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Decellularizing human colorectal cancer matrices as a 3D-organotypic model to evaluate macrophage differentiation

Marta Laranjeiro Pinto

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica da Professora Doutora Maria José Oliveira (Universidade do Porto).

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Por motivos de confidencialidade, foi omitida desta tese as informações relativamente aos protocolos de descelularização e repopulação usados e resultados específcos no que diz respeito à parte de repopulação.

Acknowledgments

The first person I have to thank is Maria for having accepted to embark with me in this crazy adventure. Without your support none of this would have been possible and I will always be extremely thankful. Apart from this, I have to thank you for all you have thought me during these almost six years and for being an example as a person and as a scientist. Even after all this time I continue to say that, with all your many flaws (and even though sometimes can be a bit nerve raking to work with you!!), that you are the best person I know.

I also have to make a special thanks to Professora Emília. We knew each other recently but you were always available to help me every time I needed. Even though I haven't been with you frequently, your enthusiasm is obvious and your dedication remarkable. I have no doubt that every student of this Masters fells the same and will always be very thankful to you.

Girls in my team, Ana and Patrícia (and also Marta that is an "adopted" member), you were always there for me (and hope continue to be) helping me in the good and in the bad. Many would say that my decision to continue this Masters was completely insane but you gave me your full support, which I will always treasure. Ana, a special thanks for being my "Word expert".

None of this would be possible without the help of Elisabete. Until this day I think you haven't understood how crucial you are for my work, but I truly mean when I call you my "guardian angel". Even though you always have thousand things to do, you have always helped me with a kind voice and a sincere smile. Thank you very much! Professora Fátima, I am also very grateful for all your help. You provided me full access to the Tumour Bank and also the possibility to collect all the material, without which I couldn't have developed this work. You are truly an example and I feel a tremendous respect and admiration for you and your work.

Professor Mário, thank you for believing in this project and for all your precious input.

Ana Silva, I will always feel in debt to you! I have no doubt I was able to do so much during this year thanks to all your previous hard work. Your passion and perseverance are contagious and I hope that we can continue with this "teamwork". To Perpétua, thank you for making this close collaboration possible and for your always pertinent questions and suggestions.

My former colleagues (and friends) "pyloris", particularly Ângela, Ana and Rui, you three welcomed me the best way possible and basically thought me most of the things I know about working in the lab. Believe me when I say that I will never forget you!

To all the people at IPATIMUP, particularly the Cancer Genetics group, for their guidance, support, friendship and for creating such a good environment to work in! Without wanting to particularize, because I'm sure I will forget someone, I have to give a special word to Cecília for your patience explaining me the challenging world of statistics!

To all the people at INEB, particularly António, for welcoming me and for all your help.

A special word to my friends and family, for always being available and for accepting my absences.

Mãe e Pai, o que sou devo-o a vocês. Mãe, amo-te pela mulher que foste, Pai, amo-te pelo homem que és. Sempre foram um exemplo de bondade, generosidade e trabalho, valores estes que desejo continuar a aplicar diariamente na minha vida. Espero poder continuar a deixar-vos orgulhosos! Maninho, adoro-te! Tenho um orgulho enorme em ter-te como irmão e na relação que temos. Apesar de estarmos juntos bastante menos vezes, estás sempre no meu coração. Obrigada por tudo!

Last but not least, I have to thank you Nuno. Many times you have been a bit neglected due to my habit of wanting to do everything, but this didn't stop you from always supporting my crazy choices. I have to thank you for your love, support, understanding and patience. There's still a long way to go but I hope that we can continue this (bumpy) ride! I love you very much.

Contents

Abbreviations list

2D - two dimensional
3D - three dimensional
ACF - aberrant crypt foci
Akt - Protein kinase B
APC - adenomatous polyposis coli
ATCC - American Type Culture Collection
AVAs - aortic valve allografts
BE - Barrett's esophagus
CA - chitosan alginate
CIN - chromosomal instability
CHAPS - 3-[(cholamidopropyl) dimethyl ammo-nio]-1-propanesulfonate
CRC - colorectal cancer
c-SRC - the cellular homologue of the Rous sarcoma virus
CSF-1 - colony stimulating factor-1
DAPI - 4',6-diamidino-2-phenylindole
ECM - extracellular matrix
EDTA - ethylenediaminetetraacetic acid
EGTA - ethylene-glycol-tetraacetic acid
EGF - epidermal growth factor
ERK - extracellular signal-regulated kinases
ESC - embryonic stem cells
EGFR - epidermal growth factor receptor
EHS - Engelbreth-Holm-Swarm
FAP - familial adenomatous polyposis
FBR - foreign body reaction
FGF - fibroblast growth factor
Gab1 - GRB2-associated binding protein-1
GAGs - glycosaminoglycans
HE - Hematoxylin-Eosin
HER - human epidermal growth factor receptor, see EGFR
HGF - hepatocyte growth factor
HGFR - HGF receptor or c-Met
HIF - hypoxic inducible factor
hMECs - human mesendodermal cells
HNPCC - hereditary nonpolyposis colorectal cancer
IFN-γ - interferon-γ
IGF - insulin growth factor
IHC - immunohistochemistry
iNOS - inducible nitric oxide synthase (or NOS2)
KU - Kunitz Units
LOX - lysyl oxidase
LPS - lipopolysaccharide
MAPK - mitogen-activated protein kinase
MCTS - multicellular tumour spheroids
MLH - MutL-homolog
MMP - matrix metalloproteinase
MMR - mismatch repair
MSCs - bone marrow-derived mesenchymal stem cells
MSH - MutL-homolog
MSI - microsatellite instability
MT – Masson's Trichrome
NOS2 - nitric oxide synthase 2 (or iNOS)
NTIRE - non-thermal irreversible electroporation
OS - overall survival
PA - polyacrylamide
PEG - polyethylene glycol
PGA - polyglycolide
PI3K - phosphoinositide 3-kinase
PKB – see Akt
PLA - polylactide
PLC-γ - phospholipase C-gamma
PLG/PLGA - Poly (lactide-co-glycolide)
PVA - poly (vinyl alcohol)
SDS - sodium dodecyl sulfate
SEM - scanning electron microscopy
SMAD - mothers against decapentaplegic homolog 1
TAMs - tumour associated macrophages
TGF -transforming growth factor
Triton - t-octyl-phenoxypolyethoxyethanol
VEGF - vascular endothelial growth factor

Abstract

Tumours are highly complex microecosystems composed of cancer cells, extracellular matrix (ECM) components and other cell types. The molecular crosstalk established between cancer cells and the surrounding environment is crucial for tumour progression. Macrophages have been described as key elements in this process, preventing the establishment and spreading of cancer cells – M1 macrophages – or supporting tumour growth and progression – M2 macrophages. Knowing that macrophages are highly plastic cells, it is possible that tumours explore this characteristic in their benefit. It is therefore important to unravel how the colorectal tumour microenvironment, namely ECM components, affects macrophage differentiation. In this work, we are particularly interested in creating a 3D-organotypic model that will allow us to elucidate how the ECM contributes to macrophage differentiation and polarization.

To achieve this goal, we started by optimizing a decellularization protocol, for both normal and tumour colorectal fragments. DNA quantification and DAPI staining confirmed the efficiency of the decellularization method. Staining with Hematoxylin Eosin and Masson's Trichrome revealed that decellularized fragments retain the histological features of the tissues. SEM analysis of normal decellularized matrices allowed the visualization of the ECM fiber meshwork, without any visible cells. In tumour matrices, the matrix was denser and seemed to be more disorganized. Nevertheless, in certain tumour decellularized fragments, it was visible the presence of some muscle fibers and a residual E-cadherin staining. Decellularization reduced significantly the glycosaminoglycans (GAGs) content in normal and tumour matrices but other ECM components, such as collagens type I and IV, laminin and fibronectin were retained. These matrices were then repopulated with freshly isolated monocytes and allowed to differentiate for different timepoints.

With this strategy, we were able to develop an innovative model that allows studying of the complex interplay established at the tumour microecosystem. In the future, we expect to help elucidating the role of tumour ECM components on macrophage differentiation and polarization, contributing to the design of novel therapeutic strategies targeting macrophages.

Key words: Colorectal cancer; extracellular matrix; macrophages; decellularization.

Sumário

Os tumores são microecossistemas complexos compostos por células tumorais, componentes da matriz extracelular (MEC) e outros tipos celulares. A interacção entre as células tumorais e os ambientes que as rodeia é crucial na progressão tumoral. Os macrófagos foram já descritos como sendo elementos essenciais no processo tumorigénico, podendo prevenir a invasão e disseminação das células tumorais – macrófagos do tipo M1 – ou promovendo o crescimento e progressão tumorais – macrófagos do tipo M2. Considerando os macrófagos como células com elevada plasticidade funcional, não é de excluir a possibilidade de as células tumorais explorarem esta característica em seu benefício. Torna-se então fundamental perceber de que forma o microambiente tumoral colorectal, particularmente componentes da MEC, influenciam a diferenciação macrofágica. Com este trabalho, pretendemos criar um modelo organotípico tridimensional que nos permita elucidar o mecanismo através do qual a MEC contribui para a diferenciação e polarização dos macrófagos.

Para isto, começámos por optimizar o protocolo de descelularização, usando fragmentos de tecido normal e tumor colorectais. A quantificação de ADN e coloração com DAPI confirmaram a eficiência do método de descelularização usado. Coloração com hematoxilina/eosina e Masson Tricrómio demonstraram que os fragmentos descelularizados retêm as características histológicas dos tecidos nativos. Análise por microscopia electrónica (SEM) em matrizes descelularizadas normais, permitiu a visualização das fibras da MEC formando uma rede, sem que fossem visíveis células. Em relação às matrizes tumorais descelularizadas, a rede aparenta ser mais densa e, eventualmente, mais desorganizada. No entanto, em alguns casos excepcionais de matrizes tumorais descelularizadas, foi possível observar algumas fibras musculares e uma quantidade residual de caderina-E. O protocolo de descelularização resultou numa redução muito significativa da quantidade de glicosaminoglicanos, em ambos as matrizes, apesar de outros componentes da MEC, nomeadamente colagénios tipo I e IV, laminina e fibronectina, estarem preservadas. Estas matrizes foram depois repopuladas com monócitos, isolados a partir de sangue humano.

Recorrendo a esta estratégia, conseguimos desenvolver um modelo inovador para estudar as interacções celulares e moleculares estabelecidas entre os elementos do microecossistema tumoral. No futuro, temos a expectativa de ajudar a esclarecer o papel dos componentes da MEC tumoral na diferenciação e polarização macrofágica, contribuindo para desenhar novas estratégias terapêuticas que tenham como alvo os macrófagos.

Palavras-chave: Cancro colorectal, matriz extracelular, macrófagos, descelularização.

Introduction

1. Colorectal Cancer

1.1. Epidemiology

Colorectal cancer (CRC) is, after lung and prostate cancer, the third most common cancer in men (663 000 new cases/year corresponding to 10% of the total cancer incidence) and constitutes, after breast cancer, the second most common cancer in women (571 000 new cases/year, corresponding to 9.4% of the total cancer incidence). Although the overall incidence starts do diminish, as a result of the introduction in developed countries of diagnosis methods, it is still worldwide the fourth cause of cancer-related deaths (608 000 deaths/year, accounting for 8% of all cancer deaths) (GLOBOCAN, IARC-International Association for Cancer Research). However, many previously developing countries that are undergoing rapid development are witnessing a dramatic increase in colon cancer incidence, which confirms the correlation between CRC development and environmental factors. Some of the risk factors include obesity and central adiposity, physical inactivity, alcohol consumption or cigarette smoking. White meat intake and fiber consumption are, on the other hand, accepted as having a protective effect[2].

1.2. Pathogenesis

In the past decades significant progress was made in order to understand the pathogenesis of CRC. Today it is accepted that only 20% of CRC have familial origin, most of them being associated with hereditary nonpolyposis colorectal cancer (HNPCC) and familial adenomatous polyposis (FAP). The other 80% are of the sporadic type being generally divided in two major groups: about 85% present chromosomal instability (CIN), with great losses or gains of chromosomal material, while the remaining 15% display accumulation of numerous mutations throughout the genome, mainly caused by inactivation of mismatch repair (MMR) genes, resulting in a phenotype known as microsatellite instability (MSI)[3]. Sporadic CRC development follows a step-wise progression of mutations in oncogenes and tumour supressors that translate into the classical adenoma-carcinoma sequence (Figure 1). 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 TGF (Transforming growth factor)-βIIR/SMAD2/4 (mothers against DPP homolog 1)-2-4 pathway and mutations in mismatch repair genes [e.g. hMLH(MutL-homolog) 1and hMSH (MutL-homolog) *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[4]. Once tumour cells accumulate these mutations they will start invading the underlying tissue and, contrary to what happens in most tumours, will most likely form distant metastasis rapidly without the common latency period. The explanation for this is that after the 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[5].

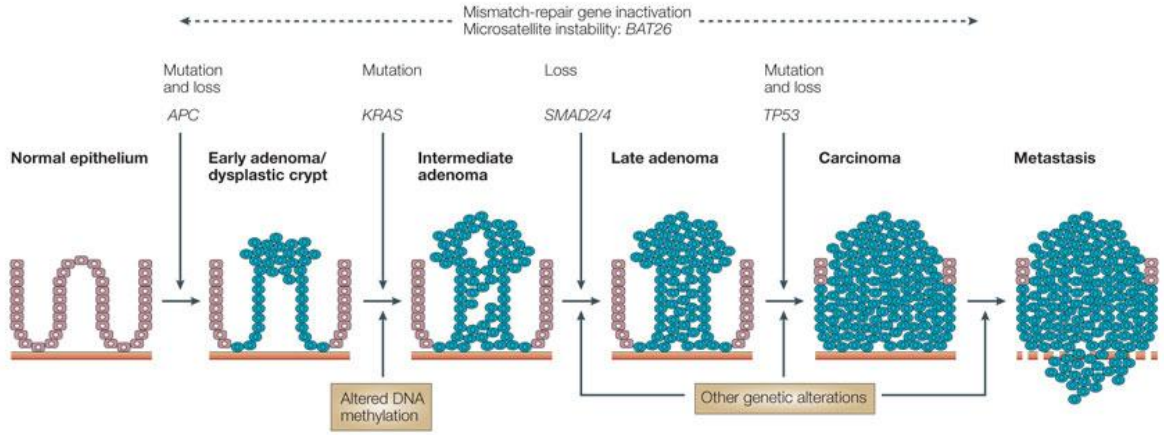

Figure 1 The colorectal adenoma-carcinoma sequence. Progression from normal epithelium through adenoma to colorectal carcinoma is characterized by accumulated abnormalities of particular genes. Mutations in mismatch-repair genes cause microsatellite instability and the successive mutation of target cancer genes, which can occur at any point in the adenoma–carcinoma sequence. Adapted from Davies *et al* (2005) *Nat Rev Cancer*[6].

2. Tumour microecosystem

Tumours are highly complex structures that besides tumour cells contain other cell types such as fibroblasts, endothelial and immune cells, and also an important non-cellular component which is the extracellular matrix (ECM). The molecular crosstalks established between cancer cells and the surrounding environment are known to have crucial impact in tumour progression.[7]

2.1. Cellular components

Fibroblasts are the major ECM producers being particularly relevant during wound healing and the embryonic development. 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 myofibroblats[8]. Several mechanisms are described to promote this differentiation namely the release of cancer cell exosomes expressing high levels of Transforming growth factor β (TGF-β) which, in turn, induce the production of fibroblast growth factor (FGF)-2 by fibroblasts[9]. Many of the interactions established at the tumour microecosystem occur through paracrine signaling as it was shown that breast cancer cells stimulate hepatocyte growth factor (HGF) production by fibroblasts which, in turn, enhances breast cancer cell HGF receptor (HGFR) activation and promotes colony formation in soft agar and facilitates tumour growth in mice[10]. Interestingly epithelial cancer cells are also able to suppress p53 expression by adjacent fibroblasts which can help overcoming its tumor suppressor function[11]. Fibroblasts were also demonstrated to increase tumor incidence, size and metastasis when injected in an orthotopic nude mouse model of pancreatic cancer showing direct involvement in cancer development[12]. Additionally, they communicate with other stromal cells, mediating the inflammatory response by secreting interleukins such as IL-1 and activating endothelial cells through the release of matrix metalloproteinases (MMPs) or of Vascular endothelial growth factor (VEGF)[13].

The reciprocal interactions established between tumour and endothelial cells have also been shown as extremely important for tumour progression. Tumour cells are able to stimulate endothelial cells and angiogenesis by secreting factors like VEGF or galectin-1[14]. This activation can also occur through direct contact, by a mechanism that involves cancer cells Jagged-1 overexpression by cancer cells, will trigger endothelial cells Notch activation, resulting in enhanced neovascularization and tumour growth.[15]

Immune cells are other major component of the tumour microenvironment. In fact it has been accepted, already for some years, that chronic inflammation predisposes individuals to various types of cancers[16] and it is generally hypothesized that inflammatory cells and cytokines, found in tumours, are more likely to contribute to tumour progression and immunosuppression[17]. This interplay is, however, extremely complex and involves both innate and adaptive immunity: antigens present in early neoplastic tissues will be transported by dendritic cells to lymphoid organs leading to activation of adaptive immune responses, and resulting in both anti-tumour and tumour-promoting effects (Figure 2)[18]. An important concept regarding immune system and tumour development is “cancer immunoediting”, which intends to describe the host-protecting and tumour-

sculpting actions of the immune system that not only prevents disease, by suppressing the formation of nascent tumours, but also shapes tumorigenesis[19]. This hypothesis is supported by studies such as the one by Scarlett and colleagues in ovarian cancer, demonstrating that phenotypic changes in dendritic cells cause a shift from immune surveillance to an accelerated malignant growth[20]. In fact, and as a result of these and other findings, inflammation was recently defined as the seventh hallmark of cancer[21]. Interestingly, it has been demonstrated that tumour infiltrating $CD4^+$ T cells promote metastasis by activating RANK-RANKL signaling in tumour cells. However, in two important studies characterizing the immune populations present in large cohorts of CRC patients, it was observed an inverse correlation between the clinical outcome and the presence of immune cells. Tumours without signs of early metastasis presented a strong immune infiltrate with a significant increase in Th1 mediators. It was also observed that a good clinical outcome was correlated with the high density of infiltrating $CD45RO^+$ cells[22]. In addition, type, density and location of immune cells in CRC showed a prognostic value that allowed prediction of clinical outcome[23].

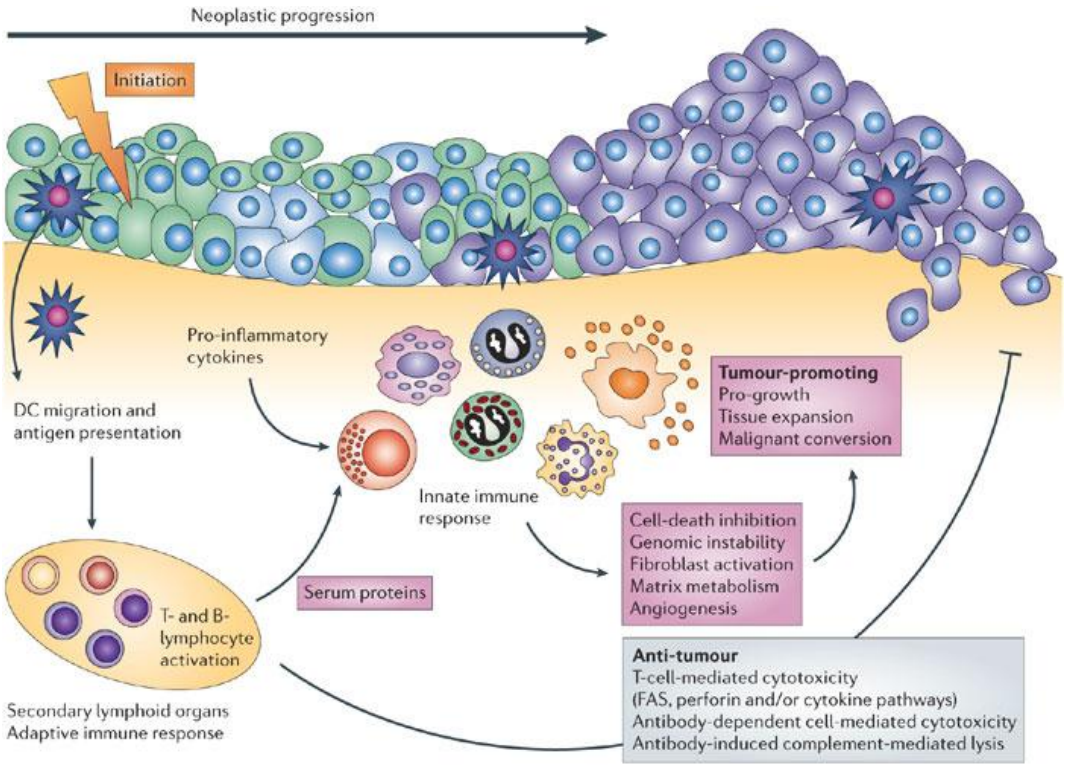

Figure 2 Antigens that are present in early neoplastic tissues are transported to lymphoid organs by dendritic cells (DCs) that activate adaptive immune responses resulting in both tumour-promoting and antitumour effects. Activation of B cells and humoral immune responses results in chronic activation of innate immune cells in neoplastic tissues. Adapted from Visser *et al.* (2006) Nat Rev Cancer[17].

2.1.1 Macrophages and cancer

Macrophages are other immune cell population that has been widely studied, particularly in breast cancer. They originate from blood circulating monocytes that are recruited in response to chemotactic factors released at the tumour microenvironment[24]. Here, they may differentiate into M1 or M2 macrophages, designation that reflects the Th1/Th2 dichotomy. In fact, macrophages are highly plastic cells and this designation refers to the two extremes of a spectrum of possible macrophage activation phenotypes. The classically activation of macrophages occurs in response to interferon-γ (IFN-γ) alone or with lipopolysaccharide (LPS) originating the M1 phenotype, characterized by being pro-inflammatory and by exerting anti-tumour activities. M2 macrophages or alternatively activated macrophages are induced by IL-10, IL-4, IL-13 or Vitamin D3 and are known for being anti-inflammatory and to contribute for tumour progression[25]. Tumour-associated macrophages (TAMs) have been shown, in various models, to be quite similar to M2 macrophages promoting tumour cell survival, proliferation, invasion and metastasis[26] and, for these reasons, are an attractive target for anticancer therapies[27]. In breast cancer it has been clearly demonstrated that macrophages and tumour cells establish a paracrine loop: while cancer cells produce Colony stimulating factor-1 (CSF-1), promoting macrophage differentiation, macrophages produce Epidermal growth factor (EGF), stimulating tumour cell migration[28] and invasion, both in vitro[29] and in vivo[30]. Additionally it was observed that macrophages were able to induce an angiogenic phenotype, the so called "angiogenic switch"[31] and that tumour cell intravasation occurred in association with perivascular macrophages[32]. Therefore, macrophages were defined as obligate partners for tumor cell migration, invasion and metastasis[33]. In an attempt to provide novel insights into the molecular crosstalk established between cancer cells and macrophages, recent work from our team focused on the influence of primary human macrophages on gastric and colorectal cancer cells, considering invasion, motility/migration, proteolysis and activated intracellular signaling pathways[34]. We demonstrated that macrophages stimulate cancer cell invasion, motility and migration, and that these effects depend on MMP activity and on the activation of epidermal growth factor receptor (EGFR) (at the residue Y^{1086}), PLC-γ (phospholipase C-gamma) and Gab1 (GRB2-associated binding protein-1). 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 colorectal cancer cell phosphorylation of Akt, c-Src and ERK1/2, and led to an increase of RhoA and Cdc42 activity. Interestingly, whereas macrophage-mediated cancer cell c-Src and ERK1/2 phosphorylation occurred downstream EGFR

activation, Akt phosphorylation seems to be a parallel event, taking place in an EGFR independent manner (figure 3). Despite all the evidences pointing to the pro-tumoral activity of TAMs, data

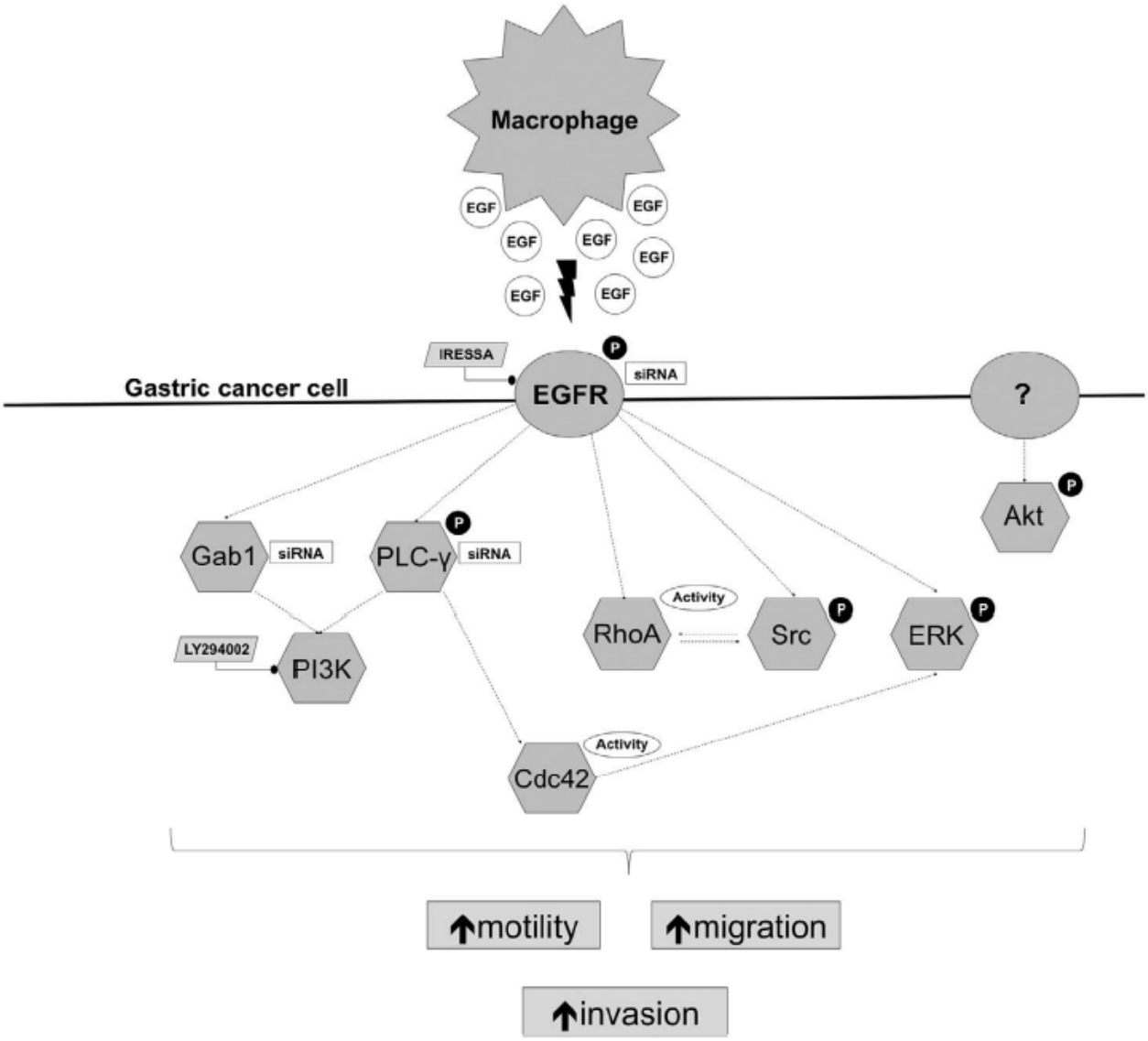

Figure 3 Model proposal of the macrophage-mediated invasion pathway. Soluble factors produced, in a paracrine manner, by macrophages and cancer cells lead to membrane-recruitment and EGFR. EFGR-interacting partners such as Gab1 and PLC-→ were demonstrated, by siRNA experiments, to be required for macrophage-mediated cancer cell invasion. Other molecules such as c-Src, ERK, Akt, and the small GTPAses Cdc42 and RhoA were demonstrated to be phosphorylated/activated. Silencing of EGFR demonstrated that macrophage-mediated cancer cell c-Src and ERK but not Akt occur downstream of EGFR signaling. Dashed arrows indicate putative interactions described in the literature. Closed circles indicate inactivation by pharmacological inhibitors. Unshaded rectangles indicate transient knocked-out expression by siRNAs. Black circles indicate increased protein phosphorylation. Elipses indicate increase in the GTP-bound form of the protein. Adapted by Cardoso *et al* (2013) Oncogene[34].

regarding macrophages has been contradictory. In lung cancer macrophage density was shown to negatively correlate with patient relapse-free survival[35] but, in other study, it was observed that macrophages were predominantly of the M1 phenotype and positively associated with survival time[36]. In CRC, the results are also ambiguous: there are reports describing the positive correlation between macrophages counts and tumour progression[37] but also others stating that the number of macrophages decreases in higher stage tumours[38]. An interesting study done in a CRC rat model showed that, even though the presence of macrophages directed tumours towards malignant histopathological differentiation, with neovascularization and matrix remodeling, they also exerted an anti-tumour effect because their depletion allowed the development of tumours with worse prognosis and poorer survival[39]. To clarify these discrepancies, a meta-analysis focusing on the prognostic significance of TAMs in solid tumour was published very recently[40]. In this study the inclusion criteria chosen were the use of CD68 as a lineage marker for TAMs, HLA-DR for M1-type TAMs and CD163 for M2-type TAMs. This study allowed concluding that in patients with various cancers namely gastric, breast, bladder, ovarian, oral and thyroid, there was a negative effect of TAMs on overall survival (OS). Conversely, in CRC patients, there was a positive correlation between high density of TAMs and longer OS. Nevertheless, this study has to be evaluated with caution due to the very restrictive guidelines followed, resulting in the exclusion of a significant number of reports. Additionally, the specificity of these markers, particularly the use of HLA-DR to identify M1-macropages, is questionable. One example of a study, excluded from this meta-analysis, was the work published by Edin and colleagues in which the distribution of macrophages with a M1 or M2 phenotype was related to prognosis and CRC molecular characteristics[41]. They accessed, by immunohistochemistry (IHC), nitric oxide synthase 2 (NOS2) (also denoted iNOS) as a marker for the M1 macrophage phenotype and CD163 as a marker for the M2 macrophage phenotype, in a total of 485 CRC specimens. The results obtained clearly revealed a positive correlation between macrophages expressing NOS2 and CD163. Moreover, improved prognosis was associated with increased infiltration of $NOS2^+$ or $CD163^+$ macrophages at the tumour front, although no differences on cancer specific-survival with different $NOS2^+/CD163^+$ ratios were described. Altogether, the results seem to suggest that the increase infiltration of macrophages with a M1 phenotype at the tumor front is accompanied by a concomitant increase in macrophages with a M2 phenotype and, in a disease stage dependent manner, correlated to a better prognosis in patients with CRC. Moreover, it is highlighted the importance of finding more specific M1 and M2 markers, particularly due to the plasticity of these cells[42].

2.2. Extracellular matrix

2.2.1 Composition and functions

Besides the various cell types present at the tumour microenvironment there is a very important non cellular component, the ECM. This is an intricate network of macromolecules 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[43]. The ECM is mainly composed by: fibrous proteins responsible for providing tensile strength and elasticity (e.g. collagens and elastins), adhesive glycoproteins (e.g. fibronectin laminin and tenascin) and proteoglycans (PGs) that adhere to the other components and to the cells within the matrix or adjacent to it[44]. Its important role in providing tissue structural support is well known but the ECM has multiple functions. Besides being fundamental in sustaining cell-matrix adhesion and signaling through interaction with the cell surface receptors integrins, the ECM has also proven to be a key player in the differentiation, proliferation, survival, polarity and migration of cells[45]. The composition, architecture and degree of crosslinking dictate the mechanical properties of the ECM and control how mechanical forces are transmitted to cells (Figure 4)[47]. Collagen fibers resist tensional forces, proteoglycans (PGs) control hydration, determining the resistance to compressive forces, while the basement membrane, a specialized ECM composed by collagen type IV, laminin, elastin and PGs, increases the mechanical stiffness. One of the most important functions of the ECM is related to its ability to arrest several growth factors including insulin growth factor (IGF), fibroblast growth factor (FGF) and hepatocyte growth factor (HGF), chemokines and other morphogenic proteins, working as a signal reservoir[46].

2.2.2 Modulation of cellular activities

The ECM network has the ability to regulate many cellular activities, such as migration, proliferation, growth and apoptosis, differentiation and invasion. It is the concerted way how these regulations occur that guarantee tissue architecture, functions and homeostasis. The role of ECM on the regulation of some of these cellular activities will be herein dissected.

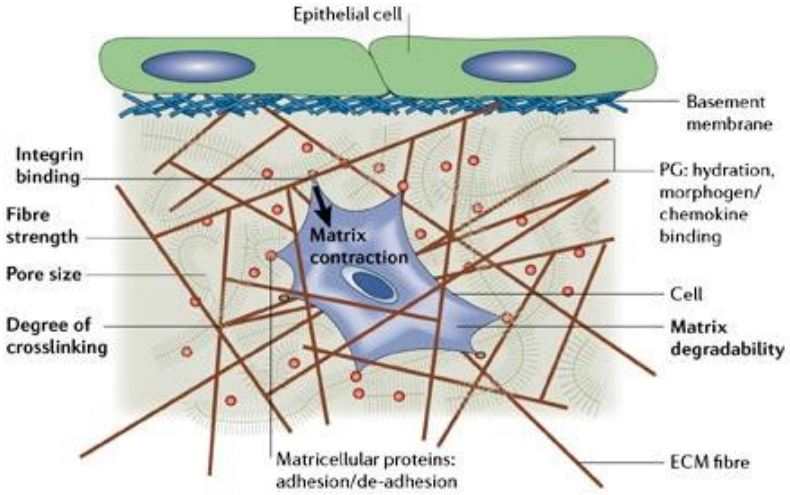

Figure 4 Importance of the 3D environment for engineering cell function: the composition, architecture and degree of crosslinking dictate the mechanical properties of the extracellular matrix (ECM) and control how mechanical forces are transmitted to cells. Adapted from Griffith and Swartz (2006) Nar Rev Mol Cell Biol[47].

2.2.2.1 Cell proliferation, growth and apoptosis

As it was mentioned previously, ECM works as a signal reservoir that sequestres and arrests many growth and survival factors involved in cell division, growth and apoptosis. Regarding proliferation, there are many reports describing multiple signaling pathways through which integrin-ECM binding promote cell proliferation[48]. More recently, the effect of matrix elasticity on cell cycle progression was studied, suggesting that ECM stiffness act as a cell-cycle inhibitor. Additionally, strong inhibition of FAK-dependent Rac activation, Rac-dependent cyclin D1 gene induction and cyclin D1-dependent Rb phosphorylation were observed[49]. Epithelial cells require interactions with the ECM to survive and avoid apoptosis. Using mammary epithelial cells, it was demonstrated the need of a basement membrane, and not just collagen, to prevent cell apoptosis. This result suggests that other non-collagenous proteins are also crucial for the induction of cell survival and emphasizes the ability of the basement membrane to sequest and provide survival stimulus[50]. Interestingly, it was also demonstrated that adhesion of small lung cancer cells to ECM confered resistance to chemotherapeutic agents as a result of β1-integrin-stimulated tyrosine kinase activation, preventing chemotherapy-induced apoptosis[51]. More recently, a different effect of ECM on apoptosis control was described: culturing pre-osteoblastic cells in collagen lead, through an epigenetic DNA-methylation process, to reduced expression of the apoptotic mediator Fas[52]. Conversely, negative regulators of cell cycle and survival are also arrested between the ECM, being TGF-β the most

studied. In thyroid cancer cell lines, TGF-β inhibited growth, migration and invasion and enhanced adhesion to some ECM components, such as fibronection or collagen type IV[53].

2.2.2.2 Differentiation

ECM is also crucial in modulating and determining cell fate. In fact, the importance of ECM on cell differentiation was described more than twenty years ago. Several reports evidence that distinct ECM components are not only able to activate and to enhance epithelial cell differentiation but also promote the transition from the epithelial to the mesenchymal phenotype[54]. In fact, many data suggests that the spacio-temporal regulation and deposition of ECM may provide permissive and even instructive differentiation signals. Concomitantly, fibronectin and laminin matrices were demonstrated to enhance human neural stem precursos (NSPCs) cells differentiation into neurons and astrocytes[55]. A different study cultured bone marrow-derived mesenchymal stem cells (MSCs) in different types of ECM specifically laminin, collagen type IV and fibronectin demonstrated that the cells cultured in laminin were the only expressing smooth muscle cell lineage markers[56]. Interestingly, besides composition, physical properties were also shown to affect cell differentiation. In a study using neuroblastoma cells cultured in polyacylamide (PA) hydrogels with different rigidities it was observed that substrate rigidity dramatically affected cell morphology, proliferation and expression of some genes such as N-Myc[57].

2.2.2.3 Migration and invasion

Cell migration plays an essential role in a variety of biological phenomena, including normal and pathological events. In adulthood cell migration is involved in processes such as immune response or repair of injured tissue but is also a determinant factor in chronic inflammatory disease development or tumour invasion and metastasis. Concerning the latter, besides tumour cell migration, endothelial cell migration and branching, required for the angiogenic process, is also extremely important[58]. Dynamic ECM-integrin interactions are known to facilitate cycles of cell adhesion and de-adhesion to the substrate. When these cycles are combined with a contractile cytoskeleton, to generate traction forces on an ECM substrate, cell locomotion occurs[59]. However, ECM is not simply a track where cells bind in order to move to a different place but is also a reservoir of multiple chemotactic factors. In fact, cell migration is a very complex and dynamic process which requires regulation and integration of multiple signaling pathways. It starts with polarization of the cell and the formation of a front protrusion, projected in the direction of

movement, followed by the binding of cell basal adhesion receptors to the surrounding ECM components, forming links to the cytoskeleton. These sites of cell–ECM adhesion serve as traction points for migration but also stabilize the protrusion via structural connections to actin filaments, mediating signaling by the Rho family of small GTPases, ERK/MAP kinases and other regulatory molecules. Finally, the cells contract from the edges towards the nucleus and the adhesion receptors are released from the ECM, detaching the cell from previous attachment sites[58] (Figure 5). ECM components are also able to interact with cells through sequestering growth factors or cytokines/chemokines, promoting intracellular signaling, mechanotransduction and cross-regulation of other cell-surface receptors, all of which can help regulating cell migration[59]. More than ten years ago it was proven that both melanoma and glioma cell adhesion and migration increased in the presence of various components of the ECM, namely laminin or fibronectin[60]. Around the same time it was also demonstrated that lung cancer cell migration was dependent on beta 1(β1) integrins-ECM components interaction and consequent receptor activation[61]. More recently, using breast cancer cell lines, it was revealed that the adhesion and migration to fibronectin and vitronectin occur through a subset of distinct integrin subunits, pointing to the importance of using multiple therapeutic agents based on integrin antagonists[62]. In prostate cancer, on the other hand, it has been demonstrated that the transcription factor Snail causes, through the MAP kinase pathway, a decrease in cell adhesion to fibronectin and collagen I,enhancing cell migration[63]. Besides epithelial cells, we cannot forget the importance of ECM in the migration of other cells types. In this regard, the work by Goethem and colleagues elegantly demonstrated the importance of matrix

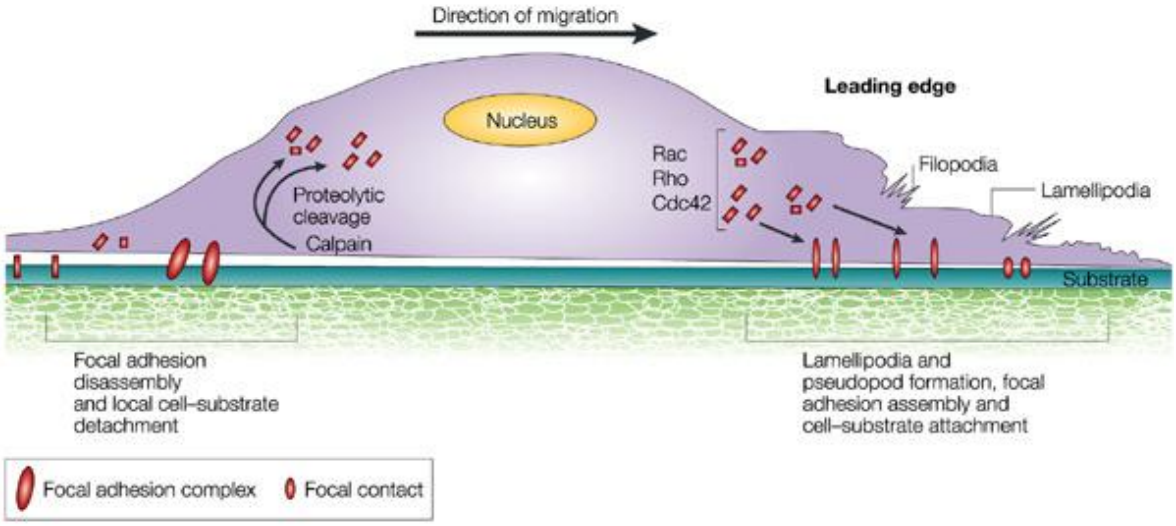

Figure 5 The dynamics of cell migration. Directed cell migration is dependent on a dynamic sequence of cell–substrate attachment at the leading edge of the cell coordinated with cell–substrate detachment at the rear. Adapted from Frame *et at* (2002) Nat Rev Mol Cell Biol[1].

architecture in the different modes of macrophage migration in 3-D[64]. Most leukocytes uses the so called amoeboid migration, characterized by a rounded cell shape and the lack of both strong adhesive interactions and proteolytic matrix degradation. An alternative migration is the mesenchymal migration, in which the cells adopt an elongated shape. By changing the composition of the matrix, using collagen I or Matrigel, they observed that macrophages adopted either the amoeboid or the mesenchymal migration, respectfully. Conversely, when they used a matrix composed of collagen I with an architecture more similar to the Matrigel matrix, the movement adopted was mainly of the mesenchymal type, suggesting that macrophages sense matrix organization. The majority of studies linking ECM and invasion focus on MMPs. Nevertheless, there are different mechanisms by which ECM controls this process. One striking example that clearly demonstrated the pivotal role of ECM on controlled invasion occurs during pregnancy establishment, specifically in the implatation process, when trophoblast invades the uterine wall[65]. Damsky and collegues developed an *in vitro* invasion model where they used blocking antibodies to different integrin subunits or ECM molecules, such as laminin, fibronectin and collagen type IV. These experiments allowed them to conclude that trophoblast undergoes an integrin switching in order to upregulate counterbalancing invasion-accelerating and invasion-restraining adhesion mechanisms, as a way to regulate its invasive capacity[66].

2.2.3 ECM alterations in cancer

As it was previously described, ECM is a highly dynamic molecular network having the ability to modulate and to be modulated by tumour and stromal cells. Several mechanisms control ECM production, degradation and remodeling, ensuring tissue homeostasis. However, if these mechanisms get deregulated, it will result in an abnormal cell, ultimately contributing to diseases such as cancer. In fact there exist already many reports describing various differences between normal and tumour ECM[67]. ECM degrading enzymes such as MMPs are known to be deregulated in cancer, leading to a defective ECM amount, composition and topography[68]. Additionally, an increased collagen deposition in some tumours[69], as well as changes in tissue architecture[70] and other physical properties such as stiffness, have been already reported. The alteration in tumour ECM stiffness has also been observed in pre-malignant tissues[71] and is mainly attributed to excess activity of lysyl oxidase (LOX), resulting in increased cross-linking of collagen fibers and of other components[72]. All these ECM alterations have been shown to affect tumour development both directly, by acting on tumour cells, or indirectly through their influence in the behavior of stromal

cells. Accordingly, collagens type I and III are described to decrease E-cadherin expression in pancreatic cell lines[73] while increased fiber crosslinking was associated to enhanced integrin signaling, promoting tumour progression[72]. In CRC, enhanced LOX activity increased proliferation and metastasis, through a mechanism dependent on Src activation[74]. Additionally, an alternative splice form of fibronectin, the extra domain A only expressed in malignancies, was shown to stimulate the production of VEGF-C by CRC cells[75]. Altogether, these examples, linking ECM alterations with cancer progression, prompt to the need of establishing realistic cancer models including ECM components as modulators of cancer cell activities and as targets of therapeutic intervention.

3. 3D in vitro models to study cancer

The environment where cells are regulates their activities and modulates the dynamic and complex crosstalks established between the distinct tumour components, being a determinant factor for cancer progression. Therefore it's crucial to find the right study model that converges, in a more faithful manner, the physiological aspects of the ECM and of the cell-ECM interface. Two-dimensional (2D) culture systems have been widely used to study intercellular interactions and cancer-related activities, and provided important contributions to understand how alterations on oncogenes or on tumour suppressor genes may lead to oncogenesis[76]. These culture systems are however limited since tissue architecture and organ context are completely lost. It is known that in three dimensional (3D) systems cells are under different mechanic and microenvironment conditions, and that their fate regarding proliferation, differentiation, apoptosis or invasion results from the summation of these multiple signals[71]. This fact might explain some of the conflicting reports frequently observed regarding cell phenotype, cellular signaling or drug response and raised the awareness regarding the importance of the 3D culture systems. For this reason, scientists are trying to develop new culture methods more similar to the *in vivo* situation.

One of the most studied 3D models comprises Multicellular tumour spheroids (MCTS) which consist of cancer cells derived either from cancer cell lines or disaggregated human tumour fragments, and include actively proliferating cells on the outside and quiescent cells in the inner nutrient deprived zone. They can be studied in suspension, using bioreactors, or in 3D matrices, closely resembling cell-cell and cell-matrix interactions *in vivo*. With proliferation, the cells in the inner part of the spheroid become necrotic probably due to limited O_2, leading to hypoxia, and nutrient availability recreating tumour heterogeneity[77]. This method is being applied with various

purposes, namely to evaluate tumour response to therapeutic agents. Using breast cancer cell lines it was possible to observe different behaviors when comparing these spheroids with 2D cultures: spheroids displayed an enhanced response to Trastuzumab, a monoclonal antibody targeting the HER-2 receptor, possibly due to an increase in human epidermal growth factor receptor (HER)-2 homodimerization and a consequent shift from phosphoinositide 3-kinase(PI3K) to mitogen-activated protein kinase (MAPK) signaling[78]. In a glioma model, however, the results were opposite with the spheroids having increased resistance to radiation, probably related to variations in hypoxic inducible factor (HIF)-1α and c-Myc expression. It was also observed an increase in the levels of both anti- (Bcl2) and pro- (Bax) apoptotic regulators, emphasizing the complex nature of endogenous as well as of induced-stress resistance that can exist in tumours, contributing to treatment failure[79]. Other study, using spheroids of pancreatic cancer cells seeded on collagen, reported a modulation in the expression of E-cadherin, β-catenin and Ezrin by stromal cells[80]. Similar models have also been used to study CRC *in vitro*, combining multicellular spheroids of cancer cells and fibroblasts (Figure 6), and were able to converge physiologic aspects and pathways described to be relevant *in vivo* in colon cancer, namely the Wnt signaling activation[81]. Several culture systems have been based on the use of ECM components naturally secreted by epithelial or stromal cells. These cells are able to produce and secrete several ECM components, forming a thin basement membrane layer composed mainly by laminin, collagen IV and elastin. This layer, termed as basement membrane, underlines the majority of the epithelial tissues, providing tissue support and nutrition, and constituting one of the first barriers that invasive cancer cells have to overpass[76]. One of the most used examples are basement membrane proteins collected from Engelbreth-Holm-Swarm (EHS) murine sarcoma cells, commercially available as Matrigel, and whose main characteristics is the formation, at 37ºC, of a 3D gel that supports cell morphogenesis, differentiation and tumour growth[82]. Two different methods have been developed to culture cells in 3D using this matrix: the 3D "embedded" assay, where cells are surrounded by Matrigel, very useful to study proliferation, viability and differentiation, and the "on top" assay, more suitable for invasion assays, time-lapse imaging and in situ immunostaining[83]. Although these type of systems can be helpful to study cell-matrix interactions, they do not fully represent the composition and structure of the tumour microenvironment, and are known to display high variability between lots[76]. As an alternative, scientists are trying to find ways to successfully engineer the 3D microenvironment by using scaffolds constructed from natural or artificial polymers. There are many natural polymers that include collagen, chitosan, and alginate[84]. Hydrogels made with collagen I and cultured with breast cancer cell lines were used to develop a bioengineered tumour

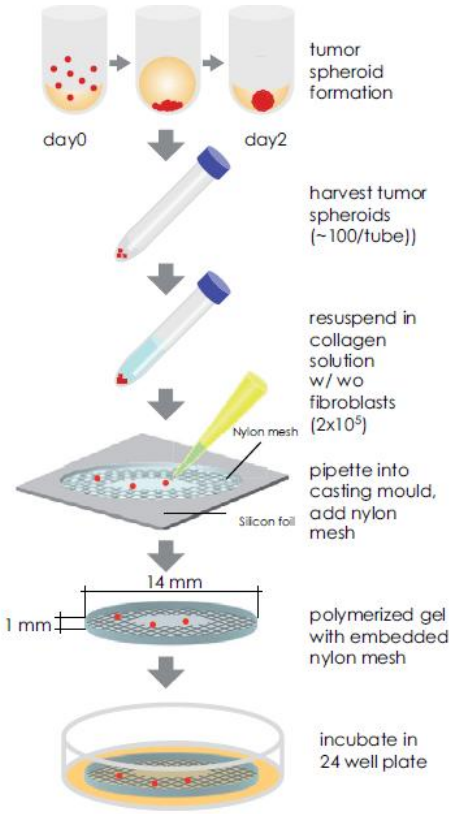

Figure 6 Workflow of spheroid embedding in collagen. Adapted from Dolznig *et al* (2011) Am J Pathol[81].

in vitro, that displayed necrosis, beyond a depth of 150-200nm, and upregulation of both HIF-1α and VEGF-A, similarly to what happens with *in vivo* tumours[85]. Matrices made with collagen are also being used to construct multicellular organotypic models with different cell types. Successfully isolated luminal cells, myoepithelial cells and fibroblasts, from normal and malignant breast tissue, were brought together in culture, in the presence of ECM components. These cells organized themselves into structures that recapitulated both normal and ductal breast carcinoma *in situ* (DCIS), with myoepithelial cells around the luminal population. Interestingly, the presence of tumour fibroblasts, contrarily to normal fibroblasts, disrupted the epithelial unit similarly to what would happen with a cancer cell, trying to invade the surrounding ECM[86]. This type of model was also employed to study premalignant lesions such as Barrett's esophagus (BE) using matrices consisting of collagen and esophageal fibroblasts cultures with BE cell lines[87]. Scaffolds made with chitosan-alginate (CA) and pre-cultured with glioma cells were used as implants into nude mice. This model recreated more accurately the malignant and invasive nature of these tumours, opening the possibility to use this system as an alternative to study anticancer therapeutics applications[88]. The same type of scaffolds also revealed to be useful for studying prostate cancer cell-lymphocyte interactions since it was able to support cell differentiation and to sustain long-term cultures[89]. An

innovative study using a matrix constructed with fibroin, a protein isolated from silk, revealed higher breast cancer viability, proliferation and MMP-9 activity, comparing with Matrigel or tissue culture plates. Once again this can be an easily manipulated microenvironment system, suitable to study some specific factors regarding cancer development and therapeutics[90]. Artificial scaffolds are also being used as alternative 3D culture systems. They can be made with different biodegradable synthetic polymers such as polylactide (PLA), polyglycolide (PGA) and co-polymers Poly (lactide-co-glycolide) (PLG/PLGA) and allow the construction of various structures like meshes, fibers or sponges. To increase cell adhesion, these scaffolds are frequently modified through either incorporating functional groups or by mixing ECM components[76]. These type of scaffolds have been particularly investigated and commonly used in the biomaterials field, namely for bone reconstruction, articular defects or fixation devices [84] but are also being studied as a possible application in cancer. Porous polymeric microparticles of PLGA and PLA were made by using several formulations and different modifications, including poly (vinyl alcohol) (PVA) or chitosan (Figure 7), and used to analyze cell adhesion and growth. Preliminary results revealed that cells are able to adhere to a greater extent to PLA/PVA microparticles, suggesting that these scaffolds could

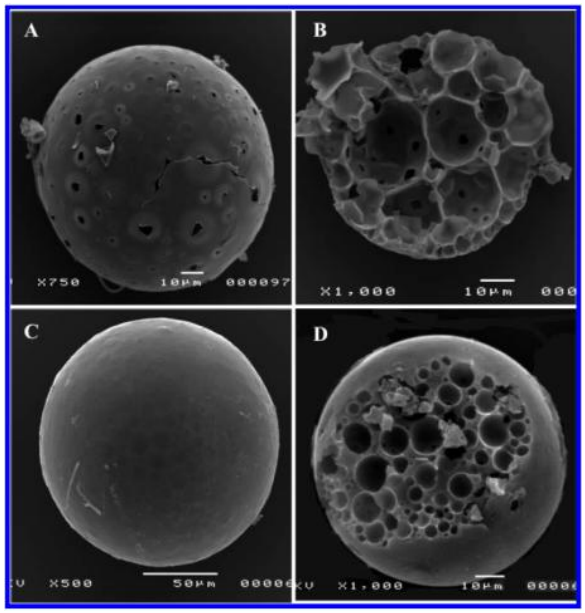

Figure 7 Scanning electron micrograph of an outside and inside of a typical PLA/PLGA microparticle formulated with (A and B) and without (C and D) sucrose in the internal phase (W1) of the primary emulsion (W1/O). Adapted from Sahoo *et al* (2005) Biomacromolecules[91].

be used to represent an alternative model for preclinical evaluation of the cytotoxic effect of anticancer agents[91]. Other study, focused on ovarian cancer cell-ECM interactions and drug resistance, constructed a synthetic hydrogel matrix from polyethylene glycol (PEG) equipped with

key biomimetic features namely cell integrin-binding motifs, and the ability to be degraded by proteases such as MMPs. Cells grown in this system showed higher survival rate to anti-drug paclitaxel when compared to 2D cell monolayers, reinforcing the idea that 2D evaluation of chemosensitivity probably does not reflect the pathophysiological events observed in patients[92].

4. Decellularization

Despite the increasing progress in 3D culture systems to study cancer, the reality is that all of them contain limitations being unable to recapitulate the tumour microenvironment. To surpass this problem, the ideal solution would be to use matrices obtained from decellularization of native tissues, retaining original ECM composition and organization. These matrices could then be functionalized and repopulated, allowing the study of tumour/stroma/ECM interactions in an environment more similar to *in vivo* as possible.

Decellularization has emerged in the last years as an innovative strategy in tissue engineering and regenerative medicine. With this method, it is possible to obtain acellular naturally occurring three-dimensional biologic scaffolds which can be subsequently seeded with either differentiated or selected progenitor cells[93]. In fact there are already available some clinical products such as demineralized bone matrix, skin grafts, bioprosthetic heart valves and acellular biologic surgical meshes that were constructed using this strategy[94]. More recently, some promising results were also obtained in whole organ engineering. Scientists were able to successfully decellularize entire organs such as heart[95], lung[96] and liver[97] while the tissue still retained its architecture, opening the possibility to used them in organ transplantation.

Objective criteria have been recently defined in order to access decellularization efficiency including (1) the absence of visible nuclear material in tissue sections stained with Hematoxylin-Eosin (HE) or 4',6-diamidino-2-phenylindole (DAPI), (2) presence of DNA fragment size below 200 bp and (3) quantitative measurement of DNA at less than 50ng/mg dry tissue weight[98]. Although these criteria have been defined on years of experience, they still constitute a limited evaluation as they are based entirely on DNA analysis, ignoring other cellular components. These parameters are clearly insufficient, particularly in situations of organ transplantation from xenografic sources due to the eventual immune response[99].

Several strategies have been developed in order to achieve decellularization with minimal alteration of tissue integrity. Decellularization efficiency strongly depends on tissue density, thickness and composition and, therefore, the protocol adopted has to have these parameters into

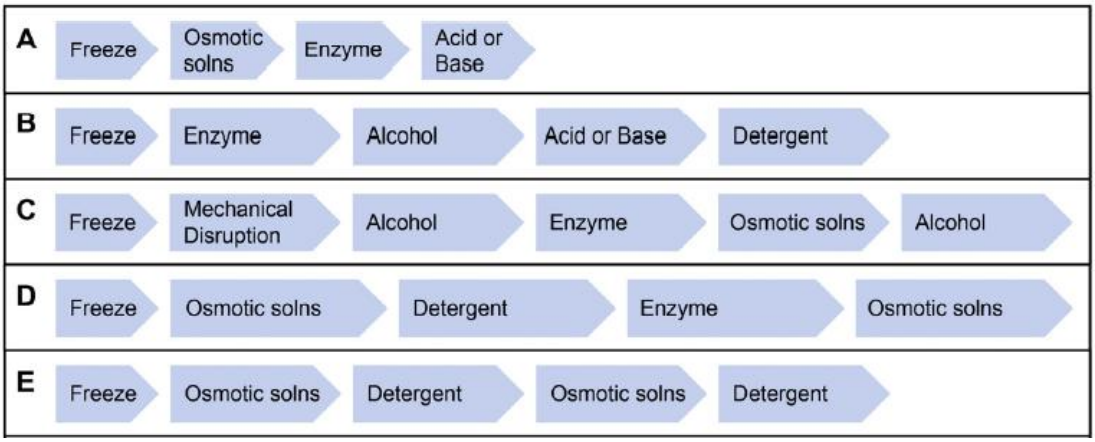

Figure 8 Example of protocols for (A) thin laminates such as pericardium, (B) thicker laminates such as dermis, (C) fatty, amorphous tissues such as adipose, (D) composite tissues or whole simple organs such as trachea, and (E) whole vital organs such as liver. Arrow lengths represent relative exposure times for each processing step. Rinse steps for agent removal and sterilization methods are not shown to simplify comparison. Adapted from Crapo *et al* (2011) Biomaterials[98].

account. It is possible to use physical methods, chemical or biological agents but experimental data shows that the highest success rate is achieved with a combination of different approaches (Figure 8)[98].

4.1. Physical methods

The physical methods most commonly used are mechanical force and pressure, changes in temperature, agitation and non-thermal irreversible electroporation (NTIRE). Applying force and pressure have been used to remove cells but these methods have shown to be too aggressive and destructive, affecting basement membrane integrity and ECM structure[100]. Sneep freezing was tried for the first time more than twenty years ago on ligament tissue from goats with little effect on tissue mechanical properties[101]. This technique causes membrane disruption and cell lysis, by the intracellular accumulation of frozen crystals, but the efficient removal of cellular components is only achieved by combining other methods[102]. NTIRE, on the other hand, is a method that doesn't rely on thermal mechanisms but on the ability of electric pulses, applied across a tissue, to increase permeability of cells by inducing formation of nanoscale pores in the membrane. Ultimately, this results in loss of tissue homeostasis leading to cell death[98]. This strategy was recently used to decellularize porcine liver and, although the results are encouraging, the amount of cellular components still needs to be evaluated[103]. Other method that is being used is agitation, most

commonly combined with emersion in chemical/biological agents, in order to facilitate cellular material removal. This strategy has been applied in different tissues including heart valves, dermis or skeletal muscle/tendon with good decellularization efficiencies. The time of agitation required varies extremely, ranging between some hours up to months, depending on the tissue thickness and density[98]. This method was already used in human tracheas, with effective removal of cell membranes but still retaining some cellular elements in cartilaginous areas. Nevertheless, tissue architecture was preserved[104].

4.2. Chemical agents

There are various types of chemical agents used in decellularization protocols including acids or bases, detergents and hypotonic or hypertonic solutions.

Alkaline and acid treatments cause cytoplasm solubilization, disruption of nucleic acids and protein denaturation[98]. In one study, blood vessels were efficiently decellularized using an alkine treatment, which was particularly effective in larger diameter veins rather than arteries, with high preservation of collagen and elastic fibers[105]. Nevertheless this type of methods, specially the use of bases such as calcium hydroxide or sodium sulfate, are reported to completely eliminate growth factors and GAGs content and to reduce mechanical strength in pig dermis[106].

Concerning detergents, they can be ionic, non-ionic or zwitterionic and their effect is based on the ability to solubilize cell membranes and to dissociate DNA from proteins[98]. In one study, using rat tail tendons, they compared t-octyl-phenoxypolyethoxyethanol (Triton X-100) with sodium dodecyl sulfate (SDS), the most used ionic and non-ionic detergents respectively. Treatment with 1% Triton, for 24 hours, wasn't enough to remove cells but caused disruption of collagen fiber structure. On the other hand, 1% SDS 24 hours treatment was sufficient to obtain an acellular tendon with structure and mechanic properties very similar to normal[107]. Nevertheless, the data regarding decellularization efficiency using Triton X-100 is not consensual, with some reports showing a complete cell-free structure. In one study performed with porcine aortic valves, cell components were totally removed but there was a decrease in collagen density as well as loss of GAGs and both laminin and fibronectin[108]. Of importance is that both the effectiveness of each method as well as the damage induced in ECM structure are most probably tissue dependent. Zwitterionic detergents, like 3-[(cholamidopropyl) dimethyl ammo-nio]-1-propanesulfonate (CHAPS), are known to exhibit properties of both non-ionic and ionic detergents. This treatment was successful in removing cell content of blood vessels but also caused ECM disruption[109].

Hypotonic and hypertonic solutions induce cell lysis through osmotic shock although are not very effective in removing cellular contents. For this reason these agents are frequently used in combination with other decellularization methods[102]. In one study, different methods to decellularize rat aortic valve allografts (AVAs) were compared, namely osmotic decellularization, through a series of hypotonic and hypertonic Tris buffers, and detergent decellularization, which also included 0.5% of Triton X-100 along with the previously mentioned solutions. Results showed that, contrary to osmotic decellularization, detergent decellularization was effective in removing cellular elements and preserving ECM architecture[110].

Other chemicals frequently used in decellularization protocols are chelating agents, such as ethylenediaminetetraacetic acid (EDTA) or ethylene-glycol-tetraacetic acid (EGTA), that are able to bind to divalent metallic ions, thus disrupting cell adhesion to ECM. These type of chemicals are always used in combination with other decellularization methods[102].

4.3. Biological agents

The biological agents more commonly used are enzymes such as nucleases, both endo- and exonucleases, trypsin, collagenases and dispase. The main advantage in the use of enzymes is their specificity although complete decellularization with this method alone is very difficult. Nucleases, like DNAses and RNAses, cleave nucleic acid sequences being helpful in the removal of nucleotides after cell lyses[98]. Trypsin is a serine protease highly specific and the enzyme most used in decellularization protocols[102]. Collagenases and dispases, on the other hand, have to be used with caution and only when maximum collagen retention is not a priority[98].

The reports regarding the various enzymes are not consensual concerning decellularization efficiency and ECM preservation. In the decellularization of aortic porcine valves, a combination of 0.5% trypsin with 0.05% EDTA and 0.2 mg/ml of DNAse was not enough to remove leaflet cells, but affected collagen network density and caused a significant decrease in fibronectin and laminin, as well as a reduction in GAGs content[108]. Conversely, in a different study done with human heart valves, they were able to obtain a cell-free scaffold using 0.5% Trypsin and 0.2% EDTA, retaining normal ECM structure namely the 3D network of collagenous fibers[111]. A study done in total fetal rat lungs combining Triton X-100, sodium chloride and DNAse revealed that, although a significant decrease in elastin, laminin and GAGs occurred, decellularization was efficient and the tissue retained its architecture, as confirmed by scanning electron microscopy (SEM) and collagen content quantification[112]. To decellularize skeletal muscle, it was used a combination of latrunculinB, to

disrupt actin fibers, osmotic shock, and DNAse I treatment. Results showed acceptable decellularization efficiency, without affecting collagen content or ECM structure. GAGs suffered a reduction of about 40% but the mechanical properties of the muscles were not significantly altered[113]. Other tissues such as human larynx were also successfully decellularized using a protocol that combined 4%SDS with 2000 KU (Kunitz Units) of DNAse, with complete cellular and nuclear material removal and without impairing hierarchical structures and biomechanical properties of the native tissue[114].

4.4. Strategies to apply decellularization agents

Physical agitation, as previously mentioned, constitutes one possible strategy to apply decellularization agents. Nevertheless there are other options available. Once again, the method chosen must have into consideration the tissue characteristics in terms of density and thickness.

All organ perfusion is the method most used to decellularize complete organs with very good results as it allows the preservation of the 3D structure of most organs. Perfusion is frequently done using the endogenous vasculature of the tissue, allowing efficient decellularization agent deliver to the majority of cells, and the simultaneous removal of the cellular material[98]. Different organs were already successfully decellularized through perfusion namely heart (Figure 9)[95], lung[96] and liver[97]. The main source of organs tested has been the rat but organs from other animals have also been used. In one study, intact porcine hearts were decellularized through pulsatile retrograde aortic perfusion with a combination of trypsin, nonionic and ionic detergents, acid solution and

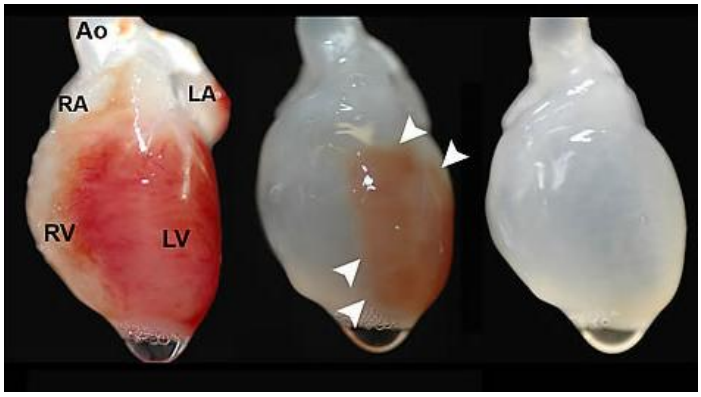

Figure 9 Perfusion decellularization of whole rat hearts with SDS over 12h. Photographs of cadaveric rat hearts mounted on a Langendorff apparatus. Ao, aorta; LA, left atrium; LV, left ventricle; RA, right atrium; RV, right ventricle. The heart becomes more translucent as cellular material is washed out from the right ventricle, then the atria and finally the left ventricle. Adapted from Ott et al (2008) Nat. Med[95].

hypotonic and hypertonic rinses. This allowed systematical removal of cellular contents, being able to remove all nuclei and muscle cells. The collagen structure remained unaltered, as confirmed by immunohistochemistry, SEM and mechanical analysis, and GAGs and elastin contents were also preserved. These results clearly show that this strategy can be applied to more complex organs, as human hearts[115]. Perfusion was also efficiently used to decellularize whole rat kidneys, combining 3% Triton X-100, DNAse and 4% SDS. Subsequent ECM analysis revealed the preservation of the cortical microstructure, without residual nuclei or intact cells. Complementary IHC analysis confirmed retention of key basement membrane proteins, such as laminin, in the cortical region, and collagen, in the medulla[116]. Other methods, such as pressure gradient have also been used to permit the efficient delivery of distinct decellularization agents. To enhance cell removal efficiency and reduce tissue incubation time, dermis originated from porcine skin was decellularized using trypsin and dispase II with a pressurized technique. The use of this system, comparing with the normal conventional stirring approach, allowed better preservation of collagen native structure and more efficient cell removal in a reduced period of time[117].

4.5. Decellularized tissue repopulation

After efficient decellularization, repopulation of the scaffold is the next challenge to face. The type of cells to use, their number and how the recellularization is done are crucial parameters to consider in order to obtain a functional tissue/organ.

Concerning the type of cells to use, and if we think about a complete organ, the diversity and heterogeneity of cells present have to be taken into consideration. Besides the parenchymal cells, responsible for the specific function of the organ, like hepatocytes in the liver or epithelial cells in the lung, there are the nonparenchymal cells, mainly endothelial cells and fibroblasts which increase organ functionality and contribute to the organization of tissue architecture[93]. There are different options regarding parenchymal cells: embryonic stem cells (ESC), known for their pluripotency, fetal cells, already committed to an end point while retaining their proliferative capacity, inducible pluripotent stem cells, which have the disadvantages of require transformation and having already acquired the epigenetic modifications of DNA and organ-derived progenitors, must still be expanded *in vitro*[93]. In one study this question was particularly addressed by comparing the differentiation potential of decellularized hearts under static culture of hESC and human mesendoderm cells (hMECs), cells derived from hESC and known for being lineage restricted progenitors and giving rise to the primary cardiac cell types. During this study, the cells

were injected to the cannulated aorta and maintained in culture for 14 days. Results show that both hESC and hMECs progressively differentiated towards the cardiac lineage, loosing stem cell markers, such as Nanog, and upregulating various markers, such as CD31. The reseeded hearts were subcutaneously implanted into immunocompromised mice and it was possible to detect cardiac markers expressing cells without having, nonetheless, the beating function. These results demonstrate that the decellularized ECM was able to direct differentiation of the stem/progenitor cells into the cardiac lineage[118]. In other study performed in decellularized heart, different types of cells including neonatal cardiomyocytes, fibrocytes, endothelial cells and smooth muscle cells were delivered through fixed injections in the anterior left ventricle. Quantification of cell number in the effluent from the myocardium immediately after injection, showed a loss of approximately 46% of the cells within 20 minutes. Afterwards, the matrix was placed in a bioreactor vessel and aortic endothelial cells were introduced directly into the heart by direct infusion into the aorta in order to "re-endothelialize" the tissue. This recellularized construct was contracting and drug responsive after 8 days of culture (Figure 10)[96]. A different group decellularized porcine pulmonary valves and then, in order to obtain viable and stable constructs, reseeded them with endothelial cells and myofibroblasts, obtained from ovine carotid heart valves. These matrices were then cultured using a hydrodynamic bioreactor system, along 9 or 16 days, and compared with valves grown under static conditions. Results demonstrated that pulsatile flow for 16 days enhances significantly the tissue formation and mechanical properties, resembling native heart valves. Histology and immunohistochemical examinations revealed a well-organized fibrous tissue with confluent coverage of viable and active secretory cells, achieving repopulation throughout the entire leaflets[112]. In a different study they recellularized a rat liver by introducing rat hepatocytes via portal vein perfusion recirculation. Engraftment efficiency was about 95.6% if they did four steps with 12.5 million cells, at each step. After seeding, the recellularized liver hearts were transferred into an

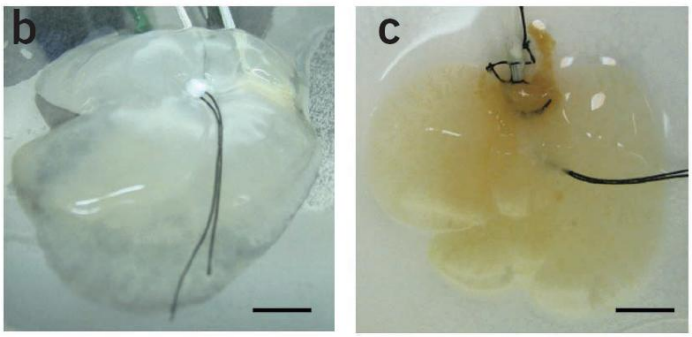

Figure 10 Repopulation of rat DLM with adult rat hepatocytes. Decellularized whole liver matrix (b) and same liver after recellularization with about 50 million hepatocytes (c). Adapted from Uygun et al (2010) Nat Med[96].

especially designed perfusion chamber for *in vitro* culture. Analysis of apoptotic cells revealed that most of the cells remained viable and that the recellularized graft was able to support liver-specific functions including albumin secretion, urea synthesis and cytochrome P450 expression at levels compared to normal liver *in vitro*. These grafts were then transplanted into rats for 8h and were able to support hepatocyte survival and function with minimal ischemic damage[97]. Recently, the same type of strategy was applied in the hope of obtaining material for corneal transplants. Porcine corneas were decellularized and then repopulated with different cells: first human corneal stromal cells were injected at different locations. After 30 days, limbal corneal epithelial cells (LCEDs) were seeded on the anterior surface of the corneas while corneal endothelial cells (CECs) were seeded in the posterior surface. Through this method, all three cell types were successfully incorporated into the scaffold. CSCs maintained a dendritic morphology forming a widespread network and expressing type I collagen and keratocan, while both LCECs and CECs showed proliferating capability with CECs staining positive for type VIII collagen. Nevertheless, *in vivo* studies are lacking to access functionality[119]. One of the most exciting results was obtained using a human trachea. After effective decellularization, the colonization was done using epithelial cells and mesenchymal stem-cell-derived chondrocytes than had been cultured from cells taken from the recipient. A bioreactor, addressing different requirements namely seeding and culturing different cell types, as well as nutrient supply and waste removal, was designed and used. Chondrocytes were applied longitudinally to the external surface matrix, with a microserynge while epithelial cells were seeded in the internal surface through a separate access. The graft was used to replace the recipient's left main bronchus and, at least in the first four months after transplantation, no complications were detected[120].

5. Emerging challenges

So far, the complexity of the carcinogenic process was emphasized. The relevance of the numerous dynamic interactions established, at the tumour site, between cellular and non-cellular components, and their contribution to cancer progression, were also referred. The limitations inherent to the models used to study cancer interactions have been appointed. Decellularization, widely investigated in the tissue engineering field, emerges as a possible way to bridge these two worlds. We believe that by decellularizing tumor tissue and repopulating it with the various cell types of interest, it will be possible to establish an organotypic culture where the microecosystem resembles more closely the *in vivo* situation.

The reports that use decellularized matrices to study cancer are very scarce. In one study they used this strategy in an attempt to understand prostate cancer metastasis to the bone. Prostate carcinoma metastasizes to the bone in about 90% of the cases, suggesting that the complex bone microenvironment offers the ideal conditions for cancer cells to grow. To address this hypothesis, decellularized matrix secreted from primary osteoblasts were used. After confirming the presence of the various components normally existent in the *in vivo* bone microenvironment, namely calcium and phosphate, a high percentage of collagen I, osteocalcin, osteonectin, osteopontin and growth factors, the matrices were cultured with two prostate cancer cell lines. When compared with cells cultured in normal plastic or just collagen I, cells grown on the decellularized matrices adhered strongly and expressed markers consistent with loss of epithelial phenotype. Additionally, cells grown on these matrices expressed genes associated with attachment, migration and osteolysis mimicking key features of prostate cancer bone metastasis. These characteristics indicate that this is a suitable model system to study tumour/bone microenvironment interactions, in homeostasis and disease[121]. A different study used decellularized rat lungs that were cultured with a human alveolar basal epithelial adenocarcinoma cell line or two lung cancer cell lines. A simplified closed-system bioreactor to culture the lung was designed, while cells were seeded into the tracheal cannula through a syringe. All three cell lines engrafted created perfusable tumour nodules and the cancer cells developed a pattern of growth similar to the original human lung cancer. These results open the possibility to use *ex vivo* models to gain a deeper understanding of the biologic processes involved in human lung cancer[122]. These two studies reinforce the idea that this decellularization/repopulation strategy is most likely the answer in the future, to gain new insights on how the molecular-interactions established at the tumour microenvironment promote tumour progression.

Materials and Methods

Clinical samples

Fresh colorectal surgical-resections were collected directly from the Pathology Department from Hospital São João few hours after the surgeries. The fragments were transported in Hanks' Balanced Salt Solution (HBSS, Sigma), at 4ºC, to the laboratory where they were processed. Briefly, fragments were cut in smaller samples, placed in square plastic containers with 2 cm side and covered with mounting medium for cryotomy (OCT compound, Thermo Scientific). Then, samples were placed in 2-methylbutane and rapidly frozen in liquid nitrogen. Containers with the fragments were stored at -80ºC until further use.

Decellularization

Decellularization protocol was optimized (not mentioned due to confidentiality reasons). Two different controls were considered: the "Not manipulated", which is a fragment stored in -80ºC immediately after sample collection, and a "Not decellularized" which is a fragments incubated with PBS during the entire protocol. Afterwards, decellularized fragments were either stored at -80ºC or 4ºC, fixed in formol or in glutaraldehyde depending on their future application.

DNA quantification

To assess total DNA content within the native tissue and decellularized matrices, DNA was extracted using PureLink Genomic DNA Mini Kit (Invitrogen) following manufacturer's instructions. Due to confidentiality reasons, details are not included.

Histological analysis

After decellularization, samples were formalin-fixed overnight at room temperature, processed to paraffin blocks and sectioned in 3µm slides. Sections were stained with 4´,6-diamino-2-phenylindole (DAPI) using Vectashield containing DAPI (VectorLab) for fluorescent staining of nucleic acids and Hematoxylin-Eosin (HE) or Masson Trichrome (MT) for histomorphological analysis. Not manipulated colon tissue was used as control. Independently on the staining used, slides were deparaffinized and rehydrated. For HE staining, slides were incubated with hematoxylin (Thermo Scientific) for 2 minutes, washed with running water and then incubated with 1.5%

ammoniacal water. After washing, slides were treated with Ethanol 95% followed by Eosin (Thermo Scientific) for 4 minutes. Samples were incubated again in Ethanol 95%, dehydrated and mounted in Richard-Allan Scientific Mounting Medium (Thermo Scientific). For MT staining, we used a kit (HT15, Sigma). Briefly, slides were incubated in Alcian Blue for 5 minutes, washed very well with distilled water and incubated 5 minutes with Hematoxylin. Afterwards slides were fixed with Bouin's solution (VWR) for 1 hour, washed, and incubated with Bierich Scarlet-acid Fuchsin Solution for 5 minutes. A new washing step was performed followed by incubation with a solution composed of 2x distilled water, 1x phosphotungstic acid and 1x phosphomolybdic acid. Finally slides were treated with Anilin Blue for 5 minutes, washed, dehydrated and mounted in Richard-Allan Scientific Mounting Medium.

Glycosaminoglycans (GAGs) quantification

GAGS from either not decellularized or decellularized colon fragments were quantified using Blyscan GAG Assay kit (Biocolor). Briefly, tissue fragments were minced with a blade and incubated with Papain Extraction Reagent 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. Not manipulated fragment was used as control. Absorbance was measured at 656 nm. Results are presented as mg of GAG per mg of fresh tissue, which corresponds to the weight of each fragment before decellularization.

Scanning Electron Microscopy

Samples were fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate for 30 minutes at room temperature under gentle agitation, washed three times in cacodylate buffer, and dehydrated in a graded ethanol-water series beginning at 50% until 100% ethanol. Fragments were critical point dried using CO_2, mounted in sticky carbon tape and covered in gold to allow visualization. Samples were then analyzed using an high resolution scanning electron microscope (FESEM JEOL JSM6301F.).

Human Monocyte Isolation

Human monocytes were isolated from Buffy coats from healthy blood donors provided by Hospital São João. Briefly, Buffy coats were centrifuged at 1200g for 20 minutes at room temperature. The whitish layer containing peripheral blood mononuclear cells (PBMCs) was then collected and incubated, during 20 minutes under continuous rotation, with the RosetteSep® Human Monocyte

Enrichment Cocktail (StemCell Technologies), following manufacturer's instructions. This mixture was then diluted (1:1) in PBS+2% FBS, carefully added over Histopaque®-1077 (Sigma), and centrifuged as previously. The intermediate layer, enriched in human monocytes, was collected and washed three times in PBS. Isolated cells were then ressuspended in RPMI1640 (Invitrogen) supplemented with 10% FBS (Lonza), 100U/ml penicillin and 100µg/ml streptomycin (Invitrogen). For the *in vitro* studies, cells were seeded in 58cm^2 tissue-culture Petri dishes at a density of 1x10^7 cells per plate, and incubated for 24 hours at 37ºC and 5% CO_2 humidified atmosphere. After this period, 3x10^5 cells were seeded into 24 tissue culture plates with coverslips. Macrophages were allowed to differentiate during 10 days and were then treated with 10ng/ml of LPS (Sigma) or IL-10 (Immunotools), for three additional days, to induce their polarization into M1 or M2 macrophages, respectively. After treatment, cells were fixed with methanol for 10 minutes at 4˚C or 4% paraformaldehyde (PFA) for 20 minutes at room temperature.

Decellularized matrices repopulation

Due to confidentiality reasons, details are not included.

Immunocytochemistry

For coverslips fixed in 4% PFA, cells were washed in PBS and quenched with 50mM NH_4Cl for 10 minutes. After washing three times with PBS, cells were permeabilized with 0.1% Triton X-100 for 5 minutes. The protocol followed from here on is common to cells fixed in methanol and PFA. Cells were washed, blocked for 30 minutes with 5% bovine serum albumin (BSA) and incubated for 1 hour with the primary antibodies CD68 (DAKO, PG-M1), CD163 (Cell Marque, MRQ-26), HLA-DR (Abcam, TAL 1B5) or Calprotectin (Fisher Scientific, MAC 387). After washing with PBS, coverslips were incubated for an additional hour with goat anti-mouse or anti-rabbit AlexaFluor-594-conjugated secondary antibodies. Samples were finally washed with PBS and coverslips were mounted on Vectashield with DAPI. Cells were visualized with a Zeiss Axiovert 200M fluorescence microscope.

Immunohistochemistry

Fifteen formalin-fixed, paraffin-embedded tissue samples from Hospital São João Tissue and Tumour Bank were stained with a panel of four antibodies: CD68, CD163, HLA-DR and calprotectin. Additionally, formalin fixed paraffin-embedded decellularized samples were immunohistochemically stained for E-cadherin (Cell Signaling, Clone 24E10), fibronectin (Sigma-

Aldrich, F3648), laminin (Sigma-Aldrich, L9393), collagens type I (Sigma-Aldrich, Col I) and IV (Millipore, AB769). Tissue 3µm-thick sections were deparaffinized and rehydrated. Antigen retrieval was performed in a water-bath at 98ºC: Tris EDTA pH9 for 20 minutes (CD68), Tris EDTA pH9 for 40 minutes (CD163), citrate buffer pH6 for 20 minutes (Calprotectin), citrate buffer pH6 for 30 minutes (HLA-DR and E-cadherin), citrate buffer pH6 for 35 minutes (fibronectin, laminin, collagens type I and IV). After cooling down for 20 minutes, slides were washed with PBS-Tween 0.02% and endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 10 minutes. To avoid nonspecific binding, slides were treated with Ultra V Block (DAKO) for 30 minutes. Tissue samples were then incubated with primary antibodies diluted in Large Volume Ultra AB Dilutent (DAKO) with the following concentrations: CD68 (1:100), CD163 (1:50), HLA-DR (1:5000), Calprotectin (1:1000), E-cadherin (1:50), laminin (1:100), fibronectin (1:400), collagen type I (1:50) and collagen type IV (1:10). As negative control, tissue samples were incubated with Large Volume Ultra AB Dilutent (Thermo Scientific), without any primary antibody. After washing, labeled polymer secondary antibody (Envision Detection System) was added to slides or, as an alternative, donkey anti-goat AlexaFluor-488-conjugated or goat anti-mouse or anti-rabbit AlexaFluor-594-conjugated secondary antibodies (Molecular Probes) (fibronectin, laminin, collagens type I and IV). Regarding these four, slides were washed in PBS and mounted on Vectashield with DAPI. For the other antibodies, peroxidase activity was detected using diaminobenzidine (DAB)-tetrahydrochloride liquid + substrate Chromogen System (DAKO), for about 2 minutes, and the reaction was stopped with distilled water. Sections were counterstained with hematoxylin and mounted in Richard-Allan Scientific Mounting Medium.

Statistical analysis

Data were analyzed with Mann–Whitney test using GraphPad Prism v6, GraphPad Software, (San Diego, CA, USA -trial version), and expressed as mean values of at least three independent experiments (±s.d.). Differences in data values were considered significant at a P-value of <0.05.

Results

1. Decellularization

The first step to create the organotypic culture was to optimize the decellularization protocol for normal and tumour tissue, in order to efficiently remove cells while retaining tissue architecture and composition. Therefore, we followed a protocol, previously developed in our group, and made some alterations in order to adjust it to our tissue. Afterwards, decellularized fragments were analysed through DAPI staining and DNA quantification, to confirm absence of DNA traces and to evaluate decellularization efficiency.

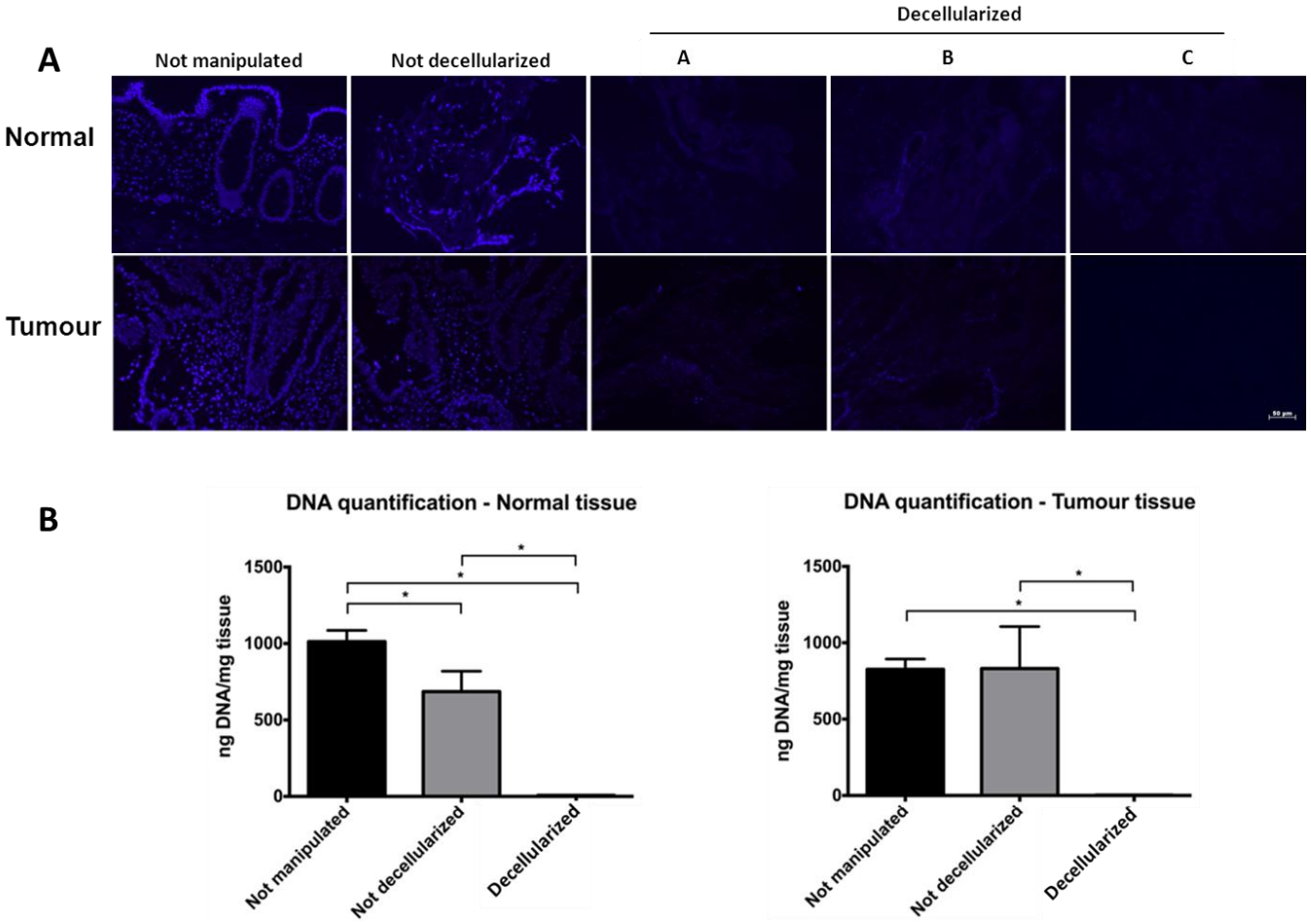

Figure 1 Decellularization effectively removes DNA content of normal and tumour colon tissues. (A) After decellularization, samples were formalin-fixed, processed to paraffin blocks, cut in 3µm sections and stained with 4´, 6-diamino-2-phenylindole (DAPI) to evaluate nuclear lost. (B) DNA quantification was done using decellularized samples stored at -80ºC. DNA extraction was done with Genomic DNA Mini Kit and PicoGreen was used to quantify DNA content. Not manipulated colon tissue was used as control. Scale bar represents 50 µm. *, significantly different at $P<0.05$.

In figure 1A, representative images of the two control conditions, not manipulated and not decellularized, and of the three decellularized ones tested (A, B and C) illustrate no visible nuclei for the three conditions used, both in normal and tumour fragments. DNA quantification (figure 1B) confirms that the established protocol effectively removes about 99% of the total DNA, in normal tissue, whereas in tumour fragments the DNA is reduced almost 300 times, with a DNA removal of 99.6%. With this specific protocol, normal but not decellularized fragments, incubated in PBS during the whole procedure, also lose a significant amount of DNA. Based on the results obtained, we chose condition A for the following experiments, for both normal and tumour colon tissues.

After decellularization protocol optimization, we preceded to the histological characterization of these normal and tumour matrices. We started by evaluating decellularized tissues histology by performing both Hematoxylin-Eosin (Figure 2A) and Masson's Trichrome (Figure 2B) stainings. Hematoxylin-Eosin, the most commonly used staining technique in histology and histopathology, particularly for diagnosis purposes, is based on the acidic/basic characteristics of each cellular component. Accordingly, basic cytoplasmic structures stain with a pink/reddish colour, given by the eosin stain, while hematoxylin, with affinity to acidic structures, stains nucleic acids and nuclei with a bluish colour. Masson's Trichrome, on the other hand, is a three-colour staining protocol, with which keratin and muscle fibers appear in red, by the action of Biebrich scarlet-acid fuchsin stains, collagens in blue, with treatment with phosphotungstic and phosphomolybdic acid followed by with aniline blue, and cell nuclei in dark brown or black, by using Weigert's iron hematoxylin. Both techniques demonstrated that decellularized fragments retain some of the histological features of colon tissue without major visible cell debris. Tumour matrices, probably due to their higher cellular density and narrowest fibre network, appeared to be the least affected by the decellularization protocol, evidencing still traces of red muscle fibers, as confirmed by the Masson's Trichrome staining. Additionally, it seems that collagens constitute the major components of the decellularized fragments.

To evaluate if the decellularized fragments still retain some of the major ECM components, we quantified glycosaminoglycans (GAGs) content. These constitute a very diverse group of long unbranched polysaccharides which are frequently bound to a core protein forming proteoglycans (PGs). Diverse functions have been attributed to GAGs, namely the ability to: i) confer resistance to tissue compression (due to their capacity to bind water and maintain matrices hydrated); ii) bind growth factors or cytokines/chemokines; iii) act as cell surface receptors; iv) and contribute to cell-

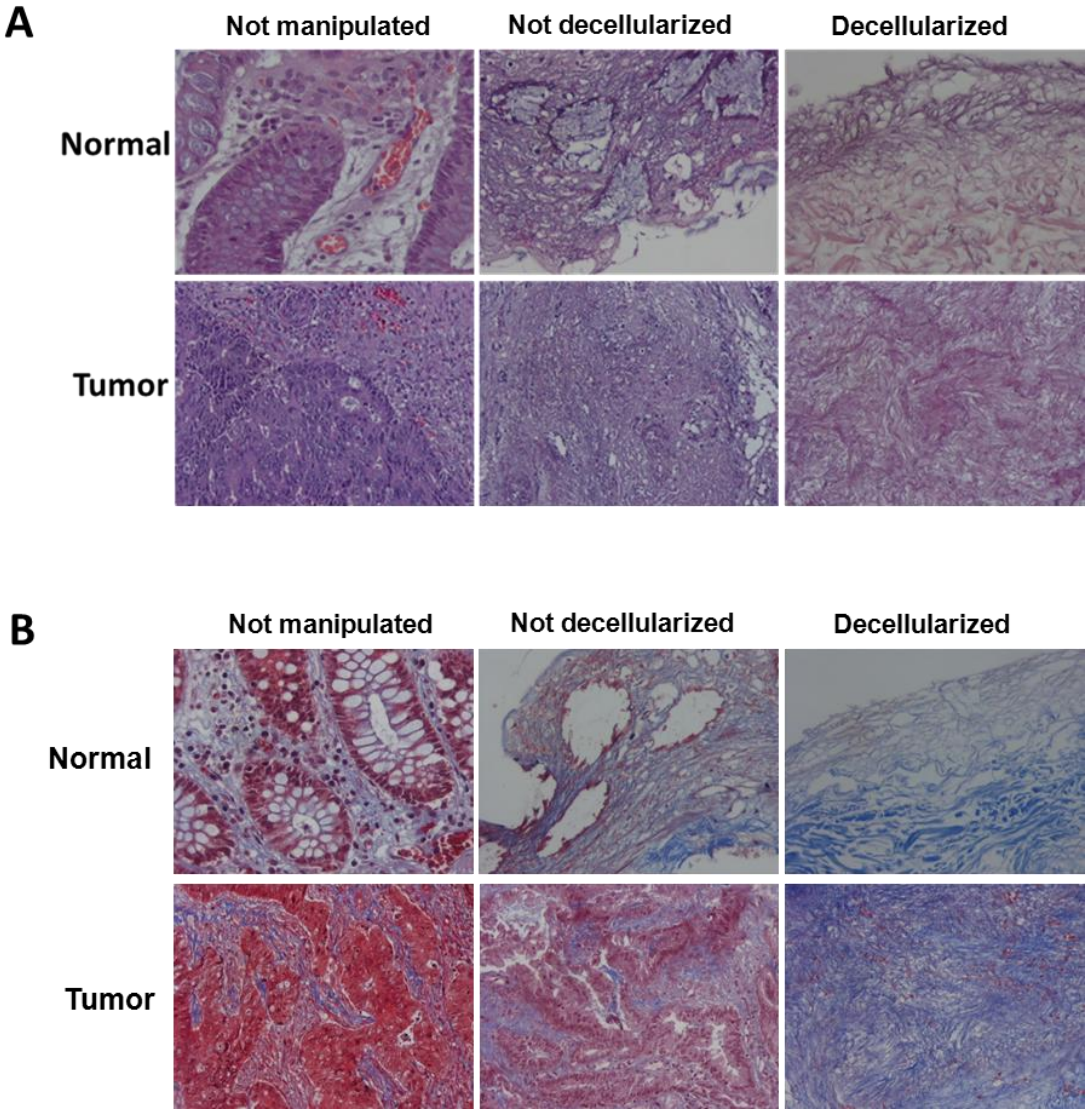

Figure 2 Decellularization retains histological features of normal and tumour colon tissues, still keeping some muscle fibers. After decellularization, samples were formalin-fixed, processed to paraffin blocks, cut in 3µm sections and stained with Hematoxylin and Eosin (40x, A) or Masson Trichrome (40x, B). Not manipulated colon tissue was used as control.

matrix attachment and cell-cell interaction. Our results evidenced that the control fragments, which weren't decellularized but instead incubated in PBS (not decellularized), already presented a reduction in GAGs content, more evident in tumour fragments (Figure 3). Additionally, both normal and tumour decellularized tissues had an evident GAGs decrease of almost tenfold.

To further characterize the molecular characteristics of these decellularized matrices, we performed immunohistochemistry (IHC) for some of the most predominant ECM components:

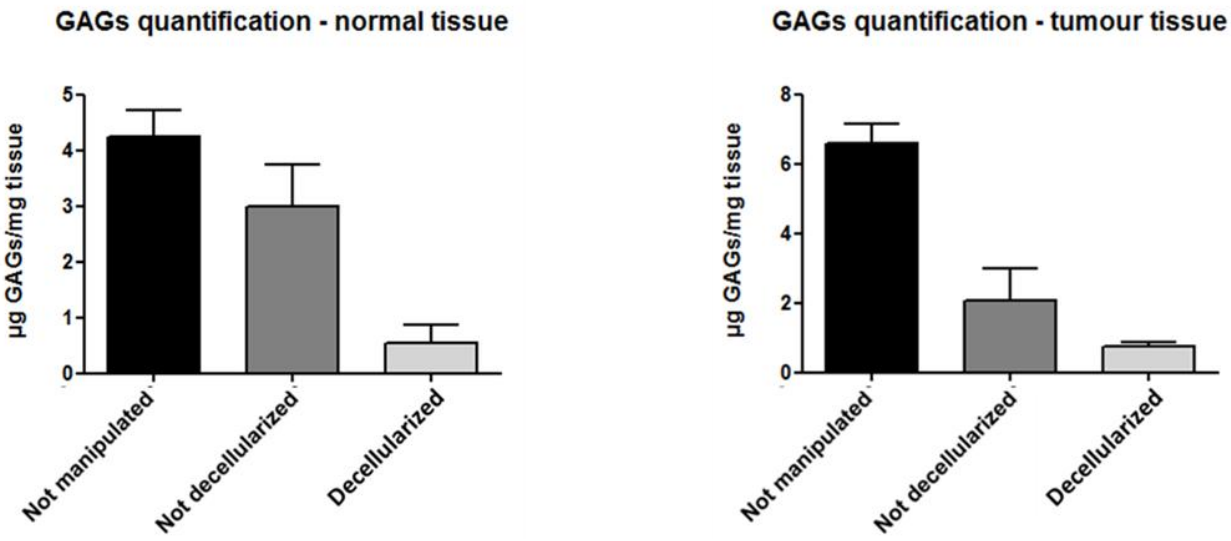

Figure 3 Decellularization decreases glycosaminoglycans content to approximately tenfold in both normal and tumour tissues. GAGs quantification was done using control (not decellularized) or decellularized samples stored at -80ºC. First GAGs were extracted and then quantified using the Sulfated Glycosaminoglycan Assay. Not manipulated colon tissue was used as control.

fibronectin, laminin and collagens type I and IV, of both normal (figure 4A) and tumour (figure 4B) fragments. Interestingly, tumour tissues seem to be less affected by the decellularization procedure, since the distribution pattern of the four ECM proteins studied was very similar to the pattern of the not manipulated control tissue. On the other hand, upon decellularization, normal fragments presented a more disrupted architecture and the glands organization is partially lost even though it is evident that some of the 3D structure is maintained.

Complementary analysis of tissue architecture was done using Scanning Electron Microscopy (SEM) in normal and tumour decellularized matrices. This is a very powerful technique, particularly useful to evaluate topography of materials and 3D architectural organization, which produces images of a sample by scanning it with a focused beam of electrons. The images obtained revealed that normal tissue, not manipulated fragments present presented a homogeneous surface with well-defined villi, whose structure is partially affected in not decellularized matrices (Figure 5). The decellularized fragment, on the other hand, presented an organized and dense fiber network without any visible cells. A higher amplification allows the observation of regions with different fiber orientation and densities. For tumour tissue it is possible to observe that the structure has other characteristics (Figure 6). The not decellularized fragment presented a more compact structure when compared to normal tissue, with what appears to be a thin biofilm of cells. On the

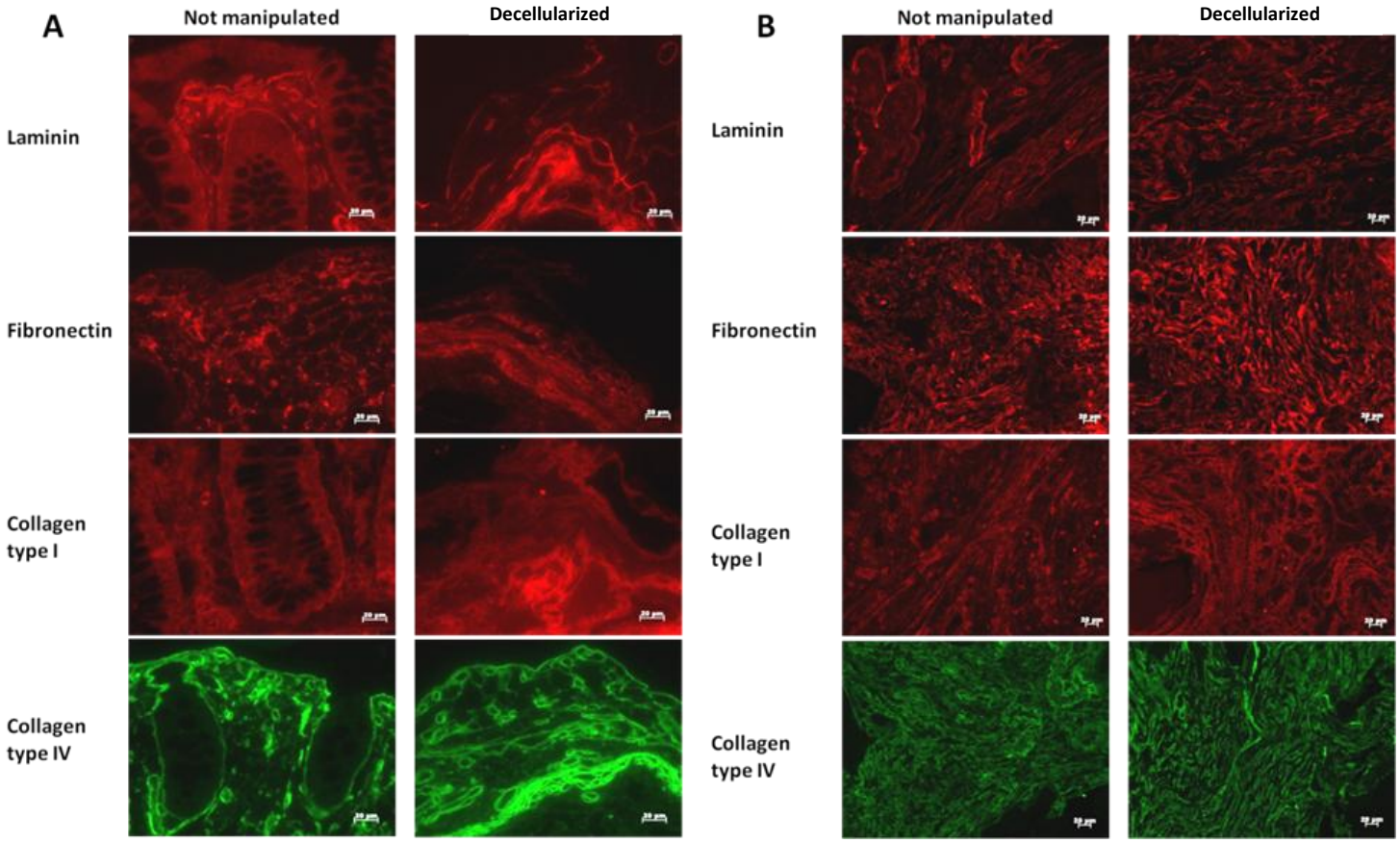

Figure 4 Decellularization retains ECM components of normal and tumour colon tissue. After decellularization, samples were formalin-fixed, processed to paraffin blocks, cut in 3µm sections and stained for laminin, fibronectin, collagen type I and collagen type IV. Not manipulated colon tissue was used as control. A – normal tissue, B – tumour tissue. Scale bar represents 20 µm.

other hand, tumour decellularized fragments displayed a more dense and disorganized fiber meshwork. Additionally, particularly when looking at magnification 5000x, it looks like there are still some cellular components present. For this reason, and to make sure cell debris were being completely removed, we decided to analyse the expression of a membrane protein, E-cadherin, by IHC. In a normal situation this cell-cell adhesion protein is expressed by the epithelial cells present in the glands of the colonic mucosa (Figure 7A). In Figure 7B is possible to observe three tumours from different patients, both not decellularized and decellularized. Not decelularized tumour

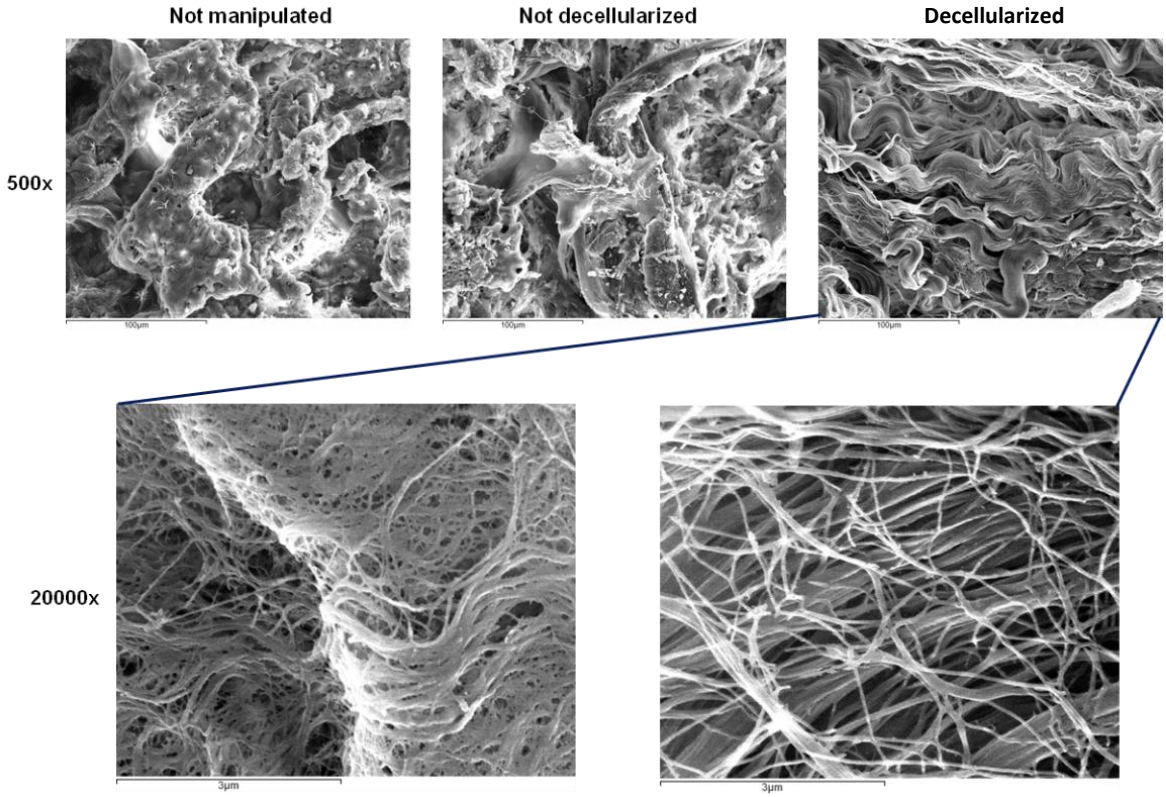

Figure 5 Normal decellularized tissues retain an organized fiber network. After decellularization, samples were fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate for 30 minutes at room temperature. After washing with cacodylate buffer, samples were dehydrated in a graded ethanol-water series to 100% ethanol, critical point dried using CO_2 and then mounted in sticky carbon taps. After this procedure, samples were covered in gold to allow visualization. Not manipulated normal colon tissue was used as control.

fragments, as expected, presented a variable lost of E-cadherin due to the different characteristics of each tumour, namely their endogenous E-cadherin expression. For the decellularized fragments, only tumour A presents some positive staining in the core part while tumours B and C are completely negative. This result seems to suggest that, in fact, what appeared to be cell debris by SEM in the tumour fragments are most probably matrix components, since they were observed on the surface part of the matrix. On the other hand, the staining observed for E-cadherin reveals that, in certain cases, some of the cellular components present in the core of the fragment are not being completetly removed with the decellularization procedure. None of the normal decellularized fragments presented any expression E-cadherin (data not shown) comfirming that most of the cellular components are being removed. The exclusive expression of E-cadherin in the core of a

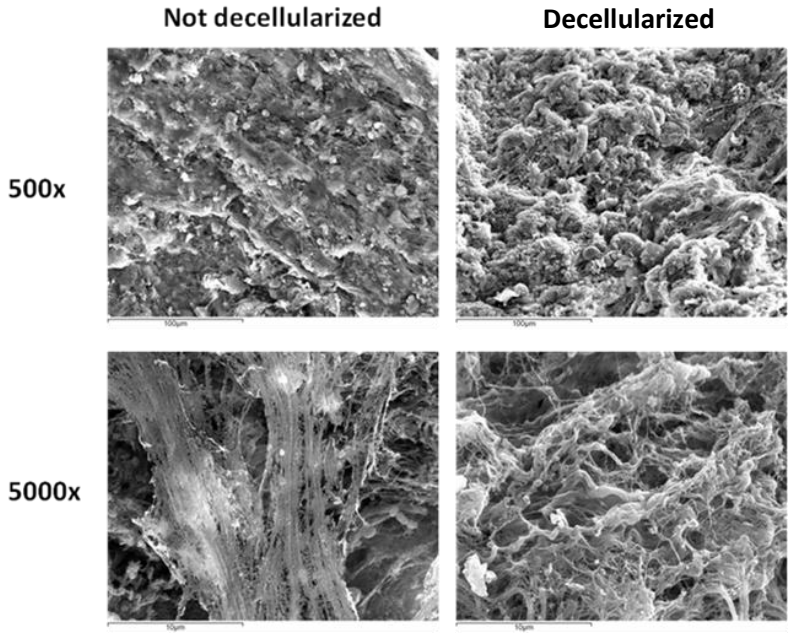

Figure 6 Tumour decellularized tissues presented a more disorganized fiber network and what appears to be some cell debris. After decellularization, samples were fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate for 30 minutes at room temperature. After washing with cacodylate buffer, samples were dehydrated in a graded ethanol-water series to 100% ethanol, critical point dried using CO_2 and then mounted in sticky carbon taps. After this procedure, samples were covered in gold to allow visualization. Not manipulated tumour colon tissue was used as control.

unique decellularized tumour fragment suggests difficulties of access due to the high cellular density and intense fiber meswork at the center of the tumour. In these cases prolonged exposure to the decellularization agents may be required.

2. Repopulation

Due to confidentiality reasons, results are not included.

Given that macrophages are highly plastic cells and can be polarized towards different phenotypes, with major consequences in what concerns their function, we did a search in the literature to know which antibodies could be used to specifically identify macrophage subtypes, M1 and M2. In what concerns M2-macrophages, the most commonly used marker is CD163, a glycoprotein belonging to the scavenger receptor cysteine rich family. For the identification of M1

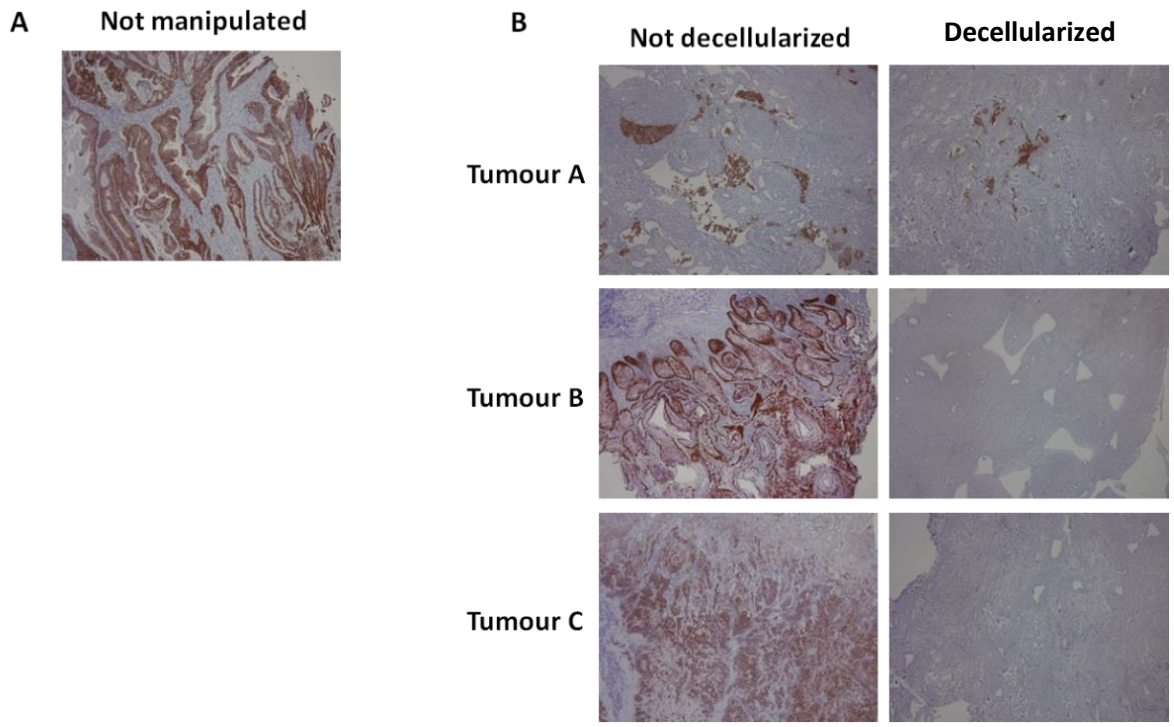

Figure 7 Decellularization does not completely remove E-cadherin from all tumour fragments. After decellularization, samples were formalin-fixed, processed to paraffin blocks, cut in 3µm sections and incubated with an anti-E-cadherin antibody (B). Not manipulated colon tissue was used as control (A). (Magnification 5x)

macrophages, different proteins have been used: HLA-DR, which belongs to the MHC II cell surface receptor; calprotectin, an endogenous molecule with antibacterial, antifungal and immunomodulating properties, released by immune cells; and inducible nitric oxide synthase (iNOS), an enzyme that catalyses the production of nitric oxide (NO) from L-arginine. To evaluate if these were reliable markers to distinguish between the two macrophage populations, we performed an *in vitro* experiment in which we isolated monocytes and plated them in 24-well plates. After 10 days of differentiation, we incubated them for an additional 3 days with LPS or IL-10, two of the standard treatments to induce macrophage polarization towards an M1 or M2 phenotype, respectively. Additionally, not treated (NT) macrophages, frequently designated as naïve were used as negative control (Figure 8). Our results evidenced that LPS treated macrophages are completely negative for CD163 whereas most IL-10 treated macrophages display CD163 staining, even though

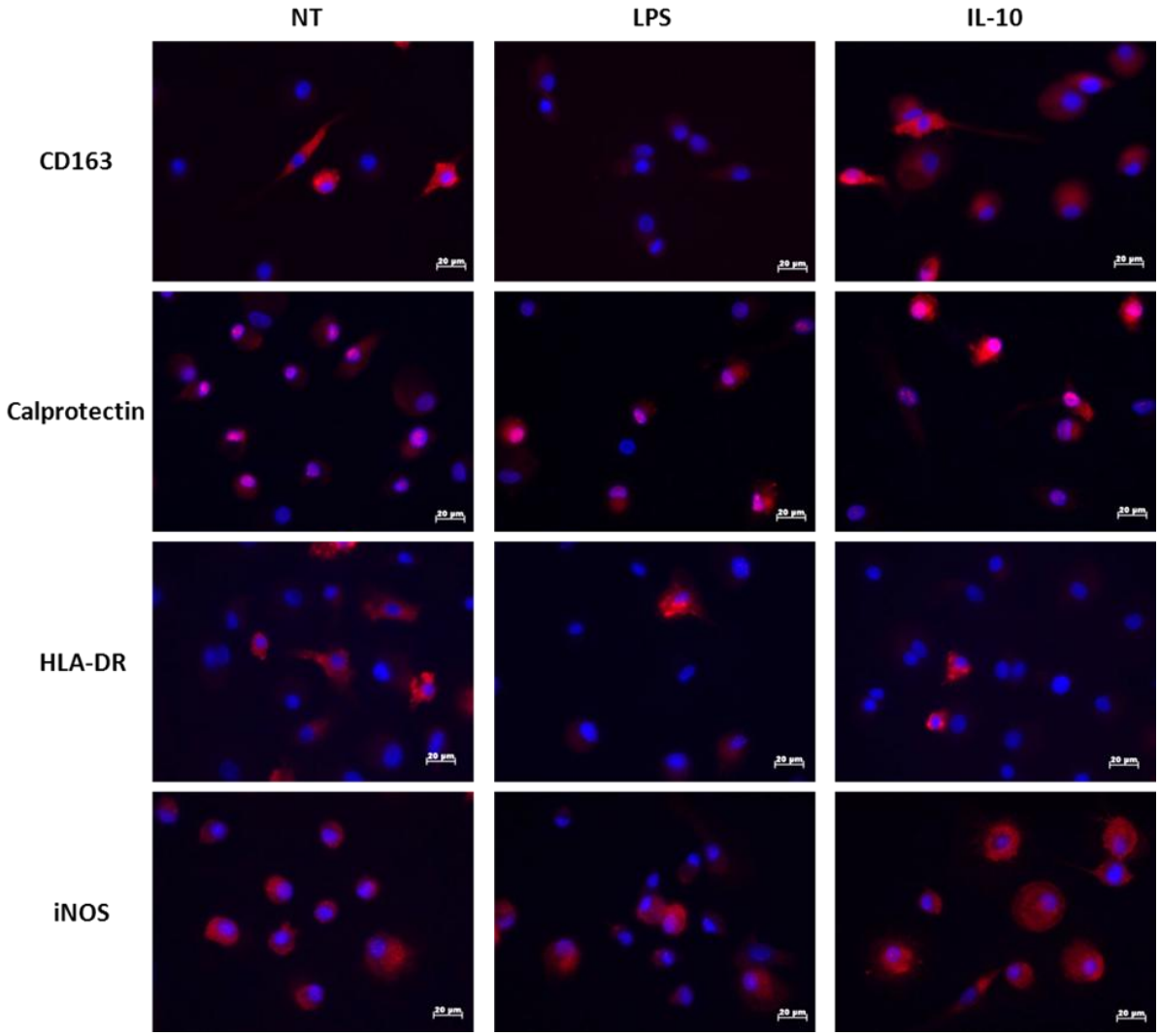

Figure 8 C163 is able to distinguish between LPS and IL-10 treated macrophages while calprotentin, HLA-DR and iNOS stains both cell populations. Monocytes were isolated from buffy coats and allowed to differentiate for 10 days. After an additional 3-day treatment with LPS or IL-10, they were fixed in either methanol of 4%Paraformaldeyde. Cells were blocked with 5% bovine serum albumin and incubated with CD163, calprotectin, HLA-DR or iNOS for 1 hour. After washing, cells were incubated for an additional hour with goat anti-mouse or anti-rabbit AlexaFluor-594-conjugated secondary antibodies and mounted on Vectashield with DAPI.

the intensity varies between cells. Not treated macrophages, as expected due to their heterogeneity, have some positive and some negative cells. These results confirm that CD163 can be used to identify M2-macrophages since it is able to distinguish between the two cell populations. In what concerns the other three markers, calprotectin, HLA-DR and iNOS, all used in previous studies to identify M1 populations, there are no significant differences in the staining pattern between NT,

LPS or IL-10-treated macrophages. Regarding calprotectin, the majority of the cells stained positively, frequently displaying nuclear staining. Analysing HLA-DR, it is evident that most of the cells stained negatively, particularly the LPS or IL-10-macrophages. On the other hand, in what concerns iNOS, the cells exhibit cytoplasmatic staining which, unexpectedly, appears to be more intense in IL-10 treated macrophages. These results seem to suggest that none of the three postulated M1 markers described in the literature can be used for this purpose. Nevertheless, assuming that, *in vivo*, macrophages are subject to additional stimuli absent in the *in vitro* experiments, we decided to evaluate these markers in CRC cases, by IHC. For all them, staining for CD68 was performed in subsequent slides in order to identify macrophages, independently of their polarization. As for iNOS, we are still optimizing the antibody for this specific technique and consequently, no results are presented. In Figure 13 it is possible to observe six different CRC cases, two of them stained for CD163, two for HLA-DR and the other two for calprotectin. Images of the same region stained with CD68 antibody are also depicted. Comparing staining for CD68 and CD163 (Figure 9A), it is evident the overlap between the two markers, clearly demonstrating that CD163 is recognizing specifically macrophages. Conversely, HLA-DR clearly stains other cell populations, negative for CD68 (Figure 9B). In case C it was possible to observe HLA-DR expression in tumour epithelial cells while, in case D, lymphocytes present in lymph nodule are also positive. Based on these results, along with the immunocytochemichal analysis, we decided to exclude HLA-DR marker. Finally, regarding calprotectin, there is also a good correlation between its expression pattern and CD68 (Figure 9C). Moreover, some intense staining is also noticed due to calprotectin secretion, in consonance with its function as an acute phase reactant.

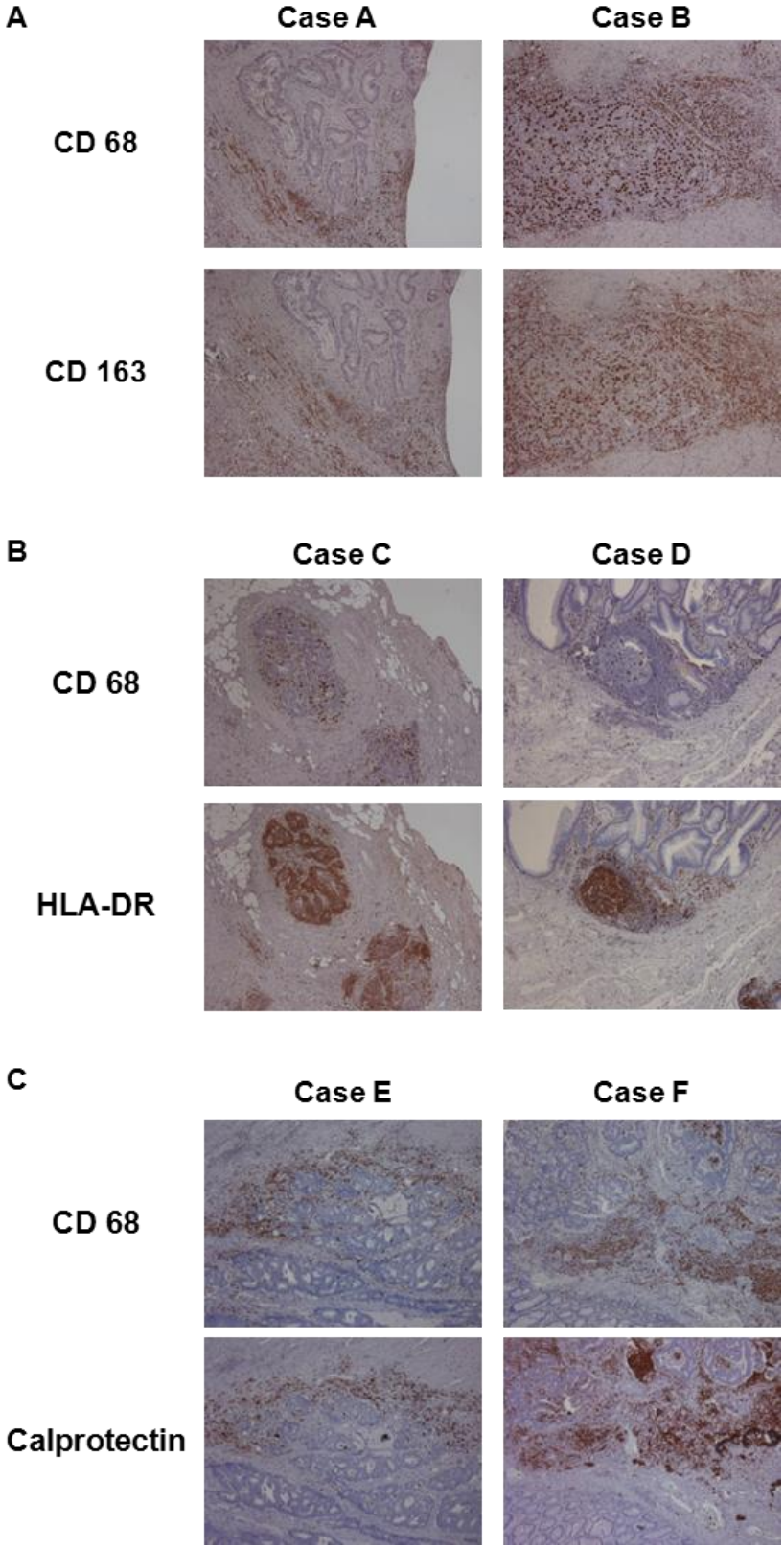

Figure 9 Immunohistochemical staining of CD68, CD163, HLA-DR and Calprotectin in colorectal tissues. Six colorectal cancer cases stained separately with CD68 and CD163 (Cases A and B), CD68 and HLA-DR (cases C and D) and CD68 and Calprotectin (Cases E and F) show different patterns of expression (Magnification 5x).

Discussion

Nowadays it is widely recognized the decisive role of the microecosystem in tumour development and progression. Having this in mind, together with the known limitations of the strategies currently in use, we adopted an innovative approach to address this problem. The work developed in this thesis is pioneer in what concerns the establishment of models to study tumour complexity, with particular focus on the extracellular matrix. We were able to efficiently decellularize both normal and tumour tissues obtained from colorectal cancer patients while keeping, at least partially, the composition and structure of the native tissues. Results regarding repopulation will not be discussed due to confidentiality reasons.

During the last decade several decellularization protocols have been described, mainly in the context of tissue engineering. In this work we used a combination of treatments, some of them are already described in the literature for this purpose. To access decellularization efficiency, we quantified total DNA and performed DAPI staining of the decellularized matrices. In normal colon fragments we obtained a reduction of approximately 100 fold in DNA content, to 6.7ng of DNA/mg tissue while, in tumour colon fragments, the reduction was almost 300 fold, to 3.1ng of DNA/mg tissue. The DNA results obtained are extremely satisfying and fulfill the criteria that were defined as fundamental to validate decellularization efficacy, namely negativity when staining with DAPI, and reduced remnants of DNA. Even though the guidelines regarding DNA quantification were defined as mg per dry tissue[9], and not mg per fresh tissue, as we did, this will not make a significant difference in the obtained results. The values we obtained are in agreement to descriptions of the literature. Decellularization of rats small bowel provided similar results, obtaining a DNA removal of about 99% to 2.9ng of DNA/mg tissue[123] while decellularization experiments using mice small intestine concluded that SDS was the most efficient decellularization method, with a final DNA content of 8.63ng DNA/mg tissue[124]. In what concerns human tissue, Bondioli *et al* used human skin and DNA results revealed a decellularization yield of 85.6%, with the decellularized fragments still retaining 120ng of DNA/mg tissue[125]. Certainly some of the differences have to do, not only with efficacy of the selected protocol, but also with the specific characteristics of the tissue as well as the thickness of the fragments used. The fact that the DNA reduction observed in tumour fragments was higher than in normal ones may be related to its higher disorganization and confirms, as expected, that both tissues have different characteristics.

The maintenance of the integrity and composition, both structural and chemical, of the decellularized matrices is crucial to validate any decellularization protocol. In this regard, the

obtained results were quite encouraging. In line to work already published, we analyzed our decellularized matrices by numerous techniques, namely HE and MT stainings, SEM, IHC and GAGs quantification.

The two histological stainings, performed before and after decellularization, confirmed the successful removal of cells, without any visible nuclei. Additionally, they allowed the assessment of matrix structure and integrity. In what concerns normal tissue, it is possible to observe that the organization is partially affected. Normal colonic epithelium has a complex architecture composed of tubular structures called crypts or glands that are arranged in parallel and embedded in a loose connective tissue called *lamina propria*. For this reason, it is not completely unexpected that we observe a partial collapse of the structure once the cells are removed. On the other hand, the fact that we are using just a small fragment of colon instead of an entire organ, surely contributes to this disruption. Conversely, the architecture of tumour tissue does not appear to be affected with the decellularization protocol. This is probably related with the higher density of ECM fibers that are able to support the 3D structure after cell removal.

Results regarding composition, specifically IHC analysis of laminin, fibronectin and collagens type I and IV are very encouraging since is possible to observe a similar organization and distribution pattern when comparing decellularized tissue with native one. This is particularly evident in tumour, whereas in normal matrices it is apparent some disorganization in the glands architecture, as previously mentioned. Despite these results, it would be important to complement this evaluation by performing a more quantitative analysis using, for example, specific kits available to quantify the different ECM components, namely collagens, following a strategy similar to the one used by Younstrom and colleagues after decellularizing horse tendons[126]. This was in fact the strategy we followed as a way to evaluate GAGs maintenance in the decellularized fragments. Nevertheless, the results are not as positive since we observe a reduction in GAGs content of approximately tenfold in both normal and tumour matrices. This result is not completely unexpected since a significant percentage of GAGs are components of cellular membranes that will inevitably be lost with the removal of cells. Surprisingly, there are a substantial number of reports, such as the one by Totonelli and colleagues, using rat intestine, or the one by Bondioli, using human skin, where no alterations in GAGs content were observed. These differences are probably related to the fact that their results are presented as µg GAGs/dry tissue instead of µg GAGs/mg initial tissue. As a consequence, since the dry weight is much smaller than the initial one, the GAGs amount in the decellularized fragments will increase. In our opinion, this is not the most accurate way of presenting these results since we are not interested in the relative amount of GAGs but in accessing

their quantity in the decellularized tissue in relation to the amount present in native one. This is even more relevant if we carefully analyse some of the published work. Very recently, Oliveira and colleagues tested a variety of decellularization methods in small intestine from mice, and claimed that GAGs content was not affected with a variety of different decellularization protocols even though it is very obvious a decrease in Alcian Blue proteoglycan staining[124]. On the other hand, this strategy of presenting data may, in fact, lead to confusing results such as the ones obtained from Baptista *et al* in which, after decellularizing livers from different animals, they observed a significant increase in GAGs content comparing to the native organ. They justify this difference by the absence of cellular components[127]. Results such as these are misleading and don't provide accurate information about the amount retained after the decellularization protocol.

The lost in GAGs content of almost 90% clearly exposes one of the limitations of this strategy and raises the possibility that other important components, namely growth factors, are also being removed with the decellularization protocol. This was confirmed by other reports where a significant reduction in some growth factors such as VEGF en dermal decellularized skin was observed[125].

When evaluating the overall results regarding the tumour fragments, particularly the Masson's Trichrome staining and the E-cadherin IHC, it is clear that, in some particular cases, residual cellular fragments are not being completely removed during the decellularization process. Consequently, we cannot exclude that these components, when not entirely removed, will have a contribution on macrophage polarization. Nevertheless, we only observed these in some particular cases and we believe that it can be avoided by using thinner tumour fragments in order to facilitate the access of the decellularizing agents. One important analysis that is missing in this thesis is the evaluation of the viscoelastic properties of the decellularized matrices. Giving the already mentioned importance of ECM biomechanical properties in tumour progression it would be essential to confirm if, after decellularization, the differences in stiffness/rigidity between normal and tumour matrices are, at least partially, retained. Ideally tumour decellularized matrices should maintain the higher rigidity when compared to normal decellularized matrices. This parameter is even more relevant based on the reports describing the influence of tissues biomechanical properties in macrophage polarization. Previous experiments using alveolar macrophages cultured on polyacrylamide gels with two concentrations of bis-acrylamide, representing low and intermediate stiffness, revealed that macrophage shape changed dramatically without generating F-actin stress fibers[128]. More recently, Patel and colleagues elegantly demonstrated that cell elasticity is modulated by substrate rigidity and stretch, and that this is a determinant factor in macrophages

function. By using macrophages of different origins and species, combined with polyacrylamide gels with different characteristics, they were able to prove that specific macrophage functions, such as phagocytosis or LPS responsiveness, are a consequence of cell elasticity and dependent from actin polymerization and rhoGTPase activity[129]. This knowledge is particularly helpful in the context of tissue engineering. It was demonstrated that Poly(ethylene glycol) (PEG) hydrogels, modified with RGD, with lower stiffness lead to reduced macrophage activation and a less severe and more typical foreign body reaction (FBR), and therefore are more suited for *in vivo* applications[130].

One critical aspect in macrophage characterization is the finding of more specific markers to distinguish between M1 and M2 populations. Nevertheless this does not present an easy task, particularly if we consider macrophage plasticity and the fact that this M1/M2 dichotomy represents two extremes of a continuum of intermediate cells[25]. Many of the studies published are based on markers that are not specific for macrophage populations, such as MMPs[37] or cytokines[131], and are not able to discriminate between both populations. In the experiments we performed, the only marker that was able to discriminate macrophage populations was CD163 and, in fact, this is the most commonly used to identify M2-macrophages[41,132]. As for M1-populations, the situation is more complex and, so far, no marker has gained consensus in the scientific community. All the three potential M1-markers described in the literature were also expressed by M2-macrophages in our *in vitro* experiments. Even though IHC, done in tissues, is completely different from immunocytochemistry, done in isolated cells, and so we cannot exclude any of these markers, the obtained results raise some concern. In fact, only HLA-DR, the marker used for the previously mentioned meta-analysis[40] proved to be a bad marker since it recognizes both colorectal epithelial cells and lymphocytes. Additionally it has also been shown to be similarly expressed by both populations[133]. As for calprotectin or iNOS we will have to try to understand the reason why they are also being expressed by IL-10 treated macrophages, since they are described to be related to pro-inflammatory populations. Based on all these results we believe that, in order to make an accurate and reliable characterization of macrophage subtypes, we will need to combine at least two cell surface markers for each population, always joint with CD68.

Conclusions

Decellularization is a strategy that is being particularly studied in the biomaterials field as an alternative for organ transplantation and for regenerative purposes. We believe that this is also a good approach to study more basic mechanisms and the interplay between the components of a certain microecosystem. Results regarding repopulation will not be mentioned due to confidentiality reasons.

The effectiveness of any decellularization protocol depends on many factors, including tissue's cellular density, fiber meshwork and thickness. The selected decellularization protocol proved to be efficient in removing DNA and cell components, while retaining part of the characteristics of the native tissues, even though some complementary evaluation of tissue biomechanical properties has to be performed.

It is important to keep in mind that, with cell removal during decellularization, the partial disruption of ECM chemical composition and structural organization are inevitable. Nevertheless, we strongly believe that, even with its limitations, this is the best method to create cultures more similar to *in vivo* as possible.

Future work

This innovative work allowed the establishment of a 3D culture system using decellularized matrices from normal and tumour, in order to study macrophage polarization. Nevertheless, as frequently happens, the results raised more questions than provided answers.

To complete the characterization of the decellularized matrices we will have to quantify components such as collagens or growth factors. Additionally, their characterization in terms of viscoelastic behavior is of great importance. We intend to perform this evaluation by dynamic mechanical analysis (DMA) which is a technique used for many years to study the viscoelastic properties of materials[139]. The technical principle consists of applying a sinusoidal deformation to a sample and, as a consequence, the sample will deform a certain amount, depending on the stress. The level of tissue deformation is related to its stiffness. As an alternative, we can use atomic force microscopy, similarly to what Goh *et al* used to characterize mice pancreas after decellularization[127].

Another very important aspect in which we will invest is the finding of good immunohistochemical markers to allow a more accurate identification of M1-macrophages. We will continue to explore which are the best candidates and, eventually, select two or more that should eventually be combined to perform a rigorous analysis.

Finally, we intend to characterize macrophage populations in colorectal cancer cases from Centro Hospitalar São João (CHSJ) Tumour Bank. Normal and tumour samples from the same patient will be profiled, as well as the transition region from one to the other. In addition, the same sections will be used to evaluate ECM components, such as laminin or fibronectin. Altogether, we propose to explore the relation between ECM/macrophage subpopulations distribution pattern and cross this data with patient clinicopathological information.

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