



Universidade do Minho
I3Bs - Instituto de Investigação em Biomateriais,
Biodegradáveis e Biomiméticos

Daniel Barreira Rodrigues

**Bioengineering strategies for cancer therapy
and modelling**

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Bioengineering strategies for cancer therapy and modelling

Tese de Doutoramento em Engenharia de Tecidos,
Medicina Regenerativa e Células Estaminais

Trabalho efetuado sob a orientação

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As once said by the American ultrarunner Dean Karnazes, “If You Want To Talk To God, Run An Ultra”, I firmly believe that the I path undertook in order to achieve this body of work required a similar mental fortitude and wouldn't have been possible without a support crew to whom I own my deepest thanks and appreciation.

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Ultimately, I would like to acknowledge the financial support from FCT for my fellowship that financed the projects behind this thesis.

STATEMENT OF INTEGRITY

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ABSTRACT

Cancer is a global pandemic with a high incidence among the world population and effective treatments are for the most part elusive. The tumor microenvironment is a highly complex and heterotypic mixture of cells that interact to regulate central control mechanisms, driving immunosuppression and promoting both survival and invasion of cancer cells into surrounding tissues. It has been this complexity that has made finding effective therapeutics such a demanding task and therefore cancer still remains a burden worldwide in health as well as in economic terms. While the progression in the field of cancer research has been clear over the years, there are still several challenges that need to be addressed.

Herein, two different sides to this disease are explored: treatment and *in vitro* models. Adoptive T cell therapy has shown impressive results, however not without its limitations. The use of the T cell mitogen IL-2 within culture systems is known to lead to early exhaustion of T cell subsets while high density of co-stimulating molecules has been linked to undesired immune responses. As an alternative, a nanoparticle system based on the natural polymer gellan-gum was proposed, with tailorable surface functionalization with co-stimulatory molecules. High levels of T cell expansion were observed over the studied period, with secreted IL-2 levels overcoming those of commercial alternatives. With this system, increased expression of cytotoxic molecules Granzyme B and Perforin were also detected *in vitro*. On the other spectrum, 3D cancer models have sustained a great number of developments observed by an increase in similarity towards native tissues; however, a requirement for even more complex architectures capable of better mimicking cellular interactions is still present. Therefore, an assembloid-based approach was proposed to develop a 3D *in vitro* melanoma model to further study cellular interactions. These heterotypic tumor assembloids presented a complex architecture capable of sustaining endothelial cell function as well as a high expression of stemness-related markers. These models were subjected to functionality assays where they showed a capacity for “cooperative invasion” which was coincident with an observed increased production of MMP-2. To further unravel the role of stromal cells in the invasive potential of cancer cells a 3D chemotaxis chamber was developed to study cellular interactions observed in the tumor microenvironment, where stem cells and fibroblasts showed to have a crucial role. Ultimately, this thesis allowed to explore biomedical engineering approaches to further contribute to the knowledge in the field opening new doors to be explored in the future.

Keywords: nanoparticles, gellan gum, T cells, heterotypic cancer models, assembloids, chemotaxis.

RESUMO

O cancro é uma pandemia global com uma elevada incidência e cujo desenvolvimento de tratamentos eficazes continua a ser difícil. O microambiente tumoral é uma mistura altamente complexa e heterotípica de células que interagem para regular mecanismos centrais que provocam imunossupressão promovendo a sobrevivência e invasão de células tumorais para os tecidos circundantes. É esta complexidade que tem tornado desafiante encontrar terapias eficazes, tornando esta doença um fardo a nível global em termos de saúde e economia. Enquanto a progressão na área da investigação oncológica tem sido clara ao longo dos anos, existem ainda vários desafios que precisam de serem encarados para permitir futuros desenvolvimentos.

Aqui, foram exploradas duas vertentes diferentes desta doença: o tratamento e os modelos *in vitro*. A terapia celular adotiva tem demonstrado resultados impressionantes, no entanto não sem as suas limitações. O uso do mitogénio IL-2 nestes sistemas de cultura é conhecido por levar rapidamente à exaustão das células T, enquanto o uso de moléculas co-estimulatórias em elevadas densidades está associado a respostas imunes não desejadas. Como alternativa, foi proposto um sistema de nanopartículas baseado no polímero natural goma gelana e funcionalizado com moléculas co-estimulatórias. Foram observados elevados níveis de expansão de células T e quantidade de IL-2 secretada superior à de alternativas comerciais. Foi ainda verificado *in vitro* um aumento de expressão das moléculas citotóxicas Granzima B e Perforina. No outro espectro, têm sido desenvolvidos modelos tumorais 3D com uma cada vez maior similaridade para tecidos nativos; no entanto, a necessidade de arquiteturas ainda mais complexas capazes de melhor representar interações celulares persiste. Assim, foi proposta uma abordagem baseada em “assemblóides” para obter modelos 3D *in vitro* de melanoma para estudar interações celulares. Estes “assemblóides” tumorais heterotípicos apresentaram uma arquitetura complexa capaz de suportar a função de células endoteliais, bem como a elevada expressão de marcadores de pluripotência. Estes modelos foram sujeitos a ensaios de funcionalidade onde mostraram a capacidade de “invasão cooperativa” que foi coincidente com uma produção aumentada de MMP-2. Para tornar mais claro o papel das células estaminais no potencial invasivo de células tumorais, uma câmara 3D de quimiotaxia foi desenvolvida para estudar as interações celulares observadas no microambiente tumoral onde as células estaminais e fibroblastos mostraram ter um papel determinante. Em última análise, esta tese permitiu explorar abordagens da engenharia biomédica de forma a contribuir para o conhecimento da área e abrir novas portas a serem exploradas no futuro.

Palavras-chave: nanopartículas, goma gelana, células T, modelos de cancro heterotípicos, assemblóides, quimiotaxia.

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LIST OF ABBREVIATIONS

#

2D – two dimensional

3D – three dimensional

A

aAPCs – Artificial antigen presenting cells

Abs – Absorbance

ACT - Adoptive cell transfer

AICD – Activation-induced cell death

a-MEM – Minimum Essential Medium

ANOVA – Analysis of variance

APC - Antigen presenting cells

ASCs – Adipose-derived stem cells

B

BSA - Bovine serum albumin

C

CAD - Computer-assisted design

CAFs - Cancer associated fibroblasts

CAMs - Cell adhesion molecules

CAR - Chimeric antigen receptor

CD – Cluster of differentiation

CED - Convection enhanced delivery

Cells/mL - Cell per milliliter

CFSE - Carboxyfluorescein succinimidyl ester

cm – centimeter

CMC - Carboxymethyl cellulose

CO₂ – Carbon dioxide

CSCs - Cancer stem cells

CTLA - Cytotoxic T-lymphocyte-associated protein

CTRL - Control

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

D

Da - Dalton

DAPI - 4',6-diamidino-2-phenylindole

DCs – Dendritic cells

DLS - Dynamic light scattering

DMSO - Dimethyl sulfoxide

DNA - Deoxyribonucleic acid

DOX - Doxorubicin

E

EC – Endothelial cells

ECM – Extracellular matrix

EDC - 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide

EDTA - Ethylenediaminetetraacetic acid

EGF - Epidermal growth factor

EGM – Endothelial Growth Medium

ELISA – Enzyme-linked immunosorbent assay

EMT - Epithelial–mesenchymal transition

EV - Extracellular vesicles

F

FAP - Fibroblast activation protein

FBS - Fetal bovine serum

FGF - Fibroblast growth factor

FIB - Focused Ion Beam

FITC - Fluorescein isothiocyanate

FTIR - Fourier-transform infrared spectroscopy

G

G' – Storage Modulus (Shear)

G'' – Loss Modulus (Shear)

GB - Glioblastoma

GC - Glycated chitosan

GG - Gellan gum

GM-CSF - Granulocyte-macrophage colony-stimulating factor

GOD - Glucose oxidase

GrB - Granzyme B

H

h – Hours

H&E – Hematoxylin–eosin

HA - Hyaluronic acid

hASCs - Human adipose derived stem cells

hDFbs – Human dermal fibroblasts

hDMECs – Human dermal microvascular endothelial cells

HIF – Hypoxia-inducible factor

HLA - Human leukocyte antigens

HPMFs - Human primary mammary fibroblasts

HR - Homologous recombination

hTERT – Human telomerase reverse transcriptase

HUVECs - human umbilical vein endothelial cells

Hz – Hertz

I

ICOS - Inducible T cell COStimulator

ID - Intradermal

IDO - Indoleamine 2,3-dioxygenase

IFN – Interferon

IGF - Insulin-like growth factor

IgG - Immunoglobulin G

IL – Interleukin

IMQs – Imidazoquinolines

ISH - In situ hybridization

ITGB - Integrin beta

K

kV – Kilovolt

L

LAG3 - Lymphocyte activation gene 3

LC - Langerhans cells

LDH - Lactate Dehydrogenase

LPA - Lysophosphatidic acid

LPS – Lipopolysaccharide

LYVE - Lymphatic vessel endothelial hyaluronan receptor

M

M – Molar

MAGS - Reduced graphene oxide

M-CSF - Macrophage Colony-Stimulating Factor

MCSs - Multicellular spheroids

MES - 2-(N-morpholino)ethanesulfonic acid

μm – Micrometer

μM – Micromolar

mg/mL – Milligram per milliliter

MHC - Major histocompatibility complex

min - Minutes

MIP - Macrophage inflammatory protein

mL – Milliliters

mmol/L – Millimole per liter

MMPs - Matrix metalloproteinases

MPL - Monophosphoryl lipid

mRNA – Messenger RNA

Ms - Mouse

MTS – 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

MW– Molecular weight

N

N - Newton

NaV - NeutrAvidin

NHS N-Hydroxysuccinimide

NK - Natural killer

nm – Nanometer

nM – Nanomolar

NOS - Nitric oxide synthases

npGG - Gellan gum nanoparticles

NPs – Nanoparticles

O

O/W - Oil-in-water

O/W/O - Oil-in-water-in-oil

ODN – Oligodeoxynucleotides

OMS - Organotypic Multicellular Spheroids

OVA – Ovalbumin

P

% – Percentage

(w/v) – Weight/volume percentage

p – Statistical level of significance

Pa – Pascal

PBMCs - Peripheral blood mononuclear cells

PBS - Phosphate buffer saline

PDGF - Platelet-derived growth factor

PDI - Polydispersity index

PDL - Programmed death-ligand

PDX - Patient-derived xenograft

Phalloidin – Phalloidin–Tetramethylrhodamine B isothiocyanate

PMA - Phorbol myristate acetate

PMMA - Polymethyl methacrylate

POU5F1 - POU Class 5 Homeobox 1

Prf - Perforin

PSR - Picro-Sirius Red

PTT - Photothermal therapy

PVA - Poly(vinyl alcohol)

Q

qPCR – quantitative real-time Polymerase Chain Reaction

R

Rb - Rabbit

RGD - Arginylglycylaspartic acid (RGD)

RHAMM - Receptor for hyaluronan-mediated motility

RNA - Ribonucleic Acid

RPMI - Roswell Park Memorial Institute

RT – Room temperature

S

SD – Standard deviation

SDS - PAGE - Sodium dodecyl-sulfate

polyacrylamide gel electrophoresis (SDS-PAGE)

SEM – Scanning electronic microscopy

SOX2 - SRY-Box Transcription Factor 2

STAT - Signal transducer and activator of transcription

STEM - Scanning transmission electron microscope

SWNT - Single-walled carbon nanotube

T

TAA - Tumor associated antigens

TDTs - Tissue-derived tumor spheres

TERM – Tissue engineering and regenerative medicine

TGF - Transforming Growth Factor

TH cells – T helper cells
TIL – Tumor infiltrating lymphocytes
TIMP - Tissue inhibitors of metalloproteinases
TLR - Toll-like receptor
TME - Tumor microenvironment
TNF – Tumor necrosis factor
Treg – Regulatory T cells
TRIS - Tris(hydroxymethyl)aminomethane
TRITC - Tetramethylrhodamine B isothiocyanate

U

UDP-glucose - Uridine diphosphate glucose
ULA - Ultra-low attachment
UM - Uveal melanoma

V

V – Volume
VEGF – Vascular endothelial growth factor
(v/v) – Volume/volume percentage

W

W/O - Water-in-oil
Wd – Dry weight
Wt - Weight
Ww – Wet weight

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SHORT *CURRICULUM VITAE*

Daniel Barreira Rodrigues was born on the 13th of December 1988, in New Jersey, USA. In 2012, he concluded his major in Biomedical Sciences (MSc) with a final mark of 17 over 20 at the Faculty of Health Sciences of the University of Beira Interior. During this period, he developed his MSc dissertation on Regucalcin regulation by extracellular calcium in prostate cells, under the supervision of Dr. Cláudio Maia and Dr. Sílvia Socorro. In result of this work, Daniel published an original research paper and co-authored a book chapter.

In 2012, Daniel joined the 3B's Research Group at the University of Minho. During the early years he participated in several different research projects in the TERM field after which he proceeded with his academic career going back to his roots of cancer related studies. In 2016, he was awarded an individual PhD fellowship from FCT (SFRH/BD/119756/2016) and initiated his studies under the PhD program in Tissue Engineering, Regenerative Medicine and Stem Cells (TERM&SC) at the University of Minho looking into novel bioengineering approaches for cancer therapy and *in vitro* modelling while under the supervision Dr. Rogério Pirraco and Prof. Rui L. Reis. During this period, he had the opportunity to work in collaboration with Dr. Gil Castro at the Life and Health Sciences Research Institute (ICVS), Braga, Portugal for several years.

From early on Daniel made different collaborative efforts in other ongoing projects, while supporting the instruction of different foreign students, taking responsibility for laboratory trainings and serving as student representative in the 3B's Research Group Scientific Council, as well as, in both the Academic Senate and the Pedagogical Council of the University of Minho.

As a result, until this date, his track record of innovation and research includes 1 patent, 8 peer-reviewed original papers (4 as first author), 3 review papers, 1 book chapter co-authored, 5 first-author oral presentations in international conferences, and several poster presentations.

LIST OF PUBLICATIONS

The work performed during the PhD period resulted in the publications listed below:

PAPERS IN INTERNATIONAL SCIENTIFIC JOURNALS WITH REFEREES (AS FIRST AUTHOR)

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Rodrigues DB, Reis RL, Pirraco RP. How are natural-based polymers shaping the future of cancer immunotherapy: a scoping review. *Submitted* (2023)

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Moreira HR, **Rodrigues DB**, Freitas-Ribeiro S, da Silva LP, Morais AS, M. Jarnalo, R. Horta, R.L. Reis, R.P. Pirraco, A.P. Marques. Spongy-like hydrogels prevascularization with the adipose tissue vascular fraction delays cutaneous wound healing by sustaining inflammatory cell influx, *Mater. Today Bio.* 17 (2022) 100496.

da Silva LP, Santos TC, **Rodrigues DB**, Pirraco RP, Cerqueira MT, Reis RL, Correlo VM, Marques AP. Stem Cell-Containing Hyaluronic Acid-Based Spongy Hydrogels for Integrated Diabetic Wound Healing, J. Invest. Dermatol. (2017).

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PATENTS

Rodrigues DB, Moreira HR, Gasperini L, Cerqueira MT, Marques AP, Pirraco RP, Reis RL. HYDROGEL-LIKE PARTICLES, METHODS AND USES THEREOF, WO/2021/064678 (2021)

ABSTRACTS PUBLISHED IN INDEXED JOURNALS

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Moreira HR, **Rodrigues DB**, Reis RL, Marques AP. (2017) Unravelling the path to create a cell sheet-based model of skin scar-like tissue. 0236, European Cells and Materials 33 (2). ISSN 1473-2262.

CONFERENCE ORAL PRESENTATIONS

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Rodrigues DB*, Moreira HR*, Reis RL, Marques AP. “Generation of an *in vitro* 3D cell sheet-based model of scar-like tissue”, 0913, World Conference of Biomechanics, Dublin, Ireland, July (2018)

*Both authors contributed equally to this work

Rodrigues DB*, Moreira HR*, Pires RA, Rui L. Reis, Marques AP. Scar-like tissue analog based on an *in vitro* 3D cell sheet model. Gene2Skin Final Conference, Porto, Portugal (2018)

*Both authors contributed equally to this work

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Moreira HR*, **Rodrigues DB***, Reis RL, Marques AP. Unravelling the path to create a cell sheet-based model of skin scar-like tissue, TERMIS EU-Chapter, Davos, Switzerland (2017)

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Rodrigues DB, Cruz-Moreira D, Gomes CF, Reis RL, Pirraco RP. Laser photoablation as a tool to trigger EMT responses in melanoma multicellular spheroids; Final Achilles Conference, Guimarães, Portugal, May (2022)

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Ma M, **Rodrigues DB**, Pirraco RP, Chen J, Reis RL. pH sensitive Gellan Gum copolymer for advanced therapies, TERM STEM, Braga, Portugal, November (2019), P22/128

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*Both authors contributed equally to this work.

Rodrigues DB, Moreira HR, Gasperini L, Cerqueira MT, Marques AP, Santos TC, Reis RL. Tailoring of an artificial antigen presenting cell system for immunotherapy. Chem2Nature, P79, Porto, Portugal (2018)

Rodrigues DB, Moreira HR, Gasperini L, Cerqueira MT, Silva LP, Marques AP, Santos TC, Reis RL. Gellan gum-based antigen presenting cells for immunomodulation through T cell priming, F087, TERM STEM/Forecast, Porto, Portugal, November (2017)

Moreira HR*, **Rodrigues DB***, Pires RA, Reis RL, Marques AP Cell sheet-based model for understanding scarring pathophysiology. The Gene2Skin Conference, Guimarães, Portugal October (2016).

*Both authors contributed equally to this work.

Rodrigues DB, Oliveira JM, Reis RL, Santos TC. Carboxymethylchitosan conjugated dendrimers as potential immune modulators. TermStem Confrence, Guimarães, Portugal October (2016).

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INTRODUCTION TO THE STRUCTURE OF THE THESIS

The field of cancer biology, while subject of constant scientific advances, is still underdeveloped when considering the end goals of both prevention and treatment with a high degree of efficacy of the different known tumor types. For this to be attainable, the body of knowledge on how tumors develop, progress, invade and metastasize and how they respond to novel therapeutics must be significantly enriched.

Throughout this thesis, several different aspects of the field of cancer biology were addressed through the development of novel strategies that focused on cancer immunotherapy as well as on the development of complex *in vitro* tumor models. Concepts underlying these two topics and their supporting literature were extensively overviewed throughout Chapters I and II. The field of immunotherapy has resorted to different materials and building blocks in order to develop novel systems that may interact with the immune system in a highly tailorable fashion. Within **Chapter I**, we make a comprehensive analysis of the different strategies used in immunotherapy with a special emphasis on the natural polymers that have been targeted as resources for the development of immunotherapeutic systems, what are their advantages when compared to alternative materials and a state-of-the-art on how these natural polymers are already being used in currently existing therapeutics. However, in order to validate such novel systems, *in vitro* tumor models capable of mimicking the tumor microenvironment are required. In **Chapter II**, we explore key aspects of cell-cell and cell-ECM interactions while performing a detailed analysis on the various methods of preparation of one of the most explored 3D *in vitro* tumor model which is the spheroid. We also delve into recent reports on the presence of those key interactions in several forms of spheroid tumor models and reflect on potential future achievements in terms of novel complex tumor models capable of better recapitulating *in vivo* cellular interactions that drive tumor progression and invasion.

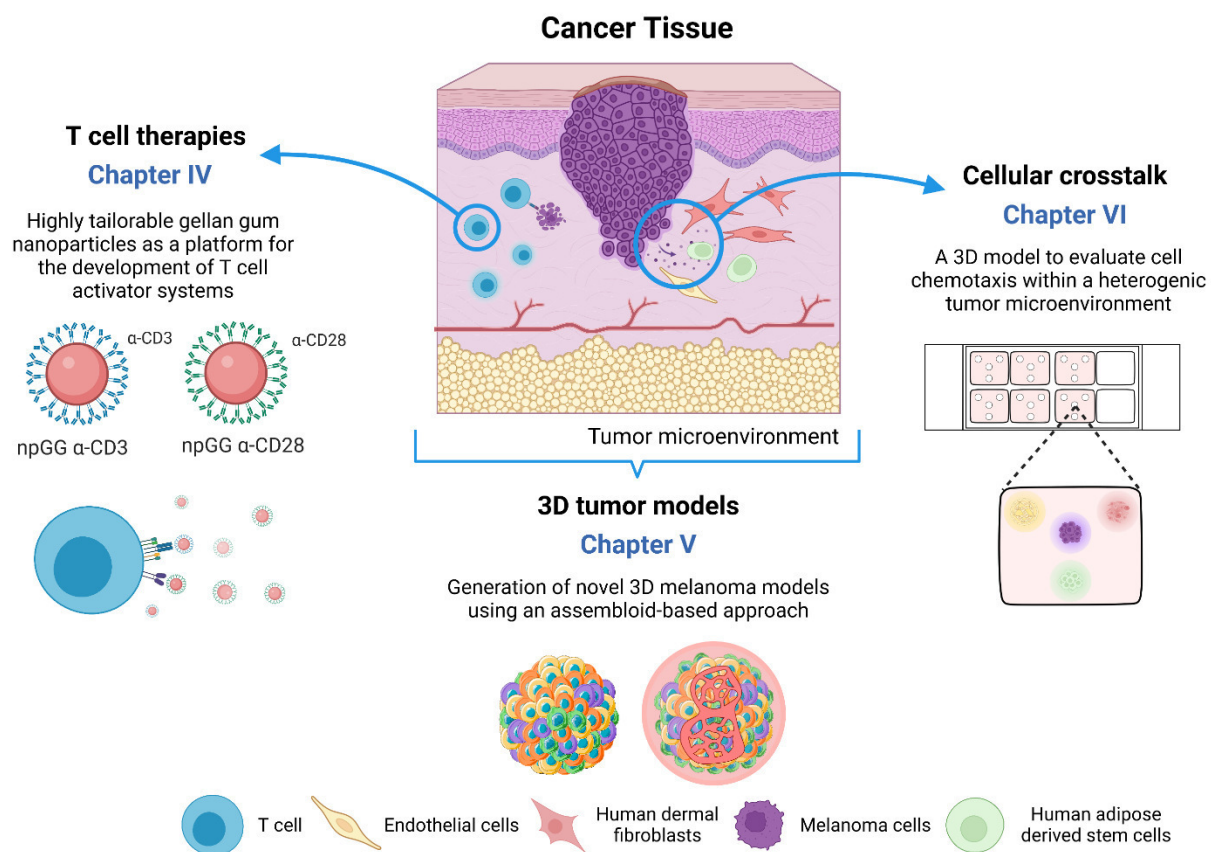
The following **Chapter III** overviews the methodologies used throughout the experimental work here presented. The purpose of this chapter is to give additional details on the experimental chapters that follow, covering the rationale behind the choice of materials as well as of the production and characterization techniques used. In sum, further insight is given to the reader on the principles behind these techniques and their role in the work herein described.

Throughout the following chapters, the main body of work done during my PhD is presented. The chapters are based on original manuscripts and patents prepared during this period. Each chapter is

structured as an adapted version of each manuscript, including its main contents (abstract, introduction, materials and methods, results, discussion, conclusions and references).

In **Chapter IV**, we describe the development of a novel platform for *in vitro* T cell activation. In this work, we tackled both the production of gellan gum nanoparticles as well as their functionalization with relevant co-stimulatory antibodies. We took a step further and studied their functionality *in vitro* in stimulating relevant T cell subpopulations. In Chapters V and VI we shift our focus to the development of a heterotypic 3D tumor *in vitro* model and the understanding of cellular crosstalk between stromal cells and tumor cells. To carry out these studies, melanoma was defined as our disease model. **Chapter V** describes how endothelial cells, fibroblasts, stem cells and melanoma cells were used to create a spherical tumor model based on the assembloid methods. Stromal compartments were produced and assembled into a more complex tumor architecture where the impact of stromal cells was studied at different levels such as 3D cell invasion. Given the obtained results, in the subsequent **Chapter VI** we carried on to develop a 3D culture platform that would allow inter-cellular communication and that would serve as a base to evaluate cell migration in response to paracrine signaling. This platform was characterized in its ability to allow the diffusion of biomolecules and cell recruitment between the different cell populations was assessed.

Finally, in **Chapter VII**, we outline the accomplishments and the significance of the presented works for their respective fields and speculate on putative future developments fueled by those.



Scheme illustrating the different approaches used throughout this thesis for the exploration of both novel therapeutic approaches and the creation of relevant 3D *in vitro* cancer models. npGG, Gellan gum nanoparticles.

*“If You Want To Run, Run A Mile.
If You Want To Experience A Different Life, Run A Marathon.
If You Want To Talk To God, Run An Ultra”*

Dean Karnazes

SECTION 1

GENERAL INTRODUCTION

CHAPTER I

HOW ARE NATURAL-BASED POLYMERS SHAPING THE FUTURE OF CANCER IMMUNOTHERAPY

CHAPTER I

HOW ARE NATURAL-BASED POLYMERS SHAPING THE FUTURE OF CANCER IMMUNOTHERAPY *

ABSTRACT

With the increasing knowledge on cancer pathophysiology, new therapeutics based on the modulation of the immune system have been developed, overcoming many of the disadvantages associated to traditional pharmaceuticals such as chemotherapeutics. Several immunotherapy systems have in fact become the preferred treatments to tackle particular types of cancer. Despite the impressive clinical results of such systems, issues such as biomolecule susceptibility to proteolytic degradation and tumor microenvironment immunosuppression need to be overcome to further increase efficacy and safety upon use in patients. Natural-based polymers have shown the potential to address some of these limitations. This type of materials, widely used in the field of tissue engineering and regenerative medicine, are being increasingly incorporated in the development of improved immunotherapeutics due to several intrinsic properties such as biocompatibility and bio-similarity. In this chapter, the novelties these polymers have brought to the field of immunotherapy and how they have been implemented to create new and more complex therapeutics is outlined. Finally, what the future may hold for natural-based polymers use in immunotherapy and identify emerging trends is discussed.

* This chapter is based on the following publications:

Rodrigues DB, Reis RL, Pirraco RP. How are natural-based polymers shaping the future of cancer immunotherapy: a scoping review. *Submitted* (2023)

I-1. CONTEXTUALIZATION

The impact of the tumor's microenvironment on the efficacy of current cancer therapeutics has become the object of increased study. It has been established that tumor extracellular matrix, intratumoral hypoxia, tumor resident cells and the immunosuppressive capacity of cancer cells may hamper currently applied treatments. Dendritic as well as T cells, responsible for conducting anti-tumoral responses, are some of the most affected cell types and this immunosuppression leads to tumor cell expansion and migration. This immunosuppressive capacity has led to the development of the concept of "Immunotherapy" which was initially introduced as a form of biological therapy in order to boost the natural immune response and to aid the host in responding against a certain pathology. This was demonstrated early on through blocking the immunosuppressive effects of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) with antibodies which in turn resulted in the rejection of tumors in immunocompetent mice models [1]. Since then, it has been widely adopted in many forms in order to stop or slow tumor growth and avoid tumor metastasis. Several different methods of immunotherapy have been developed namely immune checkpoint inhibitors, T cell transfer therapy (Adoptive Therapy), monoclonal antibodies, artificial antigen presenting systems and immunological adjuvants. The introduction of biomaterials in this field has allowed the development of novel systems comprehending greater biological similarity towards native molecules. This plays greatly in favor of increasing the biocompatibility of these systems while additionally bringing new properties to the table. Natural Polymers are among the most promising biomaterials. They are naturally occurring polymers readily available in nature or extracted from plants or animals. Some examples of these natural occurring polymers are proteins, carbohydrates or even nucleic acids. Many of these natural materials are known for their outstanding biological performance such as tensile strength, adhesive properties, superhydrophobicity, toughness, self-healing and self-assembly [2–5]. The application of biomaterials in the field of cancer immunotherapy has to some extent overlapped requirements with the field of tissue engineering and regenerative medicine. This has helped fuel the development of novel immunotherapeutics based on natural materials. Examples of these are spherical micro or nanoparticles, nano and microcapsules, micelles, artificial antigen presenting systems, hydrogels, microneedles, scaffolds and natural based immunoadjuvants. These systems have been applied in a standalone manner or in combination with more conventional therapies, such as, chemo- and radiotherapy.

Throughout this chapter we will address the main advantages of using natural based systems when compared to more conventional synthetic polymers, how these systems may play an important role

in overcoming certain key issues in immunotherapy and how they may aid in shaping the future of immunotherapy.

I-2. EVOLUTION OF CANCER IMMUNOTHERAPY

Immunotherapy may be defined as a form of treatment that utilizes the immune system, either by activation or suppression, to overcome an illness. This may encompass autoimmune diseases, hypersensitivities and different forms of cancer. Immunotherapeutics are considered a form of biological therapy, applied to direct immune responses. Some of the tools used to achieve this goal include the administration of immunomodulating agents such as interferons, interleukins, colony-stimulating factors, monoclonal antibodies and different forms of vaccines. With the field focused towards creating new more precise and effective systems, several developments have been made over the past years which can be grouped into 3 main categories: artificial antigen presenting systems, adjuvants and microenvironment immunomodulators (**Figure I-1**).

I-2.1. Artificial antigen presentation

Antigen presentation is the key mechanism of action by which the immune system develops antigen specific responses. This mechanism of action is dependent on the presentation of proteins to lymphocytes in the form of short peptide fragments or antigens. For this presentation to occur, professional antigen presenting cells (APCs) must process protein antigens, breaking these into peptides and proceed to their presentation in conjunction with either class I or class II MHC molecules on the cell surface. Once present at the cell surface, these molecules interact with the appropriate T cell receptor to generate an effective immune response. Additionally, complementary signaling is required, such as the interaction between CD80/86 on the APC and CD28 on the surface of the T cell, followed by a third signal consisting on the production of cytokines by the APC which in turn leads to the full activation of the T cell.

The understanding of how antigens are presented to immune cells and the ability of the tumor microenvironment to suppress anti-tumor responses has led to the conceptualization of artificial antigen presentation. This may be performed by one of two main strategies i) an indirect approach which consists in delivering a specific antigen to circulating antigen presenting cells, for it to be presented on their surface by MHC molecules and ii) a direct method in which a delivery system is functionalized with co-stimulatory molecules such as antigen loaded MHCI or MHCII molecules and anti-CD28 antibodies for a direct

interaction with host T cells. It was based on the latter that the concept of “artificial antigen presenting cells (aAPCs)” was created. The strategies used to develop these artificial antigen presenting systems can be of cellular or acellular nature.

Cell-based strategies may be based on various cell sources from autologous to xenogenic and even exosomal strategies. Adoptive T cell therapy (ACT) is one of the most common immunotherapy strategies and consists in the *in vitro* expansion of tumor antigen-specific T cells which are then infused back into their patients [6,7]. One of the greatest handicaps of this system is the lack of efficiency in terms of generating a great number of antitumor T cells in a short period of time. This not only renders the technique costly but also requires an exhaustive process prior to clinical application. The fate of ACT in terms of clinical efficacy is largely dependent on how the antigen-specific T cells are stimulated during the priming phase [8–10] and dependent on downstream stimulation with γ chain receptor cytokines such as IL-2, IL-7, IL-15 and IL-21 [11–13]. An inefficient priming may result in hyporesponsive or anergic T cells resulting in a lack of proliferation and/or loss of effector function when in contact with the respective antigen, rendering the treatment ineffective. However, on the contrary, excessive stimulation of the cells *in vitro* may also compromise treatment efficiency due to T cell induced cell death (AICD) [14,15]. Therefore, a fine balance is required in order to obtain a high number of effector T cells and this is where particle-based methods gain clear advantage as these are highly tunable systems with controllable properties. Alternative cell-based methods such as gene-engineered aAPCs have been developed to overcome some of these issues. K562 cells are a human erythroleukemic cell line that was derived from patients with chronic myelogenous leukemia in blastic crisis [16]. These cells prove interesting as they lack the expression of endogenous HLA class I, II or CD1d, as well as of co-stimulatory molecules such as CD86, CD83, OX40L, ICOSL or CD40L [17]. Another key aspect is the lack of expression of inhibitory molecules like PDL1, PDL2, B7H3 and B7H4 [17], which will allow for a continued effector function even in an immune suppressive microenvironment. Regarding cytokine expression, despite the lack of expression of common γ -chain receptor cytokines, these cells secrete M-CSF, IL-6, IL-8, TGF- β and MIP-1 α [17]. All these factors allow for an interesting platform in which K562 are then transduced to express HLA class I molecules and costimulatory signal 2 CD80 and/or CD86 [17]. Tumor-specific T cell expansion, through the use of autologous dendritic cells derived from patient's peripheral blood mononuclear cells (PBMCs), is another popular method to induce tumor-specific responses. Herein, immature dendritic cells (DCs) are activated and matured by stimulation with specific factors such as polarizing cytokines GM-CSF and IL-4 [18], while additional downstream re-stimulations are required to generate enough tumor infiltrating lymphocytes (TIL). However, despite the advantages of being an

autologous method, several drawbacks are still present, namely the already mentioned difficulty in generating a sufficient number of TIL with effector function. This may have several causes, namely donor variability leading to an inconsistent generation of effector memory T cells regarding function or persistence [19]. Another strategy that has been adopted is the creation of chimeric antigen receptor redirected T cells (CAR T cells), which are genetically modified T cells that encode for transmembrane chimeric molecules with dual functions: (a) immune recognition of tumor antigens expressed on the surface of tumor cells (b) active promotion and propagation of signaling events controlling the activation of the lytic machinery [20]. In order to achieve a good clinical outcome, several parameters must be taken into consideration given their direct correlation with the efficacy of the treatment: i) choice of the target epitope; ii) architecture of the produced CAR; iii) method of administration ranging from doses to frequency; iv) efficient tumor homing and survival in the tumor microenvironment; v) patient lymphocyte depletion prior to administration of CAR-T cells [21]. Despite being a promising system due to its tunability, there are still a combination of issues which make this system far from ideal, namely, the lack of specific targetable antigens and inefficient trafficking toward the tumor foci due to the unbalanced secretion of cytokines from tumor cells such as CXCL9 and CXCL10 [21], which limit the full potential of the system. Additionally, the tumor microenvironment also presents a series of obstacles related to inhospitability and inaccessibility to immune cells due to hypoxia, low nutrients and ultimately the high concentration of acidic metabolites that hamper T cell proliferation and cytokine production [21].

Regarding particle-based aAPCS, the topic of whether nano- or micro-particles are better suited for antigen delivery to T cells has been an ongoing debate over the past years [22–25]. While this debate is highly dependent on the final application of the system, studies have reported that macroparticles mimicking APC size tend to produce better results in terms of achieving stable, tight synapse-like contacts with the ligand-displaying microparticles. On the other side of the spectrum, nanoparticles offer other advantages such as surface area to volume ratio, the capacity to deliver therapeutic cargo and the ease in crossing various anatomical niches. Together with size, particle shape variance has also been a characteristic known to promote aAPCs/T cell interactions [26–28]. However, despite size, shape and core material differences, all of them share common properties such as high tunability and reproducibility in stimulating T cells across batches, which makes them ideal candidates for the preparation of aAPCs when compared to cell-based methods, which display numerous variability issues. Strategies employing nanoparticle systems, such as biocompatible iron-dextran paramagnetic particles (50-100 nm in diameter) and avidin-coated quantum dot nanocrystals (~30 nm), have been reported and used to induce

antigen-specific T cell proliferation *in vitro* and lead to effective T cell stimulation and inhibition of tumor growth *in vivo* [29].

I-2.2. Adjuvants

Immune adjuvants, also known as immunopotentiating agents, are a subset of agents with unique properties capable of increasing, improving, or extending immune response against antigens administered simultaneously, hence improving the immunogenicity of antigens by decreasing the amount and number of immunizations. Immune adjuvants have been around for decades with early versions consisting of aluminium precipitates [30]. While the field has evolved immensely, aluminium salts continue to be widely used as adjuvants with different chemical variations being developed commercially over time, such as Alhydrogel® and Adju-Phos®, intended for human use. Other adjuvants have come into play over the years, such as oil-water emulsion adjuvants (Freund's Adjuvant and Squalene), adjuvants of bacterial origin (Flagellin, bacterial membranes, Monophosphoryl Lipid A (MPL-A), Muramyl dipeptide) and adjuvants from bacterial DNA (CpG oligodeoxynucleotides). All of these systems present specific ways of interaction with the immune system in order to potentiate antigen responses. Aluminium adjuvants are known to form precipitates which promote phagocytosis of antigens by antigen-presenting cells, as well as to induce local inflammation via the NLRP3 inflammasome. Upon activation of this pathway, the secretion of mature IL-1 β and IL-18 by dendritic cells and the differentiation of T helper 2 (Th2) cells are triggered, promoting activation of B cells and the subsequent production of antibodies [31]. Oil-water emulsion, such as the case of Freund's Adjuvants, contain inactivated mycobacteria which in turn attract macrophages and other cells to the site of injection. Other oil-water based adjuvants such as GLA-SE are known to induce strong signaling through the TLR-4, caspase, IL-18, and IFN- γ pathways, leading to a Th1 response [32]. Regarding bacterial adjuvants, two have stood out, MPL-A and CpG oligodeoxynucleotides. The former consists of structurally modified lipid A, a component of lipopolysaccharide (LPS), which has been known to induce maturation of DC cells, CD4⁺ T cell clonal expansion, and Th1 responses without the inflammatory effects of LPS [33]. Regarding CpG oligodeoxynucleotides, these are synthetic oligodeoxynucleotides (ODN) that contain unmethylated CpG motifs (CpG ODN). When these CpG motifs are unmethylated they induce macrophages to secrete IL-12, which induces interferon (IFN)- γ secretion by natural killer (NK) cells and therefore may induce Th1 differentiation [34].

I-2.3. Microenvironment immunomodulators

Several hallmarks of cancer are known to regulate the tumor microenvironment, promoting tumor growth and survival. Two of the most studied are the capacity to escape immune destruction and the tumor's ability to promote angiogenesis [35]. The balance between the capacity of one's immune system to control and abolish tumor growth and the inherent ability of the tumor to evade the immune system dictates tumor aggressiveness. An imbalance in these mechanisms is known as immunoediting [36]. Two key molecules expressed on activated T cells and known as inhibitory T cell checkpoints, CTLA-4 and PD-1, are behind this mechanism. Several strategies have been developed in order to interrupt this immune suppressive capacity of tumor cells, namely through the development of checkpoint inhibitors such as antibodies with blocking potential. In the case of CTLA-4 binding, Ipilimumab has been developed for the deactivation of the inhibitory signals towards T cells [37]. Regarding the targeting of PD-1 and of PD-1L, antibodies such as nivolumab, pembrolizumab and pidilizumab have been created to block tumor T cell interactions [37].

On the other hand, tumor survival is in a big part sustained by neo-vascularization, in which tumor hypoxia has been shown to play an important role. In tumors, hyperproliferating cancer cells overgrow their blood supply and become hypoxic. This hypoxic microenvironment in turn creates an imbalance between angiogenic activators and inhibitors [38] leading to rapid and chaotic blood vessel formation which tends to be of abnormal character, underdeveloped and leaky. This continuous flow of nutrients and oxygen ensures the continued tumor cell proliferation and angiogenesis. Several key players such as hypoxia-inducible factor 1- α (HIF-1 α), PI3K/AKT/mTOR, MAPK, NF κ B and equally VEGF-A [39–41] have been tied to tumor progression and poor prognosis. With this knowledge, angiogenic inhibitors have been introduced, for instance, CRLX101 for HIF-1 α inhibition (Clinicaltrials.gov Identifier: NCT01652079), SU5416 (Semaxanib®) for the selective inhibition of VEGF receptor (Flk-1/KDR) through the decrease of HIF-1 α protein and VEGF mRNA via the PI3K/Akt pathway (ClinicalTrials.gov Identifier: NCT00005642), mTOR inhibitor Rapamycin®, known to inhibit both the stabilization and the transcriptional activity of HIF-1 α in hypoxic cancer cells [42] or monoclonal anti- VEGF-A antibodies such as bevacizumab (Avastin®) [43].

While these different strategies have shown clinical efficacy, they are not free of immune-related adverse events. Checkpoint inhibition has been shown to lead to impaired self-tolerance resulting from the loss of T cell inhibition, while much broader complications such as gastrointestinal, hepatic and endocrine toxicities are many times visible in patients. Combinatorial therapy is a promising way to

overcome these issues. By combining different therapeutic strategies, dosing may be regulated to sub toxic levels potentiating the effect of these therapeutics. Another strategy that has gained traction over the years is targeted delivery. By delivering lower doses of these drugs to the region of interest, tumor microenvironment in the case of cancer, a localized effect may be seen without systemic toxicities being achieved. Different systems have been developed over the years to accurately deliver these agents, from polymeric to metallic nano or micro-particles with varying shapes and structures.

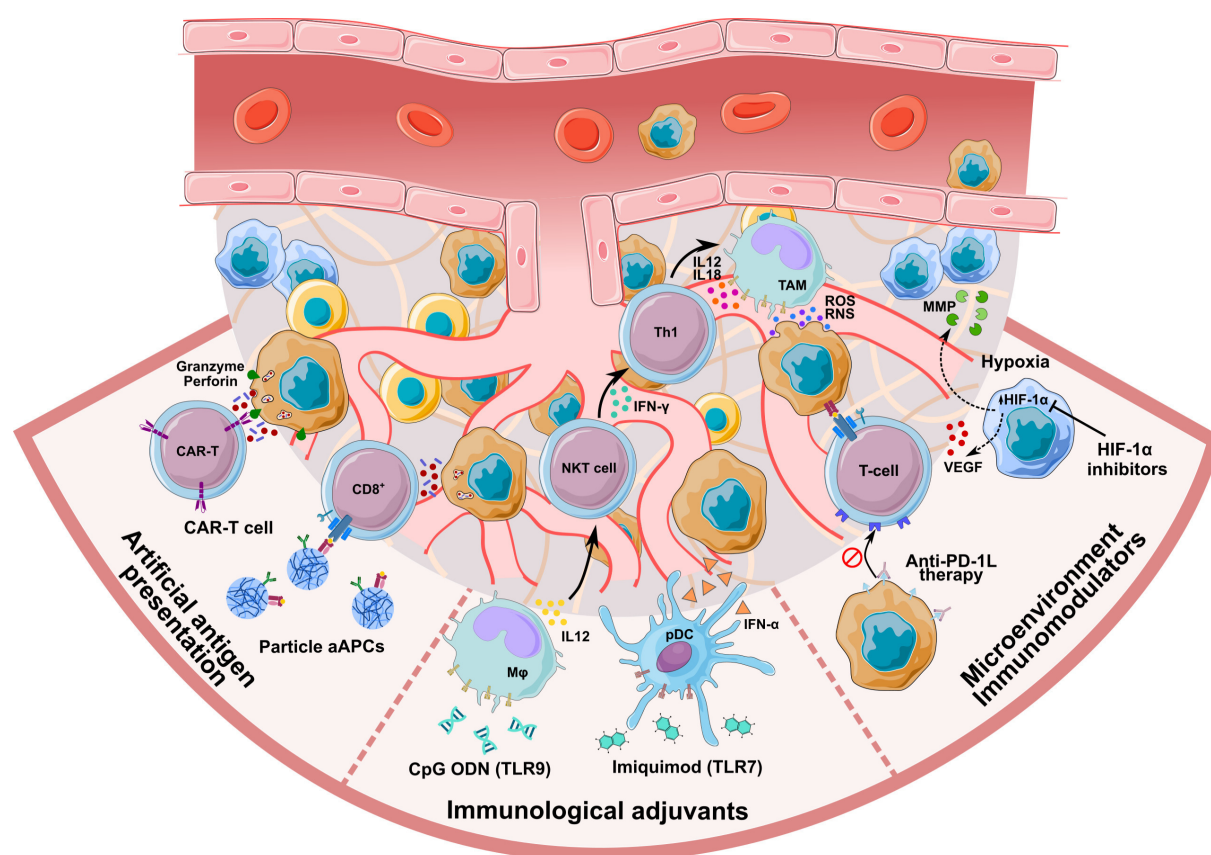


Figure I-1 Immunotherapy strategies for the treatment of cancer.

There are three main approaches used for cancer immunotherapy: (1) Antigen antigen presentation (left); (2) Immunological adjuvants (center); and (3) Microenvironment immunomodulators (right).

I-3. BASICS OF NATURAL POLYMERS

Polymers are chemical compounds made up of small molecules otherwise known as monomers, arranged in simple repeating structures bonded chemically through covalent bonds to form a large molecule or chain [44]. These compounds can be generally of three main sources, synthetic, semi-

synthetic or of natural origin. The latter occur in nature and can be extracted from plants, animals and even bacteria. Natural polymers are crucial for human existence as these encompass proteins and nucleic acids, cellulose, natural rubber, starch or even honey and wool. The advantages and disadvantages of natural vs synthetic polymers in several fields has been a recent topic of interest [45–48]. While structure controllability and reproducibility are two important features of synthetic polymers, the bio-similarity, biodegradability, biocompatibility and environmental friendliness of natural polymers are very appealing properties to the biomedical community. Several natural-based polymers have been adopted in the biomedical field, namely in tissue engineering and regenerative medicine, being also selected in the design of novel immunotherapeutics.

I-3.1. Chitin/Chitosan

Chitin is a marine-origin natural polymer and can be found in a wide variety of arthropod shells, being collected essentially from crustaceans. Structure-wise, it resembles cellulose, being a linear, high molecular weight crystalline polysaccharide composed of N-acetylated glucosamine (2-acetylamino-2-deoxy-D-glucopyranose) units linked by β -(1 $\rightarrow$ 4) glycosidic bonds [49] which contribute to its strength. Chitosan is a popular chitin derivative due to features such as hydrophilicity and ready solubility in dilute acids. It can be obtained through the partial alkaline deacetylation of chitin and displays a semi-crystalline cationic structure, comprised of β -1,4-linked 2-amino-2-deoxy-D-glucose [50]. It proves insoluble in aqueous solutions with a pH higher than 7 and soluble in dilute acids (pH 6) due to its free amine groups which become protonated. What makes it an attracting natural polymer is its non-toxicity, biodegradability, biocompatibility and antibacterial properties [51].

I-3.2. Cellulose

Cellulose is a well-known natural polymer which is heavily used in the paper and textile industries. It is widespread and can easily be found in the cellular wall of plants, more specifically within the stems, stalks or trunks [52]. Additionally, to its presence in plants, some microorganisms have been known to equally express this polymer, namely gram-negative bacterium *Acetobacter xylinum* [53]. Cellulose is composed of β -D-glucopyranose units linked by (1 $\rightarrow$ 4) glycosidic bonds which are formed through the polymerization of glucose residues from substrates such as UDP-glucose [54]. These glycosidic links possess a specific stereochemistry that creates linear glucan chains that enable precise and ordered interactions between different chains. Structurally, this polymer exists in the form of microfibrils,

consisting of various chains strongly linked by hydrogen bonds conferring this natural polymer a rigid structure [55], consequently accounting for a high degree of crystallinity, low solubility and poor degradation *in vivo*. However, features like its high strength in the wet state and its biocompatibility make it an appealing natural polymer.

I-3.3. Alginate

Alginate is an anionic natural polymer that can be found in brown algae [56]. Structurally, it comprises (1-4)-linked β -D-mannuronic acid and α -L-glucuronic acid units, organized in regions of sequential mannuronic acid units, guluronic acid units or through a combination of both [57]. One of the key elements that makes alginate such an interesting polymer for the biomedical field is its ease of gelification in the presence of divalent cations (Ca^{2+} , Mg^{2+} or Ba^{2+}). Crosslinking occurs between the carboxylic groups present in the alginate backbone, therefore forming hydrogels [58]. Its well-known structure has allowed its chemical modification through the carboxylic groups present in the guluronic acid residues, such as in the case of functionalization with RGD peptides in order to increase cell-material interactions [59]. Additionally, its low toxicity has also contributed for its increased demand as an alternative to synthetic polymers.

I-3.4. Hyaluronic acid

Hyaluronic acid (HA), or hyaluronan, is a non-sulfated linear negatively charged polysaccharide that consists of alternating repeating units of the β -1,4- D-glucuronic acid– β -1,3-*N*-acetyl- D-glucosamine disaccharide [60]. It may originate from several sources, however, the most predominant are rooster combs and bacterial expression systems in *Streptococcus* [61].

Variations in the source lead to different rheological properties. Attributes such as its solubility in water and its shear dependent viscosity, which allows for it to be injected through a small gauge needle, make this polymer interesting for biomedical applications [62]. Additionally, its amenability to enzymatic degradation [63] by HAase and papain contributes to its biodegradability which makes it compelling for *in vivo* applications.

HA is a main component of the extracellular matrix and promotes both cell motility and proliferation [64]. Two main forms of HA have been described [65], a low-molecular weight HA which triggers proinflammatory responses and a high-molecular weight HA which has been associated to anti-

inflammatory cues. Its role comes through the interaction with several types of immune cells, endothelial cells, fibroblasts and keratinocytes via cell surface receptors such as RHAMM, CD44s, TLR-4, TL2-R, Stabilin-1 (HARE), and LYVE-1 [66]. The interaction of HA with its receptor CD44 which in turn is highly expressed in many cancers and is capable of regulating the metastatic process [67,68], has allowed for the development of tumor targeting systems as the case of vaccines. Additionally, its ability to undergo enzymatic degradation makes it an enticing natural polymer for the development of several immunotherapeutic strategies. Since HA became a widely studied polymer over the years, modifications in its chemical structure were frequently introduced [69], such as the formation of esters through the esterification of its carboxylic groups [70] which promotes resistance to a range of conditions and facilitates processing into membranes, spheres, particles and porous structures.

I-3.5. Gellan gum

Gellan gum or gellan is a quite common natural polymer used in the food industry. It is a linear anionic polysaccharide composed of tetrasaccharide repeating units of 1,3- β -D-glucose, 1,4- β -D-glucuronic acid, 1,4- β -D-glucose, 1,4- α -L-rhamnose, containing one carboxyl side group [71]. This polymer is known to exist under two forms, acetylated - which is its original form produced by the *Sphingomonas paucimobilis* bacterial strain - and the deacetylated - the most widely available and able to undergo processing [72]. Similarly, to others, it is capable of ionotropic gelation. Its gelation is strongly influenced by the chemical nature and number of cations present in the solution, being promoted in a stronger manner in the presence of divalent cations when compared to monovalent cations [73]. Upon crosslinking through the presence of these divalent cations, the gelation occurs via chemical bonding between the cation and two carboxylate groups belonging to glucuronic acid molecules in the gellan gum chain [74]. However, temperature shifts induce structural changes which account for the thermoreversible nature of the polymer [75].

I-4. NATURAL POLYMERS DRIVING NOVEL DEVELOPMENTS IN CANCER IMMUNOTHERAPY

The development of novel systems for the vaccination, artificial presentation of tumor associated antigens (TAA) or the delivery of immunomodulatory molecules has proven to be challenging due to issues related with antigen or protein stability. When developing new platforms for delivery, it has to be

considered how liable these peptides are to mechanical clearance and proteolytic degradation in the microenvironment, which may lead to antigen denaturation and loss of antigenicity [76].

The use of natural polymers for the development of these system has allowed not only to exploit properties such as biocompatibility and biodegradability but also other key aspects such as ionotropic gelation capability or their structural resemblance with human extracellular matrix proteins. This allowed room for the development of both micro- and nano- particles and injectable hydrogel systems which can ensure the protection of key molecules from the hostile tumorigenic environment while potentiating immune-stimulatory effects, therefore increasing their half-life upon administration. Here, we will review some of the most recent developments in the field of immunotherapy involving the use of natural polymers (**Figure I-2**).

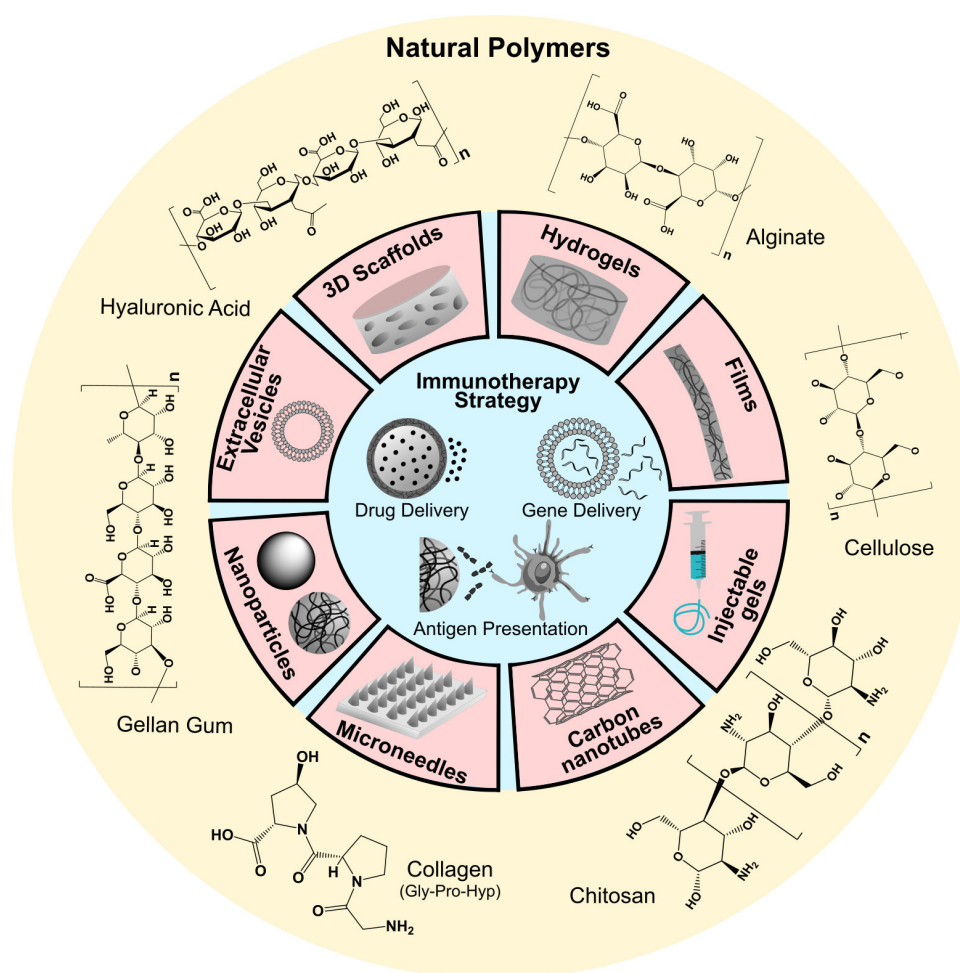


Figure I-2 Natural polymer-based systems for the development of immunotherapeutics.

I-4.1. Gel based systems

While the use of crosslinkable natural polymers in the field of TERM is widespread, this technology is fairly young in the development of immunotherapeutics. An *in situ* crosslinkable hydrogel for immunotherapeutic use has been described using alginate [77]. This polymer was used as a delivery system for catalase (Cat) and CpG oligonucleotides for cancer therapy. The rapid gelation of the system by intra-tumoral Ca^{2+} allowed for both a homogeneous distribution of ^{131}I -Cat throughout the tumor as well as a long-term entrapment of ^{131}I -Cat without leakage into the surrounding healthy tissues. Hence, decomposition of the tumor endogenous hydrogen peroxide (H_2O_2) was triggered, contributing to higher intra-tumoral oxygenation values maintained over 72 h. This originated greatly reduced levels of HIF-1 α upon Cat/ALG treatment. When assessing the potential of the system in radioisotope therapy (RIT) using a 4T1 murine tumor model, ^{131}I -Cat/ALG resulted in tumor-free animals with a prolonged survival without any deaths. A patient-derived xenograft (PDX) mouse tumor model was also used to better represent real human patient tumors where the ^{131}I -Cat/ALG led to 100% elimination of PDX tumors. When tested in a larger animal model of rabbits bearing VX₂ tumors, a similar effect was seen, where ^{131}I -Cat/ALG injection resulted in complete tumor regression within 2 weeks and sustained survival for over 150 days. When CpG oligonucleotides were mixed with the ^{131}I -Cat/ALG system and used in combination with intravenous administration of a CTLA-4 antibody, suppression of distant tumor growth was seen. Additionally, the ratio of CTL/T_{reg} was significantly increased, resulting in increased TNF- α and IFN- γ secretion. Long term immunological memory was confirmed through a higher number of effector memory T cells (T_{EM}) (CD3⁺CD8⁺CD62L⁺CD44⁺) residing in both lymphoid and non-lymphoid tissues as well as protection when animals were rechallenged with secondary tumors.

Nucleic acid-based vaccines have made their way into the field of immunotherapy due to their several advantages when compared to more traditional protein-based vaccines. This was clearly evidenced recently with the use of this technology to develop two of the first FDA approved mRNA vaccines to deal with the COVID-19 pandemic, namely the vaccines developed by BioNTech [78] and Moderna [79]. The use of pDNA or mRNA-based vaccines allow the development of CD8⁺T cell responses while not being subject to neutralization through immunosuppression, therefore allowing for repeated antigen challenging.

Natural origin-based polymers can further contribute to develop more efficient, biocompatible and biodegradable delivery systems for these vaccines. For both BioNTech and Moderna vaccines, lipid nanoparticles consisting of ionizable lipids were used for the safe and efficient delivery of mRNA encoding

SARS-CoV-2 S(2P) [80,81]. Injectable hydrogels have also aided the administration of DNA vaccines. Injectable N-succinyl chitosan (S-CS) with oxidized alginate (O-Alg) gel scaffolds have been applied for the local delivery of ovalbumin (OVA) mRNA lipoplexes [82]. Through the introduction of hydrophilic succinic anhydride side groups onto the chitosan backbone, the water solubility of the natural polymer was significantly increased. Oxidation of alginate also led to an increased solubility of the polymer. Crosslinking between the two polymers was achieved through a Schiff-base reaction followed by a lyophilization step. The Schiff-base reaction allowed for a reasonably slow sol-to-gel phase transition which in turn translated into a rehydrated gel scaffold which could easily be injected through a needle. During the rehydration step, the mRNA can be easily loaded into the system. When mRNA was used complexed to a nanoparticle system, a slower degradation rate of the gel was seen, when compared to empty gels, where a steadier release of the nucleic acids was measured overtime. These results were justified through additional crosslinking points occurring between the mRNA and the hydrogel polymer, therefore increasing the system overall stability. Regarding *in vivo* local mRNA mediated protein expression, when using a Luc reporter gene, only the conditions where the injectable gel system was applied displayed local transfection *in vivo*. Transient expression of Luc was seen to peak at approximately 8h. Subsequently, OVA mRNA was used with the system to understand whether a relevant immune response could be triggered with the system. When looking into humoral immune responses using the injectable Chitosan-Alginate-OVA system, a significant increase in OVA-specific IgG levels were detected. An alternative injectable smart hydrogel (ISH) composed of HA functionalized with levodopa- and poly(ϵ -caprolactone-co-lactide)ester was developed for the delivery of OVA-expressing plasmid and granulocyte-macrophage colony-stimulating factor (GM-CSF) for the local recruitment of dendritic cells (DCs) [83]. These displayed a slow degradation pattern while sustaining a controlled release of both polyplexes and GM-CSF *in vitro* and *in vivo*. The transfection efficiency assays showed that ISH were capable of effectively priming immune cells as seen through the expression of OVA in mice (**Figure I-3a**). Single subcutaneous injection of ISH in mice enhanced the recruitment of DCs, and of other immune cells, including macrophages and neutrophils. In turn, ISH generated strong antigen-specific humoral responses, and mice that received hydrogel-based vaccination did not develop tumors or had delayed tumor onset.

Efficient expansion of T cells while avoiding T cell exhaustion has been deemed a complex task that has captured the attention of the scientific community. Natural polymers have been applied in this regard. Alginate has been recently proposed for the development of a suspended culture method consisting of microscale hydrogel tubes (AlgTubes) [84]. In order to evaluate the potential of the system, industry approved CD3/CD28 Dynabeads from Invitrogen and tetrameric anti-CD3/CD28/CD2 antibodies from

Stem Cell Technologies were selected as controls. These tubes, while capable of protecting the cells from hydrodynamic stresses and compacting cells to ensure efficient cell mass transport, also created a cell-friendly microenvironment that allowed for high viability, low DNA damage, high growth rate, high purity and high cellular yield when compared to the respective controls (**Figure I-3b**).

Alginate has also been used for the stimulation of autologous antigen-presenting cells such as dendritic cells. Its capacity to be ionically crosslinkable has allowed for the production of 3D scaffolds embedded with reduced graphene oxide (MAGS) and loaded with OVA and granulocyte-macrophage colony-stimulation factor (GM-CSF) that have been proposed as a vaccine delivery platform for *in situ* long-term activation of antigen-presenting dendritic cells (DCs) (**Figure I-3c**). MAGS were capable of efficiently loading both OVA and GM-CSF through direct pipetting onto the surface of the scaffold and the interaction between the reduced graphene oxide and the immunomodulators was strong enough to promote a slow and sustained release over time. When implanted subcutaneously, a significantly higher CD11c⁺ DC recruitment was confirmed when GM-CSF-doped scaffolds were used, which was sustained over 30 days post implantation. Antigen presentation efficiency was also increased when GM-CSF and OVA were co-loaded, since the number of CD11c⁺SIINFEKL-MHC-I⁺ cells was significantly greater. This in turn was translated into a larger number of activated IFN- γ ⁺ CD8⁺ T cells and IFN- γ ⁺ CD4⁺ T cells in the lymph nodes of stimulated C57BL/6 mice. The MAGS-GM-CSF-OVA system also showed capability as a tool for vaccination. After completing 30 days post vaccination, animals were challenged with OVA-expressing B16 melanoma (B16-OVA) cells and a delay in tumor growth was seen with high percentages of CD11c⁺CD86⁺ cells. When determining the effect of central and effector memory T cells, mice which had been vaccinated with the system produced significantly higher numbers of these cells. Similarly to alginate, which is known for its biodegradability, the MAGS scaffolds showed *in vivo* a gradual loss of the interconnected structure over time, contributing to its biocompatibility in an *in vivo* setting [85].

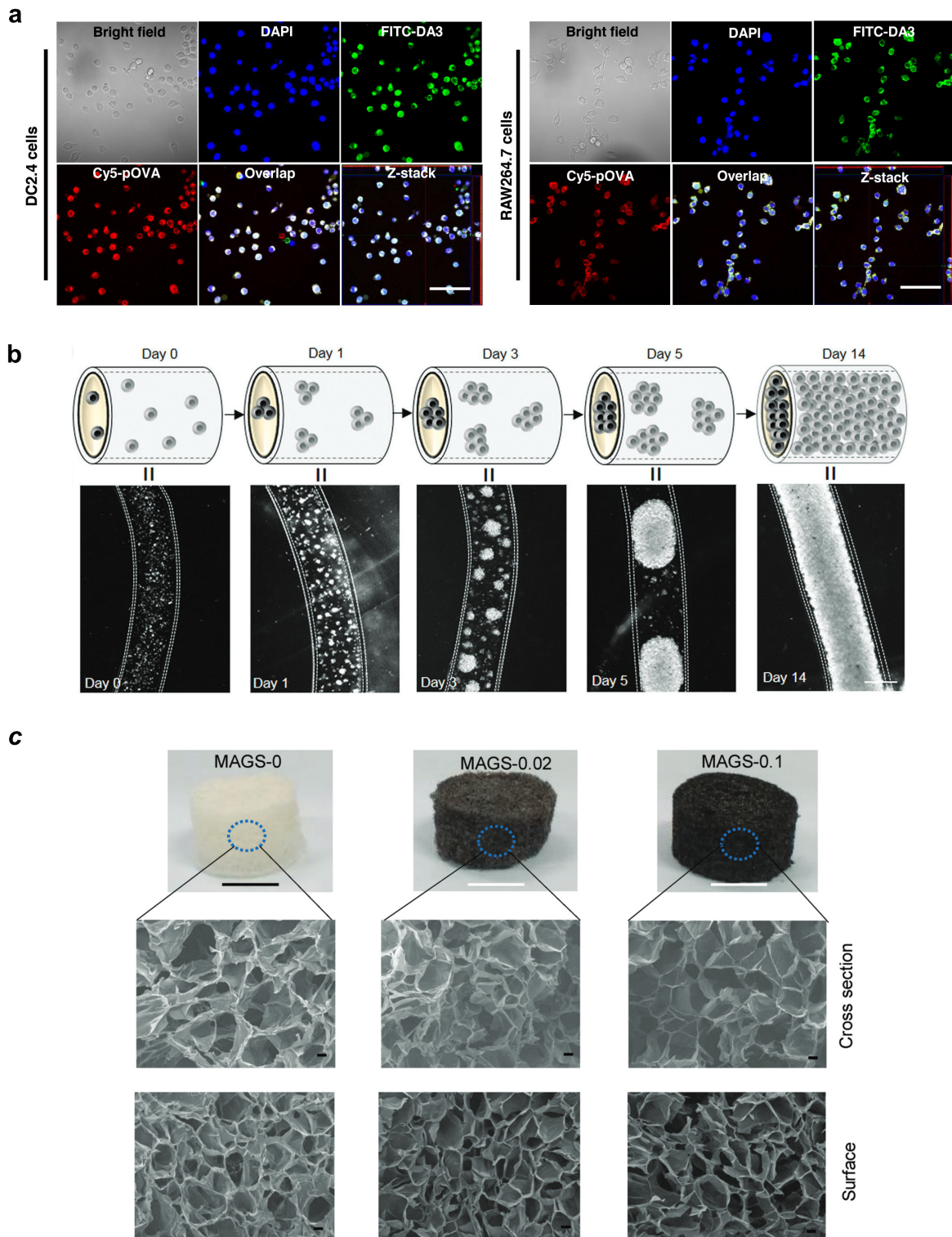


Figure I-3 Natural-based hydrogels used in immunotherapeutic strategies.

a) Polyplexes released from the hydrogels induce priming of DC 2.4 mouse DCs and RAW 264.7. Adapted with permission from [83]. Copyright 2020, Elsevier. b) T cells cultured in AlgTubes first associate to form small clusters that subsequently grow and until the tube is filled Adapted with permission from [84]. Copyright 2018, John Wiley and Sons. c) Characterization of alginate scaffolds containing various amounts of embedded MAGS. Adapted with permission from [85]. Copyright 2019, John Wiley and Sons.

Growth-factor and cytokine encapsulation using tools such as nanoparticle systems or drug releasing hydrogels have in fact been widely used for tumor therapeutics development. Over the past few years, however, increased focus has been placed into developing alternatives to these pre-loaded systems, such as cell encapsulation for the *in situ* production of cytokines at physiological concentrations. These living release systems could be used to either boost or improve immune responses or to directly combat tumors. Several of such immuno-protective cell encapsulation systems have already been reported. Alginate has been used in combination with K562 cells for the production and release of GM-CSF [86]. In other reports, for the release of IL6, genetically modified CHO cells were loaded into an alginate system to be used in a rat model of hepatocellular carcinoma [87]. Additionally, an alginate poly-L-lysinealginate (APA) copolymer was also reported for the microencapsulation of genetically modified mouse myoblasts (C2C12) for the delivery of angiostatin and IL-2 fusion protein (ssFvIL-2) [88]. HEK293 cells were transfected with several cytokines and chemokine expression vectors for GM-CSF, IFN γ and hIL-15 and encapsulated in alginate for further use in anti-tumor therapy experiments [89]. Atik et al. has described a HA-based low viscosity hydrogel to serve as a vehicle for the delivery of tumor-specific chimeric antigen receptor (CAR) T cells in convection enhanced delivery (CED) [90]. The hydrogel-based carrier presented a significantly higher CAR T cell delivery rate when compared to saline, while preserving the capacity of the CAR T cells to migrate outward of the hydrogel and therefore exhibiting a significantly superior tumor-specific killing of glioma cells versus saline after CED. Cellulose sulphate has been described as an encapsulation material for over 2 decades [91]. Cellulose when used as a mean of encapsulating mammalian cells, has shown to protect cells from immune rejection, retain cells in the site of implantation, provide long term cell survival and allow for the circulation of biomolecules [92]. A cellulose sulphate encapsulating system for Hut-78 cells to produce IL-2 for immunotherapeutic use has been reported [93]. Cellulose sulphate beads comprise a gelated membrane with interconnecting pores which restricts molecule circulation. Molecule properties such as size, structure, flexibility and charge end up playing a determinant role in their release from these particle systems. In this sense, small molecules have the upper hand regarding ease of escaping these 3D structures, while larger molecules are more dependent on their other characteristics. Salmons et al verified that upon stimulation with PMA and ionomycin, the encapsulated HUT-78 cells displayed the capacity to produce detectable levels of IL-2 as early as 3 days after encapsulation. Other authors have described similar systems comprised of gelatin hydrogels enzymatically crosslinked via microbial transglutaminase for the encapsulation of genetically engineered HEK293 to secrete human IL-2 [94].

I-4.2. Microneedles

Vaccination is an established method to explore long term immunization and is used to prevent a variety of pathologies of either bacterial or viral origin or even for the sensitization against TAAs in the case of cancer. Despite the well-known advantages of vaccination, needle-based immunization still presents issues such as risk of infection due to needle reuse and low patient compliance due to pain and fear. Microneedles (MNs) have been described as an optimal system for the dermal delivery of antigens as they can easily pierce the skin and deliver the antigen in the epidermis and dermis, ultimately in a pain-free delivery manner. MNs take advantage of the several antigen presenting cells present in skin, namely Langerhans cells (LCs) and dermal dendritic cells (dDCs) [95]. These cells display the capacity to capture antigens and migrate to draining lymph nodes where they can present these antigens to T cells, triggering a phenotypical switch to antigen specific T cells and B-cell activation. Dissolving microneedles (dMNs) in particular, consist of fast-dissolving excipients which can be polymers or sugars. When inserted in the skin, these needles dissolve and proceed to release bioactive compounds previously added to the system making them a reliable system for intradermal (ID) vaccination. With this in consideration, several works have been recently proposed regarding microneedle-based approaches for vaccination. A composite microneedle comprised of a chitosan base and a sodium hyaluronate tip was designed for biphasic release of relevant antigens [96]. Upon insertion in the skin, HA dissolves within the dermis for a rapid release of the encapsulated antigen, while the Chitosan base remains within this compartment in which it sustains a controlled release over time while undergoing biodegradation. With this system, a higher and more durable antibody response was achieved when compared to traditional two-dose or double-dose subcutaneous vaccination. Additionally, a chitosan MN system with a patch-dissolvable design has been reported for low-dose immunization [97]. Herein, an antigen-loaded chitosan MN was supported by an array patch of hydrophilic polyvinyl alcohol/ polyvinyl pyrrolidone in order to provide additional strength upon MN insertion, overcoming the skin's inherent elasticity and deformation when exerting pressure. Upon complete insertion in the skin, the supporting array would then dissolve, reducing patch-induced skin irritation. This system showed that through ID implantation, a sustained release of -OVA was verified for up to 28 days. An *in vivo* rat model showed that a low-dose immunization with this system still led to persistently high antibody levels over an 18-week period which was deemed higher than that achieved through conventional intramuscular immunization. HA has been proposed for the development of dMNs for the ID delivery of OVA [98]. Through the fine tuning of the loaded OVA peptide and the ratios of HA, a stable system was developed with an optimal penetration efficiency

followed by gradual dissolution over a period of up to 20 min. When applied *in vivo* in the skin of female BALB/c (H2d) mice, an overall increase in IgG serum levels were detected. Another example of a recently developed microneedle vaccination system comprises a sodium hyaluronate (HA)/chitosan composite as the building block. This biphasic system was designed to allow for both a rapid and sustained release of antigens mimicking more conventional prime-boost immunization regimen when compared to traditional bolus injection or to the previously described chitosan MN alone. For this effect, the authors developed a MN system composed of an antigen loaded HA tip and chitosan base, which was combined with a poly(vinyl alcohol)/polyvinylpyrrolidone (PVA/PVP) supporting structure [96]. The HA/Ch composite MN when inserted in porcine cadaver skin and rat skin showed to pierce through the stratum corneum and reach the dermal layer. The dissolvable HA tip dissolved within the skin for rapid release of the encapsulated antigens, therefore priming the immune system, while the biodegradable chitosan base remained in the dermis for a prolonged antigen release over 4 weeks, further boosting the antigen driven responses for up to 16 weeks. Immunization with the HA/chitosan MN containing OVA was shown to stimulate both T helper type 1 (Th1) and Th2 immune responses in Sprague–Dawley rats when compared to more traditional two-dose or double-dose subcutaneous vaccination.

I-4.3. Films

As aforementioned, the intradermal delivery of proteins is a good alternative to more traditional invasive delivery methods. However, this comes not without its own setbacks, as intradermal delivery through intact skin may be rather restrictive depending on the size, stability or even hydrophilic properties of the molecules to be delivered. A method to overcome some of these issues resides in the use of fractional ablative laser microporation, in which pulsed infrared lasers are used to induce a thermal ablation of tissue in micron-sized columns with a diameter of 30–200 μm . Engelke et al described water-soluble dry films to be used as a drug delivery system in laser microporated skin. Blends of PVA with carboxymethyl cellulose (CMC) were used for the production of a hydrophilic polymer with the purpose of solubilization of the films directly on laser microporated skin, given the enhanced water transport from the tissue through the porated skin into the film under occlusion. The PVA/CMC blend films were shown to dissolve within less than 6 h when attached on top of laser-generated micropores, facilitated by the generation of a liquid depot between skin surface and the occlusive tape used for film fixation, which in turn led to an efficient delivery of both RD70 and PS-particles into and through excised pig skin [99].

I-4.4. Carbon Nanotubes

The use of chitosan as an adjuvant for photothermal therapy (PTT) was recently described [100]. A single-walled carbon nanotube (SWNT) modified with glycosylated chitosan (GC) system was used in combination with α -CTLA4 as an anti-tumor therapy strategy. This system was capable of significantly increasing ROS production in 4T1 tumor cells, despite not displaying any direct cytotoxic activity. However, results showed that by the combined use of the SWNT-GC with laser irradiation a significant increase in killing of 4T1 tumor cells was visible, which resulted from a more pronounced temperature increase when SWNT-GC was used in comparison to laser alone. Regarding the immunomodulatory effect of the system, when SWNT-GC were placed in culture with dendritic cells, a higher increase in the expression of CD40 and CD80 could be verified by flow cytometry. Moreover, in the presence of 4T1 tumor cells, an even higher DC activation could be verified as well as increased secretion of TNF- α . When tested in tumor bearing mice, not only did the SWNT-GC system lead to a higher temperature increase inside the tumor under laser irradiation but also lead to a significant reduction in tumor size and number of metastases. Ultimately, when combined with checkpoint inhibition, a significant increase in mice survival time was verified as well as in the production of IFN- γ by splenocytes.

I-4.5. Particle-based systems

One of the main features of chitosan as a base system for the development of immunotherapeutic strategies is its spontaneous self-assembly in micro- or nanoparticles when ionically crosslinked with charges of opposite nature. Complexes of different nature may also be formed when using anionic crosslinking substrates. Recently, the effect of chitosan nanoparticles on V γ 9V δ 2 T cells has been studied [101]. This particular subset of T cells was shown to recognize stress-induced phosphonate antigens presented by both cancer cells and pathogen-infected cells in an MHC-independent manner. Chitosan nanoparticles were shown, as a standalone system, to be capable of enhancing the killing potential of V γ 9V δ 2 T cells via upregulation of killing molecule NKG2D expression as well as of FasL and CD56. This system was also shown to have immunomodulatory properties through the enhancement of perforin secretion which is involved in cytotoxic T cell responses. Ultimately, killing potential was confirmed *in vitro* through direct culture with leukemia cells, which showed an increased killing rate of V γ 9V δ 2 T cells when pretreated with the chitosan nanoparticle system. Other studies used this natural polymer for the development of antigen delivery tools in the form of chitosan modified PLGA nanoparticles (CS-AHPP/OVA) [102]. While retaining excellent stability, these particles were shown to induce strong cellular

immune responses as seen through the evaluation of lymphocyte proliferation, while also being capable of inducing the secretion of IFN- γ and TNF- α . T cell polarization was also assessed through the production of IgG1 and IgG2a antibodies associated respectively to Th2 and Th1-polarized immune responses. Both IgG1 and IgG2a antibodies were highly induced upon stimulation with the CS-AHPP/OVA system. The potential use of chitosan as an effective adjuvant for DNA vaccines was also explored through the development of a chitosan nanoparticle comprising human papilloma virus (HPV)-16 E7 DNA and IL-12 gene [103]. Chitosan nanoparticles as a standalone system have shown to display immunostimulatory activity [104]. Moreover, their use as co-adjuvants for cytokine therapy, was proven to increase local cytokine retention and bioactivity [105]. The chitosan-E7 DNA+IL-12 system was shown to enhance the proliferative response of T cells to the system. Moreover, an increased cytolytic activity was verified by LDH assay. Mice immunized with this system also presented a significantly higher production of IFN- γ and IL-4 and a decrease in the production of the immunosuppressive cytokine IL-10. When looking into the *in vivo* effect in a mouse tumor model, a reduction in tumor volume was seen upon immunization with chitosan-E7 DNA+IL-12 [103]. Additional chitosan-based adjuvant systems have been presented. Choi et al, described a Chitosan-RNA adjuvant system for immune modulation where nanoscale polyplexes of toll-like receptor 3-recognizing RNA adjuvants and high molecular weight chitosan (RA/CTS) were formed by ionic crosslinking [106]. Through the subcutaneous injection of the RA/CTS polyplexes in an ovalbumin tumor mouse model, it was possible to show that this system exerted a preventative effect upon challenge with B16-OVA cell line. A greater inhibitory effect was also verified when a second challenge was performed in comparison with other treatments, which was associated to a higher tumor antigen-specific humoral and cellular immune response and with a greater infiltration of CD4 helper T cells as well as CD8 T cells into tumor tissues. Other reports have also used chitosan as a delivery system for RNA targeting cancer therapy. Chitosan-coated selenium nanoparticles (SeNPs) with a folic acid targeting moiety were developed for Fluc mRNA delivery to cancer cells [107]. These particles were capable of not only successfully binding mRNA but also of conferring significant protection to the nucleic acids. This allowed for a stable delivery of the mRNA cargo when tested *in vitro*, and a further uptake of the FA-targeting system when tested in folate receptor-positive cells. Additionally, other studies have shown that chitosan may reduce the pro-oxidative activity of selenium, which was shown to lead to DNA damage. A polyethylene glycol (PEG)-chitosan-lactate (PCL) nanoparticle system has recently been developed for the delivery of A2AR-specific mRNA [108], which is known to interfere with the differentiation and function of T cells and has also been associated with a significant tumor regression in tumor-bearing mice [109]. This A2AR siRNA delivery system displayed a high transfection efficiency in T

cells while yielding low toxicity in the several cell lines that were tested and an elevated concentration in the tumor zone when biodistribution was tested. T cells stimulated with the A2AR siRNA-loaded NPs demonstrated a suppressed expression of A2AR which in turn was associated with increased T cell proliferation, reduced apoptosis, increased production of IFN- γ , while reducing the secretion of inhibitory cytokine IL-10. Additionally, differentiation of T cells into a regulatory phenotype (Treg) was blocked by using A2AR siRNA-loaded NPs in tumor-bearing mice.

Block co-polymerization has been extensively applied in the field of TERM to develop more advanced materials with more appealing characteristics and features. Block co-polymers possess a linear arrangement of 'blocks' of repeating units with varying monomer composition. Recently, this technology has made its way into immunotherapy for the development of new delivery systems. A novel co-polymer consisting of sodium alginate modified with β -cyclodextrin (Alg- β -CD), methoxypolyethylene glycol (mPEG-Fc) containing ferrocene (Fc) and α -cyclodextrin (α -CD) was developed. The non-covalent co-polymer Alg- β -CD/mPEG-Fc/ α -CD was then self-assembled into NPs for controlled drug delivery, as these nanoparticles display the capacity to disassemble in the presence of H₂O₂ [110]. This is relevant since glucose oxidase (GOD) can oxidize glucose to produce H₂O₂ and has been tied to tumorigenesis [111]. To determine the capacity of the system to be used for controlled delivery, BSA was added in the mixture prior to the complexation with α -CD yielding BSA loaded nanoparticles. A high degree of encapsulation was confirmed in the Alg- β -CD/mPEG-Fc/ α -CD when compared to the β -CD free counterpart. Regarding release profile, when tested in a solution fitted with GOD and glucose, the Alg- β -CD/mPEG-Fc/ α -CD/BSA shared a significantly higher release of pilot molecule BSA.

The combinatorial use of inorganic materials with natural origin polymers has been used to achieve certain relevant properties otherwise unachievable. Given the interaction of HA with its receptor CD44, metal-organic frameworks have been functionalized with HA for the delivery of hypoxia inducible factor signaling inhibitor (ACF) and immunologic adjuvants (CpG) [112] (**Figure I-4a**). As expected, a targeted delivery to cancer cells overexpressing the CD44 receptor was achieved as well as increased internalization. This system was applied in the context of photodynamic therapy which is known to aggravate tumor hypoxia therefore leading to tumor survival and metastasis of remaining cancer cells through the upregulation of certain factors such as VEGF, BCL-2, and MMP9. When applying the PCN-ACF-CpG@HA system, a decrease of these factors was confirmed which in turn led to a significantly lower cell viability upon laser irradiation. Furthermore, tumor suppression increased levels of intra-tumoral IL-12p70, IFN- γ , and TNF- α and ultimately more infiltrating CD8⁺ and CD4⁺ T cells were confirmed at the

tumor site. Several other systems have been developed with the targeting capabilities of HA in mind. Reports have explored the use of HA for the coating of novel polypyrrole nanoparticles using the near infrared dye IRDye800CW with camptothecin (CPT) for synergistic chemo-photothermal therapy [113] (**Figure I-4b**). These particles showed enhanced tumor targeting and when used in combination with laser irradiation and anti-PD-L1 immunotherapy, complete tumor eradication, with no recurrence during the entire 24 day observation period, could be seen, while no lung metastasis were found. Additionally, levels of immunomodulatory cytokines TNF- α , IFN- γ , IL-1 β , IL-2, IL-4, and IL-6 were significantly increased when treated by this method. Other strategies describe the use of HA for the coating of a liposomal nanoparticles system for the targeting of tumor expressing CD44 cells based on synthetic aminoxy lipids [114], which showed an increased internalization over 24 h when tested in CD44 expressing tumor cell lines (human nonsmall cell lung carcinoma H1299 and cervical carcinoma HeLa cells). Extracellular vesicles (EV) are known as an optimal system for the delivery of biomolecules as they originate from the cell membrane and therefore display fewer side effects than synthetic counterparts like liposomes. An EV-based vaccine system, referred to as HDEA@EVAT, has been achieved through coupling with HA, 3-(diethylamino)propylamine (DEAP), the immunomodulator monophosphoryl lipid A (MPLA) and mucin 1 (MUC1) antigen [115]. This system displayed a DC targeting potential derived from the high expression of CD44 in these cells, which led to improved endocytosis rates and hence a greater DC activation rate seen by increased CD86 levels and in turn translated into increased levels of TNF- α followed by higher levels of IFN- γ by CD8⁺ T cells. Other vaccine systems have also been developed considering the affinity of HA towards CD44. This was the case of pegylated HA which has been developed for the coating of multifunctional micelles loaded with OVA peptide [116] (**Figure I-4c**). These antigen delivery micelles when functionalized with HA induced high cellular uptake by B16-F10 cells when compared to non-modified particles, thus proving once again CD44 targetability. As CD44 may be expressed on the surface of several cells, other works have taken advantage of this for the targeting of macrophages. Recently, HA-decorated superparamagnetic iron oxide nanoparticles (HIONs) have been developed for the artificial reprogramming of macrophages [117], thus allowing for a higher particle internalization into macrophages leading to an increased production of inflammatory factors NOS and TNF- α and increased levels of CD80⁺ macrophages. Additionally, when tested *in vivo*, HION-reprogrammed macrophages were capable of re-educating neighbor M2 phenotype to shift toward activated M1 macrophages mainly due to the cell-to-cell communication.

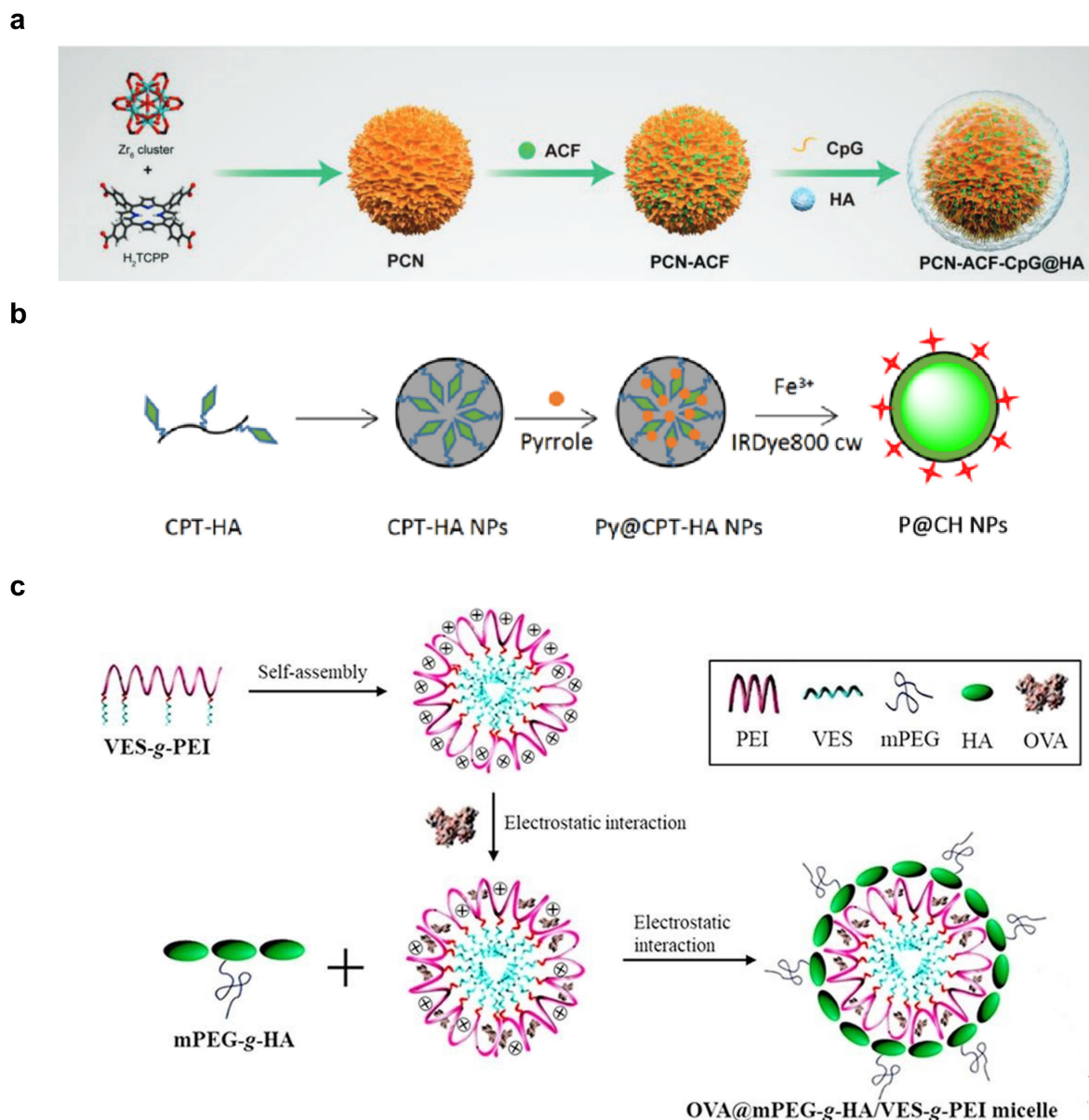


Figure I-4 HA as an adjuvant for the development of nanoparticles for immunotherapy.

a) Concept for the preparation of PCN-ACF-CpG@HA nanoparticles to be used as an in situ tumor vaccine by integrating PDT, antihypoxic signaling, and CpG adjuvants. Adapted with permission from [112]. Copyright 2019, John Wiley and Sons. b) Illustration of the formation of CD44 protein targeting P@CH nanoparticles, capable of combined chemo-photothermal therapy. Adapted with permission from [113]. Copyright 2019, Elsevier. c) Schematic illustration of the production of mPEG-g-HA/VES-g-PEI micelle for the targeted delivery of OVA and the triggering of CTL-mediated killing of tumor cells. Adapted with permission from [116]. Copyright 2019, Elsevier.

The targeted delivery of Granzyme B (GrB) has triggered interest due to its cytotoxic efficacy and action in a variety of apoptosis-inducing mechanisms [118]. Considering the alluring properties of HA for the delivery of key molecules in the tumor microenvironment (TME), nanoparticles have been developed for the targeted delivery of GrB containing a cell-penetrating peptide TAT (GrB-T) capable of inducing cell

apoptosis [119]. *In vivo*, this system showed that this mechanism led to the extracellular release of GrB-T, which enters the cell cytoplasm and triggers subsequent extrinsic apoptosis pathways, resulting in a significant anti-tumor effect. Other systems have embraced the same concept to take advantage of the biodegradable nature of HA. A nanopatform-based system comprised of Chlorin e6 (Ce6)-conjugated HA, dextro-1-methyl tryptophan (1-mt)-conjugated polylysine (PM) and anti-PD-L1 monoclonal antibodies (aPD-L1) was developed [120] as a technique to tackle, in one step, the 3 pathways that comprise the immunological cascade (antigen presentation, lymphocyte activation and proliferation/differentiation, and tumor elimination). In this case the enzyme rich TME triggered the release of the IDO inhibitor 1-mt and anti-PD-L1. This in turn promoted DC maturation, lymphocyte activation, the inhibition of the IDO pathway (enhancement of proliferation/differentiation) and the blocking of the PD-1/PD-L1 pathway (boosted tumor elimination), ultimately leading to an increased survival rate of tumor induced mice.

Imidazoquinolines (IMQs) have become of interest in the immunological field due to their ability to activate toll like receptors TLR7 and TLR8, which in turn induces the secretion of pro-inflammatory cytokines that promote innate immune responses. However, while the systemic administration of these compounds has not yet been approved, a need for novel systems capable of delivering IMQs while reducing systemic inflammation and toxicity has been identified. For this purpose, HA has been conjugated to tocopherol (vitamin E) to be used as a nanocarrier for the delivery of R848-Toco prodrug [121]. When tested *in vitro*, the R848-Toco/HA-Toco system triggered a higher TLR7 activity when compared to controls with higher levels of secreted TNF- α . Upon *in vivo* administration in a tumor mouse model, R848-Toco/HA-Toco significantly suppressed tumor growth when compared to a HA-Toco vehicle over time and generated an increase of CD8a, CD11c and CD11b.

Combination therapy, which consists on the simultaneous administration of traditional chemotherapeutics with novel immunotherapy strategies, is being explored for application in cancer therapy. Systems comprising natural polymers with biodegradable properties are being used in the development of novel and more advanced forms of applying these combinatorial therapeutics. A nanoparticle-based doxorubicin (DOX) delivery system with a MMP-sensitive peptide (CPLGLAGG) for enzyme-activated drug release comprising a HA tumor targeting moiety (HA-Psi-DOX) was described [122] to be used in combination with anti-PD-1L therapy. When tested *in vivo*, the system showed a clear homing ability, while also presenting a good retention ability when in circulation. This in turn translated into increased intra-tumoral content of IFN- γ and PD-L1, which ultimately led to a significant inhibition of

tumor growth, a higher number of tumor infiltrating lymphocytes and an improved antimetastatic capability.

Natural polymers can many times be heavily modified to yield synthetic complexes with biological response-modifying properties. Such is the case of cellulose, which has been used to develop the TLR3 agonist Poly-ICLC (Polyinosinic-Polycytidylic acid stabilized with polylysine and carboxymethylcellulose) [123]. Poly ICLC is a synthetic double-stranded RNA complex used as an immunostimulant and has been tested over several studies and clinical trials (NCT01984892, NCT03721679) and more recently has been used in combination with Flt3L and radiotherapy to yield an *in situ* vaccine (ISV) currently undergoing clinical testing (NCT01976585) [124]. Pre-clinical settings showed that upon treatment with the ISV, a marked accumulation of intratumoral cross-presenting DC was confirmed, which in turn led to a higher uptake of TAAs generating systemic tumor-specific CD8⁺ T cell responses. Through the combinatorial effect of PD1 blockade, an increased remission rate of up to 40% was verified. Patients with Indolent Non Hodgkin's Lymphoma enrolled in the phase I trial displayed similar expansion of DC subsets, upregulation of checkpoint molecules, and durable regressions of distant (untreated) tumors.

I-5. THE FUTURE OF NATURAL POLYMERS IN CANCER IMMUNOTHERAPY

While combination therapy has created advances in the field of immunotherapy through the requirement of more advanced forms of antigen presentation, key issues such as the susceptibility for proteolytic degradation of immunotherapeutics or even the effect of microenvironment immunosuppression, has raised concerns in the field. This has opened the door for new developments based on technologies typically used in other areas like polymer and tissue engineering. A chief example is the creation of polymer-based advanced delivery systems to aid in shielding biomolecules from the microenvironment. The introduction of natural polymers came naturally due to several advantages associated with them, namely their biodegradability, biocompatibility, accessibility and similarity to several ECM components, ultimately avoiding several drawbacks of synthetic polymers such as unwanted immune reactions and toxicity. However, despite the already significant presence of natural polymers in the field of immunotherapy, their use will grow even more in the future. As seen throughout this overview, several novel systems based on natural polymers have been recently developed. Many of these systems are still at a proof-of-concept stage using test molecules like -OVA to assess basic biological behavior. Upon *in vitro* and *in vivo* assessment of their efficacy with TAA, some of these systems may be in clinical

trials in the next few years in areas like vaccination. Certain natural polymers like chitosan can in fact serve as great natural platforms to develop vaccination tools due to their action as natural immunoadjuvants. Furthermore, hybrid systems taking advantage of key features of different polymers, natural or otherwise, will constitute an important portion of what the field of polymer engineering may give to immunotherapy.

As a better understanding of the basic mechanisms underlying immune-associated pathologies is achieved, the more efficiently well-known or even unexplored natural polymers be applied and new hybrid polymer systems be created. As in the case of HA and its affinity for CD44, to uncover unknown interactions of natural polymers with biological systems is a clear need.

Therefore, adopting the use of natural polymers to develop new systems or further enhance currently used ones brings clear benefits. The exploration of novel sources of these polymers, such as marine-based natural polymers [125,126], will open the doors to the creation of new materials with novel properties and characteristics that will further advance the field. However, one must keep in mind that this novelty comes with a tradeoff regarding the required path for regulatory approval. Therefore, while an effort to source for even more accessible and cheaper polymers from different natural origins is welcomed, it must be accompanied by a matching effort to establish their safety in pre-clinical and clinical trials.

I-6. REFERENCES

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CHAPTER II

MODELLING THE COMPLEX NATURE OF THE TUMOR MICROENVIRONMENT: 3D TUMOR SPHEROIDS AS AN EVOLVING TOOL

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MODELLING THE COMPLEX NATURE OF THE TUMOR MICROENVIRONMENT: 3D TUMOR SPHEROIDS AS AN EVOLVING TOOL[†]

ABSTRACT

Cancer remains a serious burden in society and while the pace in the development of novel and more effective therapeutics is increasing, testing platforms that faithfully mimic the tumor microenvironment are lacking. With a clear shift from animal models to more complex *in vitro* 3D systems, spheroids emerge as strong options in this regard. Years of development have allowed spheroid-based models to better reproduce the biomechanical cues that are observed in the tumor-associated extracellular matrix (ECM) and cellular interactions that occur in both a cell-cell and cell-ECM manner.

Here, we summarize some of the key cellular interactions that drive tumor development, progression and invasion, and how successfully are these interactions recapitulated in 3D spheroid models currently in use in the field. We finish by speculating on future advancements in the field and on how these can shape the relevance of spherical 3D models for tumor modelling.

[†] This chapter is based on the following publication:

Rodrigues DB, Reis RL, Pirraco RP, Modelling the complex nature of the tumor microenvironment: 3D tumor spheroids as an evolving tool. *Submitted* (2023)

II-1. CONTEXTUALIZATION

Over the past decade, increasing energy has been placed in the implementation of the 3 R's principle in animal experimentation, especially regarding animal replacement. Several different efforts have pushed this movement forwards including motions from the European parliament 2021/2784(RSP) which urge the acceleration of the transition to a world without the use of animals in research, regulatory testing and education. Additionally, several studies have reported that mouse models of human cancers present species-specific differences [1] such as size, life span, and organ morphology and physiology which ultimately may lead to erroneous interpretations of the efficacy of novel therapeutics during translation to human clinical application. In parallel, the growing knowledge on the role of the ECM on both physiological and pathological conditions like cancer, namely through the regulation of cell proliferation, differentiation, migration, survival and adhesion, has created the urge to adopt 3D culture platforms in detriment of non-biomimetic 2D tissue-culture plastics [2]. The effects of these 3D culture systems have shown to impact cell behavior at various levels ultimately affecting drug sensitivity through changes observed in cell morphology, cell viability and cell survival pathways among others [3].

All of this has contributed to the growing investment of the research community in building faithful cancer models that preserve the 3D architecture and the multicellular complexity of cancer tissue. Among those models are organotypic models (based on substrates like Matrigel® or Collagen I), tumoroids, organ-on-a chip and 3D scaffold-based systems [4–7].

While many of these systems have been proven advantageous for different applications such as high-throughput screening of anti-tumor drugs or to study specific cell-cell/cell-ECM interactions in detail, increasing both their fidelity and biomimetic complexity is still a major need. Additionally, key features such as ECM biomechanical properties play a crucial role in these models as not only is ECM stiffness influencing cellular attachment [8] but also tumor progression [9] and therefore requires careful tuning. In order to add to the complexity of these disease models, the cell heterogeneity observed within the tumor microenvironment [10,11] must be considered. Tumor heterogeneity has been a topic of interest in cancer research, with the first evidence of a heterotypic tumor model going back several decades [12]. The efforts to create highly complex tumor spheroid models which recreate the cellular interactions observed in the tumor microenvironment have been significant over the past years, with the use of non-cancerous cells (e.g. fibroblasts, endothelial cells, adipocytes, and immune cells) native to the tumor microenvironment key to this effort.

Spheroids emerged as 3D aggregates of cells that arrange themselves into sphere-like formations when in low-adhesion culture surfaces. These can be used to recapitulate tumor architecture when compared to conventional 2D cultures. Throughout this review, we will focus on how spheroid-based models have evolved, allowing for the development of tumor models with greater bio-similarity, how cellular interactions and heterogeneity have played a part in these advances and what can be expected for the future of these systems.

At high temperatures, GG has a coil form, which changes to a double helix upon temperature decrease. Hydrogels are formed when anti-parallel double helices

II-2. THE TUMOR MICROENVIRONMENT

In vitro tumor models have been contributing to the overall reduction of animal experimentation in the cancer field and to a more standardized use of models of higher complexity and biological relevance due to their high degree of reproducibility, high translational value and even commercial availability. However, several issues remain unresolved with a window of opportunity for new developments and advances. One of the main issues is the uniformity and reproducibility over a large number of samples, in which a uniform shape and size are difficult to attain. A second concern is defining a series of prerequisites that would qualify these systems as valid for drug screening. Ultimately, to take full advantage of these systems, high-throughput is a requirement, especially in the case of drug screening for pharmaceutical use.

To overcome these issues, a clearer understanding of the tumor microenvironment is required. Here, we will focus mainly on 2 areas of cellular processes critical in tumor biology: 1) cell-cell interactions and 2) cell-ECM interactions within the tumor microenvironment (**Figure II-1**). Attention will be given to key aspects driving tumor formation and progression, and how they may be applied during the development of novel 3D tumor models.

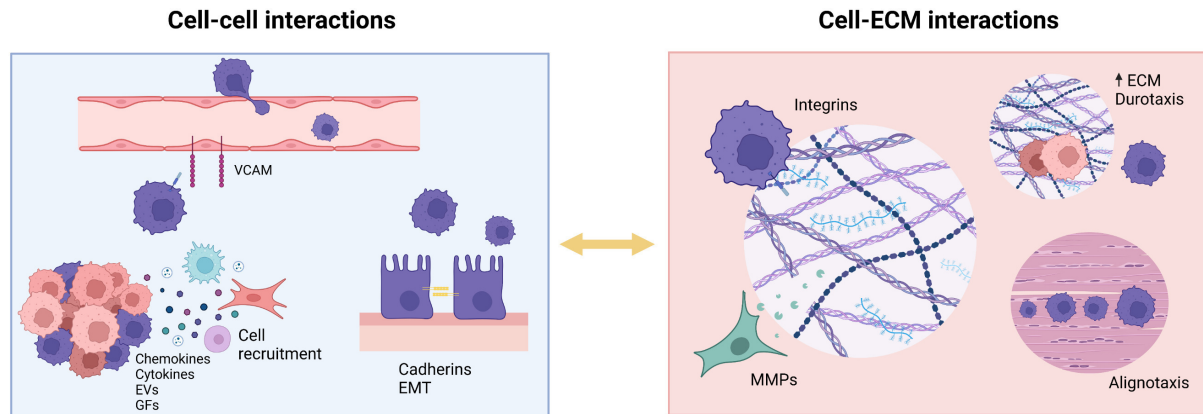


Figure II-1 Cell interactions in the tumor microenvironment shape disease progression.

The tumor microenvironment is a highly complex system that is tightly regulated by several different mechanisms. Most of these regulatory mechanisms occur in between cells, however interactions with the surrounding ECM are known to drive tumor cell migration and metastasization. A plethora of distinct biomolecules are behind these interactions which drive cell recruitment and chemotaxis, EMT, cell extravasation and cell invasion.

II-2.1. Cell-Cell interactions in the tumor microenvironment

The cellular composition of a tumor makes for a heterogeneous architecture given its distinct cell types. Communication between these different cell types allows for the regulation of the microenvironment and therefore acts as one of the driving forces promoting tumor progression [13,14]. However, to bridge the gap between native tissues and novel tumor models, it is necessary to first understand the interactions within the structural organization of current spheroid models. Variables such as time of culture and spheroid compactness contribute to how cell-cell interactions occur within these models, which in turn influences cancer cell behavior regarding proliferation, survival, and response to cancer therapeutics [15]. Within these systems, cell-cell cohesiveness is determined by key players such as cadherins or integrins [16–18] or intracellular components such as actin or microtubules [19,20].

Thus, going forward, we will split cell-cell dynamics into two distinct approaches, cell-cell interactions through cell adhesion molecules (CAMs) and secondly cell-cell signaling mediated via biomolecules such as cytokines, chemokines, growth factors and exosomes.

CAMs are a multivalent family of cell surface proteins that are involved in several roles, such as intercellular, intracellular, and cell-extracellular matrix interactions, as well as cell growth and motility regulation, signal transduction pathways, and inflammation. These proteins are further subdivided into four distinct subfamilies: immunoglobulin-like adhesion molecules, integrins, cadherins, and selectins. Several of these molecules have been implicated in cancer formation and progression.

Selectins are cell surface lectins that mediate the adhesion of circulating cells to the endothelium. This class of adhesion molecules consists of three proteins: E (endothelial), L (leukocytes), and P (platelet)-selectin. The recruitment of leukocytes through the expression of these molecules within the tumor microenvironment has aided in immune invasion, dissemination, extravasation, and the formation of a metastatic niche [21–25]. Overexpression of selectin ligands by cancer cells has also contributed to poor patient prognosis [26,27].

Similar to selectins, cadherins are responsible for cell-cell adhesion and are the most explored CAMs in the field of cancer. They are transmembrane glycoproteins involved in the maintenance of normal tissue architecture and have a particular role in organism growth. Cadherins can be further classified into several subtypes; however, E-cadherin and N-cadherin have been the most studied in the field of cancer. The expression of these classical cadherins has been associated with the epithelial-to-mesenchymal transition, a biological process in which polarized epithelial cells undergo a phenotypic switch into a mesenchymal cell phenotype [28]. Upon this switch, cells are prone to enhanced migratory capacity, invasiveness, greater resistance to apoptosis, and increased production of ECM components [28]. This is triggered by a decrease in the expression of the endothelial marker E-cadherin [29–31] followed by an increase in the expression of N-cadherin [32–34]. It is also important to highlight that this shift in phenotype may not be so black and white in which carcinoma cells may exhibit several epithelial-mesenchymal characteristics [35–37]. This mechanism is tightly regulated by a series of biomolecules, such as transforming growth factor- β (TGF- β), the fibroblast growth factor (FGF) family and epidermal growth factor (EGF) [38–45]. These soluble factors belong to a group of proteins known as growth factors which have been implicated in the constitutive activation of growth-promoting pathways, modulation of cell phenotype and promoting tumor neovascularization. Possibly one of the most studied is vascular endothelial growth factor (VEGF), a glycoprotein with an important role in endothelial cell proliferation and vascularization [46]. Within the tumor microenvironment, this molecule is produced by tumor associated macrophages as well as by cancer cells, contributing to the production of damaged, permeable and leaky neo-vessels [47][49] that nevertheless support tumor survival [48]. Other growth factors such as Epidermal growth factor (EGF) and fibroblast growth factor (FGF) have been tied to cancer cell proliferation, differentiation and survival in the case of EGF [53,55,57,59], while FGF additionally acts as a pro-angiogenic factor by synergistically acting with VEGF [61,63]. In a similar fashion, PDGF is a factor impacting cancer cell proliferation capabilities [65] that can also trigger the production of ECM proteins and support tumor angiogenesis [65,67], a process in which the tumor microenvironment supports the formation of new blood vessels crucial for tumor cell can growth, invasion and metastasization. Another

growth factor that has become a biomarker for tumor cell activity is insulin-like growth factor (IGF) [69,71] which was also implicated in inhibiting tumor cell apoptosis while stimulating their proliferation [73,75].

Several other soluble proteins have been associated with the direct regulation of the tumor microenvironment, including cytokines and chemokines. These are linked to regulating the nature of immune responses and controlling immune cell trafficking. Cytokines like tumor necrosis factor alpha (TNF- α), TGF- β , IL-6, IL-10 and IL-17 secreted in either an autocrine, endocrine or paracrine manner have assumed a pivotal role in promoting tumor survival and metastasis. This is achieved by several different pathways, may it be immune-suppression, inhibition of angiogenesis, vasculogenic mimicry and cancer cell proliferation, migration and invasion, or aggravating the inflammatory process [50–52,54,56,58,60,62]. Also crucial to the regulation of these cancer-related processes are chemokines. These are chemotactic cytokines that regulate the migration of immune cells and these have also been implicated in cancer processes [64]. Another form of soluble factor that has been tightly implicated in cancer are extracellular vesicles (EVs). EVs have gained quite some interest from the field due to their ability to transfer bioactive cargoes that have several effector functions. Linked to the transport of lipids, proteins, and nucleic acids, EVs have been implicated in the development and maintenance of tumor growth, metastasis and immune escape [66,68].

II-2.2. Cell-ECM interactions in the tumor microenvironment

While cell-cell interactions are crucial in driving cancer processes, the role of ECM is also important. This non-cellular component found within all tissues and organs is not only responsible for the structural support of cellular constituents but also has a well-known role in establishing biochemical and biomechanical cues that are required for tissue morphogenesis, differentiation and homeostasis and that additionally have been involved in driving tumor progression as previously overviewed [70,72]. Different key components contribute to the role of the ECM as a determinant factor for poor prognosis in several different cancers [70,74]. Some of the most influential are metalloproteinases (MMPs) and integrins. MMPs are a family of zinc-containing endopeptidases, which are similar among themselves both structurally and functionally. These enzymes are known for their role tissue repair and remodeling, cellular differentiation, cell mobility and wound healing [76], achieved through the cleavage of ECM components [77]. Moreover, two gelatinases (MMP-2 and MMP-9) have caught the spotlight due to their implication in several mechanisms such as angiogenesis and infiltration of cancer cells as well as metastasization [78–80]. The production of these molecules has been associated to different non-malignant stromal cells such

as fibroblasts immediately surrounding clusters of cancer cells [81,82]. An imbalance in the production of MMP and its inhibitor TIMP has been identified as a key factor in driving poor prognosis [83,84].

Changes in the structural nature of the ECM can, in turn, alter the mechanical properties of this non-cellular component and consequently lead to events such as durotaxis, in which cell migration occurs in response to gradients of extracellular stiffness [85,86]. Similarly to durotaxis, cells have been reported to undergo directed migration along aligned ECM fibers defined as alignotaxis [87]. CAFs have been implicated in this process by promoting directional cancer cell migration through the alignment of fibronectin fibers within the tumor ECM [88].

Cells interact with ECM through a series of integrins which are a class of transmembrane $\alpha\beta$ heterodimers that are responsible for the binding of extracellular matrix ligands, cell-surface ligands, and soluble factors [89]. Several domains have been identified, and at least 18 α and eight β subunits are known in humans [90,91]. Different ligands such as the collagen, laminin, fibronectin or leukocyte-specific receptors, interact with these heterodimers in a distinct fashion. In this manner, integrin function embraces several cancer processes such as guiding tumor cell migration and invasion, cancer cell survival and anoikis suppression, extravasation and enhancement of tumor stemness. For instance, $\alpha3\beta1$ is associated to the differentiation and maintenance of the CAF phenotype, while also supporting the invasion of pancreatic duct adenocarcinoma heterospheroids [92]. Other effects of integrins on fibroblasts have also been observed, with $\alpha\beta6$ and $\alpha9\beta1$ being implicated in CAF recruitment and in sustaining their survival [93,94]. Integrin $\alpha9\beta1$ has additionally been linked to promoting the migration of glioblastoma and osteosarcoma cells as well as metastatic progression [95]. In addition to cell surface expression of integrins, these have also been found in extracellular vesicles [96]. In a model of colorectal cancer, $\beta1$ integrin-rich EVs are secreted into circulation by tumor cells to activate resident fibroblasts in remote organs, which in turn induces a pre-metastatic niche and promotes metastatic cancer growth through the secretion of pro-inflammatory cytokines IL-6 and IL-8 [97]. Similarly, $\alpha2\beta1$ aided in CAF EV uptake by lung fibroblasts and consequently the activation of the TGF- β signaling pathway in these cells [98].

II-3. MODELING THE TUMOR MICROENVIRONMENT *IN VITRO*

In order to develop 3D tumor models that are able to mimic interactions that take place within the tumor microenvironment, two key aspects come into play. The first is cell source and heterogeneity, which

is an important differentiator for the different known spheroid tumor models, and the second is the level of recapitulation of key cell-cell and cell-ECM interactions that these models can achieve. Herein, we will briefly overview the different types of spheroid models that have already been described and the key features in terms of cellular interactions that have been recently studied.

II-3.1. Currently available spheroid models

The nomenclature used for 3D tumor models has been quite heterogeneous over time, with several new terms being introduced every so often. Terms like “spheroids”, “sphere”, “tumorsphere”, “oncosphere”, “organoid” or “organotypic spheroid” have been deliberately used, often making it difficult to understand the very nature of each system. These different models distinguish themselves mainly through medium composition used, culture surface, cell density, time required for formation, origin and handling.

There is however one common denominator that is the architecture of these systems. While the terms “aggregate” and “spheroids” have distinct meanings, confusion has been installed in using one term or the other. The systems that we will discuss herein are all “spheres” or “spheroids” consisting in closely compacted spherical cultures. And are not to be mistaken with “aggregates” that are loose cellular aggregates that easily detach [99]. Recently, Pasca et al., has overviewed the nomenclature of such systems in the context of nervous system models [100] while Weiswald et al. divided the classification of spherical cancer models into four main groups: 1) multicellular tumor spheroids, 2) tumorspheres, 3) tissue-derived tumor spheres, and 4) organotypic multicellular spheroids [101].

Here we will take a simplistic view to what has been used to name these systems in the context of cancer 3D models, based on the cell source and culture method (**Figure II-2**).

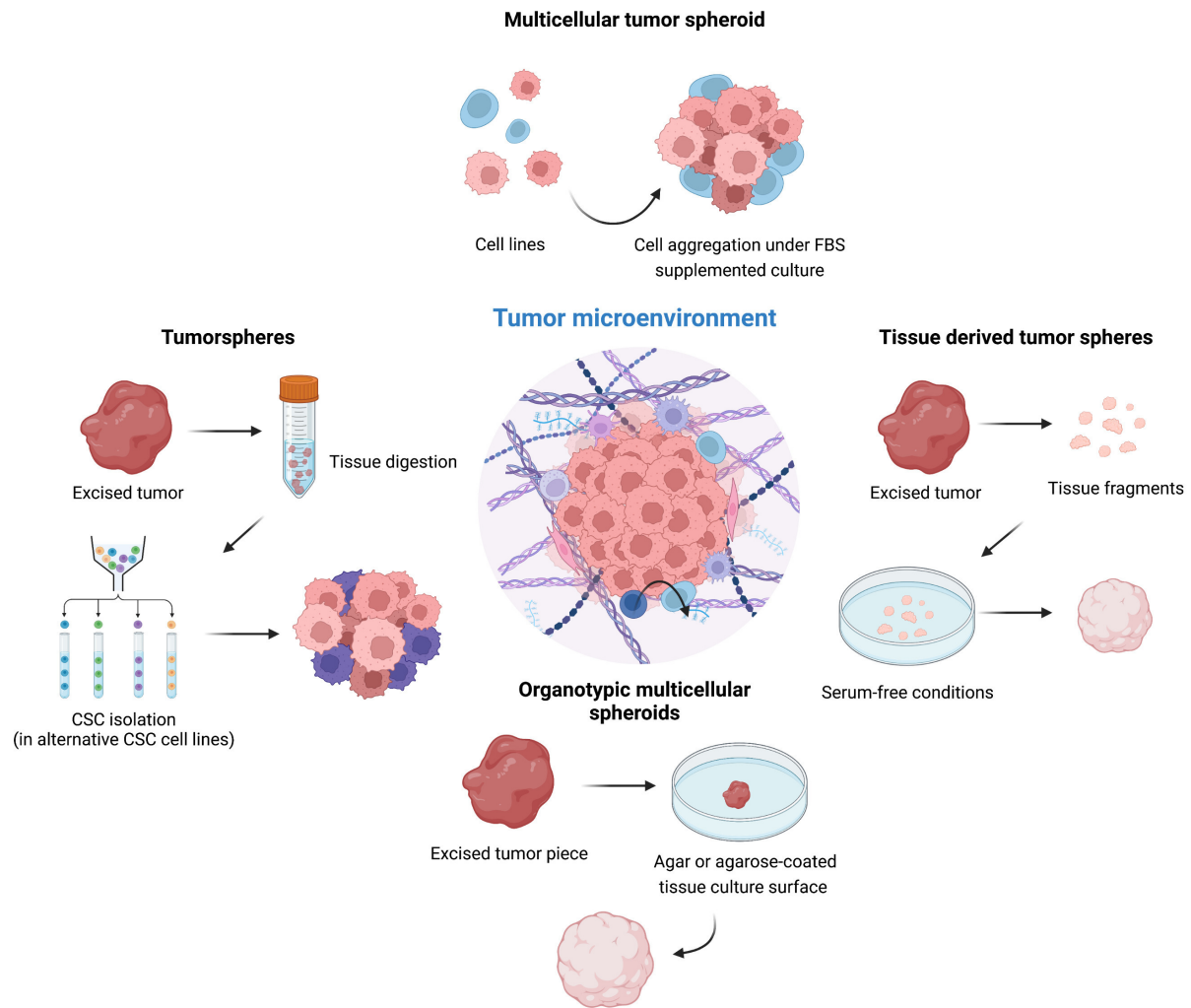


Figure II-2 3D spheroid culture models for the *in vitro* recreation of the tumor microenvironment.

Different culture methods have been adopted to attempt to recreate tissues within *in vitro* culture conditions. Spherical 3D models have been a golden standard in the field. Depending on cell-source, tissue digestion and culture methods a varying degree of complexity and homology towards native tumor tissues can be achieved.

II-3.1.1. Multicellular tumor spheroids

The main discriminator between multicellular spheroids (MCS) and traditional 2D monolayers is that MCS are grown as spheres, which promotes enhanced cell-cell and cell-ECM adhesion. These cellular spheres are generated from single-cell suspension cultures in FBS supplemented medium without the supply of an exogenous ECM and generally originate from cancer cell lines and not from dissociated tissues. Whether cells can spontaneously aggregate into spheres in the absence of a cell attachment substrate is highly dependent on the cell type, therefore it is important to highlight that not all cell lines are capable of generating compact MCS [102].

While histological resemblance of these 3D culture systems with primary cancer tissues is minimal, they present metabolic and proliferative gradients seen *in vivo* as well as relevant chemoresistance. Several other key features make MCS desirable tumor models, such as their clonality, easy maintenance and simplicity in performing genetic manipulation. From a biological point of view, MCS can expand up to sizes between 1 to 3 mm in diameter. However, above 500 μm a distinct architecture is known to take over, comprising an outer proliferating layer followed internally by a layer of quiescent cells and housing in its center a necrotic core [101]. Growth dynamics also vary from traditional 2D cultures, where an early exponential phase is observed followed by a period of delayed growth resulting from the increase in the nonproliferating and necrotic cells [103].

Several methods have been proposed for the formation of MCS [104]; however, the most adopted among the community consists in providing conditions in which the adhesive forces between the cells are greater than between cells and the substrate on which they are cultured, as is the case of ultra-low adhesion tissue culture plates.

II-3.1.2. Tissue-derived tumor spheres

While MCS have aided in recreating cell-cell interactions specific to the tumor microenvironment *in vitro*, they are still limited in their complexity when taking into consideration the distinct cellular genotypes and phenotypes that make for heterogeneity between tumors of the same histopathological subtype. More biologically representative 3D spherical tumor models are tissue-derived tumor spheres (TDTs) also called cancer tissue-originated spheroids. These systems are obtained from the partial digestion of cancer tissues into small fragments which then spontaneously form spherical structures within several hours under serum-free conditions [105]. One of the key advantages of these systems is that cell-cell contact is maintained throughout the preparation and culture process which yields spheroids consisting of highly purified and viable cancer cells [106]. This high yield in neoplastic cells may be explained by the strong cell-cell interactions observed between cancer cells, thus leading to the preservation of neoplastic cells during partial dissociation and the loss of non-neoplastic cells, resulting in TDTs-deprived stromal cells [106]. Among their advantages as a biosimilar tumor model are their capacity to recapitulate avascular tumor regions as well as the maintenance of histological characteristics, gene expression profiles, mutations in relevant genes and tumorigenic and metastatic properties [101].

II-3.1.3. Tumorspheres

Cancer stem cells (CSCs) have become a hot topic due to their important role in tumor dissemination. The capacity for self-renewal and the ability to differentiate into other specialized cell types have been identified in certain subpopulations of cells within tumors, with the added features of being capable of seeding tumors when transplanted into an animal host [107]. Tumorspheres have been created not as another model to mimic cancer tissues but to study the properties of CSCs as it has been shown that tumorspheres do not fully replicate the 3D tumor structure nor environment [108]. Tumorspheres have been known to form when CSCs are plated at low density in nonadherent conditions which promote the proliferation of these cells as clonal nonadherent spherical clusters. These cultures are usually conducted in medium devoid of FBS and supplemented with several factors that favor stem cell growth. In order to first obtain these CSCs, the mechanical and enzymatic dissociation of tumor samples into single cell suspensions is required, but ultimately CSCs culture may also be obtained through cancer cells lines.

II-3.1.4. Organotypic multicellular spheroids

Organotypic Multicellular Spheroids (OMS) are very distinct from the classical MCS as they are generally obtained from excised tumor tissues similarly to TDTs but without undergoing digestion. These tissue pieces are then transferred to agar or agarose-coated tissue culture surface in order for them to develop into multicellular spheroids [109,110]. Also important to point out is the capacity of these models to be cryopreserved while retaining their histological characteristics and being subject to only minor phenotypic and genotypic changes after thawing [111]. This model sets itself apart from other tumor models because of its high similarity to native tumor tissues, achieved by avoiding any kind of dissociation process that may interfere with the tissue architecture and cellular organization. This in turn leads to a high cellular heterogeneity similar to that of the tumor by maintaining the presence of vascular, immune and stromal fractions [112] contributing to a comparable 3D model.

II-3.2. Modelling cell-cell and cell-ECM interactions in current spheroid models of cancer

Modeling the aforementioned cell-cell and cell-ECM crosstalk in *in vitro* tumor models is a requirement for the field and therefore the creation of biomimetic models capable of modeling not only

drug behavior but also those cellular interactions within the tumor microenvironment has become a priority. In similar fashion to what is described within the tumor microenvironment, 3D tumor models must also present these cell interactions through CAMs and soluble factors while producing ECM that retains characteristics typically observed in tumor-associated ECM *in vivo*.

II-3.2.1. Intercellular adhesion

CAMs are not only crucial for the formation of spherical 3D models due to the requirement of cellular aggregation but are also one of the methods by which cells interact and communicate, which in the end becomes crucial for tumor development and invasion. Ultimately, the expression of some of these molecules may be used for targeting purposes for the delivery of novel therapeutics.

P-selectin is a CAM with a proven in tumor invasion. Glioblastoma (GB) spheroids were studied to better understand how microglia could facilitate GB invasion and immunosuppression. It was found that P-selectin expression was higher in 3D cultures and in particular when microglia was co-cultured in GB spheroids in contrast to 2D cultures (**Figure II-3a**). This change in expression mediated the role of microglia in facilitating GB proliferation and invasion by altering the activation state of microglia/macrophages [113]. This confirms the importance of a 3D structure capable of mimicking the tumor microenvironment and associated ECM. Additionally, the expression of anti-inflammatory markers and cytokines IL-10 and TGF- β by microglia/macrophages were increased similarly to *in vivo*. Hematogenous metastasis is highly dependent on cell adhesion mediated by molecules like E-selectin expressed by the endothelial compartment of blood vessels. Understanding that such regulatory mechanisms also take place in 3D tumor models demonstrates their value in comparison to traditional culture systems. Homotypic and heterotypic 3D spheroids of tumorigenic (BT20 and MCF7) and non-tumorigenic (MCF10A) mammary cell lines have also been studied regarding their capability to bind E-selectin [114]. Heterotypic 3D cultures were shown to be the best regarding the capacity to bind E-selectin (**Figure II-3b**) when compared to 2D, as well as being more invasive.

Of late, several other groups have utilized 3D spherical culture systems to study interactions around cadherins and their implications in EMT and cancer cell invasion [115–118]. Cell-cell interactions through CAMs like N-cadherin and E-cadherin, ultimately impact cell contractility and hence cell dispersion or invasion. Heterotypic tumor spheroids consisting of EMT and epithelial A549 cancer cells, demonstrated these interactions through the formation of N-cadherin/E-cadherin adhesion complexes at the interface

between highly contractile EMT cancer cells and poorly contractile epithelial cancer cells during tumor spheroid dispersion [119]. As a potential drug testing tool, multicellular spheroids of triple-negative breast cancer cells co-cultured with endothelial cells were prepared [120]. 3D culture led to an increase in the activation of the VE-cadherin pathway when endothelial cells were cultured in the presence of breast cancer cells, highlighting the importance of these models in recapitulating the tumor microenvironment *in vitro*.

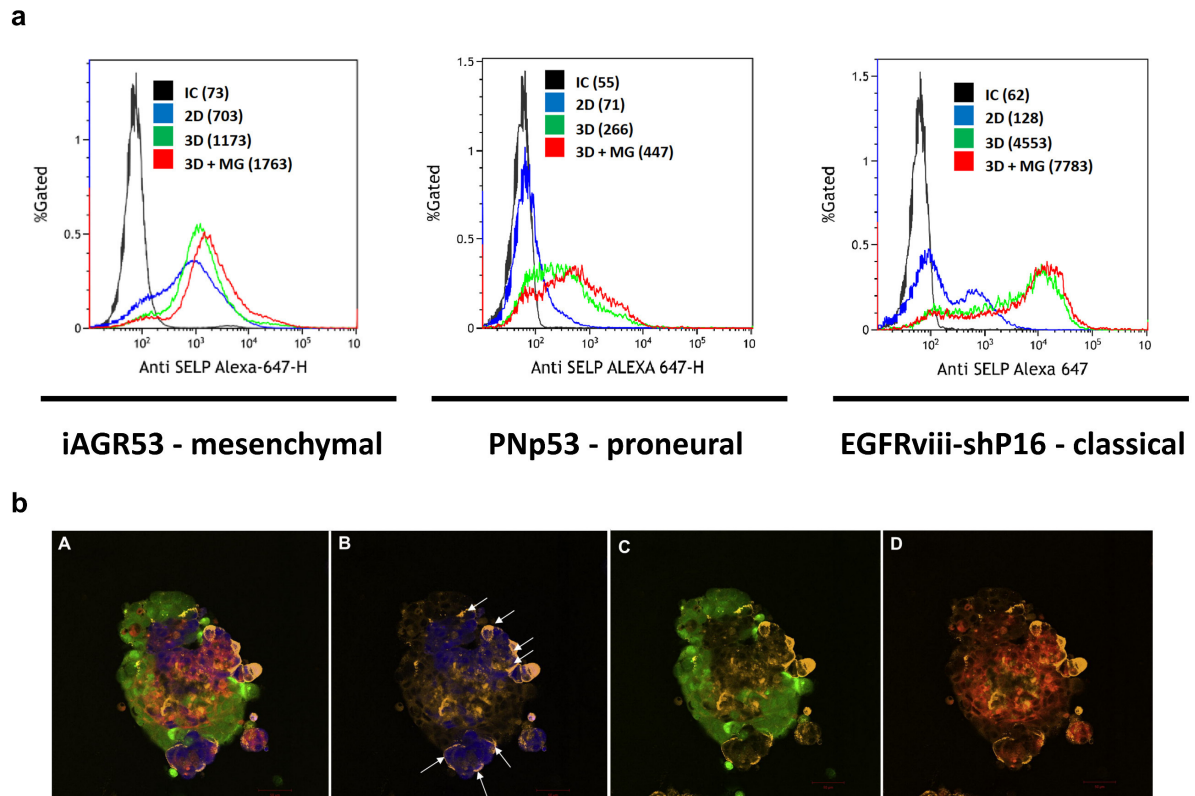


Figure II-3 *In vitro* tumor spheroids as suitable tools to study cell adhesion molecules.

a P-selectin expression was shown to be increased in multicellular spheroids of GB cells when co-cultured with murine microglia when compared to 2D controls. Adapted under the terms of CC 4.0 license from [113]. Copyright 2021, the authors. **b** Confocal microscopy images of soluble E-selectin (orange) binding to whole co-culture spheroids with BT20 (blue) MCF7 (green) and MCF10A (red) cells on PDMS (A). Superimposed images of soluble E-selectin with individual cell types in co-culture indicate preferential binding of soluble E-selectin to BT20 cells in co-culture (B) when compared to MCF7 (C) or MCF10A cells (D) in co-culture. Adapted with permission from [114]. Copyright 2012, Elsevier.

II-3.2.2. Soluble factors

The cell relies on different mechanisms through which they inter-communicate, one of these key signaling mechanisms is through the release of soluble factors which should be similar in nature to *in vivo* tumors when developing spheroid tumor models. These soluble factors can play different roles in

different pathways, one of which is the regulation of cell stemness. Given the importance of stem cell traits in cancer cells and their role in tumor development, spherical tumor models have been used to attempt to recreate the microenvironment in which these cells co-habit within the tumor. Recently, uveal melanoma (UM) cells were studied for their clonal heterogeneity in the form of non-adherent spheroid preparation [121]. Data demonstrated that ZEB1 expression levels were negatively correlated to spheroid formation from the single-cell suspension culture and to the expression of the stemness, suggesting a possible role in the suppression of cancer stem cell properties in UM (**Figure II-4a i-ii**).

While these systems can many times mimic specific cellular interactions that take place within the tumor microenvironment, they can also be created to achieve a higher resemblance with the desired cancer tissue architecture. A recent example of this was the creation of a pancreatic ductal adenocarcinoma model through the preparation of both multicellular spheroids and stratified multicellular spheroids that were produced by a 2-step process together with pancreatic CAF-Stellate Cells [122]. It was shown that this system presented stratification between cancer and stromal cells, accompanied by the expression of several soluble factors found in human pancreatic cancer such as TGF- β , FGF-2, IL-1 β , and MMP-9 (**Figure II-4b**). Additionally, in this model, de novo deposition of collagen and glycosaminoglycans was observed. In a similar fashion, others have attempted to address the question of how soluble factors play a part in tumor development and progression.

Growth factors like IGF binding protein 3 (IGFBP3) have been studied given the role of the IGF-pathway in tumor cell proliferation, metastasis, and survival. For this purpose, H1299 cell lines transfected with IGFBP3 were used to produce 3D spheroids to study both growth and invasion [123]. It was shown that IGFBP3 negatively impacted 3D spheroids growth and invasion which correlated with an inhibition in the secretion of MMP-1 and an overall decrease in total MMP activity in culture supernatants.

TGF has also been long recognized as important for the tumorigenic process, leading groups to develop new 3D culture systems in order to better understand how this cytokine interacts within the tumor microenvironment. Free floating spheroids were prepared from the human gastric cancer line MKN-45, which were then used to study the role of STAT3 activation [124]. These spheroid cultures presented higher STAT3 activity, up-regulation of TGF- β and VEGF with downregulation of IL-6. Additionally, conditioned medium from these gastric cancer spheroids were shown to polarize T cells towards a higher expression of FOXP3, TGF- β , and IL-10 indicative of a Treg phenotype. Another characteristic to consider while creating different 3D culture systems is geometry. While spheroids are valuable building blocks to mimic native tumor tissues *in vitro*, they many times lack geometrical complexity typical of tumors. To

address this issue, researchers have studied the effects of different geometries in free standing tumor models and the impact of TGF- β signaling [125]. TGF- β appeared to upregulate the expression of cell tension-related proteins for peripheral cells, and alter the sensitivity of cells to their environment. Other recently reported systems have also observed the effects of TGF- β in tumor-simulated microenvironments recreated by 3D spherical models [126–130].

VEGF, a key player in the tumor microenvironment, has been immensely studied *in vitro* through spheroid cancer models. MG-63 human osteosarcoma cells were used to develop a 3D cancer model using microfluidics in order to study the role of VEGF-A in the tumor microenvironment [131]. A distinct behavior between 3D and 2D cell culture was observed, in which VEGF-A expression decreases upon the application of external stressors (reduced serum culture and HIF inhibition) in 2D cultures but increases while in 3D. Hepatocellular carcinoma, a highly vascularized tumor, was also modelled using 3D culture models for *in vitro* studies. For this effect, monocellular or co-culture spheroids were produced by seeding of either human liver hepatoma Huh7 (p53mut) and liver cancer HepG2 (p53⁺) or the co-culture of these at a 1:1 ratio with human umbilical vein endothelial cells (HUVECs)[115]. It was observed that VEGF stimulation led to changes in both size and density of the 3D spheroids, to an increase in invasion and angiogenesis as well as an increase in the expression of EMT markers vimentin, N-cadherin 2 and Thy-1. This importance of VEGF in the tumor process has also led to different heterotypic spheroid cultures comprising tumor cells and endothelial cells. In a 3D spheroid model of malignant pleural mesothelioma, P31 epithelioid MPM cells co-cultured with HUVEC cells permitted endothelial sprouting while SPC111 co-cultured spheroids revealed endothelial sprouts leading to anisotropic sprout arborization [132]. In another heterotypic spheroid model, long-term spheroids of up to 30 days of culture were developed from HCC1954 tumor cells, human fibroblasts, and ECs [133]. This study showed that EC were maintained viable for up to 1 month of culture under agitation while maintaining the expression of key surface markers and not requiring VEGF supplementation, as this growth factor was produced endogenously. Additionally, it was shown that this long-term maintenance is tumor cell line-dependent and, in some cases, dependent on the presence of fibroblasts and agitation. Additionally, several other GFs known to play a part in the cancer microenvironment have been studied in the context of 3D spheroid tumor models *in vitro*. Drug resistance mechanisms have been recently studied in several different spheroids of both melanoma and metastatic melanoma [134], in which it was shown that the epidermal growth factor (EGF) pathway was affected by triggering protein kinase G, as seen by lower EGFR phosphorylation and reduced activation. Others have developed spheroids of human head and neck squamous cell carcinoma (HNSCC) cell lines with OECM-1 & SAS cells [135]. When stimulating these spheroids with EGF, changes to the

surrounding collagen matrix were observed by means of a strong contraction deformation and a radial alignment of the collagen fibers with respect to the center of the spheroid. Additionally, growth factors such as fibroblast growth factor (FGF) [136,137] and platelet-derived growth factor (PDGF) [138] have also been recently object of study in different 3D spheroid models underlying the importance of these models in mimicking the tumor microenvironment *in vitro* and the added value in using these systems when compared to traditional 2D cultures.

Beyond the role played by growth factors, other soluble factors such as cytokines and chemokines have been subject of study in the scope of these 3D spherical models. CXCL12 is one of these molecules, a potent chemoattractant known to be involved in several different pathologies. A 3D co-culture model between triple negative 4T1 breast cancer cells and macrophages primed with either MSCCXCL12^{+/+} and MSCCXCL12^{-/-} was performed, in which it was observed that MSC-derived CXCL12 drove macrophages to support an increase in the number and size of 4T1 multicellular spheroids [139]. TNF expression in the cancer microenvironment has been modeled with several different forms of 3D spherical cultures. This cytokine has a strong involvement in cancer development with a mixed function as tumor suppressor or tumor promoter [140]. To understand the role of this cytokine on endothelial cells, spheroids of the human endothelial cell line Ea.hy926 were prepared and incubated with both TNF and VEGF. These 3D cultures resulted in increased the expression of both pro-inflammatory and anti-inflammatory factors when compared to the 2D condition [141]. In a tumor spheroid model of human gastric adenocarcinoma cell line HGC27, TNF- α and INF- γ were used as stimulants in order to trigger the production of PD-L1 which in turn allowed to study the mechanistic effects of PD-L1 blockade [142]. Zhao and colleges [143] aimed at understanding the effects of TNF- α in the malignant transformation of intestinal stem cells (**Figure II-4c**). TNF- α proved to accelerate cell proliferation, migration and invasion, induce chemotherapy resistance and promote epithelial-mesenchymal transition.

Finally, EVs have been an object of study by the research community given their potential as targets for novel therapeutics. Several works evaluate the expression of EVs in distinct spheroid models of cancer. In one study, CABA I human ovarian cancer cells, were cultured by a hanging drop method to create spheroids [144]. In these 3D cancer models, in similar fashion to *in vivo* tumors, EVs were secreted and entrapped inside the spheroid ECM. Additionally, these spheroids presented the capability of vasculogenic mimicry, defined by the formation of tubule-like structures in an angiogenesis-independent manner, capable of supporting the metabolic needs of growing tumors [145]. Over the past few years, several other groups presented different 3D culture systems able to express EVs in a similar fashion [146–148].

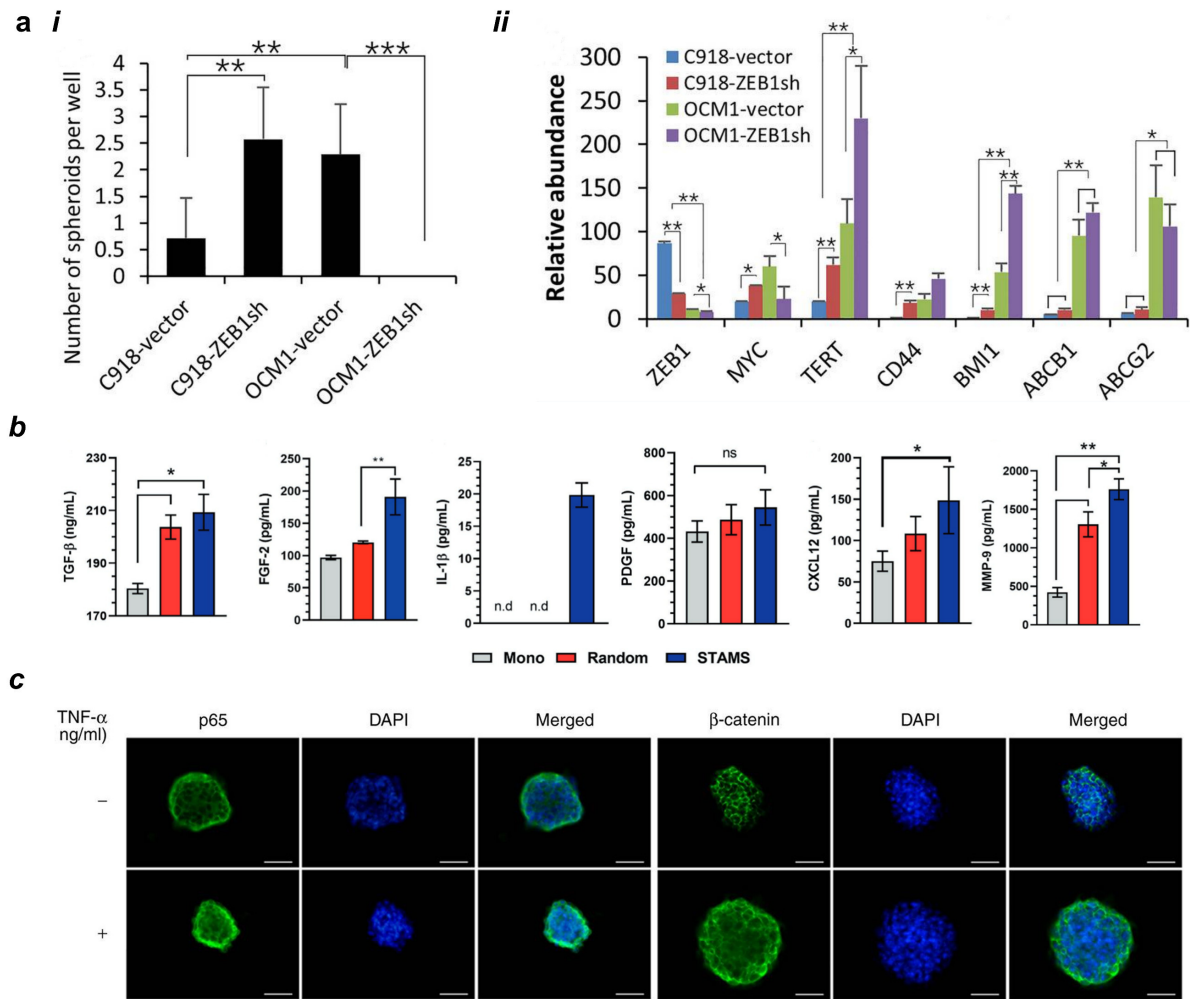


Figure II-4 Soluble factors play a role in intercellular communication within 3D spheroid systems.

a i) ZEB1 knockdown in ICM1 (OCM-ZEB1sh) leads to diminished spheroid formation, suggesting the role of ZEB1 in spheroid formation. **ii)** ZEB1 directly binds to and potentially represses expression of the TERT and ABCB1 genes. Reproduced with permission from REF. Copyright YEAR, PUBLISHER. Adapted under the terms of license CC BY-NC-ND 4.0 from [149]. Copyright 2022, the authors. **b** The secretion of EMT, chemoresistance and migratory-associated factors is heavily dependent on cellular organization in heterotypic tumor spheroids. Adapted with permission from [122]. Copyright 2021, Wiley. **c** TNF-α stimulation induces the activation of p65 and β-catenin in NCM460 cell spheroids when compared to non-stimulated spheroids. Adapted under the terms of license CC BY-NC-ND 4.0 from [143]. Copyright 2020, the authors.

II-3.2.3. ECM

Tumor-associated ECM plays a major contribution to how cells organize and distribute themselves in the microenvironment and even in regulating their phenotype. Spheroids have been used to better understand some of the mechanisms underlying ECM interactions and unveil some of the pathways that can be targeted for future therapeutics.

As discussed before, integrins are crucial for the development of tumor metastasis. In epithelial ovarian cancer, integrins play a crucial role in the detachment of malignant cells from the primary tumor and their reattachment to peritoneal surfaces. 3D spherical models of ovarian cancer have been used to shed light on the impact of both integrin beta-6 (ITGB6) and SET and MYN-domain containing 3 (SMYD3) on the activation of the TGF β 1/Smad3 pathway and downstream upregulation of N-cadherin and downregulation of E-cadherin [165]. This was indicative of the importance of the positive feedback loop between SMYD3/ITGB6/TGF β 1 in enhancing the invasion and adhesion of ovarian cancer spheroids as well as cell-cell communication in these models. Multicellular spheroids of ovarian carcinoma, prepared with the SKOV-3 cell line, have been used to better understand the role of Wnt11 on the expression of integrins and cadherin [167]. In these 3D models, Wnt11 was shown to negatively regulate ITGB2, ITGB6 and EpCAM while impeding the attachment of the multicellular spheroids to an ECM substrate, suggesting a role of this molecule in ovarian cancer progression. Other studies have also shown the importance of 3D models in studying the role of integrins in EMT. Thyroid cancer spheroids were used to study the effects of cadherin and integrins regarding motility and invasion in 3D [169]. Increased motility and a decrease in the molecular weight of integrin β 1 were observed within the 3D model as well as an upregulation of EMT signaling molecules Snail and ILK, clearly denoting the differences between 3D and monolayer cultures. Tumor heterogeneity, as observed in melanoma, may result in poor treatment response. Therefore, studying tumor tissues *in vitro* may allow to unravel some of the key features that drive tumor aggressiveness. Tissue samples from melanoma patients were used to produce spheroids in order to better comprehend tumor aggressiveness [149]. On this basis, dermal nest melanoma cells displayed *in vitro* higher expression of α 4/ α 7 integrin when compared to combined type melanoma cells. This difference among both was also visible in the expression of adhesion molecule N-cadherin, which was higher in dermal nest melanoma when compared to that of combined type melanoma. Hence, these models allowed to study the behavior of tissue derived cancer cells in specifically directed culture assays that allow to better understand the impact of certain molecular signatures and how they regulate tumor cell invasion.

Understanding microenvironment stiffness and mechanics, for instance, has become the objective of several recent reports that use tumor cell spheroids to perform mechanical analysis. Nanoindentation and microrheological experiments have been performed on spheroids of bladder cancer cell lines, which showed that cancer-induced changes lead to cell softening in T24 or HT1376 carcinoma cells when compared to HCV29 non-malignant ureter cancer cells (**Figure II-5a i-iii**) [150]. Additionally, a decrease in the rigidity index was observed between cell monolayer and 3D cultures, indicating the role that ECM

plays in microrheological properties. Other reports have equally described the use of different spheroid systems comprising multicellular or unicellular approaches to study the effects of mechanobiology on the tumor microenvironment [151–154]. Intertwined with ECM function are MMPs, which, as described above, have been associated with cancer cell invasion and are subject to various forms of regulation. The effect of intercellular communication and its impact on the production and activity of MMPs has been subject of study using 3D cancer models. Co-culture spheroids were produced using breast cancer cells (MDA-MB-231) and one of three different types of stromal cells, namely human adipose-derived stromal cells (hASCs), human bone marrow stromal cells or human dermal fibroblasts [155]. A higher deposition of both collagen type I and fibronectin was observed in the co-culture system with hASCs and this was shown to be dependent on MMPs activity which was regulated by a higher expression of tissue inhibitor of metalloproteinases-1 (TIMP-1) (**Figure II-5b**). Consequently, the presence of hASCs in the co-culture system led to a decrease in drug penetration efficacy.

The role of non-malignant tumor supporting cells within the tumor can also be addressed using 3D models. One such cell is the fibroblasts. The importance of fibroblasts within the tumor microenvironment has been evidenced in a 3D co-culture model encompassing HCT-8 colon cancer cells and NIH3T3 fibroblasts [156]. Herein, increased production of collagen type I and α -SMA was verified in the co-culture system when compared to monocellular spheroids. Increased stiffness was also observed as well as an increase in the abundance of TGF- β 1. Additionally, this model allowed to observe the decrease in drug efficiency when going from 2D monoculture to 2D co-culture and to 3D cultures. Heterotypic models like the one described by Arora and colleagues [157], comprising lung adenocarcinoma cells (A549 and NCI-H460) combined with MRC-5 lung fibroblasts and THP-1 monocytes can be used to study these mechanisms. They showed that activated fibroblasts within the heterotypic spheroids massively expressed ECM component fibronectin and smooth muscle actin stress fibers. Furthermore, CD68⁺ macrophages in late stage tumor spheroids underwent a possible myeloid lineage shift as observed through the increase in endothelial markers [157]. Additionally, highlighting the role of fibroblasts, a multicellular spheroid system was produced by the co-culture of dermal fibroblasts (NHDFs) and human primary mammary fibroblasts (HPMFs) with breast cancer-derived, tumorigenic cells (MDA-MB-231) [158]. Data showed that breast cancer cell line-conditioned medium activated both NHDFs and HPMFs, transitioning them into a CAF phenotype while contributing to a faster cellular aggregation observed by the release of MMPs. Transformed HPMFs additionally upregulated key molecules like fibroblast activation protein (FAP), vimentin, desmin, platelet-derived growth factor receptor A and S100 Calcium Binding Protein A4 (S100A4).

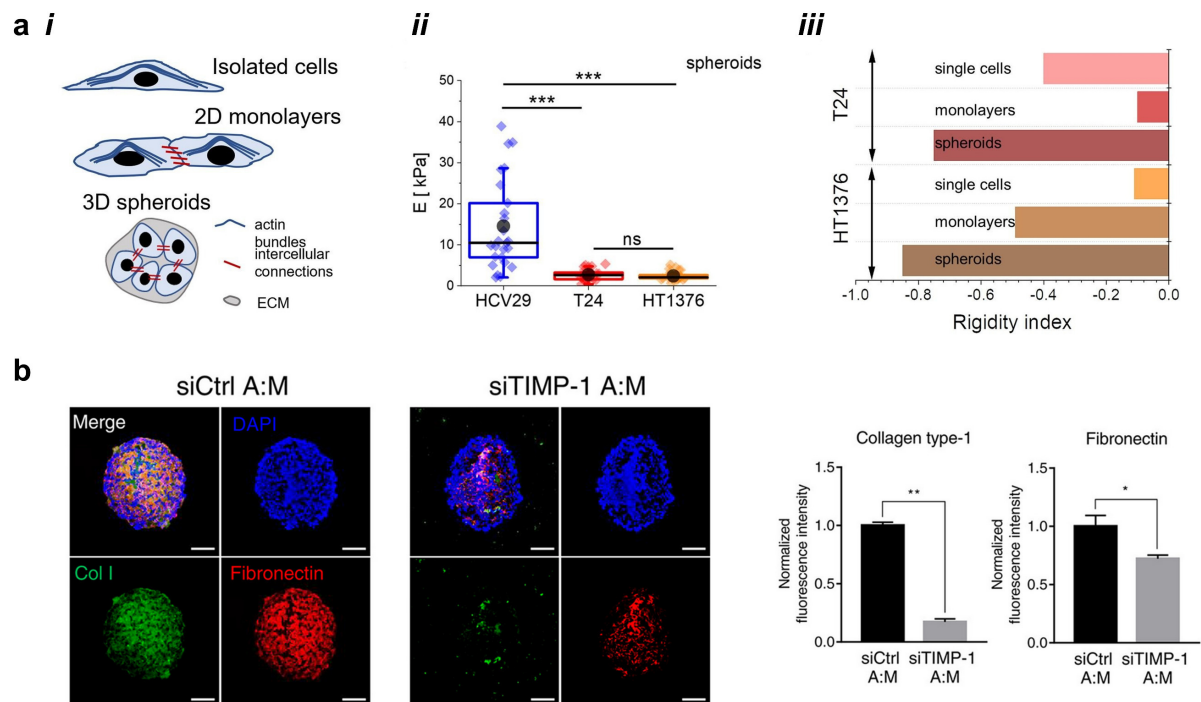


Figure II-5 *In vitro* tumor spheroid models host tumor ECM mechanics.

a i) Actin cytoskeleton, intercellular connections, and ECM contributions to the overall mechanical properties at different culture conditions. **ii)** Analogous comparison between spheroids composed of normal and cancer cells. **iii)** Spheroids present the largest softening observed through the lowest rigidity index between different culture conditions, in relation to non-malignant HCV29 cells. Adapted under the terms of license CC BY 4.0 from [150]. Copyright 2022, the authors. **b** Effects of TIMP-1 silencing in hASC spheroids co-cultured with breast cancer cells on ECM expression. Collagen type I (green) and fibronectin (red) staining of tumor spheroids. Adapted under the terms of license CC BY 4.0 from [155]. Copyright 2022, the authors.

II-4. THE FUTURE OF 3D SPHEROID CANCER MODELS

From the onset of tumor model development, using 2D monocellular cultures of cancer cells, many changes have occurred. The introduction of spheroids and their different variations has brought major developments to the field, allowing newer cancer models to become increasingly closer to mimicking the true tumor microenvironment. From monocellular tumor spheroids to more complex heterotypic patient-derived spheroids, the benefits of going 3D are convincing.

To better understand the benefits of these systems, recent studies have been performed to characterize their biomechanical similarity to native tumor ECM as well as the degree of recapitulation of native cell-cell and cell-ECM recapitulation. Establishing models that faithfully reproduce these interactions is even more important at a time where reducing the use of animal models while streamlining the testing of novel drugs and improving knowledge on key mechanisms driving tumor progression are a requirement. Growing emphasis will be placed on the necessity to unravel the role of cancer stem cells

in the development of tumors and how they regulate cellular interactions within the microenvironment. Coupled to this is the heterogeneity observed within tumors, from different cell populations to cellular clonality; these features must be reproduced in newly developed 3D models. A good way to achieve this level of complexity will be through methods such as assembloids. Paşca et al. have described assembloids as self-organizing cellular systems resulting from the combination between different types of organoids possessing different specialized cell types, that result in integration. While first being generated by combining spheroids of human pluripotent stem cells driven to differentiate into region-specific organoids resembling either the dorsal pallium or the subpallium, and subsequently fused [159–162] they have recently been used to induced hepatic stem cell spheroids [163]. This methodology harbors several advantages over more traditional systems namely, the underlying possibility of maturing and tailoring individual organoids before fusion and replicating region-specific interfaces within tissues, as well as the longer viability time due to the more native-like representation of living systems [162,164].

To additionally build the complexity of these tumor models, fusing different methodologies to simultaneously take advantage of their distinct features will also become routinely used. A form of achieving this is through the utilization of technologies like 3D printing and microfluidics or even *in silico* 3D modeling. Studies have already addressed the suitability of these fabrication technologies to develop 3D tumor analogues [166,168,170–174]. Additionally, the use of Artificial Intelligence at different stages of the development process will for sure bring for now unforeseeable benefits that cannot but leave a sense of excitement in the field.

We expect the of tumor modelling to mature over the course of the next decade, supplying the global scientific community with improved tools allowing the developing of novel therapeutics that can ease the burden of cancer on society.

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SECTION 2

EXPERIMENTAL DESIGN

CHAPTER III

MATERIALS AND METHODS

CHAPTER III

MATERIALS AND METHODS

In addition to the description of the materials and methods found in each experimental chapter of this thesis, the rationale for the selection of materials and characterization techniques is herein provided, as well as, additional and/or more detailed information.

III-1. MATERIALS

III-1.1. Gellan gum-based nanoparticles

III-1.1.1. Gellan gum

Gellan gum (GG) or gellan is a natural extracellular polysaccharide composed of tetrasaccharide repeating units of 1,3- β -D-glucose, 1,4- β -D-glucuronic acid, 1,4- β -D-glucose, 1,4- α -L-rhamnose, containing one carboxyl side group [1]. This polysaccharide is produced by the *Sphingomonas elodia* bacterial strain through the careful inoculation of a fermentation medium with this organism. Structurally, it may be found in two distinct forms, acetylated – original form produced by the bacterial organism – and the deacetylated – the most widely available and able to undergo processing [2] (**Figure III-1**). This polysaccharide is capable of undergoing ionotropic gelation which is dependent on chemical nature and amount of cations present in the solution, being promoted in a stronger manner in the presence of divalent cations when compared to monovalent cations [3]. Upon crosslinking through the presence of divalent cations, the gelation occurs via chemical bonding between the cation and two carboxylate groups belonging to glucuronic acid molecules in the gellan gum chain [4]. Additionally, to the ionotropic gelation, helix-to-coil transition occurs thermoreversibly upon cooling or heating. During the gelation process, anti-parallel self-assembled double helices form oriented bundles called junction zones then link untwined regions of extended helical chains, leading to the formation of the hydrogel. GG has a widespread use in the food and cosmetics industry [5], while additionally having been adopted in the field of drug delivery [6] and even for the development of TERM strategies [7]. For the studies in hand, deacetylated GG was purchased from SIGMA-ALDRICH® (USA) with the designation of Gelzan™ CM.

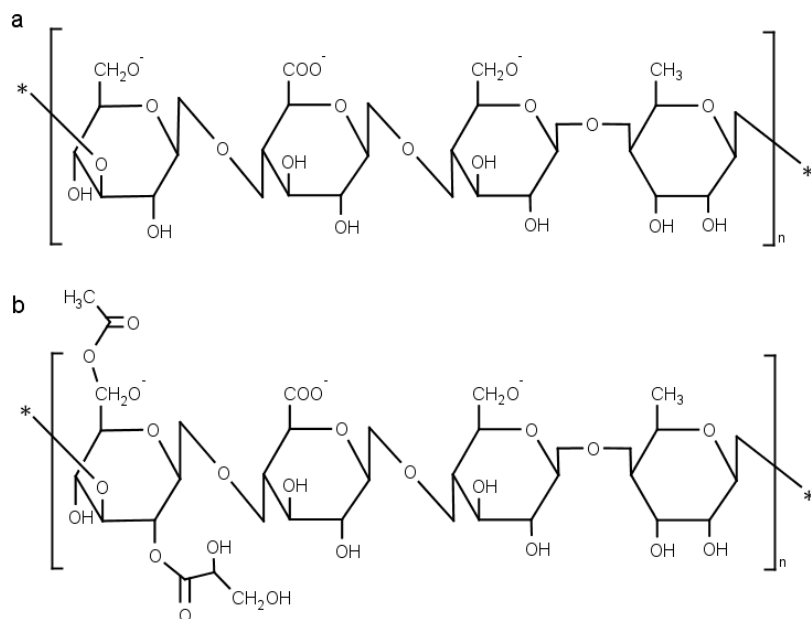


Figure III-1 Chemical structure of (a) deacylated gellan gum and (b) native gellan gum.

III-1.1.2. Fabrication of GG hydrogel-like particles

In order to create polymer-derived nanoparticles several protocols have been described, one of which being the emulsion technique. Emulsions can be performed by the mixture of two or more immiscible liquids in which one or more liquids are dispersed in another liquid. Several different forms of emulsions exist such as water-in-oil (W/O), oil-in-water (O/W), oil-in-water-in-oil (O/W/O) and water-in-oil-in-water (W/O/W) [8]. Due to their thermodynamic instability, emulsions are generally highly dependent of surfactants and stabilizers in order to stabilize the emulsion droplets, avoiding their coalescence or flocculation. Additionally, final particle properties (droplet size, stability and encapsulation efficiency) are heavily dependent on the control of homogenization conditions (temperature, pressure and cycles) [9].

For the production of GG hydrogel-like particles used, in chapter V, a W/O/W double emulsion/solvent evaporation method was chosen due to its reliability and simplicity [10]. These emulsions hierarchically are formed by the dispersion of small water droplets within larger oil droplets in an aqueous continuous phase containing a suitable emulsifier to form double emulsion (W₁/O/W₂). Removal of the volatile organic solvent leads to the formation of solid microparticles. For the choice in surfactants used, the hydrophilic-hydrophobic balance (HLB) balance was considered (**Table III-1**) and a mixture of Tween 20, which is a hydrophilic surfactant, and Span 80, a hydrophobic surfactant, was employed. Considering that these surfactants are non-ionic in nature and therefore possess no inherent

ionic affinity for interaction, makes them widely compatible and stable in several fluid systems such as ultrapure water, saline water, mild acids, alkaline substances and additionally they don't react with ionic ingredients and charged substances. Low HLB Spans favor solubility in lipophilic solvents to produce stable water-in-oil emulsion system [11]. On the other hand, Tweens are used to increase HLB range and enhance hydrophilic solubility to yield oil-in-water emulsion systems [12]. Span 80 has an HLB value of 4.3, which falls within the usual range of HLB 4–6 for water-in-oil (W/O) emulsifiers, while Tween 20 has an HLB value of 15, within the range of HLB 8–18 for oil-in-water (O/W) emulsifiers. For the oil phase, chloroform was used to allow for the solubility of Span 80 while its low boiling point facilitates the removal of residual amounts. Additionally, poly(vinyl alcohol) (PVA) was chosen to act as a cosurfactant to stabilize the W/O/W emulsion based on its good water solubility and its amphiphilic nature to maintain the stability of the double emulsion droplets.

Table III-1 Physical properties of Span 80 and Tween 20.

Surfactant appearance	HLB (unitless)	Molecular weight (g.mol ⁻¹)
Span 80	4.3	428.59
Tween 20	16.7	1227.54

In order to achieve nano-emulsification, one of two techniques is used, low-energy methods (e.g. spontaneous emulsification, solvent-diffusion, phase inversion temperature) or high-energy emulsification methods (high pressure homogenizers or ultrasound generators) [13]. For this work, a high speed homogenization system was used. The solid particles may be then separated by filtration or centrifugation and washed several times in order to eliminate the residual emulsifier and dried under vacuum or freeze-dried.

In this manner, a 1 % (w/v) of GG solution was prepared in deionized water, under stirring at 90 °C for 30 minutes (min). After dissolution, the solution was allowed to stabilize at 50 °C. The GG solution (W1) was then emulsified with Tween-20 (Sigma-Aldrich, Germany) and added to a mixture of 0.5 % (w/v) of Span®80 (Sigma-Aldrich, Germany) in chlorophorm (Sigma-Aldrich, Germany)(O) at a ratio of 1:3. The emulsion was then prepared under high speed homogenization with a T18 BASIC Ultraturrax (IKA, NC, USA) for 3 min. W1/O was then added dropwise to 15 mL of a solution of 3.5 % (w/v) poly(vinyl alcohol) (PVA)(W2) (Sigma-Aldrich, Germany) in water under continuous stirring in order to stabilize the prepared emulsion. At this point in order to gelify GG nanoparticles, ionic crosslinking of the emulsion was performed by gradually adding W1/O/W2 to 5mL of a 60 % (w/v) solution of calcium chloride (Merck

Millipore, Germany) under constant stirring. In order to remove remaining chloroform still present in the emulsion, rotary evaporation of the solvent was performed (Stuart, Staffordshire, UK). Afterwards, the particles were recovered by centrifugation of 1 hour (h) at 13'000 x g (Eppendorf, Germany). Given the size dispersity and associated issues concerning coalescence or flocculation, the produced particles were selected through a series of strainers (pluriSelect, Leipzig, Germany) ranging from 10 μm down to 1 μm to limit size dispersion. In order to remove traces of PVA and CaCl_2 from the resulting particles, additional washing steps were performed by centrifugation at 13'000 x g for 10 min with deionized water.

GG hydrogel particles were then frozen at -210 °C (liquid nitrogen) and then freeze-dried to remove the water content from these particles. Freeze drying was performed at -80 °C, 0 atm and for 3 days (LyoQuest, Telstar, Spain) to achieve complete sublimation of all the water. The freeze-dried particles were then stored away in a desiccator until further use.

III-1.1.3. Functionalization of GG hydrogel-like particles

In order to produce functional npGG, bioactive molecules capable of triggering T cell activation are required. To induce effective T cell responses, several signals have been reported as required, classically a two-signal stimulus is required in which both an antigen and secondary stimuli is required in order to achieve T cell activation. The first step is based on the recognition of a cognate peptide-MHC complex by the CD3/TCR complex which triggers the activation of kinases and phosphatases, inducing a multitude of phosphorylating and dephosphorylating events. Additionally, there is the requirement of a costimulatory second signal supplied by the engagement of the CD28 membrane protein by its ligand (CD80 or B7-1 and CD86 or B7-2) present on the antigen-presenting cell (APC) [14]. The non-existence of this co-stimulatory signal may lead to the induction of T cell anergy. The use of antibodies to achieve this form of activation has been reported back in 1985 by Tsoukas et al., where it was demonstrated that purified, resting (G0 phase) T cells incubated with monoclonal anti-CD3 antibodies proliferated in response to purified interleukin 2 (IL 2), in a lymphokine dose-dependent fashion [15]. The use of antibodies for the activation and proliferation of T cells has been extensively reportedly in the meantime [16–19]. This has also led to the development of commercial solutions for the *in vitro* expansion of T cells, such as the case of bead-based systems.

Taking all of the above into consideration, we proceeded in functionalizing our npGG system with functional grade $\alpha\text{-CD3}$ and $\alpha\text{-CD28}$ antibodies. To carry this out, carbodiimide (EDC) and N-

hydroxysuccinimide (NHS) coupling chemistry was considered to tether the functional antibodies to the nanoparticle surface. This form of coupling chemistry is commonly used to alter the surface chemistry of nanoparticles by attaching pharmaceutical agents or biomolecules to the particle surface [20–24]. N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride is a water-soluble, non-cytotoxic, and biocompatible carbodiimide which is used as a carboxyl activating agent for amide bonding with primary amines. Briefly, EDC reacts with carboxylic acid groups to form an active O-acylisourea intermediate which is easily displaced by nucleophilic attack from the primary amino groups in the reaction mixture. In turn, the primary amine forms an amide bond with the original carboxyl group, and an EDC by-product is released as a soluble urea derivative. However, EDC is not very stable in water as oxygen atoms act as a nucleophile and inactivate the cross-linker agent while failure to react with an amine also results in the hydrolysis of the O-acylisourea intermediary. Hence, N-hydroxysuccinimide (NHS) is used as a stabilizer to improve the crosslinking efficiency [20].

Given that peptides and proteins possess multiple carboxyls and amines this usually leads to random crosslinking. To overcome this concern, Biotin – Avidin was used as an alternative crosslinking methodology with the purpose of crosslinking the antibodies to the GG nanoparticles in a controlled manner. The noncovalent interaction between avidin and biotin is considered one of the strongest known interactions with a dissociation constant of 10^{-15} M, assuring the stability in the interaction between ligand and receptor when subjected to changes in pH, presence of chaotropes or during manipulation [25]. Both these molecules possess four binding sites which in turn allows for an amplification of the desired system. Due to these reasons, this form of non-covalent interaction has been sourced for the modification of a spectrum of different probes for commercial use such as the case of antibodies [26]. For this particular work, the main advantage in the use of this form of antibody binding would be the controlled orientation of our antibodies of interest as found possible in other works [27], otherwise unachievable simply through EDC/NHS as detailed above. Taking this into consideration, npGG were activated with EDC/NHS and then bound to Netravidin® through a 4 h reaction at RT after which biotinylated α -CD3/CD28 antibodies were bound to the nanoparticles as detailed in the following section. This in turn led to the formation of our T cell activator system.

III-1.2. Collagen chemotaxis chambers

III-1.2.1. Collagen

Collagen is a highly abundant structural protein which can be found in all animals and in humans makes up one-third of the total protein. As the most prevalent protein of the extracellular matrix (ECM) it has been widely resourced for the field of tissue engineering and regenerative medicine. Being a highly heterogenic protein, twenty-eight different isoforms have been identified [28], among these different types, collagen type I is the most abundant in mammals [29]. Collagen type I can be found in a variety of different tissues such as skin, tendon, bone, cornea, lung and the vasculature [30]. Additionally, and of the utmost importance for this work, is the implication of collagen type I in driving cancer cell migration [31]. Given the complex hierarchical structure of collagen, it can confer different biomechanical properties such as elasticity in skin, softness in cartilage and stiffness in bone and tendon.

Structurally, collagen is a molecule composed of three polypeptide chains in which Gly is required at every third position, resulting in the repeating amino acid motif (Gly-X-Y), where X and Y can be any aminoacid [32]. This repeat occurs in all types of collagen, however, depending on the genetic type of collagen, this motif can be a major or minor part of the molecule. In terms of 3D organization, this motif allows for the chains to form a right-handed triple-helical structure which is held together in the helical conformation by hydrogen bonds (**Figure III-2**).

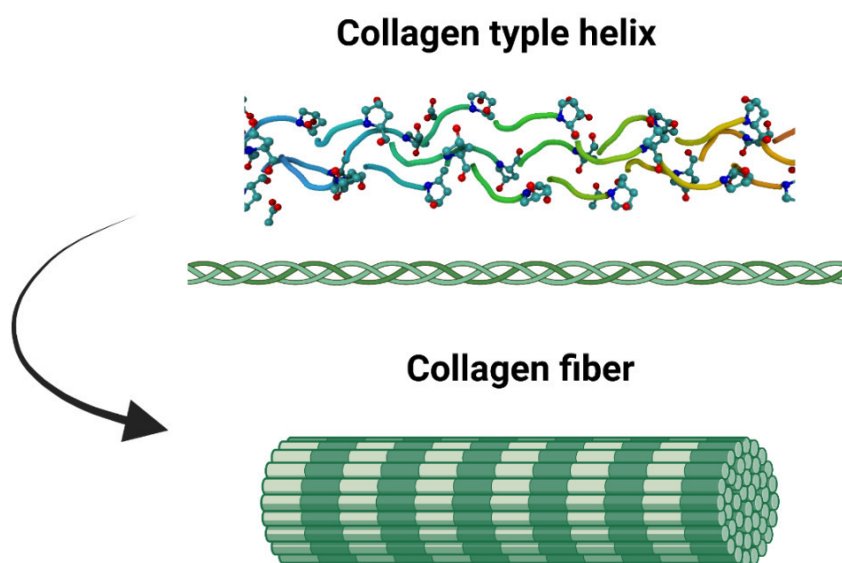


Figure III-2 Organization of individual α -chains into triple helices.

Bundles of these triple helices form into fibrils and ultimately into large collagen fibers.

III-1.2.2. Fabrication of a collagen chemotaxis chamber

Herein, we were inspired by the need felt in the field of cancer biology in how to better understand the underlying cell-cell and cell-ECM interactions that drive tumor progression and invasion. While the field has advanced immensely with the introduction of highly complex heterotypic tumor models, which are a step closer to mimicking the *in vivo* tumor microenvironment, these models may not be the optimal system to understand how cellular interplay occurs given the constraints and limitations of live cell imaging in these models. For this effect, we conceptualized a 3D collagen-based culture chamber that would allow the culture of cancer cells as well as of stromal cells and study how they regulate cell invasion through the establishment of chemotactic gradients.

For this purpose, a hydrogel molding strategy was considered, making use of commercially available μ - Slide culture chambers to facilitate manipulation during microscopic imaging. With this mold, a collagen type I hydrogel could be imprinted while undergoing gelification. The master mold was designed to imprint 4 wells per chamber, one central chamber surrounded by three equidistant ones, which in turn are equidistant among themselves. This would allow for the equal distribution of the established chemotactic gradients between the different stromal cells and to uniformly influence tumor cells in the central well. The corresponding master mold was produced in polymethyl methacrylate (PMMA) by direct micromachining (MDX-50, Roland DG) and has an overall footprint of 75 mm by 25 mm and a height of 1 cm, being each chamber 10.65 mm by 9.4 mm in which the pillars are 1.2 mm diameter each and 7 mm high. Upon imprinting the collagen hydrogel, the pillars led to the formation of microwells capable of supporting cell culture. For this current study, bovine type I collagen was purchased from SIGMA-ALDRICH® (USA). Within the next sections, additional detail on the preparation of the hydrogel chamber and characterization is provided.

III-2. METHODOLOGY FOR MATERIAL CHARACTERIZATION

III-2.1. Fourier transform infrared spectroscopy

Fourier transform infrared, otherwise known as FTIR, is one of the most widely used form of infrared spectroscopy. It is commonly used to obtain the infrared spectrum of absorption, reflection, and emission of a solid, liquid or gas and therefore can be used for the identification of organic, inorganic and polymeric materials [35]. Through changes in characteristic patterns of absorption bands it is possible to identify

changes in the composition of a material. Within the works herein described, FTIR was used to obtain information on the functional groups present on the surface of the npGG produced in chapter V as described by [36] since PVA was used as a cosurfactant to stabilize the produced GG nanoparticles and further functionalization was dependent on the maintenance of characteristic peaks of GG.

III-2.2. Micro-Bicinchoninic Acid Protein™ assay

Micro-bicinchoninic acid protein (Micro-BCA) is a colorimetric assay that quantifies protein content in a sample [37,38]. This method relies on the fact of that proteins are able to catalyze the reduction of Cu^{2+} to Cu^+ which, upon reaction with bicinchoninic acid (4,4'-dicarboxy-2,2'-bi-quinoline), forms a colored complex with bicinchoninic acid and increases in a proportional fashion over a broad range of increasing protein concentrations.

Micro-BCA assay was used to determine the amount of neutravidin bound to the npGG while also being used to determine the total amount of protein in the secretome of produced assembloids and respective control spheroids prior to zymography and ELISA analysis. Quantification was preformed according to manufacturers' instructions (Fisher Scientific, USA).

III-2.3. Scanning Transmission Electron Microscopy (STEM)

Scanning Electron Microscopy (SEM) is a form of electron microscopy that makes use of a focused beam of electrons to scan the surface of a sample and hence produce a 2D image of its surface topography in a high-resolution manner [39]. The electron beam is generally scanned in a rasterized pattern and the beam's position is combined with the reflected signal detected to produce an image which can achieve a resolution better than 1 nanometer (nm). With scanning transmission electron microscopy (STEM), a highly-focused electron probe is raster-scanned across the material, and various types of scattering are collected as a function of position. Herein, the transmitted electrons (passing through the sample) at high scattering angle can be collected to form high-resolution, chemically sensitive, atomic number (Z-) contrast images [40]. Some STEM equipment's have been capable of achieving high-resolutions of up to approximately 0.14 nm.

A High-Resolution Field Emission Scanning Electron Microscope with Focused Ion Beam (FIB – SEM) (AURIGA COMPACT, Zeiss, Germany) was used to analyze the morphology of the dried polymeric npGG at an accelerating voltage of 20 kV

III-2.4. Dynamic Light Scattering

The evaluation of both size and polydispersity are considered key when characterizing nanoparticles [41,42]. Several different techniques have been developed for this purpose, nonetheless dynamic light scattering (DLS) is considered the most versatile and useful set of techniques for measuring in situ the sizes and size distributions of nanoparticles in liquids [43,44].

Throughout the work here reported, DLS has been used to characterize the developed npGG system. This technique, otherwise known as photon correlation spectroscopy relies on the fact that light scatters when a suspension of particles or molecules undergoing Brownian motion are illuminated by a laser beam [45]. The fluctuations in the intensity of scattered light are analysed over time and depend on the positions of the particles relative to the direction of the incoming and scattered light beams. Thus, at a given instant the total scattered intensity at a given scattering angle depends on the positions of the particles and their structure. Diffusion coefficient and particle size information can be obtained from the analysis of these fluctuations.

III-2.5. Water content and water uptake

While different aspects are essential for the complete characterization of a nanoparticle system, the most important property of a hydrogel is its total water content while in equilibrium. Water content is considered a quantitative measure of the water existing in a material at a given time [46]. At intermediate times of swelling the water content is given in the form of **Equation III-1**.

$$\text{Water uptake/content (\%)} = \frac{W_w - W_d}{W_d} \times 100$$

Equation III-1 Equation of the water uptake/content.

Water content of npGG was determined by measuring the weight of dried polymeric networks before (W_d) and after (W_w) hydration with ultrapure H_2O , PBS or a-MEM solution at 37 °C for 7 days. Water uptake profile was determined using the same principle, but weighting the samples at different time points along the 7 days period. Water content or water uptake were calculated using the Equation II-2.

III-2.6. Rheology

In order to attain the viscoelastic properties of “in-house” produced collagen chemotaxis chambers, presented in chapter VI, characterization by rheology of the hydrogels was performed in order to derive their storage (G') and loss (G'') modulus. Rheology is defined as the study of flow and deformation of a material. The connection between these two is that large deformations are caused by shear forces and those shear forces cause many materials to flow [48]. To carry out these characterizations, a controlled and well-defined deformation or strain is applied to a material over time and the resulting force response is measured [49]. This given response is indicative of different parameters such as stiffness, modulus, viscosity, hardness, strength or toughness of the material.

A detailed description of the settings used can be found in chapter VI.

III-2.7. Gel electrophoresis

Gel electrophoresis is a method used for separating biomacromolecules (proteins, DNA or RNA) according to their molecular size, by the application of an electrical field [50]. This electric field pushes negatively charged molecules migrate towards the anode (positive) pole. The migration flow is determined by the speed at which molecules travel through the pores in the gel, which is inversely correlated to their size/length. This technique can be used as a solo characterization method or used in tandem with downstream processing methods such as western blotting or zymography.

In our case, gel electrophoresis was used to confirm the functionalization of the developed npGG with T cell co-stimulatory antibodies α -CD3 and α -CD28. For this purpose, sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to obtain high resolution separation of the proteins present on the functionalized system. This method initially denatures the proteins that will undergo electrophoresis therefore allowing the separation of the molecules based on their size alone and not their 3D conformation. In order to achieve a resolution capable of visualization the presence of the desired antibodies a 12 % SDS-PAGE resolving gel was chosen.

III-3. *IN VITRO* METHODOLOGY

III-3.1. Cell isolation and Culture

Several different cell types were used in the present work, some of those being established cell lines while others a mixture between both commercially available or freshly isolated primary cells. This list of cells described throughout the following chapters can be found in **Table II-2**.

Table III-2 Summary of the cell types used and their tissues of origin.

Cell name	Cell type	Medium	Abbreviation
Murine splenocytes	Primary	RPMI 1640 medium (Sigma-Aldrich, Germany)	-
Human Dermal Fibroblasts	Primary (Gibco, UK)	Alpha-MEM (Gibco, USA)	hDFbs
VMM15	Cell-line (ATCC® CRL-3227™)	RPMI 1640 Glutamax (Sigma-Aldrich, Germany)	VMM15
Human Dermal Fibroblasts	Primary	Alpha-MEM (Gibco, USA)	hDFbs
Human Adipose-Derived Stromal and Stem Cells	Primary	Alpha-MEM (Gibco, USA)	hASCs
Human Dermal Microvascular Endothelial cells	Cell-line (ATCC® CRL-4060™)	Vascular Cell Basal medium + Microvascular Endothelial Cell Growth Kit – BBE (ATCC, USA)/EGM-2 MV Microvascular Endothelial Cell Growth Medium-2 BulletKit (Lonza, USA)	hDMECs

All human biological samples were obtained from Hospital da Prelada (Porto, Portugal) and Hospital São João (Porto, Portugal), with patient's informed consent and under a protocol with the 3B's Research Group, approved by the ethics committees of all the institutions.

III-3.1.1. Splenocytes

Murine spleens were harvested from Balb/c mice after approval of all procedures by the Direcção Geral de Alimentação Veterinária, the Portuguese National Authority for Animal Health, and respected the national regulations and international animal welfare rules. The excised spleens were placed onto a strainer attached to a 50 mL conical tube. The excised spleen fragments were pressed through the strainer using the plunger end of a 5 mL syringe and the cells were washed through using excess RPMI 1640 medium (Sigma-Aldrich, Germany) supplemented with 10 % (v/v) fetal bovine serum (FBS, Gibco, ThermoFisher Scientific, Paisley, UK) 1 % (v/v) HEPES (Gibco, ThermoFisher Scientific, Paisley, UK), 1 %

(v/v) Sodium Pyruvate (Gibco, ThermoFisher Scientific, Paisley, UK) and 0.05 mM Mercaptoethanol (Gibco, ThermoFisher Scientific, Paisley, UK). Cells were collected by centrifugation at $400 \times g$ for 5 min. The cell pellet was resuspended in 1 mL of pre-warmed (37 °C) red blood cell lysis buffer (155 mM of ammonium chloride, 12 mM of potassium bicarbonate and 0.1 mM of ethylenediaminetetraacetic acid, all from Sigma-Aldrich, Germany) and incubated at 37 °C for 5 min. Murine splenocytes were then washed with excess PBS and centrifuged at $400 \times g$ for 5 min. Cells were hereon cultured with the aforementioned medium and maintained at 37 °C in a humidified incubator with 5 % CO₂ atmosphere.

III-3.1.2. Human Adipose-Derived Stromal and Stem Cells

Adipose tissue was harvested from the fat tissue of skin specimens of healthy donors undergoing abdominoplasties. Adipose tissue was digested with 0.05 % (w/v) Collagenase type II (Sigma, USA) under agitation for 45 min at 37 °C. Stromal vascular fraction (SVF) was obtained after filtration and centrifugation (800 g, 10 min, 4 °C). SVF pellet was re-suspended in red blood cell lysis buffer (155 mM of ammonium chloride, 12 mM of potassium bicarbonate and 0.1 mM of ethylenediaminetetraacetic acid, all from Sigma-Aldrich, Germany) and incubated for 10 min at RT. After centrifugation (300 g, 5 min, RT), the supernatant was discarded and the cell pellet was re-suspended and cultured in a-MEM (Invitrogen, USA) supplemented with 2.2 g/L of sodium bicarbonate (Sigma, USA), 10 % (v/v) of FBS (Invitrogen, USA) and 1 % (v/v) of antibiotic/antimycotic solution (Invitrogen, USA) and maintained at 37 °C in a humidified incubator with 5 % CO₂ atmosphere after which human adipose-derived stromal and stem cells (hASCs) were selected by plastic adherence. hASCs were further cultured in alpha-MEM (Gibco, USA) with 10% FBS (Gibco, USA) and 1% antibiotic/antimycotic (Gibco, USA), up to a maximum of 4 passages.

III-3.1.3. Human Dermal Fibroblasts

After incubation of the small fragments of skin tissue overnight at 4 °C with 2.5 U/mL of dispase (BD Biosciences, USA), the dermis (without epidermis) was digested for 3 h at 37 °C using collagenase type II (125 U/mL, Sigma, USA). The resulting cell suspension was filtered through a 70 µm cell strainer and centrifuged at 1500 rpm for 10 min at 4 °C and placed in culture. Human dermal fibroblasts (hDFbs) were cultured in a-MEM, supplemented with 10 % (v/v) FBS (Invitrogen, USA) and 1 % (v/v) antibiotic/antimycotic at 37 °C in a humidified atmosphere with 5 % CO₂. hDFbs were used up to a maximum of 4 passages.

III-4. BIOLOGICAL CHARACTERIZATION

III-4.1. Lactate dehydrogenase cytotoxicity assay

Lactate dehydrogenase (LDH) is an important enzyme of the anaerobic metabolic pathway that is present in every cell. It belongs to the class of oxidoreductases which's function is to catalyse the reversible conversion of pyruvate to lactate with the oxidation of NADH to NAD⁺ and back through the Cori cycle [51]. Upon damage to the plasma membrane, LDH leaks from cells and this extracellular LDH can be measured to determine the extent of the damage. One form of doing this is through a coupled enzymatic reaction in which LDH catalyzes the conversion of lactate to pyruvate via NAD⁺ reduction to NADH. A tetrazolium salt (INT) is then reduced by means of a diaphorase to obtain a red formazan product that can be measured at 490 nm and is directly proportional to the amount of LDH released into the medium.

In our studies, LDH quantification was used to determine the amount of cell death resulting from the treatment of tumor assembloids and control spheroids with doxorubicin in order to validate our heterotypic 3D tumor model for drug testing.

III-4.2. MTS assay

Another method to determine the cytotoxicity of a certain agent can be through changes in the cell's metabolic activity. Certain techniques have been developed to determine the overall cellular metabolic activity through enzymatic cleavage of colorimetric or fluorescent substrates.

MTS assay is a colorimetric assay that quantifies cell metabolic activity and is generally used to study the cytotoxicity of a compound. In the presence of phenazine methosulfate (PMS), this compound, is bio-reduced by viable cells into a formazan product that is soluble in culture media. This reaction is carried out by NADPH-dependent dehydrogenase enzymes in metabolically active cells and the formazan dye produced can be quantified by measuring the absorbance at 490–500 nm [52]. The production of formazan dye is directly correlated to cell metabolic activity, while cells undergoing apoptosis lose the capacity to reduce MTS.

To infer about the cytotoxicity profile of the produced npGG, metabolic activity assays were carried out at 3 and 7 days of direct stimulation of hDFbs with varying doses, using CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, USA).

III-4.3. Confocal Microscopy

Fluorescent microscopy is a non-destructive imaging technique that has become the ideal microscopy technique for the examination of biological specimens. It allows for the selective and specific detection of molecules at small concentrations with a good signal-to-background ratio rendering high resolution 2D or 3D images of fluorescent samples [53]. However, while this more traditional technique produces excellent results in thin samples, when subjected to thick or living samples, the entire sample is excited indiscriminately and most of the fluorescent photons arise from out-of-focus fluorophores [54]. Confocal scanning microscopy has solved this problem by restricting photodetection to light originating from the focal point through a spatial pinhole in the detector system, therefore eliminating out-of-focus light [55].

Given the multivalence of this technique it was used in different applications throughout the given work. Due to its high-resolution capability, it was used for the detection of signal arising from functionalized npGG on the surface of CD4⁺ murine T cells, while also being used for the acquisition of spheroid-based systems prepared by whole mount techniques to obtain 3D reconstructions of both CD31 and GP100 immunostainings. Additionally, diffusion of relevant fluorescently tagged molecules in an “in-house” collagen chemotaxis chamber were also studied over time through confocal microscopy. For these studies a Leica TCS SP8 confocal microscope was used.

III-4.4. Cell Live Tracking

Cell live tracking has become a powerful tool in the field of biology and more specifically cancer biology, due to the information that it brings regarding assessment of live cellular behaviour and therefore allowing to better comprehend cell-cell interaction undergoing in the tumor microenvironment. Several different non-toxic methods have been reported over-time [56], to undertake this analysis we have opted to use non-toxic fluorescent cell tracking dyes that allow for cell movement and localization studies. These dyes are capable of freely passing through the cell membrane where they become cell-impermeant by reacting with thiol or amine groups on proteins. Given the fact that they pass on to daughter cells allows for them to be tracked over multiple generations without transferring to neighbour cells.

In this line of work, cell live tracking was used to monitor spheroid fusion in a heterotypic cancer assembloid model. A more detailed description of the used protocol may be found in further chapters.

Fusion was analysed over time by fluorescent time-lapse microscopy with an Axio Observer microscope and Zen 3.2 (blue edition) software (Zeiss, Germany).

III-4.5. Phalloidin-TRITC Staining

Phalloidin staining is a method to visualize the cytoskeleton of cells during immunocytochemical and cytochemical analysis. Phalloidin is a member of the group of phallotoxins produced by the mushroom *Amanita phalloides*, which binds to F-actin and prevents its depolymerization, resulting in cytotoxicity [57]. Visualization of actin can be attained by incubating previously fixed cells with fluorescent Phalloidin.

Throughout this thesis, phalloidin-Tetramethylrhodamine B isothiocyanate (phalloidin-TRITC; Sigma-Aldrich, USA) or Alexa Fluor™ 488 Phalloidin (Sigma-Aldrich, USA) were employed to visualize the organization of the cells' cytoskeleton and to analyze cellular morphology, both in 2D culture and in the 3D cell-laden scaffolds.

III-4.6. Flow Cytometry

Flow cytometry is a technology that allows for rapid multi-parametric analysis of single cells in solutions. Flow cytometers base their analysis on the scattering of light and fluorescent signals emitted by cells which are detected by photodiodes or photomultiplier tubes [58]. Hence, cell populations can be analyzed and/or purified based on their fluorescence or light scattering characteristics. The scattering of visible light allows for the detection of the relative size of the cell as well as the internal complexity or granularity. To aid in the detection of specific cell populations, based on the expression of certain antigens, a series of reagents may be used such as the case of fluorescently conjugated antibodies, nucleic acid binding dyes, viability dyes, ion indicator dyes, and fluorescent expression proteins.

Throughout this thesis, flow cytometry was used for the phenotypic characterization of surface markers expressed among different cell populations, intracellular labelling of relevant immune related biomolecules, studying the proliferation profiles of cells over time and analyzing the interaction between “in-house” developed nanoparticles and T cells. Antibodies used for these analyses can be found below (**Table III-3**). Antigens were analyzed in a Fluorescence-activated cell sorting (FACS) Calibur Flow Cytometer (BD Biosciences, USA) using the CELLQuest software V3.3.

Table III-3 Antibodies used for flow cytometry.

Target	Antibody (Brand)	Ref	Host	Clonality, reactivity
Biomolecules	Perforin-APC (Biolegend, USA)	154304	Rt	Monoclonal, Mouse
	Granzyme B-PE/ Cyanine7 (Biolegend, USA)	396410	Rt	Recombinant, Mouse/Human
T cells	CD69-PE (Biolegend, USA)	104507	AH	Monoclonal, Mouse
	CD4-APC-H7 (BD Biosciences, USA)	560181	Rt	Monoclonal, Mouse
	CD8a-APC-H7 (BD Biosciences, USA)	560182	Rt	Monoclonal, Mouse
	CD45-PerCP-Cy 5.5 (BD Biosciences, USA)	550994	Rt	Monoclonal, Mouse
	CD4-AF647 (Biolegend, USA)	100424	Rt	Monoclonal, Mouse
	CD3e-PE (BD Biosciences, USA)	553064	AH	Monoclonal, Mouse
Functionalized nanoparticles	AF 488 (ThermoFisher Scientific, UK)	A-21210	Rb	Polyclonal, Rat

Legend: Rt – Rat, Rb - Rabbit, AH – Armenian Hamster

III-4.7. Enzyme-linked Immunosorbent Assay

Enzyme-linked immunosorbent assay (ELISA) is an antibody-based analytical method for the quantitative/qualitative analysis of specific biomolecules [59]. In order to detect these molecules, an antigen in the fluid phase is immobilized on a solid phase, such as a microtiter plate, and allowed to react with a specific antibody that is detected by an enzyme-labeled secondary antibody. To obtain a read out, color development is obtained by means of a chromogenic substrate from which the intensity directly correlates with the presence of the antigen. The color change is measured using a spectrometer and the antigen concentration is quantified by comparison with a standard curve.

This technique was used to quantify the secretion of IL-2 in murine T cells after stimulation with the developed T cell activator npGG, as well as the production of TIMP-1 and TIMP-2 from heterotypic 3D tumor models. ELISA kits, as follows (Table III-4), were performed following the manufacturers' procedures.

Table III-4 ELISA procedure summary.

	TIMP-1	TIMP-2	IL-2
Brand	Abcam, UK		Elabscience, USA
Reference	ab187394	ab270213	E-EL-M0042
Detection range	62.5 - 4000 pg/mL	78.125 - 5000 pg/ml	15.63-1000 pg/mL

III-4.8. Cell 3D Invasion Assays

The analysis of 3D invasion has become an important tool in understanding key mechanisms that drive tumor cell invasion and metastasis. There is still a strong need for novel therapeutics capable of avoiding metastatic spreading which can only be developed once there has been an understanding of the basic principles behind these mechanisms. Several different procedures have been developed over time to study cell invasion [60].

Within the body of work here described, two distinct strategies were used to study cell invasion. The first consisted in the use of a basement membrane extract (BME), Matrigel™ (Corning, USA), comprised of laminins, collagen IV, heparan sulfate proteoglycan, and various growth factors, cytokines, chemokines, and proteases [61]. These ECM proteins allow for the attachment and migration of invading cells, while the soluble factors present in Matrigel™ are released upon degradation contributing to further enhance the ability of invading cells to grow and spread. In our work, this BME was used for the embedding of heterotypic assembloids in which cells can then dissociate from the spheroids and invade into the hydrogel. In the second strategy, the chemotactic effect between different stromal cells present in the tumor microenvironment and their effect of the invasion of melanoma tumor cells was object of study. As detailed above, a 3D chemotaxis chamber with equidistant wells was designed “in-house” for this effect by using 4 mg/mL type I collagen from bovine skin (Sigma, USA) as the hydrogel of choice given its mechanical properties required for templating. Different stromal cells and tumor cells were seeded in the respective wells, which upon migration would allow them to invade into the surrounding 3D matrix.

The detailed description of both methods can be found in the following section. Time-lapse brightfield images were obtained over predefined time-points using an Axio Observer inverted microscope with incubation (37 °C, 5 % of CO₂), and Zen 3.2 (blue edition) software (Zeiss, Germany).

III-4.9. Quantitative Reverse Transcription Polymerase Chain Reaction

Quantitative reverse transcription PCR (RT-qPCR) is a technique that allows for the measurement of mRNA expression levels of genes of interest [62]. For this technique to be carried out, RNA must be first transcribed into complementary DNA (cDNA) by reverse transcription from total RNA or messenger RNA (mRNA). Within the qPCR reaction a nucleic acid template undergoes a succession of amplification cycles which consist in denaturation, annealing with a specific oligonucleotide primer and extension to generate a complementary strand via a thermostable DNA polymerase. Over each cycle, the exponential increase of amplicons was monitored using a fluorescent reporter, SYBR Green, from which the fluorescence intensity is plotted against the cycle number and a quantification cycle C_t value can be determined. Taqman™, while technically similar makes use of a fluorogenic probe specific to a target gene, which accumulates during PCR [63].

Within this body of work, we have used qPCR to determine several biological responses ranging from the characterization of T cell responses when stimulated with npGG activator beads to the phenotype of cells within heterotypic 3D tumor assembloids. Primers for the tested gene marker candidates and the housekeeping gene (**Table III-5**) were designed using Primer-Blast database (NCBI, USA) while Taqman sequences were commercially available (**Table III-6**). All qPCR reactions were carried out in a QuantStudio™ 5 (Applied Biosystems, Life Technologies, UK), In both cases, normalized expression values were calculated following the mathematical model proposed by Pfaffl using the formula: $2^{-\Delta\Delta C_t}$ [64].

Table III-5 Primer sequences.

Gene and accession number	Sequence (5'-3')	Amplicon size (bp)	Annealing temperature (°C)
Mouse ACTB NM_007393.5	GATCAAGATCATTGCTCCTCCTG	183	60
	AGGGTGTAACGCAGCTCA		
Mouse ACTG1 NM_009609.3	CTTACACTGCGCTTCTTGCC	78	60
	GTGCGGCGATTCTTCTTCC		
Mouse PDCD1 NM_005018.2	AACCCTGGTGGTTGGTGTC	107	55.5
	TGGCTCCTATTGTCCCTCGT		
Mouse CTLA4 NM_005214.4	CTACCTGGGCATAGGCAACG	103	57.7
	CCCCGAACTAACTGCTGCAA		
Mouse PRF1 NM_011073.3	GAGCATCCCTGACTTTCCTT	84	56.4
	ATGTTTACGCTTCGTGGCAG		
Mouse Gzmb NM_013542.3	GAAGCCAGGAGATGTGTGCT	86	56.4
	GCTCAACCTCTGTAGCGTG		
Human TP53BP1 NM_001141980.3	AAATCCTTCAGCCGTGTCGT	85	47
	AGTCACTACGACGAAAGCA-3		
Human BRCA1 NM_007294.4	GTCCCATCTGTCTGGAGTTGA	107	49
	GGCCCTTCTTCTGGTTGAGA		
Human B2M NM_004048.2	TGGAGGCTATCCAGCGTACT	108	59.5
	CGGATGGATGAAACCCAGACA		

Table III-6 Taqman references.

Gene	Reference	5' Reporter	Brand
SOX2	Hs01053049_s1	FAM™	Applied Biosystems, Thermo Scientific, UK
POU5F1	Hs00999632_g1		
NANOG	Hs04399610_g1		
SIRT1	Hs01009006_m1		
TINF2	Hs00173291_m1		
TERF2	Hs00194619_m1		
B2M	Hs00187842_m1		

III-4.10. Zymography

Zymography is a technique based on the previously described gel electrophoresis in which the activity of matrix metalloproteinases (MMPs) in complex biological samples can be analysed [65]. The main differentiator is that proteins are separated in non-reducing conditions in a polyacrylamide gel containing a proteolytic substrate which in this thesis was gelatin (Sigma-Aldrich, USA). The proteins are then renatured by the exchange of SDS with a nonionic detergent (2 % (v/v) Triton X-100) after which the gel is incubated in an appropriate development buffer the visualization of metalloproteinases 2 and 9 (50 mM Tris-HCL, 0.2 M NaCl and 5 mM CaCl_2). The gel is then stained with Coomassie Blue and the proteolytic activity of MMP's are detected as clear bands against a blue background of undegraded gelatin. Here we used zymography to study the activity of MMP-2 and MMP-9 in heterotypic assembloid models of melanoma.

III-4.11. Immunostaining

Immunostaining is a method used to determine the distribution and localization of specific proteins within individual cells or tissues using specific antibodies to detect the desired target protein [66]. This protocol makes use of a 2-step method in which a primary antibody is used against a target molecule followed by the incubation of a secondary antibody that binds to the primary antibody and is labelled with an enzyme or a fluorescent dye that allows the detection under a fluorescence microscope or through the addition of a colored substrate. Both immunocytochemistry (ICC) and immunohistochemistry (IHC) rely on this principle. Cells were incubated with primary antibodies (Table III-7).

III-4.11.1. Immunocytochemistry

ICC is a method for detection and visualization of specific antigens in cells [67]. After the desired culture period, these cells are fixed to preserve the chemical and structural state of the cellular components. Depending on the localization of the molecule to be studied, these fixed samples may be required to undergo cell membrane permeabilization to allow for the antibody to penetrate the cells which is achieved by dissolving lipids from the cell membrane. This may be achieved by one of two methods: organic solvents, such as methanol and acetone, and detergents such as saponin, Triton X-100 and Tween-20 [68]. Further conditions and concentrations can be found in a more descriptive manner in the chapters ahead. In a similar fashion, immunostaining may also be performed in a whole-mount style on

spheroids or organs which allows for 3D visualization. This however may be challenging due to the poor laser penetration of dense, opaque tissue and to refractive index mismatch. To overcome this several different methods for optical clearing have been described [69]. For the heterotypic tumor models herein described we have opted for a hyperhydration method, more specifically Scale-A2 (4 M urea, 10 % (w/v) glycerol, 0.1 % (w/v) triton x-100). These methods make use of detergent-based removal of lipids with urea-mediated hydration (in the presence of glycerol) of the remaining tissue to achieve a clear sample. Urea here is used to penetrate and partially denature the desired sample, therefore hydrating even the hydrophobic regions of high refractive index proteins. By using this clearing protocol, it is possible to reduce the overall refractive index to 1.38 which is similar to that of water. Cells were observed with a Leica TCS SP8 confocal microscope (Leica, Germany).

III-4.11.2. Immunohistochemistry

While similar to ICC, IHC involves an additional sample processing step. After being chemically preserved, samples are embedded in paraffin wax. This allows for the maintenance of the original architecture of the surrounding tissue while performing histological sectioning. Fixation and crosslinking agents render the antigens undetectable by antibodies, due to the modification of tertiary and quaternary structures and therefore antigen retrieval (AR) methods are required to retrieve the loss of antigenicity [70]. In the work here mentioned, we have used heat-induced epitope retrieval (HIER) with sodium citrate buffer (10mM Sodium Citrate, 0.05 % (v/v) Tween 20, pH 6.0)

Samples were processed and cut into 4 μ m sections prior to immunostaining. Analysis was performed using an Axiomager Z1m fluorescence microscope (Zeiss, Germany) with Zen 3.2 (blue edition) software.

Table III-7 Antibodies used for ICC and IHC.

Target	Antibody	Brand	Ref	Host	Dilution	Clonality, reactivity
T cells	CD4	Novus Biologicals, USA	NBP1-19371	Rb	1:200	Polyclonal, human/mouse
Endothelial cells	CD31	Dako, Denmark	M0823	Ms	1:30	Monoclonal, human
VMM15 tumor cells	Anti-Melanoma gp100	Abcam, UK	ab137078	Rb	1:200	Monoclonal, human/mouse
Proliferative cells	Ki67	Abcam, UK	ab16667	Rb	1:50	Monoclonal human

Legend: Ms - Mouse, Rb – Rabbit

III-4.12. Hematoxylin-Eosin and and Picrosirius Red Stainings

Hematoxylin is a natural dye that colors in blue basophilic substances, such as the nuclei of cells. Eosin Y is a synthetic dye that colors in various shades of red acidophilic substances, the acidic proteins of the cytoplasm and connective tissue. Picrosirius red (PR) staining is one of the most important stains to study collagen networks. Under polarized light, collagen bundles appear green, red or yellow, and are easily differentiated from the black background, thus allowing for quantitative morphometric analysis [71] while under brightfield collagen appears as bundles of pink to red fibers which get disturbed in pathological conditions [72].

In the subsequent chapters, both these methods were used to determine the architecture, organization and ECM deposition obtained in the developed heterotypic tumor models. For this effect, samples were processed as per immunostaining and cut into 4 μ m sections. Slices were stained with hematoxylin and eosin Y (H&E, Bio-Optica, Italy) and PR (Bio-Optica, Italy) following standard protocols. H&E and PR slices were analyzed using a LEICA DM750 microscope (Germany) coupled with the LEICA Application Suite 4.13.0.

III-4.13. Image analysis

Software-based processing and image analysis is a powerful tool that allows the analysis of large image datasets. This form of analysis allows the recognition of small variations in cell properties that occur through the activation of complex biological pathways that regulate processes as in the case of tumor invasion. Understanding these interactions and how they drive cell behaviour is important to uncover important knowledge required for the development of novel therapeutics.

Over the following chapters, different image analysis techniques have been used to aid in the understanding of cellular behaviour as well as cell invasion in 3D matrices. In the body of work here detailed, we have used software-based approaches to analyse spheroid properties such as their overall area and circularity. For this effect, brightfield images of endpoint spheroids were acquired with a LEICA DM750 microscope (LEICA, Germany). For post-processing, images were uploaded to FIJI [73] and the “Analyze Spheroid Cell Invasion In 3D Matrix, RRID:SCR_021204” macro was used for spheroid segmentation and analysis. Besides these two properties, we also used post-processing techniques to evaluate spheroid invasion in Matrigel™ hydrogels and determine parameters such as the area of the spheroid core, the maximum radius of invasion and the area of the invading edge. For this particular

case, images acquired over the course of 0, 4 and 7 days were converted to grey-scale and organized for automatic batch processing. Spheroid segmentation and analysis was performed with the INSIDIA (INvasion Spheroid ImageJ Analysis) [74] macro for FIJI. Ultimately, in order to study cell chemotaxis in our developed collagen chemotaxis chamber, we were interested in running available software image analysis tools. However, due to the architecture of the chamber and the cell number, it was difficult to use previously reported analysis macros. Hence, for this effect we describe in the following chapter an image analysis pipeline that allowed for the assessment of axial migration of stromal cells throughout the chemotaxis hydrogel. Briefly, brightfield endpoint images of cell invasion were processed with ImageMagick 6.9.11 to obtain a 1:1 ratio image centered on each well for each time point of the experiment, which was then divided into four quadrants of identical size. Pixel classification was performed with Ilastik [75] to separate background from invading cells. CellProfiler 4.2.1 [76] was then used for the cell segmentation and the calculation of the area of invasion.

Time-lapse brightfield images were obtained over pre-established time-points using an Axio Observer inverted microscope with incubation (37 °C, 5 % of CO₂), and Zen 3.2 (blue edition) software (Zeiss, Germany).

III-5. STATISTICAL ANALYSIS

GraphPad Prism 8.2.1 software (La Jolla, USA) was used to perform statistical analysis. Data were analyzed by Shapiro–Wilk normality test. A one-way analysis of variance (ANOVA) with a Tukey post-test was used to analyze the results with a normal distribution. Otherwise, data were analyzed with the Kruskal–Wallis test with Dunn’s multiple comparison post-test. The significance of data variability was determined with the Brown–Forsythe test. Results are presented as mean $\pm$ standard deviation (SD) and as coefficient of variation (reproducibility analysis) and the significance level between groups was set for * $p < 0.05$.

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SECTION 3

EXPLORING NOVEL CANCER BIOENGINEERING STRATEGIES

CHAPTER IV

HIGHLY TAILORABLE GELLAN GUM NANOPARTICLES AS A PLATFORM FOR THE DEVELOPMENT OF T CELL ACTIVATOR SYSTEMS

CHAPTER IV

HIGHLY TAILORABLE GELLAN GUM NANOPARTICLES AS A PLATFORM FOR THE DEVELOPMENT OF T CELL ACTIVATOR SYSTEMS ‡

ABSTRACT

T cell priming has been shown to be a powerful immunotherapeutic approach for cancer treatment in terms of efficacy and relatively weak side effects. Systems that optimize the stimulation of T cells to improve therapeutic efficacy are therefore in constant demand. A way to achieve this is through artificial antigen presenting cells that are complexes between vehicles and key molecules that target relevant T cell subpopulations, eliciting antigen-specific T cell priming. In such T cell activator systems, the vehicles chosen to deliver and present the key molecules to the targeted cell populations are of extreme importance.

In this work, a new platform for the creation of T cell activator systems based on highly tailorable nanoparticles made from the natural polymer gellan gum was developed and validated. Here, it is demonstrated that the developed system presented a more sustained T cell activation over time when compared to commercial alternatives. Concurrently, the expression of higher levels of key cytotoxic pathway molecules granzyme B/perforin was induced, suggesting a greater cytotoxic potential for future application in adoptive cancer therapy.

‡ This chapter is based on the following publications:

Rodrigues DB, Moreira HR, Cerqueira MT, Marques AP, Castro AG, Reis RL, Pirraco RP. Highly tailorable gellan gum nanoparticles as a platform for the development of T cell activator systems. *Biomater. Res.* 26 (2022) 48. <https://doi.org/10.1186/s40824-022-00297-z>

Rodrigues DB, Moreira HR, Gasperini L, Cerqueira MT, Marques AP, Pirraco RP, Reis RL. HYDROGEL-LIKE PARTICLES, METHODS AND USES THEREOF, WO/2021/064678 (2021)

IV-1. INTRODUCTION

Many advances have been made in the field of immunotherapy with the introduction of adoptive T cell therapy and CAR T cell therapies. These consist in the administration of either autologous T cells primed and expanded *in vitro* or genetically modified T cells that encode for transmembrane chimeric molecules with the capacity to both recognize tumor surface antigens and trigger cytotoxic machinery specifically against these tumor cells [1]. Given the significant success of these technologies, there is now a need for improved ways of expanding cells for administration.

The clinical efficacy of adoptive cell transfer (ACT) is largely dependent on how the antigen-specific T cells are stimulated during the priming phase [2–4] and dependent on downstream stimulation with γ chain receptor cytokines such as IL-2, IL-7, IL-15 and IL-21 [5–7]. In fact, a fine balance when dealing with artificial T cell stimulation is required in order to obtain a high number of effector T cells without jeopardizing cell effectiveness. An inefficient priming may result in hyporesponsive or anergic T cells, leading to a lack of proliferation and/or loss of effector function when in contact with the respective antigen, rendering the treatment ineffective. On the contrary, excessive stimulation of the cells *in vitro* may also compromise treatment efficiency due to T cell induced cell death (AICD) [8,9]. Therefore, novel methodologies to improve T cell priming are in high demand.

One such novel methodology is artificial antigen presentation which has emerged as a very promising concept due to its versatility both in terms of the platform used to build the system and the T cell activation method adopted. Over the past decades, several artificial antigen presenting cells (aAPCs) based on distinct systems have been developed with promising results in the *in vivo* expansion of specific T cells [10–12] with further evidence provided when applied to more advanced disease models of murine experimental autoimmune encephalomyelitis, collagen induced arthritis, non-obese diabetic animals [13] and cancer [14]. The base architecture of these systems has relied on the use of iron oxide nanoparticles [13], synthetic polymers [11], liposomes [15], magnetic beads [10,14,16] and cell engineering methodologies [12,17,18]. However, one of the greatest handicaps of these systems is the difficulty in generating, within a short period of time, large numbers of modified T cells of a defined phenotype and with a specified ratio of subsets [19]. Furthermore, in some cases, inorganic nano/microparticles, while qualified for applications such as diagnostics, imaging and photothermal therapies, have limitations related to their low solubility and toxicity concerns [20]. In contrast, biomaterials present characteristics such as biodegradability, biocompatibility, half-life and mechanical properties that can be used to shape

interactions with T cells and drive immune cell responses [21]. As such, biomaterials have the potential to be used in the development of novel platforms more in tune with biological systems.

Gellan Gum (GG) is a bacterial exopolysaccharide exhibiting properties such as biocompatibility, low production costs and reproducibility over batches. GG is produced by the bacteria *Sphingomonas elodea* [22] and consists of a repeating unit of α -L-rhamnose, β -D-glucose and β -D-glucuronate in the molar ratios 1:2:1 [23]. GG may either exist in its native form or upon alkaline hydrolysis originate the so called low-acyl GG [22], both forms retaining the capability to crosslink into hydrogels in the presence of mono-, di- and trivalent cations [24,25]. This polysaccharide has been proven suitable for the production of microbeads [26–28] or for the production of formulations for the sustained delivery of relevant drugs [29–32,33–35]. Due to its resemblance with the extracellular matrix glycosaminoglycan composition [36] it has gathered also important attention in the field of tissue engineering and regenerative medicine [37–40].

In this work, it has been proposed to use GG as the basis to create a multivalent T cell activator system for the controlled *in vitro* expansion of T cells. In this system, GG nano-sized particles were surface-modified with α -CD3 and α -CD28 antibodies and used to successfully prime naïve mouse T cells, generating a high number of proliferative IL-2 producing CD4⁺ T cells. Additionally, the current work shows that these cells present increased levels of T cell mediated cytotoxicity mediators Perforin 1 and Granzyme B while maintaining a low state of T cell exhaustion.

IV-2. MATERIALS AND METHODS

IV-2.1. Gellan gum particle synthesis

Gellan gum nanoparticles (npGG) were produced by the double emulsion water-in-oil-in-water technique [41]. Briefly, a 1 % (w/v) of gellan gum (Sigma-Aldrich, Germany) solution was prepared in deionized water, under stirring at 90 °C for 30 min. After dissolution, the solution was allowed to stabilize at 50 °C. A drop of Tween-20 (Sigma-Aldrich, Germany) was then added to the GG solution (W1) and each mL of the GG solution was emulsified in 3 mL of 0.5 % (v/v) of Span®80 (Sigma-Aldrich, Germany) in chloroform (Sigma-Aldrich, Germany)(O) while under stirring with a T18 BASIC Ultraturrax (IKA, NC, USA) for 3 min. W1/O was then added dropwise to a solution of 3.5 % (w/v) poly(vinyl alcohol) (PVA)(W2) (Sigma-Aldrich, Germany) in water under continuous stirring and then crosslinked by adding gradually

W1/O/W2 into 5 mL of a 60 % (w/v) solution of calcium chloride (Merck Millipore, Germany) under stirring. Chlorophorm was removed using a rotary vacuum evaporator at 37 °C (Stuart, Staffordshire, UK). The particles were recovered by centrifugation for 1 h at $13'000 \times g$ (Eppendorf, Germany) and by passing through a series of strainers (pluriSelect, Leipzig, Germany) ranging from 10 μm down to 1 μm to limit size dispersion. In order to remove PVA, 3 additional washing steps were performed by centrifugation at $13'000 \times g$ for 10 min with deionized water.

GG particles were then frozen [-210 °C in liquid nitrogen (N_2)] and then freeze-dried (LyoQuest, Telstar, Spain) to obtain dried GG particles for further modification upon rehydration.

IV-2.2. npGG characterization

IV-2.2.1. Chemical characterization of npGG

The presence of specific functional groups in the npGG was assessed by Fourier transform infrared spectroscopy (FTIR). To obtain the FTIR spectra of npGG, transparent potassium bromide (Sigma-Aldrich, Germany) pellets were prepared containing the samples to be analyzed. The readings were performed in an infrared spectroscope (IR Prestige-21, Shimadzu, Japan) with 4 cm^{-1} resolution and the results are presented as the average of 32 scans.

IV-2.2.2. npGG morphology

The npGG size distribution and surface charge were analyzed by dynamic light scattering after preparing a 1 mg/mL suspension of rehydrated GG particles in deionized water (Zetasizer Nano ZS, Malvern Instruments, UK). Acquisition was performed with the detector positioned at a scattering angle of 173°.

Particle size and morphology were analyzed with a High-Resolution Field Emission Scanning Electron Microscope with Focused Ion Beam (FIB – SEM) (AURIGA COMPACT, ZEISS, Germany). For this effect, the samples were diluted at 0.5 mg/mL in ultrapure water followed by a further dilution of 1:20 and were dispersed onto the surface of a 400-mesh carbon-coated copper grid (Ted Pella, USA) for further observation.

IV-2.2.3. Particle stability upon rehydration

To determine the stability of the produced GG particles over time, a 1 mg/mL suspension of npGG was prepared in different solutions (culture medium, phosphate buffer saline (PBS), and deionized water) and filtered through a 0.8 μm filter (Millex, Millipore, France). Particle size and dispersity was evaluated over 7 days by dynamic light scattering (DLS) (Zetasizer Nano ZS, Malvern Instruments, UK). Between data acquisition times, samples were stored at room temperature.

IV-2.2.4. Water uptake and water content quantification

The dried GG particles were rehydrated with PBS up to 7 days at 37 °C, to determine the water uptake profile. Samples were weighed prior (W_d) and after each time point (W_w) and the percentage of water uptake over time was calculated based on the **Equation III-1**. The water content of the hydrogel particles was determined through of the particles in their wet state (W_w) versus the weight in their dried state (W_d).

IV-2.2.5. Particle functionalization with antibodies

npGG in suspension were functionalized through chemical coupling by one of two methods. Briefly, 1 mg of npGG was dissolved in MES buffer at 50 mM and pH 6.5. Carboxylic groups were activated through the addition of N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride and N-hydroxysulfosuccinimide to achieve a final concentration of 17.3 μM and 18 μM respectively and left to stir for 1 h. The particles were then washed 3x by centrifugation at 18000 $\times$ g and afterwards either i) NeutrAvidin was added first and left to react over 4 h at RT under agitation after which the particles were washed again and 10 μg /20 μg of biotinylated α -CD3/CD28 antibodies (MyBioSource™, California, USA) were added or ii) 10 μg /20 μg of functional grade α -CD3/CD28 (Tonbo Biosciences™, California, USA) antibodies were added directly to the EDC/NHS activated particles and incubated overnight at 4 °C. The following day, the produced activator-npGG were washed again 3 $\times$ by centrifugation at 18'000 $\times$ g to remove unbound antibodies and particles were suspended in adequate medium.

IV-2.2.6. Density of NeutrAvidin

The density of NeutrAvidin protein on the particles surface was determined using the micro bicinchoninic acid assay (Pierce, Rockford, IL) in accordance with the manufacturer's instructions. For this effect, 1 mg of GG particles was functionalized as stated above with either 0.5 mg or 1 mg of avidin by conjugation through EDC/NHS. Experimental replicates were performed for reproducibility of the chemical conjugation and elution's resulting from washing step were stored for quantification of unbound NeutrAvidin.

IV-2.2.7. Assessment of npGG functionalization

To evaluate the binding of functional grade antibodies to npGG's, SDS-PAGE (Sodium, Dodecyl, Sulfate, Polyacrylamide Gel Electrophoresis) analysis was used (Sigma-Aldrich, Germany). To achieve protein separation, two separate gels were used, a 4.7 % stacking gel and a 12 % resolving gel. In each well, an equal amount (10 µg) of either control or modified particles were loaded after being prepared by heating at 60 °C for 30 min prior. A calibration curve of the biotinylated antibody of 0.5 µg, 1 µg and 2 µg was performed. The direct binding of the antibody to the particles was also performed and again a calibration curve of the standard antibody was equally performed 0.5 µg, 1 µg and 2 µg. The bands in each well were observed by soaking the gel with Coomassie Blue (National Diagnostics, Atlanta, USA) followed by destaining.

IV-2.3. Functionality evaluation

Functionality assays were carried out to determine the *in vitro* performance of the produced activator-npGG in terms of both surface interaction with host immune cells, as well as, the capacity to trigger T cell proliferation.

IV-2.3.1. Cell lines and culture conditions

Primary human dermal fibroblasts (hDFbs) (Gibco, UK) were obtained from Thermo Fischer and cultured in α -MEM, supplemented with 10 % (w/v) fetal bovine serum (FBS, Invitrogen, USA) and 1 % (v/v) antibiotic/antimycotic. Cells were kept in culture at 37 °C in a humidified atmosphere with 5 % CO₂.

IV-2.3.2. Cell isolation

All procedures were approved by the Direccção Geral de Alimentação Veterinária, the Portuguese National Authority for Animal Health, and respected the national regulations and international animal welfare rules. Murine splenocytes cells were obtained from Balb/c mice. Briefly, mouse spleens were excised and sliced into small pieces. The fragments were placed onto a strainer attached to a 50 mL conical tube. The excised spleen fragments were pressed through the strainer using the plunger end of a 5 mL syringe. The cells were washed through the strainer using excess cell culture medium. Cells were collected by centrifugation at $400 \times g$ for 5min. The cell pellet was resuspended in 1mL of pre-warmed (37 °C) red blood cell lysis buffer and incubated at 37 °C for 5 min. Murine splenocytes were then washed with excess PBS and centrifuged at $400 \times g$ for 5 min.

Cells were cultured from hereon with RPMI 1640 medium (Sigma-Aldrich, Germany) supplemented with 10 % (v/v) FBS (Gibco, ThermoFisher Scientific, Paisley, UK) 1 % (v/v) HEPES (Gibco, ThermoFisher Scientific, Paisley, UK), 1 % (v/v) sodium syruvate (Gibco, ThermoFisher Scientific, Paisley, UK) and 0.05 mM Mercaptoethanol (Gibco, ThermoFisher Scientific, Paisley, UK) at 37 °C in an incubator equilibrated with 5 % CO₂.

IV-2.3.3. Metabolic activity in response to npGG

To determine the metabolic activity of cells in response to npGG, hDFbs were stimulated with different npGG concentrations (2, 20, 100, 200 µg/mL) for 72h. Metabolic activity and cell proliferation were quantified after this period using CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, USA) and Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, UK), respectively as recommended by the manufacturer. Metabolic activity data was expressed as the percentage of cell metabolic activity of the stimulated cells normalized to the non-stimulated hDFbs. The concentration of DNA present in each condition was determined against a standard curve.

IV-2.3.4. Visualization of activator npGG-cell binding

For visualizing activator npGG-splenocyte interactions, murine splenocytes were stimulated for 5 days with varying concentrations of the developed system. At different time-points, cells were collected, washed and growth medium supernatant was aspirated and replaced with PBS at a concentration of $\sim 1 \times 10^6$

cells/mL. A pipette was used to gently mix and transfer 1 mL of the cell suspension into a culture plate well holding a coverslip. The culture plate was then left stationary for 30 min at room temperature to allow for the sedimentation and adhesion of cells to the coverslip. Afterwards, the PBS suspension containing remaining non-adherent cells was gently aspirated. The adhered cells were then fixed for 15 min at room temperature with ice cold methanol (Sigma-Aldrich, Germany) pipetted along the side of the well. Fixed cells were then washed with PBS and stored at 4 °C until immunolabeling.

Primary antibody CD4 (Rabbit anti-Mouse, 1:200, NovusBiologicals, U.K.) and Alexa Fluor 488-tagged Goat anti-rabbit secondary antibody (1:500, ThermoFisher Scientific, Paisley, UK.) were used for cell visualization (Supplementary Table IV-1). Nuclei were stained with DAPI (Invitrogen, U.S.) For particle visualization, a Donkey anti-rat AF594 secondary antibody (1:500, ThermoFisher Scientific, Paisley, UK) was used. An AXIOIMAGER Z1M (Carl Zeiss Microscopy GmbH, Jena, Germany) widefield microscope or a TCS SP8 Leica laser confocal microscope (Leica Microsystems, Wetzlar, Germany) were used for visualization of the samples.

IV-2.3.5. Visualization of activator npGG-cell binding

To quantitatively determine the percentage of binding of the activator-npGG system to T cells, freshly isolated murine splenocytes were placed in culture with varying concentrations of α -CD3 antibody-functionalized GG particles (1 μ g, 10 μ g, 50 μ g and 100 μ g) produced either by directly coupling the antibodies to the particles or through the NeutrAvidin/Biotin immobilization. Cultures proceeded over 5 days and, ultimately, cell-bound particles were labelled in a first step with a rabbit anti-rat IgG AF488 secondary antibody (1:500, ThermoFisher Scientific, Paisley, UK) followed by a wash step. T cells were then labelled with a rat anti-mouse AF647-conjugated CD4⁺ antibody (2.5 μ g/mL, Biolegend, London, UK) and conjugation was assessed through flow cytometry.

IV-2.3.6. T cell proliferation and activation in response to functionalized npGG

To monitor T cell expansion when stimulated with functionalized GG particles, previously isolated murine splenocytes were labeled with CFSE. In brief, 2.5×10^7 cells were incubated with 2.5 μ M of CFSE at 37 °C for 10 min. Cells were then washed three times with complete medium to quench and remove unbound CFSE and were then distributed (1×10^6 /well) in 96 well flat bottom culture plate. As controls for T cell expansion, 3 conditions were set up: Dynabead Mouse T-Activator CD3/CD28 for T cell Expansion

and Activation (Gibco, ThermoFisher Scientific, Paisley, UK), plate bound functional grade anti-CD3 antibody (eBioscience™, ThermoFisher Scientific, Paisley, UK) (2 µg/mL) or no additional stimulator.

For the coating of the 96 well plates (Cellstar M3687, Greiner Bio-One, Austria), anti-CD3 antibodies were dissolved to a concentration of 2 µg/mL and 50 µL were added per well and left to incubate for 1 h at RT. The following day, culture plate wells were washed with complete medium to block unspecific binding of antibodies still to be added.

Different np-GG α-CD3/ α-CD28 concentrations were added (1 µg/mL, 10 µg/mL, 50 µg/mL and 100 µg/mL) over a period of 7 days of stimulation in a ratio of 1:1. For plate bound antibody stimulation, anti-CD28 was added to a final concentration of 2 µg/mL to each respective well. All quantifications were performed in triplicate. The proportion of dividing cells was detected by flow cytometry based on CFSE levels of gated CD4⁺ T cells [42].

IV-2.3.7. Enzyme-linked immunosorbent assay (ELISA) IL-2 secretion assay

ELISA (Elabscience, Hubei, CN) was used to quantify the levels of IL-2 released upon stimulation with the developed activator-npGG system. Conditioned medium was collected post 7 days of stimulation with varying concentration of the npGG activator system to assess total IL-2 produced over the complete stimulation period. The procedure was carried out according to the manufacturer's instructions and the absorbance was read at 450 nm using a Synergy HT (BioTek, Vermont, USA) microplate reader. The quantity of IL-2 protein was determined by means of a standard curve. IL-2 within the control media was undetectable during the experiments and therefore was omitted from the figures.

IV-2.3.8. T cell exhaustion and cytotoxic capacity

Quantitative Real-Time Polymerase Chain Reaction (qPCR) was used to detect the expression of T cell exhaustion-related genes after exposure to 7 days of stimulation with either the activator-npGG system or the commercial activator beads. Primers for the tested genes and the housekeeping gene ACTβ were designed using Primer-Blast database (NCBI, Bethesda, MD, USA) (Supplementary Table IV-2).

qPCR reactions were carried out in a MasterCycler Realplex4 (Eppendorf, Hamburg, Germany) and primer efficiency was tested out using serial dilutions of cDNA (1, 1:10, 1:100, 1:1000). For qPCR reactions, 1 µL of synthesized cDNA was used in a 20 µL reaction containing 10 µL of PerfeCTa® SYBR

Green FastMix (Quanta Biosciences, Beverly, MA, USA) and forward and reverse primers at 300 nM. Reaction conditions comprised a 2 min denaturation at 95 °C, followed by 45 cycles of 95 °C for 10 s, a specific annealing temperature as described in Supplementary Table IV-2, for 30 s and 72 °C for 10 s. Products obtained from real-time PCR were subjected to melting curve analysis to check for the correct amplicon length and the absence of unspecific products. Transcript abundances were normalized to the expression of ACTB. Samples were run in triplicate in each PCR assay. Normalized expression values were calculated following the mathematical model proposed by Pfaffl using the formula: $2^{-\Delta\Delta Ct}$ (Pfaffl, 2001).

IV-2.3.9. Analysis of helper and cytotoxic T cell subsets

To monitor T cell activation profile, splenocytes were incubated with functionalized npGG over 7 days of stimulation. Cells were then washed, permeabilized, and stained with the following antibodies: CD45-PerCP/Cy5.5, CD4-APC-H7, CD8-APC-H7 (from BD Biosciences) and Granzyme B-PE-Cy7, Perforin-APC, CD69-PE (from Biolegend) (Supplementary Figure IV-1). For intracellular staining, cells were first incubated and fixed with Reagent A-Fix and Perm™ (Thermo-scientific, Netherlands) for 15 min, and stained with the respective conjugated antibodies diluted in Reagent B-Fix and Perm™ (Thermo-scientific, Netherlands) for 30 min at RT. After washing with PBS, cells were fixed in 1 % formalin (Sigma, USA)/PBS and then analyzed on a FACSCalibur flow cytometer (BD Biosciences, Belgium) and data was treated using Cell Quest Pro version 4.0.2 (BD Biosciences, Belgium). Cells were gated according to (Supplementary Figure IV-1).

IV-2.4. Statistical analysis

Graphpad software version 7.03 was used to perform statistical analysis. To determine if data sets fell in a normal distribution the Shapiro-Wilk normality test was performed. When a normal distribution was verified a one-way analysis of variance with a Tukey post-test was performed. Otherwise, data was analyzed with the Kruskal-Wallis test followed by the Dunn's multiple comparison post-test. Results are presented as mean $\pm$ standard deviation (SD) and the significance levels between experimental groups was set for * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

IV-3. RESULTS

IV-3.1. GG-based particles produced by double emulsion

The core GG particles were synthesized by a double emulsion technique as represented in **Figure IV-1A**. The particles were then subsequently evaluated in terms of both physical and chemical properties by characterizing the surface chemistry by FTIR and the particle morphology and size by DLS and FIB – SEM.

Through FTIR (**Figure IV-1B**), the spectrum of the GG particles was compared to those of native GG and PVA in order to check for any remaining PVA in the final GG particles after the repeated washing steps. The original spectrum of GG displays characteristic transmittance bands at 3411 cm^{-1} indicating the stretching vibration of -OH group in the gellan gum hydrogel (Ray et al., 2010). Other peaks of free-standing GG were detected at 1051.11 cm^{-1} which are assigned to the C-O stretching vibration. Additional characteristic peaks at 2414 cm^{-1} (OH-stretching), 2924.09 cm^{-1} (C-H stretching), two strong peaks at 1612 cm^{-1} and 1413 cm^{-1} for asymmetric and symmetric -COO stretching and at 1028 cm^{-1} for C-O stretching for alkyl ether were equally detected. When comparing the spectrum from the GG particles with those of free standing PVA it was verified that characteristic bands, such as, the O-H stretching from the intermolecular and the intramolecular hydrogen bonds (3550 and 3200 cm^{-1}), the vibrational band referring to the C-H stretching from alkyl groups (2840 and 3000 cm^{-1}) and the peaks regarding the C=O and C-O stretching from the acetate group ($1750\text{--}1735\text{ cm}^{-1}$) are not present or if so, in a residual manner.

Regarding size analysis by DLS, a Z-average (d.nm) of 141.9 and a Pdl of 0.206 was observed for the developed GG particles (**Figure IV-1C i**). STEM images revealed that the developed particles assumed a spherical shape in nature exhibiting a monodisperse state. Size correlating to what was obtained by DLS with variable sizes ranging from 50 nm to approximately 200 nm in diameter (**Figure IV-1C ii**), which was expected given the double emulsion protocol used.

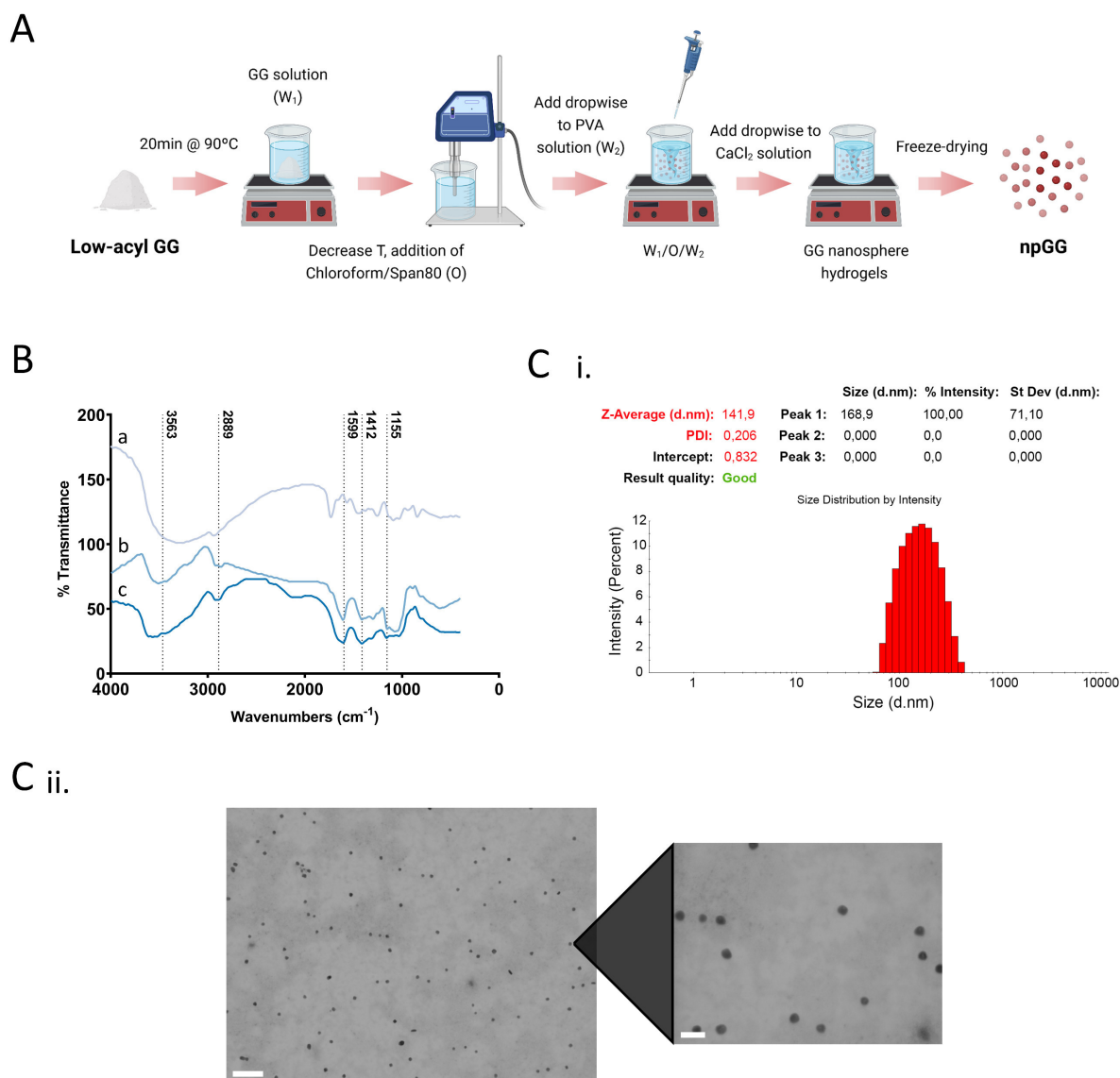


Figure IV-1 npGG production and morphological characterization.

A Schematics of the production of npGG based on a double emulsion method. **B** FTIR spectra of (a) PVA, (b) npGG, (c) GG. **C** i. Particles size distribution of npGG according to intensity when produced by a double emulsion. ii. Representative STEM images of npGG. Images were obtained with an accelerating voltage of 20 kV. The scale bars represent a size of 300 nm (left) and 100 nm (right).

IV-3.2. GG particles retain water uptake capacity and stability over time

GG solutions are known to easily crosslink in divalent cationic solutions and form hydrogels, which can withhold high contents of water. To evaluate if the developed particle system had retained this property characteristic of GG, the water uptake capacity of the particles was assessed. When the freeze-dried particles were immersed in PBS (rehydration step), a rapid weight gain of between 2700 % and 3200 % was observed due to water uptake (**Figure IV-2A**).

As the produced npGG retained the feature of significant water uptake after freeze drying and are produced only by means of physical crosslinking mechanisms, the possibility that these particles become weaker in physiological conditions due to the exchange of divalent cations by monovalent ones was present. The stability of the produced particles was then determined in 3 different solutions: deionized water (dH₂O), phosphate buffer saline (PBS) and culture medium over time by assessing the polydispersity index (**Figure IV-2B i**) and mean size of the particles (**Figure IV-2B ii**). We could observe that the polydispersity was kept in an acceptable range upon solubilization of the particles in dH₂O with a tight size variance over the 7 days period studied with an average size around 180 nm, suggesting that this system remains stable over time when in deionized liquids. Due to the high ionic content of PBS, a destabilization of the system in the form of particle agglomeration was both expected and confirmed which translated into an increased mean size and polydispersity index. Interestingly, when rehydrated in culture medium, notwithstanding the ionic content of the medium, a different behavior of the particle system was observed. Immediately after rehydration and stabilization, an increased instability of the system was verified confirmed by an increased PDI when compared to dH₂O. However, over time, a decrease in the PDI value was observed reaching similar levels to those of dH₂O, as well as, mean particle size averaging 64 nm.

In order to better determine the biological compatibility of the developed system, the metabolic activity of hDFbs was determined after 3 days of incubation with varying concentrations of the developed npGG. Values were normalized against dsDNA concentration and are presented as percent of the values for cells cultured in the absence of particles (Control) (**Figure IV-2C i**). At 3 days of incubation, metabolic activity of the hDFbs with npGG didn't differ from the control, regardless of the npGG concentration. When looking at the dsDNA content (**Figure IV-2C ii**) a slight decrease was present when increasing the concentration of npGG in the culture, but no statistically significant differences were found. To determine the impact on cell morphology at each end time-point, cells were fixed and labelled with Phalloidin-TRITC and DAPI. Upon visualization by fluorescent microscopy, cells presented a regular spindle shape regardless of npGG concentration (**Figure IV-2D**).

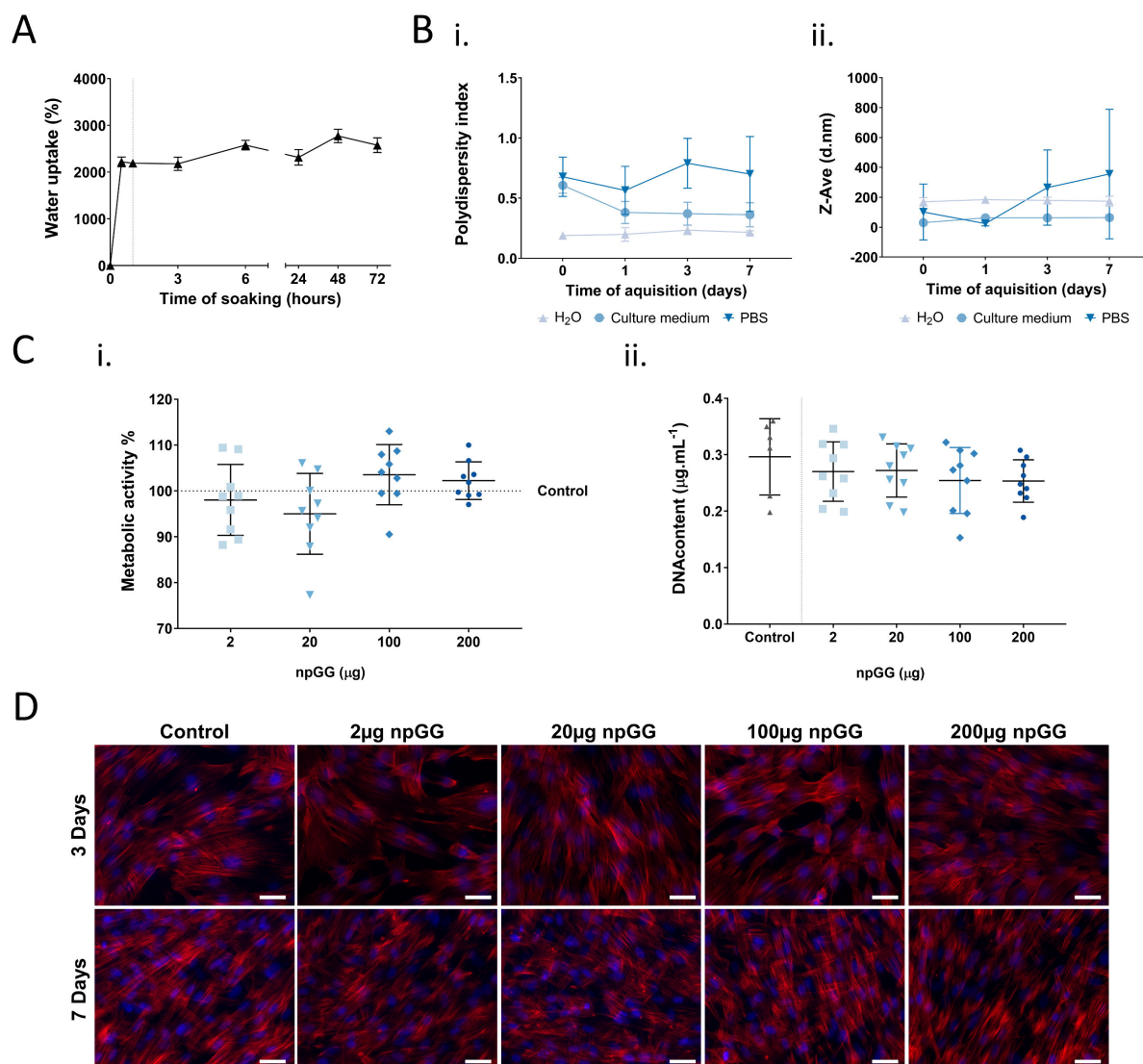


Figure IV-2 Physical and biological Characterization of npGG.

A. Water uptake of dried npGG during 72 h of rehydration in PBS. **B** Effect of different solutions on npGG regarding i. Polydispersity index ii. Average size distribution. **C** Biological performance of human dermal fibroblasts (hDFbs) stimulated with different npGG concentrations (2, 20, 100, 200 μg/mL) for 72 h i. Metabolic activity was assessed by MTS and presented as the percentage of control non-stimulated cells. ii. Cell proliferation evaluated by DNA quantification. **D** Morphological analysis of hDFbs by rhodamine-phalloidin/DAPI after stimulation with varying concentrations of npGG (red = rhodamine and blue = DAPI). Original magnification 10×, scale bar = 100 μm.

IV-3.3. GG particles may be surface functionalized with biologically relevant antibodies

The immobilization on the surface of the GG particles of two antibodies to mimic the co-stimulatory signals critical for T cell priming, was then sought. Two different methods were used to achieve this i) direct binding of the antibodies through EDC/NHS chemistry or ii) coupling of NeutrAvidin to the surface of the particles though EDC/NHS chemistry followed by non-covalent binding of biotinylated antibodies (**Figure IV-3A**).

To verify the stable binding of NeutrAvidin to the surface, protein content was quantified through micro bicinchoninic acid assay of both the functionalized particles and the elution fractions resultant from the washing steps. Results showed that opting for 1 mg of NeutrAvidin for the functionalization didn't return a significant difference in bound protein when compared to 0.5 mg with protein concentrations of respectively 45 $\mu\text{g/mL}$ and 38 $\mu\text{g/mL}$ (Supplementary Figure IV-2). Considering that that both concentrations returned a similar concentration of bound Neutravidin, a higher amount of unbound protein was expected to be found in the different elution fractions when 1mg of NeutrAvidin was used (Supplementary Figure IV-2). The low dispersion of values found over the different experimental replicates suggest stable binding of the protein and high reproducibility of the protocol.

Following, it was evaluated which of methods i) or ii) would lead to a more efficient binding of the desired antibodies. For this effect, both systems, either functionalized with NeutrAvidin or not, were reacted with two amounts of anti-CD3 antibody, 10 and 20 μg and were subsequently blotted by SDS-PAGE (**Figure IV-3B**). While a trend for a higher amount of bound antibody was observed when using 20 μg , this change wasn't statistically significant. All subsequent particles for this study were then functionalized with either 10 μg /20 μg of antibody directly bound to the particle or 10 μg /20 μg of biotinylated antibody bound to particles previously incubated with 0.5 mg of NeutrAvidin in order to determine the effects of the antibody concentration on the *in vitro* biological performance of the system.

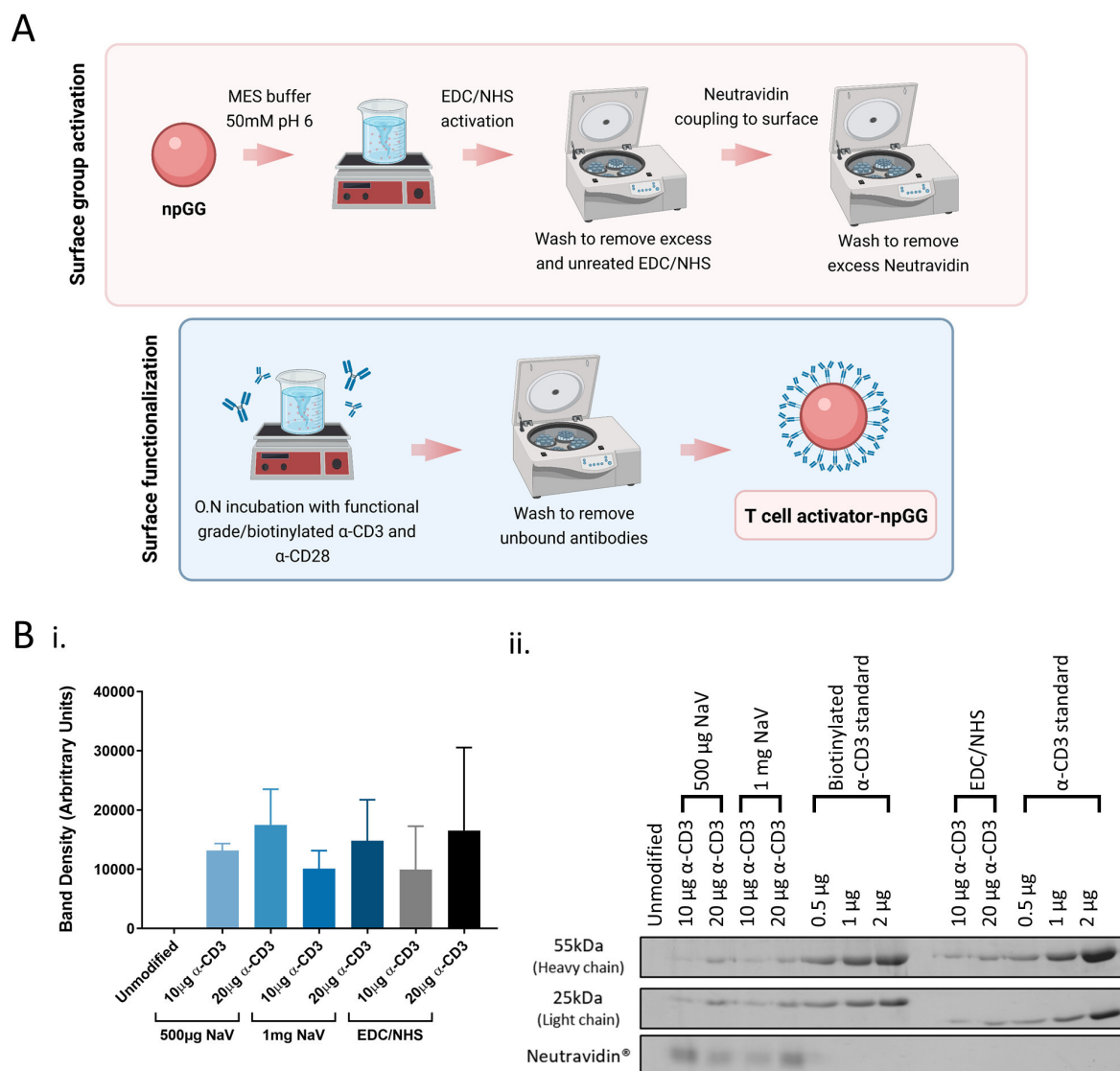


Figure IV-3 Bio-functionalization of npGG.

A Schematic illustration of the fabrication of npGG aAPCS modified with either functional grade α -CD3 and α -CD28 or biotinylated α -CD3 and α -CD28. **B i.** Quantification by densitometric analysis of the amount of both functional grade or biotinylated antibodies bound to the surface of GG particles by NeutrAvidin (NaV) or by EDC/NHS. **ii.** Surface functionalization of GG particles with α -CD3 antibody and biotinylated α -CD3 were measured by SDS-PAGE.

IV-3.4. Surface interaction between CD4⁺ T cells and activator-npGG

From a biological point of view, the key characteristic of an aAPC is its capability to specifically stimulate T cells of interest to avoid off-target inflammation and autoimmunity events. Taking this into account, the ability of the developed activator-npGG to interact with the surface of CD4⁺ T cells and bind to the CD3 receptor was evaluated (**Figure IV-4A**). By labelling both CD4⁺ and or activator-npGG, it was possible to check for double positive cells indicating particles bound to the desired T cells.

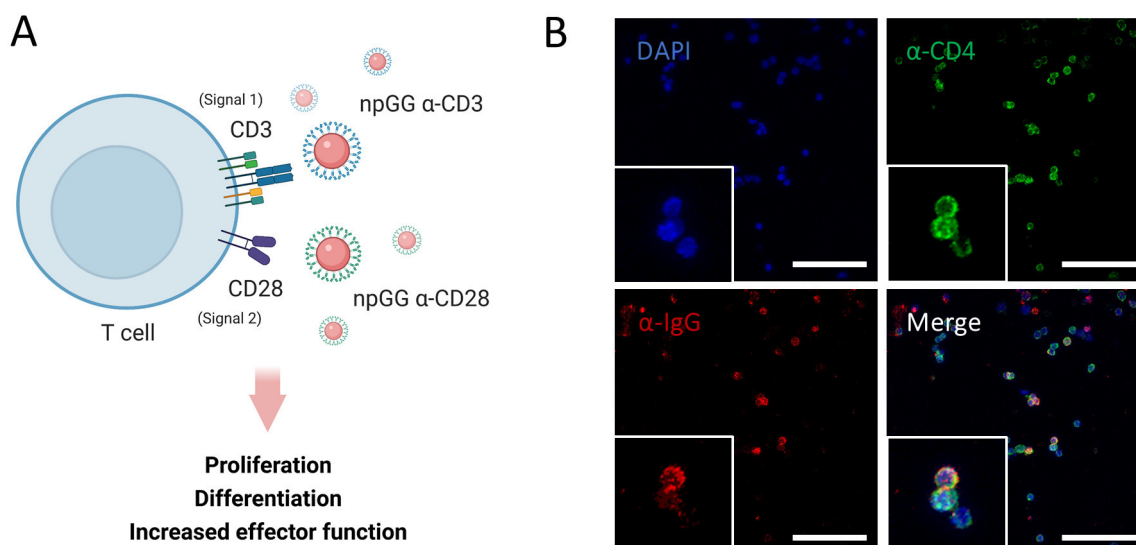


Figure IV-4 Characterization of the interaction of npGG aAPCs with murine T cells.

A npGG aAPCs interaction and T cell interaction schematics. **B** Confocal images of 100 μg of npGG- α -CD3 surface-bound to CD4⁺ T cells after 3 days of culture. Green fluorescence shows CD4⁺ T cells, while red represents npGG surface bound α -CD3, scale bar = 50 μm .

Quantitative analysis of the binding of activator-npGG to the surface of CD4⁺ regulatory T cells was performed by flow cytometry over 2 distinct time-points, 1 and 5 days of incubation (**Table IV-1**). After 24 h, a dose-dependent binding of particles to cells was verified, with increasing particle concentration leading to increased binding. A significant amount of particle-CD4⁺ T cell binding was detected in doses of both 50 μg and 100 μg when modified with 20 μg of antibody. Additionally, activator-npGG prepared by direct linkage of antibodies to the particle surface resulted in an overall lower significance ($p < 0.01$) in the number of cell bound particles when compared to the NeutrAvidin-biotin system ($p < 0.001$). Upon looking at cell interaction at day 5, a similar trend to that of the day 1 time-point was observed in which a significantly higher binding of npGG- α -CD3 to CD4⁺ T cells was observed when higher doses of the 20 μg α -CD3 were used in the NeutrAvidin-based particles.

When correlating the quantitative data with that of immunolabelling (**Figure IV-4B**) it was possible to verify a homogenous distribution of the NeutrAvidin based activator-npGG throughout the surface of CD4⁺ T cells when 100 μg of the 20 μg α -CD3 formulation was used.

Table IV-1 Surface interaction between npGG aAPCs and murine CD4⁺ T cells.

Experimental conditions	μg	%	
		Day 1	Day 5
npGG	1	1.68 (0.36)	1.1 (1.13)
	10	2.26 (0.71)	1.63 (1.39)
	50	2.4 (1.09)	1.65 (0.96)
	100	1.8 (0.53)	1.9 (1.50)
npGG 10 μg $\alpha\text{-CD3}$	1	3.71 (2.09)	8.4 (10.03)
	10	10.65 (9.88)	8.57 (6.53)
	50	22.82 (16.67)	19.53 (18.05)
	100	30.79 (2.09)	19.53 (18.05)
npGG-NaV 10 μg $\alpha\text{-CD3}$	1	3.68 (2.16)	4.98 (3.52)
	10	12.3 (9.21)	11.57 (7.86)
	50	37.57 (33.04)	19.8 (15.73)
	100	39.21 (31.75)	33.34 (23.23)
npGG 20 μg $\alpha\text{-CD3}$	1	6.65 (1.91)	6.8 (5.24)
	10	22.83 (8.35)	17.23 (4.46)
	50	38.48 (15.79)	40.4 (30.74)
	100	70.6 (0.60) **	44.1 (18.29)
npGG-NaV 20 μg $\alpha\text{-CD3}$	1	9.7 (1.67)	8.55 (3.61)
	10	35.5 (6.79)	28.13 (5.54)
	50	51.45 (17.75) ***	53.65 (8.33) **
	100	61.53 (8.04) ***	61.1 (8.08) ***

Flow cytometry data of murine splenocytes incubated with varying concentrations of npGG aAPCs over 1 and 5 days in the percentage of CD4⁺ T cells positive for nanoparticles. CD4⁺ T cells were labeled with AF647 while npGG- $\alpha\text{-CD3}$ were labelled with AF488 anti-rat IgG. Double positive labeling was translated in the % of CD4⁺ T cells with surface bound npGG- $\alpha\text{-CD3}$. Quantitative results are expressed as the mean $\pm$ standard deviation where $n = 3$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA with Kruskal-Wallis multiple comparison post-test.

IV-3.5. Activator-npGG induced a dose dependent priming of CD4⁺ T cells

Upon verification that the developed activator-npGG could efficiently bind to the surface of CD4⁺ regulatory T cells, it was hypothesized that by co-stimulating murine splenocytes with both npGG- α CD3 and npGG- α CD28 particles it would be possible to efficiently prime T cells to expand. To verify this, freshly isolated BALB/c splenocytes labelled with CFSE were co-stimulated with varying concentrations of both npGG- α CD3 and npGG- α CD28 particles in a 1:1 ratio. CFSE dilution was analyzed by flow cytometry after 7 days to determine the percentage of proliferating cells. Effective stimulation of CD4⁺ T cells only occurred at higher doses of 50 μ g and 100 μ g of both npGG- α CD3 and npGG- α CD28 particles (**Figure IV-5A i**). Stimulation happened regardless of the activator-npGG being produced by direct coupling of the antibodies or through NeutrAvidin, however, statistically significant differences were only observed when NeutrAvidin-Biotin was used to bind the antibodies. Some activation in the control condition consisting of non-functionalized particles was also evident, which may be possibly explained due to the use of a heterogenous population of cells in which host APCs may be presenting particles to T cells. The ELISA results (**Figure IV-5A ii**) demonstrated that IL-2 levels for splenocytes stimulated with npGG- α CD3 and npGG- α CD28 were much higher when treated with doses of both 50 μ g and 100 μ g of NeutrAvidin/antibody-containing particles. Moreover, these particles showed significantly higher levels of released IL-2 when compared to commercial activator beads when used as recommended by the manufacturer.

IV-3.6. Activator-npGG present diminished levels of T cell exhaustion and enhanced cytotoxicity

The potential of the npGG- α CD3 and npGG- α CD28 particles to induce a cytotoxic profile in T cells while avoiding their early exhaustion and senescence was determined using qPCR after 7 days of murine splenocyte stimulation. The mRNA expression levels for *PDCD1*, *CTLA-4*, *PRF1* and *GZMB* were studied. Results showed a significant increase ($p < 0.05$) of *PDCD1* expression (**Figure IV-5B i**) in the unmodified particles group at doses of 50 μ g. However, no significant differences in expression were found when comparing stimulated splenocytes, unstimulated splenocytes and the commercial activator beads. Interestingly, a significant ($p < 0.05$) downregulation of cytotoxic T-lymphocyte antigen 4 (*CTLA4*) expression was observed in the NeutrAvidin-Biotin activator-npGG system when bound to 20 μ g of antibody when comparing with both unstimulated cells and the commercial activator beads group (**Figure IV-5B i**) suggesting decreased repression in T cell function and therefore a more sustained immune

response upon activation with these particles. Concerning whether there was a switch to a cytotoxic T cell profile, a significant ($p < 0.01$) increase in the expression of *PRF1* (**Figure IV-5B ii**) was seen in a dose dependent manner when 20 μg of antibody was conjugated to the developed system via EDC but not via NeutrAvidin-Biotin. On the other hand, mRNA levels of *GZMB* (Figure 5Bii) were significantly increased for both activator-npGG systems when stimulating with combined doses of 50 μg and 100 μg of both npGG- αCD3 and npGG- αCD28 when compared to unstimulated cells indicative of an increased cytotoxic profile of these cells.

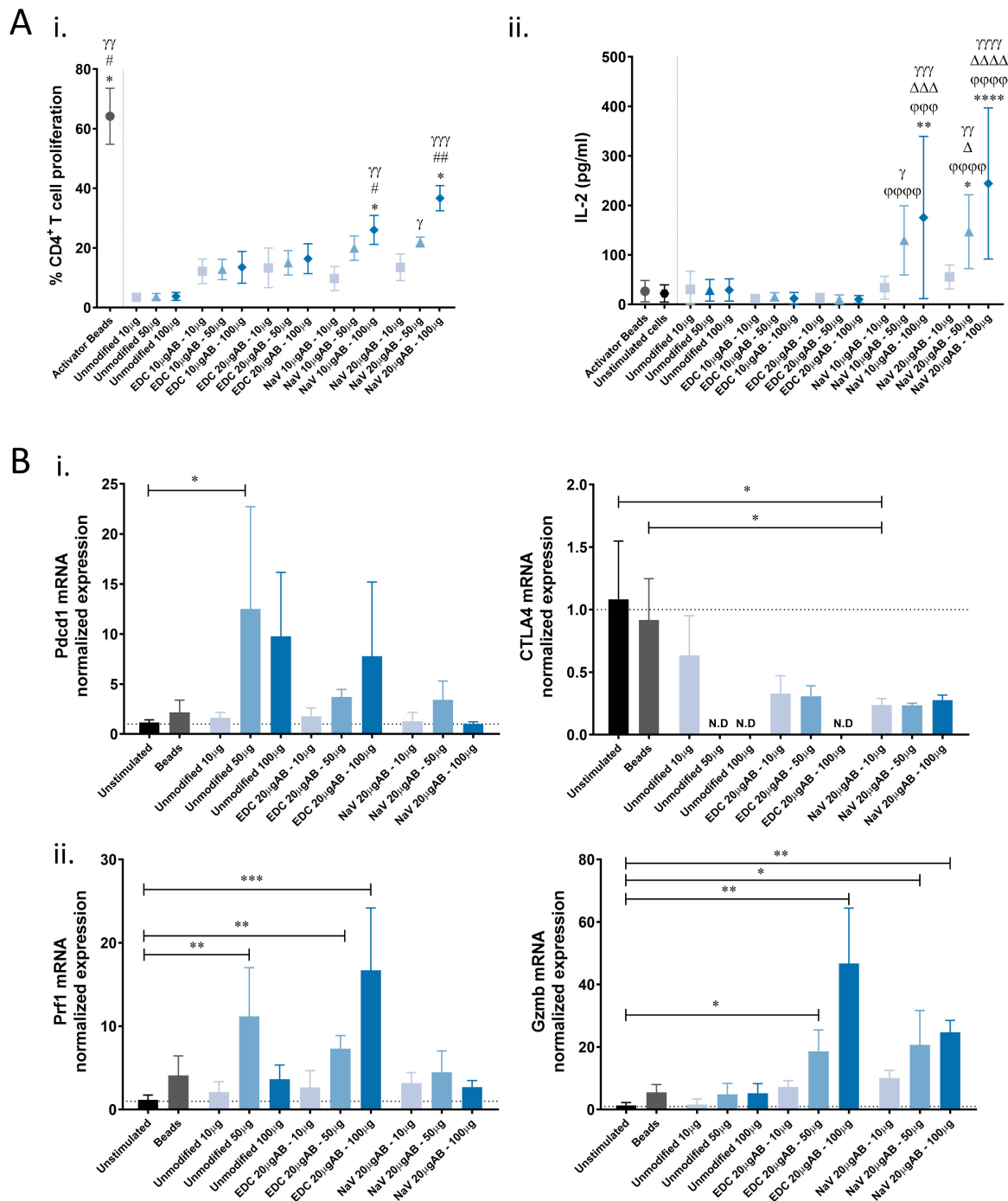


Figure IV-5 α -CD3/ α -CD28 npGG can stimulate T cells activation.

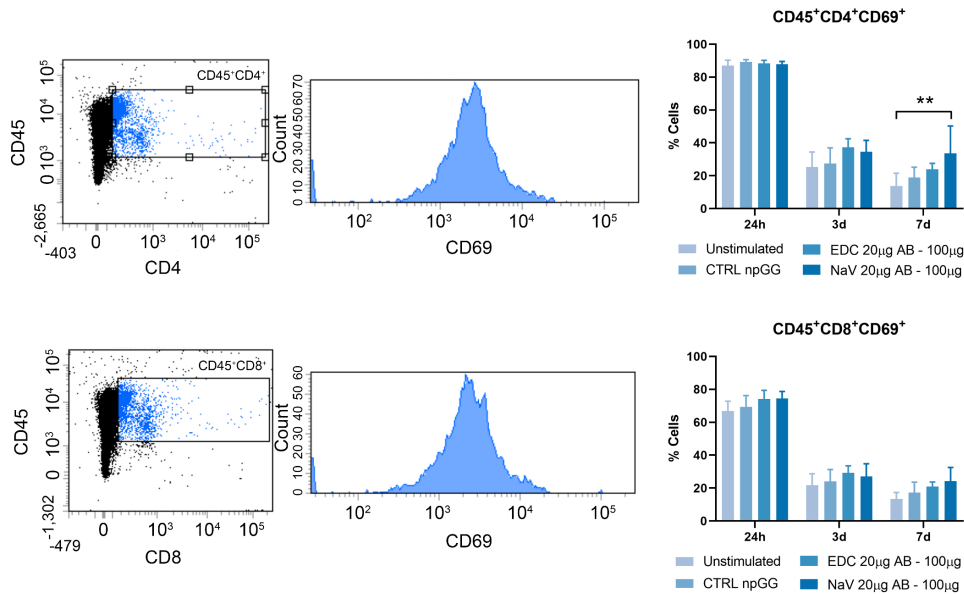
A Activation profile of murine splenocytes when stimulated with varying concentrations of npGG aAPCs. i. Measurement of CD4⁺ T cell proliferation *in vivo* by CFSE dilution. Murine splenocytes were labelled CFSE and then stimulated with both α -CD3 and α -CD28 npGG in a ratio of 1:1 for a period of 7 days. After this period cells were labelled and gated for anti-CD4 and analyzed on a BD FACS Aria™ III. Results are presented as the percentage of cells in the final population that have divided. Data are presented as means $\pm$ standard error. *, #, γ $p < 0.05$; **, ##, $\gamma\gamma$ $p < 0.01$; ***, ###, $\gamma\gamma\gamma$ $p < 0.001$ relatively to unmodified particles at 10 μ g, 50 μ g or 100 μ g respectively. ii. IL-2 production by murine splenocytes when stimulated over 7 days with both anti-CD3 and anti-CD28 GG particles in a ratio of 1:1. Control (CTRL) corresponds to the conditions in which unmodified particles were used to stimulate the cell cultures. The IL-2 contents of the control media was undetectable. Histograms show proliferation CD4⁺ T cells by CFSE-dye dilution. Data are presented as means $\pm$ standard error. *, ϕ , Δ , γ $p < 0.05$; **, $\phi\phi$, $\Delta\Delta$, $\gamma\gamma$ $p < 0.01$; ***, $\phi\phi\phi$, $\Delta\Delta\Delta$, $\gamma\gamma\gamma$ $p < 0.001$; ****, $\phi\phi\phi\phi$, $\Delta\Delta\Delta\Delta$, $\gamma\gamma\gamma\gamma$ $p < 0.0001$. * was used when pairwise comparisons are performed relative to unmodified particles at 10 μ g, 50 μ g or 100 μ g respectively. ϕ was used when comparisons are performed relative to activator beads, Δ relative to unstimulated cells and γ relatively to EDC at comparative 10 μ g or 20 μ g of antibody at 10 μ g, 50 μ g or 100 μ g respectively. **B** qPCR mRNA fold change of (i) immune checkpoint genes *PDCD1* and *CTLA4* (ii) cytotoxic genes *PRF1* and *GZMB* in murine splenocytes stimulated with activator-npGG over 7 days. Quantitative results are expressed as the mean $\pm$ standard deviation where $n = 3$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. One-way ANOVA with Kruskal-Wallis multiple comparison post-test were used.

IV-3.7. Activator-npGG favor T cell activation and helper and cytotoxic subsets

To explore the degree of T cell activation resulting from the stimulation of murine splenocytes with the activator-npGG functionalized with either EDC/NHS or NeutrAvidin/Biotin, flow-cytometric assessment was performed for the following surface markers: CD69, which is an early T cell activation marker; CD4⁺, which is a T helper cell marker (CD4⁺ T cells); CD8, which is a cytotoxic T cell marker (CD8⁺ T cells); and CD45, which is pan-leukocyte marker. Intracellular cytokine expression levels Perforin and GranzymeB (GrzB) were also examined. The percentage of CD69⁺ cells among CD4⁺ and CD8⁺ T cells were comparable at 24 h of stimulation (**Figure IV-6A**). Interestingly, a differential production of Perforin and GrzB was observed (**Figure IV-6B**). Perforin and granzymes work synergistically to induce apoptosis in target cells recognized by cytotoxic T lymphocytes. A significant increase in the expression of intracellular perforin in cells stimulated with NeutrAvidin functionalized activator np-GG was observed, while no significant differences were observed for GrzB at 24 h of stimulation. At 3 days of stimulation, despite CD69 being an early activation marker, a trend towards a greater activation of CD4⁺ and CD8⁺ T cells in both modified particles versus the control was observed (Figure 6A). Intracellular levels of GrzB showed to be increased when T cells were stimulated with activator-npGG produced via EDC/NHS, when compared to the remaining conditions (**Figure IV-6B**). After 7 days of stimulation, changes among the CD69⁺ population of CD4⁺ and CD8⁺ T cells were clear (**Figure IV-6A**). A significant increase ($p < 0.01$) in activation was evident in the NeutrAvidin functionalized activator-npGG when compared to the remaining conditions.

Regarding the expression of GrzB and perforin (**Figure IV-6B**) no statistically significant changes were verified at this timepoint although a trend similar to the one verified for the 3 day time point was observed.

A



B

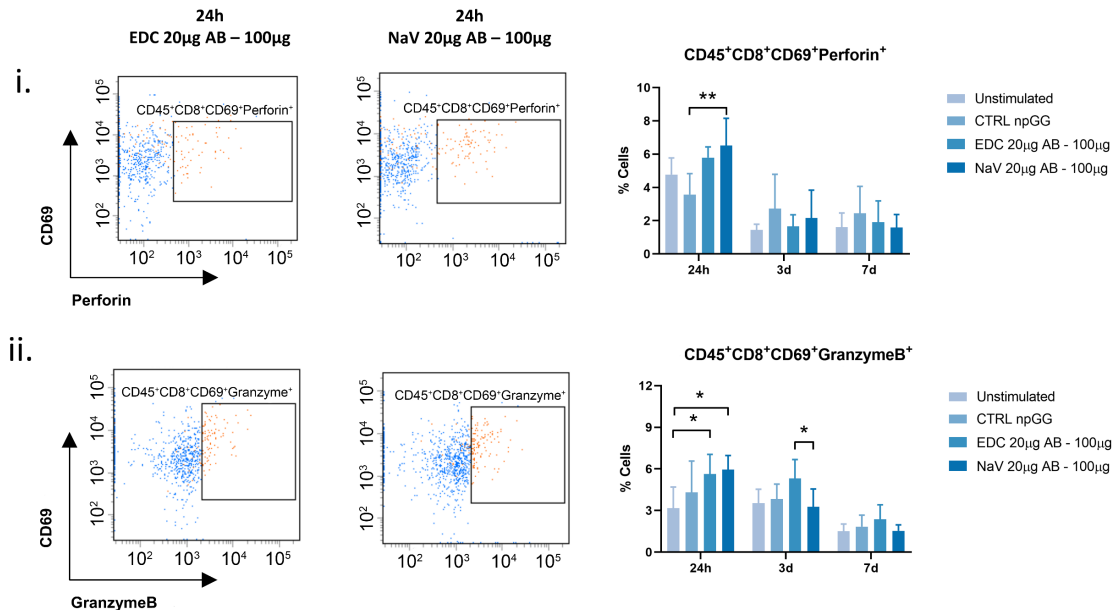


Figure IV-6 Cytotoxic T cell responses of murine splenocytes stimulated with npGG aAPCs.

A T cell activation profile of freshly isolated splenocytes stimulated with activator-npGG over 24h, 3 d and 7 d. Activated cells were gated as CD45⁺CD4⁺CD69⁺ and CD45⁺CD8⁺CD69⁺ T cells on a BD FACSARIA™ III. Summarized data is shown as bar graphs. **B** Flow cytometric plots of intracellular staining and frequencies of expression of Perforin (i) and Granzyme B (ii) in CD45⁺CD8⁺CD69⁺ T cells are shown. Quantitative results are expressed as the mean $\pm$ standard deviation where $n = 3$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, two-way ANOVA with Tukey multiple comparison post-test.

IV-4. DISCUSSION

Immunotherapy has recently been in the spotlight for the treatment of various pathologies varying from cancer to auto-immune diseases. Over the years, several different systems ranging from nanoparticles, nanogels, inorganic nanoparticles, cell derived nanoparticles to microparticles, microcapsules, as well as both living and artificial cells have been applied to develop novel immunotherapeutics. All of these possess unique characteristics with proven efficacies in several fields. A fine tailoring of these systems in order to attain specific interactions with host cells is required in order to achieve an effective and persistent T cell priming without over-activation. An imbalance within these stimulatory mechanisms regularly leads to responses of an immunosuppressive nature [8,9].

The present study demonstrates the potential of the natural origin polymer GG as a source for the development of nanogels as fully functional T cell activator systems capable of i) generating large numbers of CD4⁺ regulatory T cells, ii) preventing T cell exhaustion when compared to other commercial artificial systems, iii) being highly tailorable to carry any desired molecule of choice and iv) present the same high capacity of water uptake of previously reported spongy-like hydrogels which might be of interest for downstream uptake of biologically relevant molecules for controlled release. Furthermore, nanogels have been shown before to present improved circulation, biodistribution and adjuvancy [43]. Other nanoparticle-based systems have also been described for immunomodulation and for immunotherapeutic approaches. Some of these systems, recently reviewed by Park et al. [44] and Shin et al. [45], have been used for antigen and adjuvant delivery as well as for the modulation of the immune-suppressive tumor microenvironment. The efficacy of nanoparticles in efficiently carrying out these functions is mainly dependent on nanoparticles properties such as particle size, surface charge, shape, and hydrophobicity [44]. The developed npGG particles within this work presented a size of approximately 140 nm and a round morphology which has been shown to condition nanoparticles to ECM entrapment and restrict their circulation to lymph nodes [46], particularly suiting this current application since tumor-specific CD8 T cells are activated in the tumor-draining lymph nodes.

Having developed the above-mentioned core system, understanding its stability was of importance to determine how to store it over time while maintaining a homogenous distribution of the particles in solution. PBS was shown to clearly interact with the particles over time, which may be explained by the ionic exchange between the solvent and the particles, leading to agglomeration and an increase of the crosslinking ratio due to the introduction of new ions in the polymeric chains of the particles [38,47]. Interestingly, when rehydrated in culture medium, notwithstanding its ionic content, a different behavior

of the particle system was verified. Immediately after rehydration, increased instability of the system was observed and confirmed by increased PDI when compared to dH₂O. However, eventually, a decrease in the PDI value was observed which can be explained by the formation of a protein corona around the particles blocking further ionic exchange and therefore stabilizing their behavior. Deionized or slightly ionic solutions seem therefore the best alternatives to both swell the particles prior to use or for storage.

While relatable in terms of application with other acellular nanoparticles systems, the activator-npGG introduce a new variable which is the capability of high water uptake after freeze-drying, characteristic of GG spongy-like hydrogels [36]. This is an interesting feature as it may be used downstream for the uptake of biologically relevant molecules and their controlled release *in situ*, such as in the case of IL-1 β which has been shown to favor the expansion of both naïve and memory CD4⁺ T cells when challenged with an antigen [48]. In addition to IL-1 β , other strategies have been proposed, namely the delivery of oxygen to attenuate the hypoxic TME or the delivery of alkaline buffers to control TME pH, or even collagenases and hyaluronidase to allow for tumor ECM penetration of other therapeutic agents [45]. The delivery of adjuvants such as 3-O-desacyl-4'-monophosphoryl lipid A (MPLA), lipopolysaccharide (LPS), CpG oligodeoxynucleotides (ODNs), polyinosinic:polycytidylic acid (poly I:C), and agonists of the stimulator of IFN genes (STING) have also been used to increase immunogenicity and promote the anti-cancer immune responses [44].

To carry out the stimulation of relevant T cell subsets, a stable interaction between the particle system and the targeted cell population must be assured. As stated above, as T cell activation requires a balanced priming of the cells, the assurance that the co-stimulatory molecules are delivered equally is a requirement. First, the double emulsion protocol developed aimed at achieving an average particle size in the nanoscale (50-200 nm range). However, the recommended particle size to induce T cell priming has been widely debated. Sizes can go from the micron scale [49–51] to better mimic APCs true size, down to the nanoscale [52–54] in order to achieve a better distribution of particles to cells. The latter was then chosen, expecting to achieve a better distribution of the activator-npGG over the cell membrane of the targeted T cells due to a greater surface area-to-volume ratio [55]. Then, efficient binding of the biological relevant molecules to the particles must be achieved. One of the most recent methods to be proposed for this purpose is click chemistry as reviewed by Yi et al. for nanoparticle functionalization [56]. This method has produced promising results as seen by the work of Zhang et al. where versatile aAPCs were developed from biomimetic magnetosomes decorated with stimulatory signals by using copper-free click chemistry [57]. Other reports have made use of click chemistry for the modification of nano-gels for the delivery of doxorubicin while trying to overcome multi-drug resistance [58]. In the present work, two

different approaches have been tested to tackle this issue. First, direct coupling of functional grade antibodies, knowing this could result in a random orientation of the antibodies on the surface of the particles resulting from the unspecific binding of the -COOH groups from GG to the -NH of the antibodies. This could in turn lead to the blockade of the light chains of the antibody causing loss of functionality. To overcome this, a second approach using NeutrAvidin as an anchoring system for biotinylated antibodies was employed, as this method of binding would result in effective orientation of the microdomains, preserving their capability of interacting with their specific binding sites (CD3 and CD28 on the surface of T cells). It was found that by using NeutrAvidin, a more stable interaction between the npGG and T cells over time was favored, as shown by flow cytometry, therefore increasing the possibility of immune synapse formation, an essential prerequisite for T cell activation. In alternative to both of the proposed methods, click chemistry could be used by copper-free strategies based on the reaction of a diarylcyclooctyne moiety (DBCO, or ADIBO) with an azide-labeled antibody. This would allow to better control the reacting moieties in a site-specific manner, which is not attainable by EDC-NHS.

To determine the functionality of the npGG, it was explored whether upon interaction the system would be capable of functionally inducing T cells to undergo serial activation, proliferation, and differentiation. Considering fluorescence dilution of CFSE-labeled T cells analyzed by flow cytometry, cell proliferation directly correlated with binding results between T cells and activator-npGG's, in which a higher interaction between particles and CD4⁺ T cells translated into a higher percentage of proliferating T cells. Moreover, a single administration of the developed NeutrAvidin-based activator-npGG triggered increased proliferation of this T cell subset when applied at higher doses of 50 µg and 100 µg, albeit the percentage of proliferating cells being lower than commercial T cell activator microbeads.

T cell function and proliferation are deeply associated to effector molecules such as IL-2. This interleukin is critical for the development, regulation, proliferation and maintenance of regulatory T cells (Tregs) in peripheral tissues [59]. It is implicated in the effector activities of CD8⁺ T cells by inducing the expression of IFN-γ [60], TNF-α, and lymphotoxin α [61], by stimulating expression of the cytolytic effector molecules granzyme B, perforin, and by promoting effective target cell killing [62]. However, when delivered systemically, high doses of IL-2 or other cytokines often lead to high toxicity such as the cytokine release syndrome as well as low efficiency in terms of function [63,64]. Several methods described until now usually tether relevant biomolecules to the surface of the particles resulting in delivery but not steady release of these molecules. Others have reported encapsulation during particle synthesis which many times implies exposing these molecules to aggressive chemical agents used during the processing. The NeutrAvidin-based activator-npGG herein presented, brings the possibility of foregoing the requirement of

IL-2 supplementation for the expansion of T cell subsets as shown by the production of significantly higher levels of IL-2 over the period of 7 days, even when compared to the tested commercial activator micro-beads.

T cells are known to enter into a state of “exhaustion” when chronically exposed to an antigen-rich environment, therefore losing their ability to kill cancer cells or virus infected cells [65]. Exhaustion is a state that affects both long lived central memory and short-lived effector memory T cells, with debilitating consequences to immune functions. Cells undergoing exhaustion develop changes in cytokine synthesis, such as loss of secretion of IL-2, IFN γ and TNF α [66]. T cell exhaustion is also characterized by an upregulation in the expression of inhibitory molecules such as programmed death receptor 1 (PD1), CTLA4, T cell immunoglobulin and mucin domain-containing molecule 3 (TIM3), lymphocyte activation gene 3 (LAG3) or CD244 (2B4) [67]. This phenomenon could explain the lower levels of secreted IL-2 for the commercial activator system despite the higher levels of proliferation registered. Hence, the expression of those inhibitory molecules was analyzed in cells stimulated with both the commercial and the developed activator systems. It was possible to demonstrate that the npGG system coupled to α -CD3 and α -CD28 antibodies was not only capable of successfully inducing T cell activation but also triggered a low expression of T cell exhaustion markers CTLA-4 and PD-1. This suggests that the developed system may stimulate cells to be active for a longer period of time when compared to commercial activator beads which triggered a high expression of CTLA-4 in cells after a period of 7 days of stimulation. While maintaining a low degree of T cell exhaustion is crucial for applications such as cancer therapy, the induction of a cytotoxic phenotype is essential. In tumor surveillance, the killing of tumor cells has been associated to the release of a variety of cytolytic enzymes [68]. A subpopulation of CD4⁺ T cells that harbors cytolytic activity through perforin/granzyme and Fas-FasL pathways has been reported [69]. Granzymes A and B are among the most abundant cytolytic enzymes, capable of inducing target cell apoptosis through caspase-dependent and -independent pathways [70]. However, for these cytolytic enzymes to be effective they must be released together with pore-forming protein perforin to form cytotoxic granules. Upon T cell recognition, the release of cytotoxic granules in the immunological synapse induces apoptosis of the target cell. These findings show that the developed activator-npGG system triggered increased mRNA expression of these factors when compared to both unstimulated cells and commercial activator beads. This may be advantageous in terms of clinical application, as cells expanded *in vitro* for reinfusion will present a lower susceptibility to early exhaustion and, while capable of producing increased levels of IL-2, they will also present an increased granule-mediated cytotoxicity.

Taking into consideration the aforementioned results in terms of T cell proliferation, the differences observed between the different antibody tethering strategies were further explored. While the direct effects of free Streptavidin/Biotin in immune cells have been studied [71,72], the differences that these conjugation methods may have on a final aAPC system have never been considered in the context of helper and cytotoxic T cell activation. Given the differential expression of Prf1 and GrzB observed in the different experimental conditions, it was hypothesized whether there was a specific effect on the fate of the developed immune response in consequence of the different antibody tethering strategy used. Hence, the activation of both CD4⁺ helper or CD8⁺ cytotoxic T cells and consequently their production of perforin and GrzB for both tethering strategies was evaluated by flow cytometry. Although CD69 is considered as an early activation marker in T cells [73], within 24 h of stimulation no detectable differences were observed among the conditions analyzed in within this work, with high percentages of CD69⁺ cells for all conditions. This result comes into agreement with prior reports which show an up-regulation of CD69 in freshly isolated CD4⁺ and CD8⁺ peripheral blood lymphocytes [74]. This may be associated to a hyperresponsive state of the cells due to the isolation procedure. At day 7 of stimulation, while not statistically significant, a clear trend where particles functionalized via NeutrAvidin conjugation presented the highest levels of activated CD4⁺ and CD8⁺ T cells was observed. The fact that activation was detected at such a late time point could be explained by the fact that the splenocytes undergoing activation with the current system exhibited a more prolonged kinetics of induction, confirmed by a high percentage of proliferating CD4⁺ T cells at this time point, as well as by decreased levels of the T cell exhaustion marker CTLA4. This comes into agreement with previous reports where it was shown that CTLA-4 controls the threshold of productive TCR signaling [75]. Interestingly, the production of intracellular cytokines was noticeably higher within the first two time points of 24 h and 3 days. Real-time PCR results show that promoting the coupling of the antibodies via EDC/NHS chemistry led to higher levels of expression of the *GZMB* genes which comes into agreement with what was observed via flow cytometry. On the other hand, while these results show a higher gene expression of *PRF1* resultant from the stimulation with the EDC/NHS activator-npGG system, intracellular perforin is observed in higher amounts in NeutrAvidin as early as 24 h after stimulation suggesting that the latter system may be more effective at inducing a cytotoxic T cell phenotype while capable of equally inducing cell proliferation.

IV-5. CONCLUSION

In summary, a novel type of functional T cell activator system was successfully fabricated based on a nanoscale type of hydrogel. Considering the initial design, the surface of npGG was engineered with either α -CD3 or α -CD28 antibodies and used simultaneously to induce co-stimulation of T cells. While retaining major features of hydrogels such as bio-friendly environment and high water uptake, which could be used for small molecule controlled release, the created npGG displayed highly tailorable properties for its use as a platform for the production of aAPC systems for immunotherapy strategies.

When stimulating naïve murine splenocytes, the activator-npGG system showed favorable CD4⁺ T cell activation function, triggering significantly enhanced T cell proliferation and increased secretion of cytotoxic factors, while evading T cell exhaustion.

All these results indicate that this activator-npGG system is a promising platform for T cell activation and re-education applications such as the expansion of aspecific complete T cell populations of cancer patients.

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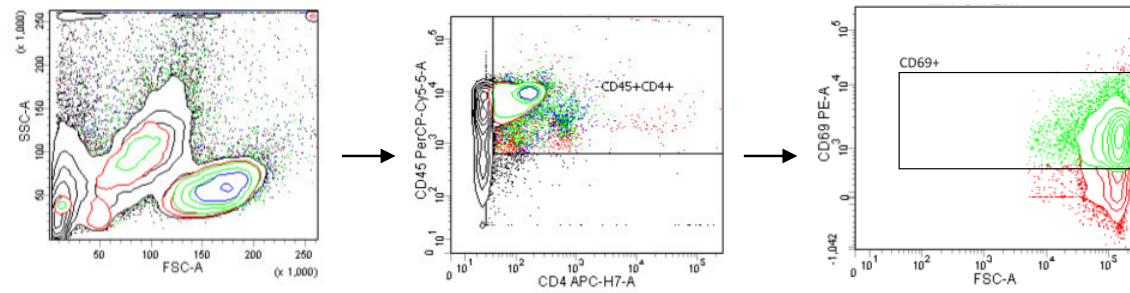
IV-7. SUPPLEMENTARY INFORMATION**Supplementary Table IV-1 Primary and secondary antibodies used for immunolabelling and particle functionalization.**

		Cat#	Host	Target
<i>In vivo</i> Ready Anti-Mouse CD3 (17A2)	Tonbo Biosciences™	40-0032-U500	Rat	Mouse
Anti-Mouse CD3 Monoclonal Antibody (BIOT conjugated) clone 17A2; Rat IgG2b, kappa	MyBioSource	MBS2534030	Rat	Mouse
<i>In vivo</i> Ready Anti-Mouse CD28 (37.51)	Tonbo Biosciences™	40-0281-U500	Hamster	Mouse
Hamster Anti-mouse CD28-BIOT, clone:37.51	MyBioSource	MBS673299	Hamster	Mouse
APC anti-mouse Perforin Antibody	Biolegend	154304	Rat	Mouse
PE/Cyanine7 anti-human/mouse Granzyme B Recombinant Antibody	Biolegend	396410	Rat	Mouse/Human
PE anti-mouse CD69 Antibody	Biolegend	104507	Armenian Hamster	Mouse
APC-H7 Rat anti-Mouse CD4	BD Pharmingen™	560181	Rat	Mouse
APC-H7 Rat anti-Mouse CD8a	BD Pharmingen™	560182	Rat	Mouse
PerCP-Cy 5.5 Rat Anti-Mouse CD45 Clone 30-F11 (RUO)	BD Pharmingen™	550994	Rat	Mouse
Alexa Fluor 647 anti-mouse CD4 Antibody	Biolegend	100424	Rat	Mouse
PE Hamster Anti-Mouse CD3e	BD Pharmingen™	553064	Armenian Hamster	Mouse
CD4 Antibody (GK1.5) [FITC]	Novus Biologicals	NBP2-26584	Rat	Mouse
CD4 Antibody (GK1.5) [PE]	Novus Biologicals	NBP2-26586	Rat	Mouse
Alexa Fluor 594 Donkey anti-Rat IgG (H+L)	Thermo Fisher Scientific Inc.	A-21209	Donkey	Rat
CD4 Antibody	Novus Biologicals	NBP1-19371	Rabbit	Mouse

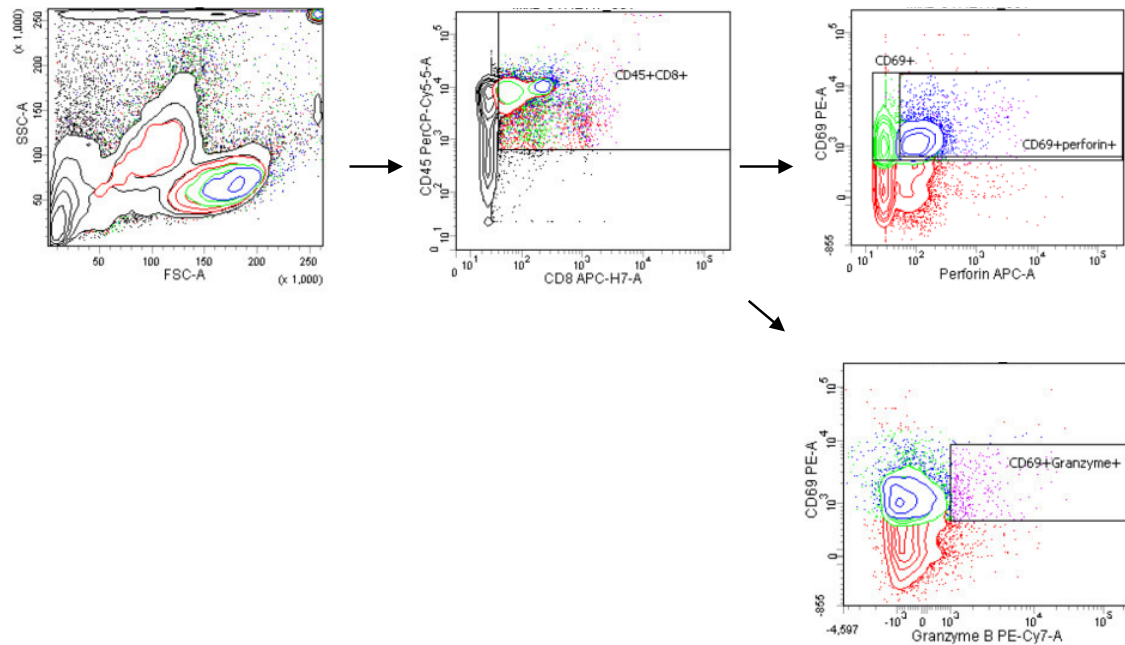
Supplementary Table IV-2 Sequence of primers used for RT-PCR studies.

Gene and accession number	Sequence (5'-3')	Amplicon size (bp)	Annealing temperature (°C)
Mouse ACTB NM_007393.5	GATCAAGATCATTGCTCCTCCTG	183	60
	AGGGTGTAACACGCAGCTCA		
Mouse ACTG1 NM_009609.3	CTTACACTGCGCTTCTTGCC	78	60
	GTGCGGCGATTTCTTCTTCC		
Mouse PDCD1 NM_005018.2	AACCCTGGTGGTTGGTGTC	107	55.5
	TGGCTCCTATTGTCCCTCGT		
Mouse CTLA4 NM_005214.4	CTACCTGGGCATAGGCAACG	103	57.7
	CCCCGAACTAACTGCTGCAA		
Mouse PRF1 NM_011073.3	GAGCATCCCTGACTTTCCTT	84	56.4
	ATGTTTACGCTTCGTGGCAG		
Mouse GZMB NM_013542.3	GAAGCCAGGAGATGTGTGCT	86	56.4
	GCTCAACCTCTTGTAGCGTG		

S1 i

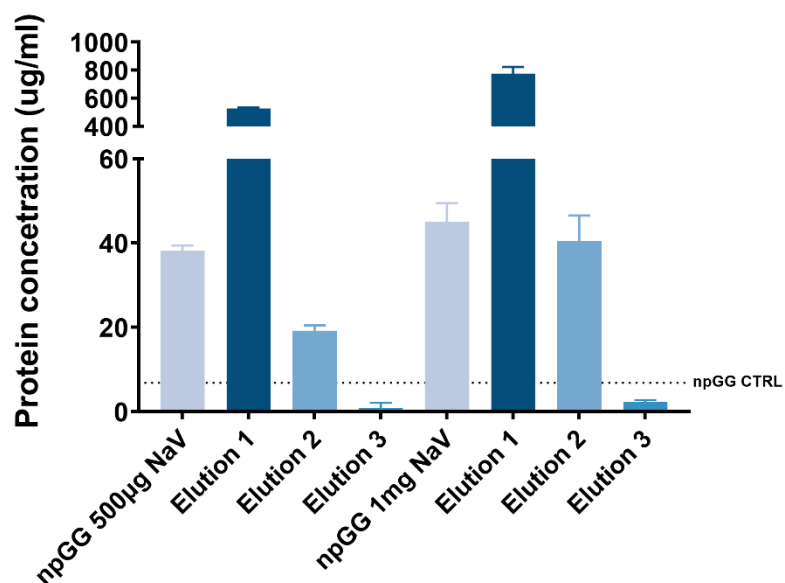


ii



Supplementary Figure IV-1 Gating strategies for CD4⁺/CD8⁺ T cell panels in murine splenocytes.

Stimulated splenocytes were stained for T cell markers CD45, CD4, CD8; activation marker CD69, and cytotoxic T cell effectors Perforin and Granzyme B. Helper T cells were identified as CD45⁺ vs CD4⁺ while Cytotoxic T cells were identified as CD45⁺ vs CD8⁺. i) From the identified CD4⁺ T cells, CD69⁺ cells were gated to determine activated CD4⁺ helper T cells. ii) From the selected CD8 T cells, dot plots for CD69 vs Perforin and CD69 vs Granzyme B were performed to determine activated cells expressing effector cytotoxic molecules.

**Supplementary Figure IV-2 Surface modification of npGG with NeutrAvidin.**

NeutrAvidin density on npGG surface after functionalization with either 500 µg or 1 mg of NeutrAvidin determined by micro bicinchoninic acid assay. Elution's obtained from each reaction were also assessed to determine unbound NeutrAvidin. Quantitative results are expressed as the mean $\pm$ standard deviation where $n = 3$.

CHAPTER V

GENERATION OF NOVEL 3D MELANOMA MODELS USING AN ASSEMBLOID-BASED APPROACH

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ABSTRACT

While 3D tumor models have greatly evolved over the past years, there is still a strong requirement for more biosimilar models which are capable of recapitulating cellular crosstalk within the tumor microenvironment while equally displaying representative levels of tumor aggressiveness and invasion. Herein, we disclose an assembloid melanoma model based on the fusion of individual stromal multicellular spheroids (MCSs). In contrast to more traditional tumor models, we show that it is possible to develop self-organizing, heterotypic melanoma models where tumor cells present stem-cell like features like up-regulated pluripotency master regulators SOX2, POU5F1 and NANOG. Additionally, these assembloids display high levels of invasiveness while embedded in 3D matrices as evidenced by stromal cell promotion of melanoma cell invasion via metalloproteinase production. Furthermore, sensitivity to anticancer drug doxorubicin was demonstrated for the melanoma assembloid model. These findings suggest that melanoma assembloids may play a significant role in the field of 3D cancer models as they more closely mimic the tumor microenvironment when compared to more traditional MCSs, opening the doors to a better understanding of the role of tumor microenvironment in supporting tumor progression.

§ This chapter is based on the following publication:

Rodrigues DB, Moreira HR, Jarnalo M, Horta R, Marques AP, Reis RL, Pirraco RP. Generation of novel 3D melanoma models using an assembloid-based approach. *Submitted* (2023)

V-1. INTRODUCTION

Skin melanoma is a malignant neoplasm that arises from the uncontrolled proliferation of melanocytes. While only accounting for about 1 % of all skin cancer cases, it is responsible for the vast majority of skin cancer deaths making it the most aggressive form of this cancer [1]. In 2022, in the US alone, 99,780 new cases of melanoma are expected to be diagnosed followed by 7,650 deaths [2].

Three-dimensional (3D) tumor models such as multicellular spheroids (MCSs), organoids and organotypic melanoma skin models [3–5] are increasingly considered as key tools in the path towards novel cancer therapies [6]. MCS, in particular, became popular in the field [7–10] due to the ease in obtaining heterotypic 3D systems that share similar traits to the native tumor and tumor microenvironment. MCSs consist in cell aggregates that exhibit complex cell-cell and cell-matrix interactions, resulting in the formation of a gradient for nutrients, gases, growth factors and signaling factors. Morphologically, they present a variable 3D architecture which is highly dependent on factors such as cell type, cell density, culture method and mechanical stress [11].

Despite this availability of 3D tumor models, several issues still remain. One must consider that melanoma progression is driven by the reciprocal interactions among the different cell types that make up the tumor microenvironment in addition to malignant cells themselves [12–14]. This accounts for paracrine interactions orchestrated among fibroblasts, endothelial cells, immune cell and other neighboring cells that contribute to the complexity and heterogeneity of this cancer, complicating the precise recapitulation of tumor features within the current models. These have critical limitations such as the lack of a vascular compartment and immune cells, the inability to perfectly recreate tissue-tissue interfaces as well as to control the spatiotemporal distribution of nutrients and waste, ultimately rendering these models unable to mimic the development factors that regulate cell growth and differentiation *in vivo* [15]. Some authors have attempted to overcome some of these issues by partitioning the formation of these 3D systems over time [16,17], by adding specific cells or molecular cues at specific timepoints but with limited success. Therefore, the need for novel complex models capable of thoroughly mimicking cell interactions to better understand tumor development and progression and to facilitate drug development, is clear.

Assembloids are considered a step forward in the development of tissue specific organoids. They were first created with the intent of combining multiple brain regions and/or cell lineages in 3D culture to model interactions among them and allow the study of cell-cell interactions as well as the assembly of

neural circuits. This conceptualization came to life through the development of separate organoids resembling dorsal and ventral forebrain and then fusing them together into a multi-region assembloid [18–21]. Several advantages of this system have been identified, namely the underlying possibility of maturing and tailoring individual organoids before fusion and replicating region-specific interfaces within tissues, and also the longer viability time due to the more native-like representation of living systems [21,22].

Here, we developed different melanoma stromal compartments by using MCSs comprising co-cultures of a human melanoma cell line (VMM15), human dermal endothelial cells (hDMECs), human adipose derived stem cells (hASCs) and human dermal fibroblasts (hDFbs) which were then fused into a melanoma-like assembloid. We dissected the impact of stromal crosstalk in melanoma 3D invasion and the active role of metalloproteinases (MMPs). Most importantly, we established a 3D model that better recapitulates the complexity and heterogeneity of human skin melanoma.

V-2. MATERIALS AND METHODS

V-2.1. Cell lines and culture conditions

hTERT Dermal Microvascular Endothelial Cell, Neonatal (ATCC® CRL-4060™) were obtained from ATCC and were cultured in Vascular Cell Basal medium (ATCC, USA) supplemented with the Microvascular Endothelial Cell Growth Kit – BBE (ATCC, USA) or EGM-2 MV Microvascular Endothelial Cell Growth Medium-2 BulletKit (Lonza, USA). Human dermal fibroblasts (hDFbs) and human adipose derived stem cells (hASCs) were harvested from human skin samples obtained from abdominoplasties after written informed consent and under the protocol Nr 477/2020 approved by the Ethical Committee of Hospital S. João (Porto, Portugal). Both hDFbs and hASCs were cultured in α -MEM, supplemented with 10 % (v/v) fetal bovine serum (FBS, Invitrogen, USA) and. Human cell line VMM15 (ATCC® CRL-3227™), derived from metastatic melanoma lesions, were grown as adherent cultures in RPMI 1640 supplemented with 10 % (v/v) FBS, L-glutamine (2 mmol/L), penicillin (100 units/mL), and 1 % (v/v) antibiotic/antimycotic. All cells were kept in culture at 37 °C in a humidified atmosphere under 5 % CO₂.

V-2.2. Generation and analysis of tumor spheroids

To produce the intended spheroids, several different cell combinations were performed to mimic independent tumor cell niches. Cell ratios and culture conditions were optimized to achieve optimal spheroid formation and growth. Co-cultures of tumor cells and hDFBs were achieved by mixing 5000 tumor cells with 15000 hDFBs which were then seeded into wells of ultra-low attachment (ULA) 96-well round-bottomed plates (Corning, Amsterdam, The Netherlands) and were thereafter cultured in standard culture conditions. Co-culture spheroids of hDMECs were achieved by culturing 10000 endothelial cells with 30000 cells of either hDFBs or hASCs. For assembloid formation, individual spheroids were allowed to stabilize for 3 days before being placed in combinatorial culture for an additional 10 days. Individual co-culture spheroids and assembloids formed without the tumor cell compartment were used as controls. Additionally, multicellular control spheroids comprising all of the cellular constituents in the same densities as used in the tumor assembloids were also established. In this condition a mixture of 5000 tumor cells, with 45000 hDFBs, 20000 endothelial cells and 30000 hASCs were added all at once at day 0 and cultured for a total of 13 days.

V-2.3. ICC and IHC

V-2.3.1. 2D co-culture immunocytochemistry/immunohistochemistry

At each relevant time-point, spheroids were fixed in methanol for 15 min and were transferred into Tissue-Tek Cryomolds (Sakura Finetek, Netherlands) wells containing HistoGel™ (Thermo Scientific, Ireland) for subsequent paraffin embedding. Samples were then sectioned to obtain 4-μm slices. Tissue sections were stained with Haematoxylin & Eosin (Sigma, USA) and Picrosirius Red kit (Bio-Optica, Italy) following routine protocols. For immunohistochemistry, paraffin tissue sections were deparaffinized in xylene, re-hydrated and boiled for 5 min in Tris-EDTA buffer (10 mM Tris Base, 1 mM EDTA solution and, 0.05 % (v/v) Tween 20, pH 9) for antigen retrieval. Afterwards, sections were permeabilized with 0.2 % Triton X-100 and unspecific staining was blocked with 3 % Bovine Serum Albumin (Sigma). For primary antibodies, we used mouse monoclonal antibody to CD31 (Dako, Denmark) at 1:30, rabbit polyclonal antibody to GP100 (Abcam, UK) at 1:300 or rabbit monoclonal antibody to Ki-67 at 1:50 (Abcam, UK) which were incubated overnight at 4 °C. For detection, secondary antibodies were incubated at RT for 1 h at 1:500 dilution. Nuclei were counter-stained with DAPI. Histological staining's were examined under

a Leica DM750 microscope, using LEICA Application Suite 4.13.0 while immunofluorescence labelling was acquired under a Zeiss Axio Imager Z1m with Zen 3.2 (blue edition).

V-2.3.2. 3D whole spheroid immunohistochemistry

To evaluate the cellular participation in the formation of capillary like structures and cell invasion, individual co-cultured spheroids or assembloids were transferred to ultra-low attachment (ULA) 96-well round-bottomed plates (Corning, Amsterdam, The Netherlands) at each respective time-point and were fixed in methanol for 15 min after which they were left to clear in Scale-A2 for 7 d. After this period samples were re-hydrated for 10 min in PBS. Whole spheroids were then permeabilized/blocked for 2 h in 0.1 % (v/v) Triton X-100 /10 % FBS (v/v)/PBS (B-PBT) and then processed by free-floating immunohistochemistry. For double staining, primary antibodies from different species were incubated simultaneously, followed by incubation with secondary antibodies. For primary antibodies, we used mouse monoclonal antibody to CD31 (Dako, Denmark) at 1:30 and rabbit polyclonal antibody to GP100 (Abcam, UK) at 1:300 diluted in B-PBT with 4',6-diamidino-2-phenylindole (DAPI) at a 1:1000 dilution overnight. Spheroids underwent two 2 h washes with 0.2 % (v/v) Triton X-100 in PBS (PBT), followed by one 2 h B-PBT wash and subsequently were incubated with secondary antibodies at 1:200 in B-PBT with DAPI at a 1:1000 dilution overnight. Spheroids underwent two 2 h PBT washes and returned to the clearing solution. Whole spheroid imaging was performed under a Leica TCS SP8 with Leica Application Suite X 3.5.7.

V-2.3.3. Sample clearing

ScaleA2 solution [23] was prepared by mixing stock solutions for a final concentration of 4M urea (Sigma, USA), 0,1 % (w/v) Triton X-100 (Sigma, USA) and 10 % (w/v) glycerol (Honeywell, Germany) according to [23]. The volume was adjusted by diluting the mixed solutions with water.

V-2.4. Real-Time PCR gene expression analysis

Total RNA was extracted from cells using RNeasy Mini Kit (Qiagen, Germany) and quantified using a spectrophotometer NanoDrop N-1000 (Thermo Fischer Scientific, USA). 250 ng of RNA was retrotranscribed in cDNA with the QScript cDNA SuperMix (Quanta Biosciences, USA). The qPCR reactions were all per-formed in triplicate. The predesigned Taqman primers (Thermo Fisher, USA) were:

SOX2 Hs01053049_s1; *POU5F1* Hs00999632_g1; *NANOG* Hs04399610_g1; *SIRT1* Hs01009006_m1; *TINF2* Hs00173291_m1; *TERF2* Hs00194619_m1; *B2M* Hs00187842_m. Data from these Taqman primers was normalized to hDMECs/hASCs MCSs due to the known expression for these markers in hASCs. Forward and reverse human specific primers used are as follows: *TP53BP1* 5'-AAATCCTTCAGCCGTGTCGT-3, 5'-AGTCACTACGACGCAAAGCA-3; *BRCA1* 5'-GTCCCATCTGTCTGGAGTTGA-3, 5'-GGCCCTTTCTTCTGGTTGAGA-3; *B2M* 5'-TGGAGGCTATCCAGCGTACT-3, 5'-CGGATGGATGAAACCCAGACA-3. These genes were normalized to VMM15/hDFbs. The cycle-threshold was automatically calculated using QuantStudio™ Design & Analysis Software v1.5.2 (Applied Biosystems, Life Technologies, UK), and *B2M* was used as an endogenous control. The relative expression levels of mRNAs were calculated using the Livak method by the equation $2^{-\Delta\Delta Ct}$.

V-2.5. Labeling of spheroids with CellTracker™ dyes

Spheroids were individually labeled for live tracking. hDMECs/hDFbs were labelled with Cell Tracker™ Green CMFDA, hDMECs/hASCs with Cell Tracker™ Orange CMRA and VMM15/hDFbs with Cell Tracker™ Blue dye. For this effect a mixture of basal serum free EGM2-MV and 10 µm CellTracker™ solution in dimethyl sulfoxide (DMSO) (VWR, USA) was prepared. To the free-floating spheroid, standard growing medium was removed from the culture plate; cells were rinsed twice with phosphate buffer (PBS, Gibco, USA) and the prepared mixture was added. After 1 h incubation at 37 °C, the spheroids were washed and left in complete culture medium for time-lapse microscopy.

V-2.6. Assembloid response to chemotherapeutic treatment

To determine the cellular cytotoxicity of a known chemotherapeutic compound on both assembloids and MCSs, spheroids were treated with Dox overtime. For this effect, at 10 d of assembloid culture, medium was switched to a solution of 40 µm Dox diluted in culture medium for an additional 4 d culture period. Cells lysates and culture medium samples were recovered to determine LDH activity. CyQUANT LDH Cytotoxicity Assay Kit (Invitrogen™, USA) was used, according to manufactures instructions, to measure the levels of the cytosolic enzyme released into the cell culture medium upon damage to the plasma membrane. Absorbance at 490 nm and 680 nm were measured using a Multi-Detection Microplate Reader (SYNERGY HT, BIO-TEK, USA).

V-2.7. Metalloproteinase production by assembloids

Conditioned media was collected from the cultures at day 4, 7 and 10 after starting the combinatorial culture of the independently formed spheroids and stored at -80 °C (pooling of samples was performed). Protein concentration in the sample was measured using Bradford method, and 20 µg of protein was electrophoretically resolved for each sample in 10 % SDS-PAGE containing 0.1 % (w/v) gelatin (Sigma-Aldrich, USA) as the MMP substrate. Gels were washed twice for 30 min in renaturing buffer to remove SDS, rinsed for 5min in developing buffer, and then incubated for 24 h in developing buffer at 37 °C. MMP activity in the gel was detected as clear bands against a dark blue background. Band intensity was quantified using the Image Studio V.5.2 software. Values were normalized to the control sample in each gel and expressed as relative densitometric units.

V-2.8. TIMP-1 and TIMP-2 production by assembloids

ELISA (Abcam, UK) was used to quantify the levels of both TIMP-1 and TIMP-2 released 10 d after starting the combinatorial culture of the independently formed spheroids. Conditioned media was collected from the cultures at day 10 and used to carry out the procedure according to the manufacturer's instructions. 5 µg of total protein was used to carry out the quantification of TIMP-1 while 20 µg was used for TIMP-2. The absorbance was read at 600 nm in a kinetic manner for TIMP-1 and for TIMP-2 absorbance was read at 450 nm using a Synergy HT (BioTek, Vermont, USA) microplate reader. The quantity of both TIMP-1 and TIMP-2 protein were determined by means of a standard curve. Both proteins within the control media were undetectable during the experiments and therefore were omitted from the figures.

V-2.9. Tumor spheroid-based Matrigel invasion assay

For 10 d cultured assembloids, 50 % of the culture medium volume was removed and replaced with an equal volume of stock Matrigel™ (BD Biosciences, Oxford, UK). The culture plate was immediately centrifuged (300 g 3 min) at 4 °C and stored for matrix-gel solidification (1 hr 37 °C). Over subsequent days (i.e. Day 0 to +7) spheroid growth and invasion was captured by bright-field microscopy (grey-scale images, Axio Observer microscope, AxioCam MR R3 camera 1388x1040 pixel resolution). Analysis settings were adjusted to achieve an optimal image segmentation that also reflects the degree of invasion.

V-2.10. Image analysis by INSIDIA macro

For post-processing image analyses of spheroid area and circularity, the FIJI [24] macro “Analyze Spheroid Cell Invasion In 3D Matrix, RRID:SCR_021204” was used. To determine assembloid and spheroid invasion in Matrigel®, grey-scale images were organized into the required file structure for automatic batch processing. Spheroid segmentation and analysis was performed with the INSIDIA (INvasion Spheroid ImageJ Analysis) [25] macro for FIJI. Considering that spheroid core size has shown to lead to misinterpretations regarding net invasion, the area of invasive edge was normalized on the area of the core.

V-2.11. Statistical analysis

Data are expressed as mean $\pm$ standard deviation of three independent experiments. Shapiro–Wilk test was performed to validate normality of data prior to statistical testing. Statistical analysis was performed using one-way or two-way analysis of variance (ANOVA), followed by either a Tukey’s or Bonferroni’s multiple comparison post hoc test. Significance was set to 0.05 (95 % of confidence interval). Significance levels were analyzed with GraphPad Prism statistical software v8.0 (GraphPad Software, USA) and indicated as p values (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$). All quantitative data refer to 3 independent experiments ($n=3$) with at least 3 replicates in each condition, in each experiment and are presented as mean $\pm$ standard deviation.

V-3. RESULTS

V-3.1. Differential 3D co-culture of endothelial cells impacts vascular network formation

To create a 3D model capable of mimicking the tumor microenvironment to a certain degree of resemblance one must understand the cellular nature of the tumor microenvironment in which cellular interplay is key. Endothelial cells have been shown to provide different cell responses dependent on different stimuli, be it from stromal fibroblasts [26], extracellular matrix [27] or stem cells [28]. Additionally, direct cell-cell contact between melanoma cells and stromal fibroblasts has been shown to drive functional interactions [29], while it has also been shown that adult mesenchymal stem cells impact tumor cell behaviour [30]. With this in consideration, we paired specific stromal cells found in the tumor

microenvironment producing three distinct MCSs which were further fused to achieve an assembloid melanoma model. Each of these subdomains are representative of distinct cellular interactions that occur within a solid tumor i) an endothelial cell/fibroblast domain (hDMECs/hDFbs); ii) an endothelial/stem cell domain (hDMECs/hASCs) and iii) a tumoral/fibroblast (VMM15/hDFbs) domain. These spheroids were characterized in terms of area and circularity. A distinct architecture was seen for each of the developed MCSs (**Figure V-1a ii**), with the hDMECs/hDFbs spheroids comprising in average an approximate area of $2.4 \times 10^5 \mu\text{m}^2$, while the combination of hDMECs/hASCs yielded a significantly greater area of $3.86 \times 10^5 \mu\text{m}^2$. Finally, the co-culture of VMM15/hDFbs yielded an area of approximately $2.85 \times 10^5 \mu\text{m}^2$. Circularity data indicated a high circularity score of approximately 0.8 regardless of the spheroid system considered, where 1 would be indicative of perfect circularity.

The hematoxylin and eosin (H&E) staining (**Figure V-1a i**) of the produced 3D spheroids after a 3-day cultivation period revealed a compact structure regardless of the co-cultured cells. Both conditions where hDMECs were present displayed a preferential alignment with densely arranged clusters of cells towards the center. The tumor MCSs displayed characteristic tumor cell nuclei including nuclear enlargement and increased nuclear-to-cytoplasm ratio. Evidence of some karyorrhexis was found towards the core region of hDMECs co-cultured MCSs. Picrosirius red (PSR) was used to provide insights regarding collagen distribution within the produced MCSs (**Figure V-1a i**). When comparing the 3 developed MCSs, it was evident that the cross-talk between the used cells were creating distinct collagen deposition signatures. The co-culture of hDMECs/hDFbs revealed dense deposition of collagen fibers within the core. Interestingly, near the surface, these spheroids displayed an aligned deposition of fibers surrounding the core. The condition comprising hDMECs/hASCs, while having a similar cellular density than hDMECs/hDFbs, presented a distinct collagen signature, with it being distributed essentially towards the spheroid core. VMM15/hDFbs on the contrary, despite fibroblasts being typically strong collagen-producing cells, presented little collagen deposition in comparison with the other conditions.

Considering the differences in collagen deposition and cellular arrangement among the developed MCSs, we investigated if this would impact the 3D architecture and organization of the hDMECs within each spheroid. Whole-spheroid confocal imaging of CD31 immunolabeled samples showed a distinct reorganization of the hDMECs compartment among both conditions analyzed (**Figure V-1b**). hDMECs/hDFbs MCSs presented dense capillary-like networks at days 7 and 14 of culture, with hDMECs concentrating in the inner core at later timepoints. However, such capillary-like networks were not seen in hDMECs/hASCs MCSs, where clusters of hDMECs were distributed throughout the spheroid.

In sum, the MCSs demonstrated differential deposition of collagen rich extracellular matrix, as well as differential organization of hDMECs with formation of capillary-like structures only in the presence of hDFbs. With this in consideration, these different subdomains were assembled into a larger heterotypic system to understand their interplay and how they assist in tumor progression.

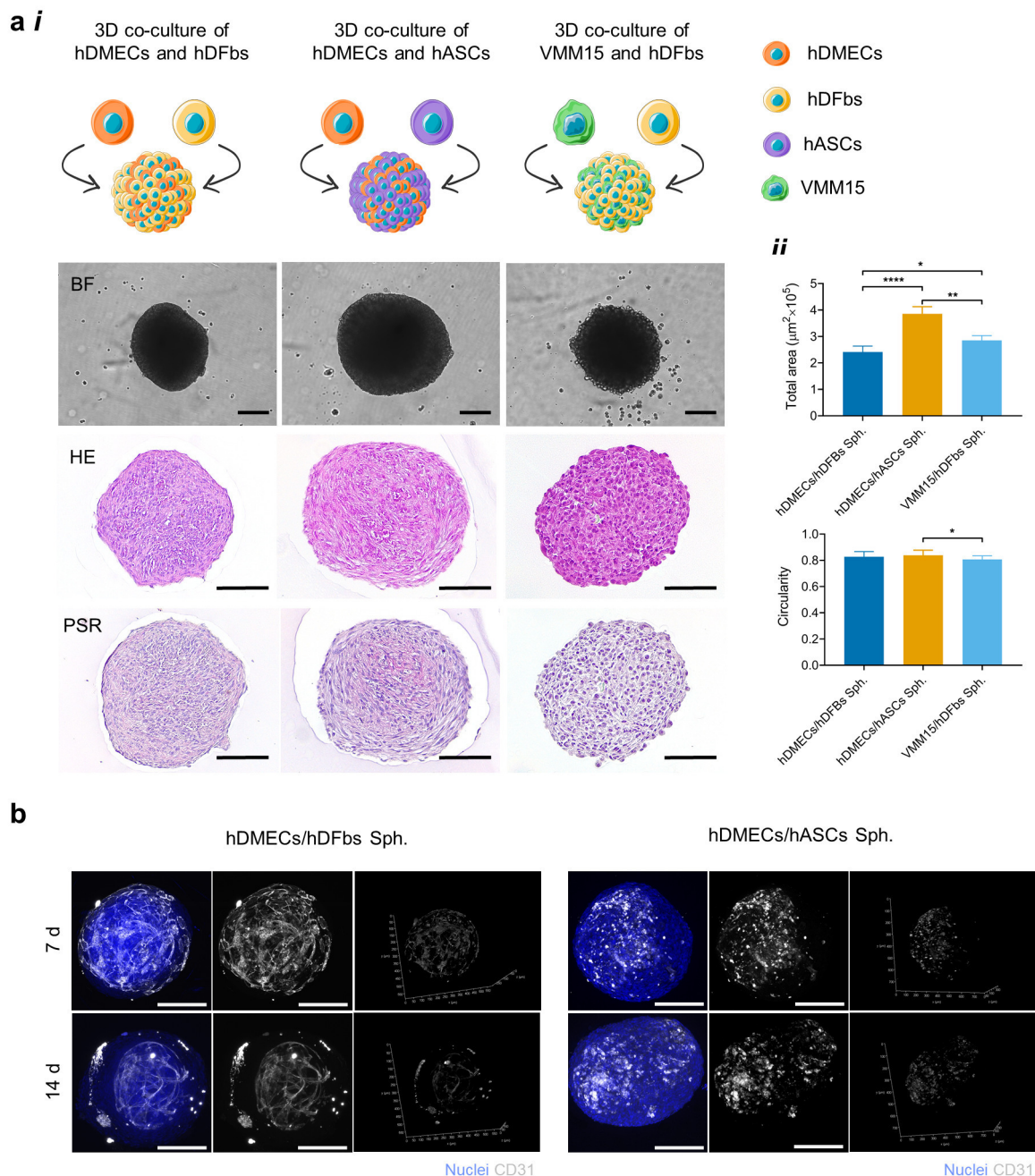


Figure V-1 Production of stromal MCS as building blocks for assembloid formation.

a i) Schematic representation of the production of MCSs. Representative hematoxylin and eosin (H&E) and PSR staining of paraffin-embedded MCSs sections. Scale bar, 200 μm . **ii)** Characterization of total area and circularity score of the described MCSs. **b** Representative immunocytochemistry images of the organization of CD31⁺ endothelial cells in MCSs sections over 7

and 14 days (d) of culture. Nuclei were counterstained with DAPI. Scale bar = 100 μm . Quantitative results are expressed as the mean $\pm$ standard deviation where $n = 3$, * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$, one-way ANOVA with Kruskal-Wallis multiple comparison post-test.

V-3.2. Obtaining complex assembloids based on combinatorial culture of MCSs building blocks

To carry out the development of the tumor assembloid system, we took into consideration the previous results and defined a 3-day priming period for each spheroid niche to self-organize without the influence of any additional cell type. After this period, the 3 MCSs were placed in combinatorial culture to interact over an additional 10-day period. Their fusion was analyzed over 7 days through time-lapse microscopy, after labelling each subset with a different colored cell tracker (**Figure V-2a**). Spheroid fusion was verified to begin as early as 24 h and was concluded around day 7 when the individual subdomains were no longer visible and a singular assembloid structure was obtained. Spheroid fusion appeared to occur in a specific order in which the hDMECs/hASCs fused around the hDMECs/hDFbs compartment and ultimately were surrounded by the VMM15/hDFbs MCS. To evaluate the cellular organization and histoarchitecture of the assembloid, HE and PSR stainings as well as immunolabelling were performed. Two different control conditions were prepared. The first consisted in hDMECs/hASCs spheroids placed in combinatorial culture with hDMECs/hDFbs spheroids (CTRL assembloid), lacking therefore the tumoral compartment of the tumor assembloid. The second control condition consisted in a multicellular spheroid (CTRL MCS) in which all cell types present in the individual spheroids used to obtain the tumor assembloid were added at the same time, at day 0. During culture, it was observed that cells in the CTRL MCS tended to form aggregates outside the main spheroid (Supplementary Figure V-1), driving an irregular distribution of cells over the outer spheroid layer (e. g. bulge formation) and increasing the heterogeneity of replicates, with likely consequences on the reliability of downstream assays.

After a total of 13 days of culture (3 days of pre-conditioning followed by 10 days of combinatorial culture), a clear layered structure was evidenced by HE staining (**Figure V-2b**) in both tumor and control assembloids, with no visible necrosis. Cell clusters arranged towards the core were comprised of CD31⁺ endothelial cells as seen by immunolabelling. Additionally, a vast deposition of collagen, stained in red by PSR, was visible in both conditions but denser in the regions where hDMECs were found to be present. In the melanoma assembloid system, an outer cell layer comprising enlarged nuclei and an increased nuclear-to-cytoplasmic ratio was visible, which overlapped with immunolabeling for the melanoma antigen GP100. These GP100 positive tumor cells distributed themselves in a manner in which they encapsulated the VMM15 assembloid, resembling an outer ring which measured approximately $39.29 \pm 3.34 \mu\text{m}$ in

thickness. When analyzing the distribution of the hDMECs within the melanoma assembloid, structures resembling lumens were found. In the CTRL MSC, an irregular distribution of tumor cells over the surface was observed by HE staining and confirmed by positive staining for GP100. Collagen staining, by PSR, showed decreased deposition of this protein when compared to both assembloid conditions. Cells positive for CD31 were few in this condition and didn't show any organization. When extending the culture period to a total of 28 days (**Figure V-2b**), a very slight increase in collagen deposition was verified for this control condition while a significant loss of GP100-positive tumor cells throughout the surface was visible. For the assembloid conditions, a more extensive reorganization of the collagen fibers in circular patterns was present in both tumor and CTRL assembloids at 28 days. This patterned deposition of collagens was coincident with an organized cellular layering as seen by HE. Lack of nuclei staining at the core is highly suggestive of necrosis. Proliferative cells were then identified in the VMM15 assembloids using Ki-67 immunofluorescence staining procedure (Supplementary Figure V-2). We observed that, at 13 days of total culture time, these assembloids displayed a homogeneous Ki-67 positive cell distribution pattern along the outermost layers, where GP100+ cells were previously shown to locate. After 28 days of total culture time, no Ki-67 positive cells were found.

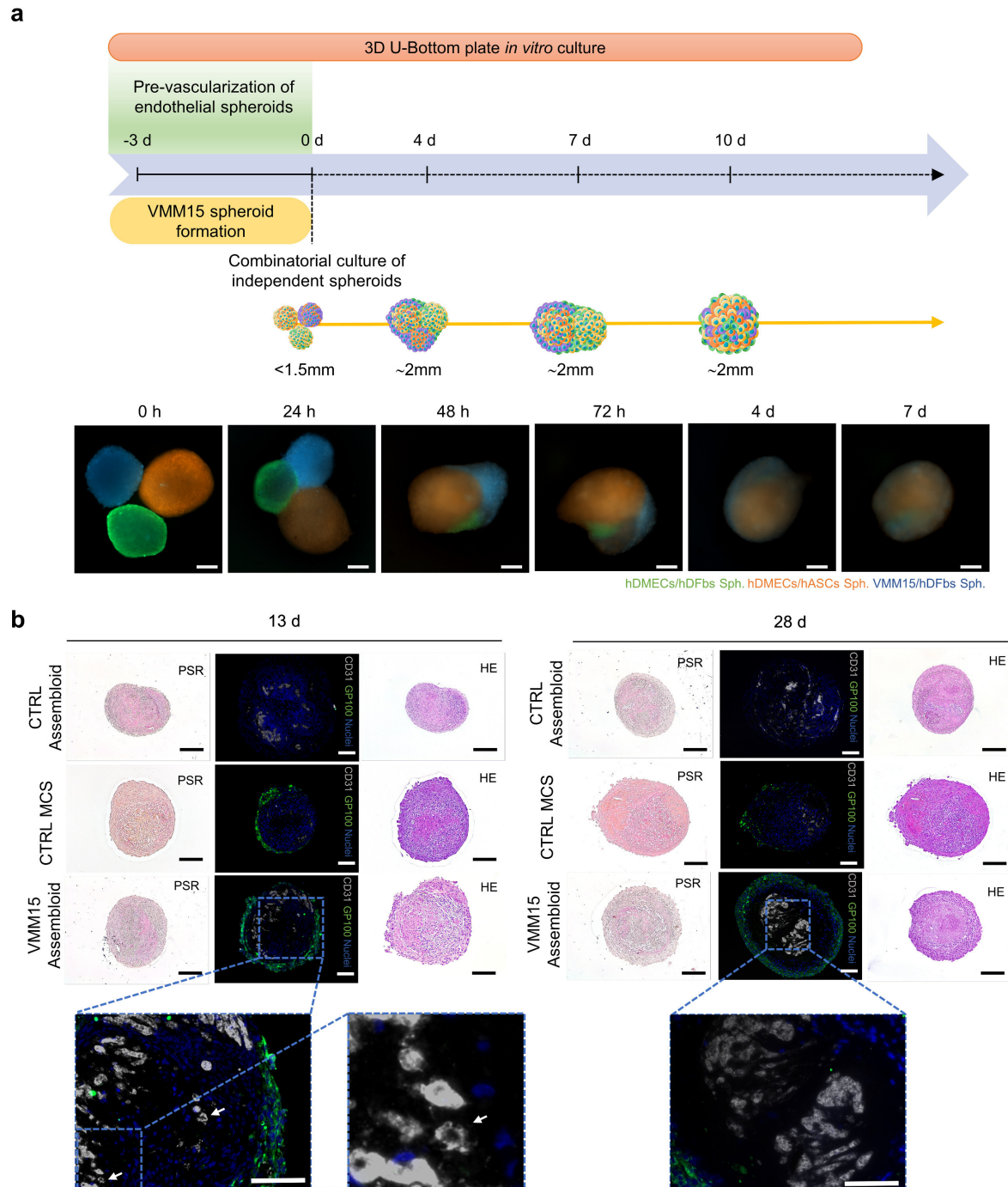


Figure V-2 Assembly of structured MCSs into complex melanoma models.

a Schematic representation of the process of production of the melanoma assembloid model. Time-lapse of MCSs fusion over 7 d. hDMECs/hDFBs were labelled with Cell Tracker™ Green CMFDA, hDMECs/hASCs with Cell Tracker™ Orange CMRA and VMM15/hDFBs with Cell Tracker™ Blue dye. **b** Representative H&E and PSR staining of paraffin-embedded MCSs sections. Scale bar, 200 μ m. Representative immunocytochemistry images of the organization of CD31⁺ and GP100⁺ cells in the developed assembloids and CTRL MCS over 13 and 28 d of culture. Nuclei were counterstained with DAPI. Arrows depict structures resembling lumens. Scale bar = 100 μ m.

Given that the VMM15 cell line is of metastatic origin, the expression of the known core pluripotency stem cell master regulators octamer-binding factor 4 (OCT4/POU5F1), SRY box-containing factor 2 (SOX2), and NANOG was then evaluated. While these factors have been associated to the regulation of self-renewal and pluripotency in adipose tissue adult stem cells [31,32], within tumors their expression has been associated to tumor transformation, tumorigenicity, tumor metastasis [33] as well as treatment resistance [34]. Firstly, the expression of these pluripotency regulators in 2D monocultures of each of the utilized cells types was evaluated (Supplementary Figure V-3), showing an overexpression of *SOX2* and *NANOG* in the VMM15 cell cultures when compared to hDFbs, hDMECs and hASCs. This suggests that VMM15 melanoma cells express high levels of these stemness-related genes, which to the best of our knowledge, is the first time such has been reported. We then questioned whether the expression of these genes would continue to appear upregulated in the multicellular models. Interestingly, we verified higher expression levels of *SOX2*, *POU5F1* and *NANOG* in conditions which included the VMM15 melanoma cells (**Figure V-3a**, Supplementary Figure V-4). The same pattern of expression was seen for all the 3 genes, with the VMM15 assembloid and the VMM15/hDFbs spheroids showing significant overexpression when compared to the remaining conditions. This is supported by previous studies showing higher expression of *POU5F1*, *NANOG*, and *SOX2* mRNA transcripts within tumor cells and cancer stem cells niches when compared to those of nontumor tissues [35–37].

We then evaluated at the transcriptional level the abundance of the NAD-Dependent Protein Deacetylase Sirtuin-1 (SIRT1) (**Figure V-3a**, Supplementary Figure V-4), which is physiologically regulated to coordinate responses to environmental stimuli and stressors. More recently, its expression was shown to be elevated in metastatic melanoma when compared with primary melanoma while also being linked to the induction of epithelial–mesenchymal transition (EMT), facilitating melanoma metastasis [38]. Here, *SIRT1* was shown to be underexpressed in the individual MCSs when compared with both of the produced assembloids.

Taking in account that spheroid size and variability of response to 3D culture have a direct impact on both viability and growth kinetics of cells, which may generate spheroid core necrosis, hypoxia-induced cellular responses and genomic instability [39], we analyzed the mRNA expression of *TINF2* and *TERF2* (**Figure V-3a**, Supplementary Figure V-4). These proteins are part of the shelterin complex, that is composed of six major proteins which are responsible for protecting the telomeric DNA from damages [40]. Higher expression levels of both these genes was evident in conditions comprising the VMM15 melanoma cell line, whereas a significant decrease in the expression of these genes was found in hDMECs/hDFBs ($p < 0.05$). Also involved in damaged DNA repair is *BRCA1*, which has been shown to be

involved in the repair of double-strand breaks and initiate homologous recombination (HR), linking the maintenance of genomic integrity to tumor suppression [41]. While no statistical difference was found, a trend of underexpression of BRCA1 was noticed in all the studied conditions when compared to the VMM15/hDFbs MCSs, especially for the VMM15 assembloid (**Figure V-3b**). Besides BRCA1, TP53BP1 plays a significant role in DNA damage response, promoting checkpoint signaling following DNA damage [42,43]. Statistically significant under expression of this gene was found for the VMM15 melanoma assembloid and the hDMECs/VMM15 MCSs in comparison with the VMM15/hDFbs MCSs (**Figure V-3b**).

The aforementioned results demonstrate that the co-culture of tumor stromal compartments in the form of MCSs allows for their complete fusion followed by their auto-organization into multilayered 3D cell models, while preserving a gene expression profile compatible with metastatic melanoma, already observed for the MCSs containing VMM15.

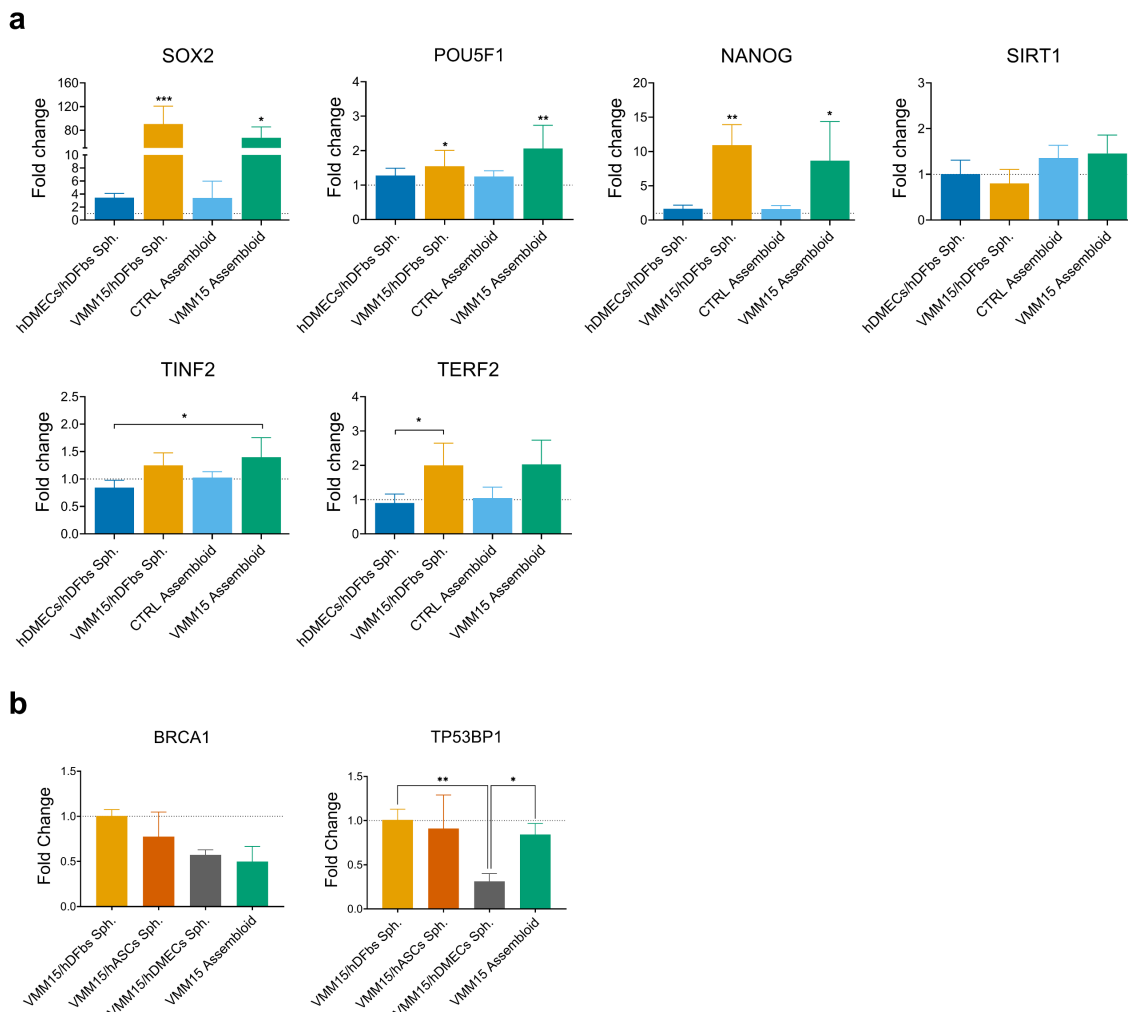


Figure V-3 Phenotypic characterization of assembloids and of their forming subunits.

a *SOX2*, *POU5F1*, *NANOG*, *SIRT1*, *TINF2* and *TERF2* mRNA expression in MCSs subdomains and developed assembloids at 13 d of total culture. Expression was determined by qPCR using $\beta 2M$ as a reference gene and normalized to the hDMECs/hASCs MCSs. Quantitative results are expressed as the mean $\pm$ standard deviation where $n = 3$. **b** *BRCA1* and *TERF2* mRNA expression in MCSs subdomains and developed assembloids comprising VMM15 melanoma cells at 13 d of total culture. Expression was determined by qPCR using $\beta 2M$ as a reference gene and normalized to the VMM15/hDFbs MCSs. Quantitative results are expressed as the mean $\pm$ standard deviation where $n = 3$. When pairwise comparisons are not directly represented, statistical significance is represented by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ when compared to hDMECs/hASCs, after a one-way ANOVA with Kruskal-Wallis multiple comparison post-test.

V-3.3. Tumor assembloids as alternative drug screening tools

Whether a 3D cancer model is capable of responding *in vitro* to known pharmaceuticals defines how well these systems can mimic *in vivo* cellular behavior and therefore how effective they are as disease models. Doxorubicin (Dox) was chosen due to its wide use as a chemotherapeutic agent recommended for the treatment of several tumors [44]. Regarding spheroid morphology (**Figure V-4a**, Supplementary Figure V-5), a clear shedding of the most superficial cells was noticed in all conditions in which VMM15 melanoma cells were present. This outer part was previously shown, in the tumor assembloid, to overlap with the location of GP100⁺ VMM15 tumor cells. This shedding was not visible in the CTRL assembloid lacking these tumor cells (**Figure V-4a**). In both VMM15 conditions, assembloid and CTRL MCS, the shedding was followed by an increase in the spheroid area in comparison to the non-treated condition (**Figure V-4b**), which is likely a consequence of cell death and detachment. In contrast, the CTRL assembloid didn't present any changes do its area over time when treated with Dox. To validate the potential use of our developed assembloid system for drug screening, a lactate dehydrogenase (LDH) assay was conducted after exposure of the models to 40 μ M Dox over 4 days. LDH release in response to Dox (**Figure V-4c**, Supplementary Figure V-6) varied for all conditions, albeit conditions comprising the VMM15 melanoma cell line presented overall higher values as expected. The VMM15 assembloid condition was shown to be the most susceptible to Dox (78.89 % of the LDH release of the positive control) followed by the CTRL MCS (72.56 %) and the VMM15/hDFbs spheroid (59.36 % LDH release), while hDMECs/hDFbs (34.73 %) and hDMECs/hASCs (29.36 %) showed significantly lower values. All of these results suggest that our final melanoma assembloid system acted as a responder to Dox, suggesting its suitability as a drug screening platform for further studies.

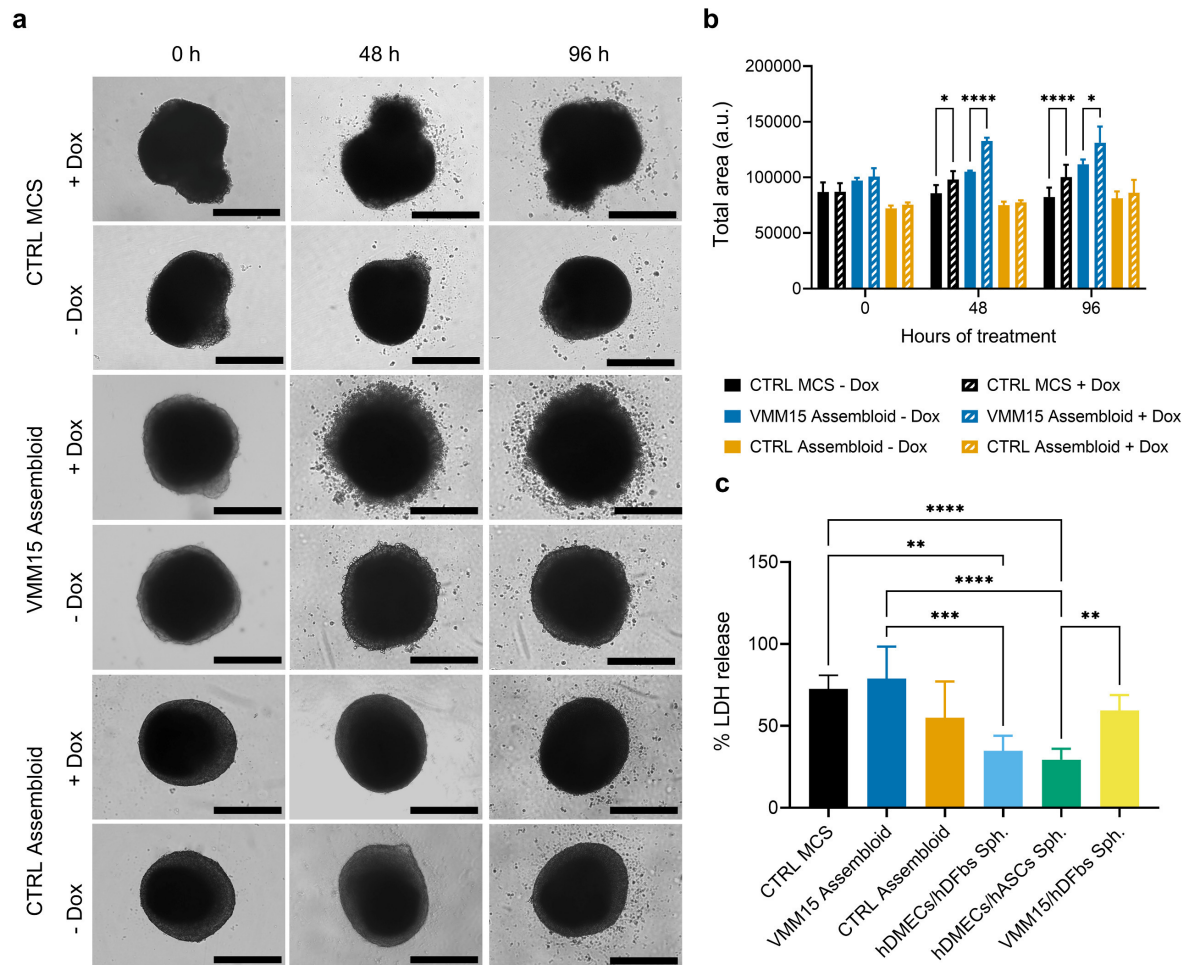


Figure V-4 Characterization of assembloid and CTRL MCS morphology and viability in response to doxorubicin treatment.

a Growth phenotype of melanoma assembloid and controls when stimulated with 40 μ m of Dox. Scale bar = 500 μ m. **b** Total area of VMM15 assembloids and respective controls over time when treated with 40 μ m of Dox. **c** Cytotoxicity evaluation by LDH release from three independent experiments of VMM15 assembloids, CTRL assembloids and CTRL MCSs exposed to Dox at 40 μ m. Quantitative results are expressed as the mean $\pm$ standard deviation where $n = 3$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA with Kruskal-Wallis multiple comparison post-test.

V-3.4. Stromal cells driving 3D tumor cell invasion in the melanoma assembloid

Cellular interplay and crosstalk within the tumor microenvironment play a role in promoting tumor growth, maintenance and invasion [45], in part through the activation of several ECM degrading MMPs. In melanoma, in particular, a critical role has been attributed to tumor stromal cells and the MMPs they produce in the promotion of tumor invasion [46,47]. Studies have shown that MMP-2 is constitutively expressed with variations implicating modest up or downregulations [48] while MMP-9 expression is highly inducible under the control of growth factors, chemokines and other stimulatory signals [49]. We proceeded in measuring the bioactivity of MMP-2 and MMP-9 in the developed spheroid models (**Figure**

V-5b, Supplementary Figure V-7,8). Results showed that pro-MMP-2 activity was detected throughout all conditions, with no significant differences found. In the single spheroid model where VMM15 was co-cultured with hDFbs, little to no MMP-2 activity was detected, with the hDMECs/hASCs having in comparison significantly increased activity ($p < 0.05$) of that enzyme over the whole culture period. Significantly higher activity of MMP-2 ($p < 0.05$) was further found in both tested assembloid models when compared to the VMM15/hDFbs spheroid. Expression of active MMP-9 was not detected throughout the tested conditions. Nonetheless, pro-MMP-9 activity was found to be significantly greater in the control assembloid in comparison to VMM15 co-cultured spheroids (Supplementary Figure V-7). These results suggest that endothelial and stromal cross-talk is important for MMP's, especially MMP-2, activity. While the constitutive gene expression of MMP is low, their expression is regulated at both the transcriptional and post-transcriptional levels. At a post-transcriptional level, the activity of these enzymes is controlled by specific protease inhibitors such as the tissue inhibitors of metalloproteinases (TIMPs). Findings have pointed to TIMP1 and -3 associating with latent or pro-MMP9 while TIMP2, -3 and -4 are capable of interaction with pro-MMP2 [50]. This considered, we were interested in understanding whether the differences seen in MMP-2 and MMP-9 activation in our developed spheroid models were being mediated by TIMP-1 and TIMP-2. ELISA (**Figure V-5c**, Supplementary Figure V-9) assays showed that TIMP-2 production was similar for all conditions with exception to the VMM15/hDFbs MCSs. Regarding TIMP-1, higher levels of this inhibitor were observed for both assembloid conditions and hDMECs/hASCs MCSs in comparison to the remaining MCSs.

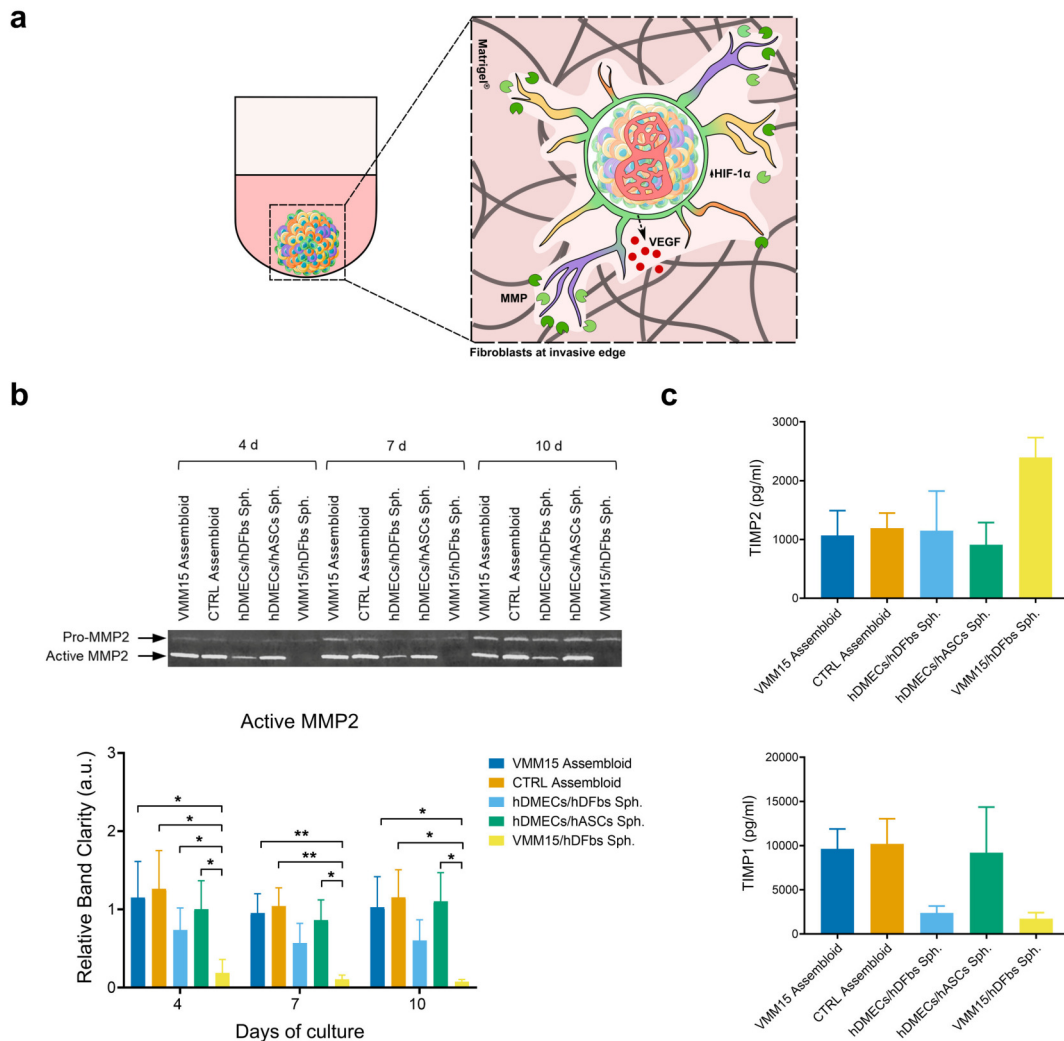


Figure V-5 MMPs as an active agent in melanoma tumor invasion.

a Schematic illustration of tumor assembloid invasion within a 3D Matrigel® assay: a single tumor spheroid per well was embedded in Matrigel®. **b** Gelatin zymography of MCSs or assembloid culture medium over 13 d of culture. MMP activity was visualized as clear bands against a dark blue background of gelatin substrate in gel. Molecular weight markers were used to estimate the molecular masses of the pro and active forms of MMP-2. Density of each band expressed as an arbitrary unit. **c** *TIMP1* and *TIMP2* secretion in MCSs subdomains and developed assembloids at 13 d of total culture. Expression was determined by ELISA. TIMP-1 was quantified in 5 µg of total protein and TIMP-2 was assessed in 20 µg of total protein. Quantitative results are expressed as the mean ± standard deviation where $n = 3$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA with Kruskal-Wallis multiple comparison post-test.

This differential expression suggests that the activity of TIMPs is linked to the different stromal cells comprising the tumor microenvironment, therefore highlighting their importance in the development of heterotypic tumor models.

Given these differences in the production of MMPs, we investigated whether this would impact overall invasion potential of the different models. We conducted a Matrigel®-based assay to evaluate both the sprouting and the invasion capability of the melanoma assembloids (**Figure V-5a**). Three parameters

considered relevant in terms of cellular invasion were assessed: area of invasion, the area of the core and the maximum radius [25]. While an increase in core size was detected for all tested conditions over the 7-day culture period, the greatest significant changes ($p < 0.001$) observed were among the different tumor single spheroids. No decreases in core size were in fact observed in any condition (**Figure V-6a**), suggesting that no necrosis was occurring over the 7-day culture period. However, the CTRL MCS displayed the lowest change in core size dynamics, suggesting hampered proliferative capacity of the cells in this group. The maximum radius is defined as the farthest distance on invasion from the center of the spheroid. When looking at these data for 7 days (**Figure V-6a**), we observed that the spheroids of hDMECs/hDFbs presented the highest invasion distance of 2.54 times the core radius at day 0, followed by the CTRL assembloid at 2.18, the melanoma assembloid at 2.06-fold and the CTRL MCS at 2.04-fold. Interestingly, all the tumor single MCSs presented no visible invasion in the 3D matrices.

When studying invasion, not only is the radius of invasion relevant but also the area of the invading edge, which consists in the area occupied by the expanding cellular mass. After 7 days of culture, the role that cross-talk among different stromal cells partakes in invasion area was clear. Single MCSs of VMM15 tumor cells co-cultured with either hDFbs, hASCs or hDMECs presented significantly less invasive potential when directly compared to all the remaining conditions (**Figure V-6b**, Supplementary Figure V-10, 11). The co-culture of hDMECs/hDFbs showed to contribute greatly to the invasive capacity of the spheroid, while the melanoma assembloid comprising all of the 4 different stromal cells showed the highest area of invasion.

Since the VMM15 assembloid presented the higher area of invasion, we proceeded to verify if VMM15 cells were present in the invasion front. For this, GP100 immunolabelling was performed in the 3D matrix used for the invasion assays (**Figure V-6c**). GP100-positive cells were found following the leading edge of the invasion front. Although the presence of randomly detached GP100-positive cells cannot be completely ruled out, results strongly suggest stromal cells remodeled the adjacent ECM to open up channels allowing the migration of the tumor cells, as described before [51].

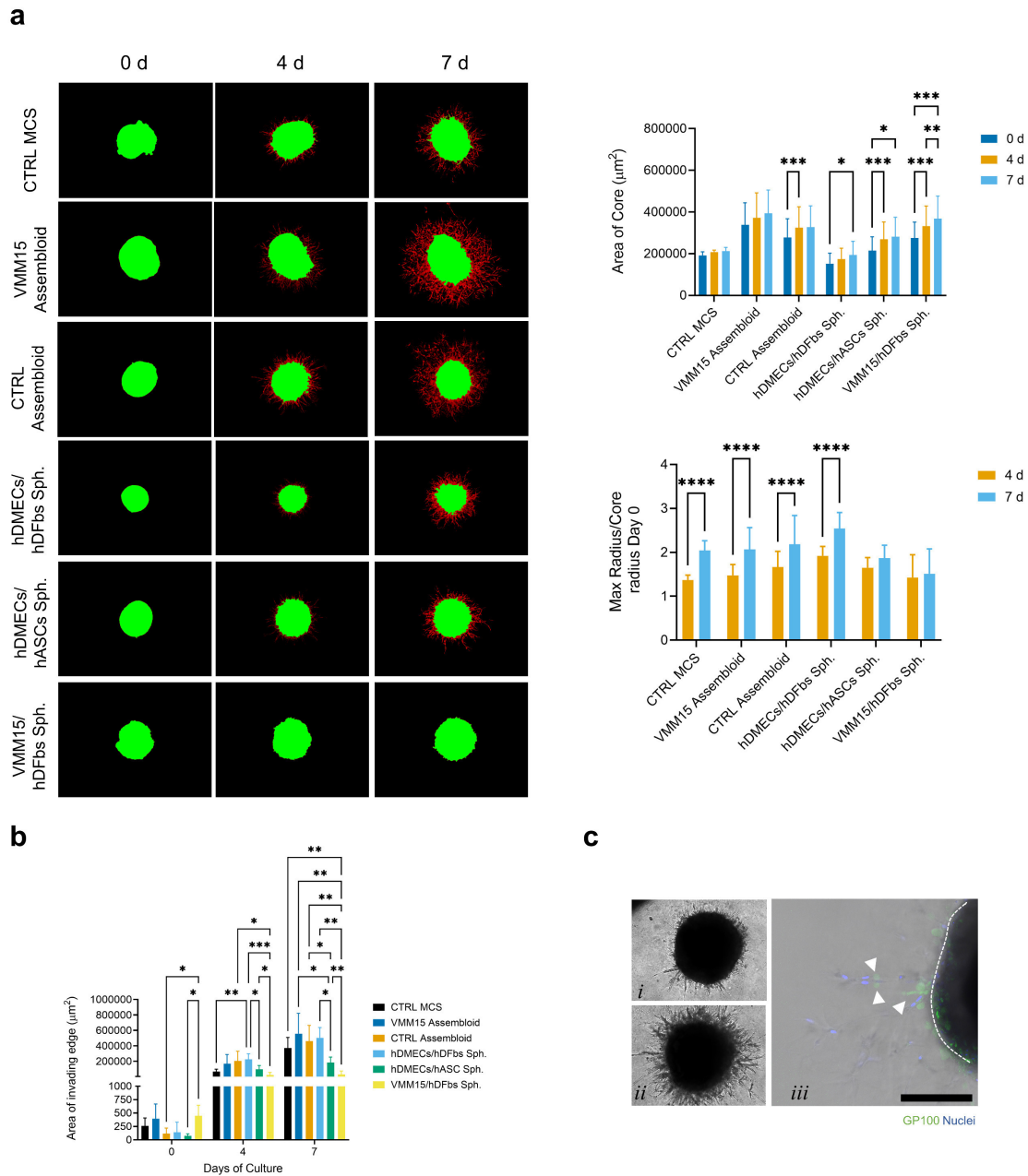


Figure V-6 3D invasion profile of assembloids and MCSs in Matrigel®.

a Segmentation profiles of MCSs and assembloid invasion in Matrigel® over 7 d. Calculation of invasion as a function of the area of the core and the longest invasive distance emanating from the spheroid normalized to core radius at day 0 of the experiment. **b** Calculation of invasion as a function of the area of the invading edge. **c** Microscopy images of assembloid invasion in Matrigel® i. Cells are seen beginning to evade from the embedded assembloid at 4 d of culture ii. Representative images of a 'starburst' invasion pattern at 7 d of invasion. iii. Details of invadopodia advancing into the matrix shown at higher magnification at 7 d. GP100⁺ labeled cells are found in green. Nuclei were counterstained with DAPI Scale bar = 200 µm. Quantitative results are expressed as the mean $\pm$ standard deviation where $n = 3$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA with Kruskal-Wallis multiple comparison post-test.

V-4. DISCUSSION

Over the past decade researchers have kept on pushing the boundaries of more traditional 3D cancer models like spheroids, MCSs and organoids. While these systems have made great advances in order to better represent native tissues they still present concerns of their own. Hybrid systems which combine different models together, like the ones reported by Boulard and colleagues [5], bridge the gap between cell-sheet systems and spheroids while capable of presenting a stratified epidermis, a basement membrane and blood and lymphatic capillaries in the engineered tissue. Similarly, organotypic models for melanoma have also been generated [52].

Recently, assembloids were proposed as novel 3D tissue models with added complexity. Originally envisioned to better study the assembly of neural circuits through the combination of organoids representing multiple brain regions, this form of 3D model allows deeper insights into tissue function, opening doors for the development of more advanced and representative cancer models [53] that will allow for a greater understanding of this disease.

The present work, provides the groundwork to unravel cell-cell and cell-ECM interactions relevant for cancer growth and invasiveness, at different stages of cancer development. Different stromal MCSs were developed to represent different stromal subdomains. Supporting this rationale was the intent of first establishing a stable endothelial cell compartment, capable of acting as a surrogate capillary bed and then combining this with a tumor-like stroma compartment encompassing VMM15 melanoma cells and hDFbs. This allowed the critical interactions between tumor and endothelial cells to take place upon combination of the MCSs into the assembloid system.

Given the importance of stromal ECM, the collagen signature in these stromal subdomains was analyzed. The spatial organization of the hDMECs appeared to be coincident with the collagen deposition seen by PSR staining, which is supported by the demonstrated role of collagen type-1 in endothelial cell morphogenesis [54]. Interestingly, while both fibroblasts and MSCs are known to support vasculogenesis through ECM deposition and secretion of pro-angiogenic factors [55–57] only in the hDFbs-containing MCS were the hDMECs organized into highly dense vascular-like network. These results are supported by reports showing that fibroblast-derived ECM favors endothelial tubulogenesis [58], while the direct co-culture between MSC and ECs has been shown to potently decrease EC proliferation and their angiogenic potential which may occur via VE-cadherin/ β -catenin interactions at the EC cell surface [59].

When the individual MCSs were assembled together, spatial organization of the different cell types in layers was evident at 10 days of combinatorial culture. Within this layered structure endothelial cells migrated toward the core of the spheroid, fibroblasts and stem cells remained in the central senescent zone and the VMM15 tumor cells migrated towards the outward proliferating zone. This organization comes in agreement with the cellular arrangement observed in other melanoma models [60], where growth factor deprivation, nutrients and oxygen gradients, and catabolites accumulation are responsible for segregating melanoma cells into a continuously proliferating subpopulation in the periphery and a G1-arrested subpopulation in the center [60]. This is similar to what is verified *in vivo* where tumor growth is characterized by a phase of exponential cell proliferation followed by a phase of declining growth rate associated with an increase in nonproliferating (quiescent) cells and necrotic cells [61]. This form of hierarchical organization was shown to be size dependent, in which MCS larger than 500 μm in diameter present a necrotic core surrounded by a viable rim, with an inner layer of quiescent cells and an outer layer of proliferating cells [62]. While comprising the same cellular constituents, the CTRL MCS failed to present a visible organization. GP100⁺ cells were irregularly distributed in the outermost layer of the spheroid and cell bulging and loose cell aggregates were frequently present. The poor spatial organization of hDFBs, hDMECs and hASCs may be related to the observed diminished collagen deposition. A previous report had already observed that when endothelial cells were seeded together with fibroblasts and tumor cells on day one, no endothelial cells were located in the microtissues [63], which comes in agreement with our results in regards to the lack of CD31⁺ cells within the CTRL MCS. This comes to show that the establishment of stromal compartments, followed by their assembly into a more complex structure such as what is herein proposed, supports the viability and function of these stromal cells.

Also deemed critical for this hierarchical organization is the deposition of ECM within these 3D models, which relays both biochemical and physical stimuli affecting all surrounding cells in terms of cell migration, differentiation, proliferation and viability [64,65]. This form of support and biochemical stimulation provided by the different cellular subsets may explain the fast fusion of the individual subdomains into one larger assembloid when placed in co-culture. The 28-day total culture period resulted in extensive necrosis at the core of the tumor assembloid when compared to the CTRL condition. It is possible that this occurred not only in function of the size of the assembloid but equally because of the dense ring of VMM15 cells that had formed around it, this way shielding the inner cells from nutrients and oxygen, which has been shown to occur *in vivo* [61].

The role that cancer stem cells, a subpopulation of tumor cells which display stem cell features, plays within the tumor microenvironment has become of high importance in understanding the mechanisms behind tumor invasiveness, metastasis, drug resistance and epithelial to mesenchymal transition (EMT) [66]. Tightly associated to this phenotype is the overexpression of pluripotency transcription factors *SOX2*, *POU5F1* and *NANOG* which have been reported to induce stemness, chemoresistance, metastatic potential, proliferation, EMT and resistance to apoptosis and are directly tied to aggressiveness and poor prognosis [67–70]. We have shown that the VMM15 melanoma cell line displays such features in light of its overexpression of pluripotency transcription factors *SOX2* and *NANOG* in 2D monocultures. Similarly, the overexpression of these transcription factors was verified both in our melanoma assembloid model as well as in the individual MCSs comprising VMM15 cells, indicating that the 3D culture supported the maintenance of this stemness profile. Tumor cells have also been shown to promote the upregulation of these stemness related genes in neighbor cells as the case of hASCs [32]. Additionally, overexpression of *POU5F1* was only verified in the 3D conditions which may be an effect of the heterogeneity of the neighboring cells which impacts cytokine levels, hypoxia, acidic pH, cancer associated fibroblasts (CAFs) and the activation of specific signaling pathways which are known to regulate cancer stem cell niches [71].

Also associated with several roles in cancer is the shelterin complex [72]. While protecting the telomere from DNA damage pathways and aging, it may act as an oncogene in cancer cells. An overexpression of the *TINF2* and *TERF2* genes has been implicated in various pro-tumorigenic mechanisms [73]. Our results are in line with these findings, where an increased expression of these genes was detected in VMM15 cells-bearing spheroids and in the melanoma assembloid. Genomic instability in our models was further studied by assessing the expression of *BRCA1* and *TP52BP1* genes. Both *BRCA1* and *TP52BP1* have been indicated as tumor suppressor genes with important roles in DNA damage repair. A loss of expression of *BRCA1* has been shown to lead to a higher tumor grade and advanced clinical stage in gastric carcinoma patients [74], while in breast cancer tissues a decrease in *BRCA1* levels led to tumor cell growth while an increase in its expression triggered growth arrest and apoptosis [75]. A similar behavior is observed with *TP53BP1* in which loss of protein expression levels were associated with high grade tumors, supporting the idea that loss of function of this gene may favor breast cancer progression and worse prognosis [76]. In patients with pancreatic ductal adenocarcinoma, loss of *53BP1* expression was also associated with decreased overall survival [77]. Our results suggest the underexpression of these DNA damage repair genes for the assembloid model in comparison with

most MCSs conditions, indicating the genomic instability typical of higher-grade tumors is preserved in this complex model.

It is nevertheless likely that the changes described in the results above are more or less pronounced depending on the studied cell population of the models. Single cell analysis would allow a more detailed characterization of the changes happening during the different stages of the study, which is a limitation of the current work.

The reaction of 3D tumor models to anti-cancer agents is of key interest to assess its potential to mimic what happens *in vivo* and be used for drug discovery. A higher sensitivity to Dox was observed in conditions comprising VMM15 melanoma cells correlating with increased cell shedding in the assembloid and MCSs and an increased LDH release. This increased sensitivity may be consequence of the cellular organization seen in this model where VMM15 tumor cells are in the proliferative zone and therefore in first contact with the drug while these cells' higher proliferation rate makes them more susceptible to apoptosis by DNA damage. Furthermore, this structured organization present in the assembloid has the potential to facilitate the unravelling of the mechanisms leading to anti-cancer therapeutic resistance in tumors [78,79] by studying cell-cell interactions within layer interfaces.

Having observed a complete overhaul in cellular organization of the 3 stromal subdomains when assembled, we questioned how this would impact cell invasion in 3D. Cellular crosstalk and paracrine signaling in the tumor microenvironment are key to drive tumor progression [80–82]. Among the major players involved, we can find MMPs which are a family of zinc-containing endopeptidases, similar between themselves both structurally and functionally. They are secreted in an inactive (latent) state, known as pro-MMPs, and require activation in order to be able to cleave ECM components [83] while their activity appears to be tightly regulated by TIMP function. Whereas MMPs are known for their important physiological functions such as tissue repair and remodeling, cellular differentiation, cell mobility and wound healing [84], gelatinases (MMP-2 and MMP-9) in particular have been implicated as key players in promoting angiogenesis, infiltration of cancer cells and metastasis [85–87]. Endothelial cells have a clear role in this mechanism through which the spread of tumor cells throughout the capillary endothelium and neovascularization are facilitated [88,89]. This effect was evident when comparing the results of MMP activity in the matrix-embedded cultures. An increased production of active MMP-2 was visible in conditions that presented a higher invasive area and maximum length of invasive edge from the spheroid core, that mostly coincided with the presence of hDMECs and hDFbs combinations. Interestingly, co-culture MCS containing VMM15 cancer cells all presented relatively low levels of MMP production and

invasion, independently of being cultured with endothelial cells, mesenchymal stem cells or fibroblasts. In humans, an inverse relationship between in situ MMP-9 expression and melanoma progression was found [90]. MMP-9 appears to be involved in the early steps of metastasis formation but not the later steps such as extravasation [91]. In most cases of tumor development and progression, MMPs are produced by nonmalignant stromal cells within the tumor microenvironment. Besides the known production of these gelatinases by endothelial cells, findings have suggested the production of MMPs to be associated to fibroblasts immediately surrounding tumor cell clusters [92,93]. This adds to the importance of having heterotypic cellular models in which crosstalk between different stromal cells is allowed, mimicking the *in vivo* situation and contributing to what is called “cooperative invasion”. The latter describes how inherently invasive cells, either malignant or nonmalignant, can induce the invasion of otherwise poorly invasive subpopulations in mixed cell populations [94]. Several mechanisms supporting this concept have been described. One in particular describes an invasive or leader cell that possesses protease activity, deposits ECM and co-invades through the 3D matrix with subpopulations of poorly invasive cells [95]. This may explain the findings in our assembloid tumor model, where GP100⁺ VMM15 tumor cells followed the invasive edge of the produced assembloids. A balance between both proteases and inhibitors is what defines the invasiveness of tumor cells within the microenvironment. Within extracellular fluids and tissues, TIMPs levels have been shown being in excess when compared to MMPs, thereby limiting their proteolytic activity to focal pericellular sites [96]. The expression of *TIMP1* within tumor correlates with a less aggressive tumor onset while an imbalance in MMP/TIMP ratio seen through the downregulation of *TIMP1* is associated with poor prognosis [97,98]. Additional studies have also pointed to the importance of TIMPs in the reduction of experimental metastasis of melanoma [99]. Additionally, active MMP-2 was shown to correlate with increased malignancy in melanoma xenograft models [47], as well as in primary and metastatic melanoma patients [100]. Our results have shown a correlation between the expression of MMP-2/TIMP-2 and Pro-MMP-9/TIMP-1. While the action of TIMP-2 in controlling the expression pattern of MMP-2 was evident, the results suggest a possible imbalance in this MMP/TIMP ratio seen through the high levels of the active form of MMP-2. On the other hand, TIMP-1 production correlated with the data obtained for Pro-MMP-9 through zymography in which no active form of this MMP was present. This may be an indication that an equilibrium in this MMP/TIMP ratio is present, inhibiting therefore the active form.

V-5. CONCLUSION

Here we have shown that melanoma assembloids present a hierarchical cellular organization with proliferative and necrotic areas and likely a quiescent area in-between, mimicking native tumor tissue. Sensitivity to Dox was demonstrated. The melanoma assembloid model furthermore favored the overexpression of key pluripotency transcription regulators, which may indicate that this model supports a more dedifferentiated state of the melanoma cells. Furthermore, the cell complexity of the model suggests that intratumoral crosstalk among stromal cells is essential in promoting 3D tumor cell invasion mediated by MMP production. Therefore, the presented assembloid-based model has the potential to be a powerful alternative to current *in vitro* 3D tumor models by allowing the study of several melanoma related traits such as anticancer drug sensitivity, pathway plasticity, dedifferentiation and stromal mimicry. Furthermore, a finer analysis of cellular and molecular events during assembloid formation, will allow to further unravel the mechanisms behind the observed cellular segregation, which may benefit cancer modelling research and also basic cancer research.

V-6. REFERENCES

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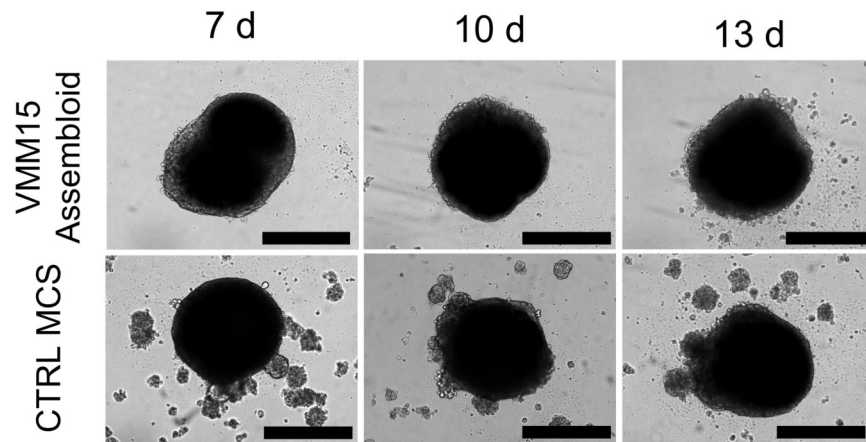
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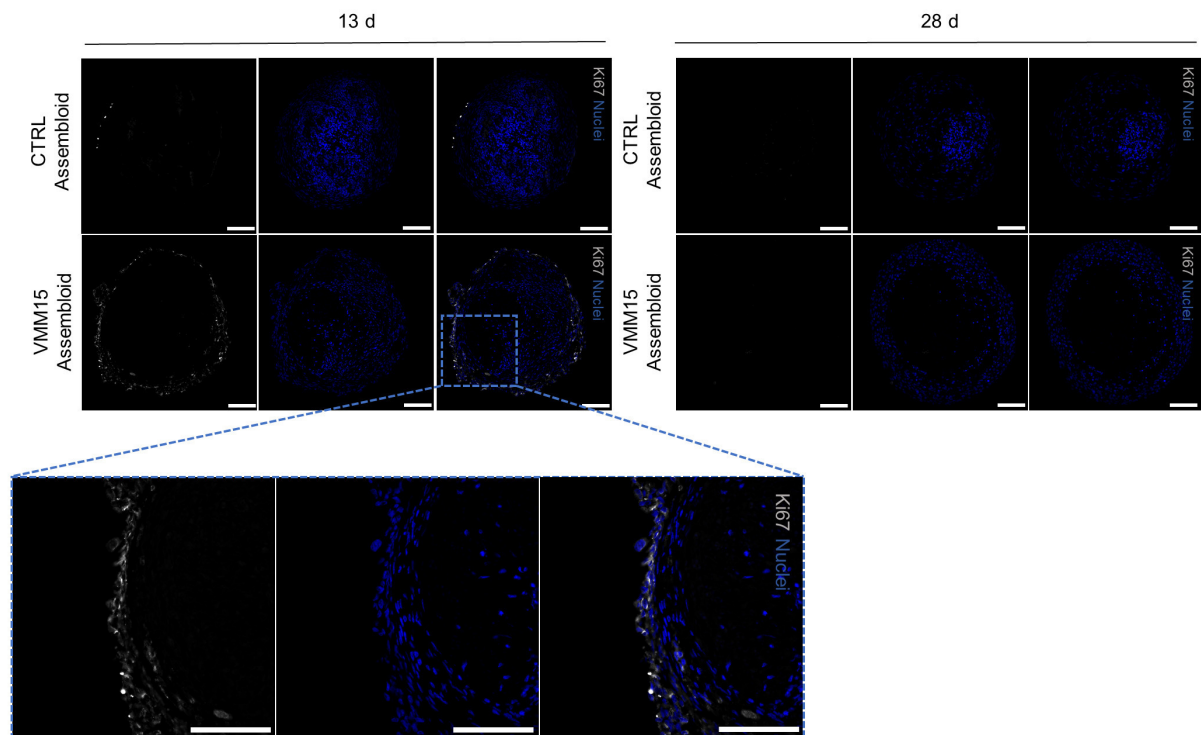
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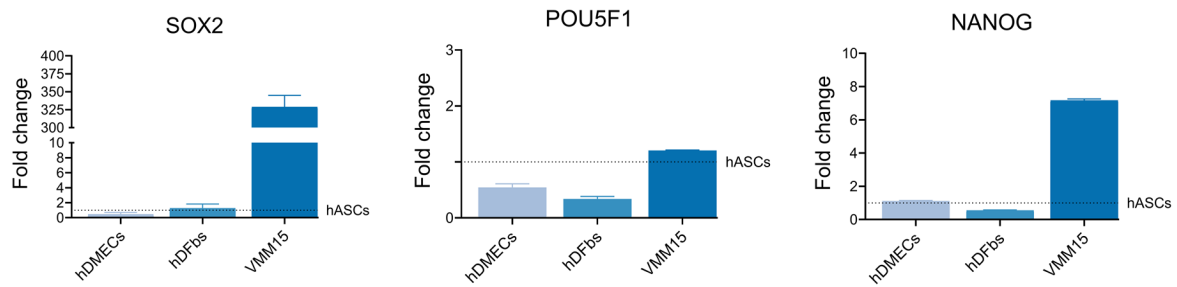
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V-7. SUPPLEMENTARY INFORMATION**Supplementary Figure V-1 Formation of VMM15 melanoma assembloids and CTRL MCS over time.**

Brightfield microscopy of VMM15 assembloid and CTRL MCS formation over 7, 10 and 13 d of total culture. Nuclei were counterstained with DAPI. Scale bar = 500 μ m.

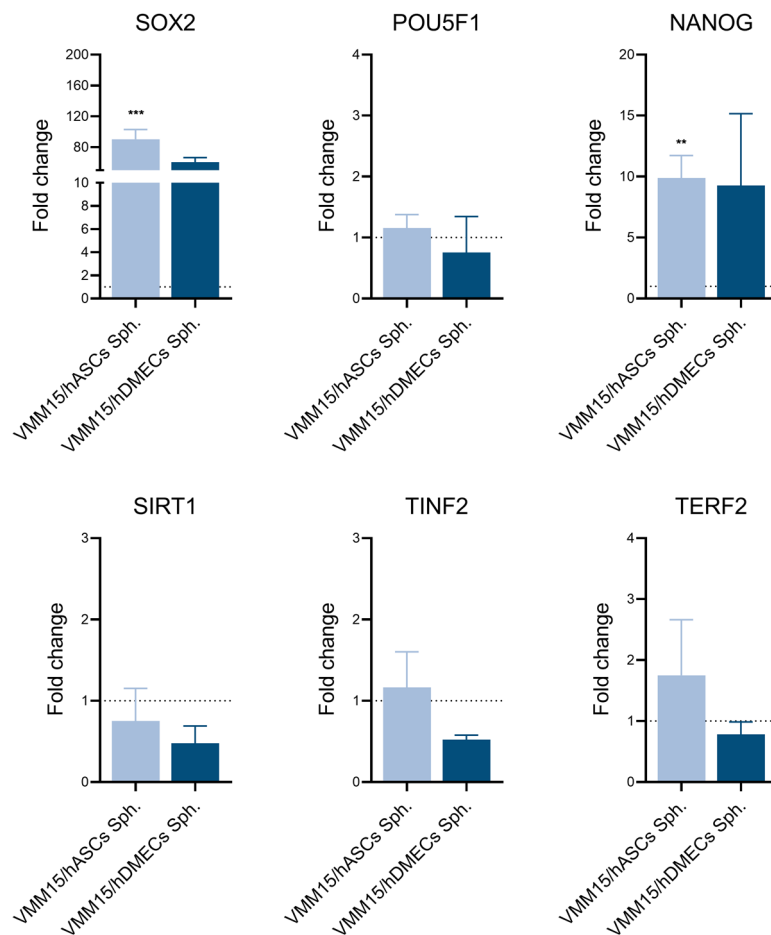
**Supplementary Figure V-2 Proliferation gradient characterization in VMM15 melanoma assembloids.**

Representative immunocytochemistry images of Ki67 positive proliferating cells in the developed assembloids over 13 and 28 d of culture. Nuclei were counterstained with DAPI. Scale bar = 100 μ m.



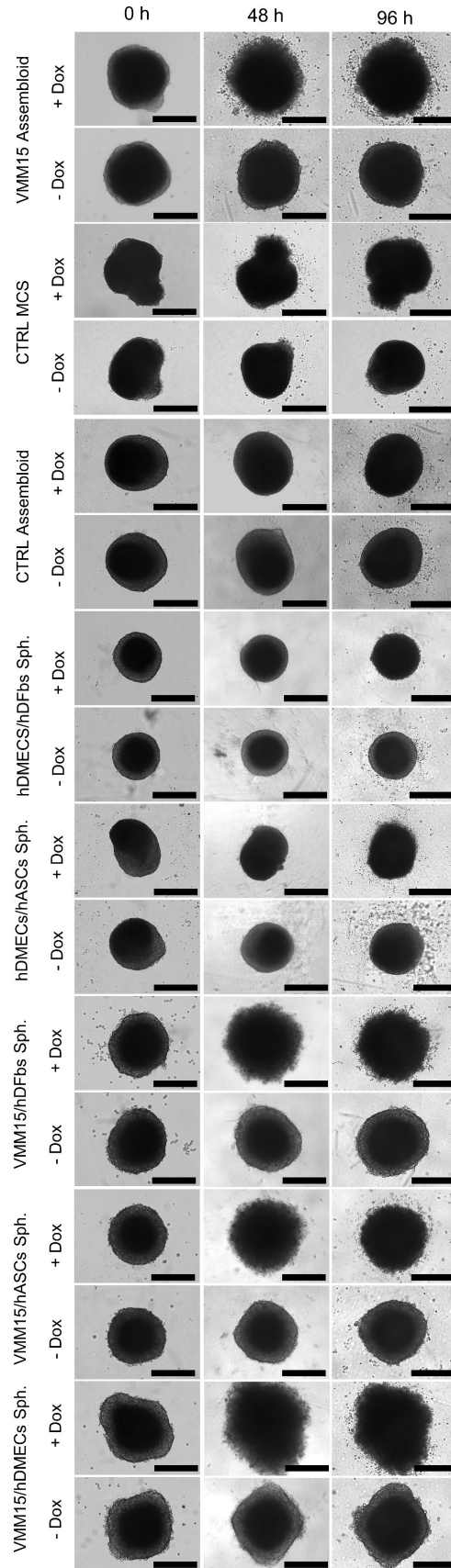
Supplementary Figure V-3 Gene expression of pluripotency transcription factors in 2D monocultures of the cellular constituents of the melanoma assembloid.

SOX2, *POU5F1* and *NANOG* mRNA expression in 2D monocultures of each cell type that comprises the melanoma assembloid. Expression was determined by qPCR using β_2M as a reference gene and normalized to hASCs.



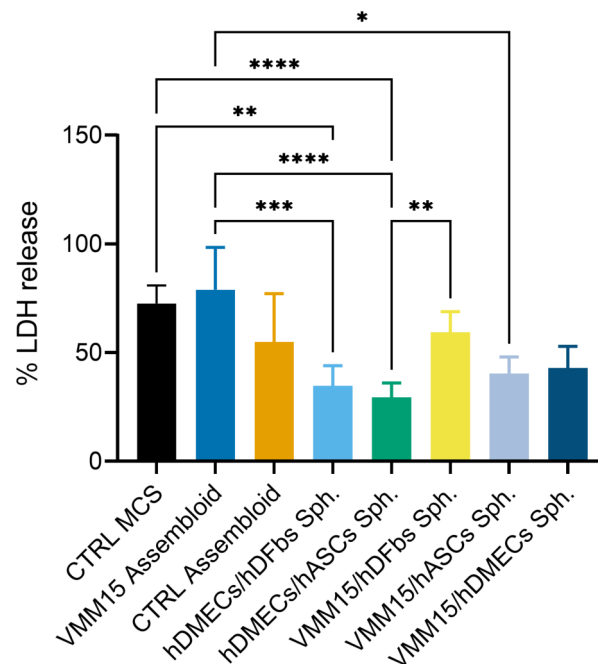
Supplementary Figure V-4 Gene expression of pluripotency transcription factors in 2D monocultures of the cellular constituents of the melanoma assembloid.

SOX2, *POU5F1*, *NANOG*, *SIRT1*, *TINF2* and *TERF2* mRNA expression in MCSs at 13d of total culture. Expression was determined by qPCR using β_2M as a reference gene and normalized to the hDMECs/hASCs MCSs. Quantitative results are expressed as the mean $\pm$ standard deviation where $n = 3$, ** $p < 0.01$, *** $p < 0.001$, one-way ANOVA with Kruskal-Wallis multiple comparison post-test.



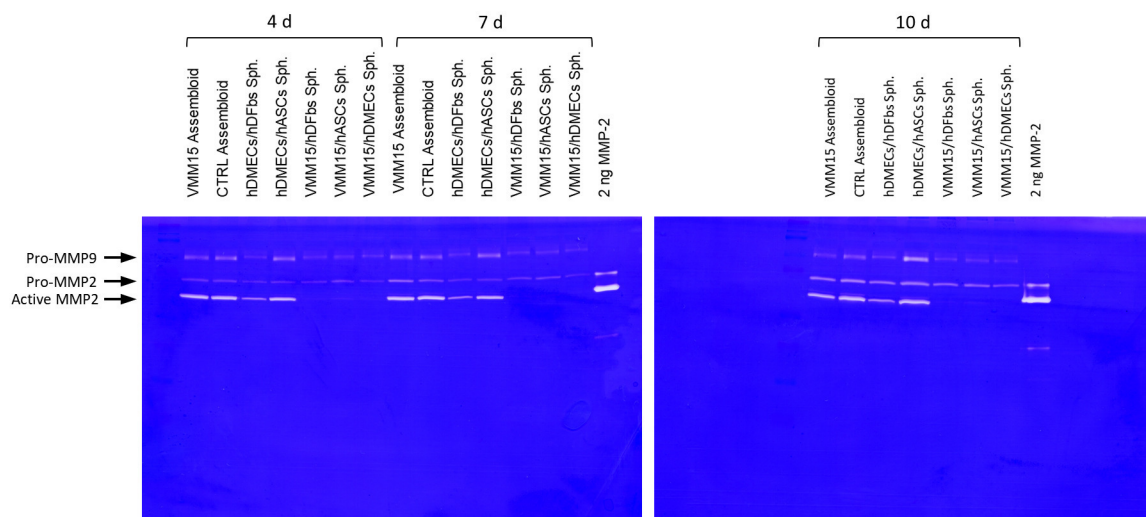
Supplementary Figure V-5 Characterization of assembloids and MCS morphology in response to doxorubicin treatment.

Growth phenotypes of assembloids and control MCSs when stimulated with 40 μ M of doxorubicin. Scale bar = 500 μ m.



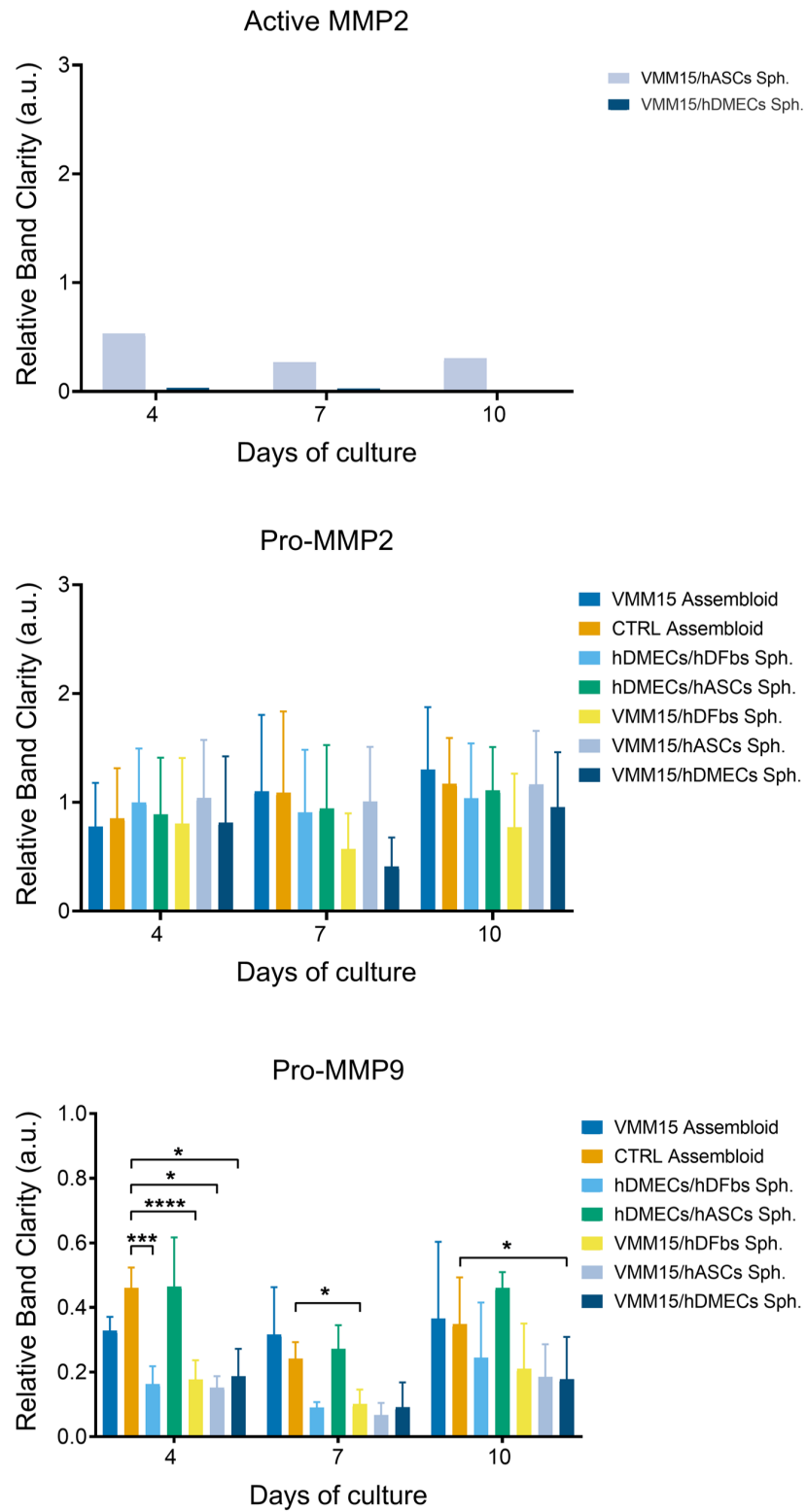
Supplementary Figure V-6 Characterization of assembloids and MCSs viability in response to doxorubicin treatment.

Cytotoxicity evaluation by LDH release from three independent experiments of the produced assembloids and MCSs exposed to doxorubicin at 40 μ M. Quantitative results are expressed as the mean $\pm$ standard deviation where $n = 3$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, two-way ANOVA with Tukey or Sidak's multiple comparison post-test.



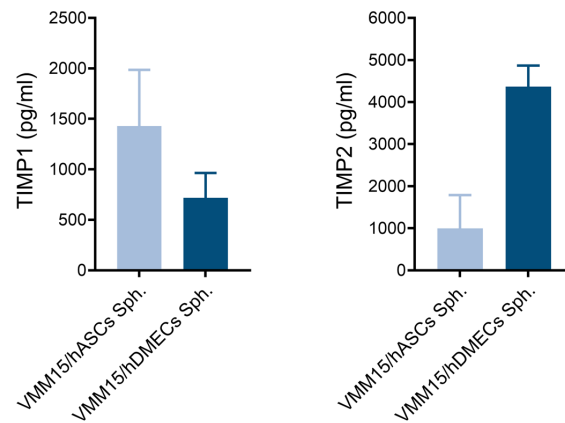
Supplementary Figure V-7 Zymography of MMP2 and MMP9 produced by melanoma assembloids and MCSs.

Gelatin zymography of MCSs and assembloid culture medium over 13 total days of culture on 10% SDS-PAGE gel containing 0.1% w/v gelatin. MMP activity was visualized as clear bands against a dark blue background of gelatin substrate in gel.



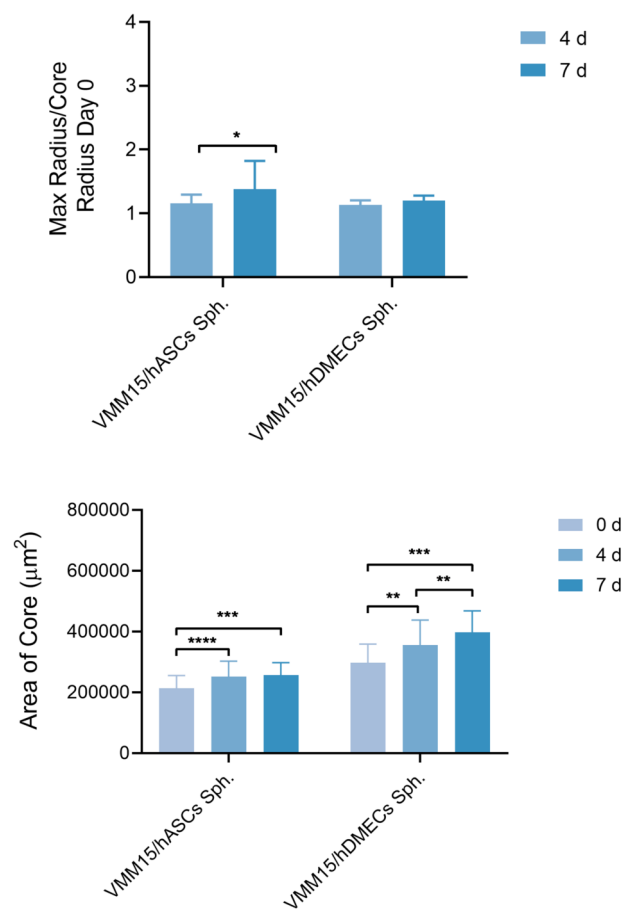
Supplementary Figure V-8 Relative quantification of Active MMP-2, Pro-MMP2 and Pro-MMP9 by melanoma assembloids and MCSs.

Band density was expressed as arbitrary units. Quantitative results are expressed as the mean $\pm$ standard deviation where $n = 3$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, two-way ANOVA with Tukey or Sidak's multiple comparison post-test.



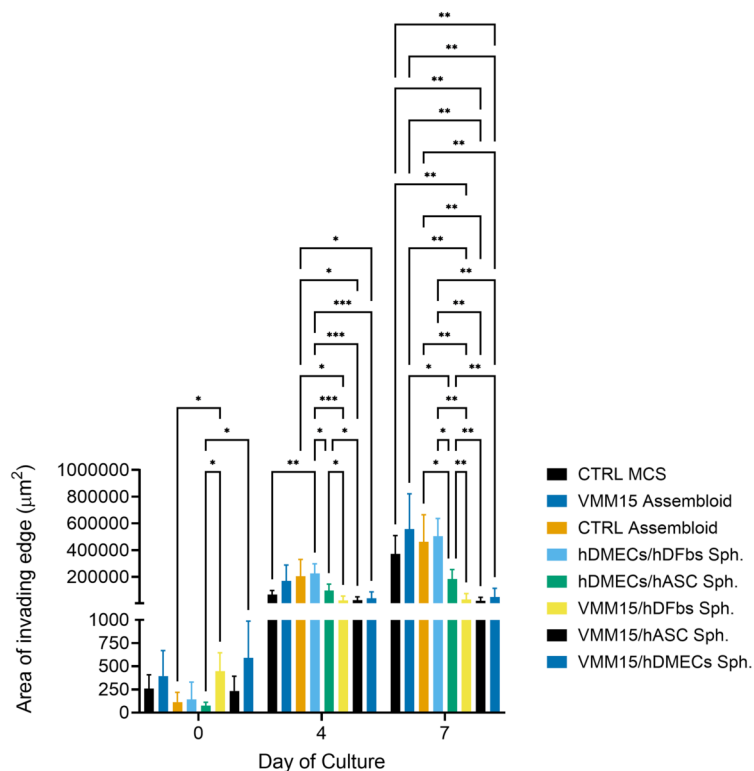
Supplementary Figure V-9 Release of TIMP-1 and TIMP-2 by melanoma MCSs.

TIMP1 and TIMP2 secretion in MCSs subdomains and developed assembloids at 13 days of total culture. Expression was determined by ELISA. TIMP-1 was quantified in 5 μ g of total protein and TIMP-2 was assessed in 20 μ g of total protein. Quantitative results are expressed as the mean $\pm$ standard deviation where $n = 3$.



Supplementary Figure V-10 3D invasion profile of MCSs in Matrigel®.

Segmentation profiles of assembloids and MCSs invasion in Matrigel® over 7 days. From top to bottom: calculation of invasion as a function of the longest invasive distance emanating from the spheroid normalized to core radius at day 0 of the experiment; calculation of invasion as a function of the area of the core. Quantitative results are expressed as the mean $\pm$ standard deviation where $n = 3$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, two-way ANOVA with Tukey or Sidak's multiple comparison post-test.

**Supplementary Figure V-11 Area of invading edge of assembloids and MCSs in Matrigel®.**

Segmentation profiles of assembloids and MCSs invasion in Matrigel® over 7 days. Calculation of invasion as a function of the area of the invading edge in all the studied conditions. Quantitative results are expressed as the mean $\pm$ standard deviation where $n = 3$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, two-way ANOVA with Tukey or Sidak's multiple comparison post-test.

CHAPTER VI

A 3D MODEL TO EVALUATE CELL CHEMOTAXIS WITHIN A HETEROGENIC TUMOR MICROENVIRONMENT

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ABSTRACT

As studies continue to bring forward data on both the complexity and heterogeneity behind the tumor microenvironment, new strategies to understand and unravel the cellular interactions that regulate tumor progression and tumor cell invasion are required. Here we present a novel and tailorable 3D culture chamber design capable of studying chemotaxis between several distinct cell types and a cancer cell population of interest. A collagen type I hydrogel as the 3D substrate allowed for a differential molecular diffusion, in which rate of diffusion was associated with molecular weight. When culturing different human stromal cells (hASCs, hDMECs and hDFbs) in the outer wells while keeping VMM15 melanoma cells within the central well it was observed that hASCs and hDFbs presented a directional invasion throughout the collagen matrix towards the tumor cells. Further analysis supported a higher area of invasion present in the hDFbs when compared to the hASCs, supporting the potential of this system to study the recruitment of supporting cells by cancer cells and how this may impact tumor invasion. demonstrates the ability of the biomaterial to trigger vasculogenesis in an integrin-specific manner, by activating essential pathways for EC survival and migration within a self-regulatory growth factor microenvironment. This strategy represents an improvement to current vascularization routes for Tissue Engineering constructs, potentially enhancing their clinical applicability.

** This chapter is based on the following publication:

Rodrigues DB, Cruz-Moreira D, Gasperini L, Jarnalo M, Horta R, Reis RL, Pirraco RP, A 3D model to evaluate cell chemotaxis within a heterogenic tumor microenvironment. *Submitted* (2023)

VI-1. INTRODUCTION

Cancer remains a major burden in modern society, with an expected 609360 deaths in the United States alone for the year of 2022 [1]. This drives the need to better understand interactions that take place within the tumor microenvironment in order to develop novel therapeutics with higher efficacy. To carry out this task, more biosimilar tumor models are required to study both the interactions that undergo within the tumor as well as to allow a more accurate prediction of drug response.

The tumor microenvironment is a highly heterogeneous milieu, comprised of tumor cells, stromal cells as the case of endothelial cells (ECs), cancer-associated fibroblasts (CAFs), mesenchymal stem cells (MSC), and pericytes and immune cells like tumor-associated macrophages and lymphocytes. Cell-cell interaction between these cells have been described [2–4] and act as a major driving force promoting tumor progression [4,5]. Additionally, interactions at cell-extracellular matrix (ECM) level also regulate tumor growth and metastasis. These interactions are mediated by a variety of different factors such as chemokines, adhesion molecules, cytokines, growth factors or even matrix metalloproteinases (MMPs), eliciting responses not only at the cellular level but also modulating both structural and biochemical properties of the ECM. A deregulation of these pathway has been found to promote tumor growth and metastasization [6–11].

It has been reported that tumor cell invasion, migration and dissemination is most efficient when they occur in a directed manner [12]. This directed cell migration may be in response to several different forms of stimuli such as chemotactic, haptotactic, electrotactic and durotactic [13]. Chemotaxis is defined as the phenomenon by which movement is directed in response to an extracellular chemical gradient and has been shown to favor tumor cell dissemination as previously reviewed [14] while haptotaxis is directional cell movement in response to a gradient of adhesion such as ECM or substrate-bound chemoattractants [15]. For instance, it has been shown that IL-8 expression by endothelial cells possesses chemotactic activity towards WM35 melanoma cells, mediated by the CXC-chemokine receptor CXCR1 [16]. Other studies have associated the outward migration of tumor cells from within the tumor microenvironment with self-generated lysophosphatidic acid (LPA) gradients. Cells create their own gradients by acting as a sink, breaking down locally present LPA, and thus forming a gradient that is low in the tumor and high in the surrounding areas [17,18]. In other works, ECM molecules laminin, fibronectin, and type IV collagen have been associated to both chemotaxis and haptotaxis of the A2058 human melanoma cell line, depending on whether the ligand was in solution or bound to a substrate [19]. Additionally, vitronectin has also been implicated in chemotaxis and haptotaxis in A2058 human

melanoma cells, mediated by integrin $\alpha v \beta 3$ [20]. These aforementioned studies, while contributing with worthwhile findings, are still based on 2D approaches which lack the complexity observed in the tumor microenvironment from its 3D nature to its cellular heterogeneity. Therefore, there is a pressing need for models better representing that native complexity to allow a more comprehensive understanding of cell behavior and cell communication.

Considering the limited systems available to study these cellular interactions, representative of the heterogeneity of the tumor microenvironment, in such a complex manner, a collagen I-based 3D culture system was developed, where the formation of chemotactic gradients from 3 different stromal cellular types was allowed in order to better understand how each individual cell type interacts with 3D melanoma cells in a directly comparative manner.

VI-2. MATERIALS AND METHODS

VI-2.1. Design and fabrication of master pattern via direct micromachining

A collagen type I chemotaxis chamber was created by hydrogel molding and integrated within μ – Slide culture chambers for ease of manipulation during microscopic imaging. The conceptualization of the chamber is shown in **Figure VI-1** and the schematic diagram of the mold is shown in Supplementary Figure VI-1. The master mold counterpart acts as a cover for the μ – Slide culture chamber, imprinting the collagen hydrogel while it undergoes gelification. The master mold was designed and built to produce wells in eight independent chambers. Each chamber contains a central well that is equidistant from three others, which in turn are equidistant among them. The master mold has an overall footprint of 75 mm by 25 mm and a height of 1 cm, being each chamber 10.65 mm by 9.4 mm. The pillars are 1.2 mm diameter each and 7mm high. The master mold layout was drawn using computer-assisted design (CAD) software (Fusion 360, Autodesk Inc.). The corresponding master mold was produced in polymethyl methacrylate (PMMA) by direct micromachining (MDX-50, Roland DG) using a ZHS-100 tool (480 mm/min, 15000rpm) in both roughing (0.16 mm cutting-in, 0.60 mm path interval, 0.16 mm finish margin) and finishing (0.05 mm cutting-in, 0.05 mm path interval, 0mm finish margin) steps. Features of the pattern were sharply contoured in order to enable good pattern resolution. After milling, the micromachined acrylic structures were cleaned off and their surface polished by exposure to stream of dichloromethane vapor and sterilized by UV radiation. The pillars, upon imprinting in the collagen solution, gave rise to microwells capable of supporting cell culture.

VI-2.2. Hydrogel production and characterization

Type 1 collagen from bovine skin (Sigma) was prepared as recommended by the manufacturer. Briefly, 10× PBS was placed in a sterile tube on ice followed by 1N NaOH to adjust the pH of the solution. The required amount of sterile ice-cold water was then added to the mixture in order to achieve a final concentration of 4 mg/mL of collagen. Finally, the calculated volume of 6 mg/mL of collagen I was added to the mixture, mixed and left on ice. Prior to adding to the mold, the solution was degassed to remove any air bubbles incorporated into the mixture. For this effect the solution was placed under vacuum, during cycles of 20 s until there was no longer formation of cavitation bubbles. Sample evaporation was considered and followed closely. Afterwards, 400 μ L of the type I collagen solution was added to each well of μ – Slide 8 well chambers (Ibidi) and the chemotaxis mold was added on top. The chambers containing the collagen mixture and the mold were placed at 37 °C for 2 h for gelification to occur.

Rheological characterization of the resulting collagen I chemotaxis chambers was performed on a Rheometer (Malvern Kinexus Pro+). For the derivation of storage and loss moduli, hydrogels were loaded into the rheometer, and the geometry was moved into contact with the hydrogel until a very small resistance (0.05 N) was detected, point at which loading was complete. The hydrogel chambers were then subjected to a strain amplitude sweep (from 0.01 to 10% strain amplitude), at a fixed 1 Hz shear rate, in order to find the linear viscoelastic region. A fixed strain within that region was then selected to perform a frequency sweep (from 0.1 to 10 Hz) and then derive the storage (G') and loss (G'') shear moduli profile of the hydrogels. For quantifying the different moduli, the G' and G'' values recorded at 1 Hz were selected.

VI-2.3. Characterization of diffusion

Food dyes were injected simultaneously into the the outer wells (yellow) and the inner well (blue) and color diffusion was followed over time. To better understand the solution gradients within the developed system, 10 μ M solutions of both Albumin–fluorescein isothiocyanate conjugate (Sigma) and Rhodamine B isothiocyanate–Dextran wt ~10,000 (Sigma) were prepared in PBS and 2.5 μ L were added to each well to be analyzed.

Fluorescence diffusion throughout the gels were observed under an SP8 Leica laser confocal microscope (Leica Microsystems, Wetzlar, Germany). Time-lapse images were taken at 0 min, 10 min, 20 min, 30 min and 40 min. The fluorescent intensities were quantified using Leica Application Suite X

ver. 3.7.2.23383 (Leica Microsystems). Briefly, for each well, 4 different line profiles of 500 μm were drawn from the most outer edge of each axes (north, south, east and west) outwards into the gel. The fluorescent intensities were recorded throughout each ROI and an average in function of distance was calculated per well.

VI-2.4. Cell lines and culture conditions

hTERT Dermal Microvascular Endothelial Cell, Neonatal (ATCC® CRL-4060™) were obtained from ATCC and cultured in EGM-2 MV Microvascular Endothelial Cell Growth Medium-2 BulletKit (Lonza, USA). Human dermal fibroblasts (hDFbs) and human adipose derived stromal/stem cells (hASCs) were harvested from human skin samples obtained from abdominoplasties after written informed consent and within the scope of a collaboration protocol approved by the ethical committees of both institutions for this work (Comissão de Ética do Hospital de S. João/University of Minho: 217/19; CEICVS 008/2019). Both hDFbs and hASCs were cultured in α -MEM, supplemented with 10 % (v/v) fetal bovine serum (FBS, Invitrogen, USA) and 1 % (v/v) antibiotic/antimycotic (Sigma, USA). Human cell line VMM15 (ATCC® CRL-3227™), derived from metastatic melanoma lesions, were grown as adherent cultures in RPMI 1640 Glutamax supplemented with, 50 μM 2-Mercaptoethanol (Gibco, Scotland, UK) 10 % (v/v) FBS and 1% (v/v) antibiotic/antimycotic (Sigma, USA). All cells were kept in culture at 37 °C in a humidified atmosphere under 5 % CO_2 .

VI-2.5. Seeding of cells on collagen gels cell isolation and culture

To best determine the correct cellular confluency to be used in the produced chemotaxis culture system, four densities of respectively 1×10^3 , 5×10^3 , 10×10^3 and 15×10^3 were seeded in the collagen I chemotaxis chamber in a total volume of 2.5 μL . Cell were left to adhere to the bottom of the well for 1 h at 37 °C, after which 175 μL of EGM-2MV culture medium was added to the whole chamber. Cell confluency was followed overtime through bright-field microscopy (Axio Observer inverted microscope, Zeiss, Germany). The cell density that yielded a suitable confluency to allow for cell growth was chosen to carry out the remaining work.

To evaluate the impact of different chemotactic gradients produced by stromal cells on VMM15 tumor cell invasion, cells were seeded within the chemotactic chamber in a manner in which the VMM15

tumor cells seeded within the center chamber would be subjected to all three of the chemotactic gradients formed by the hDMECs, hDFbs and hASCs.

VI-2.6. Cell migration and cell tracking

To evaluate the impact of different chemotactic gradients produced by stromal cells on VMM15 tumor cells invasion, cells were strategically seeded within the produced chemotactic chamber in a manner in which the VMM15 tumor cells seeded in the center chamber would be subject to all 3 of the formed chemotactic gradients by the hDMECs, hDFbs and hASCs. For this effect 10000 cells of each cell type were added to each well as described above. Cell migration was followed over time on an Axio Observer inverted microscope with incubation (37 °C, 5 % of CO₂), and Zen 3.2 (blue edition) software (Zeiss, Germany) at every 30 minutes intervals for 3 days.

Brightfield images were first cropped using ImageMagick 6.9.11 to obtain a 1:1 ratio image centered on each well for each time point of the experiment. The spheroid was cropped to leave only migrating cells and the image was then divided into four quadrants of identical size and labeled with intercardinal directions (NW, NE, SW, SE). The Ilastik pixel classification tool 1 was used to separate background and foreground pixels obtaining a probability map of all processed images. The probability map is a grey scale image where the probability of each pixel belonging to the foreground is proportional to its intensity (e.g intensity 1 or white corresponds to 100 % probability while black corresponds to 0 %). The probability map was then used in CellProfiler 2 for the segmentation of cells and the calculation of the area they occupy on the image.

VI-2.7. Immunocytochemistry

After cell migration assays, gels were fixed in 4 % (v/v) paraformaldehyde solution (Pierce, USA) at 4°C for 48h. For immunocytochemistry, samples were permeabilized with 0.2 % (v/v) Triton X-100 and unspecific staining was blocked with 3 % (w/v) Bovine Serum Albumin (Sigma). Subsequently, the gels were incubated in PBS with 0,2 % (v/v) Triton X-100 (Sigma), 1:250 fluorescein phalloidin (Invitrogen, Netherlands) and 1:1000 DAPI (Biotium, USA). Samples were examined under a Zeiss LSM980 AiryScan 2 Confocal Microscope, Zen 3.6 (blue edition) software (Zeiss, Germany).

VI-2.8. Statistical analysis

Data are expressed as mean $\pm$ standard deviation of three independent experiments. Shapiro–Wilk test was performed to validate normality of data prior to statistical testing. Statistical analysis was performed using one-way or two-way analysis of variance (ANOVA), followed by either a Tukey's or Bonferroni's multiple comparison post hoc test. Significance was set to 0.05 (95 % of confidence interval). Significance levels were analyzed with GraphPad Prism statistical software v8.0 (GraphPad Software, USA) and indicated as p values (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).

VI-3. RESULTS

VI-3.1. Characterization of collagen type I chemotactic chambers

To create a 3D chemotaxis chamber, a master mold was designed and conceived to be compatible with commercially available 8 well slides (Ibidi) (**Figure VI-1a**). The central pillar of the mold was designed to create a well to accommodate tumor cells, whereas the three remaining pillars, equidistant to the center wells, would host stromal cells. The mold was produced by microdrilling a 75 mm x 25 mm x 10 mm PMMA piece in which the obtained pillars presented an average height of 6.95 ± 0.05 mm and an average diameter of 1.63 ± 0.05 mm. Within the mold design, an additional hole was placed in each chamber to prevent hydrogel overflow. Having obtained the master mold required to produce the chemotaxis chambers, the obtained hydrogels could be further characterized before validating their use to study chemotaxis.

While the elastic nature of cancer tissues has already been described [21,22], biological components, such as the extracellular matrix (ECM) also possess viscoelastic responses to varying stimuli. Viscoelasticity, as the term implies, is the property of a material that exhibits mechanical traits of both elastic solids and viscous fluids. This viscoelastic behavior of both ECM and cancer cells has been shown to play a crucial role in the progression of cancer [23,24]. Considering this, we assessed the rheological properties of the developed 3D chemotaxis chambers. We observed that collagen I hydrogel chambers at a concentration of 4 mg/mL presented an average storage modulus (G') of 460.78 ± 141.09 Pa, while the average loss modulus (G'') presented a value of 74.69 ± 32.96 (**Figure VI-1b**).

To determine whether the developed collagen hydrogels would be suitable for the formation of chemotactic gradients, we assessed diffusion throughout the gels. Two dyes, yellow (outer wells) and blue

(center well) were injected into the wells, in which over time, the color in the wells changes concentrically from distinct yellow (outer wells) and blue (inner well) to a green tint where both interfaces unite (**Figure VI-1c i**) suggesting that the produced collagen hydrogel allows for diffusion. To further quantify generated gradients, two fluorescently labelled molecules with distinct molecular weights were used, 66'000 Da albumin and 10'000 Da (nominal MW) dextran, representative of the range of sizes that constitute chemokines and cytokines [25,26]. Fluorescent intensities in the chamber were measured over time and compared (**Figure VI-1c ii**). Albumin displayed a higher fluorescent intensity closest to the well indicative of a higher degree of retention in the collagen chambers. When compared to dextran, the latter showed increased levels of diffusion as seen by the steady levels of fluorescence measured throughout the hydrogel immediately after injection.

These results confirm the potential of the developed collagen chemotaxis chamber to be used as a system to study cell – cell interactions paracrine signaling.

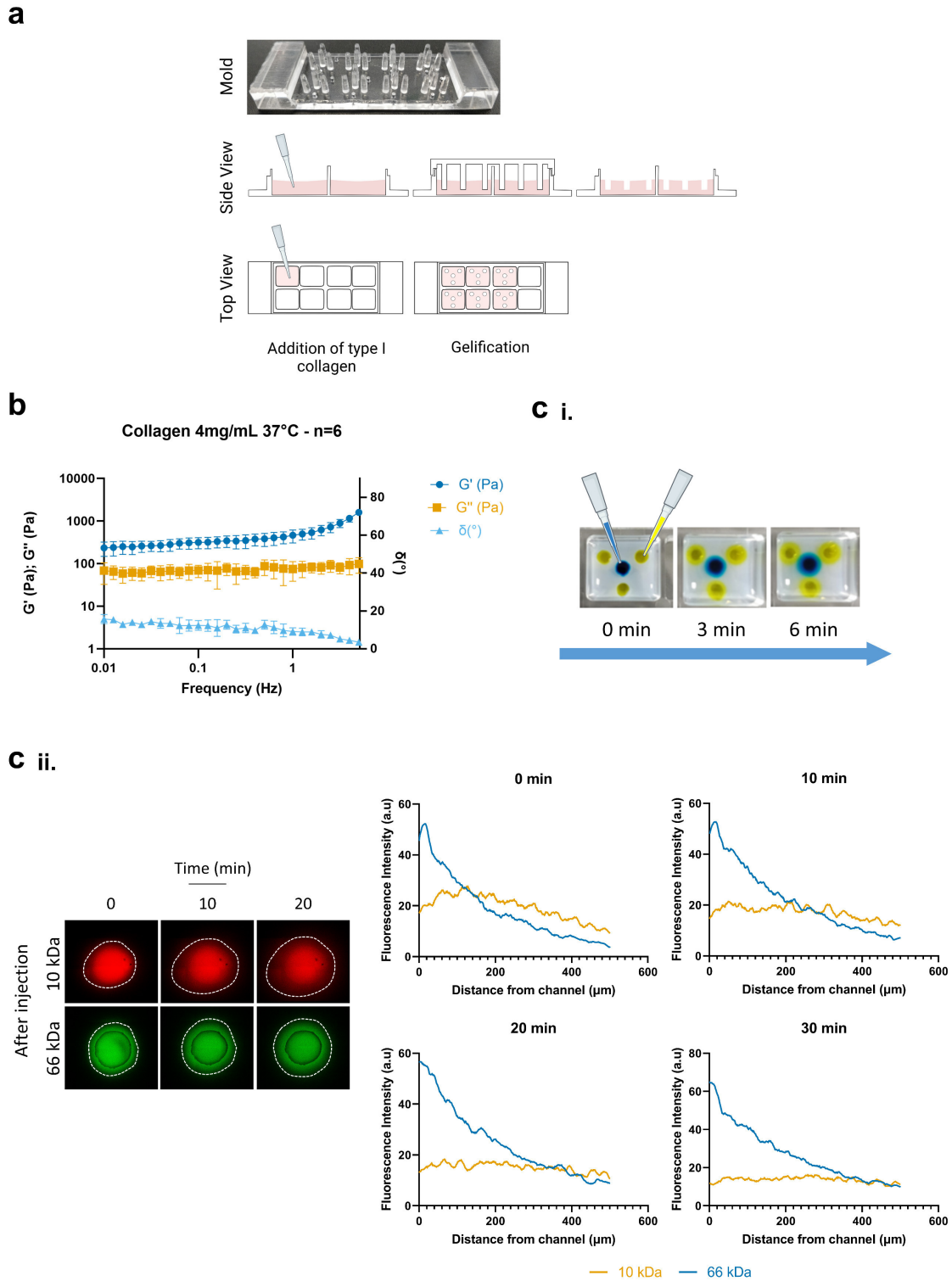


Figure VI-1 Chemotaxis chamber design and characterization.

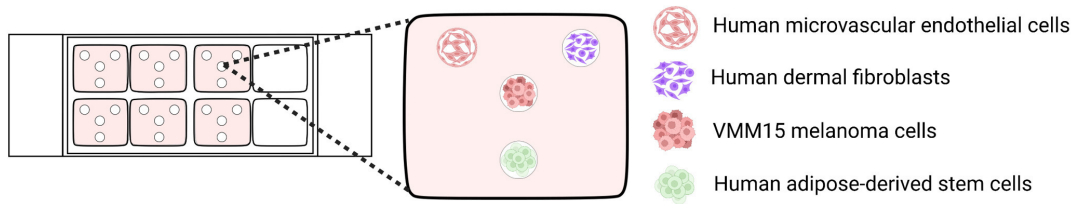
a Schematics of the development of collagen chemotaxis chambers. **b** Storage and loss moduli, as well as, phase angle as function of frequency for collagen gel (4 mg/mL) ($T = 37^\circ\text{C}$). **c i**) Yellow and Blue dyes were injected into the wells. A merge between both colors was seen at the interface between both wells. **ii**) Fluorescent imaging of both Albumin–fluorescein isothiocyanate conjugate and Rhodamine B isothiocyanate–Dextran wt ~10,000 Da in the chemotaxis chamber. Fluorescence intensity was used to determine diffusion rates throughout the hydrogels.

VI-3.2. Cell migration in response to chemotactic gradients

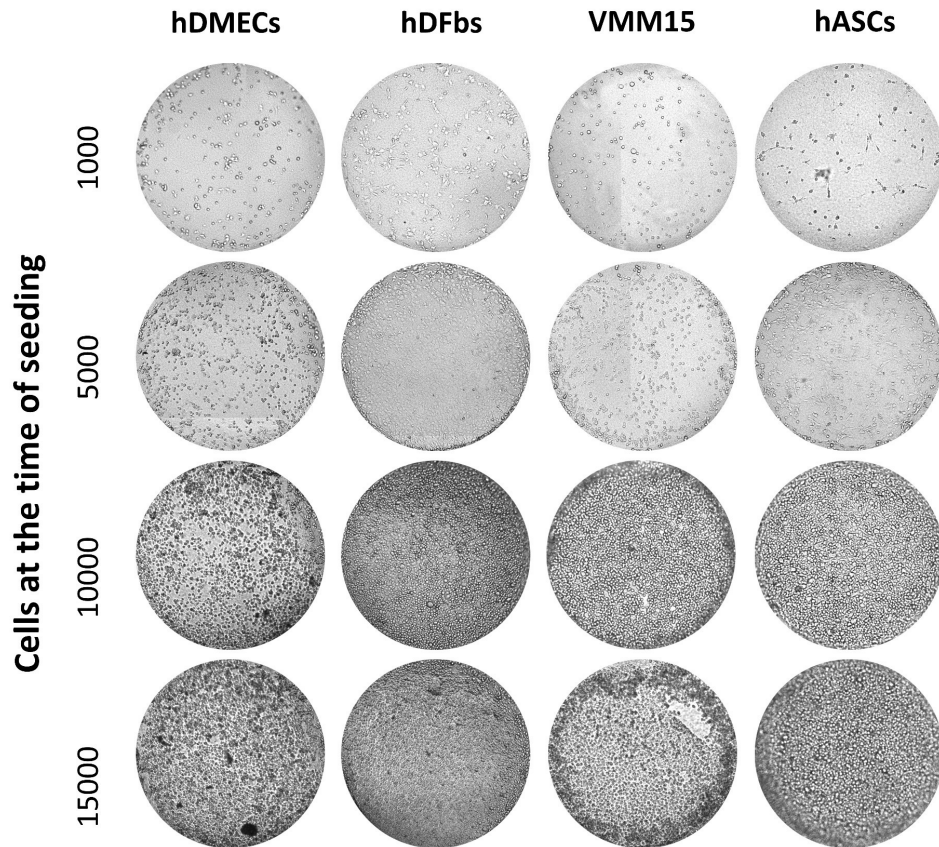
Chemotaxis has long been described to play a crucial role in tumor progression and to play a key role in directed migration of cells within the tumor microenvironment. These migration patterns have been shown to be dependent of the type of cancer and the surrounding factors within the tumor microenvironment. Previous reports have suggested that stromal cells lead the tumor invasive front, opening channels within the stroma that allow for the migration of tumor cells. However, it is still not clear whether these tumor stromal cells may also have a paracrine effect in the invasion of the tumor cells through these channels.

To test the developed chambers, cells were seeded in the wells in order to assess the putative effects of cellular crosstalk between tumor cells and stromal cells on migration patterns. Chemotactic gradients were expected to form and regulate cell migration directionality within the chamber. VMM15 melanoma cells were placed in the center chamber in order to influence all of the surrounding cells equally, while also being under the effect of the signals secreted by the stromal cells. In the outer chamber wells, human dermal microvascular endothelial cells (hDMECs), human adipose derived stromal/stem cells (hASCs) and human dermal fibroblasts (hDFbs) were seeded (**Figure VI-2a**). A titration assay was first carried out to understand the correct cellular confluency required for the gels. Four different densities of 1×10^3 , 5×10^3 , 10×10^3 and 15×10^3 cells/well were tested. Upon analyzing cells in the wells 2h after seeding, the density of 5×10^3 was found to be the best since above that density the wells appeared overcrowded for an additional culture period of 3 days.

a



b



c

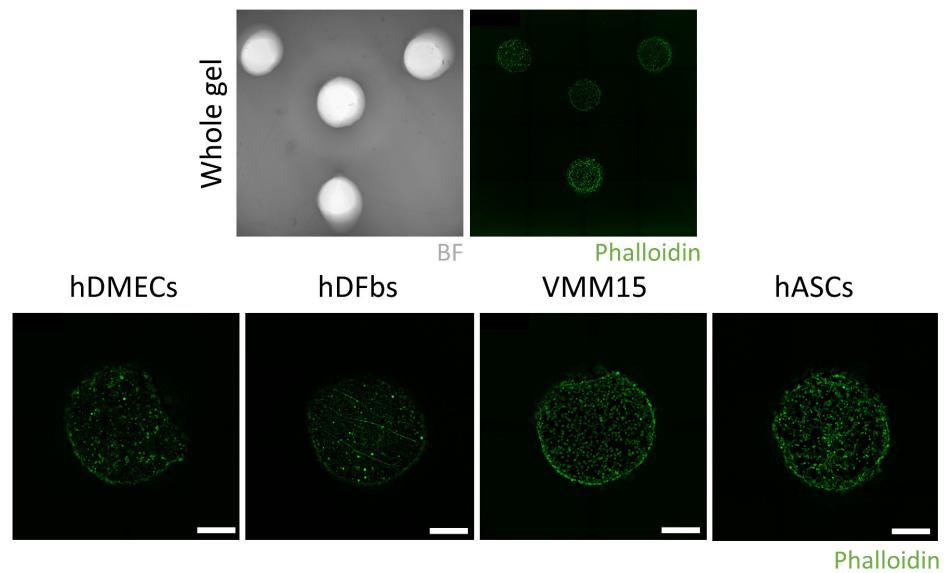


Figure VI-2 Chemotaxis chamber design and characterization.

a Schematics of cell organization within the collagen chemotaxis chambers. **b** The phase-contrast images of VMM15, hDMECs, hASCs and hDFBs cells after 2h of seeding in the collagen chemotaxis chambers at increasing cell densities as described **c** Morphological analysis of VMM15, hDMECS, hASCs and hDFBs, 24h after seeding at a density of 5×10^3 , by fluorescein isothiocyanate-phalloidin/DAPI (green = phalloidin and blue = DAPI). Scale bar = 400 μm .

Observation of cell migration in these chambers by time-lapse microscopy was performed (**Figure VI-3b**). Segmentation of the adjacent area to the culture well showed that hDMECs and VMM15 melanoma cells lacked any form of migration. hASCs and hDFBs, on the other hand, presented clear evidence of directional migration throughout the collagen I chamber (**Figure VI-3b i**). Characterization of directional migration was attained by quantifying the area occupied by cell migration in an 8 – axis template (**Figure VI-3b ii**). An increased area of migration in the direction of the VMM15 tumor cells was shown for hDFBs, represented by a shift towards the -90° axis. In what regards hASCs, an increased migration towards the VMM15 tumor cell well was also observed seen by an increase in the area on migration towards 0° . A control group encompassing chemotactic chambers lacking VMM15 cells resulted in greatly decreased migration (**Figure VI-3b ii**).

Considering the directed migration that hASCs and hDFBs were undergoing, we considered relevant to evaluate cell morphogenesis during the observed migration. Cellular morphogenesis characteristic of a mesenchymal migration type was observed through a spindle-shaped morphology, followed by an extension of lamellipodia and filopodia and the attachment of the protrusions to the surface (**Figure VI-3bc**). As clearly observed in the invading edge of the fibroblast condition, a higher density of actin within the cell cytoskeleton correlates with the directionality towards the VMM15 cell chamber.

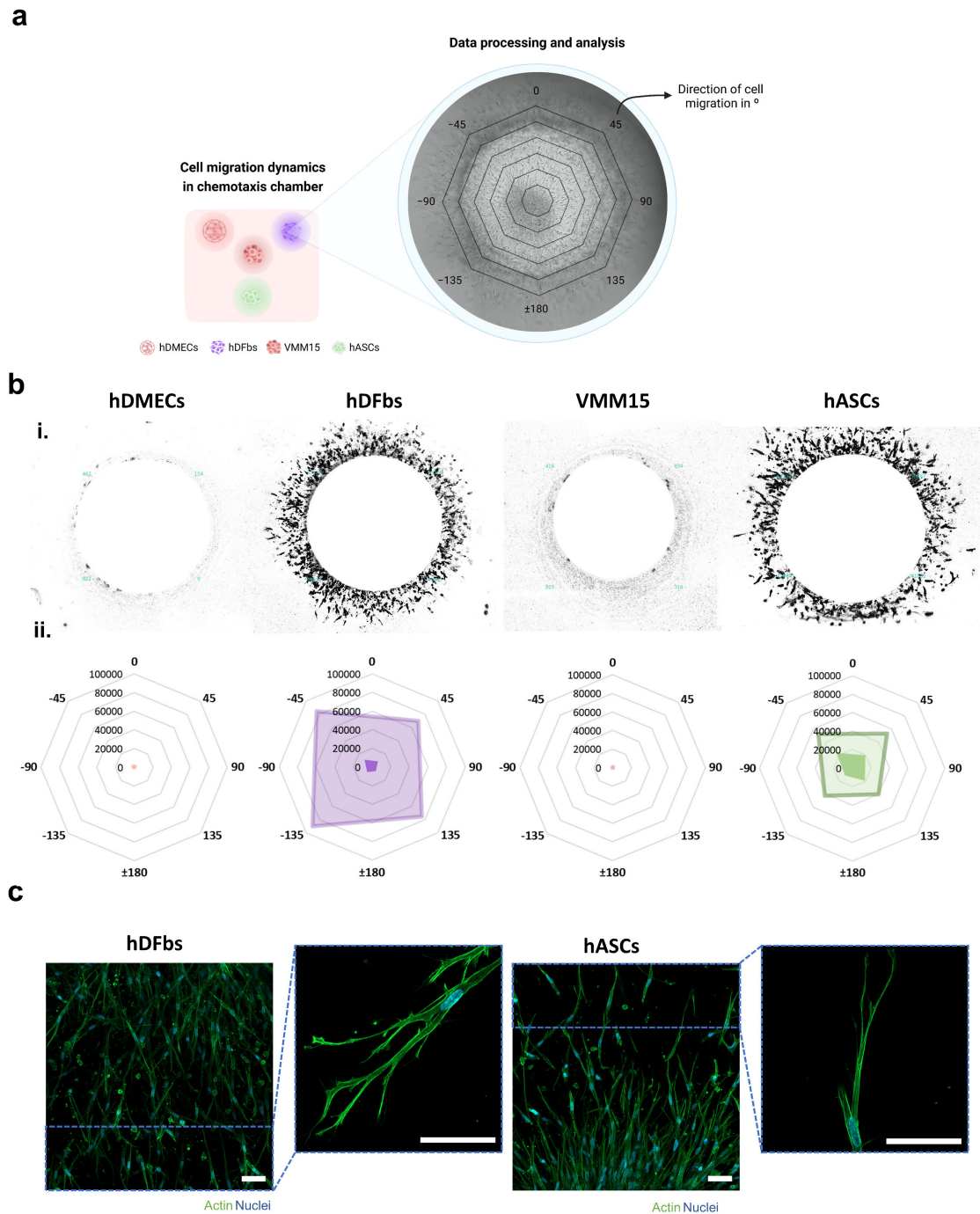


Figure VI-3 Chemotaxis of stromal cells in a 3D tumor microenvironment model.

a Schematics of cell organization within the collagen chemotaxis chambers. **b** i Segmentation of migrating cells in the chemotaxis chamber wells. ii Quantification of the migration profile of hDMECs, hDFbs, VMM15 and hASCs after time-lapse microscopy. **c** Double-labeling showing the distribution of F-actin (green) and cell nuclei (blue). Scale bar = 50 μ m.

VI-4. DISCUSSION

Chemotaxis has long been described as playing a critical role in tumor progression. By definition, it is the directed movement of cells in response to an extracellular chemical gradient and has been associated to several different types of cancer [14]. Hence, it is a critical feature to be modelled in 3D culture systems that study cancer biology. In order to efficiently benefit from the culture chamber, a chemotactic gradient should be formed in between each cell type cultured in the outer wells and the central chamber comprising the melanoma VMM15 cells. In light of the above, we considered collagen type I for the development of the chamber given that collagen gels have been proven to serve as better mimic in terms of molecule diffusion for the development of new models [27] especially considering the high abundance of this protein in the extracellular matrix of tumors [28]. Our results show that the implemented chamber organization allowed for molecule diffusion dependent on molecular weight, with a faster diffusion occurring for molecules of lower molecular weight. Taken as known that the chosen molecular weights fall in the range of sizes corresponding to chemokines and cytokines, it can be assumed that molecules that display a chemotactic role may will be able to diffuse throughout the gel in a similar fashion to the fluorescent tagged albumin and dextran.

One must consider that mechanical properties of biomaterials impact cellular behavior [29,30] therefore, there is a need to meet physiologically relevant parameters. Additionally, the viscoelastic nature of the tumor microenvironment is ever clearer [24,31,32], which makes it of upmost importance to take into consideration such features when modeling tumor cell behavior *in vitro*. Hydrogels tend to present higher a G' while displaying a lower G'' , this is indicative of the materials potential to store deformation energy with small dissipation therefore behaving predominantly like solids [33]. From the presented amplitude sweeps, not only was this behavior well evident as it also is representative of the behavior for a range of soft tissues [34].

Given the demonstrated molecule permeability throughout the collagen I matrix, the chamber was next populated with relevant stromal cells that would allow the study of their chemotactic response in the presence of tumor cells. For the stromal compartment, ECs, stem cells, and fibroblasts were chosen to represent the tumor microenvironment and were seeded in the outermost wells while melanoma tumor cells were cultured in the central chamber.

Several different forms of migration, “taxis”, have been described overtime [35]. Here, the focus was essentially on cell migration driven by chemical gradients, but it must be considered that given that the

developed chemotaxis chamber is an *in vitro* assay in which cells are exposed to a given concentration of collagen which in turn determines the mechanical properties of the system, the observed cell migration may also be impacted by other mechanisms such as durotaxis and alignotaxis. In these mechanisms, cell migration is driven preferentially toward and within substrates with a higher stiffness [36] and mediated by the reorganization of collagen from curly, coiled fibers to strait aligned fibers promotes the directed, rapid and persistent migration of tumor cells [37,38].

A dynamic interplay is known to take place between tumor cells and stromal cells within the tumor microenvironment. Different cell-cell interactions take place giving rise to the recruitment of endogenous stromal cells into the tumor microenvironment by tumor cells [39–42] or, on the other end of the spectrum, to the migration of tumor cells following a chemotactic gradient set by noncancerous cells [43,44]. Several different elements are known to play a part in this guided migration as the case of cell adhesion molecules (CAMs), chemokines, cytokines and growth factors [45–49]. Interplay with the ECM has also been shown to promote tumor progression and poor prognosis [50,51]. An example of one of the contributors to this are MMPs which are tied to the infiltration of cancer cells and equally metastasization [6–8].

In the context of this work, a directed migration of the tumor cells from the inner well outward towards the outer wells would be possible as suggested by studies showing that tumor cells are capable of creating local gradients of matrix metalloproteinase-mediated degradation of ECM to drive their directed migration [52–54]. However, it was verified that it was the fibroblasts and the stem cells that were migrating towards the center of the well. This comes into agreement with previous works showing that VMM15 melanoma cells benefit from the assistance of stromal cells, namely hASCs and fibroblasts, to facilitate the invasion of a 3D basement membrane matrix-embedded heterotypic tumor model. Furthermore, several other studies have demonstrated that tumor cells possess a chemoattractant capacity towards stromal cells [55–58]. These results support that in fact chemotaxis gradients are formed within the tumor microenvironment and that tumor cells play an important role in how stromal cells, such as, fibroblasts and stem cells migrate. To further evidence this observation, understanding the key molecules responsible for this behavior is crucial and should be of focus in future studies. Understanding intracellular communication is of utmost importance to obtain answers in how to address cancer invasion metastasization, which can be achievable with this 3D culture platform.

While cell morphogenesis has always been linked to cell migration, a direct correlation between how cells undergo migration under chemotactic gradients has been missing. A while back, filopodia

distribution and their dynamics were shown to be dictated by the gradient of the Cxcl12a chemokine [59]. They observed that a graded distribution of Cxcl12 determined the number, distribution and dynamics of the formed filopodia protrusions. While herein, we haven't performed such in depth analysis, the correlation of the observed directional migration with that of VMM15 melanoma cell secretome would be of high relevance while analyzing whether a similar behavior to that reported by Meyen et al. [59] is reproduced. This could in turn, open the path for novel therapeutics that could limit non-tumor cell recruitment to the microenvironment and therefore hamper tumor progression.

VI-5. CONCLUSION

Here, a 3D collagen chamber capable of supporting the establishment of chemotactic gradients between several known cells present in the tumor microenvironment was developed, providing a tool to better understand cellular cross-talk, how it regulates cell migration and recruitment. The formation of diffusion gradients was observed using model biomolecules and these were dependent of the molecular weight of the biomolecule. Upon seeding of stromal and melanoma cells in the different wells, differential migration of fibroblast and stem cells was observed towards the VMM15 melanoma cells suggesting that chemotactic gradients were established, potentially mimicking intratumoral crosstalk between stromal and tumor cells. This demonstrates the potential of the developed chamber to be used as a tool to study and characterize mechanisms underlying tumor cell migration and metastasis.

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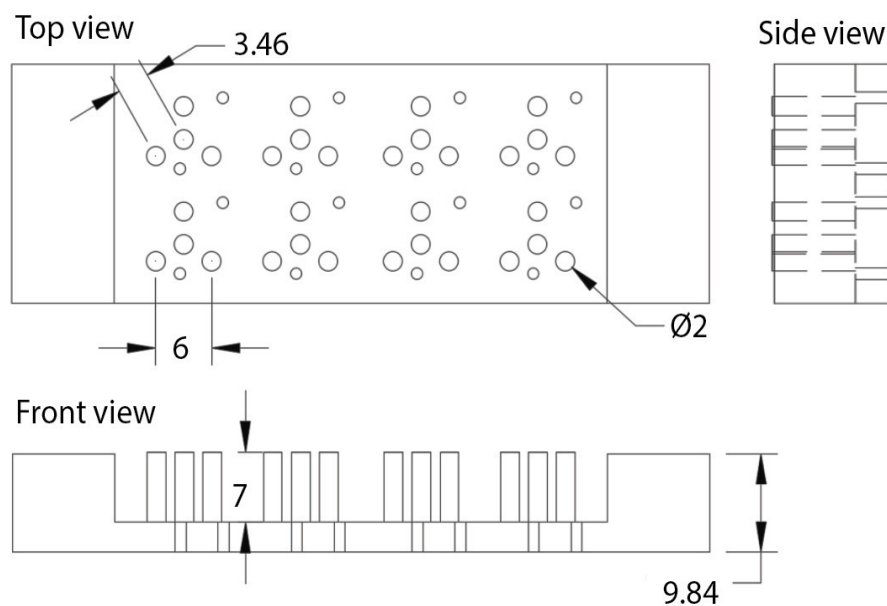
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VI-7. SUPPLEMENTARY INFORMATION



Supplementary Figure VI-1 Computer-aided design of chemotaxis chamber master mold.

2-D view of top, side and front view of the master mold used to imprint the hydrogel chemotaxis chambers. Dimensions are in mm.

SECTION 4

CONCLUDING REMARKS

CHAPTER VII

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

CHAPTER VII

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

The tumor microenvironment is a highly complex system with several mechanisms that support tumor progression and invasion, making the treatment of this disease a significant challenge. In this context, different strategies were herein proposed in order to push forward the fields of cancer therapy and *in vitro* 3D cancer models.

One of the most difficult issues to address in cancer therapy is the capability of cancer cells to undergo immune evasion through the inhibition and modulation of anti-tumoral responses while displaying a high susceptibility for genome instability and mutation. The field has addressed these difficulties with the introduction of different immunotherapeutic strategies such as combinatorial treatments and T cell transfer therapy. The success of these adoptive therapies depends on the methods used for ex vivo outgrowth and expansion of T cell populations, as in the case of IL-2, which is used to trigger the rapid proliferation of these cells and may ultimately lead to T cell exhaustion. In addition to the rapid expansion of cells, other parameters such as differentiation, activation and function are of the utmost importance for the effective anti-tumor activity of cells when reinfused into the patient as undesirable metabolic phenotypes may impact cell activity. In Chapter IV, we describe the production of gellan gum nanoparticles for application in *in vitro* T cell expansion. Although gellan gum has been used in the field of tissue engineering and regenerative medicine, in this study, we explored its potential for the expansion of T cells. Through the method described here, we obtained a proliferative population of CD4⁺ cells that produced high levels of IL-2 when compared to commercially available alternatives while maintaining low levels of CTLA4, a well-known immune checkpoint protein, contributing to the extended functionality of our activator bead system. These activated cells also displayed an increased production of cytotoxic molecules granzyme B and perforin, reaffirming the potential of this system for the *in vitro* production of viable and tumor-challenging lymphocytes and once again proving the potential of natural-based polymers. We also explored how different mechanisms used for the attachment of functional molecules to these systems can influence their effectiveness which has been a topic lacking in the literature until this point. We have shown that the use of biotin-avidin, which allows for the orientation of molecules when tethered to a desired system, triggers a better cytotoxic T cell profile at the protein level,

as suggested by the increased production of perforin and granzyme B. Additional functionality studies using tumor cell models are of great importance for further validation of this system. Additionally, we have overcome two nanoengineering problems involving construct design, the first being antibody orientation and the second being the use of certain conjugation procedures that may reduce antibody functionality. Antibody density, however, remains an issue that may be addressed through the use of alternative antibody formats lacking the Fc region, some of which are Fabs, diabodies, single-chain variable fragments, bispecific antibodies and variable domains.

Being the identification of novel therapeutics undoubtedly crucial for the treatment of cancer, biomimetic tumor models capable of faithfully representing the tumor microenvironment are crucial tools in the initial steps of testing such therapeutics. The field of 3D cancer models has sustained a great number of developments with heterotypic cell models bearing a high level of similarity to previously described cellular interactions in the native cancer tissue; however, there is still a requirement for models with a more complex architecture that better mimic cell-cell and cell-ECM crosstalk. With advances in the development of these 3D tumor models, several different strategies such as organotypic 3D models and microfluidic systems have been used. However, spheroids remain one of the most widely used systems for testing novel therapeutic systems in the laboratory. In Chapter V, we attempted to add to the field by developing a heterotypical 3D tumor model by exploring the novel methodology of assembloid-based spheroid systems applied in the development of 3D melanoma tumor models. We observed that co-culture of hDMECs and hDFbs at a 1:4 ratio allowed for the establishment of highly complex endothelial networks observed up to 14 d in culture. When these endothelial networks were used as one of the three cellular compartments with the addition of co-cultured hDMECs and hASC, as well as VMM15 melanoma cells and hDFbs, a structured organization of the cellular constituents in a layered fashion was observed. Endothelial cells appeared to organize centrally in the core, originating lumen-like structures with an increased deposition of collagen in their surroundings, whereas GP100⁺ melanoma cells were limited to the surface of the assembloid structure, enveloping all of the remaining cells. This combinatorial culture additionally led to an increased invasive potential of this system in a 3D invasion model observed by the area of invasive edge accompanied by a higher production of MMP-2, confirming the role of stromal cells in “cooperative invasion” of tumor cells. While altogether these results point to the superiority of the assembloid methodology as a stronger model to study cellular interactions in a simulated tumor microenvironment, there are still several topics to address such as a more comprehensive analysis of the individual gene signatures specific to each cell type, as well as looking into earlier time points to better

comprehend how these cells organize themselves in this tumor model and how this impacts downstream tumor cell behavior.

Finally, in Chapter VI, given the results obtained through the combinatorial culture of individual stromal compartments for the assembly of a 3D melanoma tumor model, we proceeded to unravel the mechanisms behind the cooperative invasion observed earlier and whether chemotaxis plays a role in the invasion of VMM15 tumor cells. For this effect, as described above, a 3D chemotaxis chamber with equidistant wells that allowed for the culture of four distinct cell types was created. Differential molecular diffusion was observed when two distinct molecular weights were studied, with a faster diffusion rate observed for molecules with a lower molecular weight. When performing the culture of hDMECS, hDFbs and hASCs in the outer wells while keeping the VMM15 melanoma cells in the central well, it was observed that hASCs and hDFbs presented a directional invasion throughout the collagen matrix towards the tumor cells. Additionally, it was observed that hDFbs presented a greater area of invasion when compared to the hASCs, thus shedding some light on how tumor cell invasion may occur *in vivo*, mediated via a chemotactic effect observed towards hASCs and hDFbs, which then allows for the cooperative invasion of tumor cells. The open platform of this system allows the personalization of different basement membrane matrix (BMM) alternatives, mechanical properties, well shapes and dimensions supporting the introduction of other cells types into the platform, as in the case of immune cells known for their role in immunosuppression and angiogenesis contributing to tumor survival.

The paths we have explored throughout this body of work have opened new doors in the exploration of different cancer-related topics. The advantages of using natural-based polymers for the development of therapeutic platforms has been addressed, highlighting their high tunability for the rapid expansion of cells for adoptive therapy. Additionally, the development of 3D tumor models based on the assembly of smaller stromal multicellular spheroids proved to be a strong alternative to the currently used *in vitro* tumor models with plenty of room for future studies, while the creation of novel 3D culture systems allows for a better understanding of cell interactions and dynamics that occur *in vivo*. The knowledge provided by the field of tissue engineering and regenerative medicine may therefore allow us to take a step closer towards more effective forms of cancer therapeutics and *in vitro* tumor models that will drive the future of cancer studies.