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Colorectal tumor microenvironment: unravelling the interplay between macrophages and the extracellular matrix

Marta Laranjeiro Pinto

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

MARTA LARANJEIRO PINTO

COLORECTAL TUMOR MICROENVIRONMENT: UNRAVELLING THE INTERPLAY BETWEEN MACROPHAGES AND THE EXTRACELLULAR MATRIX

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Sumário

Os tumores não são apenas células com a capacidade de proliferar de forma descontrolada e que, após adquirirem determinadas características, irão ter a capacidade de migrar, invadir os tecidos adjacentes e eventualmente metastizar para nódulos linfáticos adjacentes e órgãos distantes, culminando numa doença maligna. A verdade é que o processo tumorigénico é extremamente complexo e envolve vários elementos, celulares e não celulares, presentes no microambiente tumoral.

De entre destes, os macrófagos representam as células imunes predominantes e estão ativamente envolvidos no desenvolvimento e progressão tumorais. Vários estudos epidemiológicos têm, na generalidade, descrito uma correlação entre uma elevada infiltração de macrófagos associados ao tumor (TAMs) e pior prognóstico. Múltiplos trabalhos, realizados tanto *in vitro* como *in vivo,* demostraram a capacidade dos macrófagos em promoverem a proliferação, sobrevivência, migração e invasão das células tumorais. Os macrófagos são, na verdade, células extremamente plásticas com capacidade de adotarem diferentes perfis, de acordo com os estímulos externos. Hoje em dia, é aceite que existe um contínuo de perfis de diferenciação entre duas populações extremas: os macrófagos pro-inflamatórios ou M1, ativados classicamente, e os macrófagos anti-inflamatórios ou M2, ativados alternativamente. Para além das várias células presentes no estroma tumoral, existe um componente não-celular muito importante denominado matriz extracelular (ECM). Trata-se de uma rede de macromoléculas de elevada complexidade que aprisiona vários elementos envolvidos na sinalização celular, incluindo fatores de crescimento e citocinas/quimiocinas. Assim sendo a ECM, além de providenciar suporte às células e tecidos, modela também vias de sinalização, desempenhando múltiplas funções como a regulação da diferenciação e migração celulares. Os tecidos tumorais apresentam uma ECM com composição, organização e propriedades biomecânicas anómalas, mas com um papel fulcral no desenvolvimento e progressão tumorais. Apesar do reconhecido papel pro-tumoral dos TAMS noutros tumores, existe ainda algum debate sobre o seu papel no cancro colo-retal. Por conseguinte, **o objetivo geral deste trabalho foi de clarificar a importância dos macrófagos, e de definir o perfil de distribuição de subpopulações macrofágicas de distintos perfis inflamatórios em tumores de doentes com cancro colo-retal. Simultaneamente, tendo em conta a capacidade da ECM em modelar o comportamento celular, procurámos compreender o papel da ECM tumoral colo-rectal na polarização macrofágica e quais as possíveis consequências para a invasão celular mediada pelos macrófagos. O derradeiro objetivo foi a identificação de novos alvos moleculares para o desenvolvimento de estratégias terapêuticas mais eficientes dirigidas à modelação do microambiente tumoral.**

Procurando definir o perfil dos TAMs no CRC, os macrófagos foram avaliados quantitativamente numa série de casos de CRC, incluindo a análise de diferentes subpopulações, identificadas pelo CD80, expresso por macrófagos pro-inflamatórios, e o CD163, expresso por macrófagos do tipo anti-inflamatório. Neste estudo, incluímos ainda a mucosa normal adjacente ao tumor, proveniente do mesmo paciente, que foi usada como termo de comparação. Esta estratégia permitiu demonstrar que há uma completa inversão na proporção de macrófagos do tipo pro- e anti-inflamatório entre os tecidos normais e tumorais, maioritariamente devido a um desaparecimento quase completo de células positivas para o CD80 nos tecidos neoplásicos. Em tumores colo-rectais do estadio III, uma infiltração mais elevada de macrófagos e uma redução do rácio CD80/CD163 revelaram estar associado a menor sobrevida. Por outro lado, a expressão de CD80 apresentou-se como tendo um efeito protetor na prevenção da recorrência ou recidiva loco-regional.

Após descrever a alteração do perfil macrofágico em tumores colo-rectais, procurou-se clarificar qual o papel da ECM na polarização dos macrófagos. Por forma a obter ECM que recapitulasse com exatidão o tecido nativo, fragmentos humanos obtidos a partir de ressecções cirúrgicas de pacientes com CRC foram descelularizados e caracterizados a nível estrutural, bioquímico e biomecânico. Posteriormente, estas matrizes foram repopuladas com monócitos humanos. Após diferenciação, revelou-se que, nas matrizes derivadas de tumores, os macrófagos adotavam um perfil mais do tipo anti-inflamatório, com uma produção mais elevada de TGF-β, IL-10 e CCL18 e uma expressão reduzida de TNF e CCR7. Por outro lado, estes macrófagos condicionados pelas matrizes tumorais induziram a invasão de linhas celulares colo-rectais, um processo mediado pelo CCL18. Finalmente, mostrou-se que o CCL18 ativa uma cascata de sinalização que envolve a fosforilação da FAK, EGFR, Akt, Src, ERK e p38, e induz uma transição epitélio-mesenquimal parcial.

Como conclusão, esta tese providenciou novos conhecimentos sobre o papel dos macrófagos no carcinoma colo-retal. Os nossos resultados realçam o papel do microambiente tumoral, mais especificamente da ECM, na modelação da polarização macrofágica num fenótipo do tipo anti-inflamatório. Por outro lado, perante o papel protetor das células $CD80^{+}$ na prevenção da recorrência/recidiva loco-regional combinado com o efeito pro-invasivo do CCL18 proveniente dos macrófagos, este trabalho reforça a relevância de modelar os TAMs pelas novas estratégias terapêuticas, nomeadamente a imunoterapia.

Abstract

Tumors are not simply a group of cells proliferating uncontrollably which, upon acquiring specific features, will be able to migrate, invade the adjacent tissues and eventually metastasize to regional lymph nodes adistant organs, culminating in malignant disease. The reality is that the tumorigenic process is much more complex and involves several players, cellular and non-cellular, present within the tumor microenvironment.

Among these, macrophages represent the predominant immune cells and are actively implicated in tumor development and progression. Epidemiological studies have described an association between increased tumor-associated macrophages (TAMs) infiltration and worst prognosis. In addition, extensive *in vitro* and *in vivo* work reported the capacity of macrophages to promote tumor cell proliferation, survival, migration and invasion. Macrophages are extremely plastic cells and can adopt different profiles according to the external environment and, nowadays, it is accepted that there is a continuum of polarization status between two extreme populations: the pro-inflammatory, M1 or classically-activated macrophages, and the anti-inflammatory, M2 or alternatively-activated macrophages.

Aside from stromal cells, there is also an important non-cellular component within the tumor microenvironment designated as the extracellular matrix (ECM). The ECM is a complex network of macromolecules with several arrested signaling molecules, namely growth factors and cytokines/chemokines, which, besides providing support to cells and tissues, also performs other functions, such as regulating cell differentiation and migration. Cancer tissues are known for having an abnormal ECM, specifically in composition, organization and biomechanical characteristics, and it is recognized that this aberrant ECM is also involved in cancer development and progression. Despite the known pro-tumoral role of macrophages in cancer, there is still great debate on their role in colorectal cancer (CRC). Therefore, **the overall aim of this thesis was to clarify the relevance of TAMs in CRC. Simultaneously, given the ECM role in shaping cell behavior, we sought to unravel the role of colorectal tumor ECM on the modulation of macrophage polarization and the implications on macrophage-mediated cancer cell invasion. The ultimate goal was to identify novel targets for the development of more efficient therapeutic strategies focusing on the modulation of the tumor microenvironment.**

In order to profile TAMs in CRC, we quantitatively assessed macrophages in a series of well-characterized CRC cases, including the analysis of different subpopulations, identified by CD80, expressed by pro-inflammatory macrophages, and CD163, expressed by their anti-inflammatory counterparts. In the present study, we have also included the tumor adjacent

normal mucosa from the same patient for comparison. This strategy enabled the demonstration that there is a complete inversion in the proportion of pro- and anti-inflammatory macrophages between normal and tumor tissues, mainly due to an almost complete disappearance of $CD80^+$ cells in neoplastic tissues. In stage III tumors, higher macrophage infiltration and decreased CD80/CD163 ratio associated with decreased survival. Moreover, CD80 expression provided a protective role in preventing relapse and locoregional recurrence.

After describing the alterations on macrophage profile within colorectal tumors, the role of the tumor ECM on macrophage polarization was also assessed. To obtain an ECM which accurately resembled the native tissue, human fragments originated from CRC patients' surgical resections were decellularized and subsequently characterized regarding their biochemical, structural and biomechanical properties. These matrices were then repopulated with human monocytes derived from healthy blood donors. Their characterization revealed that, in tumor-derived ECM, macrophages adopted a more anti-inflammatory profile, with increased production of TGF-β, IL-10 and CCL18 and decreased expression of TNF and CCR7. Moreover, tumor ECM-educated macrophages stimulated CRC cell invasion, a process mediated by CCL18. Finally, the chemokine CCL18 was shown to activate a signaling cascade involving FAK, EGFR, Akt, Src, ERK and p38, and to induce a partial epithelial to mesenchymal transition.

Taken together, this thesis provided new insights regarding macrophages in CRC. Our findings highlight the role of the tumor microenvironment, specifically the ECM, on the modulation of macrophage polarization towards an anti-inflammatory phenotype. Additionally, given the protective role of $CD80^+$ cells and the pro-invasive role of macrophage-derived CCL18, this PhD work further supports the relevance of targeting macrophages by new therapeutic approaches, namely immunotherapy.

Abbreviations

3D - three dimensional

A

ACF - aberrant crypt foci

AM - alveolar macrophages

ANM - adjacent normal mucosa

APC - adenomatous polyposis coli

APCs - antigen presenting cells

ANG2 - angiopoietin-2

B

bFGF - basic FGF

BMDC - bone marrow-derived cells

C

CAFs – cancer-associated fibroblasts

CAM-RR - cell-adhesion-mediated-radio-resistance

CAR - chimeric antigen receptors

CCL - chemokine (C-C motif) ligand

CCR2 - C-C chemokine receptor type 2

CIMP - CpG island methylator phenotype

CIN - chromosomal instability

CNS - central nervous system

CRC - colorectal cancer

CSF-1/M-CSF - Colony stimulating factor 1/Macrophage colony-stimulating factor

CSFR1 - colony stimulating factor receptor 1

CTCs - circulating tumor cells

CTLA-4 - cytotoxic T-lymphocyte protein 4

CXCL - chemokine (C-X-C motif) ligand

CD – cluster of differentiation

D

DCs - dendritic cells

E

ECM - extracellular matrix

EGF - epidermal growth factor

EGFR - epidermal growth factor receptor

EMT – epithelial-mesenchymal transition

F

FACITs - fibril-associated collagens with interrupted triple helices

FAK - focal adhesion kinase

FDA - Food and Drug Administration

DFS - disease free survival

FGF - fibroblast growth factor

FOXP3 - forkhead boxP3

G

GAGs - glycosaminoglycans

GPR30/GPER1 - G protein-coupled estrogen receptor 1

H

HA - hyaluronic acid/hyaluronan

HB EGF - heparin-binding EGF-like growth factor

HCC - hepatocellular carcinoma

HGF – hepatocyte growth factor

HGFR/c-Met - HGF receptor

HIF1α - hypoxia-inducible factor 1-α

HNPCC - hereditary nonpolyposis colorectal cancer

HSC - hematopoietic stem cells

I

IF - tumor invasive front

IFN - interferon

IGF - insulin growth factor

IL - interleukin

IRA % - percentage of the immunoreactive area

IT - intratumoral region

J

JAK - Janus kinase

L

LPS – lipopolysaccharide

LOL - lysyl oxidase

LOXL - LOX-like

LXs - lipotoxins

M

MACITs - membrane-associated collagens with interrupted triple helices

MCP1 - monocyte chemoattractant protein-1

MDSCs - myeloid-derived suppressor cells

MHC - major histocompatibility complex

miRNA - microRNAs

MMPs - matrix metalloproteinases

MMR - mismatch repair

MPS - mononuclear phagocyte system

MSCs - mesenchymal stem cells (MSCs)

MSI - microsatellite instability

MULTIPLEXIN - multiple triple-helix domains and interruptions/endostatin-producing collagens

N

NF-κB - nuclear factor-κB

NK - natural killer cells

NO - nitric oxide

NOS2/iNOS - NO synthase 2/inducible NO synthase

O

OPN - osteopontin

P

PAMPs - pathogen-associated molecular patterns

PAR1 - protease-activated receptors 1

PBMCs - peripheral blood mononuclear cells

PDAC - pancreatic ductal adenocarcinoma

PD1 - programmed cell death protein 1

PDGF - platelet-derived growth factor

PD-L1 - programmed death-ligand 1

PEMs - polyelectrolyte multilayers

PGA - chitosan/poly γ-glutamic acid

PGE2 - prostaglandin E2

PGF - placental growth factor

PGs - proteoglycans

PITPNM3 - Membrane-associated phosphatidylinositol transfer protein 3

PPRs - pattern recognition receptors

Pyk2 - proline-rich tyrosine kinase 2

PyMT - polyoma middle T

R

RGD - Arg-Gly-Asp

S

SDF-1 - stromal cell-derived factor-1

SEM - Scanning electron microscopy

SLRP - small leucine-rich proteoglycans

STAT3 - signal transducer and activator of transcription 3

STI1 - stress inducible protein 1

T

TAMs - Tumor-associated macrophages

TEMs - TIE2-expressing monocytes

TF - tissue factor

TGF - Transforming growth factor

Th1 - T helper type I

Th2 -T helper type II

TILs - tumor-infiltrating lymphocytes

TIMPs - tissue inhibitors of metalloproteinases

TLR - Toll-like receptors

TMEM - tumor microenvironment of metastasis

TNC - tenascin-C

TNF - tumor necrosis factor

TNM - tumor/node/metastasis

Tregs - regulatory T cells

U

UICC - Union for International Cancer Control

uPA - urokinase-type plasminogen activator

V

VEGF - vascular endothelial growth factor

VCAM-1 - vascular cell adhesion molecule-1

Thesis aims

In many malignancies, including melanoma, breast or ovarian cancer, the presence of high levels of tumor associated macrophages (TAMs) correlates with more aggressive disease and worst prognosis. TAMs are actively involved in tumor progression, since they may promote tumor growth, survival, angiogenesis, cancer invasion and metastasis. Nevertheless, in colorectal cancer (CRC), the data regarding macrophage's clinicopathological importance is contradictory, with some studies describing an association between macrophage infiltration and decreased survival and others reporting the exactly the opposite. Therefore, in this PhD thesis, we sought to clarify the significance of TAMs within CRC. Subsequently, the focus of our research was centered on the role of the extracellular matrix (ECM), present at the tumor microenvironment, on the modulation of macrophage inflammatory profile but also on identifying the molecular mechanisms through which macrophages enhance cancer cell invasion.

Accordingly, the subsequent specific objectives were established:

1. **Characterize the macrophage populations, specifically the inflammatory subtypes, present in CRC tissues, and explore possible associations with prognosis and clinical outcome**

   Many studies have addressed the relevance of macrophage infiltration in CRC but, probably due to disparities in the selected markers and in the methodologies used across studies, the available results are conflicting. For this reason, we performed a quantitative characterization of macrophages, including different subpopulations, across CRC tissues. Our approach consisted on profiling macrophage subtypes in 150 CRC cases (dated from 2007-2012) from Centro Hospitalar São João Tumor Bank. Importantly, all tumor fragments analyzed also contained the adjacent normal mucosa. Consecutive sections were stained with specific antibodies for CD68, a macrophage lineage marker, CD80, a co-stimulatory molecule expressed by pro-inflammatory macrophages, and CD163, a scavenger receptor expressed by anti-inflammatory macrophages. Following immunohistochemistry, tissue slides were digitalized and the immunoreactive area for each marker was quantified, using Fiji software, in three different regions: normal adjacent mucosa, intratumoral region and tumor invasive front. For each region, 10 distinct areas were randomly selected for each marker quantification and analysis. The data was crossed with patient's clinicopathologic information in an attempt to unravel the clinical relevance of the distinct macrophage

subpopulations in CRC, and hopefully help to discriminate which patients might benefit from immunotherapies targeting macrophages. These results are included in Chapter 2.

2. Recreate an organotypic 3D culture system that resembles the colorectal tumor ECM

Having shown, in the previous chapter, that there is an alteration in macrophage profile within colorectal tumors, we sought to unravel whether tumor ECM, being the most prevalent element at the tumor microenvironment, had any role in this differential macrophage polarization. Due to the biochemical and biomechanical complexity of the human ECM, difficult to recreate by bioengineered scaffolds, we decided to take advantage of human colorectal samples and use decellularization as a method to obtain reliable ECM that accurately resembles the native tissue. Accordingly, using fragments obtained from CRC patients' surgical resections, we optimized a decellularization protocol able to remove DNA and cell debris from both normal and tumor colorectal tissues. Furthermore, to ensure that decellularized tissues preserved native tissues' characteristics, their composition, architecture, and biomechanical properties were monitored through immunohistochemistry, scanning electron microscopy and rheometer analysis, respectively. These results are included in Chapter 3.

3. Dissect the role of tumor extracellular matrix on human macrophage polarization

Knowing that macrophages adjust their phenotype according to microenvironmental factors, and that the tumor ECM displays modifications in its biochemical and physical properties, we assessed the effect of such altered ECM on macrophage polarization. Therefore, decellularized normal and tumor matrices, described in the previous objective, were repopulated with human monocytes derived from healthy blood donors. After 14 days of differentiation within such matrices, macrophages were characterized in terms of their morphology, RNA expression for specific cell surface receptors and pro- and anti-inflammatory cytokine/chemokine production. Furthermore, the effect of such ECM-educated macrophages on CRC cell invasion was also evaluated. These results are included in Chapter 3.

4. Unravel the effects of CCL18 on colorectal and gastric cancer cell invasion

Our group has previously reported that macrophages, particularly the anti-inflammatory, stimulate gastric and CRC cell invasion through the secretion of factors, as EGF or MMPs. In Chapter 3, we report that macrophages differentiated in tumor- derived matrices secrete high levels of CCL18, an anti-inflammatory and immunosuppressive chemokine. Moreover,

we describe that these tumor-ECM educated macrophages stimulated CRC cell invasion through a process mediated by CCL18. Therefore, it became important to unravel the CCL18 mechanism of action which was leading to this increased pro-invasive capacity. To address this question, CRC cells were stimulated with CCL18 and the activated signaling pathways were evaluated. Additionally, the effect of CCL18 on the expression of some epithelial-mesenchymal transition (EMT) related genes was assessed. Due to previous work from our group regarding the effect of macrophages on gastric cancer cell migration and invasion, a gastric cancer cell line was also included in this work. These results are included in Chapter 4.

CHAPTER 1

General Introduction

1. Hallmarks of cancer

Cancer was known for a long time as a genetic disease. Alterations in genes, either by somatic or germinal mutations, resulted in uncontrolled cell growth, escape to apoptosis, enhanced migration or ability to migrate across basement membranes, which ultimately could lead to metastatic disease. The central role of the neoplastic cell was well illustrated in the review from 2000 by Hanahan and Weinberg, describing the capabilities that cells had to acquire during the carcinogenic process, which they coined as the six hallmarks of cancer [1]. These included the self-sufficiency in growth signals, limitless replicative potential, insensitivity to growth-inhibitory signals, evasion of apoptosis, sustained angiogenesis and tissue invasion and metastasis. At the time, it was already recognized that, albeit the unquestionable fundamental role of the cancer cell, a tumor was much more complex than initially though. In fact, neoplastic disease develops in a complex microenvironment also composed by normal non-malignant stromal cells, namely fibroblasts, endothelial cells, adipocytes and immune cells, from both the innate and adaptive immune system (*Figure 1.1*). All these cells intercommunicate and influence each other's behavior through direct interaction or by exchanging a myriad of soluble factors, including growth factors, cytokines or matrix metalloproteinases (MMPs) [2]. Moreover, supporting the cellular components, there is also a complex network of macromolecules called the extracellular matrix (ECM). Once largely neglected, nowadays the ECM is recognized to be extremely dynamic and to have a major impact in cell behavior, in both health and disease [3, 4]. This evolution in cancer biology knowledge culminated in a 2011 revised version of the hallmarks of cancer, according to which tumors are in fact considered intricate organs with multiple components with active roles in the carcinogenic process [5].

2. Tumor microenvironment

2.1. Tumor cells

Carcinomas are malignant tumors that originate from epithelial cells. Accumulation of mutations in specific genes confers a growth advantage to cells enabling them with the capacity to resist the control mechanisms which would cause their elimination [6]. In fact, errors during DNA synthesis are not a rare event, but there exist specific mechanisms, namely the mismatch repair (MMR) system, which assure the correction of the majority of these mistakes before resulting in fixed mutations [7]. Those cells in which the mutations are not efficiently corrected are generally directed to cell death, through induction of apoptosis or autophagy. It was proposed that cancer cells arise from an evolutionary process, where mutations and a process of natural selection act together [8]. Epigenetic alterations, namely disruptions in DNA methylation and histone modifications, add another layer of complexity to this process and, in

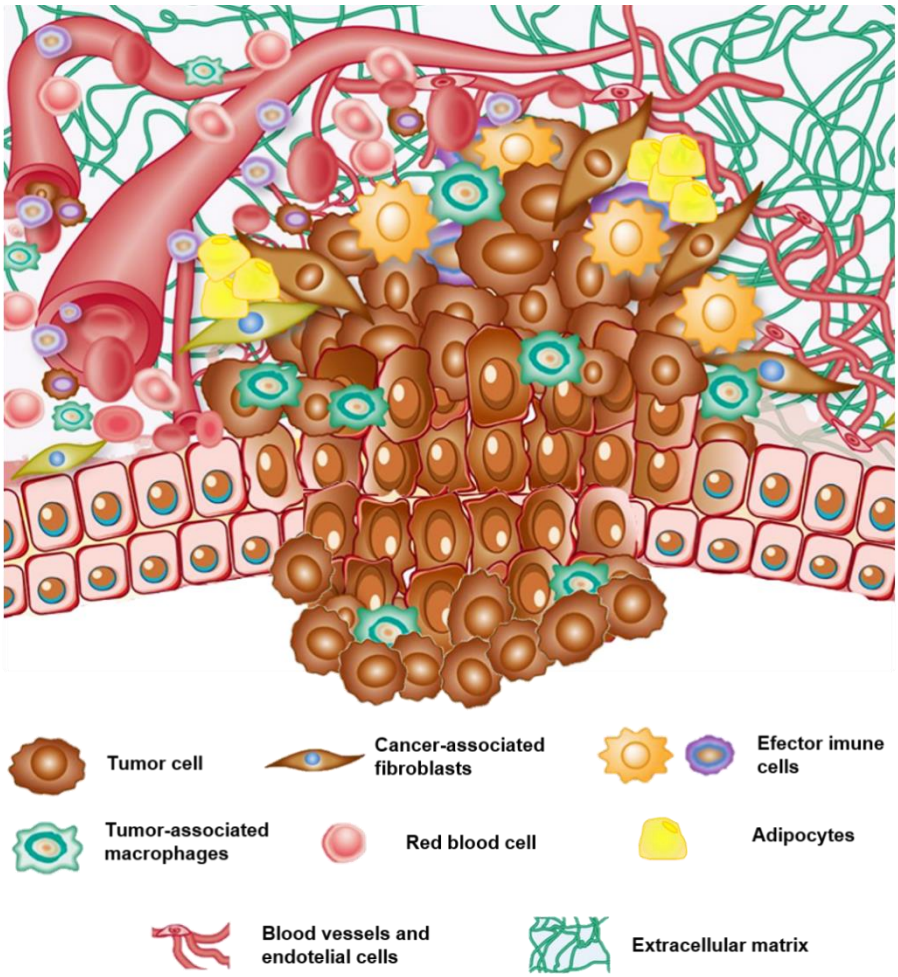


Figure 1.1. Schematic representation of the primary tumor microenvironment. Tumor cells, derived from normal epithelial cells, are surrounded by a complex microenvironment formed by stromal cells, namely fibroblasts, immune cells, such as macrophages and lymphocytes, adipocytes and endothelial cells, and by a complex non-cellular component that is the extracellular matrix. *Adapted from Berindan-Neagoe I, Clin Can Res, 2014, with permission from AACR* [9].

fact, cannot be dissociated from mutations [10]. The combination of all these alterations represents an advantage to the cells, providing them the capacity to migrate and invade adjacent basement membranes. Once tumor cells reach a blood vessel, they will enter the bloodstream, a process named intravasation, which will allow their transport to a secondary site. There, they will leave the blood vessel, or extravasate, and colonize the new organ forming a metastasis [11].

In the specific case of colorectal cancer (CRC), the pathogenesis of the disease is fairly well established. Nowadays it is accepted that only 20% of CRC have familial origin, some being associated with hereditary nonpolyposis colorectal cancer (HNPCC) or familial adenomatous polyposis (FAP), while others remain without known mechanism. The other 80% are sporadic, being generally divided in three groups: about 85% present chromosomal instability (CIN), with great losses or gains of chromosomal material, others are characterized by epigenetic instability presenting the CpG island methylator phenotype (CIMP) pathway while others

display accumulation of numerous mutations throughout the genome, mainly caused by inactivation of MMR genes, resulting in a phenotype known as microsatellite instability (MSI), or [12].

Sporadic CRC development follows a step-wise progression of mutations in oncogenes and tumor suppressor genes that translate into the classical adenoma-carcinoma sequence (*Figure 1.2*). The earliest genetic change is, most frequently, the mutation and/or loss of the *adenomatous polyposis coli* (*APC)* gene that mediates the transition of single preneoplastic cells to aberrant crypt foci (ACF). The exact sequence of acquired genetic changes, accumulated subsequently to inactivation of *APC* is variable. *K-ras* mutations are found in about 50% of CRC and are thought to be relatively early events which correlate, in terms of histology, with the transition from early to intermediate adenomas. Disruption of the transforming growth factor (TGF)-βIIR/mothers against DPP homolog (SMAD)-2-4 pathway and mutations in MMR genes [e.g. MutL-homolog (hMLH) 1 and MutS-homolog (hMSH) *2*] have also been identified as key factors in the development and progression of CRC, while p53 mutations are believed to mark the transition from adenoma to carcinoma [13]. Once tumor cells accumulate these mutations they will start to invade the underlying tissue and, contrary to what happens in most tumors, will most likely form distant metastasis rapidly without the common latency period. One plausible explanation is that, after cells acquire the mutations needed to invade, very few, if any, genetic alterations are required to be able to colonize other organs, namely the liver [14].

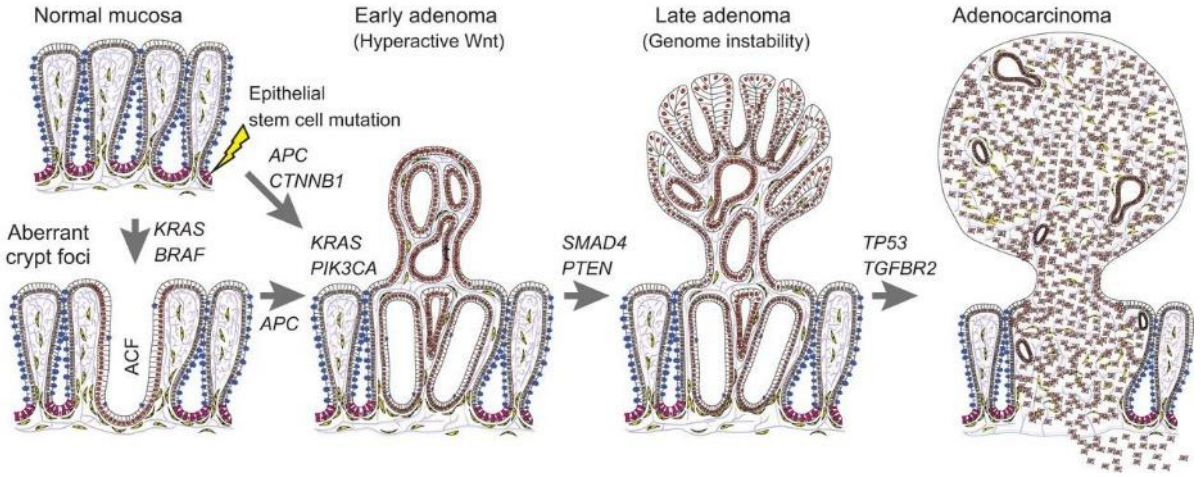


Figure 1.2. Step-wise progression in sporadic colorectal cancer. Upon initial mutations in the epithelial stem cells, most frequently in the *adenomatous polyposis coli (APC)* gene, there will be accumulation of mutations in both oncogenes and tumor-suppressor genes, resulting in sequential development of pre-malignant lesions, culminating in an invasive adenocarcinoma. BRAF, B-Raf proto-oncogene, serine/threonine kinase; KRAS, Kirsten rat sarcoma viral oncogene homolog; CTNNB1, catenin beta-1; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; SMAD4, Mothers against DPP homolog family member 4; PTEN, phosphatase and tensin homolog; TGFBR2, transforming growth factor beta receptor 2; TP53, tumor protein p53. *From Strubberg AM, Dis Model Mech 2017* [15].

2.2. Fibroblasts

Fibroblasts are the major ECM producers, being particularly relevant during wound healing, embryonic development and tissue repair and regeneration. In cancer, fibroblasts get activated similarly to what happens in a wound situation, gaining increased expression of α-smooth muscle actin, which leads to their differentiation into myofibroblasts [16]. Several mechanisms are described as being involved in this differentiation, namely the release of cancer cell exosomes expressing high levels of TGF-β which, in turn, induces the production of fibroblast growth factor (FGF)-2 by fibroblasts [17]. Indeed, many of the interactions established at the tumor microenvironment seem to occur through paracrine signaling. Breast cancer cells were described to stimulate hepatocyte growth factor (HGF) production by fibroblasts which, in turn, enhance breast cancer cell HGF receptor (HGFR/c-Met) activation, promoting colony formation in soft agar and facilitating tumor growth in mice [18]. Interestingly, de Wever *et al.* demonstrated that myofibroblasts isolated from human colon tumors, and contrarily to what happened with fibroblasts isolated from adjacent normal tissue, induced colon cancer cell migration through the secretion of HGF and Tenascin-C (TNC). Importantly, both of the pro-invasive signals were required, but not sufficient, for such stimulation, with HGF acting through Rac activation and TNC through RhoA inactivation [19]. Importantly, recent work in prostate cancer revealed that TNC is a marker of CAFs and a predictor of poor prognosis [20]. Moreover, epithelial cancer cells were shown to suppress p53 expression in fibroblasts, a mechanism not dependent of cell-cell contact. Also in this specific case, human cancer-associated fibroblasts (CAFs) were more susceptible to this suppression than normal fibroblasts [21]. Given the non-cell-autonomous effects of stromal p53, namely by inhibiting cell growth [22] and angiogenesis [23], this mechanism may contribute to overcome the fibroblast-mediated tumor suppression. MMPs are another player in this crosstalk. One such example is Stromelysin-3, a MMP mainly produced by fibroblasts, which was shown to promote the homing of malignant breast cancer cells in a mouse model, a process dependent on the presence of ECM-associated growth factors [24]. Fibroblast-derived MMP-1 was also reported to induce breast cancer cell migration by binding to protease-activated receptors 1 (PAR1) in tumor cells, cleaving it and triggering PAR1-Dependent Ca^{2+} signaling [25]. Fibroblasts were also demonstrated to increase tumor incidence, size and metastasis when injected in an orthotopic nude mouse model of pancreatic cancer, demonstrating direct involvement in cancer development [26].

Additionally, fibroblasts communicate with other stromal cells. By secreting various cytokines and chemokines, namely chemokine (C-C motif) ligand (CCL)-2 [27], osteopontin (OPN), chemokine (C-X-C motif) ligand (CXCL)-1, CXCL2, interleukin (IL)-6 and IL-1β [28] they recruit immune cells, which mediate the inflammatory response. Moreover, they are involved in

angiogenesis through the release of vascular endothelial growth factor (VEGF) [29] or stromal cell-derived factor-1 (SDF-1), which induce the recruitment of endothelial progenitor cells [30].

2.3. Endothelial cells

The reciprocal interactions established between tumor and endothelial cells are also extremely important for tumor progression [31]. The so called “angiogenic switch”, describing the moment when pro-angiogenic factors are predominant over the anti-angiogenic ones, ultimately resulting in an abnormal vascularization of tumors, is a key event during carcinogenesis [32]. As a result, primary tumor cells will have access to nutrients and oxygen, which enables their growth [33], will more easily intravasate through the fenestrated new blood vessels, reaching the circulation, and will be able to disseminate and metastasize to distant organs. Tumor cells, on their turn, are able to stimulate endothelial cells by secreting factors such as VEGF, a potent pro-angiogenic factor involved in the induction of endothelial cell survival, proliferation, migration and branching. Tumor cells were also shown to secret other factors such as galectin-1, which will be uptake by endothelial cells and promote Ras signaling, resulting in the activation of the Ras/Mek/ERK cascade, ultimately leading to endothelial cell proliferation and migration [34]. The action of cancer cells can also occur through direct contact, by a mechanism involving Jagged-1 overexpression which will trigger endothelial cells Notch activation, resulting in enhanced neovascularization and tumor growth [35]. In addition, specific microRNAs (miRNA) are involved in endothelium activation, namely miR-132 which was shown to downregulate p120RasGAP, a crucial negative regulator of vascular development and remodeling. As a result, there is an increase of Ras activity leading to endothelial cell proliferation and increased tube formation, ultimately resulting in neovascularization [36]. Endothelial cell proliferation, branching and migration is also promoted by other stromal cells within the tumor microenvironment, namely fibroblasts, as mentioned above, bone marrow derived myeloid cells, through the production of IL-1β which will act on endothelial cells [37], or macrophages, a topic which we discussed in detail in the following chapters [38].

Moreover, endothelial cells are reported to promote tumor cell invasion independently of angiogenesis. Similarly to VEGF, conditioned medium from head and neck tumor cells enhance Bcl-2 expression in endothelial cells. On the other hand, endothelial cells transfected with Bcl-2 promote tumor cell invasion, a process involving CXCL1 and CXCL8 secretion by endothelial cells which will bind CXCR2 on tumor cells [39].

2.4. Adipocytes

Adipocytes are another stromal cell type within the tumor microenvironment that has been gaining increased attention by the scientific community [40]. In fact, the association between an increased body-mass index and cancer incidence is not new and has been reported for many cancers [41]. Nevertheless, the mechanisms through which adipose tissue contributes to tumor development, growth and metastization are only now being fully understood [42]. Adipocytes within tumors, termed cancer-associated adipocytes, present an "activated" phenotype characterized by secretion of cytokines and other inflammatory mediators, growth factors and hormones, called adipokines [43]. One example is the secretion of leptin by adipocytes which will act in an autocrine way, leading to aromatase production and estrogen levels increase, resulting in breast cancer cell growth [44]. Additionally, leptin can act directly on cancer cells as it was reported in a murine model of colon cancer in which leptin promoted tumor cell proliferation by binding to leptin receptor, and subsequently activating the signal transducer and activator of transcription 3 (STAT3) [45]. Conversely, adiponectin, other hormone also secreted by adipocytes, was reported to inhibit cell proliferation by selectively binding to growth factors, specifically platelet-derived growth factor (PDGF)-BB, basic FGF (bFGF), and heparin-binding EGF-like growth factor (HB EGF), and thus preventing their interaction with the respective receptor [46]. Interestingly, adiponectin is decreased in several cancers [40]. The involvement of IL-6 was also shown to be a key player in both breast cancer cell invasion [47] and radioresistance mediated by adipocytes [48]. Indeed, the link between adipocytes and inflammation, namely due to the close interplay with immune cells such as macrophages, emerged as crucial in the establishment of a permissive microenvironment, supportive of tumor growth and progression [49].

2.5. Immune cells

Immune cells are responsible not only for the detection and elimination of foreign agents that may cause an injury or infection but also for the removal of death or mutated cells from a given organism. When DNA repair mechanisms or cell cycle check points fail and do not conduct damaged cells towards apoptosis or autophagy, immune cells work as a second line of defense. Myeloid cells, namely macrophages, dendritic cells (DCs), mast cells and granulocytes (eosinophils, basophils and neutrophils), and lymphoid cells, as γδT and natural killer (NK) T cells, are mediators of primary innate immune responses, which are not specific for a given pathogen or insult. In contrast, T and B cells exert specific functions towards an antigen exposed by antigen presenting cells (APCs) and mediate the latter adaptive immune response [50]. The notion that immune cells are involved in cancer development is not new. Already in 1863 Rudolf Virchow, a German pathologist, reported a strong leukocyte infiltration

in tumors and suggested a relation between this finding and the occurrence of the disease [51]. Since then, his theory has been extensively proven by a series of epidemiological studies and, nowadays, it is unquestionable that chronic inflammation predisposes individuals to various types of cancers. In fact, chronic infection and inflammation have been proposed to contribute to about 25% of all cancers worldwide [52], as in the case of *Helicobacter pylori* for gastric cancer, and inflammatory bowel disease for CRC [53]. Besides these extrinsic factors, which favor the mutational rate and may lead to cancer initiation, there is also an intrinsic pathway involving genetic alterations of oncogenes and tumor-suppressor genes, ultimately resulting in an inflammatory microenvironment [54]. But how does it work? (*Figure 1.3*) Regardless of being caused by extrinsic or intrinsic factors, there are specific transcription factors that become activated in pre-malignant or tumor cells, namely nuclear factor-κB (NF-κB), STAT3 and hypoxia-inducible factor 1-α (HIF1α). Of these, NF-κB was proposed to be the master regulator linking cancer and inflammation [55], being involved in processes such as

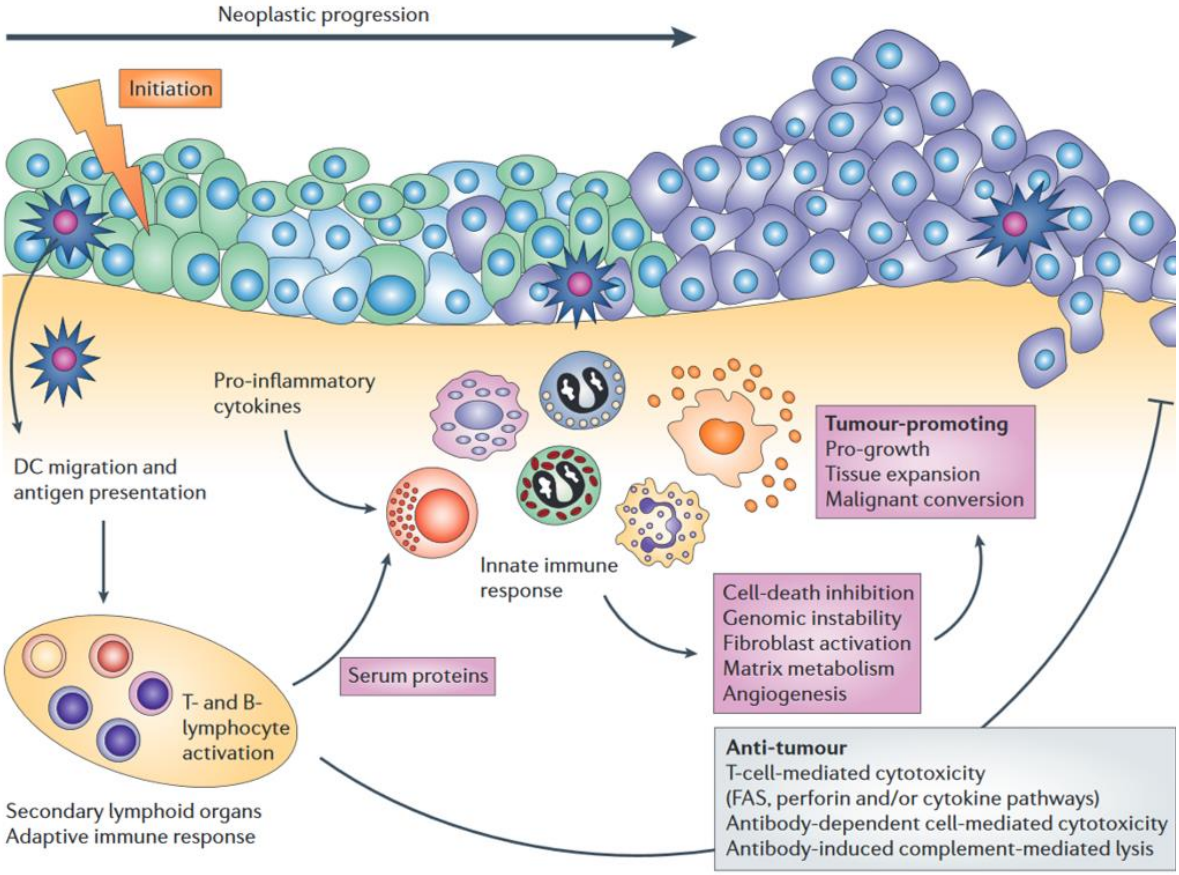


Figure 1.3. Involvement of innate and adaptive immune cells in inflammation associated cancer development. Antigens present in early neoplastic tissues will be transported to lymphoid organs by dendritic cells (DCs), resulting in the activation of adaptive immune responses, ultimately leading to either tumor-promoting or anti-tumor effects. Activation of B cells and humoral immune responses results in chronic activation of innate immune cells in neoplastic tissues, namely mast cells, granulocytes and macrophages, leading to the production of pro-survival, pro-angiogenic and tissue remodeling factors. On the other hand, adaptive immune cell activation also mediates tumor cell killing, either by T-cell mediated cytotoxicity, by activation of perforin/granzyme or Fas/FasL pathways, or by antibody-dependent cell- mediated cytotoxicity. Reprinted by permission from Springer Nature on behalf of: de Visser KE, Nat Rev Cancer 2006 [59]

tumor cell proliferation, apoptosis inhibition, angiogenesis, metastasis [56] and orchestration of both innate and adaptive immune surveillance [57]. Once upregulated, these transcription factors will induce the expression of a series of chemokines, cytokines and inflammatory enzymes, such as cyclooxygenase-2, by tumor cells, resulting in the recruitment of cells of the innate immune system, specifically dendritic cells, macrophages, neutrophils and NK cells. These will also produce inflammatory chemokines and cytokines, namely tumor necrosis factor (TNF)-α and IL-6, MMPs and growth factors further sustaining the inflammatory environment. Additionally, upon the immune cells attack, the secretion of reactive oxygen species will also contribute to DNA damage and mutations [58], further supporting a mutagenic environment. Some of the acquired genetic alterations may result in immunogenic peptides which will be presented at the surface of early neoplastic cells by major histocompatibility complex (MHC) class Ia molecules in the form of neoantigens, and will be recognized by the immune system as “non-self”. This information will be transported by dendritic cells to lymphoid organs leading to the activation of adaptive immune responses, specifically T- and B- lymphocyte responses, resulting in anti-tumor effects through T-cell and antibody-dependent cell-mediated cytotoxicity. At the same time, there will be chronic activation of innate immune cells within tumors which will secret pro-survival and pro-angiogenic factors [59].

One important concept regarding immune system and tumor development is cancer immunoediting, which intends to describe the host-protecting and tumor-sculpting actions of the immune system that not only prevent disease, by suppressing the formation of nascent tumors, but also shapes tumorigenesis. Cancer immunoediting encompasses three phases: elimination, which relies on immune surveillance, equilibrium, based on immune selection, and escape [60, 61] (*Figure 1.4*). In the elimination phase, there is involvement of cells from both innate and adaptive immunity. The first cells to arrive to the tumor site are neutrophils, followed by macrophages, which will exert an unspecific phagocytic activity against mutated cells, while releasing several pro-inflammatory cytokines (IL-1β, TNF-α IL-6, IL-8, and IL-12), chemokines, leukotrienes, prostaglandins, and complement proteins. These will regulate the adaptive immunity by recruiting and activating T cells. Macrophages, and particularly DCs, act as antigen presenting cells. At the tumor site, DCs recognize neo- or mutated antigens, become activated, maturate and migrate to peripheral lymph nodes, where they synthesize MHC and express co-stimulatory CD80 and CD86 molecules, in order to effectively present the antigens to naïve T cells. At this time, additional T cell co-stimulatory molecules, CD4 (for $CD4^+$ T cells) or CD8 (for $CD8^+$ T cells), maximize the interaction between the T cell receptor (TCR)-CD3 complex and the MHC-II molecule expressed on the antigen presenting cell surface, strengthening the immunological synapse. Activated T cells may then return to tissues to exert their immune functions. CD4 T cells may differentiate into T helper type I (Th1), producing

interferon (IFN)-γ, TNF-α, IL-2 and activating macrophages, or into T helper type II (Th2) cells, producing IL-4, IL-10 or IL-13 and reducing macrophage pro-inflammatory activity. In the presence of anti-inflammatory mediators, CD4 T cells may differentiate into forkhead box P3 (FOXP3) regulatory T cells (Tregs), creating an immunosuppressive environment. On their turn, CD8 T cells exert cytotoxic activity directly against the tumor cells, inducing perforin/granzyme or Fas/FasL-mediated apoptosis. Finally, the coordinated action between innate and adaptive immune cells will recognize transformed cells ultimately leading to their eradication through the activation of transcription factors and IFN-γ dependent pathways [63].

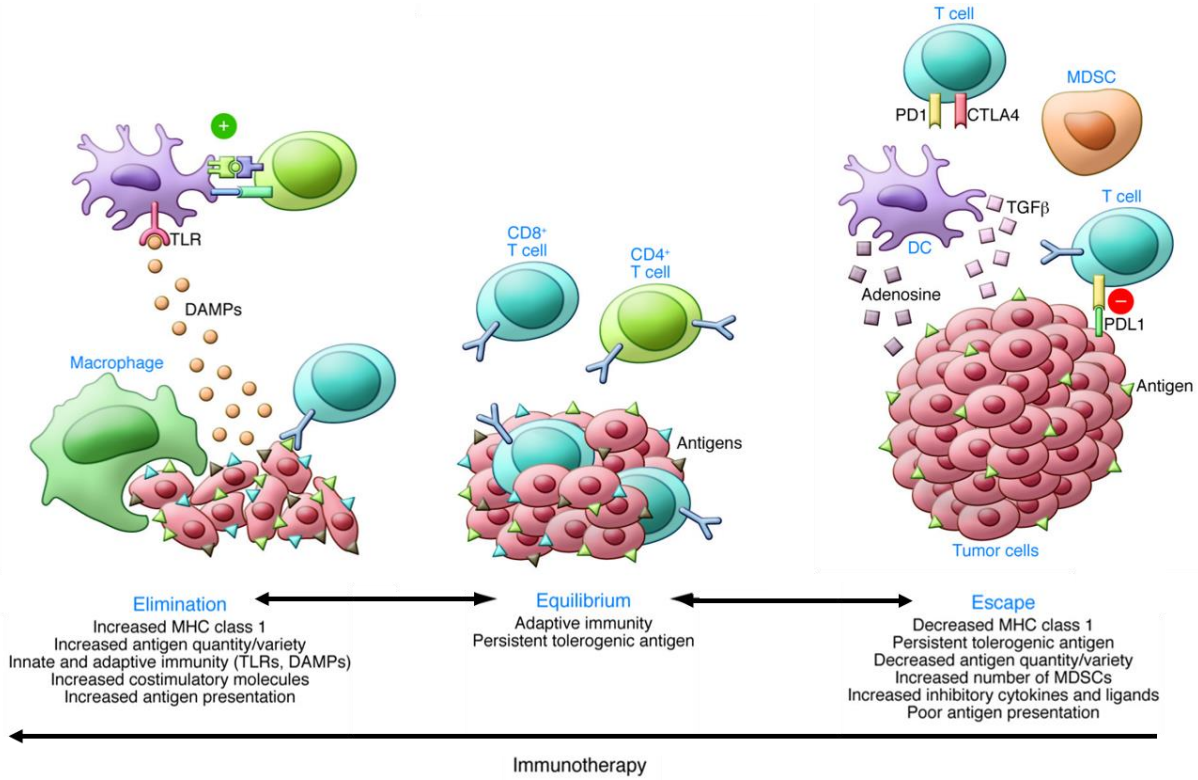


Figure 1.4. Immunoediting hypothesis encompassing three stages: the elimination, the equilibrium and the escape. The elimination phase usually occurs in the early stages of tumor development and relies on the expression of neo- or mutated antigens by tumor cells. These will be recognized by dendritic cells and macrophages that will activate the adaptive immune cells, which will ultimately lead to tumor cell killing. If this process is not completely effective, some tumor cells will survive and will be maintained in a dormant state, coexisting with immune cells from both innate and adaptive immune system, in a delicate equilibrium. After a series of adaptations by the tumor cells, namely by decreasing antigen presentation or by releasing immunosuppressive cytokines, they will be able to surpass the immune system, escape their control and eventually proliferate and form malignant disease. Republished with permission of American Society for Clinical Investigation, from *Kalbasi A , JCI 2013*; permission conveyed through Copyright Clearance Center, Inc *[62].*

Nevertheless, there is the possibility that some cells might survive and will be kept in a dormant state, in a delicate equilibrium with the immune system. At this stage, as a result of constant selection pressure caused by the presence of immune mediators, or by therapeutic intervention, tumor cells undergo a series of genetic and epigenetic alterations originating tumor variants highly resistant and with the ability to escape the immune attack [64]. Two of the most frequent strategies developed by tumor cells to escape the immune system is the

decrease of antigen presentation and the induction of an immunosuppressive environment, namely by expressing proteins such as programmed death-ligand 1 (PD-L1). Finally, in the last phase, these selected cells will be able to proliferate and eventually invade the adjacent tissues and form metastasis [65].

Contrary to what happens with the infiltration of cells involved in chronic inflammation, high number of lymphocytes, namely T cells ($CD3^+$), cytotoxic T cells ($CD8^+$), or memory T cells ($CD45RO^+$), correlate with good prognosis in different tumors namely melanoma, breast and non-small cell lung cancer, among others [66]. Similar results were obtained by Galon and colleagues in CRC. By characterizing the infiltrating immune cells, focusing on markers of inflammation, Th1 adaptive immunity, and immunosuppression, they reported that the type, density and location of immune cells may predict clinical outcome. Moreover, this characterization was more accurate and reliable in predicting patient survival than the classical histophatological methods used, specifically the tumor/node/metastasis (TNM) classification established by the Union for International Cancer Control (UICC) [67]. As a result, an immune-classification named Immunoscore, based on the numeration of two lymphocyte populations (CD3/CD45RO, CD3/CD8 or CD8/CD45RO) quantified within the core of the tumor and on the invasive margin, was created. This was proposed to be introduced in the clinical setting, specifically in CRC, in order to improve prognosis and help to determine therapy response [68-70]. An international consortium is currently ongoing to validate the immunoscore relevance and eventually add this new component to cancer classification, ultimately including other cancer types [71]. Indeed, a meta-analysis by Fridman *et al.* comprising 124 studies validated the transversal positive association between specific T cells and survival. Conversely, regarding other immune populations, such as B cells, NK cells, myeloid-derived suppressor cells (MDSCs), macrophages and a subset of T-helper populations (Th2, Th17, Treg cells), the prognostic impact varied according to the tumor type [72]. One such example are the $FoxP3^+$ Tregs, which high density was associated with better survival and improved prognosis in CRC [73] whereas, in breast cancer, was correlated with increased relapse and shorter survival [74].

The importance of neoantigen expression and their recognition by the immune system in the prevention of tumor development and progression is well illustrated in the specific case of MSI colorectal tumors. Given the defects in MMR genes, these tumors have an abnormally high mutational load leading to an increased expression of neoantigens. As a result, tumor cells will most likely be detected by the immune system. Interestingly, these tumors also present a strong infiltration of cytotoxic T cells and, when both factors are combined, allow the identification of patients with favorable prognosis [75]. Also in melanoma, high mutational load is associated with improved survival [76]. Besides the number of neoantigens, their quality,

meaning their immunogenicity calculated by the probability of TCR recognition, is also extremely important. Recently, Balachandran *et al.* showed that the combination between highest antigen quality and the most abundant $CD8^+$ T cell infiltrates, stratified patients with the longest survival in pancreatic cancer [77].

Given the great advances in immuno-oncology, namely the capacity of the immune system to recognize and reject tumors combined with the increased knowledge on the strategies acquired by tumors cells to evade immune destruction, a new era in immunotherapy emerged. Adoptive immune therapy, using either natural host cells with antitumor reactivity, such as tumor-infiltrating lymphocytes (TILs), or genetically engineered ones, specifically chimeric antigen receptors (CAR) T-cells, have shown very positive results in different cancers [78]. As a result, CAR T-cell therapy was recently approved by Food and Drug Administration (FDA) for pediatric and young adult patients with acute lymphoblastic leukemia or advanced lymphoma [79]. On the other hand, the observation that blocking cytotoxic T-lymphocyte protein 4 (CTLA-4), a receptor expressed by T cells involved in their inhibition when bound to CD80 and CD86, enhanced antitumor immunity [80], opened new perspectives in immunotherapy. The introduction of immune-checkpoint inhibitors, targeting not only CTLA-4 but also the PD-L1/Programmed cell death protein 1 (PD1) pathway as a strategy to boost T cell activation, has contributed to revolutionize the field (*Figure 1.5*). Due to successful results obtained in clinical trials performed with monoclonal antibodies targeting either CTLA-4 or PD-1/PDL-1, these have also been approved by FDA to be used in different cancers [81, 82]. Despite the great enthusiasm in the field, the reality is that the number of patients that respond to such therapies, even in combination with conventional approaches as radio or chemotherapy, is still limited and quite unpredictable. Thus, in this era of personalized medicine, the attention is also directed towards the discovery of new biomarkers that will help to discriminate which patients will truly benefit from such therapeutic regimens [83]. Accordingly, Luksza and colleagues created a fitness model based on immune interactions of neoantigens with T cells, which predicts survival of patients with melanoma and lung cancer using anti-CTLA-4 and anti-PD-1, respectively [84]. In this constantly evolving “omics” period, high-throughput experimental designs and technologies are certainly contributing to increase the knowledge quickly translatable into the clinics. Rivzi *et al.*, by performing whole-genome sequencing of non-cell lung cancers treated with an antibody targeting PD-1, revealed that higher non synonymous mutation burden was associated with improved objective response [85]. In the specific case of melanoma, genomic and transcriptomic analysis of pretreated biopsies enabled the discovery of a transcriptional signature associated with anti-PD-1 resistant tumors. At the same time, patients with increased mutations in the DNA repair gene BRCA2 were the ones with increased response [76]. Furthermore, whole-exome sequencing

of melanoma samples, before and after anti-PD-1 treatment, led to the discovery of inactivating mutations of Janus kinase (JAK) 1 and JAK2 associated genes related to acquired resistance to PD-1 blockade [86]. Other strategies currently ongoing involve the study of MHC-associated peptidome aiming at finding predictive neoantigens suitable of being targeted by immunotherapies [87-89]. Finally, genomic, transcriptomic and proteomic analysis of single cells, specifically T cells after cancer treatment, are also expected to contribute to therapy

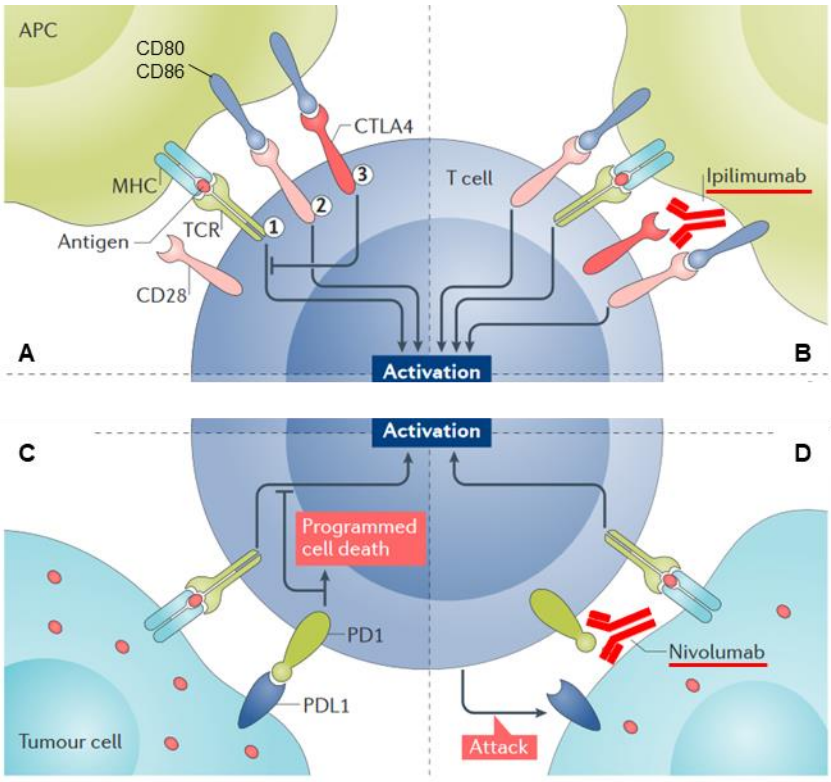


Figure 1.5. Immune checkpoint blockade in cancer therapy. (A) Normal activation of a T-lymphocyte by an antigen presenting cell requires binding of the major histocompatibility complex (MHC) presenting an antigen in the APC with T cell receptor, and interaction between CD28 and co-stimulatory molecules CD80 and CD86. CTLA4 in T cells compete with CD28 for CD80/CD86 ligands, preventing the interaction with CD28, and thus inhibiting T cell activation. **(B)** By neutralizing CTLA4 receptor with a monoclonal antibody, Ipilimumab, it will no longer bind to CD80/CD86, and the latter will be available to interact with CD28 resulting in T cell activation. **(C)** In order to evade T cell detection, tumor cells start to express the immune checkpoint activator programmed cell death ligand 1 (PD-L1), which will bind to PD1 in T-cell, preventing their activation. **(D)** Blocking PD1 or PD-L1 using a specific monoclonal antibody, Nivolumab, enables tumor cell detection by T-cells. *Adapted by permission from Springer Nature on behalf of: Byun DJ, Nat Rev Cancer 2*017 [90].

decision [91]. Indeed, besides the immunogenicity of these antigens, the immune contexture of each tumor has also proven to be determinant for the success of immunotherapies, particularly of the immune check point inhibitors [92]. In this sense, Chen and Mellman have recently proposed a cancer classification based on the immune phenotype they present: the immune-desert phenotype, the immune-excluded phenotype and the inflamed phenotype. The first two can be considered as non-inflamed tumors, being characterized by the expression of cytokines related with immune suppression or tolerance. What distinguishes both types is that, while the immune-desert has a diminished or absent infiltration of CD8-carrying T cells, the

immune-excluded phenotype is abundantly infiltrated by immune cells but these are retained in the stroma, and isolated from the tumor cells by a fibrotic capsule. For these reasons, both tumor types present a very low responsiveness to anti-PD-L1/PD-1 agents. On the other hand, the inflamed type is the one that presents higher infiltration of different immune cells, contains pro-inflammatory cytokines and, despite some exceptions, generally correlates with higher response rates to anti-PD-L1/PD-1 therapy [93]. Moreover, Fridman and colleagues have suggested the integration of immune, vascular and stromal gene-expression signatures of tumors in order to stratify patients for the most appropriate form of immunotherapy [92].

Albeit the fact that immunotherapy is mostly based on T-cells, the reality is that there are other immune populations within the tumor microenvironment, namely macrophages, with crucial roles in the tumorigenic process and hence are also a subject of intensive research [94].

2.5.1 Macrophages

2.5.1.1 Origin

Macrophages are present in virtually all tissues within the body and are key regulators in development, tissue repair, angiogenesis, homeostasis and disease, namely host defense mechanisms, inflammatory diseases and cancer.

Macrophage origin has been a topic that has witnessed great evolution in the last years. In 1970, Furth and colleagues included highly phagocytic cells and their precursors in a system called “mononuclear phagocyte system” (MPS), encompassing promonocytes, monocytes and macrophages. These cells had a common origin, being the promonocytes the most immature cells that differentiated sequentially into monocytes and these into macrophages, and shared specific characteristics, namely morphology and function [95]. The idea that circulating monocytes, originated from hematopoietic progenitors, were continuously replenishing macrophages within tissues lasted for many years and only in the last decade there was a complete shift in this paradigm. Nowadays, it is known that, in fact, tissues have tissue-resident macrophages [96] which, in the majority of cases, are able to self-maintenance independently of circulating monocytes. By using chimeric animals obtained by parabiosis, Ajami and colleagues were able to prove that microglia was maintained in the brain independently of bone-marrow derived progenitors, in both healthy and central nervous system (CNS) degenerative disease. In fact, microgliosis resulted exclusively from the expansion of CNS-resident cells [97]. This was further validated by work from Ginhoux *et al* who revealed that the adult microglia had a distinct ontogeny than the mononuclear phagocyte system and that these cells derived from primitive yolk sac macrophages [98]. More recently, it was shown that the same was true for other organs, namely lung, splenic red-pulp, peritoneal and bone marrow

[99]. Conversely, in the specific case of the intestine, yolk sac-derived macrophages, characterized by being F4/80^{hi}CD11b^{lo}, seem to be lost in adulthood and are the circulating monocytes, Ly6C^{hi}, which sustain macrophage populations in this organ, through a mechanism dependent of the CCR2 chemokine receptor. Interestingly, macrophage proliferation is mostly detected in neonatal colon but decreases gradually with age, concomitantly with the arrival of circulating monocytes [100]. Despite the recognized complexity regarding macrophage origin within adult tissues, the most updated model describes three major sources. It all begins early in embryogenesis, being the first macrophages derived from early embryonic progenitors during primitive hematopoiesis in the yolk sac. Once the circulatory system is established, they will colonize different organs in the embryo, namely brain, liver, kidney, spleen, lung, and skin. These yolk sac macrophages will also migrate to the fetal liver where, together with hematopoietic stem cells (HSC), originate myeloid progenitor cells. Once differentiated in fetal liver-derived monocytes, these cells will also populate the previously mentioned organs, contributing to the resident macrophage population, characterized by self-renewal capacity and high proliferative ability when homeostasis is lost. Apart from the CNS, in which microglia is exclusively originated from the primitive yolk sac derived macrophages, in the other tissues the main contributors are the fetal-derived monocytes. Additionally, within the fetal liver, HSC suffer an expansion and colonize the bone marrow. These cells will be the source of circulating monocytes in the blood, which will then be recruited to tissues with high turn-over, such as the intestine, as previously described, and in the case of infection or disease [96, 101-106].

2.5.1.2 Functions and classification

Macrophages were first reported by Élie Metchnifoff and his work won him the Nobel Prize for Physiology or Medicine in 1908. He discovered the process of phagocytosis and named the responsible cells as phagocytes [107]. Only later the term macrophage which, in fact, means big eaters, was adopted. Indeed, macrophages are described as professional phagocytes having a key role in maintaining tissue homeostasis.

Depending on the organ they are in, macrophages present different characteristics, perform specific functions and have precise nomenclatures, e.g. microglia in the brain, Kupffer cell in the liver, osteoclast in the bone, Langerhans cell in the spleen, alveolar macrophage in the lung, etc. [108]. Macrophages are innate immune cells responsible for the clearance of apoptotic or necrotic cells in tissues such as the respiratory system, gastrointestinal tract or central nervous system [108], and for the removal of red blood cells in the liver or spleen [109]. Macrophages are also important cells in bone morphogenesis and in ductal branching during the mammary gland development [110]. These cells benefit from their strategic location in the body, working as sentinels constantly searching for danger signals, particularly pathogens.

Expression of specific receptors by macrophages, namely pattern recognition receptors (PRRs) such as Toll-like receptors (TLR) or scavenger receptors, assures the specificity of the phagocytic process [111]. Upon a bacterial infection, specific structures present in many pathogens, known as pathogen-associated molecular patterns (PAMPs), will bind to PPRs in macrophages triggering the activation of a complex signaling pathway. Although there's a wide range of receptors involved is this process, including non-PPRs, TLRs are certainly the most studied ones [112], namely the binding of TLR4 to lipopolysaccharide (LPS) [113, 114]. This macrophage activation triggered by bacterial infections was first described by Mackaness *et al*. in the 1960s, in which they used mice infected by *Listeria monocytogenes*, *Brucella abortus*, or *Mycobacterium tuberculosis* [115]. They were able to prove that the antibacterial mechanism mediated by macrophages was non-specific and was highly dependent on IFN-γ or bacterial LPS. These macrophages activated by bacterial infection were described as being classically activated [116]. Upon activation, macrophages produce high levels of reactive oxygen intermediates and inflammatory cytokines, namely TNF-α, IL-1, IL-12 and IL-23, leading to an oxidative environment and to the recruitment of another immune cells, namely bone-marrow derived monocytes, neutrophils and T cells, which ultimately result in the killing of the microorganism [117]. Moreover, macrophages are able to directly kill microorganisms by engulfing them through a phagocytic process. The phagosome will then fuse with lysosomes, resulting in the phagolysosome which contains hydrolytic enzymes and toxic radicals [118]. This inflammatory response must be tightly controlled in time and space to avoid unwanted side effects, namely systemic inflammation, autoimmune disorders, or tissue damage. Mechanisms such as chemokine depletion, either by MMP degradation or sequestration by decoy receptors, and the clearance of apoptotic neutrophils by macrophages via efferocytosis are also key to stop the inflammatory response [119]. At this stage, macrophages completely change their cytokine secretion profile [120] switching to what has being described as a resolution-phase macrophage, characterized by the production of anti-inflammatory cytokines, namely IL-10 and TGF-β1, and also of VEGF [119]. Contrarily to what happens with leukotrienes and prostaglandins, known for their important role in promoting the inflammatory process, lipid metabolites such as lipotoxins (LXs) were shown to be important mediators in the resolution process [121].

Macrophages are probably the most plastic cells in the body, meaning that they are able to respond to external cues, constantly adjusting their phenotype and behavior. This amazing capacity has contributed, over the years, to a continuous evolution regarding macrophage nomenclature [122]. In the early 1980s, several groups reported the effect of specific cytokines, namely colony-stimulating factor (CSF-1) and IFN, on macrophage differentiation [123, 124]. Important work performed with *Leishmania* infected macrophages revealed that, comparing

with non-parasitized macrophages, these were less efficient in activating Th1 T cells but were able to increase Th2 T cell activation [125]. In 2000, Mills and colleagues proposed the terms M1 and M2 to describe macrophage polarization, a designation that intended to reflect the Th1/Th2 dichotomy. They made use of Th1- and Th2-type mouse strains, characterized by having lymphocytes that, in response to concanavalin, produced high IFN-γ/low IL-4 or the inverse, respectively. By using macrophages from these mouse strains they proved that, while Th1-macrophages produced nitric oxide (NO) in response to IFN-γ and/or LPS, Th2-macrophages produced ornithine, reflecting a differential arginine metabolism. TGF-β1 secretion was also shown to be the opposite in macrophages from both strains, being inversely proportional to NO production, and thus having an impact on the inflammatory response and macrophage functions [126]. Since then, macrophage polarization was proven to be considerably more complex than initially thought. Given the amount and diversity of environmental factors influencing macrophage polarization, ranging from cytokines and chemokines, growth factors, PRRs or hormones, it was proven that, in fact, these cells could display a series of functional phenotypes. More importantly, in many cases, macrophage plasticity was shown to be reversible [127]. In an attempt to include these new data in macrophage classification, Mantovani *et al.* suggested that M2 macrophages should be further divided into 3 groups depending on the stimuli they responded to: M1a, derived from IL-4 and IL-13 stimulation, M2b, induced by immune complexes together with TLR or IL-1R agonists, and the M2c, induced by IL-10 stimulation. These populations expressed different cell surface receptors and cytokines and, consequently, had specific functions: M2a were involved in Th2 inflammatory processes and parasite killing, M2b in immunoregulatory processes while M2c were a more “deactivated state” characterized mainly by being involved in matrix deposition and tissue remodeling. M1 macrophages, on the other hand, were a consequence of TNF or IFN-γ combined with LPS and led to type I inflammatory processes and intracellular pathogen killing. Despite this more strict classification, it was already recognized that the M1/M2 dichotomy in fact represented two extremes of a continuum of polarization status [128]. Also in disease, it was proposed that macrophage characteristics evolved together with the pathology and in fact mixed populations could co-exist in the same environment [129]. Recognizing macrophage complexity and plasticity, Mosser and Edwards suggested an alternative nomenclature represented by a colored wheel in which macrophages are grouped according to their functions in homeostasis, specifically host defense (classically activated macrophages), wound healing (wound-healing macrophages) and immune regulation (regulatory macrophages) [130].

Regardless of the classification used to define macrophages and besides their key role in maintaining tissue homeostasis, macrophages are implicated in the development of many

diseases, directly or indirectly related with chronic inflammation, namely atherosclerosis, diabetes, arthritis, asthma and cancer (*Figure 1.6)* [129, 131].

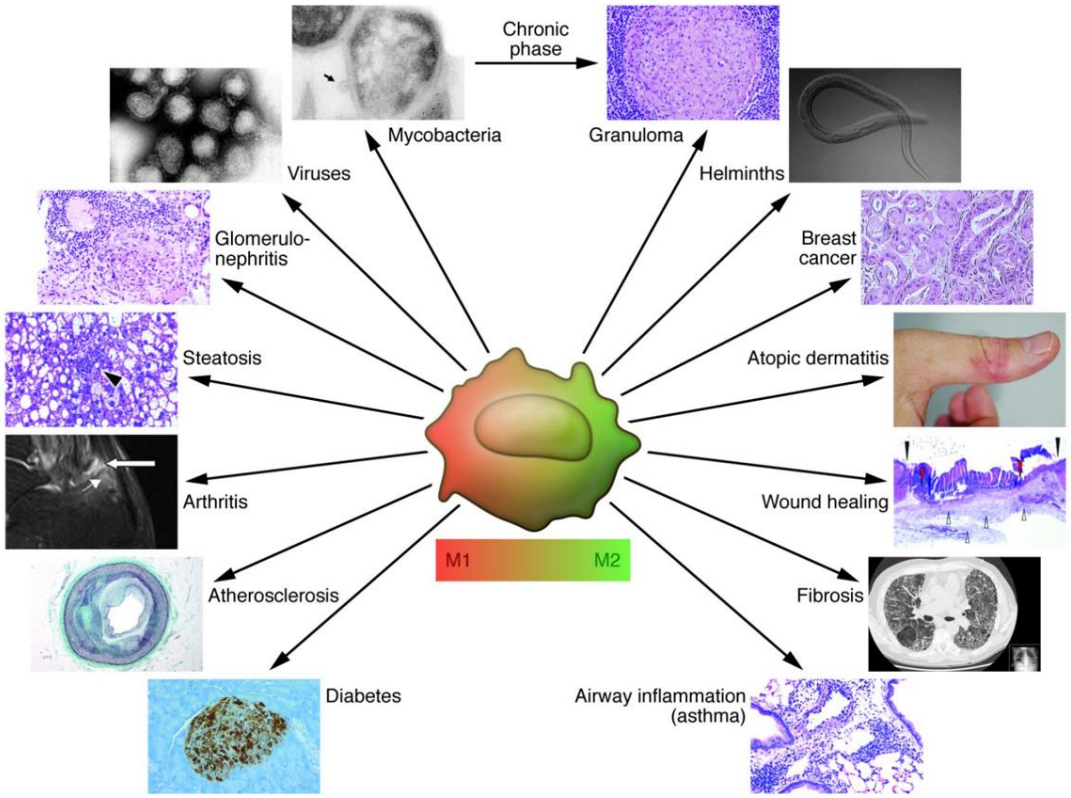


Figure 1.6. Macrophage plasticity in disease. Macrophages are actively involved in many diseases and their polarization changes from the M1 to the M2 phenotype with chronic inflammation. Additionally, in some situations, it is likely that different subpopulations coexist in the same environment. Republished with permission of American Society for Clinical Investigation, from *Sica A, JCI 2012*; permission conveyed through Copyright Clearance Center, Inc [129].

2.5.1.3 Tumor-associated macrophages

Tumor-associated macrophages (TAMs) are the most abundant cells within the immune infiltrate in tumors [132]. Several epidemiological studies have reported a clear association between TAM infiltration and advanced disease, worst prognosis and poorer outcome [133, 134], particularly in breast cancer [135-137], bladder cancer [138], hepatocellular carcinoma [139], melanoma [140], Hodgkin's Lymphoma [141], leiomyosarcomas [142] and gastric cancer [143]. On the other hand, in cases such as lung cancer, there is some conflicting data: TAMs infiltration has been associated with both worst [144, 145] and better prognosis [146, 147]. Also in CRC, some reports associate high macrophage infiltration with lower liver metastasis [148] and with improved survival specifically in the colon [149], while others state that, in fact, high TAMs correlate with worse outcome [150] and are associated with CRC progression [151]. For long, TAMs were described as originating from circulating monocytes [152, 153] which were recruited to the tumor site, specifically the $LYC6^{+}$ expressing monocytes [154]. More

recently, the spleen was also shown to be an important reservoir of monocytes constantly supplying the tumors [155]. This monocyte recruitment is promoted by chemotactic factors produced by both stromal and tumor cells. Factor chemotactic for mononuclear phagocytes was the first one described by Bottazzi and colleagues [156], which was later renamed monocyte chemoattractant protein-1 (MCP1), also known as chemokine CCL2 [157]. CCL2 has been shown to be critical for monocyte recruitment in a variety of tumors [158-160] and high CCL2 expression by tumors has been associated with poor prognosis [161]. This specific process is dependent on C-C chemokine receptor type 2 (CCR2) expression by monocytes [155] and, due to the importance of the CCL2/CCR2 axis in cancer, this is now seen as a potential target for therapeutic intervention [162]. Other factors such as monocyte colony stimulating factor (M-CSF) [163], VEGF [164, 165] and angiopoietin-1 [166] are also involved in monocyte recruitment to the tumor site. New data on the ontogeny of TAMs has shown that this is an heterogeneous population which, besides originating from circulating monocytes, can also be a result of resident macrophages derived from either embryonic progenitors, in brain, liver or lung, or from monocytic origin, namely in the intestine or mammary gland (Figure 1.7) [167]. Interestingly, by using the polyoma middle T (PyMT) oncogene-driven mouse model of breast cancer, Franklin *et al.* demonstrated that TAMs originated from circulating $Ly6C^{+}$ monocytes had a high proliferative capacity and their number was not affect using a *Ccr2*$^{-/-}$ genetic model [168]. Using a different spontaneous mammary mouse model, Tymoszuk and colleagues also reported high *in situ* macrophage proliferation, a mechanism dependent on CSF-1 [169]. In pancreatic ductal adenocarcinoma (PDAC) it was recently reported that TAMs have a heterogeneous origin, with tissue-resident macrophages derived from embryonic progenitors being a major source. Moreover, these embryonically derived macrophages were shown to expand in PDAC through *in situ* proliferation and, interestingly, exhibited a pro-fibrotic transcriptional profile [170].

Irrespectively of their origin, TAMs polarization is determined by the cytokines, chemokines and growth factors present at the tumor microenvironment. Even though it is plausible to think that, in the initial stages of tumor development, macrophage recruitment is intended to contribute to tumor eradication in fact, due to the strategies developed by cancer cells to evade the immune response, the immunoediting process will allow tumor escape. One of these strategies is the ability of tumor cells to produce immune suppressive mediators, namely IL-10 and TGF-β, which will subvert macrophage polarization towards an anti-inflammatory and pro-tumor phenotype. It has been suggested that TAMs share many characteristics of the M2 macrophages [171], namely the low production of pro-inflammatory cytokines, such as IL-12, and high levels of the anti-inflammatory IL-10 [172]. This specific characteristic was shown to be caused, at least partially, by a decreased activity of NF-kB due to an overexpression of p50

NF-kB in TAMs nucleus, which was associated with the inhibition of M1 inflammatory responses, in particular, with a defective IL12p70 production, and gain of tumor resistance [174]. Furthermore, TAMs express high levels of scavenger receptor A [175], have low cytotoxic activity as a result of a reduced production of nitric oxide synthase [176] and are predominantly immunosuppressive. They produce CCL17 and CCL22 [177], both agonists of CCR4 [178, 179], involved in the recruitment of cells without cytotoxic activity such as Th2 cells and Tregs. They also secrete CCL18, another immunosuppressive chemokine, which recruits naïve T cells [180] that contribute to immune regulation and tolerance. Additionally, TAMs themselves secrete IL-10 and TGF-β involved in the suppression of T cell activation and proliferation [181-183]. Finally, and similarly to what happens with M2 macrophages involved in wound healing and tissue repair, TAMs are high producers of pro-angiogenic factors, such as VEGF, bFGF, CXCL8 and urokinase-type plasminogen activator (uPA) [184-188] and proteases, namely MMPs and specific cathepsins [189-191].

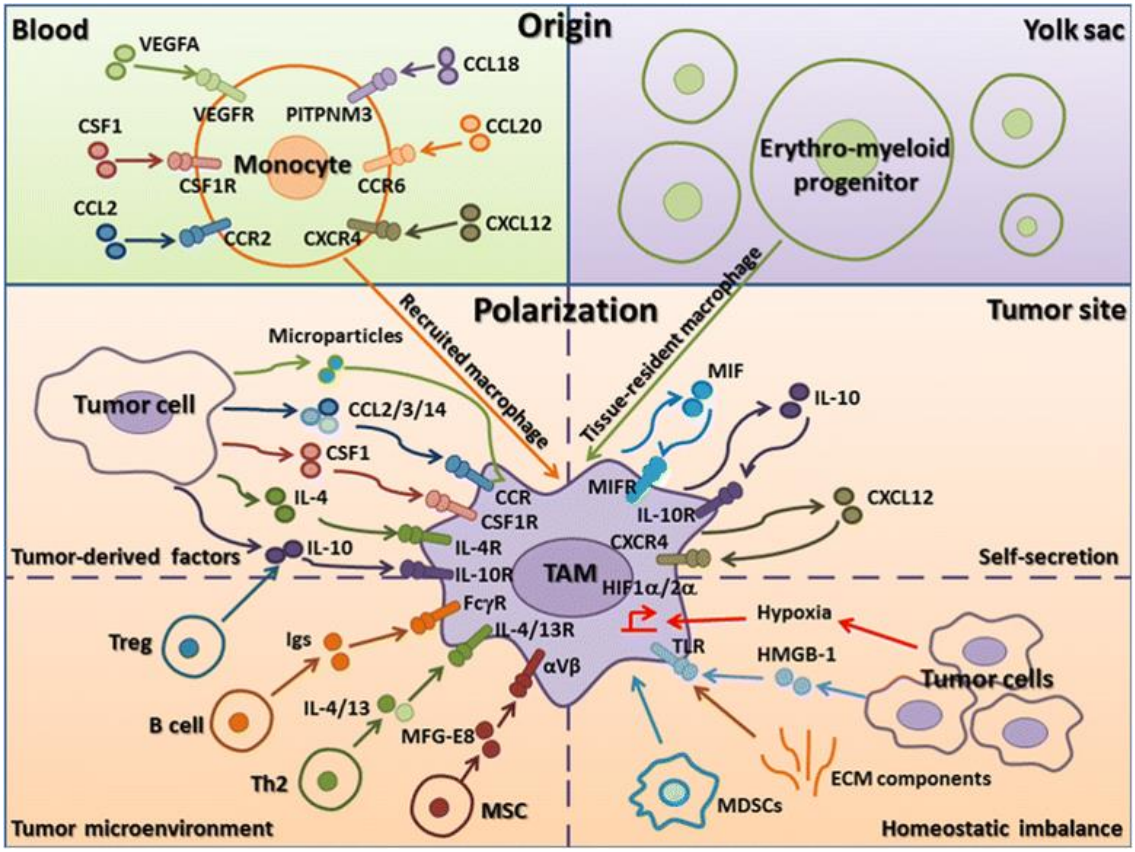


Figure 1.7. Representation of tumor-associated macrophages (TAMs) origin and the factors within the tumor microenvironment that are involved in their polarization. TAM originate from tissue-resident macrophages (purple), from embryonic progenitors, or from blood-derived monocytes (green). Regardless of their origin, their differentiation and polarization is dependent of the factors secreted by both tumor and stromal cells, namely immune cells including macrophages themselves, such as IL-10, CCL2, CSF1, VEGFA, CCL18, CCL20, and CXCL12. Moreover, factors such as hypoxia or aberrant ECM are also important players in TAM polarization. *From Yang L, J Hematol Oncol 2017* [173].

Nowadays it is unquestionable the role of macrophages during all steps of the tumorigenic process, ranging from tumor growth, invasion and metastasis (*Figure 1.8*) [192]. In different models, macrophages were shown to produce factors that stimulate tumor cell proliferation, such as IL-1 and IL-6 in colon cancer [193, 194], prostaglandin E2 (PGE2) in melanoma [195] and stress inducible protein 1 (STI1) in glioblastoma [196]. Other mitogenic factors secreted by macrophages, specifically epidermal growth factor (EGF) [197] and PDGF [198], were also reported to induce tumor cell proliferation. TAMs are also key regulators of the angiogenic switch [199]. Work by Lin *et al.* using the PyMT breast cancer mouse model revealed that macrophage infiltration occurred prior to the angiogenic process and, more importantly, that macrophage depletion inhibited vessel formation [200]. Tumor blood vessels are characterized by an abnormal morphology, organization, fenestration and function. Contrarily to what

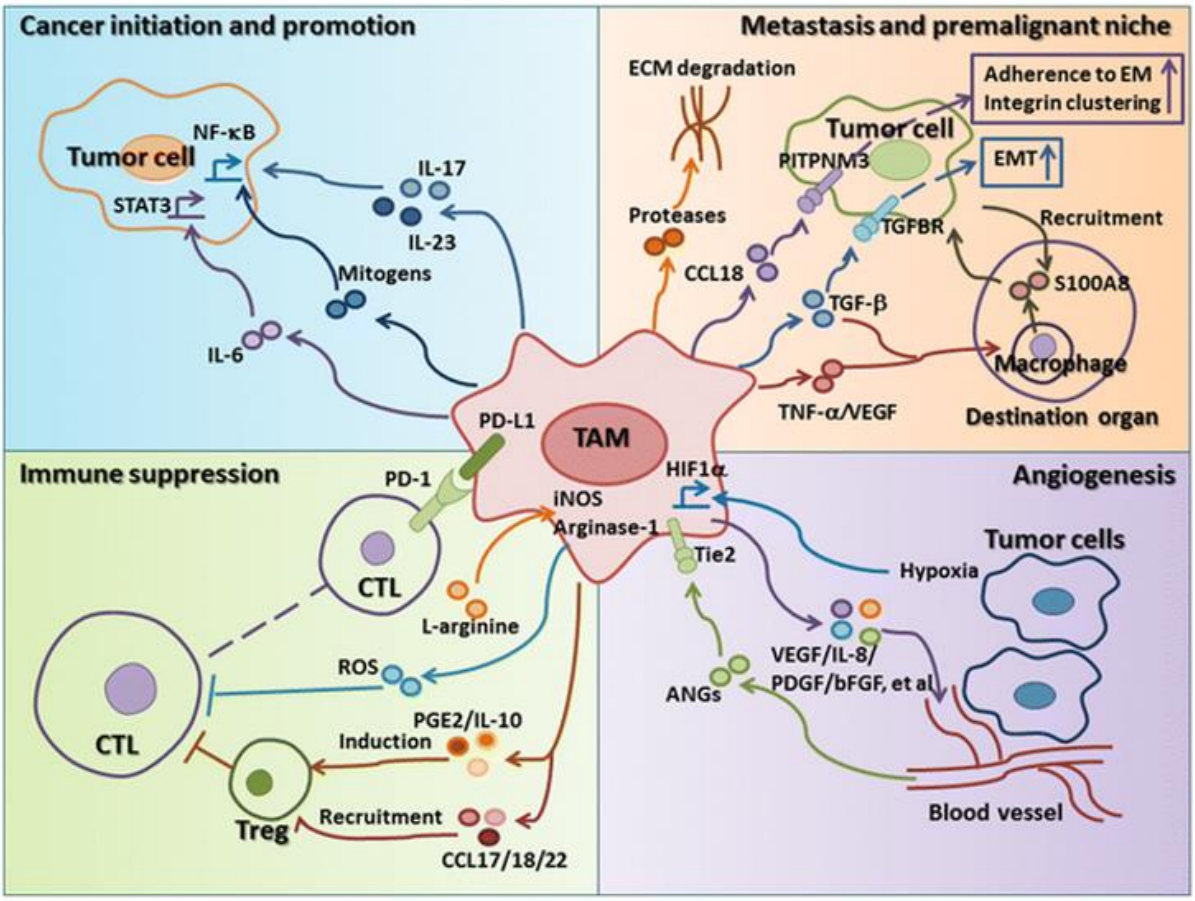


Figure 1.8. Tumor-associated macrophages (TAM) role in tumor development and progression. TAM are involved in several steps in the carcinogenic process mainly through the secretion of a variety of soluble factors. Release of factors such as IL-6 and EGF contribute to tumor cell proliferation (blue). By expressing PD-L1, TAMs are able to directly suppress T-lymphocyte activation. Moreover, release of factors such as IL-10, CCL17/18/22 promote Treg recruitment which further inhibit T cells (green). TAMs are key regulators of the metastatic process, by contributing to ECM remodeling, through MMPs release, promoting epithelial-to-mesenchymal transition, by TGF-β, and stimulation cell migration. Moreover, they are also involved in pre-metastatic niche formation and tumor cell recruitment (orange). Due to the hypoxia tumor microenvironment, TAMs will release many pro-angiogenic factors which will act of endothelial cells resulting in a dense and abnormal vasculature (purple). *From Yang L, J Hematol Oncol 2017* [173].

happens to the normal vasculature, the newly formed tumor vessels do not exhibit a hierarchical organization but, instead, have irregular branching patterns, altered lumen sizes and chaotic distribution [201]. Moreover, they are quite leaky due to an abnormal distribution of endothelial cells and pericytes resulting in the impairment of the barrier function [202]. One of the main reasons for this anomalous vasculature is the imbalanced production of pro-angiogenic factors, particularly members of the VEGF family, such as VEGF-A and placenta growth factor (PGF), and FGF. As a result, and although there is an increased density of blood vessels, tumors present many hypoxic regions with very low oxygen tensions [203]. So far, a clear association between macrophage infiltration and higher vasculature density in cancers such as breast [135], oral [204], cervical [205] and lung [144] has been reported. Additionally, macrophages are preferentially recruited to hypoxic areas within tumors [186, 206] and tumor cell-derived cytokines, namely oncostatin M and eotaxin, are described to prime macrophages towards a pro-angiogenic phenotype with high production of factors such as VEGF [207]. Indeed, the tumor hypoxic environment will up-regulate both HIF1 and HIF2 in TAMs, resulting in the production of a series of pro-angiogenic factors, as previously mentioned [208, 209]. Moreover, macrophages will also produce proteases, such as MMP9, which will release angiogenic factors, namely VEGF, from extracellular reservoirs, making them bioavailable to act on endothelial cells [210]. Interestingly, De Palma *et al.* showed that there is a specific monocyte population in the bone marrow which expresses Tie2, an angiopoietin receptor, named TIE2-expressing monocytes (TEMs), that are recruited to tumors and are essential for the angiogenic process and the establishment of the angiogenic switch [211]. These TEM population accounts for less than 7% of the peripheral blood mononuclear cells (PBMCs) and, besides being detected in blood from cancer patients, are present in human tumors, including breast, renal, colon and lung carcinoma. Conversely, this population is almost absent in the non-neoplastic tissues from the same patients [212]. Furthermore angiopoietin-2 (ANG2), a Tie2 ligand secreted by endothelial cells, was shown to induce chemotaxis in human monocytes [213]. Mazzieri and colleagues reported that, by targeting the ANG2/TIE2 axis, specifically through the blocking of ANG2 in endothelial cells, were able to reduce tumor angiogenesis and, as a consequence, inhibit tumor growth and progression. Additionally, knockdown of Tie2 gene in TEMs, did not affect macrophage number within tumors but was sufficient to decrease tumor angiogenesis [214].

The ability of tumor cells to start migrating and invading the adjacent tissues is what tips the balance towards malignant disease. Importantly, macrophages are considered critical elements in this complex process. In breast cancer it has been clearly demonstrated that macrophages and tumor cells establish a paracrine loop: while cancer cells produce CSF-1, promoting macrophage differentiation through binding to colony stimulating factor receptor 1

(CSFR1), macrophages produce EGF, stimulating tumor cell migration [215] and invasion by binding to epidermal growth factor receptor (EGFR), both *in vitro* [216] and *in vivo* [217]. Moreover, Yamaguchi and colleagues have shown that EGF regulates the invadopodia formation in cancer cells while CSF-1 controls podosome formation in macrophages [218]. As a result, there is a directional migration of both cell types towards blood vessels. Macrophage contribution to the invasion process is also related with the secretion of several proteases, such as cathepsins and MMPs, which will degrade and remodel the ECM, making it more permeable to the invading cancer cells [189, 219, 220]. In an attempt to provide novel insights into the molecular crosstalk established between cancer cells and macrophages, recent work from our group focused on the influence of primary human macrophages on gastric and CRC cells, considering invasion, motility/migration, proteolysis and activated intracellular signaling pathways [221]. We demonstrated that macrophages stimulate cancer cell invasion, motility and migration, and that these effects depend on MMP activity and on the activation of EGFR, specifically at the residue Y^{1086}. EGF-immunodepletion impaired macrophage-mediated cancer cell invasion and motility, suggesting that EGF is the pro-invasive and pro-motile factor produced by macrophages. Macrophages also induced gastric and CRC cell phosphorylation of Akt, c-Src and ERK1/2, and led to an increase of RhoA and Cdc42 activity. Moreover, by polarizing human macrophages with LPS or IL-10, to induce a M1 or M2 polarization, we confirmed that, indeed, IL-10 stimulated macrophages were more efficient in stimulating cancer cell invasion, as a result of a higher MMP-2 and MMP-9 secretion [222].

Besides the invasive capacity of cancer cells, the intravasation and extravasation processes are also required for a successful metastization. In this regard, elegant work by Wyckoff and colleagues, using multiphoton microscopy, revealed that tumor cell intravasation occurred in association with perivascular macrophages and correlated with their amount [223]. The direct apposition of an invasive carcinoma with a macrophage and an endothelial cell was named the tumor microenvironment of metastasis (TMEM), and the density of these microanatomic structures were shown to predict distant metastasis in breast cancer [224, 225].

Once tumor cells enter the circulation, they will have to survive in the harsh environment that is the blood stream until reaching the secondary organ where they will extravasate and form the metastasis, culminating in a malignant disease. Although the metastatic process is quite inefficient, since the majority of tumor cells in circulation, the so called circulating tumor cells (CTCs), are not able to survive, the reality is that some cells are successful in such process [226]. The concept of the pre-metastatic niche defines the particular environment, distant from the primary tumor, which presents the most favorable characteristics suitable for the homing of the CTCs [227]. Interestingly, the metastasis location is not randomly distributed. Instead, each tumor type has specific organs more commonly colonized by its CTCs, highlighting the

specificity of this process [14, 228]. Importantly, macrophages were proven to be critical elements contributing to the success of this complex event [229]. Kaplan and colleagues have reported that cells derived from the bone marrow, specifically hematopoietic progenitors expressing VEGFR1, cluster in pre-metastatic niches before the arrival of tumor cells, both in mice and human. They also reported that the conditioned media secreted by tumor cells had a role in the specificity of metastasis location [230]. Accordingly, it was shown that factors produced by hypoxic mammary tumor cells, namely CCL2, CSF-1, VEGF and MMP-9, promote the recruitment of bone marrow-derived cells (BMDC), including macrophage precursors, to the lungs and, as a consequence, promote metastasis formation [231]. Indeed, CCL2 has been implicated both in the extravasation of breast cancer cells in the lung, mediated by $CCR2^{+}$ macrophages [160], as well as in an increased retention of these metastasis-associated macrophages by the activation of CCR1 through CCL3. Tumor-derived tissue factor (TF), known for its procoagulant role, was also shown to be important in the pre-metastatic niche formation, by establishing a platelet clot involved in the recruitment of monocytes and macrophages and in metastatic cell survival [232]. Moreover, interesting work by Chen *et al*. revealed that macrophages contribute to the survival of breast cancer cells invading the lungs by binding to vascular cell adhesion molecule-1 (VCAM-1) overexpressed by tumor cells, triggering survival mechanisms, including Akt signaling [233].

All these data regarding macrophage involvement in the tumorigenic process supported the potential of TAMs as attractive targets for therapeutic intervention, either alone or in combination with conventional treatment approaches (*Figure 1.9*) [234, 235]. The efforts of targeting TAMs becomes even more relevant taking into consideration that these cells are radioresistant, which means they are not killed by the most common form of cancer treatment, radiotherapy [236]. Furthermore, our group has recently shown that, although a cumulative dose of 10Gy resulted in an alternation of macrophage profile towards a pro-inflammatory-like phenotype, these cells are still able to stimulate angiogenesis and CRC cell invasion *in vitro* [237]. Interestingly, using a glioblastoma model, Leblond *et al.* revealed that M2 macrophages are more resistant to radiation than M1 macrophages in both normoxia and hypoxia conditions [238]. In this regard, different strategies are being studied in order to target TAMs and several clinical trials are currently ongoing [173]. One possible approach is to prevent monocyte recruitment by targeting, for example, the CCL2/CCR2 axis [239, 240]. As an alternative, TAMs

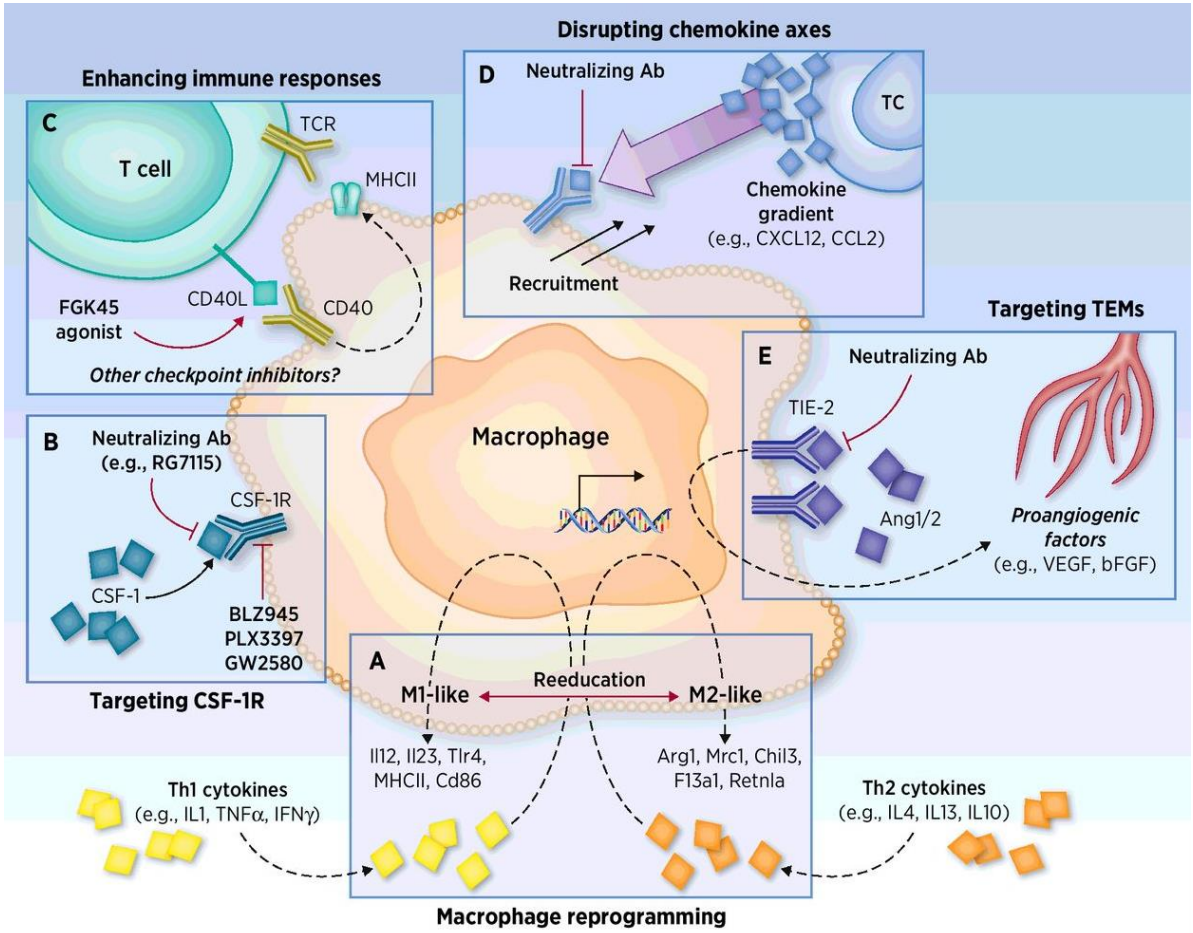


Figure 1.9. Strategies targeting tumor associated macrophages. Several approaches are being considered directed to macrophages within tumors, specifically **(A)** Reprogram macrophage polarization from the M2-type to the M1-type, **(B)** neutralize CSF-1R in order to deplete macrophages in tumors, **(C)** increase macrophages antigen presenting capacity in order to promote anti-tumor immune responses, **(D)** prevent macrophage recruitment by blocking chemokine gradients and **(E)** blocking specific pro-tumoral macrophage activities namely the inhibition of pathways involved in their pro-angiogenic role. *Reprinted from Quail DF, Clin Canc Res with permission from AACR* [241].

can be targeted directly by inhibiting their activation using CSF1/CSF1R inhibitors. Ries and colleagues created an anti-CSF-1R inhibitory monoclonal antibody that led to a decrease of $CD163^{+}$ TAM infiltration and, more importantly, resulted in a reduction of tumor burden in patients with diffuse-type giant cell tumors [242]. Macrophage surface receptors such as CD206 or CD52 are also being analyzed as possible therapeutic targets [173]. Finally, other attractive possibility is to make use of both the high macrophage number within tumors together with their plasticity [235]. In this sense, several groups are trying to successfully reprogram TAMs towards an anti-tumor pro-inflammatory phenotype by targeting CD40 or STAT3. Biospecific antibodies directed towards Ang-2/VEGF revealed promising results in different glioblastoma mouse models, resulting in prolonged survival with a concomitant shift on macrophage polarization towards an anti-tumoral M1 phenotype [243]. Besides using chemical

inhibitors or monoclonal antibodies, engineered nanomaterials are also alternatives being considered. In this sense, our group has recently shown that polyelectrolyte multilayers (PEMs) containing IFN-γ modulated IL-10-stimulated macrophages towards a more pro-inflammatory phenotype, inhibiting their ability to induce cancer cell invasion [244]. The same result was achieved when we used nanoparticles composed of chitosan/poly γ-glutamic acid (PGA). Additionally, these NPs-stimulated macrophages presented an increased expression of co-stimulatory molecules and had an impaired capacity to stimulate CRC cell invasion [245]. Regardless of the undeniable potential of macrophages in cancer therapy, the most successful results will most likely rely in combinatory strategies targeting multiple factors involved in disease progression.

2.6 Extracellular matrix

The ECM is an intricate network of macromolecules tightly structured both in its composition and three dimensional (3D) organization. ECM concept and definition evolved from the mid-1800s, prompted by major contributions of histology, light and electron microscopy, which led to the observation of a “ground substance” and organized interstitial fibers in the extracellular space [246]. From the 1970s, new technologies allowed the isolation and characterization of ECM components, unraveling its diversity and complexity.

In its composition there are mainly fibrous proteins, namely collagen and elastin, which provide tissue strength and flexibility, adhesive glycoproteins, such as fibronectin and laminin, which interlink the other ECM components and establish their interaction with cells, proteoglycans (PGs) and glycosaminoglycans (GAGs) which provide compression strength, lubrication and hydration to the ECM [247]. There are two major types of ECMs: the interstitial and the pericellular matrices (*Figure 1.10*) [248]. Their important role in providing tissue structural support and nutrition is well known, but the ECM has multiple functions. Besides being fundamental in sustaining cell-matrix adhesion and intracellular-extracellular signaling through direct interaction with cell surface receptors such as integrins, the ECM modulates cell differentiation, proliferation, survival, polarity, migration and invasion [249]. Moreover, the ECM is able to arrest several growth factors, including insulin growth factor (IGF), FGF, EGF and HGF, hormones, cytokines, chemokines and other morphogenic proteins, working as a signal reservoir [250]. Importantly, the composition, architecture and degree of crosslinking between its components dictate the mechanical properties of the ECM and control how mechanical forces are transmitted to cells [251].

One important concept regarding the ECM is its specificity, meaning that each organ presents a well-defined ECM in terms of composition, organization and topography [252, 253]. Each tissue and organ within the body executes different functions and is structured in a way to

maximize its potential. In this sense, cells are subjected to specific signals and cues which will result in the secretion of certain ECM components and enzymes, ultimately contributing to the creation of a distinct favorable niche. This dynamic crosstalk between cells and their environment, specifically the ECM, will have a critical impact in determining cell behavior [254-256].

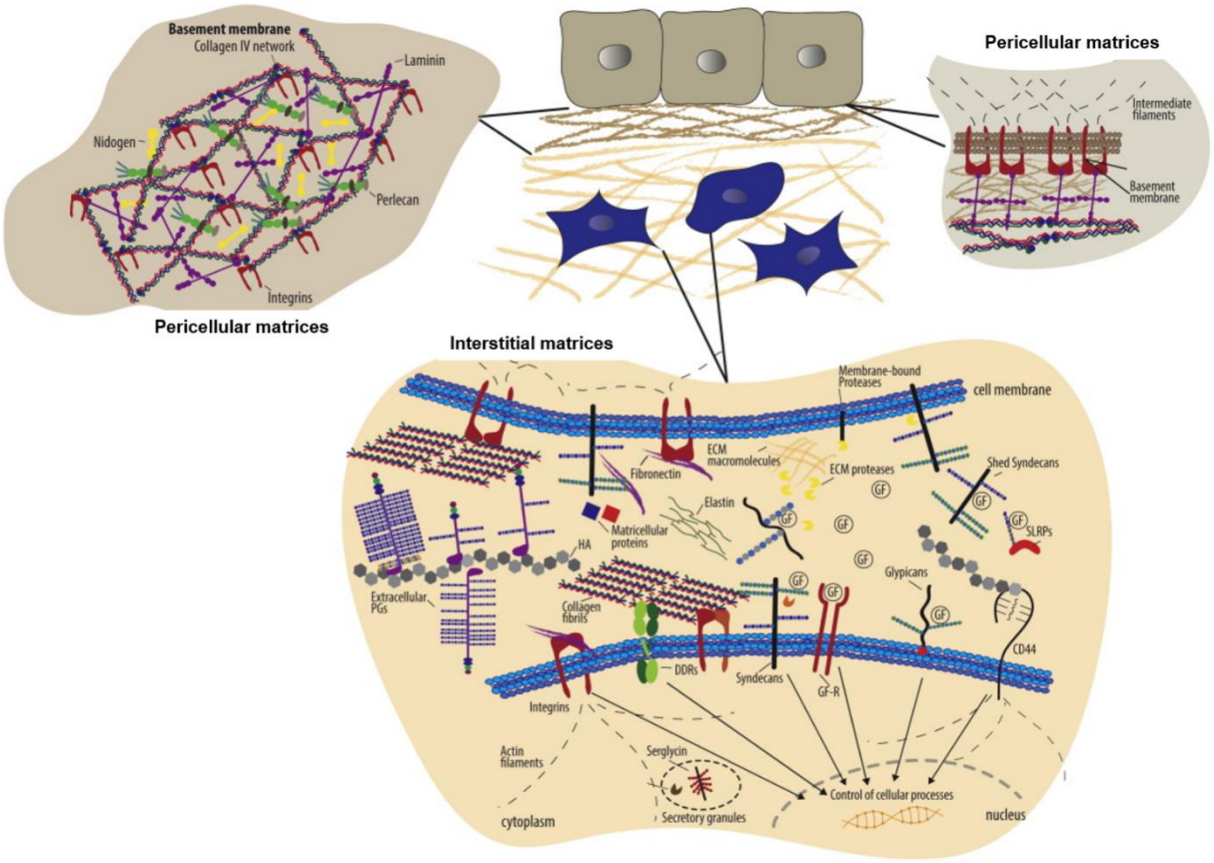


Figure 1.10**. Schematic representation of the interstitial and pericellular extracellular matrices.** Epithelial cells are attached to basement membranes through interactions between integrins and components from the pericellular matrices, namely laminin, collagen type IV and fibronectin. Interstitial matrices are characterized by presenting a highly complex network of a variety of macromolecules, such as collagen fibrils and proteoglycans/glycosaminoglycans. These are characterized by having many arrested growth factors that, when released, activate several receptors within cells, culminating in the activation of signaling pathways and in the modulation of cellular functions*. Adapted from Theocharis AD, Adv Drug Deli Rev 2016 with permission from Elsevier* [248].

2.6.1 Composition

The ECM is mainly formed by proteins and polysaccharides, secreted locally and assembled into an organized meshwork, through a pathway which was shown to be highly conserved in eukaryotes (*Table 1.1*) [257].

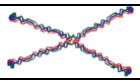
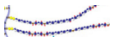
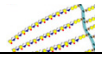
Collagen is ubiquitously expressed and is the most abundant protein in humans, representing about 30% of the total protein content. There are 28 different forms of collagen composed by, at least, 46 polypeptide chains, or α chains, which form a triple helical chain in at least one

region. Collagen construction occurs in the extracellular space mainly by self-assembly, followed by a cross-linking process meditated by lysyl oxidase (LOX) and LOX-like (LOXL) enzymes, which will strengthen the collagen fibrils. The distinct 28 collagens are organized into different families, depending on their characteristics, namely common domain homology, structure and functions: fibrillar collagens (I, II, III, V and XI), network-forming collagens (IV, VIII and X), fibril-associated collagens with interrupted triple helices (FACITs) (IX, XII and XIV), membrane-associated collagens with interrupted triple helices (MACITs) (XIII, XVII and XXIII), anchoring fibrils, beaded filament-forming collagens and multiple triple-helix domains and interruptions/endostatin-producing collagens (MULTIPLEXIN) [258-261]. Fibrillar collagens, which main function is to provide tensile strength, are targets of many post-translational modifications and form supramolecular networks, being mostly present in fibrous stromal matrices such as skin, bone, tendons and ligaments [262]. The network forming collagens are characterized by having interruptions in their triple helical structures, a feature which confers them a great flexibility and allow the interaction with each other, resulting in extensive networks. Of these, collagen type IV is highlighted since it is one of the major constituents of basement membranes, where it forms a three dimensional structure [263]. The FACITs, on their turn, are characterized by being shorter and more flexible collagens. They are frequently linked to fibrillar collagens, specifically collagens type I and II, contributing to the integrity of some tissues such as cartilage. MACITs are generally inserted in the plasma membrane. Besides being present in hemidesmosomes in epithelia, they work as cell surface receptors, suffering proteolytic cleavage and being shedded from the cell membrane, originating soluble reactive forms [248].

Elastin is another fibrous ECM protein and, as the name indicates, confers elasticity to tissues. Comparing with collagen, elastin is about 1000 times more flexible and is mainly present in tissues such as arteries, lung, bladder, ligament, tendon, skin, and elastic cartilage [248]. Elastin, together with microfibrils, form the elastic fibers which are very stable structures with low turnover. Elastin results from the crosslinking of the soluble precursor, tropoelastin, through the activity of enzymes such as LOX. Microfibrils are mainly composed by fibrils, specifically fibrillin-1, -2 and -3, and some have interesting motifs such as EGF-like domains or the tripeptide Arg-Gly-Asp (RGD) motifs, which bind integrins [264, 265].

Fibronectin has a very high molecular weight and is composed by two subunits covalently connected by disulfide bonds, each one composed of three repeating modules, termed Type I, II and III. Being a glycoprotein, it has a high carbohydrate percentage, from 4 to 9%, depending on the tissue [258, 266]. This protein is ubiquitously expressed and, although it results from a single gene, alternative splicing gives rise to at least 20 isoforms [267].

Table 1.1. ECM components, including their location, function and structure.

Family	Protein			Location	Function	Structure
Fibrous proteins	Collagen	Fibrillar collagens		Fibrous stromal matrices such as skin, bone, tendons and ligaments	Provide tensile strength, structural support and flexibility and contribute to maintain tissues' integrity	
		Networking forming collagens		Basement membranes		
		FACITs		Fibrous cartilage and bone		
		MACITs		Inserted in the plasma membrane		
		Anchoring fibrils		Into the basal lamina		
		Beaded filament-forming collagens		Found in most tissues		
		MULTIPLEXIN		Vascular and epithelial basement membranes		
	Elastin			Arteries, lung, bladder, ligament, tendon, skin, and elastic cartilage	Confers elasticity to tissues	
Adhesive glycoproteins	Fibronectin			Ubiquitously expressed	Involved in cell adhesion, growth, migration, and differentiation	
	Laminin			Basement membranes		
Proteoglycans	Core protein	Extracellular proteoglycans	Hyalectans (aggrecan; versican)	Ubiquitously expressed in ECMs within connective tissue, cell surfaces and intracellular compartments	Provide hydration to tissues, namely cartilage, cornea, brain and skin, confer resistance to compressive forces and maintain tissue's integrity. Involved in determining cell behavior	
			SLRPs (decorin; biglycan			
		Pericellular-basement membrane proteoglycans (perlecan; agrin)				
		Cell surface proteoglycans (syndecans, glypicans, CD44)				
		Intracellular proteoglycans (serglycin)				
	GAGs	Dermatan sulfate				
		Keratan sulfate				
		Heparin				
		Heparan sulfate				
		Chondroitin sulfate				
		Hyaluronic acid or hyaluronan				

Fibronectin is known for acting as an extracellular glue due its ability to bind to numerous molecules, ranging from other ECM components such as collagens, to cell membrane receptor as integrins, and signaling molecules namely growth factors, namely VEGF [268]. One interesting characteristic regarding fibronectin is the fact that it can exist both in the fibrillar insoluble state as in a soluble form present in high concentrations in the plasma, the latter having a role in clot formation [269, 270].

Laminin is another important high molecular weight adhesive glycoprotein within the ECM. It forms a family of heterotrimeric proteins composed by α, β and γ-chains. So far, there were identified five α-chains, four β-chains and three γ-chains, given rise to 16 different laminins isoforms [271]. Similarly to fibronectin, laminin also harbors EGF-like domains and RGD sequences, interacting with other ECM components, cell receptors and growth factors [272]. Together with collagen type IV, laminins are predominant elements of the basement membrane, contributing to the formation of the polymer network by self-assembly. Similarly to other ECM proteins, laminins can also be cleaved by proteases originating bioactive fragments [273] known as matrikines [274].

Proteoglycans are generally composed of a multidomain core protein with GAGs chains covalently attached. Besides being present in the ECM, proteoglycans can be found both intracellularly or at the cell membrane. GAGs are linear polysaccharides negatively charged which can be divided into 6 different types: dermatan sulfate, keratan sulfate, chondroitin sulfate, heparin, heparan sulfate and hyaluronic acid or hyaluronan (HA). Unlike other GAGs, HA does not appear covalently bound to any proteoglycan but, instead, exists in a protein-free form and can interact with other ECM proteins. Additionally, rather than being synthesized in the Golgi apparatus, as the other GAGs, it is synthesized at the cell membrane [275]. Proteoglycans have the ability to interact with cell surface receptors, many growth factors, cytokines and other ECM components, either directly or through the GAG chain. They are divided into four families according to their location: extracellular proteoglycans, namely aggrecan, versican, which belong to hyalectans subfamily, decorin and biglycan, included in the small leucine-rich proteoglycans (SLRP), the pericellular-basement membrane proteoglycans, such as perlecan and agrin, the cell surface proteoglycans namely syndecans, glypicans or CD44, and the intracellular proteoglycans, specifically serglycin [276]. Proteoglycans are important regulators of cell behavior and contribute to the organization of the ECM [277]. Moreover, due to the high negative charge provided by some GAGs, proteoglycans can hold large amounts of water, providing hydration to tissues, such as cartilage, cornea, brain and skin, and conferring resistance to compressive forces [278].

2.6.2 ECM in homeostasis and disease

Seminal work by Mina Bissell in breast morphogenesis clearly uncovered the ECM role in determining cell behavior, specifically by the regulation of gene expression. Through a mechanism called “dynamic reciprocity”, Bissell, in the early 1980s, proposed that biomechanical cues provided by the ECM to cells were transmitted to the nucleus via cell surface receptors interconnected with the actin cytoskeleton, ultimately resulting in alterations of gene expression. Moreover, these alterations contributed to a reciprocal modification of the ECM, which further affected other cells [279]. Indeed, by culturing primary epithelial cells on reconstituted basement membrane (derived from Engelbreth-Holm-Swarm murine tumor), Bissell and collaborators observed the formation of functional alveoli-like structures, able to secret milk into the lumina [280]. These results indicate that elements of the basement membrane recapitulate and promote the expression of tissue-like morphogenesis. Conversely, when the cells were cultured on plastic or attached collagen gels, neither these structures formation nor milk secretion was observed [281]. Notably, if the same cells were collected and cultured in the presence of basement membrane matrix, but not collagen type I, β-casein gene was re-expressed [282]. At the same time, the substratum was shown to regulate the expression of ECM components synthetized by cells. When in plastic, cells increased mRNA expression of laminin and collagen type IV without, nonetheless, being able to deposit these proteins. Conversely, in the presence of basement membrane components, cells downregulated their own ECM protein expression, presumably through a negative regulatory mechanism [283].

ECM importance begins early during embryo development, being synthesized and secreted by embryonic cells. During development, the ECM provides a track for guided cell migration, being crucial in processes such as mesodermal cell migration, neural tube formation and heart tube assembly [284, 285]. Accordingly, many studies have described embryonically lethal ECM mutations. More than 30 years ago, Lohler *et al.* reported that mutations in collagen type I gene resulted in mouse embryo death between day 12 and 14 of gestations, due to aortic rupture [286]. Since then, mutations in genes such as fibronectin, collagen types III and IV and different laminins were also shown to be lethal for the embryo due to vascular, CNS, renal, heart or muscular defects [285, 287]. Interestingly, it has been reported that the morphogenic movement is not exclusively caused by cell migration but also relies on ECM movement, reflecting its dynamic characteristics [288].

The ECM also plays an important role in stem cell niche maintenance and stem cell differentiation [289, 290]. The instructive cues provided by the ECM are determined not only by a combination of specific components, but also by their biomechanical characteristics, as elegantly shown by Engler and colleagues. By culturing mesenchymal stem cells (MSCs) on

matrices with different rigidities, specifically soft, stiffer and rigid, they were able to direct lineage specification towards neurons, myoblasts, and osteoblasts, respectively [291]. This mechanobiology is controlled by cell surface receptors, such as integrins, which work as sensors, regulating the outside-in signaling, since they bind to the ECM and are associated with the intracellular cytoskeleton [292].

Besides being involved in development, morphogenesis and stem cell fate, the ECM has being implicated in processes such as angiogenesis, autophagy and inflammatory regulation [293-295]. Given the pleiotropic role of the ECM in homeostasis combined with its dynamic characteristics, it is inevitable that deregulation in either its synthesis or remodeling will ultimately contribute to disease development [296]. One such example occurs in the wound healing process during which, following an inflammatory response, there is ECM deposition and remodeling. Nevertheless, frequently due to chronic inflammation and excessive activation of repair mechanisms, the collagen synthesis is excessive, resulting in abnormal scar formation and pathological fibrosis [297]. Notably, it has been reported that fibrotic ECM activates a profibrotic program in fibroblasts, through a positive feedback mechanism, resulting in exacerbated ECM deposition [298]. Indeed, fibrosis is a major contributor for impaired organ function, namely the lung, kidney or heart [299]. Similarly, also after CNS injury, the formation of the glial scar will impede axon regeneration and nervous stimuli transmission [300]. ECM alterations have also been implicated in cancer and, given the similarities between the wound healing process and tumor development, it was postulated that, in fact, tumors are comparable with wounds that never heal [301].

2.6.2.1 ECM and cancer

Many tumors are characterized by an excessive fibrosis, known as desmoplastic reaction [302]. Interestingly, excessive collagen deposition also increases the risk for cancer development, namely in women with higher mammographic density [303], or in patients with cystic fibrosis [304]. This abnormal tumor ECM is a consequence of increased deposition and of post-translational modifications combined with altered organization. As a result, the biochemical and biomechanical cues provided by this atypical ECM will affect the behavior of both cancer and stromal cells. In fact, it has been proposed that the ECM is able to modulate the hallmarks of cancer [4], having an active role on the oncogenic transformation and on tumor progression (*Figure 1.11*) [3].

One of the main changes regarding tumor ECM is its abnormal dynamics caused by an excessive production or reduced turnover, leading to alterations in its composition and biomechanical properties. Indeed, by using a bi-transgenic tumor model characterized by

increased stromal collagen in mouse mammary tissue, it was possible to demonstrate that collagen density promoted tumor initiation, intravasation and metastasis [305, 306]. Increased collagen deposition, mainly collagens type I and III, has been reported in different tumors, including ovarian, colon, breast and pancreatic cancer [307-310]. Although being mainly produced by CAFs, tumor cells also secret their own ECM, specifically some laminin components of the basement membrane and HA. Interestingly, both tumor cell-derived as well

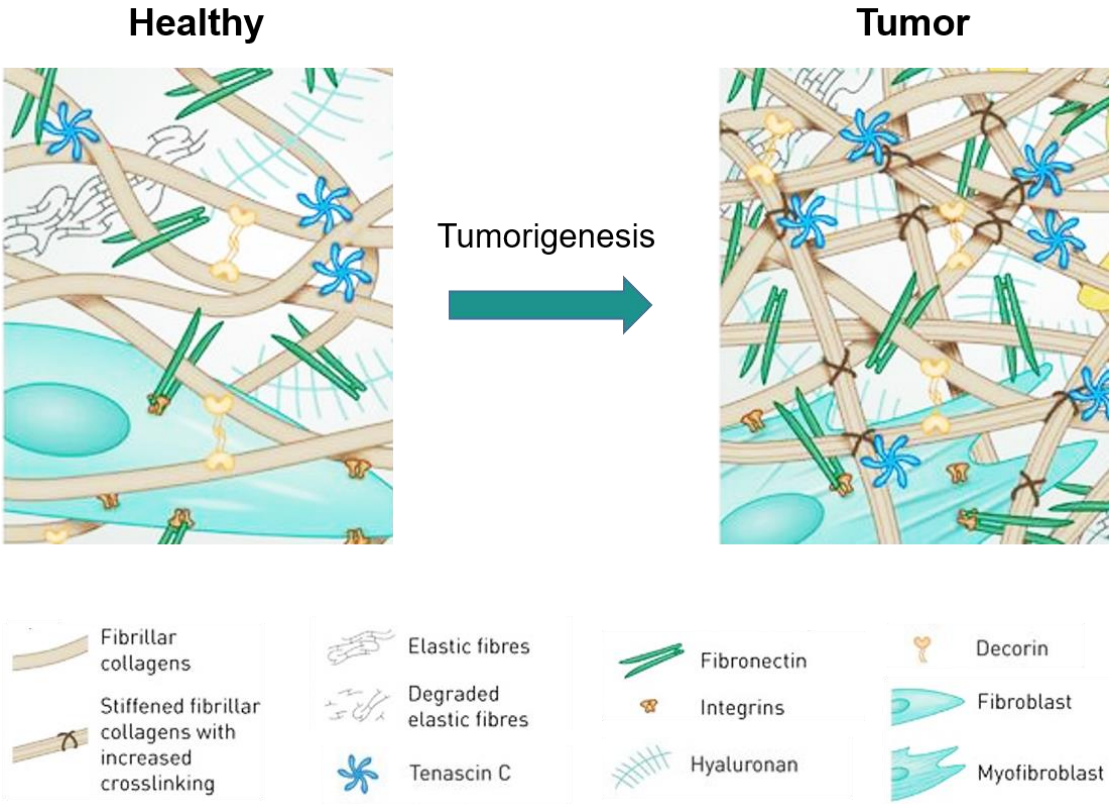


Figure 1.11. Representation of the ECM alterations in tumor tissue. During the tumorigenic process, the ECM undergoes several changes, namely increased deposition and crosslinking, resulting in altered biochemical and biomechanical properties, which have an impact on cells behavior. *Reproduced with permission of the © ERS 2018. European Respiratory Journal Jul 2017, 50 (1) 1601805; DOI: 10.1183/13993003.01805-2016* [311].

as stromal-derived ECM differed according with the tumor metastatic potential [253, 312]. In the specific case of hepatocellular carcinoma (HCC), *LAMC1*, the gene that encodes for laminin subunit γ1 chain, was shown to be increased in tumor tissue compared with matched normal liver and its expression correlated with decreased survival. In addition, LAMC1 silencing resulted in a reduction in proliferation, migration and invasion, *in vitro* [313]. TNC is another extracellular matrix glycoprotein which has been gaining attention in the cancer field. TNC was shown to be upregulated in different cancers, being produced by both tumor and stromal cells, preferentially fibroblasts [314], and its overexpression is correlated with poor prognosis and recurrent disease [315, 316]. Besides stimulating cell proliferation [317], TNC induces epithelial–mesenchymal transition (EMT) [315, 318], promotes tumor cell invasion [319] and favors the metastatic niche colonization [320]. Upregulation of other ECM

components have been reported in cancer, namely of the proteoglycan biglycan in prostate cancer, which increased expression correlated with poor prognosis [321].

Concomitantly with increased ECM production, there is also a dysregulation of the ECM remodeling enzymes in cancer tissues. Of these, MMPs are clearly the most studied, but other enzymes, namely tissue inhibitors of metalloproteinases (TIMPs), adamalysins, heparanases and cathepsins were appointed as important for extracellular matrix dynamic assembly and disassembly [296, 322]. ECM proteolytic cleavage results in the formation of bioactive cryptic fragments and neo-epitopes, known to be involved in processes such as tumor cell migration [323, 324], angiogenesis [325] and immune cell recruitment, namely innate immune cells as monocytes and neutrophils [326, 327]. Moreover, MMPs, heparanases and other enzymes are also known to release growth factors and cytokines arrested within the ECM. The relevance of these proteolytic enzymes is highlighted in the work by Poola *et al.* in which *MMP1* expression was proposed to be a putative predictive marker for breast cancer, since it allowed the stratification of atypical ductal hyperplasia into benign and pre-malignant lesions [328].

Alterations in ECM dynamics and matrix remodeling enzymes results in an impaired basement membrane and consequent loss of tissue polarity in cancer [329]. Indeed, in CRC, the transient loss of basement membrane is linked to EMT and metastasis formation [330]. In addition, the basement membrane surrounding tumor blood vessels was also shown to present alterations in laminin and collagen type IV subunits, which might result in less differentiated and leaker blood vessels and promote cancer cell attachment [331].

Architectural alterations is another feature of tumor ECM, namely the shift from relaxed collagen fibers, characteristic of normal tissues, to linearized fibers observed in tumors. Interestingly, tumor cells themselves are able to convert a random collagen organization to a radially aligned one, which they use for migration and invasion [332]. By creating an engineered microenvironment within a microfabricated chip, Han and colleagues revealed that aligned collagen fibers enhance the intravasation process [333]. Interestingly, this orientation of collagen fibers perpendicularly to tumor boundaries correlated with poor survival in breast cancer [334]. Indeed, work performed with intravital imaging combined with multiphoton microscopy confirmed that tumor cells from metastatic tumors associate with collagen fibers and display linear locomotion *in vivo* [335, 336].

Furthermore, tumors are characterized by alterations in the biomechanical and biophysical properties. With tumor progression, there is, generally, an increase in tissues stiffness [337, 338], mainly caused by an excessive activity of the LOX or the LOXL enzymes. In different tumors, LOX is upregulated and its expression correlates with worst prognosis and disease progression [339, 340]. Moreover, LOX inhibition leads to a reduction in tissue fibrosis, tumor

incidence, volume and progression [338]. This increase in tumor rigidity is more than just a natural outcome of disease progression, but rather an active contributor to all steps of the oncogenic process. In stiffer matrices, cells adhere more strongly to the ECM causing up-regulation of integrin signaling, promoting cell proliferation, survival and inhibiting apoptosis [341-344]. Increased tumor stiffening was also shown to be an important player in the angiogenic process, namely by promoting angiogenic outgrowth, invasion, and neovessel branching, being the vessels characterized by disrupted cell-cell junctions and, as a result, impaired permeability [345]. Furthermore, stiffer matrices induce VEGFR2 expression, a VEGF receptor, by affecting the balance between two transcription factors: TFII-I and GATA2 [346]. It was also described that LOXL2 expression induced by hypoxia resulted in sprouting angiogenesis, through collagen type IV assembly [347]. Interestingly, Reid and colleagues reported that matrix stiffness altered endothelial cells proteome, namely the upregulation of the CCN1 protein, which promoted tumor cell adhesion to blood vessels [348]. Increased tumor stiffness is also a key player in tumor cell invasion and metastasis. Substrate rigidity modulates invadopodia formation [349] and regulates the EMT process promoted by TGF-β1 [350]. Additionally, both matrix stiffness and confinement promote tumor cell migration, being the latter a result of increased actomyosin traction forces [351]. Higher crosslinking and stiffness induced by fibroblast-derived LOX have also been implicated in metastasis formation [352], and LOX inhibition reduced metastatic colonization [353]. Moreover, tissue stiffening promotes integrin clustering, focal adhesion formation and enhances growth factor-dependent PI3K activation, leading to increased invasion in a premalignant mammary epithelium, *in vitro*, and tumor progression, *in vivo* [338]. Besides having an impact on tumor cells, endothelial cells and fibroblasts, ECM rigidity was shown to decrease human T cell activation and proliferation [354] and to promote neutrophil directional chemotactic movement [355].

Notably, also the pre-metastatic niche presents ECM alterations which favor tumor cell colonization [356]. Tumor cells are able to induce fibronectin overexpression by stromal fibroblasts at premetastatic sites, shown to be important in the recruitment of VEGFR1-positive haematopoietic bone marrow progenitors [230]. Moreover, by using two models of pulmonary fibrosis in immunocompetent mice with orthotopic breast cancer, Cox and colleagues observed an increase in metastasis formation in a LOX-depended manner. Similar results were observed when liver fibrosis was induced. Both *in vitro* and *in vivo* work enabled the conclusion that collagen type I crosslinking by fibroblast-derived LOX at the metastatic site supports initial survival and persistence of tumor cells, specifically by activating Src, and thus promotes metastatic colonization [357]. LOX secretion by hypoxic breast tumor cells are also involved in the recruitment of $CD11b^+$ cells to pre-metastatic lung tissue, where they will cleave the crosslinked collagen, ultimately resulting in recruitment of BMDC [358]. Interestingly,

fibronectin is able to bind LOX and activate it by proteolytic cleavage, and thus is reasonable to speculate that both mechanisms mentioned above are related [359]. LOX is further involved in bone metastasis by breast and colorectal tumor cells, since it disrupts the balance of bone homeostasis, specifically by favoring osteoclast resorption, resulting in the formation of osteolytic bone lesions, prone to be colonized by circulating tumor cells [360, 361].

Finally, ECM alterations are also involved in tumor resistance to therapy, namely in breast cancer in which stiffer tumors were shown to be less responsive to neo-adjuvant chemotherapy [362]. One of the suggested explanations is the fact that tumor stroma can act as a physical barrier, affecting the chemotherapeutic compound delivery, as it was shown by the vascular impairment due to excessive HA in a pancreatic cancer mouse model [363]. Enzymatic targeting of HA inhibited proliferation, increased apoptosis and improved survival [364]. Moreover, malignant mammary cells acquire an apoptosis-resistant phenotype in the presence of basement membrane laminins, leading to the formation of polarized three-dimensional structures dependent on beta4 integrin, with consequent NF-kB activation [365]. Cell binding to the ECM is also involved in radioresistance, a process named cell-adhesion-mediated-radio-resistance (CAM-RR) [366]. Lung carcinoma cells grown in 3D present an increase in highly condensed chromatin, or heterochromatin, resulting in a reduced number of DNA double strand breaks and increased radiation survival [367]. Moreover, in such conditions, cell adhesion promoted β1 integrin clustering which favored irradiation resistance [368].

Altogether, these data clearly evidences that tumor ECM dysregulation results in abnormal tissue polarity, architecture and organization, ultimately contributing to epithelial cell transformation, invasion, escaping the immune system surveillance, and favoring metastasis. Simultaneously, stromal cell behavior also reflects these ECM changes, contributing to a pro-tumoral and pro-angiogenic phenotype. For these reasons, the ECM has become an attractive therapeutic target and different strategies are currently being considered [369]. Of these, HA targeting using a PEGylated recombinant human hyaluronidase has shown encouraging results in a randomized phase II clinical trial in pancreatic cancer patients, specifically by significantly improving progression-free survival [370]. Given the antitumor and antimetastatic effect of heparanase inhibitors in preclinical models, specifically heparan sulfate mimetics, several clinical trials are currently ongoing in different tumor types [371]. In addition, LOX2 inhibition with a monoclonal antibody is also being tested and the pre-clinical studies published so far are quite promising [372, 373]. TNC inhibition, either by monoclonal antibodies or peptides, showed encouraging results and clinical trials are ongoing [372, 374].

2.6.3 ECM and macrophages

Similarly to other cell types, and given their plasticity, macrophages are extremely responsive to the extracellular environment, namely ECM chemical and mechanical characteristics [375]. Additionally, besides secreting proteases that contribute to ECM remodeling, macrophages are also producers of ECM components. In the 1980s, it was reported that macrophages produced fibronectin [376] which worked as a chemoattractant for fibroblasts [377, 378]. Laminin was also described as being produced by macrophages and to localize specifically at cell surface [379]. Interestingly, macrophages were shown to express the laminin receptors α6β1 integrin [380]. Weiktkamp *et al.* were the first ones describing collagen production by macrophages, specifically collagen type VIII in the atherosclerotic plaque [381]. Since then, *in vitro* work revealed that macrophages express all known collagen mRNAs and also secret collagen type VI [382]. Macrophage secretion of proteoglycans has also been reported, particularly the intracellular serglycin and the extracellular versican [383, 384]. In the specific case of CRC, recent work by Afik *et al.* using an orthotopic mouse model revealed that macrophages are major contributors for tumor ECM, namely by increasing the expression of molecules associated with collagen synthesis, stability, assembly, and cross-linking. Moreover, TAM-deficient colorectal tumors displayed alterations in both collagen density and organization and, interestingly, cancer associated fibroblasts from these tumors had a reduced expression of collagen types I and XIV [385].

Over the years, several studies addressed the ECM effects on macrophages. Collagen type I, one of the main components of the atherosclerotic matrix, was shown to promote macrophage differentiation and led to an increase in MMP-9 secretion and intracellular accumulation of modified lipoproteins when compared to uncoated polystyrene [386]. Collagen type I also promoted M2 differentiation in alveolar macrophages (AM) from both healthy controls and patients with idiopathic pulmonary fibrosis. When in collagen type I, AM up-regulated CCL2, CCL18, IL-1ra and CD204. Interestingly, by inhibiting CD204 with a neutralizing antibody, collagen type I effects were no longer observed [387]. Conversely, it was reported that soluble fibronectin, rather than fibronectin-coated plates, promoted macrophage-mediated cytotoxic activity using a melanoma and a renal carcinoma cell lines [388]. Laminin, on the other hand, increased macrophage MMP-9 and uPA, being the latter dependent of the α6β1 integrin [389]. Some authors have reported that low molecular HA fragments, usually produced in an injury situation, induced a pro-inflammatory macrophage activation [390, 391], but these must be analyzed with caution since it is speculated that they might be a result of endotoxin contamination [392]. Given macrophage importance in the wound healing process, Hsieh *et al.* addressed the effect of fibrin and fibrinogen on macrophage activation. They showed that fibrinogen induced TNF-α and NO synthase 2 (NOS2) expression, while fibrin resulted in an

up-regulation of IL-10 and arginase 1. Interestingly, fibrinogen-induced inflammatory activation was inhibited when macrophages were simultaneously in the presence of fibrin [393]. Macrophages were also shown to recognize damaged collagen fibrils in tendons subjected to a mechanical overload. Scanning electron microscopy (SEM) images revealed that, when compared with macrophages in intact tendons, cells formed more aggregates, presented an increased membrane ruffling and a decrease in MMP9 secretion [394].

As previously mentioned, not only chemical factors are important in shaping cell differentiation and behavior. In this sense, elegant work by Van Goethem using matrices with different properties, namely composition, architecture and stiffness, revealed that macrophage migration is mainly determined by ECM architecture rather than composition [395]. Moreover, McWhorther and colleagues clearly showed that macrophage phenotype is reflected in their shape. By using a micropatterning approach which forced macrophage elongation, they were able to induce a M2 phenotype with increased expression of arginase-1, CD206 and YM-1, and reduced secretion of pro-inflammatory markers such as CD54, IFN-γ, and macrophage inflammatory protein-1α (MIP-1α). Interestingly, this shape-induced polarization required myosin-dependent cytoskeletal contractility [396]. Cell elasticity was also described as having a critical impact on macrophage function. LPS and IFN-γ promoted macrophage phagocytic capacity concomitantly with an increase in cell elasticity, a process dependent of actin polymerization and of enhanced Rho GTPase activity. Interestingly, increased substrate rigidity, by itself, led to a higher macrophage phagocytosis, as a result on an increased cell elasticity. Finally, modulation of cell elasticity by increasing substrate rigidity modulated macrophage proteomic profile and affected LPS response, resulting in a decrease of TNF-α secretion [397]. Also in 3D hydrogels, macrophages were shown to adopt a less round morphology concomitantly with a decreases expression of TNF-α, IL-6 and IL-1β [398].

Macrophage response to extracellular stimuli is also being investigated in the tissue engineering field with the perspective of using bio-fabricated tissues or organs, created with synthetic polymers or natural materials derived from decellularized organs. In this sense, recent work revealed that, while solubilized urinary bladder up-regulated macrophage PGE2 secretion, brain solubilized ECM induced macrophage expression of TNF-α and NO and promoted their phagocytic activity, pointing to the acquisition of a pro-inflammatory phenotype. Interestingly, HA content was similarly shown to be critical for this different polarization. Urinary bladder had increased HA levels than brain tissue and, upon hyaluronidase treatment, the PGE2 secretion was inhibited while the NO increased [399]. Macrophages stimulated with solubilized ECM bioscaffolds derived from small intestinal submucosa, esophageal tissue and colon, and contrarily to what happen with liver, also presented an anti-inflammatory, pro-remodeling phenotype [400]. Decellularized mouse kidneys were also shown to skew

macrophage polarization towards a M2 phenotype but this effect was dependent on the 3D-structure [401].

Altogether, these data clearly exposes the complexity regarding the various features of the ECM effect on macrophage activity, including composition, organization and biomechanical properties, which undoubtedly strengths the need of using systems able to accurately recreate native microenvironments in research.

CHAPTER 2

Profiling macrophage populations in human colorectal cancer

Macrophages are the most prevalent immune population within the microenvironment of solid tumors and their infiltration is generally associated with decreased patients' survival and worst prognosis. Macrophage pro-tumor effects have been demonstrated, both *in vitro* and *in vivo*, and include: i) the promotion of tumor growth, provided by the production of pro-survival and growth factors; ii) the stimulation of invasion, supported by the release of pro-invasive factors and the activation of cancer cell pro-invasive pathways as the EGFR-signaling cascade; iii) the induction of angiogenesis, sustained by the secretion of VEGF; iv) and the promotion of metastasis, promoted by the creation of an immunosuppressive environment receptive to the colonization at distant sites. Nevertheless, in CRC, the studies addressing the clinicopathological significance of macrophages present contradictory results and, thus, still no consensus was achieved.

In the present work, we proposed to profile macrophages in a series of 150 CRC cases, using a computer-assisted approach, which enabled an accurate quantification. Knowing that macrophages can present either pro- or anti-inflammatory features, three different markers were used: CD68, a macrophage lineage marker, CD80, a pro-inflammatory marker, and CD163, an anti-inflammatory marker. Importantly, macrophage populations were assessed in three different regions, specifically the tumor adjacent normal mucosa, the intratumoral region and the tumor invasive front.

Macrophage infiltration, analyzed by CD68 staining, increased in both tumor regions comparing with the normal mucosa, contrarily to $CD80^+$ cells, which were almost exclusively located in the normal mucosa. When analyzing macrophages according to tumor location, tumors in the right colon presented increased positivity for CD68 and CD163 in the intratumoral region, suggesting an enhanced infiltration of anti-inflammatory macrophages. Interestingly, CD80 was more prevalent in T1 tumors, whereas CD68 and CD163 expression was higher in stage II tumors. Regarding survival analysis, the only associations were found in stage III tumors and revealed that patients with a higher CD68 or a lower CD80/CD163 ratio presented a decreased overall survival. In agreement, a higher CD80 expression was associated with a decrease risk for relapse/locoregional recurrence, suggestive of a protective role provided by the expression of the co-stimulatory molecule CD80.

This extensive work brought new insights into the complexity of macrophages within CRC. It is, according to our knowledge, the first time that the distribution and profiling of macrophage populations in human tumors is quantified in comparison to the adjacent normal mucosa and the invasive front. The results obtained regarding overall survival in patients with stage III tumor deserves further investigation, since it suggests that this specific subset of patients might benefit from therapeutic strategies targeting the modulation of existing macrophages towards a pro-inflammatory phenotype. Moreover, the apparent contribution of CD80 cells in

decreasing the risk of tumor relapse, which is a frequent problem in CRC management, also justifies confirmation in other cohorts and may also be a potential target for immunotherapy.

Profiling macrophage populations in human colorectal cancer

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Manuscript to be submitted

Introduction

Within the complex tumor microenvironment, a variety of non-malignant stromal cells are active players in cancer progression [5]. Specifically in solid tumors, TAMs are one of the most represented populations [132] and have important roles in the invasive, angiogenic and metastatic processes [11, 192].

Macrophages are extremely plastic cells, able to respond and adapt to external stimuli [122]. In the last decade, macrophage classification as evolved and nowadays the most accepted model describes a myriad of polarization status between two extreme populations: the M1-like or pro-inflammatory, and the M2-like or anti-inflammatory. In the presence of factors such as LPS, IFN-γ or TNF-α, macrophages adopt a pro-inflammatory phenotype, with high antigen presenting capacity and production of cytokines such as IL-6, IL-12, TNF- α, IFN-γ and ROS. These cells are known for their bactericidal and pro-inflammatory functions. On the other extreme of the spectrum are the M2-macrophages, induced by factors such as IL-4, IL-13, IL-10 or glucocorticoids, which produce anti-inflammatory cytokines, specifically TGF-β and IL-10 [128]. They are characterized by their scavenger, angiogenic and pro-invasive properties [11, 192]. As a consequence of the immunosuppressive tumor microenvironment, namely due to high IL-10 and TGF-β levels [402, 403], TAMs are reported to adopt features common to M2-like macrophages. They generally produce growth factors, chemokines and MMPs, which act directly on cancer cells or in other stromal cells, ultimately leading to tumor growth, invasion and metastasis [192].

A variety of clinical and epidemiological studies have described a strong association between TAMs infiltration, worst prognosis and shorter survival in melanoma, breast and ovarian cancer [134-136, 138, 140]. Nevertheless, in CRC, there is still disagreement in the scientific community. Some studies conclude that, indeed, higher macrophage infiltration correlates with more advanced tumor stages [151] and worst prognosis [150], while others report that TAMs are in fact associated with improved survival, specifically in the colon [149] and with reduced liver metastasis [148]. Importantly, the majority of these studies were solely based on CD68, a macrophage lineage marker, expressed by all macrophages, without taking into consideration differences amongst the distinct pro- or anti- inflammatory subpopulations. Recognizing the importance of macrophage polarization, some authors have started to analyze markers which discriminate between the M1 and the M2 subpopulations. In this sense, Igars and colleagues have proposed that, in fact, macrophage role evolves during the carcinogenic process and that their influence on survival is determined by the type and distribution of TAMs. They reported that, in less advanced tumor stages, macrophage infiltration is associated with improved disease free survival (DFS), while, in stage IV CRC, the high number of CLEVER-1/Stabilin-11 positive cells, used as an M2-marker, correlates with shorter DFS [404]. By using

NOS2, as a marker for M1 macrophages, and CD163, as a marker for M2 macrophages, an association with improved prognosis was reported for both subpopulations. Nevertheless, no significant differences in survival were observed when analyzing the ratio between $NOS2^+$ and $CD163^+$ cells [405]. By using the same markers, Koelzer *et al* also did not find any significant association between improved survival and the presence of NOS2 or $CD163^+$ cells [406]. Nevertheless, although NOS2 has been frequently used to identify pro-inflammatory macrophages in mice, many groups have argued that there are significant differences in human nitric oxide metabolism and thus this is not an appropriate marker to identify M1 macrophages [407-409]. Other limitations of the published studies are related with the use of tissue microarrays, which may not accurately represent the characteristics of the complete tumor, the evaluation of hotspots, an approach that already presents some bias in the analysis, and the use of a semiquantitative scoring, which inevitably results in a more subjective and less sensitive method.

In the present work we performed a quantitative evaluation of the distinct macrophage subpopulations present in CRC, using three markers: CD68, CD80, and CD163 in consecutive histological slides. The quantification was done, not only in the intratumoral region (IT) and in the tumor invasive front (IF), but also in the tumor adjacent normal mucosa (ANM) of the same patient. Importantly, our analysis benefit from the existence of a detailed clinicopathological information which permitted the association of the distinct profiles with tumor stage and location, and with tumor relapses and patient overall survival. Using this strategy, we hope to shade some light in the conflicting data regarding the clinical impact of macrophage subpopulations within colorectal tumors, and hopefully help to discriminate which patients might benefit from immunotherapies targeting macrophages.

Materials and Methods

Clinical samples

A series of 150 cases retrieved from the files of the Pathology Department from Centro Hospitalar São João (CHSJ, Porto, Portugal) were included in the study. The material was collected during primary tumor surgical resections between 2007 and 2012. Relapses and synchronous tumors were not included.

All histophatological evaluations, including stage, grade, tumor type and lymphocytic infiltrate, were performed by experienced pathologists from the CHSJ Pathology Department. The study was approved by the CHSJ Ethics Committee for Health (References 259 and 260/11), in agreement with Helsinki declaration. Informed consent was obtained from all patients.

Immunohistochemical staining

Specimens were fixed in formalin and embedded in paraffin in accordance with the routine protocol implemented in the Pathology Department from CHSJ. In order to evaluate macrophage subpopulations, sequential 5μM sections were stained with antibodies against CD68 (Dako, PG-M1), CD80 (R&D, MAB140) and CD163 (Novocastra, MRQ-26). Briefly, tissues were deparaffinized, hydrated and then endogenous peroxidase activity was blocked with 3% methanol in hydrogen peroxide for 10 min. Following antigen retrieval in the water bath at 98°C with Tris EDTA, pH9 (CD68, 20 min) or citrate buffer, pH6 (CD80, 20 min; CD163, 40 min), primary antibodies were incubated as follows: CD80 overnight (1:50) at 4°C, CD68 30 min (1:100) and CD163 30 min (1:100), both at room temperature. After washing, labeled polymer secondary antibody (Envision Detection System, Dako) was added to slides and peroxidase activity was detected using diaminobenzidine (DAB) –tetrahydrochloride liquid plus substrate Chromogen System (Dako). Reaction was stopped with distilled water and sections were counterstained with hematoxylin and mounted in Richard-Allan Scientific Mounting Medium (ThermoFisher).

Quantification

Following immunohistochemistry, the slides were digitalized using a NanoZoomer 2.0HT Hamamatsu camera (Meyer Instruments). For each marker, ten random areas of the tumor adjacent normal mucosa (ANM), intratumoral region (IT) and tumor invasive front (IF) were photographed (20x magnification). Using Fiji software, the immunoreactive area (IRA) for each cell surface marker and each region was calculated on basis of red, green and blue segmentation, and represented as a percentage of the immunoreactive area (IRA%). Afterwards, the mean of the 10 distinct microscopic fields was calculated for each marker in each region. Importantly, the pictures of the 3 markers were acquired in the same area, keeping in mind that consecutive sections were used.

Statistical analysis

Statistical analyses were conducted in STATA version 12.0 (StataCorp, College Station, Texas) or GraphPad Prism Software v5 (GraphPad-trial version) and graphics were done using GraphPad Prism Software. Descriptive statistics included count and frequencies for categorical and median with interquartile range for continuous variables. Departure from normality was determined using the Shapiro-Wilk test. Comparison of macrophage's populations between and within locations in the tumor region was performed using Friedman's test followed by multi-group comparisons with Wilcoxon test. Comparisons according to colon side, meaning left and right, were performed with Mann-Whitney U test while Kruskal-Wallis with Dunns multiple

comparisons was applied in the analysis according to stage and primary tumor invasiveness. Survival curves comparison was performed with log-rank test. Associations were testes using Spearman's rank correlation. Association between macrophages' type and localization with relapse followed a robust statistical procedure. First, empirical analyses with unconditional logistic regression with adjustment for age and gender, were undertaken to uncover the relevant clinicopathological and macrophage characteristics variables to be included in subsequent multivariate models (p for retention > 0.05). Then, multivariate logistic regression was conducted to assess the independent strength of association of macrophage's characteristics in predicting risk for CRC progression. Lastly, in order to confirm the strength of association of emerging results from multivariate analysis Bootstrap analysis were done using Monte Carlo simulations (n=1000).

Results

Descriptive data of the Patient Characteristics

One hundred and fifty CRC cases, collected from the Pathology Department between 2007 and 2012, and containing in the same histological sections the intratumoral region, invasive front and tumor adjacent normal mucosa, were included in this study. Relapses and synchronous tumors were excluded. Of these, 83 were males and 67 were females, aged between 22 and 93 years old (median of 70.5 years). The available clinicopathological information, including tumor stage and location, lymphocytic infiltration, the existence of tumor relapses, the therapeutic scheme and patient overall survival are included in Table 2.1. In this cohort, only five patients received pre-operative chemotherapy, of which 3 also received pre-operative radiotherapy. From the initial cohort, clinical data for survival was obtained for 136 patients.

$CD68^+$ and $CD163^+$ cells are predominantly found in the tumor invasive front whereas $CD80^+$ cells are mainly located in the tumor adjacent normal mucosa

Given the difficulty in accurately assessing macrophage number using the classical approach of counting cells in the microscope, macrophage populations were evaluated by digitally quantifying the percentage of immunoreactive area (IRA %), for each of the markers, and within three distinct regions (Supplementary Figure 2.1), similarly to what was done by other groups [410, 411]. Three markers were used to characterize macrophages: CD68, a macrophage lineage marker broadly used to identify these immune cells [149, 151, 404], CD80, a co-stimulatory molecule expressed by pro-inflammatory macrophages [412], and CD163, a scavenger receptor associated with anti-inflammatory macrophages [413]. Quantifications were performed in three regions: the tumor adjacent normal mucosa (ANM),

Table 2.1 Patient's clinicopathological information

Characteristics	No. of patients (%)
Age, *median (IQR)*	*70.5 (62.0-79.0)*
Gender, M/F	83 (55.3)/67 (44.7)
Anatomic tumor region	
Cecum	11 (7.3)
Ascending colon	25 (16.7)
Transverse colon	21 (14.0)
Descending colon	11 (7.3)
Sigmoid	53 (35.3)
Rectum	29 (19.3)
Pathological stage, TNM	
Tumor	
T1	9 (6.0)
T2	25 (16.7)
T3	93 (62.0)
T4	23 (15.3)
Nodes	
N0	85 (56.7)
N+	65 (43.3)
Metastasis	
M0	121 (80.7)
M+	29 (19.3)
Clinical stage	
I	26 (17.4)
II	51 (34.0)
III	44 (29.3)
IV	29 (19.3)
Lymphocytic infiltration	
Absent/mild	92 (61.3)
Moderate/strong	58 (38.7)
Adjuvant radiotherapy	
No	135 (90)
Yes	14 (9.3)
Unknown	1 (0.7)
Adjuvant chemotherapy	
No	81 (54)
Yes	69 (46)
Relapse	
No	132 (88.0)
Yes	17 (11.3)
Missing	1 (0.7)
Survival	
Alive	76 (50.7)
Death	60 (40.0)
Unknown	14 (9.3)
Cause of death	
Cancer-related	29 (19.3)
Other causes	27 (18)
Missing	4 (2.7)

Abbreviation: IQR, interquartile range

the intratumoral region (IT) and the invasive front (IF) (Figure 2.1). Importantly, since IRA % was calculated by averaging the specific positive reactive area for each selected marker in 10 random areas in each region, the contribution of eventual existent hotspots is minimal. As previously reported by others, macrophages are mainly located at the IF of colorectal tumors

comparing with the IT region (median IRA 5.23% vs 2.59 %) (Figure 2.2A and Table 2.2) [149, 414], being this infiltration higher than what is detected in the ANM (IRA 2.27%). Regarding CD163, and contrary to what was observed for CD68, ANM exhibit a higher density of $CD163^+$ cells than the IT (IRA 1.04% vs 0.63%). Nevertheless, the majority of these anti-inflammatory macrophages were also found at the IF (IRA 1.65%) (Figure 2.2A and Table 2.2). Interestingly, CD80 was almost exclusively located in the ANM (1.31%). In both tumor regions analyzed CD80 staining is very low and, similarly to the other markers evaluated, the expression is

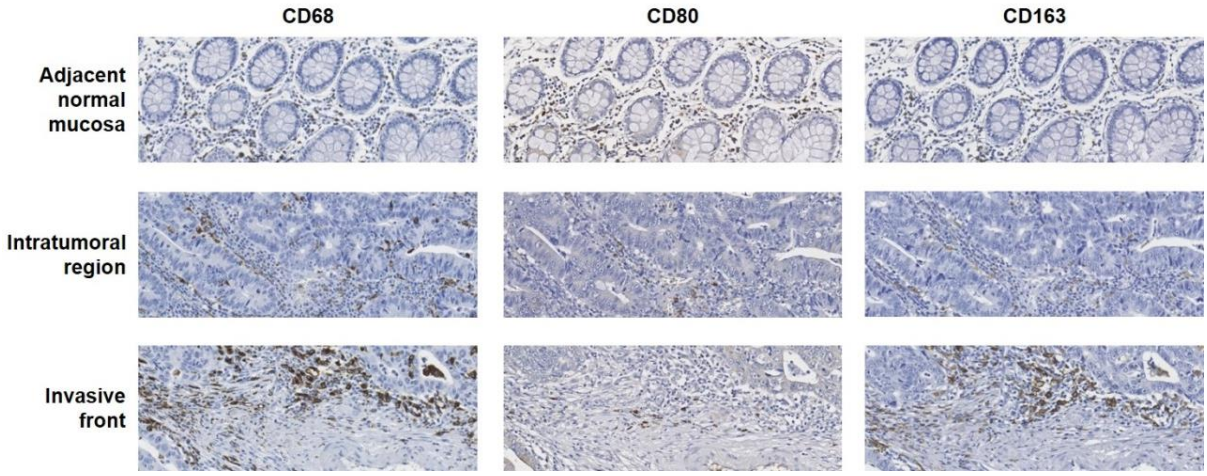


Figure 2.1. Immunostaining of CD68, CD80 and CD163 in the tumor adjacent normal mucosa, intratumoral region and invasive front of colorectal cancer, in consecutive paraffin-embedded sections.

higher in the IF than in the IT (IRA 0.12% versus 0.04%) (Figure 2.2A and Table 2.2). Spearman's rank correlation test revealed a moderate association, meaning $r_s>0.5$, between CD68 and CD163 staining, in the three regions analyzed, suggesting that the cases that have higher levels of CD68 were also the ones with higher infiltration of $CD163^+$ cells. Moreover, there was also a positive association in CD163 positivity between IT and IF (Supplementary Table 2.1), suggesting that the cases of higher CD163 expression at the IT region are also the ones that exhibit higher expression of this scavenger receptor at the IF. Since the quantifications for each marker were performed in consecutive sections in the same area, the percentage of pro-inflammatory and anti-inflammatory cells among the overall macrophage population was assessed by calculating the ratio between CD80 or CD163 and CD68 (Figure 2.2C and Table 2.2). Interestingly, at the ANM, CD80 staining represents almost 75% of the total CD68 staining. Of note, some of the cases studied have a higher CD80 IRA % compared with CD68, suggesting that CD80 is not exclusively expressed by macrophages. Within the IT and IF, the percentage of cells expressing CD80 relatively to CD68 decreased to approximately 2 and 3.45%, respectively. As for CD163, its expression represents about 50% of the total CD68 staining in ANM. Interestingly, despite the increase of CD163 IRA % at the IF relatively to CD68 expression, their percentage is still lower than what was observed in ANM (38.7% versus 52%). Altogether these observations clearly demonstrate that there is a

significant number of macrophages at the IF and at the IT regions that do not express neither CD80 nor CD163. Spearman's test showed a direct correlation in CD80/CD68 between IT and IF (r_S=0.52, p=6.4E^{-12}), similarly to what was observed in CD163/CD68 ratio between the same regions (r_S=0.63, p=6.1E^{-18}).

Moreover, the ratio between CD80 and CD163 was also calculated to evaluate the proportion of pro-inflammatory macrophages relatively to the anti-inflammatory ones (Figure 2.2C). In the

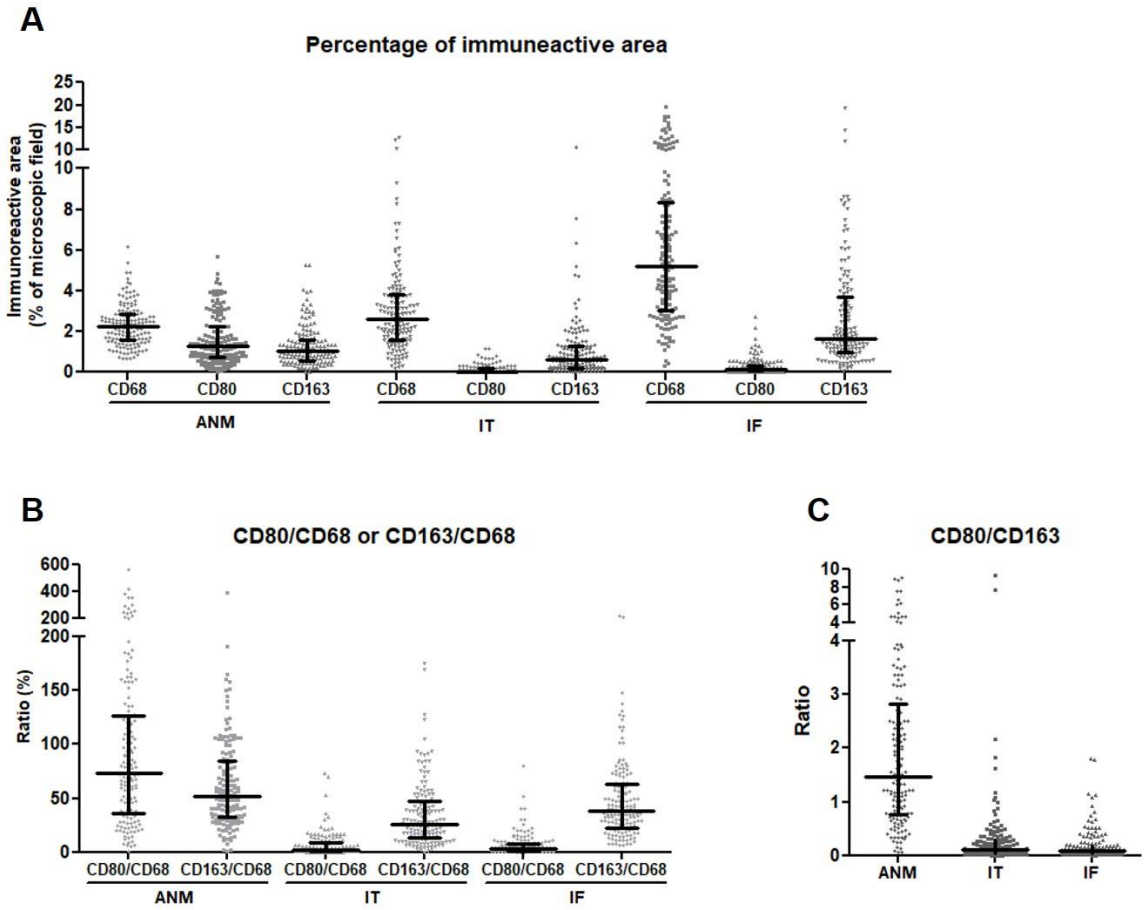


Figure 2.2. Quantifications of CD68, CD80 and CD163 in the 150 colorectal cancer cases. (A) Percentage of immunoreactive area (IRA %) of CD68, CD80 and CD163 in the tumor adjacent normal mucosa (ANM), intratumoral region (IT) and invasive front (IF). **(B)** Percentage of CD80/CD68 and CD163/CD68 ratios in the ANM, IT and IF calculated from the IRA %. **(C)** CD80/CD163 ratio in the ANM, IT and IF calculated from the IRA %. Each dot represents one patient, calculated by averaging the quantification of 10 areas.

ANM, CD80 positivity is about 1.5 times higher than CD163. Conversely, in both IT and IF, there is a shift in the quantity of both populations, with CD163 positivity being 10 times higher than CD80, without differences between the 2 tumor regions. Spearman's test revealed a positive association in CD80/CD163 ratio between IT and IF (r_S=0.57, p=1.79E^{-14}), suggesting that the cases that presented a lower CD80/CD163 ratio at the IT region, are also the ones with a lower ratio of CD80/CD163 at the IF.

Adjacent normal mucosas and tumors in the right colon exhibit higher macrophage infiltration

Given the known differences between the right and left-sided colon, not only in terms of anatomy and genetic alterations but also considering the present microbiota [415], macrophage populations in both locations were compared (Table 2.3). Interestingly, CD68,

Table 2.2. Immunoreactive area percentage for CD68, CD80 and CD163, and CD80/CD68, CD163/CD68 and CD80/CD163 ratios in the adjacent normal mucosa, intratumoral region and invasive front.

	Adjacent normal mucosa	Intratumoral region	Invasive front	P*
CD68 (IRA %)	2.27 (1.56-2.83)	2.59 (1.60-3.79)	5.23 (3.05-8.34)	<0.0001[a]
CD80 (IRA %)	1.31 (0.73-2.26)	0.04 (0.01-0.17)	0.12 (0.04-0.31)	<0.0001[b]
CD163 (IRA %)	1.04 (0.57-1.57)	0.63 (0.20-1.26)	1.65 (0.96-3.70)	<0.0001[c]
CD80/68 ratio (%)	73.75 (35.64-127.05)	2.06 (0.70-8.22)	3.45 (1.12-7.91)	<0.0001[d]
CD163/68 ratio (%)	51.98 (32.50-84.32)	26.16 (13.47-47.17)	38.69 (22.72-62.87)	<0.0001[e]
CD80/163 ratio	1.47 (0.76-2.82)	0.10 (0.03-0.28)	0.09 (0.04-0.19)	<0.0001[f]

Data presented as median and inter-quartile range. * Friedman's test. Multi-group comparisons using the Wilcoxon test: [a]ANM vs. IT ($P=4.70x10^{-4}$), ANM vs. IF ($P=3.65x10^{-22}$), IT vs. IF ($P=4.80x10^{-19}$); [b]ANM vs. IT ($P=8.11x10^{-26}$), ANM vs. IF ($P=2.36x10^{-25}$), IT vs. IF ($P=2.22x10^{-9}$); [c]ANM vs. IT ($P=5.36x10^{-5}$), ANM vs. IF ($P=1.55x10^{-11}$), IT vs. IF ($P=5.21x10^{-21}$); [d]ANM vs. IT ($P=2.30x10^{-26}$), ANM vs. IF ($P=3.05x10^{-26}$), IT vs. IF ($P=0.089$); [e]ANM vs. IT ($P=1.97x10^{-13}$), ANM vs. IF ($P=1.46x10^{-5}$), IT vs. IF ($P=7.95x10^{-9}$); [f]ANM vs. IT ($P=2.45x10^{-24}$), ANM vs. IF ($P=2.76x10^{-26}$), IT vs. IF ($P=0.155$).

CD80 and CD163 infiltration was higher in the ANM of tumors in the right colon than in the left one. The same observation was true for CD68 and CD163 in the IT. Nevertheless, regarding the IF, no differences were observed between left and right colon in any of the macrophage markers analyzed.

Stage II tumors have higher infiltration of CD68 and CD163$^+$ cells whereas CD80 cells are more frequent in T1 tumors

Macrophage scorings were then assessed according to the tumor stage (Figure 2.3A). For the three markers analyzed, there were no differences in the ANM among the distinct CRC stages. The same was not true when evaluating the tumor and its respective IF. Interestingly, CD68 and CD163$^+$ macrophages were significantly more abundant in both tumor regions of stage II tumors, specifically comparing with stage IV tumors. Conversely, no differences were observed for CD80.

To further complement this analysis, macrophage populations were separately analyzed based on the primary tumor depth of invasion (Figure 2.3B). Interestingly, $CD80^+$ cells were more frequent in the IT and IF of the less invasive T1 tumors. This was not observed in CD68 nor in $CD163^+$ cells, which appear to predominantly infiltrate T3 tumors, although no statistical significant differences were detected.

Table 2.3. Immunoreactive area percentage of CD68, CD80 and CD163 in the adjacent normal mucosa, intratumoral region and invasive front in the right and left colon.

Region	Marker	Right colon, N=52	Left colon, N=98	P value
Adjacente normal mucosa	**CD68**	2,56	2,20	0,022
	CD80	2,02	1,40	0,002
	CD163	1,71	1,04	0,0004
Intratumoral region	**CD68**	3,66	2,65	0,004
	CD80	0,15	0,12	0,466
	CD163	1,36	,86	0,0705
Invasive front	**CD68**	5,82	6,37	0,392
	CD80	0,29	0,25	0,122
	CD163	3,08	2,54	0,158

Data are represented as mean of immunoreactive area percentage. P values were obtained by Mann-Whitney U test.

Higher CD68 expression in stage III colorectal tumors is associated with decreased overall survival

In CRC, the data regarding macrophage infiltration and patient survival is contradictory, with some studies reporting an association between increased overall survival with higher macrophage infiltration and others describing exactly the opposite [149, 150]. In order to perform this analysis, the IRA for each one of the markers was divided into 2 categories according to the median, resulting in the low-expressing and high expressing cases. When all patients were included in the survival analysis, no differences were observed regardless of the marker or region analyzed (data not shown). Also no differences were observed when evaluating the association in survival according to the CD80/CD163 ratio (data not shown). Moreover, when the same analysis was performed exclusively with colon cancer patients, excluding the rectum, also no differences were detected (data not shown). Given that our cohort includes all tumor stages, which present completely different prognosis, the association between macrophages and survival was evaluated considering stages I + II, stage III and stage IV, separately. Importantly, specifically in stage III tumors, higher infiltration of $CD68^+$ cells in

the IT region was clearly associated with decreased overall survival (Figure 2.4). This was no longer observed in the IF, nor regarding CD80 or CD163 expression. Importantly, the association between patients OS and the ratio CD80/CD163 was similarly assessed.

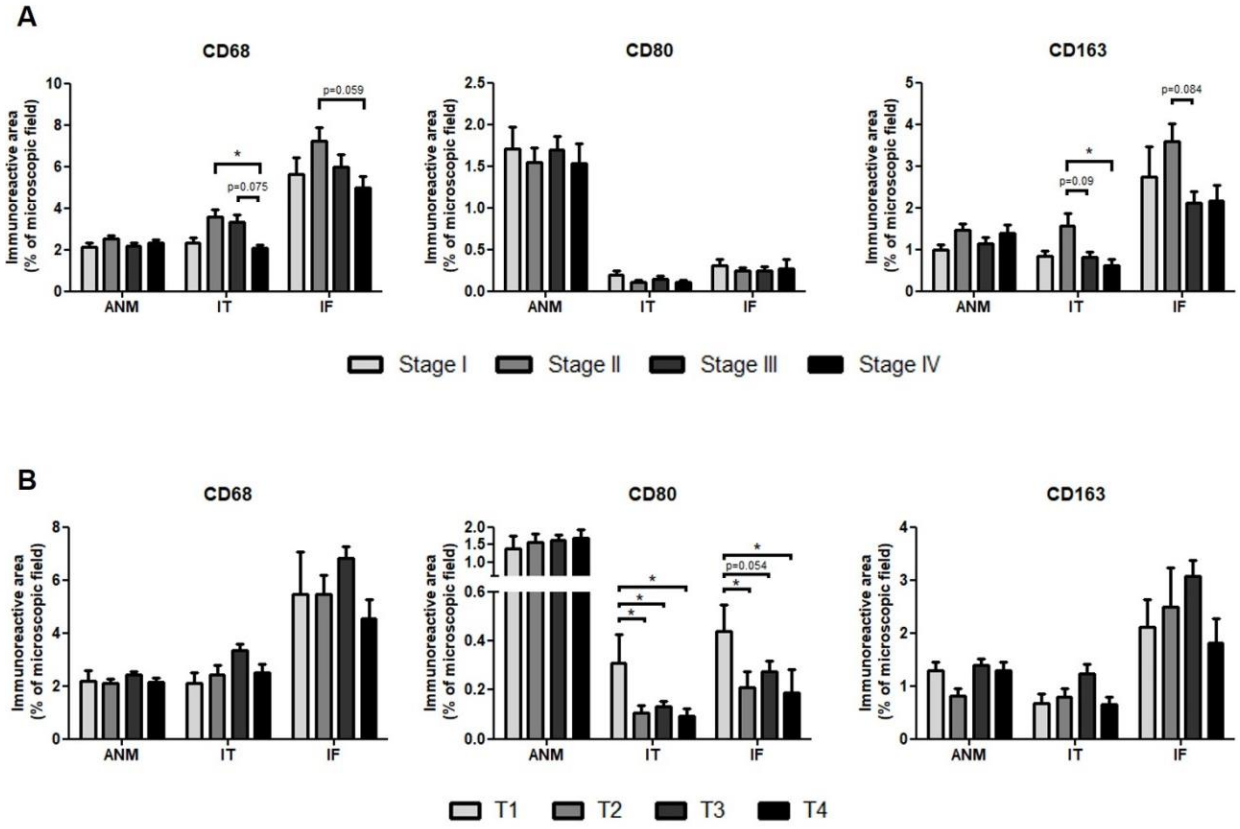


Figure 2.3. Percentage of Immunoreactive area of CD68, CD80 and CD163 in the adjacent normal mucosa (ANM), intratumoral region (IT) and invasive front (IF) according to (A) tumor stage or (B) primary tumor invasive depth. Results are presented as mean and standard error of the mean (SEM). (*) p<0.05, Kruskal-Wallis with Dunns multiple comparisons.

Interestingly, also in stage III tumors, a higher ratio CD80/CD163 in the IF associated with better overall survival, although not reaching the established significance limit, probably due to the relatively low number of stage III tumors in our series (Figure 2.5). This result suggests that, specifically in stage III, a higher proportion between pro-inflammatory cells relatively to anti-inflammatory ones, might represent a survival advantage.

Lower CD80 infiltration is associated with increased relapse

Local recurrence and relapse are frequent problems in CRC treatment [416] and efforts are being made in order to discover factors that might help to predict the risk of such event [417]. Among the 150 cases of our series, 17 experienced relapse. No differences were detected in the percentage of CD68 or CD163 macrophage infiltration between patients with or without relapse, in the three regions analyzed. Conversely, the cases without relapse, presented a significantly higher CD80 IRA % in both the IT (p=0.016) and in the IF ($p=1.16E^{-7}$). Univariate

logistic regression revealed an association between higher CD80 at the IF and a decreased risk for relapse (Supplementary table 2.2). From this, multivariate logistic regression including only variables with significant risk, confirmed the previously mentioned association. This result was further validated by bootstrap analysis using Monte Carlo simulations (Table 2.4). Altogether, these results clearly support a protective role of CD80 cells in the IF of colorectal tumors, specifically regarding relapse.

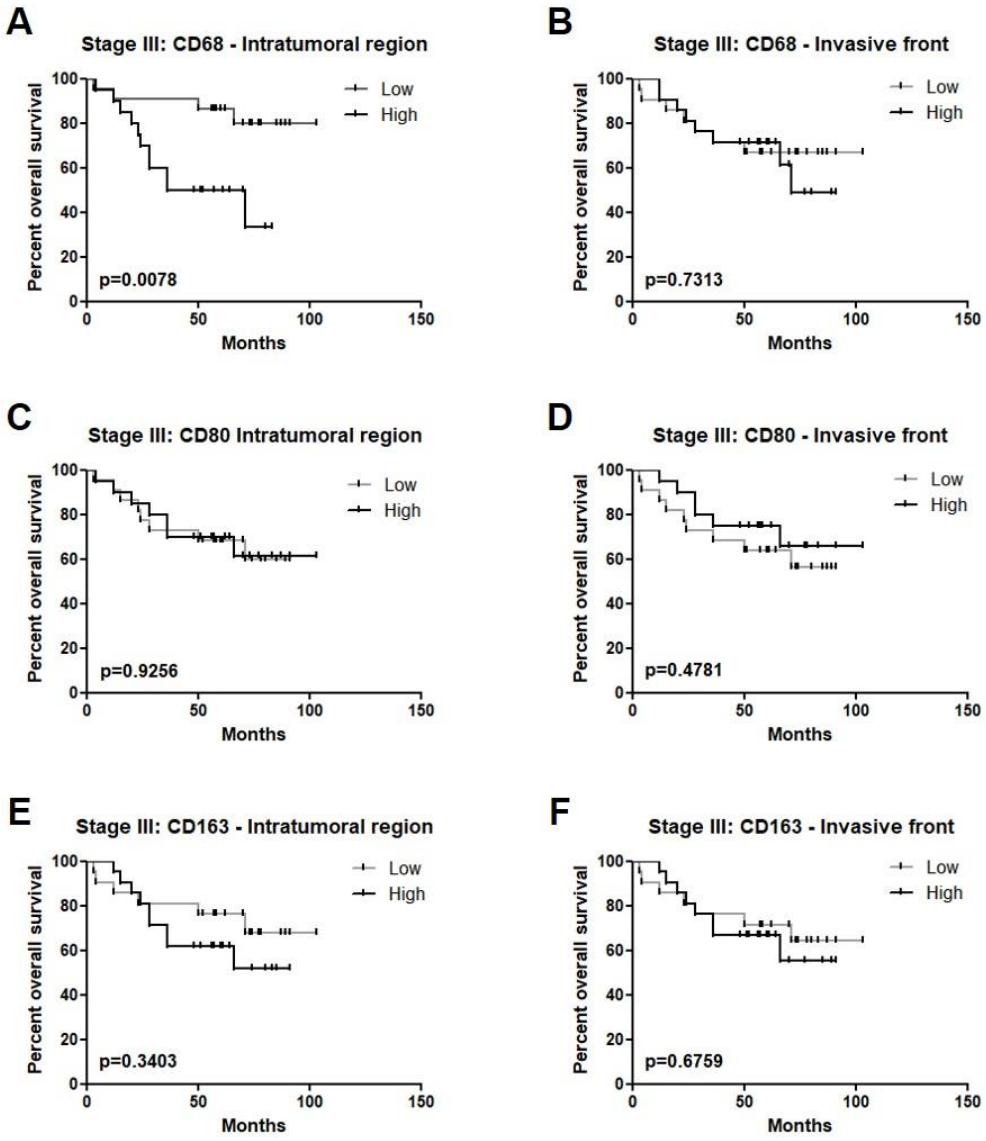


Figure 2.4. Overall survival curves for stage III colorectal cancer patients. Forty-four stage III CRC patients were divided into two groups, low and high, according to the median of immunoreactive area percentage for each marker: (A) CD68 (C) CD80 and (E) CD163 in the intratumoral region and (B), CD68 (D) CD80 and (F) CD163 in the invasive front. P values were obtained through Log-rank test.

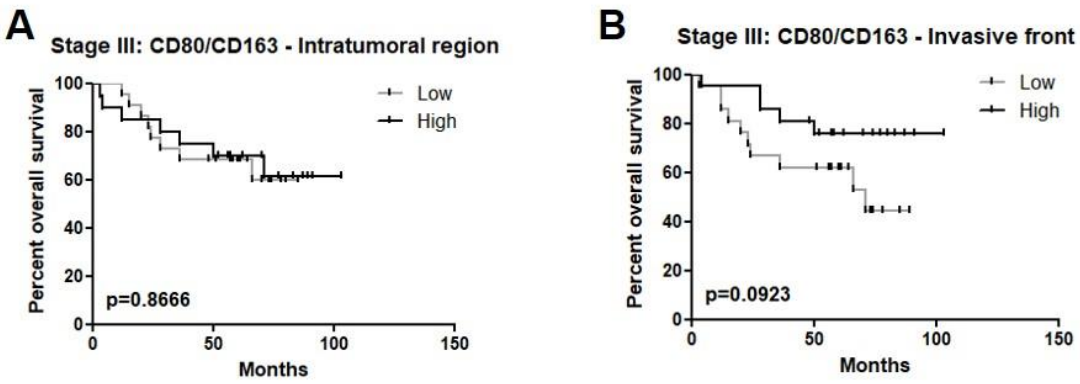


Figure 2.5. Overall survival curves for stage III colorectal cancer patients. Forty-four stage III CRC patients were divided into two groups, low and high, according to the median of CD80/CD163 ratio in **(A)** intratumoral region and **(B)** invasive front. P values were obtained through Log-rank test.

Curiously, also radiotherapy showed a very significant association with increased risk of relapse both in multivariate and bootstrap analyses. This might be related, not with the therapy itself, but with the specific characteristics of the colorectal tumor, which led it to be a candidate for this therapeutic approach.

Table 2.4. Multivariate stepwise logistic regression (pr<0.05) including only variables with significant risk from univariate analysis (Supplementary table 2.3). In order to confirm the strength of association of emerging results from multivariate analysis Bootstrap analysis using Monte Carlo simulations (n=1000) was performed.

	Multivariate			**Bootstrap**	
	OR (95 CI)	P value		OR (95 CI)	P value
Radiotherapy	**17.77 (4.83-65.29)**	**<0.0001**		**19.93 (2.55-155.49)**	**0.004**
IF_CD80*	**0.001 (0.00-0.92)**	**0.047**		**0.001 (0.00-0.45)**	**0.028**

OR, odds ratio; 95 CI, 95% confidence interval; IF, tumor invasive front; * analyzed as continuous variables.

Discussion

In CRC, similarly to what was reported for other cancers, increased infiltration of lymphocytic cells correlates with improved clinical outcome. Specifically, higher infiltration of T cells ($CD3^+$), cytotoxic T cells ($CD8^+$) and memory T cells ($CD45RO^+$) was associated with a longer disease-free and/or overall survival [67]. Moreover, the so called Immunoscore, based on the quantification of two lymphocyte populations (CD3/CD8, CD3/CD45RO, or CD8/CD45RO), both in the core of the tumor and at the invasive margin, revealed to be a more robust and superior prognostic tool than the classical TNM classification by the UICC, specifically in stages I-III. As a result, this immune-classification is currently being introduced in routine clinical settings in order to improve prognosis and help to determine therapy response [68-70].

Importantly, albeit being the most represented immune population within solid tumors, macrophages have not been considered in the mentioned immune-classification, probably due to the contradictory results in the studies addressing the clinicopatologic significance of macrophage infiltration in CRC.

In the present work macrophage profiling was assessed by quantitatively evaluating CD68, a macrophage lineage marker, CD80, a marker of pro-inflammatory macrophages, and CD163, a marker of anti-inflammatory ones. The latter has been frequently used in the literature for this purpose, including in studies performed in CRC, being the marker which gathers more consensus [406, 418-420]. Regarding the identification of an ideal marker of pro-inflammatory macrophages, it has been more challenging. Although several published works used NOS2 [405, 406, 421], it is becoming more evident that, although being specifically expressed in pro-inflammatory macrophages in mice, the same is not true in humans [408, 409]. CD80 is commonly referred in the literature as being expressed by M1 macrophages [171] and has been used to identify pro-inflammatory macrophages in oral squamous cell carcinoma, by immunohistochemistry [422]. Nevertheless, one must keep in mind that none of these markers is completely specific and thus it is expected that some of the quantified immunoreactive area might be due to other populations, namely monocytes, dendritic cells or activated B cells.

The present work clearly showed an increase of CD68 infiltration in tumors comparing with ANM, supporting the idea that these cells are attracted to the tumor site by chemotactic signals [423, 424]. Interestingly, it was possible to observe a complete inversion in the ratio CD80/CD163 between normal and tumor regions, particularly as a result of an almost complete disappearance of $CD80^+$ cells in neoplastic tissues. This result is not supported by other studies, in which M1 macrophages were reported to be approximately 60% of all macrophages, which is probably related with the fact that NOS2 was used to identify this macrophage subpopulation [406]. As reported by others, we confirmed that the IF of colorectal tumors is densely infiltrated by macrophages. Nevertheless, of these, less than 40% were $CD163^+$ cells, which is in accordance with the literature [406], and only 3.5% stained positively for CD80, meaning that more than half of the macrophage population is not expressing any of the polarization markers used in this work. Moreover, we cannot exclude that some macrophages might be expressing both M1 and M2 markers, as reported by Edin and colleagues [405]. Macrophages are known for their plasticity and ability to shift between polarization status according to the stimuli present in their environment and, as a result, macrophage characterization represents a true challenge. Nevertheless it would be fundamental to determine which macrophage subpopulations are represented in the $CD68^+$ cells not identified by CD80 nor CD163 antibodies, in order to identify other macrophage subpopulations present at the tumor microenvironment that could have a putative relevant prognostic role or could be potential targets for therapeutic modulation.

Importantly, when macrophage populations were evaluated according to the tumor stage, CD68 and CD163 expressing cells were shown to be more prevalent in stage II tumors and less in stage IV, similarly to what was observed by Sickert *et al*., in a work performed with TMAs from 100 patients [423]. Conversely, Bailey and colleagues reported a higher macrophage infiltration in stages III and IV but this study only included 22 patients [425]. Regarding CD80 staining, in our series, no differences were observed among the tumor stages. Over the years it has been proposed that, in the initial steps of tumor development, macrophages recruited to the tumor site acquire a pro-inflammatory and anti-tumoral activity and then, as a result of the high IL-10 and TGF-β levels within the tumor microenvironment, their polarization shifts towards a pro-tumoral anti-inflammatory phenotype [171]. We know now that other mechanisms may support the modulation of a tumor tolerogenic or immunosuppressive microenvironment that may favor cancer cell immune escape [85]. For this reason, macrophage populations were separately analyzed according to the primary tumor invasive depth. Interestingly, we observed that $CD80^{+}$ cells were predominant in T1 tumors, contrarily to what happened with CD68 and CD163, supporting, to some extent, the previously mentioned theory.

One important feature of the large intestine is the fact that there are significant differences between the right and left colon, partially explained by their different embryological origin: while the right colon derives from the midgut, the left one is originated in the hindgut [426]. Work by Glebov *et al*., reporting gene expression analysis of the ascending and descending normal colon mucosa from the same subject, revealed clear differences in the expression of genes involved in the control of many cellular functions, namely cell proliferation, adhesion, death and signal transduction. Moreover, by including fetal samples in their study, they concluded that, although significant differences are indeed already established in embryonic colon, additional alterations in gene expression arise in postnatal development [427]. The microbiome present in the gut has also been a subject of deep investigation and it is now known that the amount and type and bacteria in the right and left colon are not the same [428]. Among the various factors which contribute to these differences, food habits are one of the most important. Strikingly, Lawrence and colleagues demonstrated that alterations in the diet, namely the ingestion of animal or plan products, altered the gut microbiome in mice within 2 days [429]. Importantly, these bacteria also have an impact on immune responses, both in health and disease [430]. Interesting work by O'Keefe *et al.* revealed that African Americans rendered to an American diet, comparing with Native Africans maintaining their native food habits, had an increased incidence of colon cancer due to a higher intake of animal products, which resulted in higher colonic populations of potentially toxic hydrogen and secondary bile-salt-producing bacteria [431]. Furthermore, there are molecular, pathological and clinical differences in CRC according to the location side [432]. A recent meta-analysis by Petrelli and colleagues, which

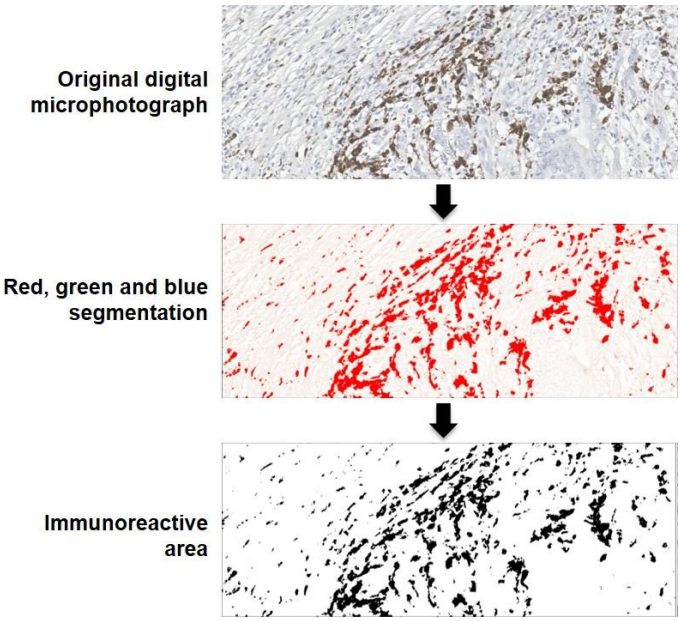
included 66 studies and more than 1.4 million patients, reported a 20% reduction in the risk of death for CRC located on the left side. These authors propose that the side in which the tumor arises should be recognized as a criterion for establishing prognosis in all stages of disease [433]. Importantly, the tumor location also seems to predict therapy response, as reported by Brulé *et al*., in a work showing an improved progression free survival with cetuximab in patients with wild type KRAS only in left-sided tumors [434]. All these differences are reflected in our results, since CD68, CD163 and CD80 macrophages were more prevalent in the normal mucosa of tumors on the right side of the colon. The same was observed in the intratumoral region for both CD68 and CD163. Interestingly, these differences were lost when analyzing the tumor invasive front, suggesting that, in this specific region, the tumor is able to shape the immune response regardless of the environment in which it originates.

Different strategies targeting macrophages are currently being explored, specifically to be applied in breast or ovarian cancer [234]. Of these, inhibiting either monocyte recruitment [239] or their activation [242] are the most studied approaches. More recently, the possibility of re-programming M2 macrophages towards the M1-type is also been considered [243]. In this sense, recent work by our group described the immunomodulatory capacity of PEMs containing IFN-γ and of nanoparticles composed of PGA, specifically in reverting the pro-invasive capacity of IL-10-stimulated macrophages [244, 245].

In terms of prognosis, our results clearly indicate that, within stage III tumors, higher CD68 infiltration in the IT region is associated with decreased OS. Moreover, a higher CD80/CD163 ratio at the tumor IF correlates with improved survival. This work strengths the need to establish the inflammatory profile of the existing populations and to perceive their distribution along the tumor microenvironment for an accurate survival prediction. It is also plausible to speculate that, specifically these patients, might benefit from the latter strategy which would result in an increase of M1 macrophages with a concomitant decrease of M2 subpopulations. Additionally, the fact that a lower infiltration of $CD80^+$ cells strongly associated with increased risk of relapse following surgery, further supports the potential advantage in increasing the number of such cell populations. Nevertheless, since this is first report describing the protective role of $CD80^+$ cells in preventing CRC relapse, it is imperative to validate, in the future, our results in additional cohorts.

Altogether, this work contributed to increase the knowledge regarding macrophage profile in CRC. The association of lower CD68 infiltration and higher CD80/CD163 ratio with increase OS within stage III CRC supports the need for further validations and suggests that it might be beneficial to include such markers in the already established immunoscore. Furthermore, the possible protective role of $CD80^+$ cells in preventing relapse might also open new perspectives in the immunotherapy field.

Supplementary data



Supplementary Figure 2.1. Scheme representing the steps followed for the quantification of the percentage of immunoreactive area. Example of computer assisted quantification of the immunoreactive area by red, green and blue (RGB) segmentation from an original photograph. The immunoreactive area percentage is automatically quantified in relation to the total image area.

Supplementary Table 2.1. Spearman's rank correlation for the percentage of immunoreactive area for CD68, CD80 and CD163 in the adjacent normal mucosa (ANM), intratumoral region (IT) and invasive front (IF).

		ANM			IT			IF		
		CD68	CD80	CD163	CD68	CD80	CD163	CD68	CD80	CD163
ANM	CD68		0,143	**0,508** p =3,4e-011	0,349	-0,109	0,209	0,25	-0,004	0,232
	CD80			0,4120	0,099	0,182	0,202	-0,097	0,273	0,074
	CD163				0,212	0,093	0,411	0,011	0,148	0,278
IT	CD68					0,193	**0,548** p =3,7e-013	0,456	0,145	0,396
	CD80						0,39	-0,074	0,481	0,066
	CD163							0,266	0,273	**0,6149** p = 5,7e-017
IF	CD68								0,103	**0,639** p = 1,4e-018
	CD80									0,347
	CD163									

Spearman Rank-order Coefficient (rs) are presented. In the cases in which rs>0.5, p values are also included.

Supplementary Table 2.2. Empirical univariate analysis of independent variables, clinicopathological and macrophage markers, in association with risk for disease relapse with adjustment for age and gender.

	Risk for disease relapse	
Variables	**OR (95 CI)**	**P value**
Tumor anatomic	**1.60 (1.04-2.40)**	**0.032**
Clinical stage	0.68 (0.30-1.90)	0.461
Radiotherapy	**18.2 (5.30-61.90)**	**<0.0001**
Chemotherapy	3.00 (0.94-9.50)	0.062
ANM *		
CD68	0.92 (0.55-1.55)	0.757
CD80	0.85 (0.53-1.37)	0.502
CD163	0.91 (0.52-1.61)	0.757
IT *		
CD68	1.09 (0.87-1.36)	0.453
CD80	0.02 (0.00-4.40)	0.153
CD163	0.74 (0.40-1.38)	0.346
IF *		
CD68	0.93 (0.81-1.08)	0.365
CD80	**0.001 (0.00-0.48)**	**0.030**
CD163	0.82 (0.61-1.13)	0.191

OR, odds ratio; 95CI, 95% confidence interval; ANM, adjacent normal mucosa; IT, intratumoral region; IF, invasive front; * analyzed as continuous variables.

CHAPTER 3

Decellularized human colorectal cancer matrices polarize macrophages towards an anti-inflammatory phenotype promoting cancer cell invasion via CCL18

Given the results obtained in the previous chapter, describing the different macrophage populations in both normal colon and CRC, combined with the knowledge that tumor tissue presents an abnormal ECM, the question of whether the tumor ECM had any effect on macrophage polarization emerged.

Taking into consideration the complexity of the ECM, not only in terms of composition but also regarding its structure and biomechanical properties, it was not possible to entirely mimic its characteristics using an artificial scaffold. Therefore, we chose to use both normal and tumor human colon tissue, which we decellularized in order to remove DNA and cell debris while retaining the tissue's ECM native characteristics. Following extensive characterization of these decellularized matrices by immunohistochemistry, scanning electron microscopy and rheology, they were repopulated with primary human monocytes. Differentiated macrophages were then characterized and their pro-invasive capacity was evaluated.

The optimized decellularization protocol effectively removed DNA and cell debris from both normal and tumor tissues. Moreover, major ECM components such as collagen type I and IV, fibronectin, laminin and hyaluronic acid were retained, concomitantly with the maintenance of tissue architecture. Mechanical properties, specifically the rigidity, were also partially preserved. Macrophage characterization revealed that tumor ECM induced a more anti-inflammatory macrophage polarization, since these cells presented an increased secretion of IL-10, CCL18 and TGF-β and a decreased expression of TNF and CCR7. Furthermore, macrophages differentiated in tumor-derived matrices stimulated cancer cell invasion contrarily to the ones differentiated in normal-derived matrices. Invasion assays performed in the presence of a specific neutralizing antibody led us to conclude that the invasion induced by tumor ECM-educated macrophages was mediated by CCL18. Importantly, we demonstrated that CCL18 was present at higher levels at the invasive front of more advanced tumors.

Altogether, this study strengthens the use of decellularized tissues as a suitable approach when trying to recreate complex environments. This strategy allowed the conclusion that there are differences between normal and tumor ECM determinant for macrophage polarization, reinforcing the crucial role of this frequent neglected component of the tumor microenvironment on shaping cell behavior. Additionally, CCL18, known as an immunosuppressive molecule, emerged as a macrophage-derived chemokine with pro-invasive capacities, and thus as a potential target for new therapeutic interventions.

Decellularized human colorectal cancer matrices polarized macrophages towards an anti-inflammatory phenotype promoting cancer cell invasion via CCL18

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Decellularized human colorectal cancer matrices polarize macrophages towards an anti-inflammatory phenotype promoting cancer cell invasion via CCL18

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ABSTRACT

Macrophages are frequently identified in solid tumors, playing important roles in cancer progression. Their remarkable plasticity makes them very sensitive to environmental factors, including the extracellular matrix (ECM). In the present work, we investigated the impact of human colorectal tumor matrices on macrophage polarization and on macrophage-mediated cancer cell invasion. Accordingly, we developed an innovative 3D-organotypic model, based on the decellularization of normal and tumor tissues derived from colorectal cancer patients' surgical resections. Extensive characterization of these scaffolds revealed that DNA and other cell constituents were efficiently removed, while native tissue characteristics, namely major ECM components, architecture and mechanical properties, were preserved. Notably, normal and tumor decellularized matrices distinctly promoted macrophage polarization, with macrophages in tumor matrices differentiating towards an anti-inflammatory M2-like phenotype (higher IL-10, TGF-β and CCL18 and lower CCR7 and TNF expression). Matrigel invasion assays revealed that tumor ECM-educated macrophages efficiently stimulated cancer cell invasion through a mechanism involving CCL18. Notably, the high expression of this chemokine at the invasive front of human colorectal tumors correlated with advanced tumor staging. Our approach evidences that normal and tumor decellularized matrices constitute excellent scaffolds when trying to recreate complex microenvironments to understand basic mechanisms of disease or therapeutic resistance.

1. Introduction

The tumor microenvironment has been widely studied in the last decades. The recognition that tumors are comprised by extracellular matrix (ECM) components and different cell populations, including immune cells, fibroblasts and endothelial cells, opened new perspectives for the understanding of cancer biology and the development of more efficient therapies [1,2].

In this context, macrophages emerged as key players in cancer progression, with the capacity to modulate cancer cell migration, invasion and metastasis [3]. These highly plastic immune cells may adopt a myriad of distinct polarization phenotypes, according to the

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microenvironment context. In a rather simplistic vision, macrophages were initially described as being either pro-inflammatory (M1-like) or anti-inflammatory (M2-like). M1-like macrophages are induced by factors such as LPS, interferon-gamma (IFN-γ) or TNF-α. They exhibit high antigen presenting capacity, produce high levels of IL-6, IL-12 and IL-23 and toxic intermediates, and are known for their tumoricidal properties. Conversely, M2-like macrophages are induced by factors such as IL-10, IL-4, IL-13 or glucocorticoid hormones. They produce low levels of pro-inflammatory cytokines and high levels of anti-inflammatory cytokines, e.g. IL-10, having an important role in tissue repair, angiogenesis and in tumor progression [4]. In the last decade, macrophage polarization was recognized to be more complex and a continuum of polarization status between these two extreme populations was proposed [5,6].

Together with these cell populations, the ECM was described as being of pivotal importance for cancer progression [7,8], modulating the behavior of both cancer cells and other populations within the tumor microenvironment, namely immune and endothelial cells. The ECM is a highly dynamic and complex network of macromolecules, consisting of a reservoir of numerous bioactive domains and arrested growth factors [9]. It has critical functions, both in disease and homeostasis, development, morphogenesis and stem cell fate [10,11].

Since it has been clearly demonstrated that cells behave differently in 2D or 3D cultures [12], efforts have been made to develop new models that recapitulate as accurately as possible the native tumor microenvironment. These include organotypic explant cultures, multicellular tumor spheroids, or the use of engineered scaffolds composed of natural and/or synthetic components [13,14].

In the last decade, tissue decellularization, a process that enables cell removal without affecting the ECM structure and composition, emerged as an alternative technique in the field of tissue engineering and regenerative medicine [15]. Organs such as heart [16], lung [17] and liver [18] have been successfully decellularized and re-colonized by cells, constituting a promising solution for end-organ failure. Decellularization has also been recently applied in an attempt to unravel the complex role of ECM on metastasis and tumor progression [19–21].

In the present work, we developed a model that mimics the native tumor microenvironment, aiming to elucidate the role of tumor ECM on macrophage polarization and how this impacts cancer cell invasion. To achieve this goal, we used paired samples of human colorectal cancer (CRC) and non-neoplastic mucosa, which were efficiently decellularized and repopulated with primary human monocytes. Notably, although derived from the same patient, normal and tumor matrices distinctly modulated macrophage polarization. In tumor decellularized matrices, macrophages differentiated towards an anti-inflammatory phenotype, stimulating CRC cell invasion through a mechanism involving the CCL18 chemokine. In agreement, we observed that CCL18 was highly expressed at the invasive front of more advanced CRC cases. Overall, this 3D-organotypic approach, using tumor decellularized matrices as native human ECM source, provided new insights into the intricate crosstalks established at the tumor microenvironment, and contributed to the discovery of a putative new target for therapeutic intervention.

2. Materials and methods

2.1. Study approval

In the present study, both normal and tumor human samples were obtained from the Pathology Department from Centro Hospitalar São João (CHSJ, Porto, Portugal). Human monocytes were isolated from buffy coats from healthy blood donors, also from CHSJ. All studies using these human samples were approved by CHSJ Ethics Committee for Health (References 259 and 260/11), in agreement with the Helsinki declaration. Informed consent was obtained from all subjects.

2.2. Clinical samples

Fresh colorectal surgical specimens were collected directly from the Pathology Department from the Pathology Department from CHSJ within 1 h after surgery and transported in HBSS (Sigma-Aldrich), at 4 °C, to the laboratory where they were processed. Briefly, fragments were cut in smaller samples, placed in plastic containers and covered with mounting medium for cryotomy (OCT compound, Thermo Scientific). Samples were rapidly frozen in liquid nitrogen cooled 2-methylbutane and stored at −80 °C until further use.

2.3. Decellularization

Normal and tumor frozen colorectal specimens, from the same patient, were washed in PBS, cut in similar sizes, weighed and placed in a 24-well plate. Colorectal samples were decellularized based on the protocol described elsewhere [22]. After incubation with hypotonic buffer A (10 mM Tris, 0.1% EDTA, pH 7.8) for 18 h, tissue fragments were washed with PBS and decellularized for 24 h with 0.1% SDS. Following three washes with hypotonic buffer B (10 mM Tris, pH 7.8), a 3 h digestion, at 37 °C, was performed using 50 U/mL DNase (Applichem) prepared in 20 mM Tris and 2 mM $MgCl_2$, pH 7.8. The protocol was performed under constant agitation (165 rpm) in presence of 10 μg/mL of gentamicin (Gibco). Two different controls were considered: the "Native", a fragment stored at −80 °C immediately after sample collection, and the "Non-decellularized", a fragment incubated with PBS and refreshed every time a new solution was added to the samples undergoing decellularization. The experimental setup is represented in Supplemental Fig. S1.

2.4. DNA evaluation

After decellularization, samples were formalin-fixed, processed to paraffin blocks and sliced into 3 μm-thick sections. These were counterstained with Vectashield containing DAPI (VectorLaboratories) for fluorescent staining of nucleic acids. To assess total DNA content within the native tissue and the decellularized and non-decellularized matrices, DNA was extracted using PureLink Genomic DNA Mini Kit (Invitrogen) according to the manufacturer's instructions. DNA was quantified using Quant-iT™ PicoGreen® dsDNA kit (Invitrogen) and the fluorescence was measured with excitation at 480 nm and emission at 520 nm. Results were presented as ng of DNA per mg of tissue.

2.5. Histological analysis

3 μm-thick sections from paraffin-embedded samples were processed and stained with Hematoxylin and Eosin (H&E) and Masson's Trichrome for histomorphological analysis.

2.6. Immunohistochemistry

Imunohistochemistry analyses were performed for ECM proteins fibronectin, laminin and collagens type I and IV. Antigen retrieval was performed in a water-bath at 98 °C for 35 min with citrate buffer pH6, following blocking using Ultra V Block (Thermo Fisher Scientific) for 30 min. Tissue samples were then incubated

for 1 h and 30 min with primary antibodies diluted in Large Volume Ultra AB Diluent (Thermo Fisher Scientific) according to the following concentrations: laminin (Sigma-Aldrich, L9393; 1:100), fibronectin (Sigma-Aldrich, F3648; 1:400), collagen type I (Sigma-Aldrich, Col I; 1:50) and collagen type IV (Millipore, AB769; 1:10). After washing, donkey anti-goat Alexa-Fluor-488-conjugated, goat anti-mouse or anti-rabbit Alexa-Fluor-594-conjugated secondary antibodies (Life Technologies) were added to slides for 1 h, washed in PBS and mounted on Vectashield with DAPI.

2.7. Hyaluronic acid staining

Native and decellularized normal and tumor sections were blocked using Ultra V Block, for 20 min and then incubated with Hyaluronic Acid Binding Protein (Millipore, 385911, (1:200)), overnight, at 4 °C. After washing with PBS, Streptavidin Alexa-Fluor-555-conjugated (1:500) was added for 30 min and slides were mounted on Vectashield with DAPI.

2.8. Scanning Electron Microscopy (SEM)

Samples were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate for 30 min at room temperature under gentle agitation (50 rpm), washed with cacodylate buffer and dehydrated in a graded ethanol-water series from 50% to 100% ethanol. Fragments were critical point dried using CO_2, mounted in sticky carbon tape and coated with a Au/Pd thin film, by sputtering, using the SPI Module Sputter Coater equipment. The SEM/EDS exam was performed using a High resolution Scanning Electron Microscope with X-Ray Microanalysis: JEOL JSM 6301F/Oxford INCA Energy 350 (Tokyo, Japan).

2.9. Viscoelastic properties

The viscoelastic properties of colorectal tissue samples were analyzed using a Kinexus Pro (Malvern, UK) rheometer. Normal and tumor native and decellularized matrices from three different patients were cut using a 4 mm diameter biopsy cylinder punch (Integra® Miltex®) and analyzed. All measurements were performed inside the rheometer hood, in a humidified and temperature-controlled environment (37 °C), using 4 mm sand-blasted parallel plates. The linear viscoelastic regions (LVR) of the tissue samples were determined by iteratively performing frequency and amplitude strain sweeps. Three to six matrices of each native normal, native tumor, decellularized normal and decellularized tumor samples (*per* patient) were analyzed.

2.10. Human monocyte isolation

Human monocytes were isolated from buffy coats from healthy blood donors as previously described [23]. Isolated cells were ressuspended in RPMI1640 (L-glutamine) (Invitrogen) supplemented with 10% FBS (Lonza), 100U/ml penicillin and 100 μg/mL streptomycin (PS) (Invitrogen). Using this procedure, 90%–95% of CD14-positive cells are usually obtained, as previously validated by flow cytometry analysis [23].

2.11. Decellularized matrices repopulation

Before repopulation, decellularized matrices were washed with PBS and incubated overnight with RPMI1640 (L-glutamine) supplemented with 10% FBS and PS. Normal or tumor-derived matrices were then transferred to a 96-well plate and a plastic O-ring was placed on top of each matrix. Subsequently, 1.5×10^6 freshly isolated monocytes were ressuspended in 20 μL of culture medium, carefully placed inside each ring and incubated for 18 h in humidified conditions. The following day, matrices were transferred to a new well with 200 μL of medium, which was changed every 2/3 days. Monocytes were allowed to differentiate for 8 or 14 days at 37 °C and 5% CO_2 humidified atmosphere. A schematic representation of the repopulation setup is included in Supplementary Fig. S2. For each assay, matrices from at least 3 different patients were used. In some assays, matrices from the same patient were used twice, in independent experiments (independent decellularization and repopulation with monocytes from different blood donors). Monocytes from the same blood donor were sometimes used to repopulate matrices from different patients. In each figure legend the number of matrices and monocytes used, as well as the final number of pairs (each pair corresponds to a unique combination of matrix from a specific patient repopulated with monocytes from a specific blood donor) are included.

2.12. Repopulation assessment

To evaluate differences in the repopulation efficiency, DNA was extracted from repopulated matrices as described in a previous section. Experiments were performed using two experimental replicates to minimize repopulation variability. DNA from not repopulated normal and tumor decellularized matrices was also quantified and subtracted to the values obtained in repopulated matrices. Normal and tumor-repopulated decellularized matrices were formalin-fixed and processed for IHC using a specific CD68 (Dako, PG-M1) antibody. Briefly, antigen retrieval was performed in a water-bath at 98 °C using Tris EDTA pH9 for 20 min and the antibody was incubated for 30 min (1:100). After washing, labeled polymer secondary antibody (Envision Detection System, Dako) was added to slides and peroxidase activity was detected using diaminobenzidine (DAB)-tetrahydrochloride liquid plus substrate Chromogen System (Dako) for 2 min. Reaction was stopped with distilled water and sections were counterstained with hematoxylin and mounted in Richard-Allan Scientific Mounting Medium (ThermoFisher). Repopulated matrices were also analyzed by SEM, as above described.

2.13. Morphology evaluation

To evaluate macrophage morphology within the decellularized matrices, cell cytoskeleton was stained for actin (Invitrogen) and tubulin (Sigma-Aldrich). Matrices were fixed with 4%PFA-5%sucrose for 20 min and permeabilized with 0.5%Triton X-100. After blocking for 30 min with Ultra V Block, matrices were incubated for 2 h with a monoclonal anti-α-tubulin antibody (Sigma-Aldrich, T9026) and washed with PBS-Tween$^{0.02\%}$. Anti-mouse Alexa-Fluor-488-conjugated secondary antibody was incubated for 1 h and 30 min (Life Technologies). After a new washing step, Alexa-Fluor-647-Phalloidin (Life Technologies) was added for 1 h. Finally, samples were incubated with DAPI (Sigma-Aldrich) and mounted on Mowiol mounting medium. Repopulated matrices were photographed using a Spectral Confocal Microscope Leica TCS-SP5 AOBS (Wetzlar, Germany). For shape analysis, ICY software was used [24]. Stack images were thresholded with the Threshold plugin and 3D regions of interest (ROIs) were extracted with Connected components plugin (developed by Alexandre Dufour). Extracted objects were then filtered by size and only ROIs between 2500 and 50000 μm^3 were considered for further analysis. 3D Analysis plugin (developed by Thomas Boudier) was used to calculate several cell shape parameters including macrophage volume, sphericity, compactness, flatness and elongation [25]. Matrices from 4 patients were analyzed in randomly selected regions, considering at least 60 cells per matrix.

2.14. ELISA

Fourteen days after repopulation, conditioned media from repopulated matrices were collected and centrifuged at 1200 rpm for 5 min to remove cell debris. These were then analyzed by ELISA for IL-6, IL-10, CCL18 and TGF-β levels, in accordance with the manufacturers' instructions (BioLegend, and Abcam, in the case of CCL18). Cytokine levels were normalized to the repopulation efficiency. As control, IL-6, IL10 and CCL18 were also evaluated in conditioned media from non-repopulated matrices.

2.15. Cell culture and reagents

RKO cells, derived from a colon carcinoma (CRL-2577), SW420 (CCL-228), SW620 (CCL-227) and HCT15 (CCL-225) were purchased from the American Type Culture Collection (ATCC, USA) and were maintained at 37 °C under a 5% CO_2 humidified atmosphere in RPMI1640 (L-glutamine) (or DMEM, in the case of HCT15) supplemented with 10% FBS and PS.

Recombinant Human MIP-4 (CCL18, Peprotech) was used at 1 ng/mL.

2.16. Matrigel invasion assay

In order to evaluate the ability of macrophages differentiated in normal or tumor decellularized matrices to stimulate RKO cell invasion, 5×10^4 cells were added to the upper compartment of Matrigel-coated inserts of 8-μm pore size (BD Biosciences). At the lower compartment, two decellularized matrices, either normal or tumor, repopulated or not, were added for 24 h. A schematic representation of the invasion assay is included in Supplementary Fig. S3. The same assay was also performed with recombinant human CCL18, in the presence or absence of the neutralizing anti-CCL18 antibody (R&D, AF394, 2 μg/mL). Furthermore, CCL18 was tested in Matrigel invasion assays using SW480, SW620 and HCT15 cells. Additionally, a positive control, a coverslip with 13 days-differentiated macrophages was included in each assay [23]. After 24 h, invasive cells were counterstained with DAPI and counted using a fluorescence microscope.

2.17. CCL18 evaluation in CRC

A total of 68 CRC cases from CHSJ were immunohistochemically stained with anti-CCL18 antibody (Peprotech, 500-P108, 1:200). Antigen retrieval was performed with Citrate Buffer, pH 6, for 20 min in a water bath at 98 °C, and the antibody was incubated for 45 min. The remaining protocol was performed as previously described. CCL18 staining was evaluated at the invasive front and assessed as absent/few positive cells (score 1), less than 40% (score 2), between 40 and 80% (score 3) and more than 80% of the invasive front (score 4). Each specimen was evaluated twice, independently and blindly regarding tumor stage. Discordant cases were re-analyzed and a definite decision was taken.

2.18. Statistical analysis

All graphs and statistical analysis were performed using GraphPad Prism Software v.5 (GraphPad-trial version). Data was analyzed for Gaussian distribution using the D'Agostino and Pearson normality test, when $n \geq 8$. Wilcoxon paired test or Mann-Whitney test were used for non-parametric samples, whereas *t*-test (either paired *t*-test or one sample *t*-test) was used for parametric data or when $n < 8$. For other analyses, One-way ANOVA with Dunnett's multiple test correction or Kruskal-Wallis test corrected with the Dunn test for multiple comparisons were performed. The association of CCL18 expression with tumor stage was assessed using a two-sided Fisher's exact. Univariate and multivariate ordinal regressions were performed in SPSS v.23 to test for variable independence. The multivariate regression model included the independent variables age, gender, stage, tumor location (colon vs rectum and right vs left side) and morphology. A P-value <0.05 was considered statistically significant.

3. Results

3.1. Decellularization efficiently removes DNA content and cell debris from normal and tumor colon matrices

Paired samples of similar weights, encompassing normal mucosa and tumor, were retrieved from CRC patients' surgical specimens. Both tissues were decellularized using a protocol that we developed and is described in detail in the Materials and Methods section, which combines the use of a hypotonic buffer with a detergent of anionic surfactant properties and DNAse. For control purposes, part of the normal and tumor fragments were immediately frozen and stored at −80 °C ("native") or kept in PBS ("non-decellularized") during the decellularization protocol (Supplementary Fig. S1). Macroscopically, throughout the procedure, decellularized fragments became more white and transparent than native tissues, suggesting cellular removal (Fig. 1A). To evaluate decellularization efficiency, samples were analyzed by DNA staining (DAPI) and DNA quantification [26]. No visible nuclei were observed in normal and tumor matrices after decellularization (Fig. 1B). DNA quantification (Fig. 1C) confirmed that the established protocol efficiently removed about 99.3% of the total DNA (1012.2 ± 73.7 ng/mg native vs. 6.7 ± 0.4 ng/mg decellularized) in normal tissue and 99.6% (826.2 ± 163 ng/mg native vs. 3.1 ± 1.5 ng/mg decellularized) in tumor fragments. Hematoxylin and Eosin (H&E) staining (Fig. 1D) confirmed the absence of cell remnants in both normal and tumor decellularized tissues. In addition, Masson's Trichrome staining (Fig. 1E) evidenced that native tissues have a predominant red and blue colors corresponding to cell cytoplasm and collagens, respectively. Conversely, decellularized fragments displayed an almost exclusive blue staining which indicates loss of cellular components with preservation of a collagen-rich ECM network.

3.2. Decellularized normal and tumor matrices retain major ECM components and preserve ECM architecture

To further characterize the impact of decellularization on the composition and distribution of major ECM components, immunohistochemistry for fibronectin, laminin and collagens type I and IV was performed in both normal and tumor native and decellularized matrices (Fig. 2A). In normal native tissues, the four proteins presented a typical organized distribution, delimiting the glands, being mostly adjacent to the cell basement membrane. Upon decellularization, it was still possible to detect these four proteins, although presenting an apparent disrupted architecture, with partial loss of glands organization. On the other hand, native tumors exhibited a denser ECM network and the distribution pattern of the four proteins was preserved after decellularization. The presence of hyaluronic acid (HA), a non-sulfated glycosaminoglycan, was also evaluated using a specific hyaluronic acid binding protein (Fig. 2A). In both normal and tumor native tissues, HA is highly abundant but with distinct patterns: in normal tissue it is possible to observe a more reticulated organization whereas tumor presents a more diffuse staining, probably related with the expression of HA of lower molecular weights. Upon decellularization, the matrices still present HA with similar organization as in

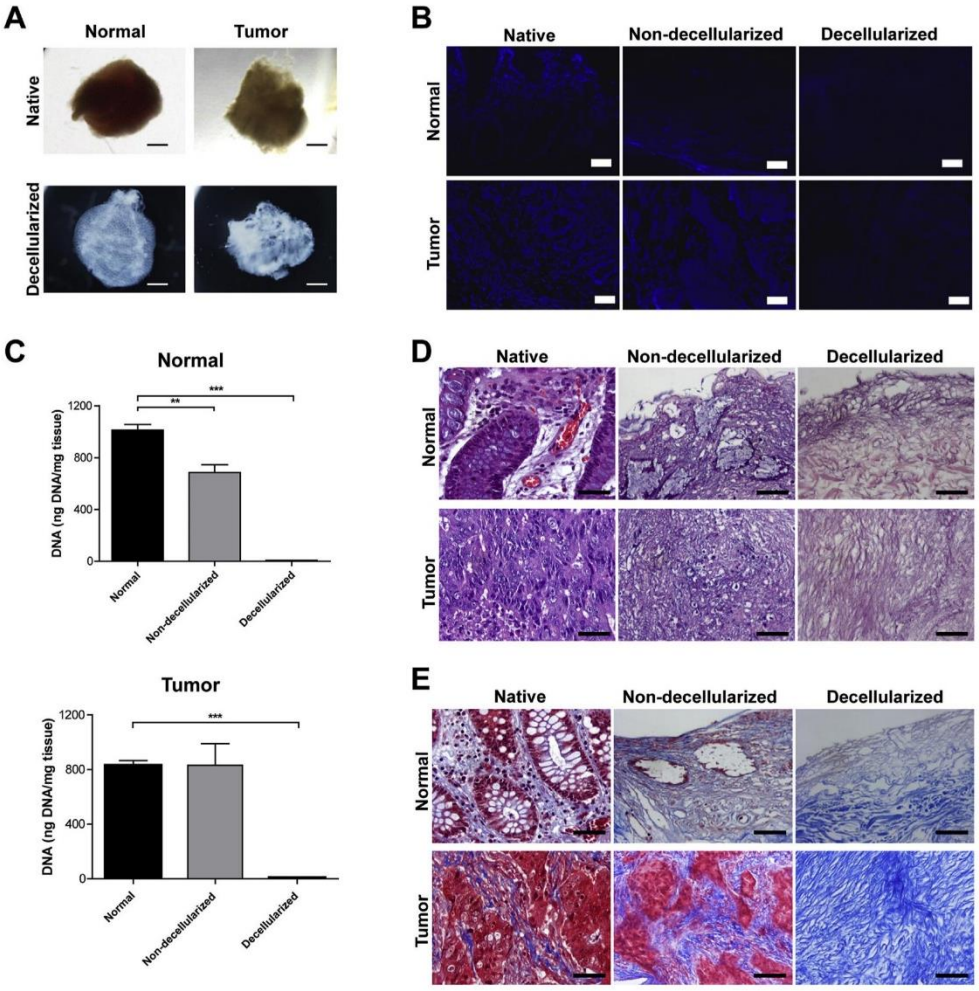

Fig. 1. Decellularization protocol efficiently removes DNA and cell debris from normal and tumor colon matrices. **(A)** Human normal and tumor colorectal matrices, obtained from surgical specimens, before and after decellularization. Scale bar = 1 mm. **(B)** Native, non-decellularized and decellularized sections from formalin fixed paraffin embedded tissues were stained with DAPI. Scale bar = 50 μm (Normal) and 100 μm (Tumor). **(C)** PicoGreen DNA quantification of native, non-decellularized and decellularized matrices (mean ± SEM); $^{**}P < 0.01$, $^{***}P < 0.001$, One-way ANOVA with Dunnett's multiple test correction. **(D)** Histological sections stained with H&E and **(E)** Masson's Trichrome. Scale bar = 50 μm. Representative images and quantifications of matrices from 5 different patients.

the native tissues. Conversely, sulfated glycosaminoglycans (GAGs) were similarly affected in normal and tumor matrices by the decellularization process. Both tissues retained approximately 12% of total GAGs amount detected in the native tissues (4.23 ± 0.49 μg/mg native vs. 0.54 ± 0.33 μg/mg decellularized in normal matrices; and 6.48 ± 0.5 μg/mg native vs. 0.78 ± 0.1 μg/mg in decellularized tumor matrices) (Supplementary Fig. S4).

Complementary analysis of tissue architecture was performed by Scanning Electron Microscopy (SEM) (Fig. 2B) revealing that normal native matrices exhibited a homogeneous surface organization with well-defined colon crypts. Upon decellularization, the ultrastructure was preserved without visible tissue alterations, suggesting that the modifications previously observed through immunohistochemistry were caused by the required manipulation necessary for the inclusion of the samples in paraffin blocks. Native tumor matrices, on the other hand, presented a more compact and disorganized structure in comparison to healthy colon tissue. Nevertheless, decellularized tumor matrices resembled the native tumor tissue morphology.

3.3. Tumor decellularized matrices preserve higher stiffness than normal decellularized matrices

In order to evaluate the impact of decellularization on the viscoelastic properties of normal and tumor matrices, rheological analyses were performed to compare native and decellularized samples' properties. Frequency and amplitude strain sweeps were iteratively performed on the different matrices (Supplementary Fig. S5) and showed that their linear viscoelastic region (LVR) ranged from 0.01 Hz to 0.1 Hz and from 0.1% to 2% strain (highlighted in light-grey). For comparison purposes (Fig. 3), we retrieved the complex shear modulus (G*) data from the LVR of the

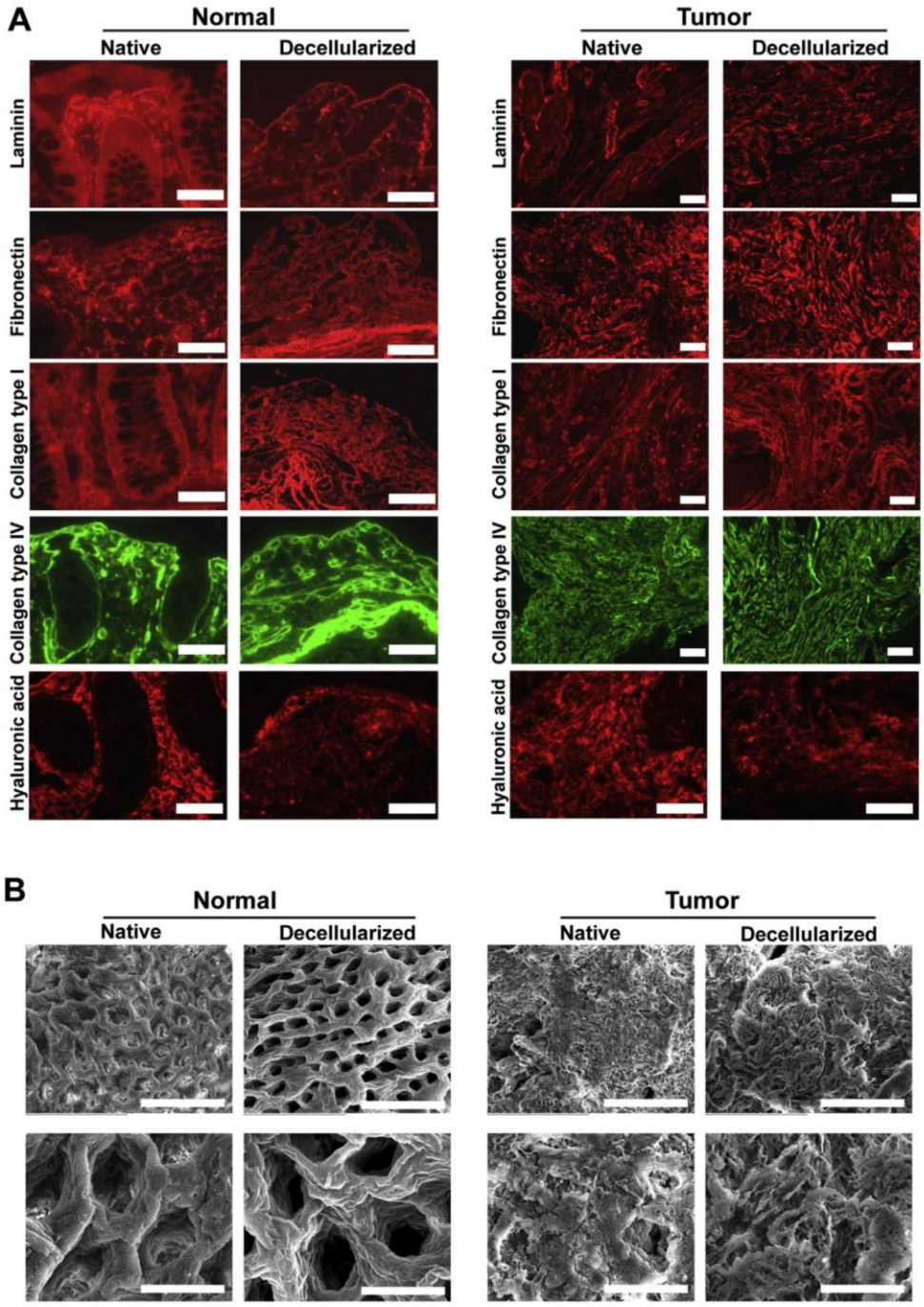

Fig. 2. Decellularized normal and tumor colorectal matrices preserve major ECM components and architecture. (A) Immunohistochemical expression of laminin, fibronectin, collagens type I and IV in native and decellularized normal and tumor sections. Hyaluronic acid was stained using a Hyaluronic Acid Binding Protein. Scale bar = 20 μm. **(B)** Scanning Electron Microscopy of native and decellularized normal and tumor matrices. Scale bar = 400 μm (upper images) and 100 μm (lower images). Representative images of matrices from 4 different patients.

frequency sweeps (at ≈0.03 Hz and 0.5% for the native matrices and 1% for the decellularized matrices). This showed that tumor native tissues are stiffer than the normal ones. Moreover, the observed differences were preserved after decellularization in all cases, despite the decrease in tumor matrices' stiffness in two out of three patients (Fig. 3).

Altogether, our decellularization protocol efficiently removed the cellular components, while maintaining major ECM proteins, tissue architecture and viscoelastic properties, thus generating bioscaffolds that conserved native tissues differences observed between normal and tumor colon ECM.

3.4. Decellularized matrices promote monocyte differentiation

To evaluate the capability of tumor ECM to modulate macrophage differentiation, normal and tumor decellularized matrices were repopulated with monocytes, freshly isolated from buffy coats from healthy blood donors. Eight days after monocyte repopulation, DNA quantification revealed a 22% reduction in tumor matrices in comparison to normal ones (Fig. 4A). Since we did not observe any differences concerning proliferation and apoptosis in the repopulated matrices (Supplementary Figs. S6 and S7), our data suggest that the disparities in the DNA are caused by a lower monocyte repopulation efficiency. This was taken into consideration in further analyses, specifically in ELISA quantification and zymography.

Efficient monocyte repopulation was evidenced by the observation of nuclei in both normal and tumor repopulated matrices (data not shown), not visible in not repopulated controls. Immunohistochemistry for CD68, a monocyte/macrophage lineage marker, confirmed that monocytes were able to migrate through the ECM network since they were found deep within the repopulated matrices (Fig. 4B). The elongated cell morphology suggested monocyte polarization and differentiation into macrophages. SEM analysis allowed the visualization of several cell protrusions, indicative of close cell-matrix interactions (Fig. 4B).

Knowing that macrophage morphology can reflect distinct polarization status [27], we assessed possible morphological differences between macrophages differentiated in both decellularized matrices. By doing a 3D reconstruction of macrophage morphology, combining actin and tubulin stainings, analysis of morphometric parameters (e.g. cell sphericity, flatness or elongation) was performed. No significant differences on any of these characteristics were found between macrophages differentiated in normal and in tumor-derived matrices (Fig. 4C and D).

3.5. Tumor decellularized matrices polarize macrophages towards a more anti-inflammatory (M2-like) phenotype

To evaluate whether normal and tumor decellularized matrices had the ability to differently modulate macrophage polarization, gene expression and cytokine/chemokine secretion of pro- and anti-inflammatory markers were analyzed by quantitative real-time PCR (Fig. 5A) and ELISA (Fig. 5B), respectively. The expression profile of macrophages, derived from the same blood donor, differentiated in normal decellularized matrices was compared with the ones differentiated in tumor decellularized matrices from the same CRC patient. Results consistently indicate that macrophages differentiated in tumor decellularized matrices significantly express less *CCR7* and *TNF*, both characteristic markers of pro-inflammatory macrophages, and more *CCL18*, a chemokine associated with anti-inflammatory macrophages. No differences were observed regarding the expression of other macrophage surface receptors, namely *MRC1*, *CD163* and *CD80*, nor ECM components like fibronectin and versican. On the other hand, *MMP1*, a matrix metalloproteinase frequently attributed to M2-like macrophages, was significantly less expressed in cells repopulating tumor decellularized matrices (Fig. 5A). To complement this analysis, the levels of IL-6, a pro-inflammatory cytokine, and IL-10, TGF-β and CCL18, which are anti-inflammatory molecules, were evaluated in the conditioned medium of repopulated matrices, by ELISA. As control, IL-6, IL-10 and CCL18 levels were also analyzed in the conditioned media of non-repopulated normal and tumor decellularized matrices (Supplementary Fig. S8), revealing very low concentrations when compared with the repopulated counterparts. A decrease in IL-6 levels was observed in macrophages repopulating tumor matrices, although without statistical significance ($P = 0.07$). Conversely, the secretion of the anti-inflammatory IL-10, TGF-β, and CCL18 was significantly increased in cultures with macrophages differentiated in tumor-derived matrices than their counterparts differentiated in normal matrices (Fig. 5B), suggesting the acquisition of a more anti-inflammatory phenotype.

Given the fact that MMPs are important players in cancer, the amount of pro- and active forms of these gelatinolytic enzymes

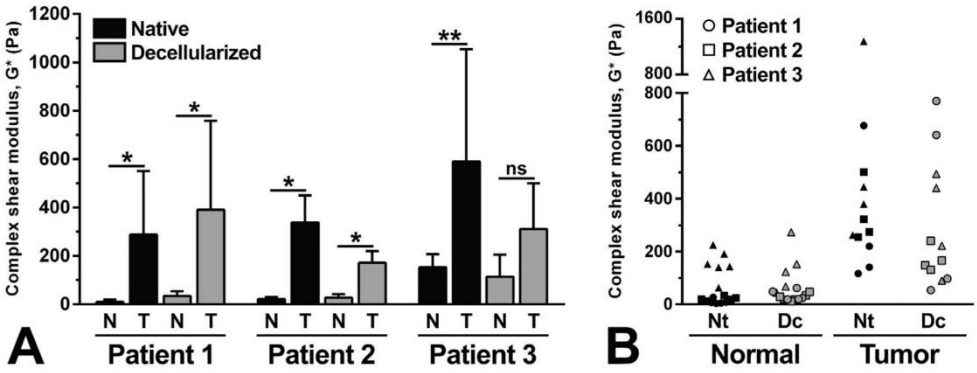

Fig. 3. Tumor decellularized matrices exhibit a higher stiffness than normal decellularized matrices. (A) Viscoelastic properties assessment by rheological analysis. Complex shear modulus (G*) values (at 0.03 Hz, retrieved from the linear viscoelastic region (LVR) of the performed frequency sweeps, in Supplementary Fig. S5) of the different colorectal tissue samples from 3 different patients (5 or 6 matrices, for normal tissues, and 4 matrices, for tumor tissues). "N" refers to normal and "T" refers to tumor. **(B)** Scatter dot plot combining the viscoelastic data of all the matrices analyzed. Circle, square and triangle symbols were used to identify the matrices from each of the 3 patients, respectively. "Nt" refers to native and "Dc" refers to decellularized. *$P < 0.05$, Mann-Whitney test. ns, not significant.

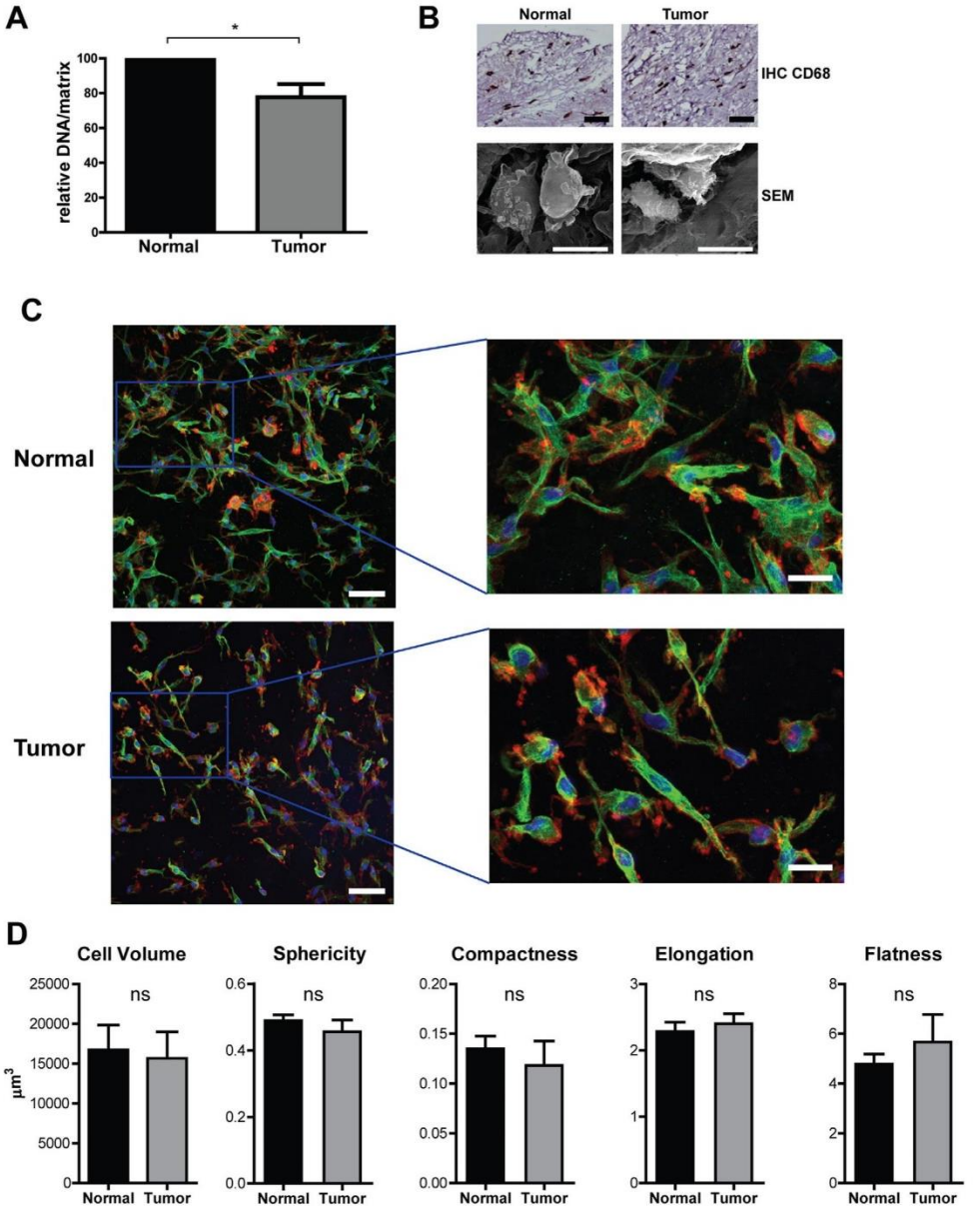

Fig. 4. Decellularized matrices promote monocyte differentiation (A) PicoGreen DNA quantification of normal and tumor repopulated decellularized matrices (mean ± SEM, matrices from 5 different patients). $^{*}P < 0.05$, paired 2-tailed Student's *t*-test. **(B)** Representative images of CD68 lineage marker immunohistochemistry (scale bar = 50 μm) and Scanning Electron Microscopy analysis (scale bar = 10 μm) from normal and tumor repopulated matrices. **(C)** Representative confocal images of tubulin (green), actin (red) and nuclei (blue) staining of macrophages repopulating normal and tumor decellularized matrices. Scale bar = 50 μm or 20 μm. **(D)** Tridimensional shape analysis of macrophages cultured in the designated decellularized matrices, namely cell volume, sphericity, compactness, elongation and flatness. Matrices from 4 different patients were analyzed, with a minimum of 60 macrophages in each matrix. ns, not significant.

were analyzed through gelatin zymography in conditioned media from macrophages differentiated in normal or tumor-derived matrices (Fig. 5C). The recombinant pro-MMP9 and the respective PMA activated form were run in parallel and used as a molecular weight reference. Consistently, we observed an enhanced secretion of pro-MMP9 by macrophages differentiated in normal matrices when compared with the ones differentiated in tumor matrices.

Altogether our data demonstrates that, although derived from the same patient, normal and tumor matrices distinctly modulate macrophage inflammatory profile.

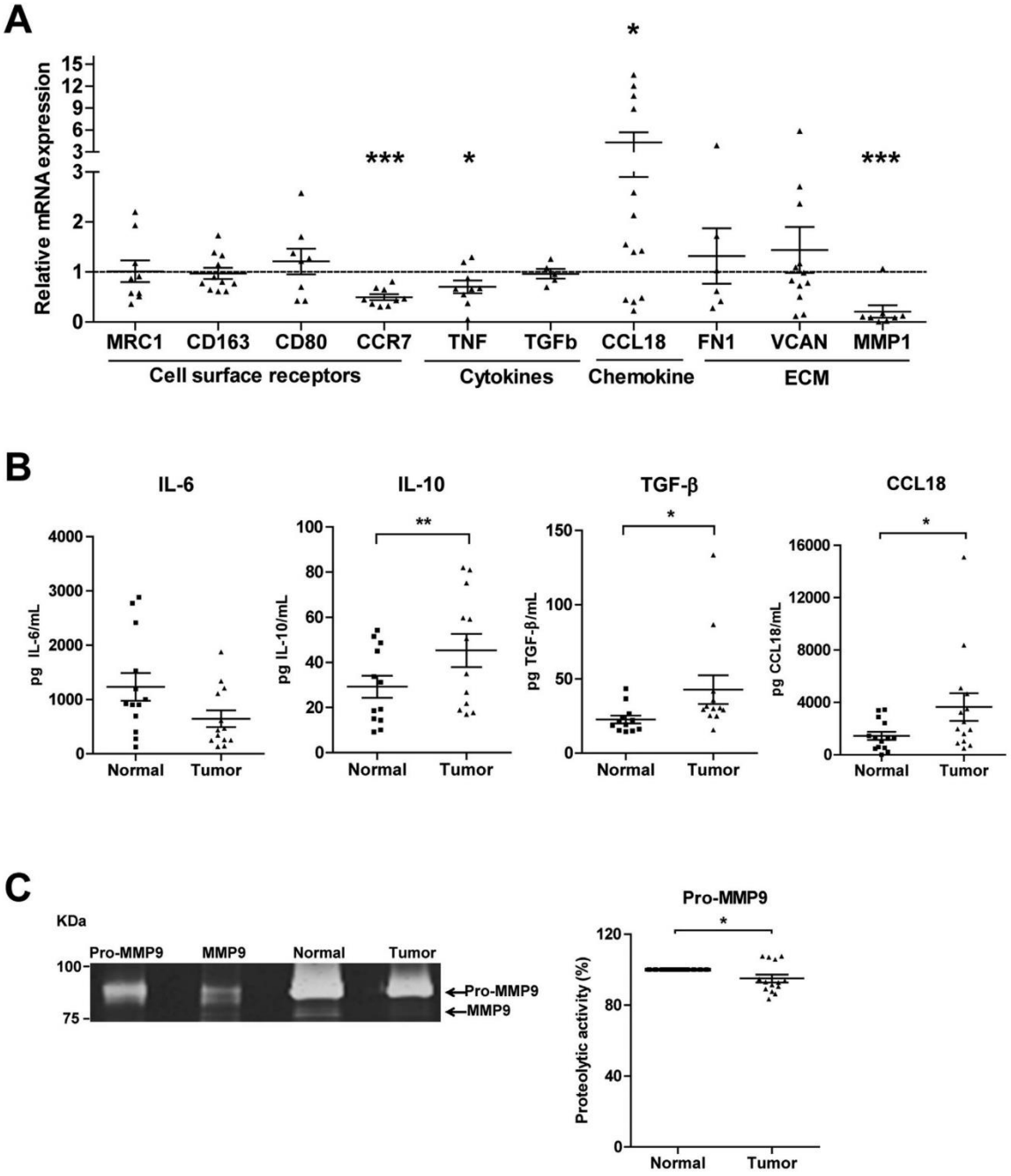

Fig. 5. Macrophages differentiated in tumor decellularized matrices present a more anti-inflammatory M2-like phenotype. (A) mRNA expression of pro-(*CD80*, *CCR7*, and *TNF*) and anti-inflammatory (*MRC1*, *CD163*, *TGFb1*, *CCL18*, *VCAN* and *MMP1*) genes evaluation by quantitative real-time PCR. Gene expression data from tumor repopulated samples were normalized relatively to normal repopulated matrices (represented as dotted line), using matrices from 3 to 6 different patients and monocytes from 5 to 9 blood donors (final combinations between 5 and 13, depending on the gene). β-actin was used as housekeeping gene. Wilcoxon signed rank test was used to compare the median of each dataset against a hypothetical median value of 1. **(B)** Secretion levels of the pro-inflammatory cytokine IL-6, and the anti-inflammatory cytokines IL-10, TGF-β and CCL18 evaluated by ELISA in conditioned media of normal and tumor repopulated matrices (matrices from 6 different patients and monocytes from 8 or 9 different blood donors, in a final combination of 12, 13 or 14). **(c)** MMP-9 secretion was evaluated by gelatin zymography in both samples in study. Representative image of the proteolytic activity present in the conditioned media of normal and tumor repopulated matrices revealed by with Coomassie Blue staining. Recombinant human Pro-MMP9 and its PMA activated form were loaded as control of pro and active MMP9 forms, respectively. Graph with the quantitative data of MMP-9 proteolytic activity (matrices from 6 different patients and monocytes from 8 or 9 different blood donors, in a final combination of 14). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, paired 2-tailed Student's *t*-test.

3.6. Macrophages repopulating tumor decellularized matrices promote colorectal cancer cell invasion through CCL18

Based on previous works, showing that macrophages cultured in 2D induce CRC cancer cell invasion [23] and that M2-like macrophages are more efficient in such stimulation [28], we evaluated the impact of these macrophage-repopulated matrices on colorectal cancer cell invasion. Accordingly, RKO cells were confronted with normal/tumor decellularized matrices, repopulated or not with human macrophages, using an *in vitro* Matrigel-based invasion assay (Fig. 6). As positive control, naïve macrophages seeded on glass coverslips were used (Fig. 6A). We observed that normal/tumor not repopulated matrices induced a slight increase in cell invasion, although not statistically significant, which can be probably attributed to the release of some soluble factors retained in the decellularized matrices. Interestingly, macrophages differentiated in tumor-derived matrices were very efficient in stimulating RKO cell invasion, similarly to macrophages differentiated on glass coverslips. In contrast, macrophages differentiated in normal-derived matrices did not affect colorectal cancer cell invasion (Fig. 6B).

Since it was previously described that CCL18 stimulates breast cancer cell invasion [29], and given its increased release by macrophages differentiated in tumor decellularized matrices, we hypothesized that CCL18 could be contributing to the invasion process mediated by these macrophages. For this reason, invasion assays involving repopulated matrices in the presence of an anti-CCL18 neutralizing antibody were performed (Fig. 6A and 6B). Strikingly, the increased invasion induced by macrophages repopulating tumor matrices was reduced to basal levels, similarly to what happened with control macrophages incubated in the presence of such neutralizing antibody. To further validate that CCL18 was indeed the factor responsible for this pro-invasive activity, we performed invasion assays using, as single stimuli, recombinant human CCL18 chemokine, in the presence or absence of the neutralizing anti-CCL18 antibody (Fig. 6C). Our results demonstrate that CCL18, by itself, stimulates RKO cell invasion, being this stimulation suppressed by the respective neutralizing antibody. The same assay using CCL18 was extended to three additional cell lines - SW480, SW620 and HCT15 – and shown to be enough stimuli to induce HCT15 cancer cell invasion (Supplementary Fig. S9).

3.7. CCL18 is highly expressed at the invasive front of more advanced CRC cases

To validate the clinical relevance of our organotypic model, where CCL18 was shown to stimulate CRC cell invasion, we decided to evaluate its expression, by immunohistochemistry, at the invasive front of human CRC specimens (Table 1). CCL18 expression was scored according to four categories, from 1 to 4, being 1 the negative/very low expressing cases and 4 corresponding to cases where more than 80% of the invasive front was positively stained (Fig. 7A). We found that lower CCL18 expression (scores 1 and 2) were mainly found in tumors of stages I and II, whereas higher expression was mostly found in stages III and IV. Interestingly, the higher positive cases (score 4) were only found in tumors of stages III and IV (Fig. 7B). Our results evidenced an association between tumor staging and CCL18 staining (Fisher exact test, P = 0.033; multivariate analysis, P = 0.035) (Fig. 7C, Supplementary Table 1), with higher CCL18 expression present in more advanced CRC stages. Additionally, through a double immunohistochemistry of CCL18 with CD68 or CD163, we confirmed that part of the cells expressing CCL18 are indeed macrophages, particularly of the M2 type, since they are positive for both CD68 and CD163. (Supplementary Fig. S10). However, it is evident that CCL18 was not expressed by

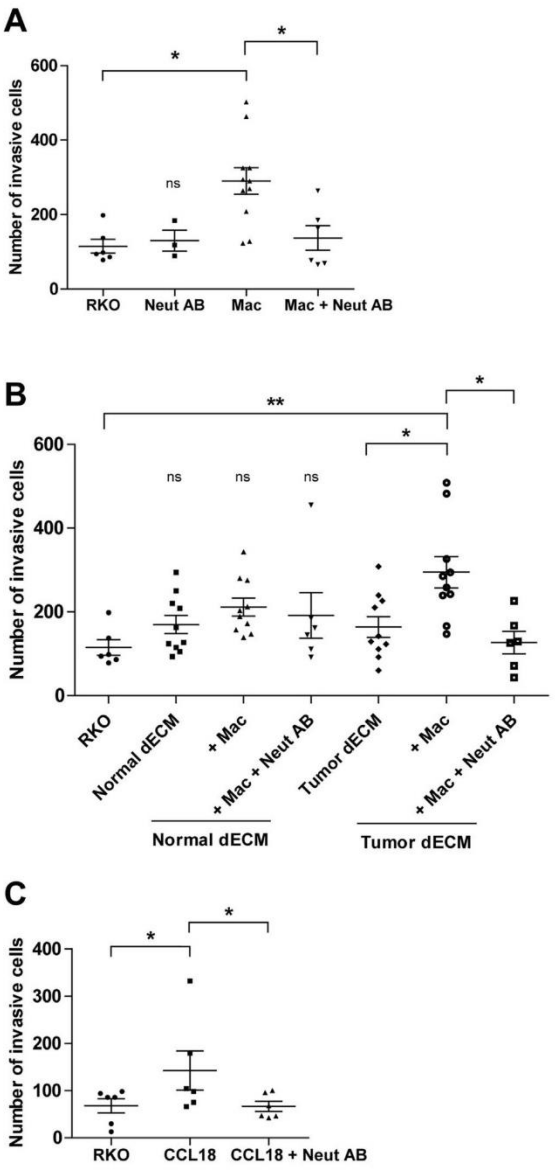

Fig. 6. Macrophages repopulating tumor decellularized matrices promote colorectal cancer cell invasion through CCL18. **(A)** Quantification of Matrigel invasion assays where RKO cells were incubated in the upper compartment while CCL18 was incubated in the lower compartment. Invasive cells were counterstained with DAPI and counted in the microscope. **(B)** The same invasion assays where RKO cells were incubated in the upper compartment and decellularized normal (Normal dECM) or tumor (Tumor dECM) matrices repopulated or not with human macrophages (Normal dECM + Mac, Tumor dECM + Mac), in the presence or absence of a neutralizing anti-CCL18 antibody (CCL18 neut AB), in the lower compartment (matrices from 6 different patients and monocytes from 9 different blood donors – for the experiments without neutralizing antibody – and matrices from 5 different patients and monocytes from 6 different blood donors – when neutralizing antibody was included). **(C)** The same invasion assay using recombinant CCL18, in the presence or not of the anti-CCL18 neutralizing antibody (n = 6). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, Kruskal-Wallis test corrected with the Dunn test for multiple comparisons. ns, not significant.

Table 1
Characteristics of the CRC patients studied.

Characteristic	Frequency (n[%])
Patients (n)	68
Gender	
Male	35 (51.5%)
Female	33 (48.5%)
Age, y	
Mean	68.8 ± 13.7
TNM stage	
I and II	39 (57.4%)
III and IV	29 (42.6%)
Tumor location 1	
Colon	55 (80.9%)
Rectum	13 (19.1%)
Tumor location 2	
Right	23 (33.8%)
Left	45 (66.2%)
Tumor morphology	
Adenocarcinoma	61 (89.7%)
Mucinous adenocarcinoma	7 (10.3%)
Average CCL18 score	
Stage I	1,75
Stage II	1,96
Stage III	2,37
Stage IV	2,40

all CD68/CD163 macrophages and that macrophages were not the only cells expressing CCL18. Moreover, it was possible to observe that these $CCL18^{+}CD68^{-}$ cells are frequently in close vicinity to macrophages, suggesting the existence of a CCL18 axis at the tumor microenvironment.

Overall, these data reinforce the potential of using human decellularized matrices as valid organotyic models able to recapitulate the tumor microenvironment.

4. Discussion

The ECM is described to provide critical chemical and physical cues that determine cell differentiation, polarity, growth, survival, proliferation and fate, both in physiological and pathological conditions, such as cancer [9]. Nevertheless, most of the research published in the field is based on oversimplified and artificial systems that do not resemble native human tumors.

Aiming at understanding the role of ECM components, present at the tumor microenvironment, on macrophage polarization and on their ability to modulate cancer cell activities, we created a novel organotypic model that combines human decellularized matrices, derived from surgical resections from CRC patients, with human monocytes isolated from healthy blood donors. Accordingly, we developed a protocol that combines the use of a hypotonic buffer with a detergent of anionic surfactant properties and DNase. Using this protocol, recently reported by our team as preserving cardiac tissue characteristics [22], we demonstrated that, in contrast to normal matrices derived from the same patient, tumor matrices polarize macrophages towards an anti-inflammatory (M2-like) phenotype. Additionally, we revealed that these tumor ECM-educated macrophages stimulate CRC cell invasion through a mechanism involving the anti-inflammatory CCL18 chemokine. Finally, CCL18 expression at the tumor invasive front correlated with more advanced CRC stages, suggesting that our organotypic system is a reliable model that recapitulates the tumor microenvironment.

In this study, paired normal mucosa and CRC tissues were decellularized using 0.1% SDS as main chemical agent. SDS is one of the detergents most frequently used throughout distinct decellularization protocols and it has been applied in a variety of tissues including heart [16], liver [18] or intestine [30]. In our particular case, SDS, in combination with DNase treatment, was proven to be a good decellularization method since DNA and cellular remnants were efficiently removed. Histological, immunohistochemical and ultrastructural evaluations confirmed the preservation of native tissues architecture and of major ECM components (as laminin, fibronectin, collagens type I and IV and hyaluronic acid). On the other hand, GAGs content significantly decreased after the decellularization procedure, similarly to what was observed by other groups [31,32]. This is not completely unexpected since GAGs, apart from being easily leachable components of the ECM, are also present in cell membranes, being partially removed with the loss of cellular components.

Previous studies regarding macrophages and cancer cells were mostly performed in 2D cultures, with recognized limitations [12], or in immunocompromised animal models which do not entirely recapitulate human disease. Additionally, the use of engineered biomaterials, either natural, as matrigel, or synthetic, as polyacrylamide or poly (ethylene glycol), was proposed as a good alternative to dissect the biochemical and mechanical cues influencing cell fate [33]. To date, there are few studies addressing the effect of decellularized human ECM on macrophage behavior. Moreover, most of these studies were developed in the context of tissue engineering, as an alternative to other regenerative/repair therapies [34,35]. In our study, using normal and tumor decellularized matrices, monocytes differentiated as macrophages, expressed CD68 and repopulated these 3D scaffolds in their full depth. The 22% difference observed in the repopulation efficiency between both matrices, not justified by distinct macrophage proliferation or apoptosis, is probably related with the structural characteristics of each tissue. Since matrices were repopulated using passive cell seeding, it is not surprising to have more macrophages in normal matrices, where the porosity resulting from the intestinal crypts is higher.

The literature provides solid evidences that cell morphology has a clear impact in cell function [36], with macrophages being no exception. *In vitro* studies reported that macrophages, when polarized towards an M1-phenotype, adopted a rounded morphology, whereas M2 macrophages presented an elongated cell shape. Additionally, by using micropatterned surfaces that forced macrophage shape, McWhorter and colleagues demonstrated that elongation, by itself, was enough to drive the expression of M2 markers and to reduce the secretion of pro-inflammatory cytokines [27]. However, in our study, using human-derived scaffolds, no major differences in terms of cell morphology were observed. The fact that our analysis was performed in 3D matrices may justify the absence of macrophage morphometric alterations, in contrast with previous published work.

Notably, our results evidenced that macrophages differentiated within patients' tumor matrices exhibit an increase of IL-10, TGF-β and CCL18 (anti-inflammatory markers), whereas the ones differentiated within normal matrices expressed higher CCR7 and TNF and secreted more IL-6, all pro-inflammatory M1 markers. From a clinical point of view, the fact that the tumor ECM is able, by itself, to drive macrophage polarization towards a M2-like phenotype, emphasizes that this component of the tumor microenvironment cannot be disregarded, namely when thinking about strategies to "re-educate" macrophages and to modulate their phenotype [37].

The differences observed in macrophage polarization can be attributed to the chemical characteristics of normal and tumor matrices, their mechanical/structural properties, or the combination of both. Similarly to what has been described for the majority of tumors [38], we confirmed that CRC tissues are indeed stiffer than the normal colon, even after the decellularization process. This supports the hypothesis that the behavior of macrophages

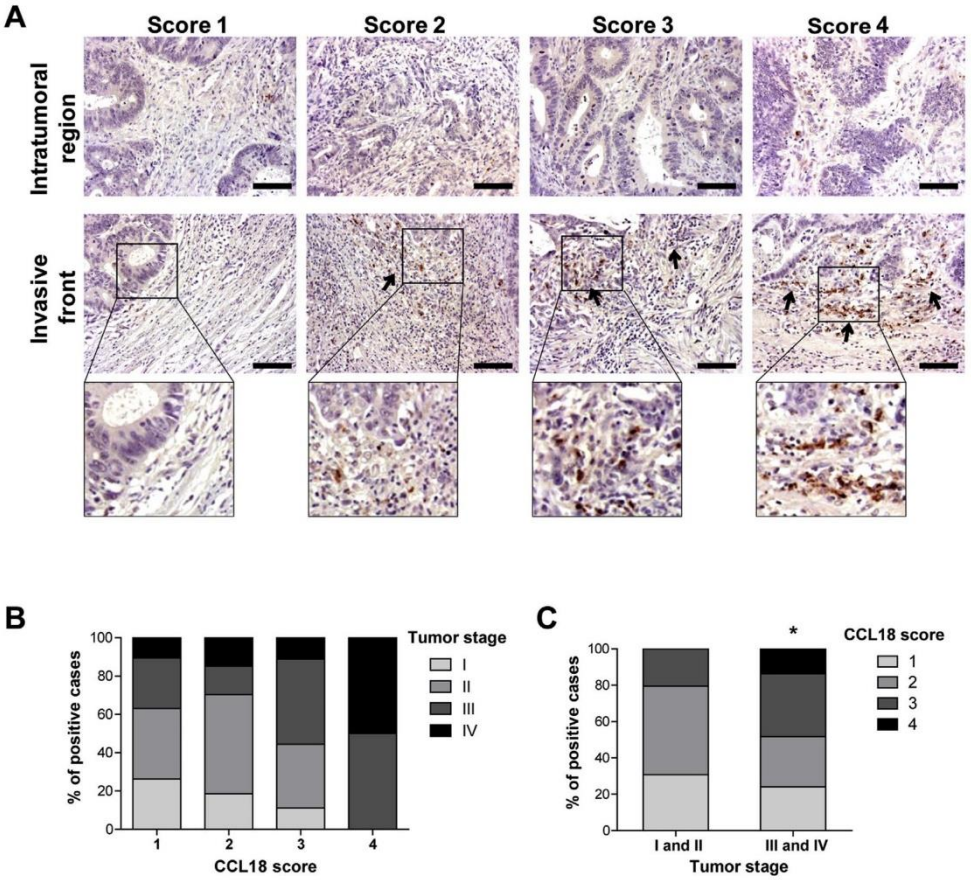

Fig. 7. CCL18 is highly expressed at the invasive front of more advanced CRC cases. (A) Sixty-eight CRC cases were immunohistochemically stained with anti-CCL18 antibody and scored accordingly to the staining at the invasive front: absent/few positive cells (score 1), less than 40% (score 2), between 40 and 80% positivity (score 3) and more than 80% (score 4). Arrows indicate positive areas (scale bar = 100 μm). **(B)** CCL18 expression by score and stage. **(C)** The association of CCL18 expression with tumor stages I and II vs. III and IV was assessed using a two-sided Fisher's exact test (*$P = 0.033$) and multivariate ordinal regression (*$P = 0.035$).

repopulating decellularized tissues might have been influenced by the differences in stiffness presented by normal and tumor scaffolds. Accordingly, macrophages in stiffer hydrogels were shown to be less round and presented a decreased expression of TNF-α, IL-6 and IL-1β [39]. It has also been previously reported that stiffer hydrogels inhibited both MMP secretion and activity in primary human fibroblasts [40], similarly to our findings of reduced MMP expression and activity in macrophages repopulating the stiffer tumor matrices. Interestingly, the work of Pirilä *et al.*, using a human 3D hypoxic myoma-based model, also showed a decreased secretion of MMP-9 by M1-like THP-1 macrophages in comparison with their M2 counterparts, although the M2 macrophages were the ones that stimulated the invasion of a tongue carcinoma cell when in co-culture [41]. The increase in tumor ECM stiffness is caused, in part, by an increased activity of enzymes such as lysyl oxidase (LOX), responsible for the crosslinking between collagen and elastin. Similarly to other cancers, LOX was described to be upregulated in CRC comparing to normal colon [42] and was shown to promote CRC progression [43]. Together with stiffness alterations sensed by macrophages, modifications regarding tumor ECM composition may also account for the different macrophage polarization observed. In CRC tumors, an increase of type I collagen [44] and *de novo* expression of stromal collagens type V and XI [45] have been described. More recently, Naba and colleagues [46] performed a proteomic study that allowed the identification of the matrisomes of colorectal tumors and matched normal and metastasis samples, permitting the definition of an ECM signature characteristic of each tissue.

It has been reported that IL-10 (M2-like) stimulated macrophages were more efficient in promoting gastric and CRC cell invasion than LPS (M1-like) stimulated ones [28]. In the present work, we have demonstrated that macrophages differentiated in tumor decellularized matrices were able to stimulate RKO cell invasion, reinforcing their features of M2-like polarization. CCL18 neutralization clearly identified this chemokine as having a major role in the invasion process. In lung, CCL18 was shown to be upregulated in alternatively activated anti-inflammatory alveolar macrophages (AMs) [47]. Co-culture experiments with fibroblasts revealed that they induced CCL18 secretion by AMs, similarly to AMs monoculture in collagen I. Additionally, AMs from patients with idiopathic pulmonary fibrosis stimulated collagen I production by fibroblasts, more than AMs from healthy controls, partially

mediated by CCL18. This feedback loop between AMs and fibroblasts was proposed as being, at least partially, responsible for sustaining pulmonary fibrosis [48]. Interestingly, the presence of ECM components, such as HA, causes a significant increase in CCL18 secretion by macrophages, even in the absence of additional stimuli. In fact, HA was described as being able to regulate different immune cells in pathological conditions [49] and, in CRC, tumor-cell HA was associated with a poor survival rate [50]. Nevertheless, CCL18 role in cancer remains controversial: in prostate and breast cancer it was described as correlating with malignant progression and poor survival [29,51], whereas in gastric and CRC it was defined as an independent favorable prognostic biomarker [52,53]. Our results from the immunohistochemistry analysis confirm that CCL18, although not exclusively, is indeed being produced by macrophages, and in particular by the anti-inflammatory ones. More importantly, they clearly evidence an association between more advanced CRC staging and a higher CCL18 at the invasive front, the region that is described as being the most densely infiltrated by macrophages [54].

5. Conclusions

Even though it is well recognized the critical role of the ECM on determining cell behavior, this component of the tumor microenvironment continues to be neglected in the majority of the *in vitro* studies performed in the field. In the present study, by using decellularized paired normal and colorectal tumor tissues from the same patient, we were able to preserve the complexity of both environments, namely in what concerns their composition and tissue architecture. Despite both scaffolds supported monocyte to macrophage differentiation, direct comparison regarding the impact of healthy and diseased ECM on macrophage polarization clearly evidenced that tumor ECM harbors biochemical and biomechanical properties critical for a differential macrophage polarization. This is the first report showing that tumor ECM-educated macrophages, contrary to macrophages within normal matrices, displayed anti-inflammatory like features, namely higher IL-10, TGF-β and CCL18 and lower CCR7 and TNF expression. More importantly, these macrophages stimulated cancer cell invasion, a property attributed to tumor associated macrophages. Finally, CCL18 was shown to be a key player in this process and its expression was shown to correlate with more advanced CRC stages. Altogether, we were able to demonstrate that human decellularized tissues, either healthy or cancer-derived, retain their native characteristics responsible for governing cell differentiation. For this reason, we believe that they may constitute excellent bioscaffolds when trying to recreate complex microenvironments to understand basic mechanisms of disease or therapeutic resistance.

Author contributions

MLP, PPO and MJO conceptualized the project. MLP and ER collected the material. MLP, ACS, SCN, HRC and FAC performed experiments. MLP, SCN, HRC, FAC, CCB and NCS performed formal analysis. ER, ATP, APC, CCB, NCS provided technical and material support. CD performed the statistical analyses. MLP wrote the original draft. ACS, SCN, HRC, ATP, DSN, MAB, FC and MJO reviewed and edited the manuscript. MJO acquired funding. MAB, FC and MJO supervised the project.

Conflicts of interest

None.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2017.02.004.

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Supplementary Data

Supplementary Methods

Glycosaminoglycans (GAGs) quantification

GAGs were quantified using Blyscan GAG Assay kit (Biocolor), following manufacturer's instructions. Briefly, tissue fragments were minced and incubated with Papain Extraction Reagent (Sigma-Aldrich) for 3 hours at 65ºC, with occasional vortexing. Aliquots of each sample were mixed with 1,9-dimethyl-methylene blue dye, followed by incubation with Dissociation Reagent provided by the GAG assay kit. Absorbance was measured at 656 nm. Results are presented as µg of GAGs per mg of tissue.

Proliferation/apoptosis evaluation

Normal and tumor repopulated matrices formalin fixed paraffin embedded sections were stained with Ki-67 antibody (Dako, MIB1, 1:100). Briefly, after antigen retrieval with Citrate Buffer, pH 6, for 20 minutes and blocking with Ultra V Block for 30 minutes, Ki-67 was incubated for 2 hours. Detection was performed as previously described.

Apoptosis was evaluated using ApopTag® Fluorescein In Situ Apoptosis Detection Kit (Millipore, S7110), following manufacturer's instructions.

RNA extraction and real-time PCR

After 14 days of culture, RNA was extracted from repopulated matrices using mirVana isolation Kit (ThermoFisher Scientific) following the manufacturer's instructions. cDNA was prepared using 150 U of SuperScript™ II Reverse Transcriptase, 1× first strand buffer, 10 mM DTT 0.1 M (Invitrogen), 0.5 mM dNTPs 10 mM (Bioron, Germany), 8U of rRNasin (Promega, WI) and RNase/DNase free water (Gibco). Real-time was performed using TaqMan Universal PCR Master Mix (Applied Biosystems) and probes for *actin*, *MRC1*, *CD163*, *CD80*, *CCR7*, *TNF*, *TGFb1*, *CCL18*, *FN1*, *VCAN* and *MMP1* (Applied Biosystems). At least 4 ng of cDNA were used per reaction together with 0.5µL of Taqman in a 10 µL reaction.

Zymography

Normal and tumor repopulated matrices conditioned media was analyzed by gelatin zymography. Protein concentration was determined by Dc Protein kit (Bio-Rad) and 25ug of protein were mixed with sample buffer (10% SDS, 4% sucrose and 0.03% bromophenol blue in 0.5M Tris–HCl, pH 6.8) and separated on 10% polyacrylamide gels containing 0.1% gelatin (Sigma-Aldrich) as substrate. After electrophoresis, gels were washed twice with 2% Triton X-100 and incubated for 16h at 37ºC in 50mM Tris–HCl, pH 7.5 and 10mM $CaCl_2$. Gels were stained with 0.1% Coomassie Brilliant Blue R-250 (Sigma-Aldrich), 50% methanol and 10%

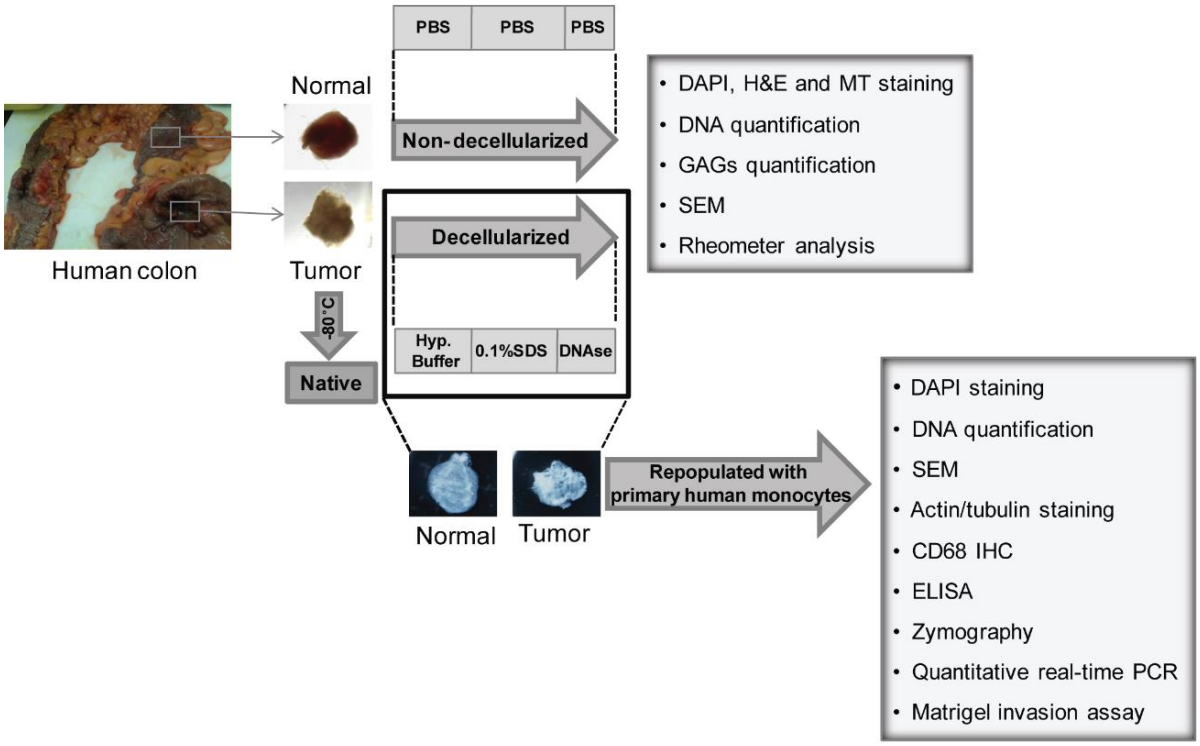
acetic acid (Merck, Germany). MMPs activity was estimated by densitometric analysis (QuantityOne, Bio-Rad). MMP levels were normalized to the repopulation efficiency. The recombinant pro-MMP9 and the respective PMA activated form were run in parallel and used as a molecular weight reference.

Fluorescent immunohistochemistry

CRC cases were stained for CD68 and CCL18 or CD163 and CCL18. After antigen retrieval with Citrate Buffer, pH 6, for 30 minutes, in a water bath at 98ºC, slides were incubated with 0.1% Sudan Black B in 70% ethanol, for 20 minutes, to reduce autofluorescence. After blocking with Ultra V Block for 30 minutes, CCL18 and CD68 or CCL18 and CD163 (Cell Marque, MRQ-26, 1:50) were incubated simultaneously, for 45 minutes, following incubation with goat anti-rabbit Alexa-Fluor-647-conjugated secondary antibody (1:200) and anti-mouse biotinylated antibody (1:250), in the dark, for an additional hour. Finally, Streptavidin Alexa-Fluor-555-conjugated (1:500) was added for 20 minutes and slides were mounted on Vectashield with DAPI. Images were acquired using an Axio Imager Microscope Zeiss (Oberkochen, Germany).

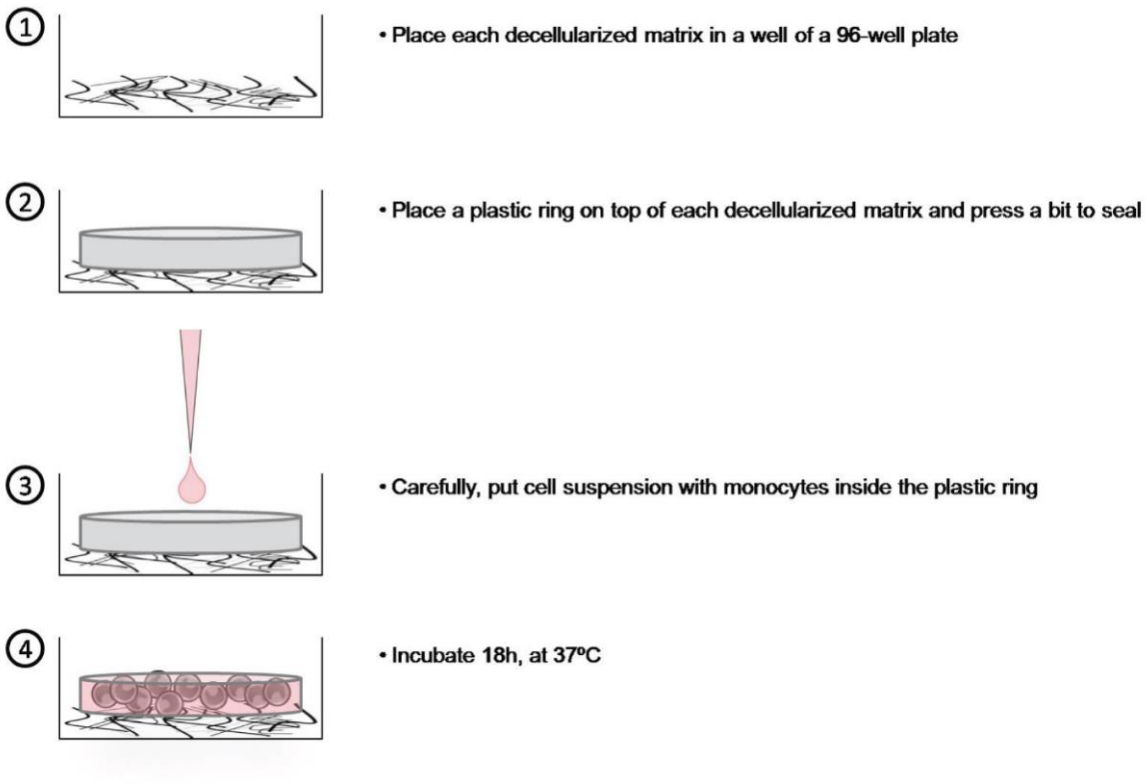
Supplementary Figures

Supplementary Figure S1



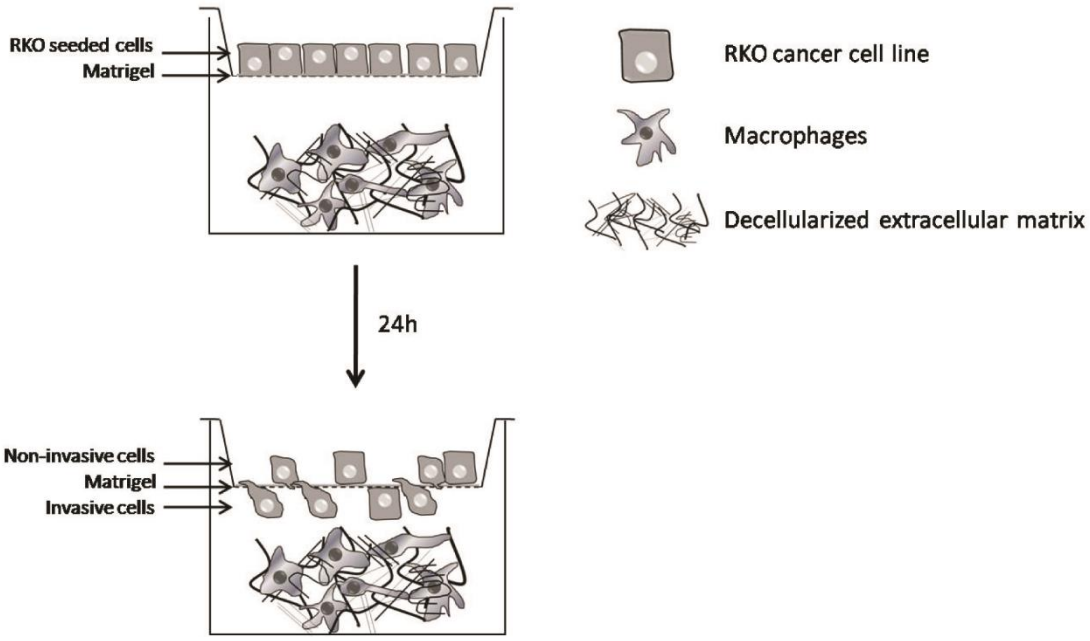
Supplementary Figure S1. Schematic representation of the methodology used in the present study. Normal and tumor colorectal fragments, obtained from CRC cancer patients' surgical specimens, were stored at -80°C upon material collection (native), kept in PBS (non-decellularized) or decellularized using a combination of hypotonic buffer, 0.1% SDS and DNase treatment. These fragments were then evaluated by DAPI/H&E and Masson's Trichrome (MT) stainings, DNA and GAGs quantification, immunohistochemistry (IHC), scanning electron microscopy (SEM) and in the rheometer. Afterwards, normal and tumor decellularized matrices were repopulated with primary human monocytes, and allowed to differentiate in macrophages for 8/14 days. Repopulated matrices were evaluated for DNA content, stained with DAPI/CD68 and actin/tubulin. Analyses by SEM, quantitative real-time PCR, ELISA and zymography were also performed. Finally, these repopulated matrices were used in a Matrigel invasion assay.

Supplementary Figure S2



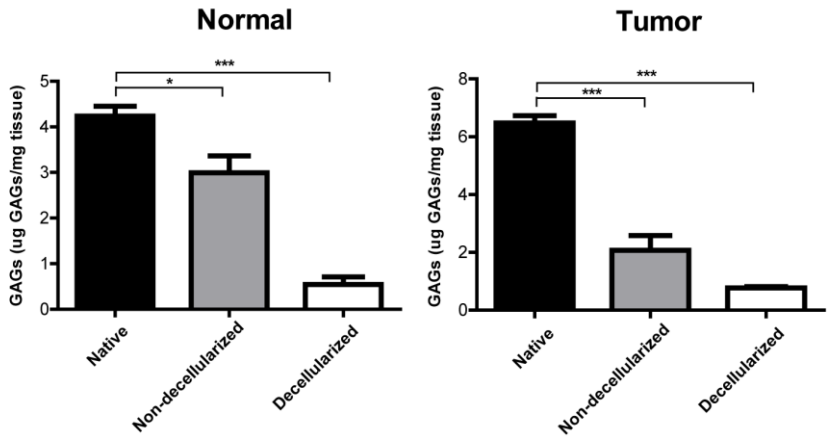
Supplementary Figure S2. Schematic representation of the repopulation setup applied in the present study. Each normal or tumor-derived matrix was placed in well of a 96-well plate and a plastic O-ring was positioned on top. After pressing a bit to seal, $1.5x10^6$ freshly isolated monocytes, ressuspended in 20 µL of culture medium, were carefully placed inside each ring and incubated for 18 h in humidified conditions. Regarding non-repopulated matrices, the same protocol was followed but only culture medium was placed inside the ring.

Supplementary Figure S3



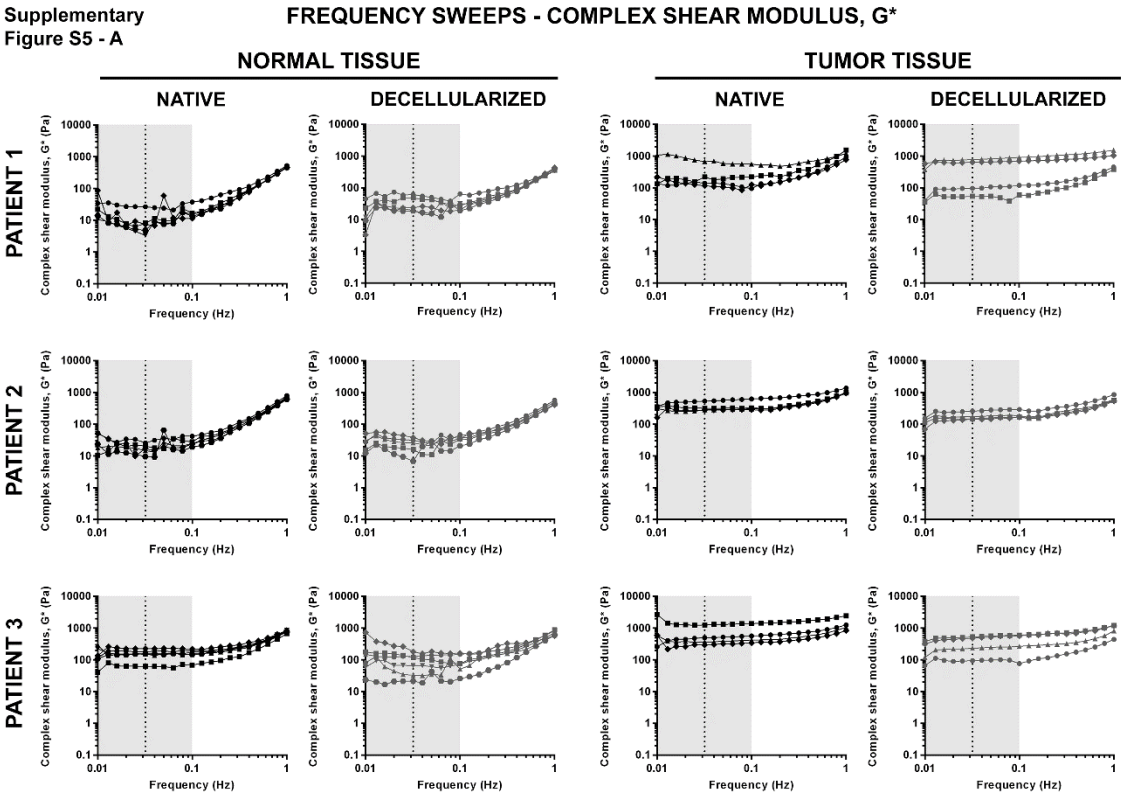
Supplementary Figure S3. Schematic representation of the Matrigel invasion assay used in the present study. Two decellularized normal or tumor matrices, which have been previously repopulated, or not, with monocytes for 13 days, were placed in a well of a 24-well invasion plate. RKO cells were added to the upper compartment of a Matrigel-coated inserts of 8-µm pore size. The same assay was also performed in the presence of neutralizing antibody. After 24 hours, invasive cells were counterstained with DAPI and counted on the fluorescence microscope.

Supplementary Figure S4

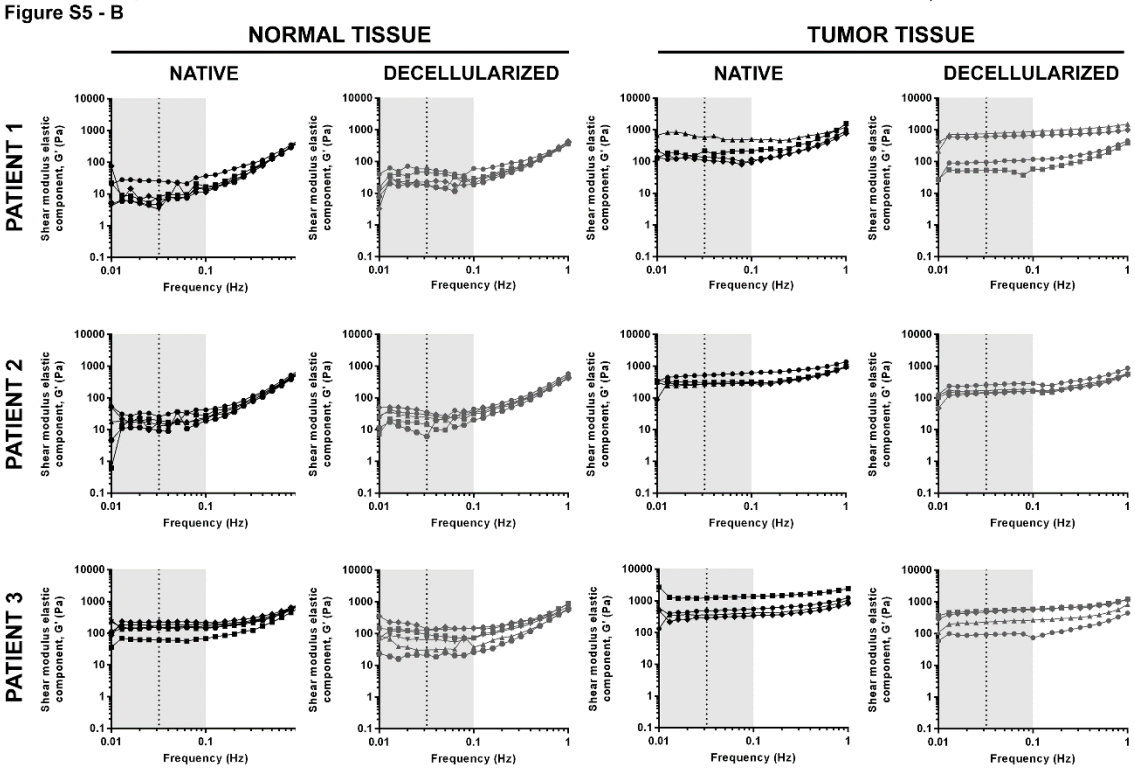


Supplementary Figure S4. Decellularization decreases GAGs content to approximately tenfold in both normal and tumor decellularized matrices. GAGs quantification in native, non-decellularized and decellularized normal and tumor samples using the Sulfated Glycosaminoglycan Assay. (Mean ± SEM, n = 4). *P < 0.05, ***P < 0.001, One-way ANOVA with Dunnett's multiple test correction.

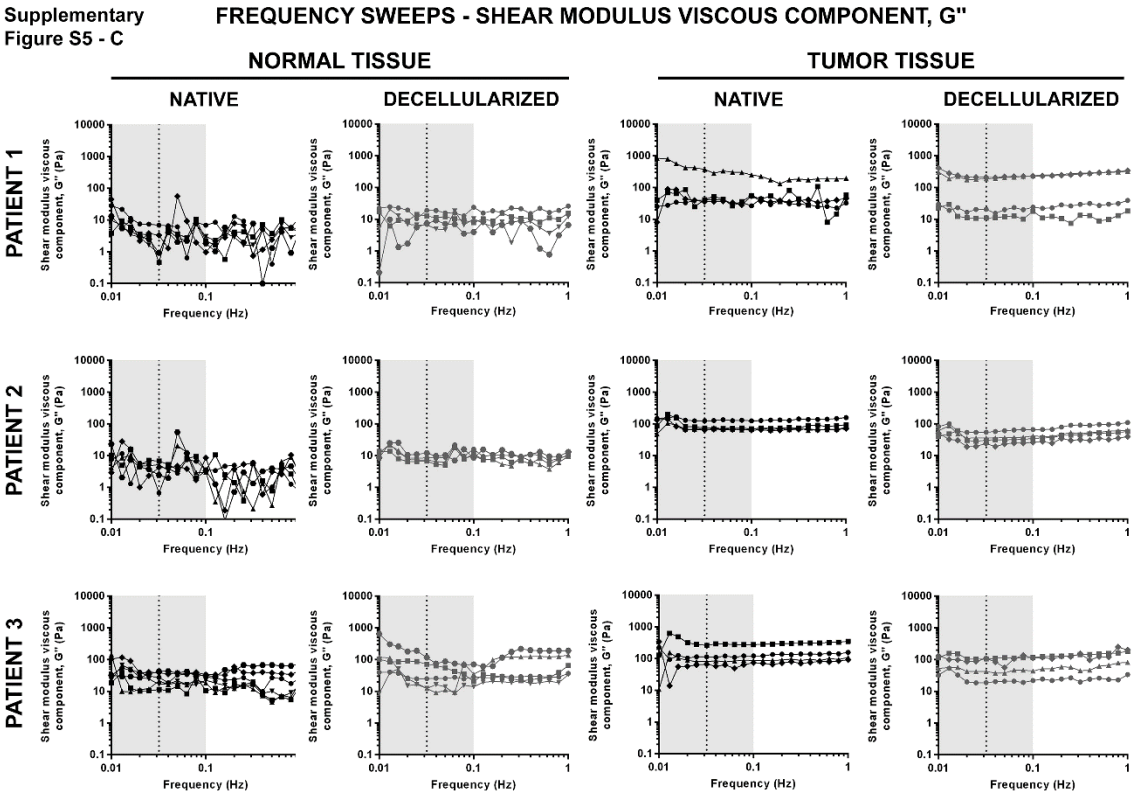
Supplementary Figure S5 - A



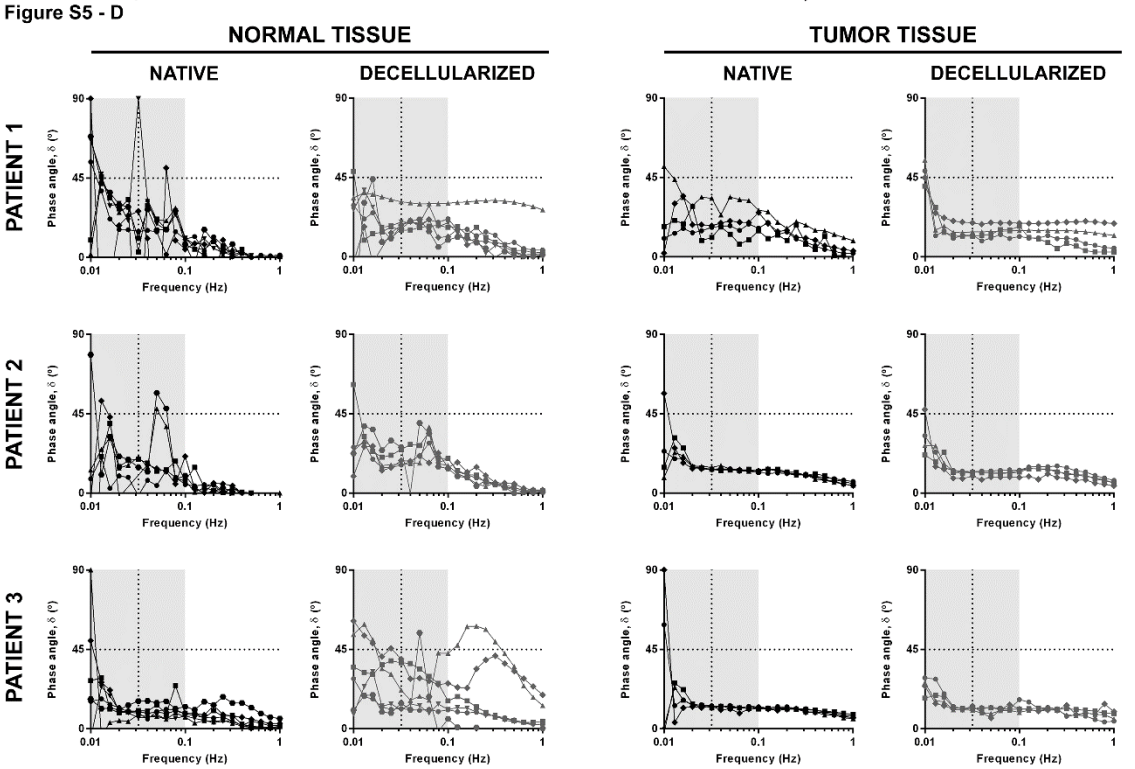
Supplementary Figure S5 - B



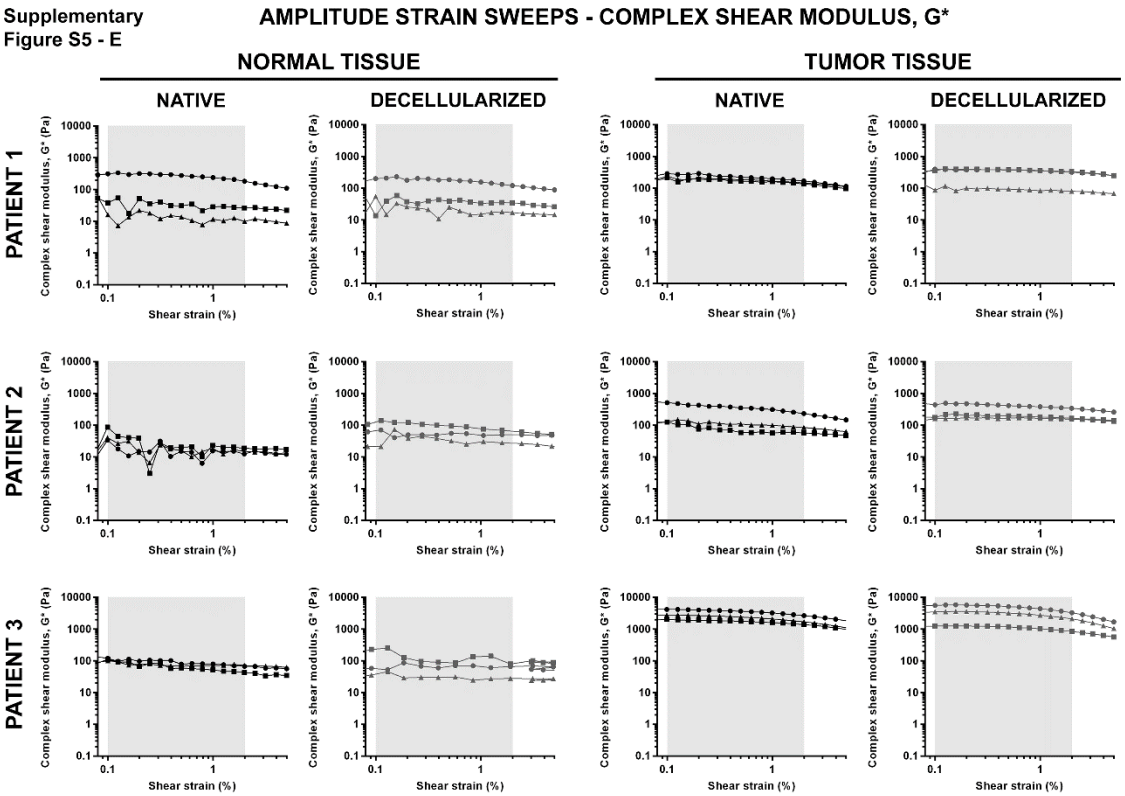
Supplementary Figure S5 - C



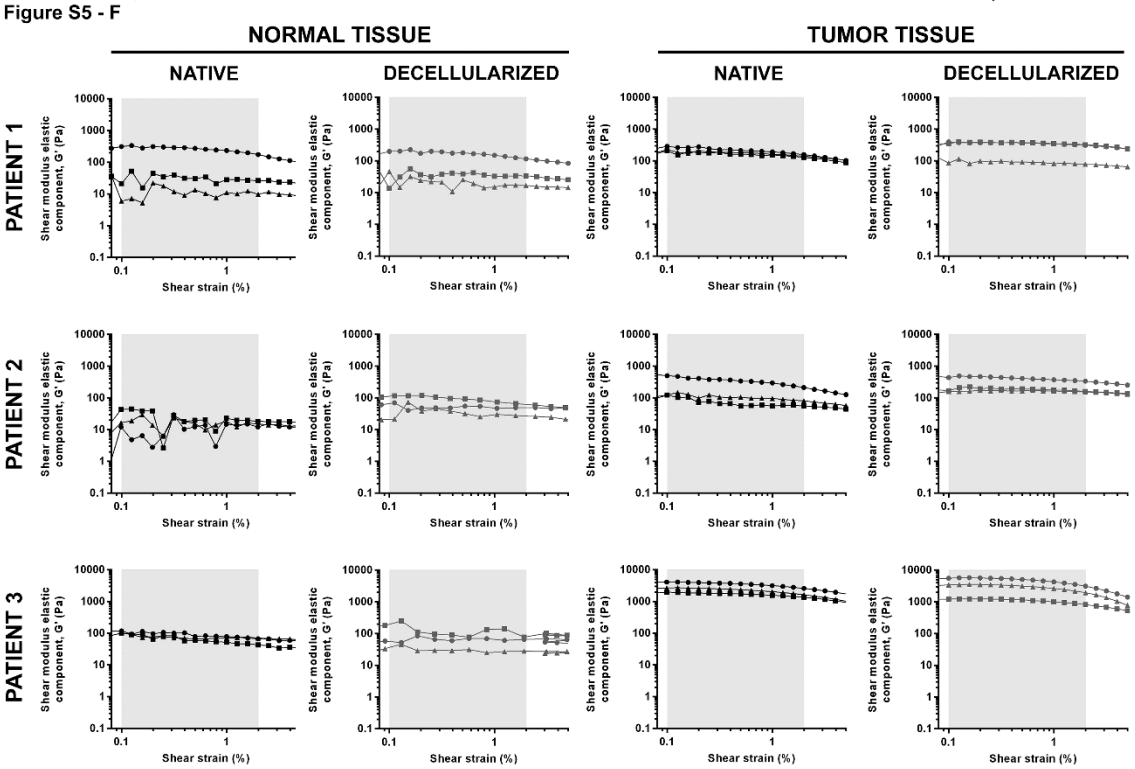
Supplementary Figure S5 - D



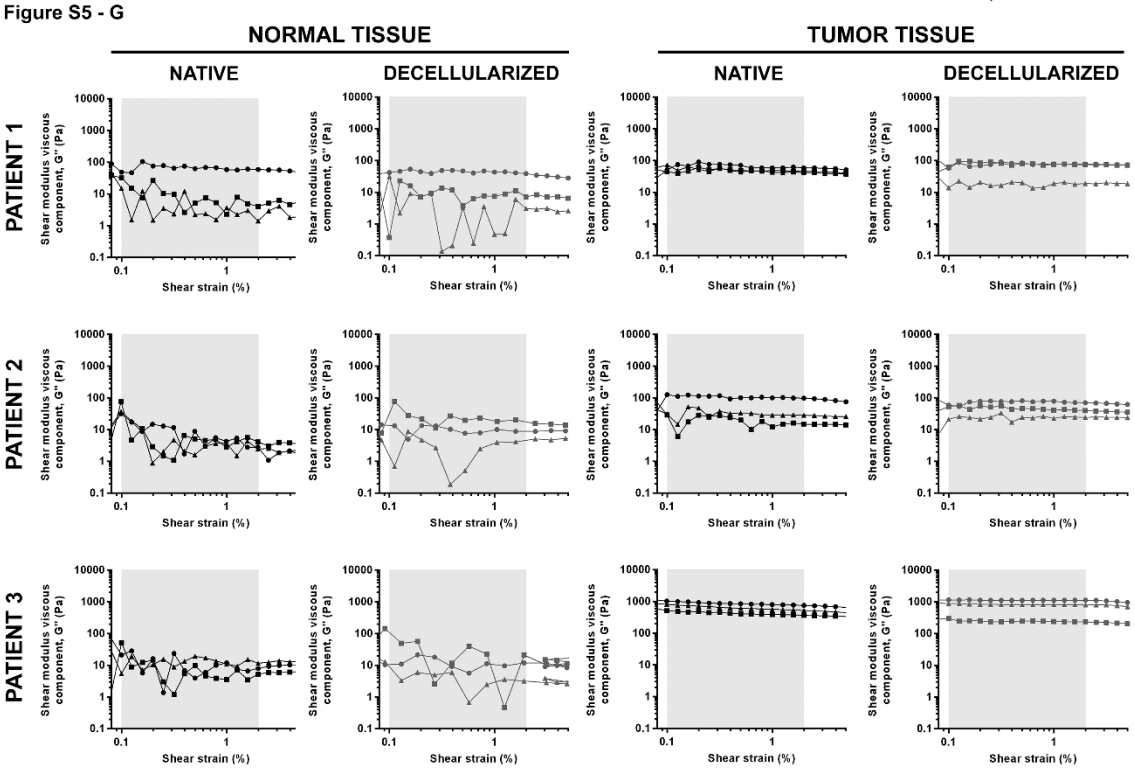
Supplementary Figure S5 - E



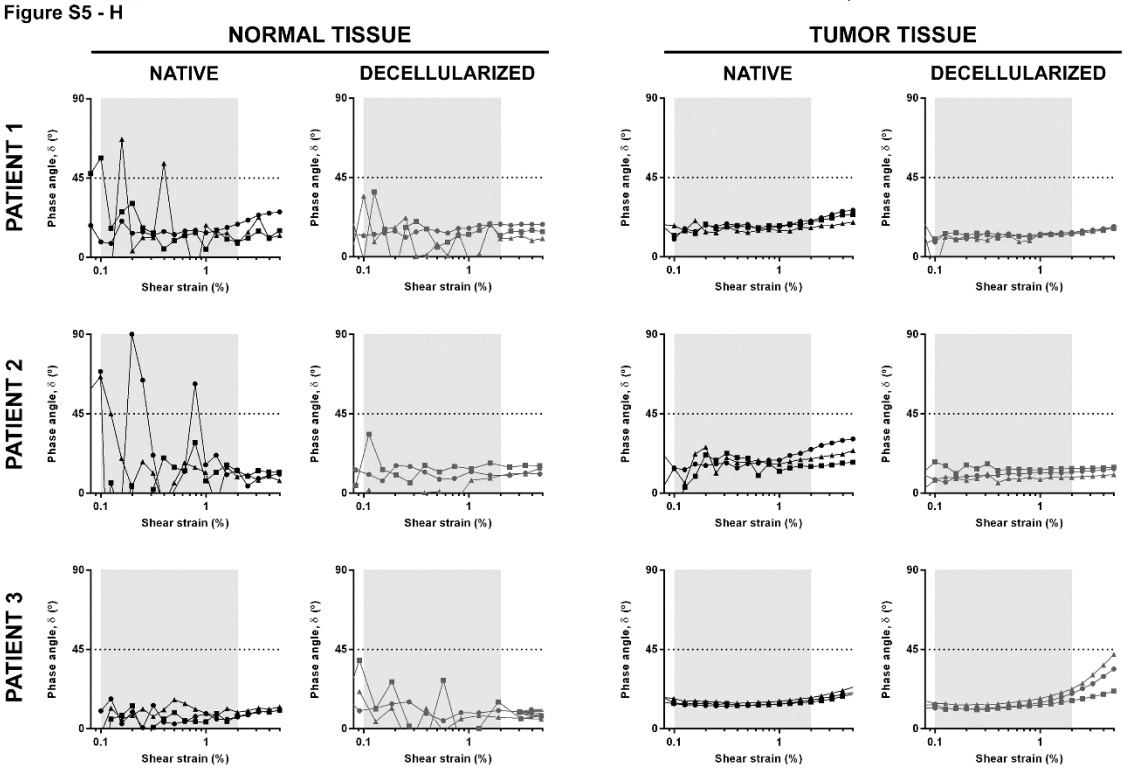
Supplementary Figure S5 - F



Supplementary Figure S5 - G

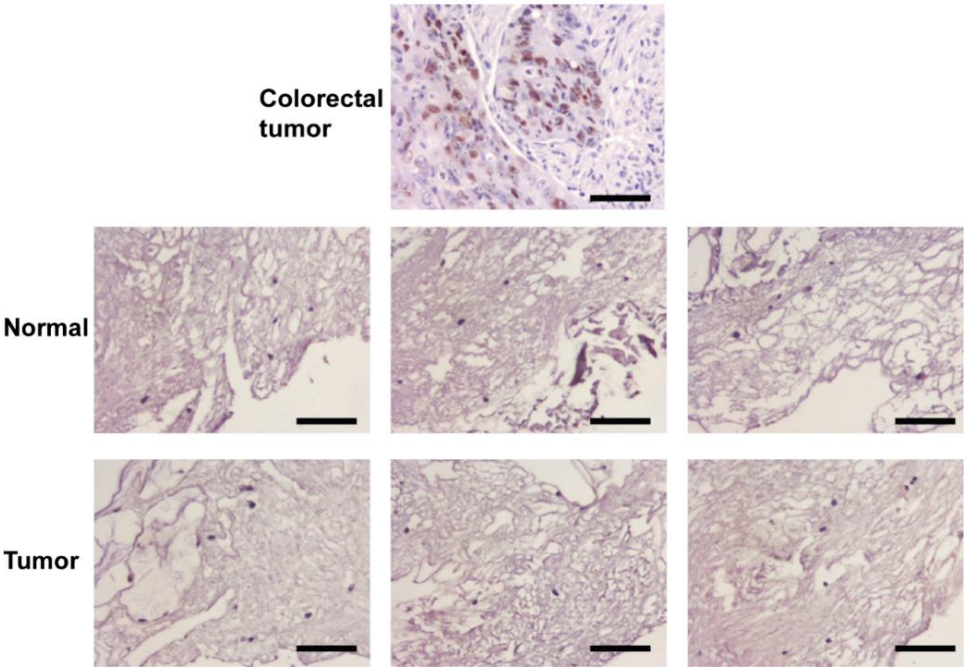


Supplementary Figure S5 - H



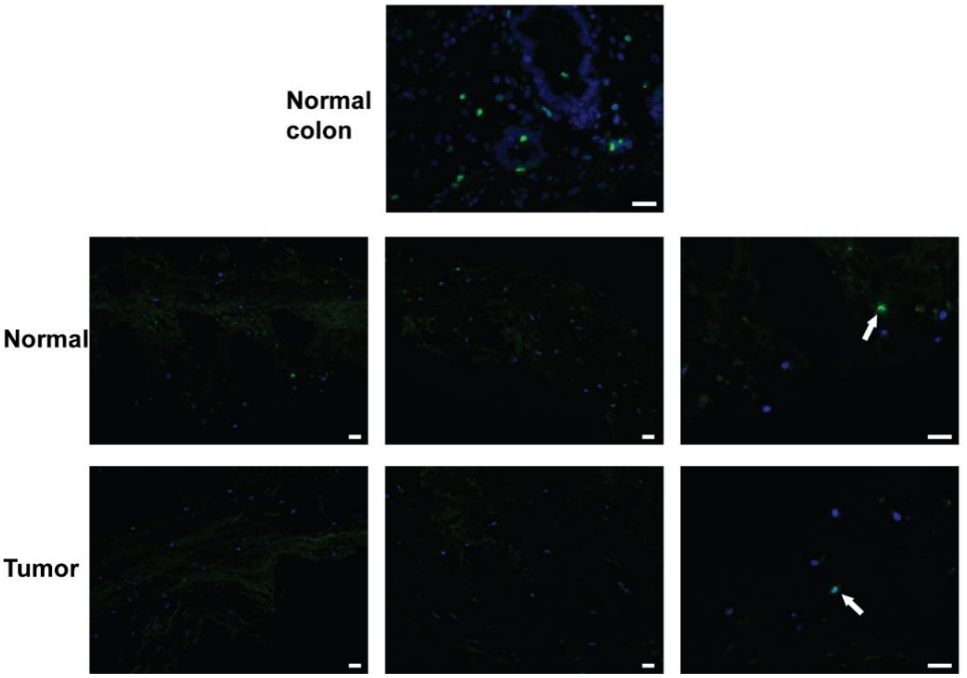
Supplementary Figure S5. Viscoelastic data from rheological analysis performed to the tissue samples of the three patients. (A-D) Data from the frequency sweeps (performed at 0.5% shear strain for the native tissues and at 1% shear strain for the decellularized tissues). (E-H) Data from the amplitude strain sweeps (performed at ≈ 0.03 Hz).

Supplementary Figure S6



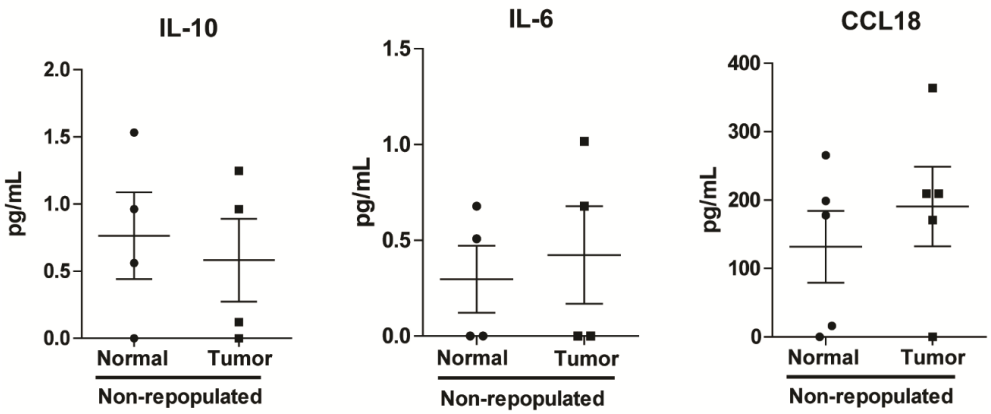
Supplementary Figure S6. Macrophages do not proliferate in normal and tumor decellularized matrices. Ki-67 immunohistochemistry of normal and tumor repopulated matrices. A native tumor colorectal section was used as positive control. Scale bar = 50 µm.

Supplementary Figure S7



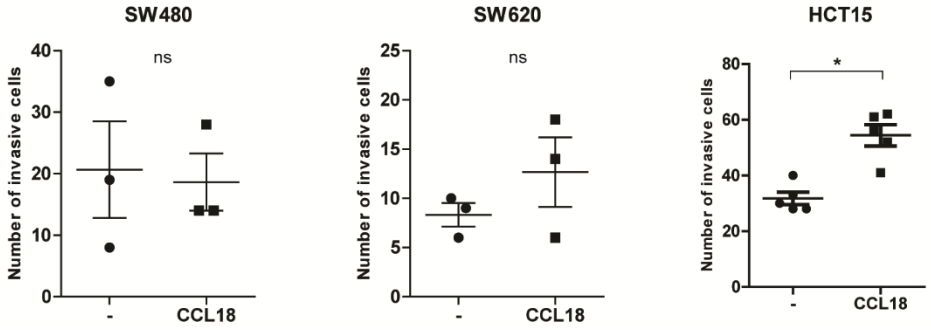
Supplementary Figure S7. Macrophages repopulating normal and tumor decellularized matrices exhibit no differences in apoptosis. Apoptotic macrophages detected in situ by the indirect TUNEL method in normal and tumor repopulated matrices. A native normal colorectal section was used as positive control. Arrows indicates apoptotic cells. Scale bar = 20 µm.

Supplementary Figure S8



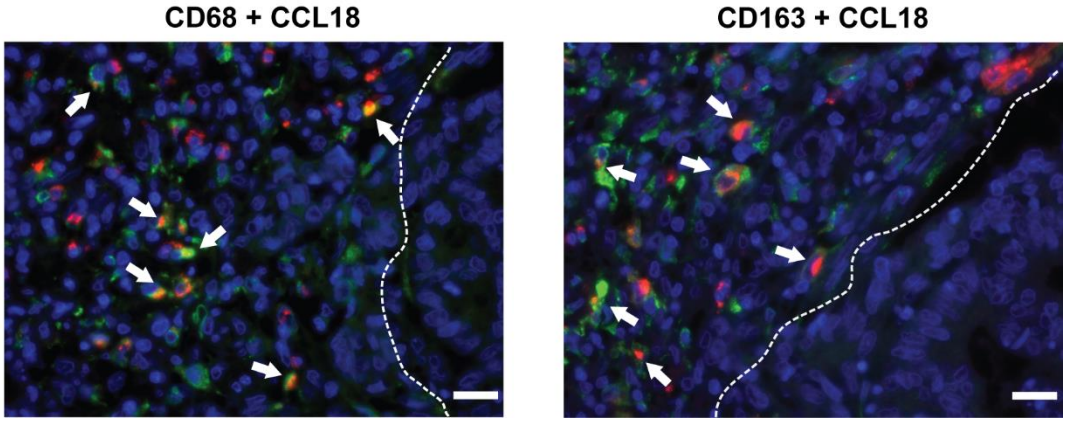
Supplementary Figure S8. Conditioned media of normal and tumor not repopulated matrices present low levels of IL-6, IL-10 and CCL18. ELISA evaluation of IL-6, IL-10, and CCL18 in the conditioned media of normal and tumor not repopulated matrices (matrices from 4 different patients, for IL-6 and IL-10, and 5 different patients, for CCL18).

Supplementary Figure S9



Supplementary Figure S9. CCL18 stimulates invasion of HCT15, but not SW480 and SW620. Quantification of Matrigel invasion assays where SW480, SW620 or HCT15 cells were incubated in the upper compartment of Matrigel-coated inserts while CCL18 was incubated in the lower compartment (n=3 for SW480 and SW620 and n=5 for HCT15). *P< 0.05, Mann-Whitney test. ns, not significant.

Supplementary Figure S10



Supplementary Figure S10. CCL18 is expressed, although not exclusively, by macrophages, namely the anti-inflammatory ones, at the invasive front of CRC. Representative images of the invasive front of CRC sections simultaneously stained with anti-CD68 and anti-CCL18 or anti-CD163 and anti-CCL18 antibodies. Arrows indicate cells positive for both CD68 (green) and CCL18 (red) or CD163 (green) and CCL18 (red). Dashed lines indicate the interface between the normal tissue of the host and the tumor mass. Scale bar = 20 µm.

Supplementary Table I: Association between CCL18 positivity and CRC patients clinicopathological characteristics.									
		CCL18 immunoreactive area [category (%)]				Univariate analysis		Multivariate analysis	
		1	2	3	4	odds (95% CI)	P	odds (95% CI)	P
		(absent-few positive cells)	(few positive cells-40%)	(41-80%)	(>80%)				
Age at diagnosis (years [sd])		65.1 (14.1)	69.1 (14.6)	72.3 (11.6)	68.0 (14.1)	1.02 (0.99-1.06)	0.176	1.02 (0.98-1.05)	0.378
Stage, n (%)									
	I and II	12 (63.2)	19 (70.4)	8 (44.4)	0	1.00*		1.00*	
	III and IV	7 (36.8)	8 (29.6)	10 (55.6)	4 (100)	2.65 (1.07-6.56)	0.036	2.86 (1.08-7.60)	0.035
Gender, n (%)									
	Female	13 (68.4)	12 (44.4)	6 (33.3)	2 (50.0)	1.00*		1.00*	
	Male	6 (31.6)	15 (55.6)	12 (66.7)	2 (50.0)	0.42 (0.17-1.02)	0.055	2.60 (1.01-6.72)	0.048
Location 1, n (%)									
	Colon	15 (78.9)	20 (74.1)	16 (88.9)	4 (100)	1.00*		1.00*	
	Rectum	4 (21.1)	7 (25.9)	2 (11.1)	0	0.56 (0.18-1.72)	0.309	0.63 (0.18-2.22)	0.470
Location 2, n (%)									
	Right	7 (36.8)	7 (25.9)	9 (50.0)	0	1.00*		1.00*	
	Left	12 (63.2)	20 (74.1)	9 (50.0)	4 (100)	0.96 (0.38-2.40)	0.923	1.27 (0.44-3.62)	0.657
Morphology, n (%)									
	Adenocarcinoma	18 (94.7)	21 (77.8)	18 (100)	4 (100)	1.00*		1.00*	
	Mucinous adenocarcinoma	1 (5.3)	26 (22.2)	0	0	0.66 (0.16-2.78)	0.570	0.72 (0.15-3.39)	0.678
Proportional odds, 95% confidence intervals (95% CI) and P values were calculated considering univariate and multivariate ordinal regression models; P<0.05 was considered statistically significant; *reference value; sd: standard deviation.									

CHAPTER 4

CCL18 stimulates gastric and colorectal cancer cell invasion: dissecting the underlying molecular mechanisms

Following the results obtained in chapter 3, in which the pro-invasive role of macrophage-derived CCL18 on CRC cells was demonstrated, a preliminary study was conducted in order to unravel some of the mechanisms of action.

Accordingly, two colorectal cancer cell lines were stimulated with recombinant human CCL18, for different timepoints, and the activation of a series of proteins was assessed. Given the results previously published by our group, describing the ability of macrophages to promote gastric cancer cell invasion through an EGFR-dependent signaling, the gastric cancer cell line AGS was also included in this study.

Indeed, besides RKO and HCT15, CCL18 stimulated AGS cancer cell invasion and induced the phosphorylation of FAK, Src, ERK, Akt and p38, although the latter was not observed in AGS cells. Additionally, CCL18 also induced the phosphorylation of EGFR at the tyrosine residue Y1086, similarly to what we have previously reported with macrophage conditioned media, suggesting the activation of the same signaling cascade. In addition, the capacity of CCL18 to induce cancer cell EMT was also evaluated. Our results evidence that CCL18 enhanced the expression of Snail, Slug, Zeb1 and vimentin genes, suggesting that this chemokine might be inducing a partial EMT phenotype.

Despite the fact that these results are still preliminary and require further investigation, it is clear that CCL18 activates a signaling cascade in both gastric and colorectal cancer cell lines that culminates in the promotion of cancer invasion and thus strengthens the need to proceed with this study. Moreover it further supports the likelihood of CCL18 being a relevant player in tumor progressing and, as a consequence, a plausible attractive target for combinatory therapeutic approaches.

Introduction

CCL18 was identified in 1997, in a study by Hieshima et *al.*, in which they searched the GenBank expressed sequence tag (EST) database for the MIP-1α cDNA sequence. This strategy led to the discovery of a chemokine, located in chromosome 17, which shared great homology with MIP-1α and was constitutively expressed at high levels in human lungs, and less in the lymph nodes, thymus and appendix, and thus was called pulmonary and activation-regulated chemokine (PARC). Chemotaxis experiments revealed that PARC was chemotactic for T lymphocytes but not monocytes nor neutrophils [435]. A similar strategy was followed by a different group that reported the identification of a cDNA encoding a chemokine called macrophage inflammatory protein-4 (MIP-4) [436], without a murine ortholog [437]. Almost simultaneously, Adema and colleagues described a chemokine specifically produced by GM-CSF/IL-4-induced monocyte-derived dendritic cells, which they named as DC-CK1, meaning dendritic cell C-C chemokine. *In vitro* work revealed that this chemokine induced the recruitment of T cells, preferentially naïve T cells [438]. Later that year, Kodelja *et al*. characterized what they referred to as a novel human CC-chemokine highly produced by human macrophages and hence called it alternative macrophage activation-associated CC-chemokine (AMAC)-1. Despite the high homology with MIP-1α, they had opposite expression patterns: while MIP-1α was activated by LPS, AMAC-1 expression was induced by IL-4, IL-13 and IL-10, and inhibited by IFN-γ [439]. Recombinant or synthetic MIP-4 induced calcium mobilization in naive and activated T lymphocyte subpopulations, *in vitro*. Injection of synthetic MIP-4 into the peritoneal cavity of mice lead to the accumulation of both $CD4^+$ and $CD8^+$ T lymphocytes, but not monocytes or granulocytes [440]. Later, in 2000, a new classification system renamed PARC/MIP-4/DC-CK1/AMAC-1 as CCL18 [441].

CCL18 has been reported to be increased in a series of inflammatory disorders, including atopic dermatitis [442], rheumatoid arthritis [443], chronic obstructive pulmonary disease [444] and sarcoidosis [445]. Besides being produced by dendritic cells and macrophages in response to factors such as IL-4, IL-10 [438, 446, 447], its production is also increased in the presence of infectious agents [443] and allergens [448], clearing supporting its role in immune responses. Since the first study describing the ability of CCL18 to attract naive $CD4^+$ T cells [438], it was shown that it can also recruit skin-homing memory T cells [442] and immature dendritic cells [446]. Concomitantly with its chemotactic role, CCL18 promotes dendritic cell differentiation with tolerogenic features, specifically increased IL-10 production and the ability to suppress effector $CD4^+CD25^-$ cell proliferation [449]. Chang and colleagues have also reported that CCL18-treated memory $CD4^+$ T cells increase the expression of IL-10 and TGF-β and, more importantly, are converted to $CD4^+CD25^+Foxp3^+$ regulatory T cells [450]. Additionally, CCL18 attracts, both *in vitro* and *in vivo*, a specific subset of regulatory T cells,

characterized by being $CD25^{+}CD127^{low}$ and $FoxP3^{-}$, which exerts a regulatory function through the production of IL-10 and the inhibition of $CD4^{+}CD25^{-}$ effector cell proliferation [451]. More recently, the CCR8 was shown to be a CCL18 receptor, specifically in immune cells. By using CCR8 transfected cells and Th2 cells, Islam *et al.* revealed that CCL18 induces cell migration and calcium flux, and leads to CCR8 internalization, competing with its known ligand CCL1 [452].

CCL18 has been widely studied in lung diseases. Its expression was shown to be increased in bronchoalveolar lavage from scleroderma patients, a disease characterized by excessive fibrosis [453]. Interestingly, fibroblasts stimulated with CCL18 increased collagen type I expression, a process dependent of ERK [454] and specificity protein 1 (Sp1) activation [455], revealing the direct involvement of CCL18 in the fibrotic process. In fact, a positive feedback loop involved in pulmonary fibrosis has been proposed: alveolar macrophages (AMs) from patients with pulmonary fibrosis produce higher CCL18 levels which act on fibroblast leading to collagen I expression. On the other hand, collagen I stimulates CCL18 expression in AMs perpetuating the fibrotic process [456]. Moreover, macrophages from idiopathic pulmonary fibrosis patients were reported to have higher CD204 expression, which was further increased by *in vitro* stimulation with collagen type. By blocking CD204 with a neutralizing antibody, collagen type I no longer induced CCL18 expression [387].

Since the study by Schutyser *et al.*, in 2002, describing increased CCL18 levels in ascites from ovarian cancer patients, numerous groups have reported the up-regulation of this cytokine in many other malignancies, namely in bladder, cervical, ovarian, breast and non-small cell lung cancer, either by real-time PCR or immunohistochemistry in tumor samples, or ELISA in the plasma, serum, urine or bronchoalveolar lavages (Annex Table 4.1, page 111). Importantly, besides being generally associated with advanced disease and correlated with decreased survival, CCL18 levels are reported to be increased in some benign lesions comparing with healthy controls, as in the ovary and breast [457, 458].

Within the tumor microenvironment, CCL18 is mainly produced, although not exclusively, by macrophages, identified by immunohistochemistry as the $CD68^{+}$ cells [180, 459], specifically the anti-inflammatory $CD163^{+}$ or $CD209^{+}$ macrophages [460, 461]. By isolating fibroblasts from breast tumor tissue or paired normal mammary tissue, it was shown that CCL18 is also upregulated in CAFs [462]. In the case of oral squamous cell carcinoma, Jiang and colleagues revealed that CCL18 is produced by tumor cells, rather than TAMs, and acts in an autocrine manner, inducing tumor cell proliferation, both *in vivo* and *in vitro*, migration and invasion. Using a PI3K inhibitor, the *in vivo* tumor growth and *in vitro* invasion mediated by CCL18 was partially reduced [463]. Importantly, *in vitro* work performed with gastric cancer cell lines also showed a CCL18 overexpression comparing with immortalized cells from human gastric epithelium, and CCL18 silencing or overexpression results in either a decrease or increase of

cancer cell invasion, respectively [464]. Similarly, ovarian cancer cell transfected with CCL18 promoted metastasis formation in an orthotopic transplantation mouse model, with up-regulation of mTOR signaling [465]. Elegant work by Chen *et al.* revealed the pro-migratory and pro-invasive role of CCL18 in breast cancer, specifically by promoting integrin clustering and enhancing cell adherence to the ECM. Membrane-associated phosphatidylinositol transfer protein 3 (PITPNM3) was identified, for the first time, as being a functional CCL18 receptor and the downstream signaling pathway described to involve the activation of focal adhesion kinase (FAK), proline-rich tyrosine kinase 2 (Pyk2), and Src. Importantly, PITPNM3 silencing abrogated CCL18-induced metastasis in mice [466]. More recently, a positive feedback loop between TAMs and mesenchymal-like cancer cells was reported to be crucial for breast cancer metastasis. Breast cancer cell lines of the mesenchymal type induced a TAM-like phenotype in macrophages via GM-CSF, namely with increased expression secretion of IL-10 and CCL18. On their turn, GM-CSF-activated macrophages induced EMT in breast cancer cell lines, via CCL18. Using a humanized mouse model, it was shown that the GM-CSF-CCL18 loop promoted breast cancer metastasis, specifically to the liver and to the lungs. Finally, double IHC revealed increased $CCL18^+$ cells in cancers with higher GM-CSF expression, and both were independent markers for worst prognosis in breast cancer patients [467]. The pro-invasive role or CCL18 was also reported in hepatocellular carcinoma, pancreatic, ovarian, lung and gastric cancer, the latter being a process dependent of ERK and NF-κB activation [464, 465, 468-470]. Despite PITPNM3 expression has been mainly attributed to tumor cells, Shicheng *et al.* recently described its expression by naive $CD4^+$ T cells, recruited to breast tumors through macrophage-derived CCL18. Additionally, by using an orthotopic humanized breast tumor mouse model with $CD4^+$ cells knockdown for PITPNM3, there was a reversion of the immunosuppressive environment, evidenced by the decrease of naïve $CD4^+$ T cells and Tregs and increase of cytotoxic $CD8^+$ T cells, leading to a reduction in tumor growth and metastasis [471]. Interestingly, *in vitro* work showed that CCL18 also promotes a M2 macrophage polarization. Furthermore, ECM components as HA and vitronectin were reported to stimulate CCL18 secretion by macrophages, a process not mediated by PITPNM3 [472], suggesting the involvement of other receptors and mediators. In this sense, work performed in acute lymphocytic leukemia (ALL) revealed that CCL18 was able to bind to G protein-coupled estrogen receptor 1, known as GPER1 or GPR30, acting as an antagonistic and thus blocking CXCL12 activities on pre-B ALL cells, specifically calcium mobilization and chemotaxis [473].
Besides acting directly on cancer or immune cells, leading to their proliferation, invasion and metastasis, CCL18 has other roles within the tumor microenvironment. In the specific case of breast phyllodes tumors, a type of tumor that develops in the connective tissue, TAMs-derived CCL18 induced myofibroblast differentiation, proliferation and invasion, a process mediated by PITPNM3 and NF-kB signaling [461]. Moreover, CCL18 promoted angiogenesis in breast

cancer, namely by acting synergistically with VEGF in the induction of endothelial cell migration. By binding to PITPNM3 in HUVECs, CCL18 induced the endothelial-mesenchymal transformation and activated ERK and Akt/GSK-3β/Snail signaling [474].

Altogether, it becomes clear the CCL18 potential as a biomarker in different cancers. Additionally, its premature detection in patients' blood, namely in pre-malignant lesions, is a possibility that would certainly help the early diagnosis and thus requires further investigation. Nevertheless, in the specific case of CRC, the available data is still very scarce and the only published work is not in line with our results, since it describes CCL18 as an independent favorable prognostic marker [475]. Given the previously presented ability of CCL18 to stimulate colorectal cancer cell invasion *in vitro* (Chapter 3), it is important to unveil the underling mechanisms, specifically the receptor involved and the downstream signaling pathways, on the hopes of finding potential targets for therapeutic intervention.

Materials and Methods

Matrigel invasion assay

5 x 10^4 AGS cells were added to the upper compartment of Matrigel-coated inserts of 8-µm pore size (BD Biosciences) while 1ng/mL of recombinant human CCL18 (Peprotech) was incubated in the lower compartment. After 24 h, filters were washed, fixed in 10% methanol. Invasive cells were counterstained with DAPI and counted using a fluorescence a Zeiss Axiovert 200M fluorescence microscope (Carl Zeiss). Invasive ratio was calculated relatively to unstimulated cells.

Western blot

For the evaluation of the activated signaling pathways, RKO, HCT15 and AGS cells were treated for 15 min, 30 min, 60 min, 1h, 3h and 6h with 1ng/mL of human recombinant CCL18 (Peprotech, 1ng/mL). Regarding the assessment of cell receptors activation, the three cell lines were stimulated with CCL18 for 30 minutes, 1h and 3h. Afterwards, cells were washed with PBS and lysates prepared in cold lysis buffer [50 mM Tris-HCl-pH 7.5, 1% IGEPAL, 150 mM NaCl, 2 mM ethylenediaminetetraacetate, 3 mM sodium orthovanadate, 20 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin and leupeptin]. Following protein concentration quantification using the Dc Protein kit (BioRad), 35 µg of protein were loaded on a 7.5 % or 10 % SDS-polyacrylamide gel. Membranes were blocked with 5% milk or 4% bovine serum albumin (BSA) in PBS+0.5 % Tween-20 (Sigma-Aldrich) and incubated overnight, at 4°C, with rabbit polyclonal antibodies against phospho-EGFR (Y1086) (Invitrogen), Akt, FAK, Src, p38 ERK1/2, phosphor-FAK (Tyr397), phospho-Src (Y416), phospho-ERK1/2 (T202/Y204), phospho-p38 (Thr180/Tyr182), (Cell Signaling), α-tubulin (Sigma-Aldrich), anti-

GPR30 (ThermoFisher, PA5-28647) and PITPNM3 (ThermoFisher, PA5-21903) or with a mouse monoclonal antibody against EGFR (Transduction), phospho-Akt(S473). Donkey anti-rabbit or sheep anti-mouse-HRP-conjugated secondary antibodies (GE Healthcare) were used, followed by ECL Detection (GE Healthcare).

RNA extraction and real-time PCR

RKO, HCT15 and AGS cells were incubated with 1ng/mL of CCL18 for 1, 4 and 7 days. RNA extraction was performed using the Trizol. cDNA was prepared using 150 U of SuperScript™ II Reverse Transcriptase, 1× first strand buffer, 10 mM DTT 0.1 M (Invitrogen), 0.5 mM dNTPs 10 mM (Bioron, Germany), 8U of rRNasin (Promega, WI) and RNase/DNase free water (Gibco). Real-time was performed using TaqMan Universal PCR Master Mix (Applied Biosystems) and probes for Snail, Slug, Zeb1, Zeb2, Vimentin, Fibronectin, Epcam and actin (Applied Biosystems). 0.5µL of Taqman were used in a 10 µL reaction for the real-time PCR reaction.

Immunocytochemistry

RKO, HCT15 and AGS cells were stimulated with 1ng/mL of CCL18 for 15 minutes, at 37°C, or 2h20, at 4°C. Following washing with cold PBS, cells were fixed with 4% paraformaldehyde and quenched with 50mM NH4Cl for 10 minutes. After washing three times with PBS, cells were blocked for 45 minutes with 5% BSA and incubated overnight, at 4°C, with the primary antibodies PITPNM3 and GPR30. Cells were then washed with PBS and were incubated for 1 hour with the goat anti-rabbit AlexaFluor-594-conjugated (ThermoFisher) secondary antibody. Samples were finally washed with PBS and coverslips were mounted on Vectashield with DAPI (VectorLab). Cells were visualized with a Zeiss Axiovert 200M fluorescence microscope.

Results

CCL18 induces AGS gastric cancer cell invasion

Previous work from our group revealed that CCL18 stimulates colorectal cancer cell invasion, specifically of RKO and HCT15 cancer cells (Chapter 3) [476]. Moreover, we have also shown that macrophages promote gastric (AGS) and colorectal (RKO) cancer cell invasion via EGFR phosphorylation at the tyrosine residue Y^{1086}, and subsequent tyrosine phosphorylation of c-Src, ERK and p38 [221]. These results led us to evaluate if CCL18 also had the ability to promote AGS cell invasion. Indeed, in the presence of CCL18, the number of invading AGS cells increased (Figure 4.1), although to a lower extent than what we observed previously with RKO CRC cells.

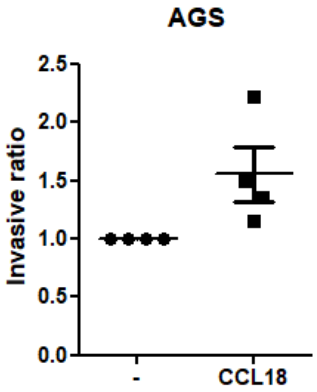


Figure 4.1. CCL18 stimulates AGS cell invasion. Quantification of Matrigel invasion assays in which AGS cells were stimulated with 1ng/mL of CCL18. After 24h, invasive cells were counterstained with DAPI and counted in the microscope. Graphic includes data from 4 independent experiments with standard error.

CCL18 induces the phosphorylation of EGFR (Y1086), FAK, c-Src, Akt, ERK and p38 in gastric and colorectal cancer cells

In order to understand which signaling pathways were activated by CCL18, the three cell lines that invaded in the presence of this chemokine were stimulated with CCL18 for different time points. From 15 minutes to 6 hours, the phosphorylation of EGFR, FAK, c-Src, Akt, ERK and p38 were evaluated using specific antibodies recognizing the total or the phosphorylated protein forms (Figure 4.2). Interestingly, similarly to what we have previously described for macrophages [221], CCL18 induces cancer cell EGFR (Y1086), c-Src, ERK and p38 tyrosine phosphorylation, although the latter was only observed for the colorectal cancer cells. Additionally, FAK was also activated in the presence of CCL18. The majority of the effects were already observed at 15 minutes after stimulation, and further increased until 1 or 3 hours after CCL18 addition. Following 6 hours, as expected, phosphorylation of all proteins returned to basal levels.

CCL18 induces on colorectal cancer cell lines a partial Epithelial to Mesenchymal transition

Several reports have described the CCL18 ability to induce EMT in different cancer cell lines, including breast and pancreatic cells [469, 477]. Therefore, and given the pro-invasive role of CCL18, RKO, HCT15 and AGS were stimulated with CCL18 for 1, 4 and 7 days. Preliminary quantitative real-time PCR for some EMT related genes, namely Snail, Slug, Zeb1 and vimentin, revealed that CCL18 induces a slight increase in these genes (Figure 4.3). Regarding HCT15, Slug amplification was only successful in half of the experiments and, thus, we did not

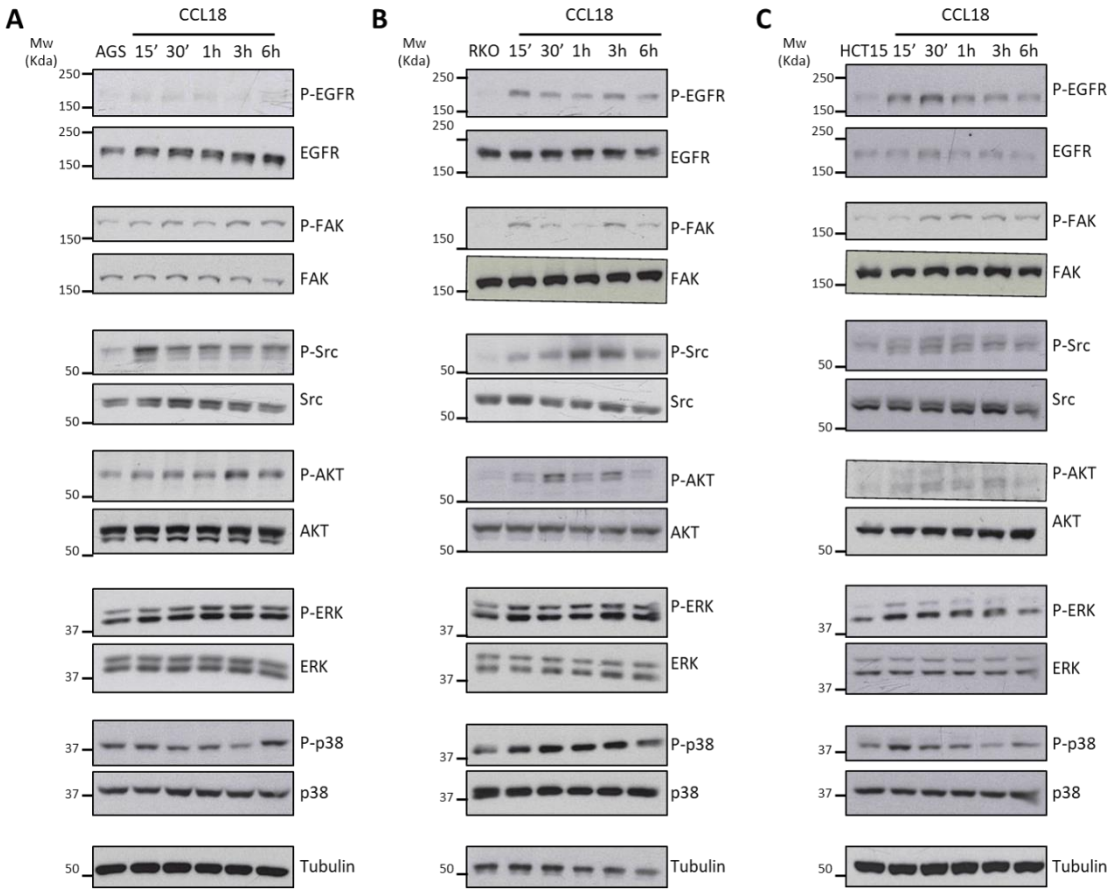


Figure 4.2. CCL18 induces activation of EGFR, FAK, Src, Akt, ERK and p38. (A) AGS, **(B)** RKO and **(C)** HCT15 were stimulated with CCL18 for different time points, ranging from 15 minutes to 6 hours, and western blot was performed for phosphorylated and total EGFR, FAK, Src, Akt, ERK and p38. Tubulin was used as loading control. Images are representative of 4 independent experiments.

include the results. On the other hand, there was a significant increase of vimentin at day 4. In the specific case of AGS cells, there was no amplification of Slug and the effect on the other genes was less evident than in the CRC cell lines. Fibronectin, Zeb2 and Epcam were also analyzed but no amplification was detected.

RKO, HCT15 and AGS cells express PITPNM3 and GPR30 but nor their amount nor location was altered upon CCL18 stimulation

Three receptors have been proposed to interact with CCL18: PITPNM3 [466], GPR30 [473] and CCR8 [452], the latter being responsible for CCL18 chemotaxis of Th2 immune cells.

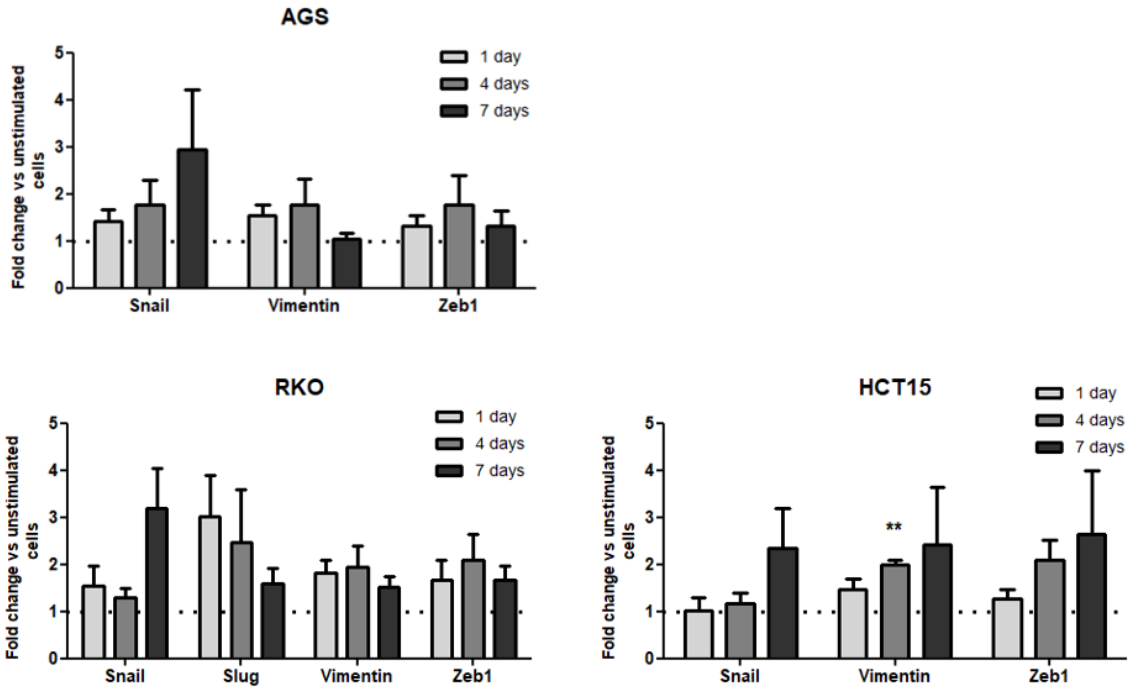


Figure 4.3. CCL18 induces a partial increase of some EMT genes in AGS, RKO and HCT15. mRNA expression of Snail, Slug, Vimentin and Zeb1 evaluated by quantitative real-time PCR in AGS, RKO and HCT15 stimulated with CCL18 for 1, 4 or 7 days. Gene expression data from stimulated cells was normalized relatively to unstimulated ones and β-actin was used as housekeeping gene. Graphs include data from 4 independent experiments.

Cancer Genome Atlas enabled the confirmation that RKO do not express CCR8 and so this was excluded as the putative receptor responsible for the above described CCL18 activities. Western blot revealed that the three cell lines express both PITPNM3 and GPR30. Furthermore, their expression is not altered following CCL18 stimulation up to 3 hours (Figure 4.4A). In order to evaluate if there were any modifications in the localization of any of the receptors, cells were stimulated with CCL18 for 15 minutes, at 37°C, or 2h20 minutes, at 4°C. Immunocytochemistry performed in non-permeabilized cells confirmed that CCL18 does not induce any alteration in either receptor in the three cell lines (Figure 4.4B and C). These experiments did not provide any hint to which of the receptors is being directly activated by CCL18 and further investigations are required.

Discussion

TAMs are key players in tumor progression, namely due to their pro-migratory and pro-invasive roles [192]. Their action relies predominantly on the release of a series of growth factors, cytokines and chemokines which affect tumor cell behavior [478]. Among these, CCL18

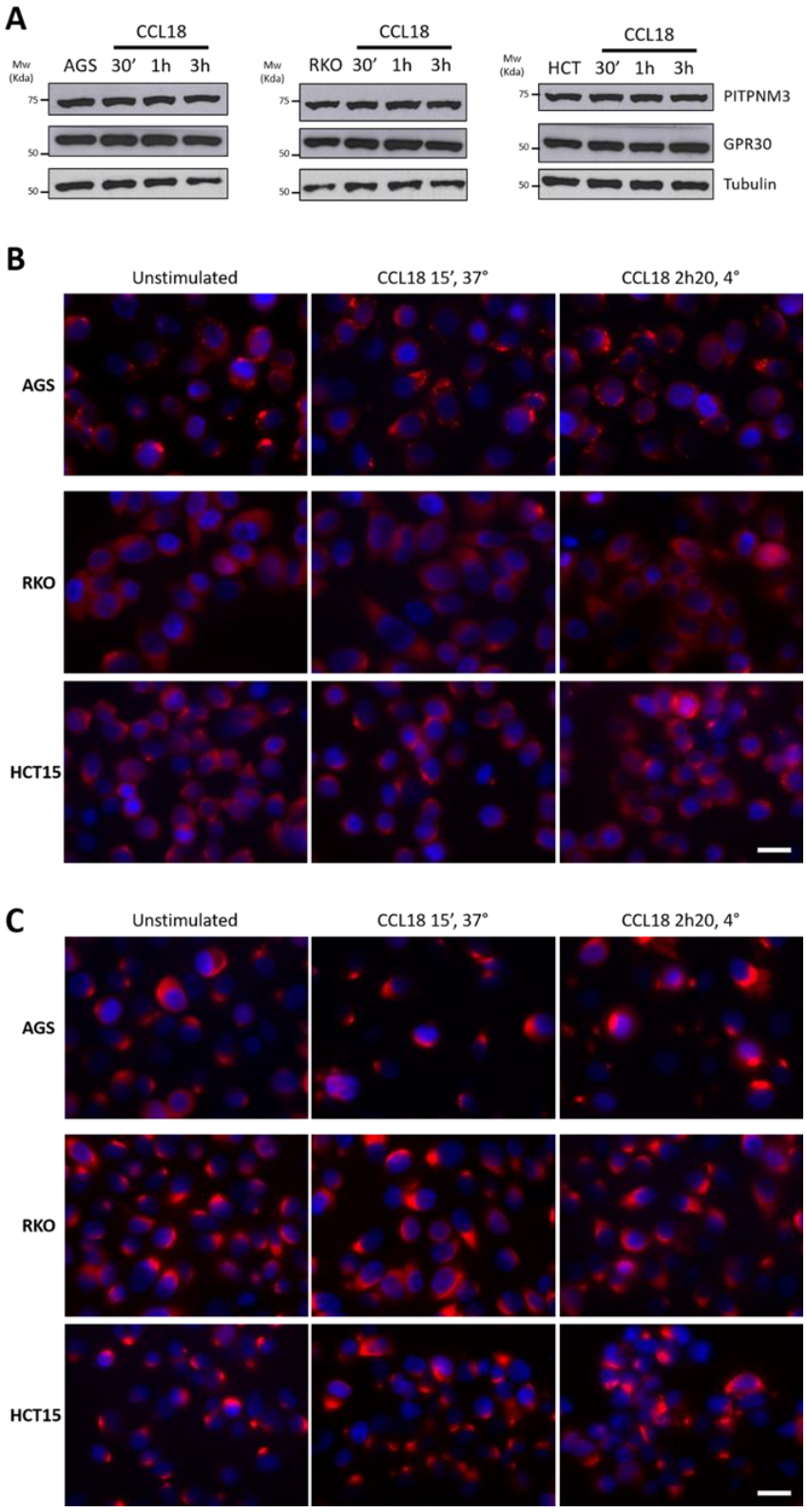


Figure 4.4. CCL18 stimulation does not induce alterations in neither the amount nor the location of PITPNM3 and GPR30. (A) AGS, RKO and HCT15 cells were incubated with CCL18 for 30 minutes, 1h and 3h and total PITPNM3 and GPR30 were analyzed by western blot in total lysates. **(B)** Immunocytochemistry for GPR30 in non-permeabilized AGS, RKO and HCT15 stimulated with CCL18 for 15 minutes, at 37°C, or 2h20, at 4°C. **(C)** Immunocytochemistry for PITPNM3 in non-permeabilized AGS, RKO and HCT15 stimulated with CCL18 for 15 minutes, at 37°C, or 2h20, at 4°C. Scale bar represents 20μm.

emerged in the last years as a chemokine mainly produced by anti-inflammatory macrophages within the tumor microenvironment [460], being involved in the metastatic process [466]. Now, through this preliminary work we showed, for the first time, that CCL18 leads to the phosphorylation of FAK, EGFR, c-Src, ERK, Akt and p38 in two CRC cell lines, similarly to we have previously described to be induced by macrophages. Additionally, CCL18 appears to induce a partial epithelial to mesenchymal transition, given the enhanced expression of genes associated to a mesenchymal profile. These effects were also observed, to a lower extent, in a gastric cancer cell line.

Until the work by Catusse *et al*., describing the binding of CCL18 to GPR30 in pre-B ALL cells blocking CXCL12 activity [473], there was no identified receptor for this chemokine. Even though this is the only report describing such interaction, GPR30 has been implicated in different cancers. In endometrial carcinoma, GPR30 expression correlated with more aggressive disease and decreased survival [479] while, in breast cancer, was positively associated with the presence of distant metastasis [480]. Also in ovarian cancer, nuclear expression of GPR30 was a predictor of poor survival [481]. In the specific case of CRC, GPR30 expression was significantly associated with poor survival in female patients in stages III and IV. Moreover, it mediated the pro-proliferative and pro-migratory effect of estrogen [482]. PITPNM3 was reported to be a functional CCL18 receptor, being upregulated in breast cell lines and tissues, and mediating the CCL18 pro-invasive activity [466]. Similar results were observed in lung cancer cell lines and tissues, in which CCL18-PITPNM3 interaction activates the ELMO1/DOC180 signaling pathway, resulting in F-actin polymerization, β1 integrin phosphorylation and Rac1 activation [470]. CCR8 was also shown to be a CCL18 receptor, specifically in immune cells [452], but it was excluded from our study since it is not expressed in RKO cells. So far, we could not identify which of these two receptors, GPR30 or PITPNM3, are being directly activated by CCL18, and thus we are exploring both possibilities. Accordingly, we are currently conducting siRNA experiments directed to GPR30 or PITPNM3 that will, hopefully, clarify which of these receptors is responsible for CCL18 signaling and pro-invasive activity.

Albeit not knowing to which receptor CCL18 is binding, our results reveal that this chemokine induces EGFR phosphorylation at the same tyrosine residue (Y1086) and phosphorylates a very similar signaling pathway as the pro-invasive macrophage conditioned medium. Interestingly, it was reported the EGFR transactivation by GPR30, specifically via the release of heparin binding-EGF (HB-EGF) by metalloproteinases [483]. Additionally, Filardo and colleagues revealed that ERK1/2 activation by estrogen happening downstream of EGFR phosphorylation, requires GPR30 and is mediated via Gβγ-subunit-Src-Shc signaling [484]. In the case of ovarian cancer cells, GPR30 activation led also to EGFR-Akt signaling [485]. The direct interaction between GPR30 and EGFR was also described in breast cancer cell lines,

which is further potentiated by zinc chloride stimulation [486]. Given all these data, it is plausible to speculate that EGFR activation by CCL18 might be occurring, directly or indirectly, via GPR30, eventually leading to the common phosphorylation of the MAPK cascade.

PITPNM3 interaction with CCL18 was also reported to activate FAK and Pyk2 [466]. Additionally, Pyk2 silencing inhibited CCL18-mediated Src activation, and either Pyk2 or Src silencing inhibited CCL18 mediated FAK activation, α5β1 integrin clustering, migration and invasion [487]. Both malignant ascites and CCL18 stimulated ovarian cancer cell migration by inducing Pyk2 phosphorylation, and women with phospho-Pyk2 positive tumors have a significantly shorter progression-free survival than women with phospho-Pyk2 negative tumors [488]. Although Pyk2 was not evaluated in our experiments, we reported that CCL18 induced FAK and Src activation in both colorectal and gastric cell lines, thus we cannot exclude that this is occurring downstream of PITPNM3.

CCL18 binding to PITPNM3 is described to induce EMT. Mechanistically, CCL18-PITPNM3 interaction in breast cancer cells stabilizes Snail via the Akt/GSK3β signaling pathway, a process dependent of PI3K [477]. Snail upregulation by CCL18 was observed in other models, such as pancreatic cancer [469], while, in oral squamous cell carcinoma, CCL18 induced E-cadherin downregulation concomitantly with Slug overexpression [489]. More recently, Haiyan and colleagues showed that AMAP1 acts downstream of PITPNM3 and Pyk2 and its abrogation impairs CCL18 effect in stimulating breast cancer cell EMT and invasion [490]. In our preliminary study, there seems to be a partial induction of EMT, more evident in the colorectal cancer cell lines, although further studies are required to confirm this observation. Nevertheless, it is important to mention that the CCL18 concentration that we are using in our experiments is considerably lower than the one used in the previously mentioned works. There aren't any studies addressing a possible EMT induction by CCL18 in CRC cells, while the only published work in gastric cancer cells describes a Slug upregulation and an E-cadherin downregulation [464]. Nevertheless, these authors used the MGC-803 gastric cancer cell line, whereas we used AGS where no Slug expression was detected.

Despite the numerous reports describing the increase of CCL18 in cancer, the data regarding gastric cancer is contradictory. One study based on cDNA microarray technology described an association between increased CCL18-expressing macrophages with improved overall survival [459], while other two reports, in which they analyzed CCL18 in the serum, by ELISA [491], or in tumor samples, by immunohistochemistry [492], showed opposite results. On the other hand, in the specific case of CRC, the only published work evaluated CCL18 staining in 371 samples from CRC patients and revealed that it was an independent favorable prognostic biomarker [475]. These results are not in accordance to what we recently published, showing an increase on CCL18 expression at the invasive front of more advanced CRC stages [476]. Nevertheless, it is noteworthy to mention one study performed with bladder cancer samples

describing an increase in CCL18 levels in tumor patient's urine, particularly in muscle invasive bladder and high-grade disease, although no differences were observed in tumor samples by immunohistochemistry [493]. This study clearly demonstrates that there isn't necessarily a correlation between what is observed histologically in primary tumor samples and what is systemically detected in patient's blood, serum or urine.

Our preliminary results further support the importance of dissecting the CCL18 mechanisms of action, namely the clarification of the receptor involved that may lead to the activation of the signaling pathways above described. Moreover, it would be extremely important to evaluate the systemic CCL18 levels in CRC patients' serum, at different stages of disease, but also on patients with pre-malignant lesions. Such detailed analysis will clarify if CCL18 can be appointed as a biomarker in this specific type of cancer and eventually contribute to early diagnosis, or at least, as predictor of disease outcome or therapy response. Depending on the results, it could also be interesting to monitor the alterations in CCL18 serum levels during all stages of treatment, with the purpose of unravel if it could be an indicator of therapeutic response/resistance. In this regard, we are currently developing a device with high sensitivity that will enable the detection of CCL18 in patients' samples. Altogether, based on the published data together with our preliminary results, CCL18 targeting, specifically by neutralizing its activity, seems to be an attractive strategy in anticancer immunotherapy that would promote anti-tumor immunity and eventually impair metastasis formation. In this sense, PITPNM3 inhibition might also be a plausible approach, namely in breast cancer, although, in CRC, GPR30 still has to be considered as the possible receptor mediating CCL18 activities.

Annex Table 4.1. CCL18 in cancer.

Cancer type	Samples	Methodology used	Major conclusions	Reference
Pediatric Acute Lymphoblastic Leukemia (ALL)	- 13 acute myeloid leukemia (AML), - 38 ALL (11 T-ALL and 27 precursor B-lineage patients (16 prepreB-ALL, 11 preB-ALL) - 21 pediatric healthy controls	ELISA (Serum)	CCL18 significantly increased in serum of patients with T-ALL and Prepre-B ALL comparing with healthy controls (85.2 ng/mL versus 44.7ng/mL versus 30.9ng/mL). In AML samples, CCL18 levels were similar to controls (37.8ng/mL).	[494]
B-Cell Chronic Lymphocytic Leukemia (B-CLL)	- 47 B-CLL - 39 healthy controls	ELISA (Serum)	CCL18 significantly increased in the serum of B-CLL patients compared with the healthy controls (approximately 100 ng/mL versus 200ng/mL).	[495]
Cutaneous T-cell lymphoma (CTCL)	- 5 mycosis fungoide (MF) tumors - 5 MF patch/plaque - 4 parapsoriasis en plaque (PEP) - 8 atopic dermatitis (positive control) - 6 healthy controls	ELISA (Serum) Real time PCR (Skin samples) IHC	CCL18 significantly increased in the serum of MF patients compared to healthy controls (59 ng/mL versus 18ng/mL). 18% increase of CCL18 mRNA in MF-tumor compared to healthy skin. CCL18 expressed in MF-plaque/patch and MF-tumor but not in healthy skin. Its expression is co-expressed by $CD209^+$ and $CD163^+$ macrophages.	[460]
	- 38 CTCL (patch, plaque, tumor and erythroderma) - 20 healthy controls	ELISA (Serum) Real time PCR (Skin biopsy) IHC (Only in 16 CTCL and 7 controls)	CCL18 increased in the serum of CTCL compared with controls (798 ng/mL vs 154.7ng/mL). Levels were higher in erythroderma, then in tumor and finally in patch. Patients with levels higher than 1000 ng/mL had worst prognosis. CCL18 mRNA was higher in CTCL than healthy controls CCL18 increased in tissues with disease progression but the differences were only significantly different in tumors compared with healthy controls.	[496]
Non-Small-Cell Lung Cancer (NSCLC)	- 241 NSCLC	IHC	CCL18 staining correlated with more advanced stages, specifically lymph node and distant metastasis. Additionally, it correlated with decreased survival. Nir1 expression much higher in tumors and positively correlated with CCL18.	[470]

	- 170 NSCLC (70 adenocarcinoma, 54 squamous carcinoma (SCC) and 46 with mixed histology) - 31 healthy controls	ELISA (Serum)	CCL18 higher in serum of NSCLC patients than controls (150ng/mL versus 32ng/mL). Moreover, SCC had higher levels than adenocarcinoma (187ng/mL versus 143ng/mL). CCL18 was increased in stages III and IV (210 ng/mL and 182 ng/mL) compared with stage I (119ng/mL). Lower levels associated with increased survival only in adenocarcinoma.	[497]
	- 37 NSCLC (24 adenocarcinomas, 11 SCC and 2 large-cell car)	ELISA (bronchoalveolar lavage (BAL)) (Serum from diferente cohort, n =25).	Increase CCL18 in BAL from patients with bigger tumors and lymph node metastasis (N0 – 988pg/mL; N+ - 2585pg/mL). In the serum there were no associations with tumor size.	[498]
Bladder cancer (BCa)	- 64 urothelial cancer - 63 controls	ELISA (Urine)	Higher levels of CCL18 in the urine of tumor patients than controls (637 pg/mL versus 4.81 pg/mL).	[499]
	- 102 BCa - 206 controls (47 with voiding symptoms, 44 with urolithiasis, 9 with gross hematuria, 14 with urinary tract infection and 92 without any diagnosed condition)	ELISA (Urine) IHC	CCL18 increased in serum from Bca patients than controls (52.8 pg/mL versus 11.1 pg/mL). Moreover, higher levels in muscle invasive Bca compared than non-invasive (90.7 pg/ml vs. 44.7 pg/ml) and in high-grade compared to low-grade disease (79.6 pg/ml vs. 38.1 pg/ml). No differences observed by IHC.	[493]
Prostate cancer (PCa)	- 46 PCa - 42 benign prostate hyperplasia (BPH)	Antibody microarray (Serum)	Higher CCL18 levels in serum of PCa patients than BPH (217pg/mL versus approximately 40 pg/m).	[500]
Oral squamous cell carcinoma (OSCC)	- 60 OSCC - adjacent normal tissues	IHC	CCL18 increased in tumor tissues (all OSCC positive for CCL18 and 70% displayed strong expression). Moreover, there was a positive association with higher grade tumors, specifically stages III and IV. CCL18 did not colocalize with CD68.	[463]
Cutaneous basal cell carcinoma (BCC)	- 18 facial BCC and peritumoral skin. - Controls :buttock skin from the same patient	qRT-PCR (NGS) (Biopsies)	Comparing with buttock skin CCL18 mRNA was increased 30x in peritumoral skin and 60x in tumor (60x) samples.	[501]
Cervical cancer	- 28 invasive cervical cancers. - 8 cervical Intraepithelial neoplasia (CIN): 4CIN3/CIS and 4 CIN1/CIN2. - 5 normal cervix tissues	Microarray technique and validation by Real Time PCR (biopsy)	CCL18 expression increased in tumor and CIN3 samples comparatively to normal tissue. Moreover, CIN1 samples presented lower CCL18 mRNA levels.	[502]

Ovarian cancer (OC)	- 12 OC - 12 nonovarian carcinoma (3 benign gynecological disorder (ovarian cyst, fibroma and mucinous adenoma) and 9 nongynecological problems (peritonitis, pancreatic carcinoma, pancreatic carcinoma with liver metastasis, osteosarcoma with liver metastasis, metastasized breast carcinoma, or liver cirrhosis (4)).	ELISA (Ascitic fluids) IHC	CCL18 increased in ascites from OC patients than non-tumor samples (124ng/mL versus 44ng/mL). Its expression colocalized with $CD68^+$ macrophages.	[180]
	- 51 OC (27 serous, 14, endometrioid and 10 mucinous) - 27 benign lesions (9 mature cystic teratoma, 7 serous cystadenoma, 5 ovarian fibroma, 4 mucinous cystadenoma and 2 simple serous cyst). - 27 healthy controls	ELISA (Serum)	CCL18 increased in the serum of tumor patients comparing with benign lesions or healthy controls (77ng/mL versus 40.8ng/mL verses 25ng/mL). Additionally, CCL18 significantly higher in early OC stages (I and II) compared to late stages (III and V).	[503]
	- 41 OC - 32 benign pelvic mass - 41 healthy controls (Validation in 535 serum specimens: 130 OC, 64 benign ovarian masses, 36 lung cancer, 60 gastric cancer, 55 nasopharyngeal carcinoma, 48 hepatocellular carcinoma, and 142 healthy controls)	Proteomics (Serum) ELISA (Serum)	CCL18 overexpressed in serum of OC patients.	[504] ABSTRACT
	- 59 OC - 64 benign ovarian tissue (34 serous cyst adenomas and 30 mucinous cyst adenoma)	RNA from cancer cells by laser capture microdissection(real time PCR) ELISA (Serum)	CCL18 increased in the serum of cancer patients comparing with benign disease (150 ng/mL versus 35.8 ng/mL). Moreover, higher CCL18 levels correlated with increased tumor size, pelvic metastasis and decreased survival. CCL18 mRNA about 25x higher in cancer cells than adjacent ones and 1.9x higher comparing with RNA from whole ovarian carcinoma sample.CCL18 mRNA almost absent in normal ovary or benign lesions. CCL18 positive cells scattered in the tumors, without staining in cystic cells, adjacent non-neoplastic epithelia or benign lesions.	[465]

	- 187 epithelial OL (EOC) - 126 benign pelvic mass - 118 healthy controls	ELISA (Serum)	Comparing with healthy controls, CCL18 increased in the serum of patients with benign pelvic mass and EOC (32.98 ng/mL versus 49.3 ng/mL versus 144.8ng/mL). Specifically in EOC, higher CCL18 associated with decreased overall survival.	[457]
Osteosarcoma	- 2 aggressive tumors and 1 curable tumor	Whole exome sequencing of genomic DNA	CCL18 significantly overexpressed in aggressive tumors.	[505]
Glioma	- 8 brain tumors - 4 “normal samples” as controls: 2 post-mortem biopsies of preterm babies, 1 patient with diabetes and 1 with lung pneumothorax hemorrhage	Biopsy – qRT-PCR and IHC	Detected by real time and IHC in tumor samples (colocalizes with CD68)	[506]
	-297 glioma samples (170 low grade gliomas and 127 glioblastomas	Whole genome microarray expression data (Biopsy)	Increased CCL18 expression associated with decreased overall survival (combined with other genes)	[507]
Breast cancer	- 562 primary ductal carcinomas (215 localized and 347 metastatic) - 61 cystic fibrosis of the breast with or without atypical epithelial hyperplasia	ELISA (Serum) Real time PCR and Western blot in 39 samples (isolated TAMs and peripheral blood monocytes (PBM) IHC	CCL18 increased in serum of patients with metastatic tumor than localized tumor or benign disease (approximately 220pg/mL versus 60pg/mL vs 5pg/mL). Higher CCL18 levels associated with decreased overall survival. CCL18 mRNA and protein levels higher in TAMs than PBM, particularly in lymph node positive patients $CCL18^{+}$ cells scattered in the tumor stroma of 505 out of 562 cases of invasive breast carcinomas. No staining in neoplastic cells and adjacent non-neoplastic epithelia. CCL18+ cells absent in benign breast tissues.	[466]
	- 58 breast cancers - 42 benign breast tumors - 30 healthy controls	ELISA (Plasma)	CCL18 increased in serum samples from cancer and benign tumor patients than controls (34.9 ng/mL versus 25 ng/mL (?) versus 17.4 ng/mL).	[458]
	- 103 breast cancers (5 carcinoma in situ, 74 infiltrating ductal carcinoma, 12 infiltrating lobular carcinoma, 5 mixed carcinoma, 4 tubular and mucinous and 3 with other types. - 47 benign disease - 29 adjacent samples from patients with benign lesions	IHC	CCL18 only present in breast cancer tissue, specifically in 84 samples, scattered in the margins of the tumor and not in the center. CCl18 higher in bigger tumors, in more advanced tumor stages and in cases with higher number of metastasized lymph nodes.	[508]

	- 102 malignant breast tumors - 42 benign breast tumors - adjacent non-tumor tissue from all tumor patients - 55 healthy controls (plasma)	cDNA microarray (laser-capture microdissected specimens). Quantitative protein array (Plasma)	CCL18 expression higher in tumor tissues than in adjacent non-tumor tissue. CCL18 increased in serum of tumor patients comparing with benign tumors or healthy controls (42.9ng/mL versus 34.6 ng/mL versus 28.7 ng/mL). Significant differences only when comparing healthy with benign or healthy with cancer.	[509]
	- 207 breast cancer - 126 benign breast tumors - 93 healthy women	ELISA (Serum)	CCL18 increased in serum of tumor patients comparing with benign tumors or healthy controls (290pg/mL vs 170 pg/mL versus 119 pg/mL). Higher CCL18 levels associated with advanced tumor stage and decreased overall survival.	[510] ABSTRACT
Breast phyllodes tumor (PT)	- 68 malignant PT - 36 borderline PT - 167 benign PT - 10 breast normal tissues (only for rea time PCR)	ELISA (Serum) Fresh frozen tissues – mRNA IHC	CCL18 increased in the serum of patients with malignant PT comparing with benign or borderline PT (approximately 500pg/mL versus 200pg/mL versus 20pg/mL). Higher CCL18 levels correlated with decreased survival. CCL18 mRNA progressively increased from normal breast to malignant PT. CCL18 increased in malignant tissues versus benign and borderline PTs, and colocalized with CD163.	[461]
Pancreatic ductal adenocarcinoma (PDAC)	- 62 PDAC - 8 healthy controls	ELISA (serum (only 24 out of the 62) IHC	CCl18 higher in serum from PDAC patients than healthy controls (65.3ng/mL versus 17.3 ng/mL). Increase of CCL18 in PDAC tissues, in mesenchymal and/or cancer cells. CCL18 expression correlated with more advanced tumor stages, lymph node metastasis and decreased survival. Its expression colocalizes with CD68.	[469]
Gastric cancer (GC)	1st set used for microarray analysis: - 90 primary gastric adenocarcinomas, - 14 lymph node metastases - 22 nonneoplastic gastric mucosa 2nd independent set (for real time PCR): - 59 adenocarcinomas	cDNA microarray technology (Tissue) IHC	Higher CCL18 expression levels associated with prolonged survival. CCL18 mainly produced by $CD68^+$ cells, mainly at the tumor invasive front.	[459]
	- 60 patients GC - 20 healthy controls	ELISA (Serum)	CCL18 increased in the serum of patients with GC comparing with healthy controls (116 pg/mL versus 42.5 pg/mL).	[491]
	-134 GC - 87 paired normal (PN) - 25 apparently normal gastric tissue (AN)	IHC (TMA)	Epithelial CCL18 expression lower in GC tissues comparing with AN (9/113, 8% versus 8/24, 33.3%). Conversely, stromal CCL18 expression elevated in GC compared with AN and PN (85.0% versus 50.0% versus 48.8%).	[492]

Colorectal cancer (CRC)	- 371 CRC	IHC	Tumors with higher CCL18 expression presented increased survival. When evaluating each stage separately, CCL18 only associated with increased survival in stage IV tumors. CCL18 colocalizes with CD68.	[475]

CHAPTER 5

General Discussion and Future Perspectives

Decades of research have contributed to increase the knowledge regarding tumor biology, namely on the determinant factors for cancer initiation and progression. Tumors are now known for being multidimensional, arising, not simply as a consequence of mutations one permissive environment, but rather from the combination of genetic and environmental factors, which modulates the crosstalk between mutated cancer cells and their microenvironment, determining the fate of progression [11, 511]. The awareness of such complexity, as always in science, uncovered many questions which, until this day, remain unanswered. In this sense, this PhD thesis aimed at clarifying the role of the abnormal colorectal tumor ECM, the most abundant element at the tumor microenvironment, on macrophage polarization and on macrophage-mediated CRC cell invasion. Furthermore, we proposed to profile the macrophage subpopulations present in human colorectal tumors, in an attempt to infer their prognostic role and to discriminate subgroups of patients which might benefit from new therapeutic strategies targeting immune cells, specifically macrophages.

Regarding the latter aim, described in chapter 2, the decision to quantitatively evaluate macrophages, including different inflammatory subsets, enable an unbiased assessment of the macrophage landscape in CRC. Furthermore, the rare approach of profiling paired normal mucosa adjacent to the tumor allowed the evaluation of changes on macrophage distribution pattern specifically induced by the tumor. Macrophages are known for their plasticity, which results in a myriad of polarization phenotypes, not clearly defined as black and white, but rather through numerous shades of grey [512]. Nevertheless, it was somewhat surprising to observe that the majority of $CD68^+$ cells present in colorectal tumors do not express neither CD80 nor CD163, markers herein used to identify the pro- and anti-inflammatory subpopulations, respectively. Indeed, this is a limitation of the present study since it remains to be identified the other macrophage subpopulations predominantly infiltrating colorectal tumors. Precisely due to the challenging task of identifying macrophage subsets, and knowing that there are distinct M2-macrophage populations which express different cell surface markers, Waniczek and colleagues decided to infer the amount of M2 macrophages by subtracting, to the overall CD68 population, the $NOS2^+$ cells, which they considered to be the pro-inflammatory ones. Nevertheless, having observed the very low expression of NOS2 cells, they choose to consider the total tumor $CD68^+$ cells as being M2 macrophages [421]. We believe that this is not the most accurate approach, not only because of the previously mentioned reservations regarding the use of NOS2 as an M1 macrophage marker, but also due to the existence of other macrophage subpopulations that do not express the classical markers, as clearly described in this thesis. To correctly and thoroughly assess macrophage phenotype in such complex environments, a combination of approaches must be applied. The isolation, from formalin-fixed paraffin-embedded tissues by laser capture microdissection, of $CD68^+CD80^-CD163^-$ cells, followed by gene expression analysis focusing on a panel of macrophage specific markers, is

a strategy of focus in the near future [513, 514]. Alternatively, macrophages may also be isolated from fresh tumor samples and analyzed by flow cytometry, which allows the simultaneous staining of several markers in the same cells [515]. The use of multiplexed fluorescence microscopy method, which enables high-level multiplexing of protein detection and quantitation in a single formalin-fixed paraffin embedded tissue section, may also be an alternative method to simultaneously profile distinct macrophage receptors [516].

Despite the previously mentioned limitation of the present work, there are some important observations and conclusions that deserve to be further discussed. The strategy of quantifying $CD80^+$ and $CD163^+$ cells in consecutive slides enabled an accurate assessment of the proportions between the two macrophage subpopulations amongst the distinct regions. By calculating the CD80/CD163 ratio, it was possible to observe a clear inversion in the distribution of both cell populations between tumors and the adjacent normal mucosas, which might be explained by two factors that can act simultaneously. On one side, it is clear that the tumor microenvironment is able to shape macrophage phenotype, possibly through the action of both tumor and stromal cells, either by direct cell-cell contact or through the release of soluble factors which act in a paracrine way. Work by Elpek and colleagues, in which they analyzed different tumor mouse models, including breast and melanoma, revealed that both TAMs and tumor-associated neutrophils (TANs) displayed significant differences in gene expression profile and function according to the tumor type. Moreover, they concluded that these differences resulted from the specific microenvironment of each tumor type [517]. Additionally, by stimulating macrophages with conditioned medium from breast cancer cell lines with different aggressiveness features, Sousa *et al.* demonstrated that the most aggressive cell line skewed macrophage polarization towards the anti-inflammatory M2c phenotype [518]. This capacity of the tumor microenvironment is further validated by the observation that the differences in macrophages between the left and right colon described in this work are lost when analyzing the tumor invasive front. Concomitant with the tumor microenvironment, the dysbiosis of the gut microbiota, meaning an imbalance in the present bacteria, may also affect the proportion between pro and anti-inflammatory macrophages. In this regard, it was already shown that there are alterations in the bacteria composition within the stools of CRC patients comparing with people with a normal colonoscopy [519]. It would also be interesting to evaluate if there is any association between macrophage profile and tumor mutational load, frequently augmented in MSI tumors with a consequent increase in neoantigen expression. At the moment, the higher neoantigen burden in MSI tumors seems to explain the enhanced number of tumor-infiltrating lymphocytes, in particular of $CD8^+$ T cells, as well as of activated Th1 cells expressing IFN-γ [520]. Probably by exhibiting a more robust immunoediting and efficient immune response, MSI tumors exhibit better prognosis than MSS tumors [521]. In fact, CRC patients with MSI-high and MMR-deficient tumors, harboring higher

mutational load were the most responsive to immune therapies blockade [522] and recently approved by FDA as elected for immunotherapy (FDA News Release, 2017).

Albeit the low number of $CD80^+$ cells in the tumor invasive front, our results clearly suggest a protective role of these cells in the prevention of relapse. Despite being the first description of such association in CRC, it is a promising result which may have an impact in terms of prognosis but also of therapeutic intervention. Interestingly, in hepatocellular carcinoma, an increase in M1 macrophages, evaluated by CD86 staining, another T cell co-stimulatory molecule, associated with increased time until recurrence [523], which corroborates, at least partially, our results. Although we are still not able to explain this association, recent work by Malesci and colleagues revealed that when CRC cells were co-cultured with M1 macrophages there was an increase in cell death. Conversely, in the presence of naïve, unstimulated macrophages, cell death remained unchanged or even decreased, depending on the cell line. Moreover, the cytotoxic effect of M1 macrophages synergized with 5-FU conventional therapy, specifically enhancing the expression of the pro-apoptotic cytokines TNF-α and TRAIL [411]. In a recent study, Li and co-authors developed computational methods to estimate the significance of tumor-infiltrating immune cells in human cancers. They analyzed the abundance of distinct immune cells (B cells, CD4 T cells, CD8 T cells, neutrophils, macrophages, and dendritic cells) over 10,000 RNA-seq samples across 23 cancers, recurring to The Cancer Genome Atlas (TCGA) databases. Interestingly, this study revealed that in melanoma, colorectal and cervical cancers, patients with higher primary tumors $CD8^+$ T cell infiltration exhibited a significantly lower risk of developing a second tumor [524]. Considering this data, it will be important to evaluate, in the future, whether the suggested protective role of $CD80^+$ cells in the prevention of tumor relapse might be associated with the enhanced infiltration of cytotoxic $CD8^+$Tcells or with the expression of cell death markers, namely caspases, PARP or TRAIL.

When analyzing the association between the different macrophage populations and overall survival, no differences were observed when all patients were included. On the other hand, by separately evaluating stage III tumors, higher CD68 infiltration and lower CD80/CD163 ratio associated with decreased overall survival. These results, again, highlight the putative protective role of a higher CD80 expression, which requires further elucidation. Interestingly, in ovarian cancer, a lower M1/M2 macrophage ratio was also associated with decreased survival [525]. Nevertheless, the previously mentioned study by Malesci and collaborators, conducted with stage III CRC, evidenced that high CD68 infiltration associated with increased overall survival, but only in patients treated with 5-FU. In this study, authors even proposed the measurement of TAMs in metastatic lymph nodes, as an independent and powerful tool to predict chemotherapy efficacy [411].

Given the aforementioned differences in the gut microbiome according to each person's life style, specifically diet characteristics, and knowing that there is a close crosstalk between bacteria and the immune system [526], it is plausible to speculate that some of the controversy regarding macrophages and CRC might be due to diet or microbiota differences among populations. Therefore, it would be interesting to perform a study using cohorts from different countries, characterized by diverse nutritional habits and differences regarding intestinal flora, in order to unravel if, in the specific case of macrophages, it is possible to draw general conclusions that suit everyone or if, in fact, there are associations related to each population specificities. If so, there might be important to conceive therapeutic approaches suitable for some specific populations and not necessarily to others. Albeit this controversy, we believe our results further support the need for continuing with the research that focuses on the best way to develop therapeutic strategies to reprogram macrophages towards the pro-inflammatory and tumoricidal phenotype [235].

Having shown that the tumor microenvironment is able to shape macrophage polarization, we sought to unravel which was the role of the tumor ECM on this process. The decellularization of both normal and tumor human colorectal tissues was chosen has a strategy to obtain the native matrices since it enables the most accurate recreation of the endogenous tissue characteristics [527]. Although decellularization was initially intended to be applied in the fields of regenerative medicine and tissue engineering [528, 529], nowadays it is recognized for its potential in basic research. In the specific case of cancer research, there are recent studies in which decellularized matrices were used as a tool for the *in vitro* recreation of the complex tumor microenvironment, in an attempt to unravel new mechanisms involved in disease development and progression. Moreover, given their biomimetic properties, these decellularized matrices are also being applied in studies focusing on therapeutic research, namely drug resistance [530-533]. In our specific work, described in chapter 3, we confirmed that the developed and optimized protocol retained the majority of the analyzed ECM characteristics, namely protein composition, architecture and biomechanical properties, being GAGs the exception. Furthermore, we reported that the tumor-derived ECM, contrarily to the normal one, induced macrophage polarization towards an anti-inflammatory profile [476]. Interestingly and in agreement with this observation, our results described in chapter 2 clearly revealed a higher proportion of anti-inflammatory macrophages relatively to the pro-inflammatory ones in colorectal tumor tissues. Nevertheless, it is worth mentioning that no differences were observed in both CD80 and CD163 expression between macrophages differentiated in normal and tumor decellularized matrices. Altogether these results suggest that, although the ECM is unquestionably involved in macrophage polarization, other components from the tumor microenvironment, not present in the decellularized matrices, are also important for macrophage polarization. These factors might be both tumor and/or stromal

cells, and even soluble factors that might have been lost upon the decellularization process. It remains to be addressed which is/are the factor/s from the tumor ECM responsible for skewing macrophage profile towards an anti-inflammatory phenotype. It is likely that a multitude of physical and chemical elements are involved in such process, and we cannot exclude that each specific macrophage characteristic might be caused by one specific ECM feature. We consider to explore, in the near future, the impact of altering tumor ECM chemical properties maintaining its biomechanical characteristics, and of altering the ECM stiffness without affecting is biochemical composition, on macrophage polarization and macrophage ability to induce cancer cell invasion. In addition, we are conceiving proteomic studies comparing tumor and native decellularized matrices to identify the major ECM components or adsorbed soluble factors that may affect macrophage polarization.

Specifically regarding CCL18 increased expression by tumor-ECM educated macrophages, we believe that HA is a plausible factor involved in this process which will be further explored in the future. In fact, this ECM protein is known for being involved in cancer initiation and progression [534, 535] and, in the specific case of CRC, increased levels of HA are associated with decreased survival [536]. To tackle this problem and unravel whether HA is mediating the CCL18 production by tumor ECM-educated macrophages, tumor decellularized matrices could be treated with hyaluronidase in order to remove HA, similarly to what was done by Meng and colleagues [399]. Afterwards, hyaluronidase-treated tumor decellularized matrices or non-treated ones, could be repopulated with monocytes and their polarization profile compared.

Our results revealed an interesting association between higher CCL18 in the invasive front of more advanced colorectal tumors, specifically in stages III and IV. Moreover, by double immunohistochemistry, we showed that macrophages, specifically the $CD163^+$ cells, were expressing CCL18 in this specific region. Nevertheless, they were not the only stromal cells positive for CCL18. This observation is further supported by the results obtained in chapter 2, in which we showed that both $CD68^+$ and $CD163^+$ cells were predominant at the invasive front, in particular of stage II tumors. For this reason, it would be important to further explore which other stromal cells are also expressing CCL18 at the tumor invasive front, also due to the described pro-invasive role of this cytokine. So far, CCL18 is reported as being produced by dendritic cells [446] but is likely that other stromal cells are also secreting this cytokine in the tumor microenvironment. In the near future, we will explore the possibility of other macrophage subpopulations (as $CD206^+$ cells), dendritic cells and fibroblasts are also producing CCL18 in CRC, both by immunohistochemistry and by laser capture microdissection followed by gene expression analysis, as previously explained.

Unfortunately we were not able to identify the receptor directly activated by CCL18. Three different receptors have been described for this chemokine, specifically CCR8, GPR30 and PITPNM3 [452, 466, 473]. Knowing that our CRC cells did not express CCR8 receptor, multiple

attempts were performed, without success, to identify which of the other two receptors were directly activated by CCL18 and involved in CCL18-mediated cell invasion. Based on the literature describing the PITPNM3 activation by CCL18 in different cancers, PITPNM3 is the most likely possibility. Nevertheless, we cannot exclude the involvement of other receptors, in particular GPR30, given the ability of this receptor to activate EGFR and the fact that we discovered that CCL18 was also activating EGFR and EGFR-related signaling partners in both colorectal and gastric cancer cells [473, 537]. Interestingly, as described in chapter 3, the CCL18 pro-invasive ability was not observed in all CRC cell lines: RKO and HCT15 responded to CCL18, while SW480 and SW620 did not. Therefore, it would be important to identify the different molecular and genetic characteristics that might exist between CCL18-responsive and non-responsive cells, in an attempt to unravel possible tumor features that might help to discriminate patients' sensitivity to an increase of CCL18 at the tumor microenvironment. In this sense, Ahmed and colleagues work revealed that RKO and HCT15 cells are MSI and present phosphoinositide 3-kinase (PI3K) mutations while SW480 and SW620 cells are MSS and harbor a wild type PI3K [538]. Understandably, these are mere observations but they strengthen the need for further investigation in order to clarify the relevance of these differences regarding CCL18 effect.

Having in mind the results described in chapter 3, specifically the pro-invasive ability of CCL18 and its increased expression at the invasive front of more advanced CRC tumors, combined with chapter 4 preliminary data, namely the ability of CCL18 to activate proteins known to be involved in cancer invasion, we believe that it is essential to conduct a study focusing on the measurement of CCL18 levels in CRC patients serum. Many reports have described an increase in CCL18 serum levels in different types of cancer [457, 466, 497] but, to the best of our knowledge, this chemokine has still not been evaluated in the serum of CRC patients. By analyzing patients in different stages of the disease, eventually also including patients with pre-malignant lesions, it would allow the clarification of whether CCL18 could be used as a prognostic biomarker in CRC. Albeit the obtained promising results and the potential of CCL18, the reality is that it has one important limitation, which is the lack of specificity. Given its increase in many malignancies, it could never be applied in the diagnosis of a specific tumor type, but at least as an indicator of prognosis of CRC, as it is already suggested for breast and ovarian cancer [465, 499]. On the other hand, CCL18 might have the potential to be used as a sensor which may help monitoring the therapy response. In this sense, it would be crucial to conduct a study based on the collection of serum samples from patients in different phases of the disease and treatment, starting at the diagnosis, surgery and following all therapeutic steps. Despite being a challenging project that requires the commitment of numerous people, we believe that it has the potential to improve patients follow up. Accordingly, we are currently submitting a protocol to IPO-Porto Ethical Committee to initiate such prospective study which

may elucidate the role of CCL18 both as a prognostic marker in CRC and as a predictor of therapeutic response on patients ongoing radiotherapy, conventional chemotherapy, targeted therapy or immunotherapy.

This PhD work contributed to clarify important topics regarding macrophages and the extracellular matrix at the colorectal tumor microenvironment. Simultaneously, and recycling a thought stated in the beginning of the present chapter, it culminated in a series of pertinent unanswered questions which require and open the horizons of further research. As major conclusion, one can state that the tumor ECM revealed to be a crucial element in macrophage differentiation and should always be taken into consideration when studying the tumor microenvironment. Furthermore, in this era of personalized therapy, rather than the amount of macrophages *per se*, their polarization profile is probably the most relevant parameter that must be thoroughly scrutinized to further improve patient's treatment and cancer management.

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