande-Garcia, S. Hooper, R. P. Jenkins, S. I. Chaudhry, K. Harrington, P. Williamson, E. Moeendarbary, G. Charras, and E. Sahai, "Mechanotransduction and YAP-dependent matrix remodelling is required for the generation and maintenance of cancer-associated fibroblasts," *Nat Cell Biol*, vol. 15, no. 6, pp. 637–646, 2013.

[65] S. Lamouille, J. Xu, and R. Derynck, "Molecular mechanisms of epithelial-mesenchymal transition.," *Nat. Rev. Mol. Cell Biol.*, vol. 15, no. 3, pp. 178–96, 2014.

[66] T. Brabletz, "EMT and MET in Metastasis: Where Are the Cancer Stem Cells?," *Cancer Cell*, vol. 22, no. 6. pp. 699–701, 2012.

[67] M. Zeisberg and J. S. Duffield, "Resolved: EMT produces fibroblasts in the kidney.," *J. Am. Soc. Nephrol.*, vol. 21, pp. 1247–1253, 2010.

[68] C. Feig, J. O. Jones, M. Kraman, R. J. B. Wells, A. Deonarine, D. S. Chan, C. M. Connell, E. W. Roberts, Q. Zhao, O. L. Caballero, S. a Teichmann, and T. Janowitz, "Targeting CXCL12 from FAP-expressing carcinoma- associated fibroblasts synergizes with anti – PD-L1 immunotherapy in pancreatic cancer," *Proc Natl Acad Sci U S A*, vol. 110, no. 50, pp. 20212–20217, 2013.

[69] M. Kraman, P. J. Bambrough, J. N. Arnold, E. W. Roberts, L. Magiera, J. O. Jones, A. Gopinathan, D. A. Tuveson, and D. T. Fearon, "Suppression of antitumor immunity by stromal cells expressing fibroblast activation protein-alpha.," *Science*, vol. 330, no. 6005, pp. 827–30, 2010.

[70] A. Lochter, S. Galosy, J. Muschler, N. Freedman, Z. Werb, and M. J. Bissell, "Matrix metalloproteinase stromelysin-1 triggers a cascade of molecular alterations that leads to stable epithelial-to-mesenchymal conversion and a premalignant phenotype in mammary epithelial cells," *J. Cell Biol.*, vol. 139, no. 7, pp. 1861–1872, 1997.

[71] A. Boire, L. Covic, A. Agarwal, S. Jacques, S. Sherifi, and A. Kuliopulos, "PAR1 is a matrix metalloprotease-1 receptor that promotes invasion and tumorigenesis of breast cancer cells," *Cell*, vol. 120, no. 3, pp. 303–313, 2005.

[72] Y. P. Bai, K. Shang, H. Chen, F. Ding, Z. Wang, C. Liang, Y. Xu, M. H. Sun, and Y. Y. Li, "FGF-1/-3/FGFR4 signaling in cancer-associated fibroblasts promotes tumor progression in colon cancer through Erk and MMP-7," *Cancer Sci.*, vol. 106, no. 10, pp. 1278–1287, 2015.

[73] Y. Hu, C. Yan, L. Mu, K. Huang, X. Li, D. Tao, Y. Wu, and J. Qin, "Fibroblast-derived exosomes contribute to chemoresistance through priming cancer stem cells in colorectal cancer," *PLoS One*, vol. 10, no. 5, pp. 1–17, 2015.

[74] G. Ishii, A. Ochiai, and S. Neri, "Phenotypic and functional heterogeneity of cancer-associated fibroblast within the tumor microenvironment," *Adv. Drug Deliv. Rev.*, vol. 99, pp. 186–196, 2016.

[75] W. Matthijs Blankesteijn, "Has the search for a marker of activated fibroblasts finally come to an end?," *J. Mol. Cell. Cardiol.*, vol. 88, pp. 120–123, 2015.

[76] H. Sugimoto, T. M. Mundel, M. W. Kieran, and R. Kalluri, "Identification of fibroblast heterogeneity in the tumor microenvironment," *Cancer Biol. Ther.*, vol. 5, no. 12, pp. 1640–1646, 2006.

[77] R. Kalluri, "The biology and function of fibroblasts in cancer," *Nat. Rev. Cancer*, vol. 16, no. 9, pp. 582–598, 2016.

[78] S. Gordon, P. Annette, and F. Martinez Estrada, "Macrophage heterogeneity in tissues: Phenotypic diversity and functions," *Immunol. Rev.*, vol. 262, no. 1, pp. 36–55, 2014.

[79] S. Gordon and P. R. Taylor, “Monocyte and macrophage heterogeneity.,” *Nat. Rev. Immunol.*, vol. 5, no. 12, pp. 953–64, 2005.
[80] C. D. Mills and K. Ley, “M1 and M2 macrophages: The chicken and the egg of immunity,” *Journal of Innate Immunity*, vol. 6, no. 6. pp. 716–726, 2014.
[81] A. Mantovani, A. Sica, S. Sozzani, P. Allavena, A. Vecchi, and M. Locati, “The chemokine system in diverse forms of macrophage activation and polarization,” *Trends in Immunology*, vol. 25, no. 12. pp. 677–686, 2004.
[82] G. J. Nau, J. F. L. Richmond, A. Schlesinger, E. G. Jennings, E. S. Lander, and R. A. Young, “Human macrophage activation programs induced by bacterial pathogens,” *Proc. Natl. Acad. Sci.*, vol. 99, no. 3, pp. 1503–1508, 2002.
[83] F. O. Martinez and S. Gordon, “The M1 and M2 paradigm of macrophage activation: time for reassessment.,” *F1000Prime Rep.*, vol. 6, no. March, p. 13, 2014.
[84] K. A. Kigerl, J. C. Gensel, D. P. Ankeny, J. K. Alexander, D. J. Donnelly, and P. G. Popovich, “Identification of Two Distinct Macrophage Subsets with Divergent Effects Causing either Neurotoxicity or Regeneration in the Injured Mouse Spinal Cord,” *J. Neurosci.*, vol. 29, no. 43, pp. 13435–13444, 2009.
[85] F. O. Martinez, S. Gordon, M. Locati, and A. Mantovani, “Transcriptional Profiling of the Human Monocyte-to-Macrophage Differentiation and Polarization: New Molecules and Patterns of Gene Expression,” *J. Immunol.*, vol. 177, no. 10, p. 7303 LP-7311, Nov. 2006.
[86] A. Sica and A. Mantovani, “Macrophage plasticity and polarization: In vivo veritas,” *Journal of Clinical Investigation*, vol. 122, no. 3. pp. 787–795, 2012.
[87] T. H. Sulahian, P. Högger, A. E. Wahner, K. Wardwell, N. J. Goulding, C. Sorg, A. Droste, M. Stehling, P. K. Wallace, P. M. Morganelli, and P. M. Guyre, “HUMAN MONOCYTES EXPRESS CD163, WHICH IS UPREGULATED BY IL-10 AND IDENTICAL TO p155,” *Cytokine*, vol. 12, no. 9, pp. 1312–1321, 2000.
[88] H. W. L. Ziegler-Heitbrock and R. J. Ulevitch, “CD14: Cell surface receptor and differentiation marker,” *Immunol. Today*, vol. 14, no. 3, pp. 121–125, 1993.
[89] P. Italiani and D. Boraschi, “From monocytes to M1/M2 macrophages: Phenotypical vs. functional differentiation,” *Frontiers in Immunology*, vol. 5, no. OCT. 2014.
[90] A. Mantovani, B. Bottazzi, F. Colotta, S. Sozzani, and L. Ruco, “The origin and function of tumor-associated macrophages.,” *Immunol. Today*, vol. 13, no. 7, pp. 265–270, 1992.
[91] A. Mantovani, “B cells and macrophages in cancer: yin and yang,” *Nat. Med.*, vol. 17, no. 3, pp. 285–286, 2011.
[92] A. P. Cardoso, M. L. Pinto, A. T. Pinto, M. I. Oliveira, M. T. Pinto, R. Gonc, J. B. Relvas, C. Figueiredo, R. Seruca, and A. Mantovani, “Macrophages stimulate gastric and colorectal cancer invasion through EGFR Y 1086 , c-Src , Erk1 / 2 and Akt phosphorylation and smallGTPase activity,” no. December 2012, pp. 1–11, 2013.
[93] M. L. Pinto, E. Rios, A. C. Silva, S. C. Neves, H. R. Caires, C. C. Barrias, M. J. Oliveira, and F. Carneiro, “Biomaterials Decellularized human colorectal cancer matrices polarize macrophages towards an anti-in fl ammatory phenotype promoting cancer cell invasion via CCL18,” vol. 124, 2017.
[94] R. Noy and J. W. Pollard, “Tumor-Associated Macrophages: From Mechanisms to Therapy,” *Immunity*, vol. 41, no. 1, pp. 49–61, 2014.
[95] T. Hanada, M. Nakagawa, A. Emoto, T. Nomura, N. Nasu, and Y. Nomura, “Prognostic value of tumor-associated macrophage count in human bladder cancer,” *Int. J. Urol.*, vol. 7, no. 7, pp. 263–269, 2000.
[96] I. Marech, M. Ammendola, R. Sacco, G. Sammarco, V. Zuccal??, N. Zizzo, C. Leporini, M. Luposella, R. Patruno, G. Filippelli, E. Russo, M. Porcelli, C. D. Gadaleta, G. De Sarro, and G. Ranieri, “Tumour-associated macrophages correlate with microvascular bed extension in colorectal cancer patients,” *J. Cell. Mol. Med.*, vol. 20, no. 7, pp. 1373–1380, 2016.
[97] A. T. Pinto, M. L. Pinto, S. Velho, M. T. Pinto, and A. P. Cardoso, “Intricate Macrophage-Colorectal Cancer Cell Communication in Response to Radiation,” pp. 1–20, 2016.
[98] A. T. Pinto, M. L. Pinto, A. P. Cardoso, C. Monteiro, M. T. Pinto, A. F. Maia, P. Castro, R. Figueira, A. Monteiro, M. Marques, M. Mareel, and S. Gomes, “Ionizing radiation modulates human macrophages towards a pro-inflammatory phenotype preserving their

pro-invasive and pro-angiogenic capacities," no. June 2015, pp. 1–15, 2016.

[99] A. Mantovani and P. Allavena, "The interaction of anticancer therapies with tumor-associated macrophages," *J. Exp. Med.*, vol. 212, no. 4, pp. 435–445, 2015.

[100] C. B. Williams, E. S. Yeh, and A. C. Soloff, "Tumor-associated macrophages: unwitting accomplices in breast cancer malignancy," *npj Breast Cancer*, vol. 2, no. September 2015, p. 15025, 2016.

[101] M. Herrera, A. Herrera, G. Domínguez, J. Silva, V. García, J. M. García, I. Gómez, B. Soldevilla, C. Muñoz, M. Provencio, Y. Campos-Martin, A. García de Herreros, I. Casal, F. Bonilla, and C. Peña, "Cancer-associated fibroblast and M2 macrophage markers together predict outcome in colorectal cancer patients," *Cancer Sci.*, vol. 104, no. 4, pp. 437–444, 2013.

[102] A. B. Shekhter, G. N. Berchenko, and A. V Nikolaev, "[Macrophage-fibroblast interaction and its possible role in regulating collagen metabolism during wound healing].," *Biull. Eksp. Biol. Med.*, vol. 83, no. 5, pp. 627–630, 1977.

[103] T. Oka, K. Ohta, T. Kanazawa, and K.-I. Nakamura, "Interaction between Macrophages and Fibroblasts during Wound Healing of Burn Injuries in Rats.," *Kurume Med. J.*, pp. 3–10, 2016.

[104] P. Chiarugi, "Cancer-associated fibroblasts and macrophages: Friendly conspirators for malignancy.," *Oncoimmunology*, vol. 2, no. 9, p. e25563, 2013.

[105] X. Q. Liu, R. Kiefl, C. Roskopf, F. Tian, and R. M. Huber, "Interactions among lung cancer cells, fibroblasts, and macrophages in 3D co-cultures and the impact on MMP-1 and VEGF expression," *PLoS One*, vol. 11, no. 5, 2016.

[106] D. Rama-Esendagli, G. Esendagli, G. Yilmaz, and D. Guc, "Spheroid formation and invasion capacity are differentially influenced by co-cultures of fibroblast and macrophage cells in breast cancer," *Mol. Biol. Rep.*, vol. 41, no. 5, pp. 2885–2892, 2014.

[107] M. Miyake, S. Hori, Y. Morizawa, Y. Tatsumi, Y. Nakai, S. Anai, K. Torimoto, K. Aoki, N. Tanaka, K. Shimada, N. Konishi, M. Toritsuka, T. Kishimoto, C. J. Rosser, and K. Fujimoto, "CXCL1-Mediated Interaction of Cancer Cells with Tumor-Associated Macrophages and Cancer-Associated Fibroblasts Promotes Tumor Progression in Human Bladder Cancer," *Neoplasia*, vol. 18, no. 10, pp. 636–646, 2016.

[108] O. Hashimoto, M. Yoshida, Y. ichiro Koma, T. Yanai, D. Hasegawa, Y. Kosaka, N. Nishimura, and H. Yokozaki, "Collaboration of cancer-associated fibroblasts and tumour-associated macrophages for neuroblastoma development," *J. Pathol.*, vol. 240, no. 2, pp. 211–223, 2016.

[109] C. Herrera, M., Herrera, A., Larriba, M. J., Ferrer-Mayorga, G., Herreros, A. G., Bonilla, F., Baulida, J. and Peña, "Colon Cancer-associated Fibroblast Establishment and Culture Growth," *Bio-protocol 6(7)*, vol. 6, no. 2, pp. 3–8, 2016.

[110] A. Teresa Pinto, M. Laranjeiro Pinto, A. Patrícia Cardoso, C. Monteiro, M. Teixeira Pinto, A. Filipe Maia, P. Castro, R. Figueira, A. Monteiro, M. Marques, M. Mareel, S. G. Dos Santos, R. Seruca, M. Adolfo Barbosa, S. Rocha, and M. José Oliveira, "Ionizing radiation modulates human macrophages towards a pro-inflammatory phenotype preserving their pro-invasive and pro-angiogenic capacities.," *Sci. Rep.*, vol. 6, no. November 2015, p. 18765, 2016.

[111] T. Oida and H. L. Weiner, "Depletion of TGF-β from fetal bovine serum," *J. Immunol. Methods*, vol. 362, no. 1–2, pp. 195–198, 2010.

[112] P. J. Murray, J. E. Allen, S. K. Biswas, E. A. Fisher, D. W. Gilroy, S. Goerdt, S. Gordon, J. A. Hamilton, L. B. Ivashkiv, T. Lawrence, M. Locati, A. Mantovani, F. O. Martinez, J. L. Mege, D. M. Mosser, G. Natoli, J. P. Saeij, J. L. Schultze, K. A. Shirey, A. Sica, J. Suttles, I. Udalova, J. A. vanGinderachter, S. N. Vogel, and T. A. Wynn, "Macrophage Activation and Polarization: Nomenclature and Experimental Guidelines," *Immunity*, vol. 41, no. 1. pp. 14–20, 2014.

[113] A. P. Cardoso, M. L. Pinto, A. T. Pinto, M. I. Oliveira, M. T. Pinto, R. Gonçalves, J. B. Relvas, C. Figueiredo, R. Seruca, A. Mantovani, M. Mareel, M. a Barbosa, and M. J. Oliveira, "Macrophages stimulate gastric and colorectal cancer invasion through EGFR Y(1086), c-Src, Erk1/2 and Akt phosphorylation and smallGTPase activity.," *Oncogene*, vol. 33, no. 16, pp. 2123–33, 2014.

[114] R. D. Barber, D. W. Harmer, R. A. Coleman, and B. J. Clark, "GAPDH as a housekeeping gene: analysis of GAPDH mRNA expression in a panel of 72 human

tissues," *Physiol. Genomics*, vol. 21, no. 3, pp. 389–395, 2005.

[115] K. Shiga, M. Hara, T. Nagasaki, T. Sato, H. Takahashi, and H. Takeyama, "Cancer-associated fibroblasts: Their characteristics and their roles in tumor growth," *Cancers*, vol. 7, no. 4. pp. 2443–2458, 2015.

[116] A. P. Sappino, O. Skalli, B. Jackson, W. Schürch, and G. Gabbiani, "Smooth muscle differentiation in stromal cells of malignant and non malignant breast tissues," *Int. J. Cancer*, vol. 41, no. 5, pp. 707–712, 1988.

[117] H. Chen, W.-W. Yang, Q.-T. Wen, L. Xu, and M. Chen, "TGF-beta induces fibroblast activation protein expression; fibroblast activation protein expression increases the proliferation, adhesion, and migration of HO-8910PM [corrected].," *Exp. Mol. Pathol.*, vol. 87, no. 3, pp. 189–194, 2009.

[118] F. O. Martinez, A. Sica, A. Mantovani, and M. Locati, "Macrophage activation and polarization.," *Front. Biosci.*, vol. 13, no. 4, pp. 453–61, 2008.

[119] J. Boyle, "Molecular biology of the cell, 5th edition by B. Alberts, A. Johnson, J. Lewis, M. Raff, K. Roberts, and P. Walter," *Biochem. Mol. Biol. Educ.*, vol. 36, no. 4, pp. 317–318, 2008.

[120] J. A. Burger and T. J. Kipps, "CXCR4: A key receptor in the crosstalk between tumor cells and their microenvironment," *Blood*, vol. 107, no. 5. pp. 1761–1767, 2006.

[121] J. Wyckoff, W. Wang, E. Y. Lin, Y. Wang, F. Pixley, E. R. Stanley, T. Graf, J. W. Pollard, J. Segall, and J. Condeelis, "Wyckoff Condeelis 2004 A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors.," pp. 7022–7029, 2004.

[122] F. Y. McWhorter, T. Wang, P. Nguyen, T. Chung, and W. F. Liu, "Modulation of macrophage phenotype by cell shape," *Proc. Natl. Acad. Sci.*, vol. 110, no. 43, pp. 17253–17258, 2013.

[123] J. Wang, H. Chen, A. Seth, and C. A. McCulloch, "Mechanical force regulation of myofibroblast differentiation in cardiac fibroblasts," *Am. J. Physiol. - Hear. Circ. Physiol.*, vol. 285, no. 5, pp. H1871–H1881, 2003.

[124] M. J. Dalby, D. Giannaras, M. O. Riehle, N. Gadegaard, S. Affrossman, and A. S. G. Curtis, "Rapid fibroblast adhesion to 27 nm high polymer demixed nano-topography," *Biomaterials*, vol. 25, no. 1, pp. 77–83, 2004.

[125] H. Kurahara, H. Shinchi, Y. Mataki, K. Maemura, H. Noma, F. Kubo, M. Sakoda, S. Ueno, S. Natsugoe, and S. Takao, "Significance of M2-polarized tumor-associated macrophage in pancreatic cancer," *J. Surg. Res.*, vol. 167, no. 2, 2011.

[126] A. Sica, T. Schioppa, A. Mantovani, and P. Allavena, "Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: Potential targets of anti-cancer therapy," *Eur. J. Cancer*, vol. 42, no. 6, pp. 717–727, 2006.

[127] M. Sporn, A. Roberts, L. Wakefield, and R. Assoian, "Transforming growth factor-beta: biological function and chemical structure," *Science (80-.).*, vol. 233, no. 4763, pp. 532–534, 1986.

[128] B. Bierie and H. L. Moses, "TGF-beta and cancer.," *Cytokine Growth Factor Rev.*, vol. 17, no. 1–2, pp. 29–40, 2006.

[129] D. Gong, W. Shi, S. J. Yi, H. Chen, J. Groffen, and N. Heisterkamp, "TGFbeta signaling plays a critical role in promoting alternative macrophage activation," *BMC Immunol*, vol. 13, p. 31, 2012.

[130] D. Pohlers, J. Brenmoehl, I. Löffler, C. K. Müller, C. Leipner, S. Schultze-Mosgau, A. Stallmach, R. W. Kinne, and G. Wolf, "TGF-β and fibrosis in different organs - molecular pathway imprints," *Biochim. Biophys. Acta - Mol. Basis Dis.*, vol. 1792, no. 8, pp. 746–756, 2009.

[131] R. K. Assoian, B. E. Fleurdelys, H. C. Stevenson, P. J. Miller, D. K. Madtes, E. W. Raines, R. Ross, and M. B. Sporn, "Expression and secretion of type beta transforming growth factor by activated human macrophages.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 84, no. 17, pp. 6020–6024, 1987.

[132] A. Mantovani, S. Sozzani, M. Locati, P. Allavena, and A. Sica, "Macrophage polarization: Tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes," *Trends in Immunology*, vol. 23, no. 11. pp. 549–555, 2002.

[133] P. Chen, Q. Mo, B. Wang, D. Weng, P. Wu, and G. Chen, "Breast cancer associated

fibroblasts promote MCF-7 invasion in vitro by secretion of HGF," *J. Huazhong Univ. Sci. Technol. - Med. Sci.*, vol. 32, no. 1, pp. 92–96, 2012.

[134] A. J. Daly, L. McIlreavey, and C. R. Irwin, "Regulation of HGF and SDF-1 expression by oral fibroblasts - Implications for invasion of oral cancer," *Oral Oncol.*, vol. 44, no. 7, pp. 646–651, 2008.

[135] P. C. Ma, M. S. Tretiakova, V. Nallasura, R. Jagadeeswaran, A. N. Husain, and R. Salgia, "Downstream signalling and specific inhibition of c-MET/HGF pathway in small cell lung cancer: implications for tumour invasion," *Br. J. Cancer*, vol. 97, no. 3, pp. 368–377, 2007.

[136] E. C. Woenne, W. Lederle, S. Zwick, M. Palmowski, H. Krell, W. Semmler, M. M. Mueller, and F. Kissling, "MMP inhibition blocks fibroblast-dependent skin cancer invasion, reduces vascularization and alters VEGF-A and PDGF-BB expression," *Anticancer Res.*, vol. 30, no. 3, pp. 703–712, 2010.

[137] K. P. Sundararaj, D. J. Samuvel, Y. Li, J. J. Sanders, M. F. Lopes-Virella, and Y. Huang, "Interleukin-6 released from fibroblasts is essential for up-regulation of matrix metalloproteinase-1 expression by U937 macrophages in coculture: Cross-talking between fibroblasts and U937 macrophages exposed to high glucose," *J. Biol. Chem.*, vol. 284, no. 20, pp. 13714–13724, 2009.

[138] S. Sakaguchi, J. I. Shono, T. Suzuki, S. Sawano, J. E. Anderson, M. K. Q. Do, H. Ohtsubo, W. Mizunoya, Y. Sato, M. Nakamura, M. Furuse, K. Yamada, Y. Ikeuchi, and R. Tatsumi, "Implication of anti-inflammatory macrophages in regenerative moto-neuritogenesis: Promotion of myoblast migration and neural chemorepellent semaphorin 3A expression in injured muscle," *Int. J. Biochem. Cell Biol.*, vol. 54, pp. 272–285, 2014.

[139] K. Shimizu and N. Oku, "Cancer anti-angiogenic therapy.," *Biol. Pharm. Bull.*, vol. 27, no. 5, pp. 599–605, 2004.

[140] M. Pàez-Ribes, E. Allen, J. Hudock, T. Takeda, H. Okuyama, F. Viñals, M. Inoue, G. Bergers, D. Hanahan, and O. Casanovas, "Antiangiogenic Therapy Elicits Malignant Progression of Tumors to Increased Local Invasion and Distant Metastasis," *Cancer Cell*, vol. 15, no. 3, pp. 220–231, 2009.

[141] K. Y. Aguilera, L. B. Rivera, H. Hur, J. G. Carbon, J. E. Toombs, C. D. Goldstein, M. T. Dellinger, D. H. Castrillon, and R. A. Brekken, "Collagen signaling enhances tumor progression after anti-VEGF therapy in a murine model of pancreatic ductal adenocarcinoma," *Cancer Res.*, vol. 74, no. 4, 2014.