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Dissertação no âmbito do Mestrado em Investigação Biomédica, no ramo de Oncobiologia, orientada pela Professora Doutora Cristina Maria Godinho Pires João e pela Professora Doutora Ana Bela Sarmento Antunes da Cruz Ribeiro e apresentada à Faculdade de Medicina da Universidade de Coimbra

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Ernesto Maciel (Vovô Nené) | à beira-mágoa

Acknowledgments

To everyone who, directly or indirectly, contributed to the attainment of this project, thank you.

First of all, I would like to thank my supervisor, Professor Cristina João, who always challenges us to be better and reach further. Thank you for all the trust, guidance, availability and patience and, above all, for all the calmness and sense of security transmitted, even in the most hectic times. It was a real pleasure to be able to work and learn in an environment fulfilled with knowledge. I would also like to thank Professor Ana Bela Sarmento-Ribeiro for the kindness, promptitude and availability in accepting to be part of this journey and this project.

Thank you to the HematoGirls, Ana, Bruna, Carolina, Emilie, Filipa and Joana, who welcomed me and made me feel included from the first day, with smiles, and spirited conversations, and who were always available to answer my questions and help me, even in-between tasks. Thank you to Raquel, who advised, listened and encouraged me, and whose passion for science motivated me, even without realizing it (and for the countless car rides, thank you!).

I would also like to thank Doctor Adriana Sanchéz Danés and Sofia, for all the help and troubleshooting. Likewise, I want to thank Cátia, for always receiving me and my questions with good mood and a happy smile on her face.

I would also like to thank all the Champalimaud Foundation collaborators, who were always available to help and share their knowledge, making this project better and more complete. Especially, I would like to thank the Flow Cytometry; Histopathology; Advanced BioImaging and BioOptics; and Glass Wash and Media Preparation platforms. I also thank the doctors, nurses and administrative staff of the Champalimaud Foundation Hematology Unit, and, of course, all the patients who accepted to be part of this study and allowed it to be carried out.

To my Parents, I want to thank you for raising me surrounded by love, affection, joy and education. Thank you for all the trips, tours, visits to museums and activities we always did, and for instilling in me a joy for learning and the notion that we should work towards our goals. Thank you for never putting obstacles in my way and my dreams. I love you.

To my brother, who, although not really knowing what I was working on, asked me several times how the experiments were working “over there in the laboratory”. Thank you for all the clever jokes you drop here and there, and for always looking at me with pride and admiration.

A very special thank you to my Grandparents, Beti and Nené, without whom none of this would be possible, for the beautiful house by the beach, that became my home in the last year, for all the support and motivation, for all the phone calls to know if I was resting and eating well, and for all the emails wishing me a good week at work. To Grandpa Tó, thank you for all the stories and lessons, and for always welcoming me with a cheerful smile.

To Luís, who listens to my stories and dilemmas every day and with his natural lightness makes me feel like I can overcome all obstacles. To my favorites, Carlote and Curado, who, although far away, always root for me and fill my heart with happiness.

Everyone who has been present during this journey gave me, in one way or another, the strength to face this year, that has been both the best and the hardest year of my life. It has been full of everything one can imagine it may happen while doing research: challenges, setbacks, disappointments, and a lot of work; but most of all, it has been a year full of opportunities, discoveries, and learning. As I left my comfort zone, I have grown, as a scientist, and also as a person, and the thesis book you are holding is the rewarding result of everything I have accomplished during this work.

So, to all of you, thank you.

Agradecimentos

A todas as pessoas que, direta ou indiretamente, contribuíram para a realização deste projeto, obrigada.

Antes de mais, quero agradecer à minha Orientadora, Professora Doutora Cristina João, que nos desafia sempre a sermos melhores e a chegar mais longe. Obrigada por toda a confiança depositada, por toda a orientação, por toda a disponibilidade e paciência e, acima de tudo, por toda a calma e segurança transmitidas, mesmo nas alturas mais agitadas. Foi um verdadeiro prazer poder trabalhar e aprender num ambiente tão repleto de conhecimento. Quero também agradecer à Professora Doutora Ana Bela Sarmento-Ribeiro, pela simpatia, prontidão e disponibilidade com que aceitou fazer parte deste percurso e deste projeto.

Obrigada às HematoGirls, Ana, Bruna, Carolina, Emilie, Filipa e Joana, que me acolheram e fizeram sentir incluída desde o primeiro dia, com sorrisos e conversas animadas, e que sempre se mostraram disponíveis para responder às minhas perguntas e ajudar, mesmo entre tarefas. Obrigada à Raquel, que me aconselhou, ouviu e encorajou, e cuja paixão e gosto pela ciência me motivaram, mesmo sem se dar conta (e pelas inúmeras boleias, obrigada!).

Quero também agradecer à Doutora Adriana Sanchéz Danés e à Sofia, por toda a ajuda e discussão de ideias. Igualmente, agradeço à Cátia, por receber sempre as minhas perguntas com boa disposição e um sorriso alegre.

Agradeço também a todos os colaboradores da Fundação Champalimaud, que sempre se disponibilizaram para ajudar e partilhar os seus conhecimentos, contribuindo para que este projeto se tornasse ainda melhor e mais completo. Em especial, quero agradecer às plataformas de Citometria de Fluxo; de Histopatologia; de Advanced BioImaging and BioOptics; e de Glass Wash and Media Preparation. Igualmente, agradeço aos médicos, enfermeiros e administrativos da Unidade de Hematologia da Fundação Champalimaud, e claro, a todos os doentes que aceitaram fazer parte deste estudo e permitiram a sua concretização.

Aos meus Papás, quero agradecer por me terem criado rodeada de amor, carinho, alegria e educação. Obrigada por todas as viagens, passeios, visitas a museus e atividades que sempre fizemos, e por me terem incutido o gosto em aprender e a noção de que devemos trabalhar pelos nossos objetivos. Obrigada por nunca terem posto entraves às minhas vontades e aos meus sonhos. Adoro-vos.

Ao meu Maninho, que mesmo sem saber bem o que é que eu andava a fazer, me perguntava como é que estavam a correr as experiências “aí no laboratório”. Obrigada por todas as piadas inteligentes que vais largando aqui e ali, e por olhares sempre para mim com orgulho e admiração.

Um obrigada muito especial aos meus Avós, Beti e Nené, sem os quais nada disto seria possível, pela bonita casa ao pé da praia, que se tornou a minha base no último ano, por todo o apoio e motivação, por todos os telefonemas para saber se andava a descansar e a alimentar-me bem, e por todos os emails a desejar uma boa semana de trabalho. Ao Avô Tó, obrigada por todos os ensinamentos, e por me receber sempre com um sorriso bem-disposto.

Ao Luís, que todos os dias ouve as minhas histórias e dilemas e com a sua leveza natural me faz sentir capaz de ultrapassar todos os obstáculos. Aos meus preferidos, Carlote e Curado, que mesmo estando longe torcem sempre por mim e me enchem o coração.

Posso dizer que todos aqueles que estiveram presentes ao longo deste percurso me deram, de uma forma ou de outra, força para encarar este ano, que foi o melhor, mas também o mais difícil da minha vida. Este ano teve tudo aquilo que se imagina que possa acontecer quando se faz ciência: desafios, contratempos, desilusões, e muito trabalho; mas, acima de tudo, muitas oportunidades, descobertas e aprendizagens. Ao deixar a minha zona de conforto, cresci, enquanto cientista e enquanto pessoa, e a tese que têm nas mãos é o resultado recompensador de tudo aquilo que consegui alcançar.

Por isso, a todos, obrigada.

Acquired Competence

I started to work in the Myeloma and Lymphoma Research Group in August 2021, setting the preliminaries for the development of my Master Thesis Project. During this period, I learned about different techniques and studied about the current knowledge on the field of interest of this project. After approval, I began to carry out the present study, and it was a pleasure to develop this effort. During this year, I was able to expand my knowledge on theoretical and practical aspects of laboratory research and science.

I had the opportunity to enroll on several activities and further develop soft skills, including time management, teamwork and problem solving. I have improved my critical thinking and verbal and written communication abilities and practiced how to process information and make connections.

I was able to grow and improve my experimental techniques capabilities, thanks to routine tasks in the laboratory context and courses that I took part in:

I attended the *Practical Course on Animal Use for Functions A, C & D (Rodents)*, recommended by the Federation of European Laboratory Animal Science Association, obtaining my personal accreditation to work with rodent models. This allowed me to provide support in experiments with six- to eight-weeks old BALB/CByJ female mice, maintained in a specific and opportunistic pathogen free facility of Champalimaud Foundation. Briefly, I was responsible for the constant monitoring of the mice, that were known to eventually develop paraplegia of the hind limbs after injection with a mouse multiple myeloma cell line, and giving them the adequate care (e.g., placing food pellet on the floor of the cage for easier access; changing lids of the water bottles from regular to longer ones). I was also able to assist on procedures, that included intravenous injections of tumor cells in the tail, and mice sacrifice, with organ collection (e.g., femurs and tibias) and bone marrow (BM) flushing, for characterization of the tumor niche.

Later, I participated in a *Necropsy Workshop*, held at Instituto Gulbenkian da Ciência, Oeiras, under the umbrella of COLife, which is a partnership between the Lisbon and Oeiras institutes. This workshop aimed at providing biomedical scientists with basic knowledge of animal anatomy and histology, as well as necropsy procedures. It included a very complete practical component, where each participant was able to train, question and test different procedures, that included injections, cardiac puncture and thoracotomy with blood sampling, organ isolation and dissection, whole body perfusion and lung inflation. Organ isolation methods ranged from simple procedures, such as dissection of thoracic organs and hind limbs, to more complex ones, such as the isolation of the brain, eye and optic nerve, and techniques like the "Open Swiss-Roll", for complete intestinal tissue examination.

I also participated on the first edition of the hands-on *Advanced Course on Organoid Models*, at i3S – Institute for Research and Innovation in Health, Porto. This 4-day intensive course mainly focused on the potentialities of organoids in the fields of cancer and infection in biomedical research. It comprised a theoretical component with experts on the field, and practical lessons, using gastric, intestinal and lung organoids as examples for applying different

culture set-up and maintenance procedures. Additionally, insight on how to plan the experiments and analyze the obtained data was given. The methods focused on the processing of organ tissue for culture, stem cell handling principles, recovery of organoids, and readout techniques such as confocal imaging, histology, and molecular biology (e.g., Reverse Transcription-Polymerase Chain Reaction).

Furthermore, during the time of my project, I was able to learn the basic principles of different equipment for samples analysis, including flow cytometry and microscopy hardware. I also proceeded to the analysis of the collected data during the whole duration of the experiments related to my thesis project, using different software, such as FlowJo™ v10.8.0 (FlowJo, LLC; BD Bioscience), for analysis of flow cytometry data, ZEN 3.4 (blue edition; ZEISS) and ImageJ (Fiji; National Institutes of Health), for confocal and brightfield imaging analysis. I also conducted statistical analysis of the obtained results, using GraphPad Prism (Dotmatics), stimulating my capacity to comprehend how to select the appropriate statistical test to apply in each situation, and understand and interpretate the meaning of the information collected.

During my master thesis' preparation time, I also collaborated in other research projects of the group, which resulted in my first co-authoring of a peer-reviewed scientific paper entitled "Multiple Myeloma-derived Extracellular Vesicles Modulate the Bone Marrow Immune Microenvironment" (Lopes et al., 2022), published in *Frontiers in Immunology*. Our group recently showed that the content of extracellular vesicles (EVs) in Multiple Myeloma (MM) patients significantly differs from healthy subjects. Specifically, the level of EVs cargo (protein/particle ratio) is associated to MM overall survival. Moreover, functional enrichment analysis revealed that the protein content of EVs from MM patients were mainly related to immunoparesis (Ferreira et al., 2022). This work was published in *Frontiers in Oncology*. This led us to decipher the immune alterations induced by MM-derived EVs using a MM mouse cell line called MOPC315.BM (Hofgaard et al., 2012). This cell line has tropism to the BM and faithfully replicates the major characteristics of MM disease seen in humans (Hofgaard et al., 2012; Lopes et al., 2022; Ziouti et al., 2020). In this study, I helped performing proteomic functional analysis using adequate software to determine the protein expression profile of EVs that were previously isolated from the mouse MM cell line MOPC315.BM, and of EVs from the BM supernatant of both control and MOPC315.BM-bearing mice. For this, I used the rodent UniProt® database in FunRich, which is a functional enrichment analysis tool software. Altogether, we showed that MM-derived EVs promote immune suppression, through modulation of lymphocytes, strengthening the interest on further studies on EVs as targets for MM treatment.

Related to my master thesis project, I was given the opportunity to write and to be the first author of a review manuscript on three-dimensional (3D) multiple myeloma models, entitled "Patient-derived Multiple Myeloma 3D Models for Personalized Medicine", that is currently in submission to *International Journal of Molecular Sciences* (MDPI), for peer review. In this review, we critically analyzed and explained the strengths and limitations of currently existing patient-derived *ex vivo* three-dimensional multiple myeloma models. We also dissected their biochemical and biophysical properties, molecular and cellular characteristics,

and their potential for drug testing and molecular therapeutic targets discovery. Lastly, we discussed the remaining challenges in the field and gave some understanding on how to achieve a more biomimetic and accurate 3D MM BM *ex vivo* model. Part of this review is included in Chapter 2 of this thesis (State of the Art).

Finally, I had the chance of presenting my work to the Champalimaud Research community, on the Champalimaud Internal Seminar Series (CISS), which allowed me to practice public speaking and interaction with an audience. More recently, I submitted an abstract about the work we have been developing with the 3D MM model, to the Champalimaud Research Symposium 2022, Lisbon, which has been selected for a poster presentation and I will be presenting it in October 2022.

Abstract

Multiple myeloma (MM) is characterized by the clonal expansion of terminally differentiated B cells in the bone marrow (BM), overproduction of monoclonal immunoglobulins and end-organ damage, such as hypercalcemia, renal insufficiency, anemia, or bone lesions (CRAB). Accounting for 10% of all hematological malignancies, this is a challenging, progressive, and heterogeneous disease, that still remains without a cure. A significant part of the patients evolves between periods of remission and relapse, and most of them develop drug resistance to all therapies and maintain a poor overall survival. Despite the progresses made in the last years, there are still several aspects of cellular and molecular nature that need to be further studied and addressed, to fill the gaps regarding MM pathophysiology. This is crucial to assess the personal MM characteristics of each patient and the best, safest and most relevant treatment approach. MM cells grow within the BM niche, and the crosstalk between these tumoral cells and its microenvironment plays a critical role in disease progression, allowing for immune evasion, proliferation, migration, and survival. Current research performed *in vitro* or using MM animal models often leads to poor predictions when compared to clinical outcomes. The *in vitro* models are mainly two-dimensional and use cell line monocultures that have lost their bone marrow dependence, representing an unfaithful recapitulation of tissue architecture, biochemical intrinsic properties, cell interactions, or the personal heterogeneity aspects of the disease. Mouse models also have limitations, as the murine immune system varies significantly from the human one. Immunodeficient mice impede the study of immune and cancer cells interactions in the microenvironment, and humanized models are expensive and time-consuming. In this project, we developed and characterized a three-dimensional *ex vivo* co-culture model of MM, capable of mimicking each patient's BM microenvironment with its cellular, biophysical and biochemical cues. Whole samples were obtained from BM aspirates of MM patients, and cells were embedded in a Matrigel matrix, after lysis of red blood cells. After determining the optimal culture conditions of our model, multiparametric flow cytometry was used to identify and quantify the different cell populations present inside the matrix. Confocal imaging was used to spatially assess cellular distribution and death within the model. Resistant and sensitive MM cell lines were cultured in 3D and treated with lenalidomide, to validate the use of the model for studying drug response. This patient-derived 3D *ex vivo* model was able to sustain 60% viability of seeded cells after 14 days of culture. After 7 days in culture, BM cellular subpopulations presented similar proportions to patient sample on day 0, including fibroblasts, myeloid and T cells, and MM cells, indicating a good recapitulation of the *in vivo* MM-BM setting. MM cell lines responded to lenalidomide treatment according to their cell line characteristics. Resistant cells kept viability, and proliferative and migrative activity, and sensitive cells started to die, and lost their activity, compared to the control group. This model may provide a powerful tool for patient-individualized profiling and holds potential for easy and low-cost research and clinical applications.

Key words: multiple myeloma, bone marrow microenvironment, 3D model, *ex vivo* model, personalized therapy

Resumo

O mieloma múltiplo (MM) é caracterizado pela proliferação anormal de células B diferenciadas, na medula óssea (MO), aumento da produção de imunoglobulinas monoclonais e lesões em diferentes órgãos, incluindo hipercalcemia, insuficiência renal, anemia e lesões ósseas (CRAB). O MM representa 10% das neoplasias hematológicas, sendo uma doença heterogénea e progressiva, para a qual ainda não existe cura. A maior parte dos doentes oscila entre períodos de remissão e recaída, acabando por desenvolver resistência às terapias, com uma baixa taxa de sobrevivência. Apesar dos notáveis avanços feitos nos últimos anos, existem ainda vários aspetos relacionados com a natureza celular e molecular do mieloma que requerem uma maior compreensão. Esta informação é crucial para poder determinar as características fisiopatológicas pessoais de cada doente, bem como selecionar a melhor opção terapêutica, em termos de segurança e eficácia. As células do mieloma crescem no microambiente medular. A interação entre as células tumorais e o ambiente envolvente desempenha um papel crítico na progressão da doença, permitindo a evasão imunológica, proliferação, migração e sobrevivência. Atualmente, a investigação feita *in vitro* ou com modelos animais de MM, acaba por resultar em previsões pouco precisas, quando comparadas com os resultados clínicos. Os modelos *in vitro* são maioritariamente culturas 2D e monoculturas com linhas celulares, que perderam a sua dependência medular característica, constituindo uma representação imperfeita da arquitetura do tecido, propriedades bioquímicas, interações celulares ou aspetos da heterogeneidade pessoal da doença. Os modelos animais também apresentam limitações, uma vez que o sistema imunitário murino varia significativamente do humano. Murganhos imunodeficientes impedem o estudo das interações entre as células imunes e tumorais no microambiente medular, e os modelos humanizados são caros e demorados. Neste projeto, desenvolvemos e caracterizámos um modelo tridimensional de co-cultura *ex vivo* de MM, capaz de mimetizar o microambiente da MO de cada doente, com as suas propriedades celulares, biofísicas e bioquímicas. Amostras de aspirado medular foram recolhidas de doentes com MM, e as células foram incorporadas numa matriz de Matrigel, após lise dos eritrócitos. Depois de determinar as condições ideais de cultura do nosso modelo, as diferentes populações celulares presentes na matriz foram identificadas e quantificadas, através de citometria de fluxo. A distribuição e morte celular no modelo foram avaliadas espacialmente, através de microscopia confocal. Linhas celulares de MM, resistentes e sensíveis a tratamento, foram incluídas no modelo 3D e tratadas com lenalidomida, para validar o uso do modelo em testes de resposta a agentes terapêuticos. Este modelo 3D *ex vivo*, derivado de amostras de doentes, manteve 60% de viabilidade das células cultivadas, durante 14 dias. Depois de 7 dias em cultura, as subpopulações celulares da medula óssea apresentaram proporções semelhantes às da amostra do doente ao dia 0, incluindo fibroblastos, células T e mielóides, e células de mieloma, indicando uma boa representação do cenário medular *in vivo*, em contexto de mieloma múltiplo. As células do mieloma responderam ao tratamento com lenalidomida de acordo com as características da linha celular. As células resistentes mantiveram a viabilidade e a sua atividade

proliferativa e migratória, e as células sensíveis começaram a morrer, perdendo a sua atividade, em relação ao grupo controlo. Este modelo pode assumir-se como uma ferramenta relevante para caracterização individualizada dos doentes e apresenta potencial para estudos e aplicações clínicas, fáceis e de baixo custo.

Palavras-chave: mieloma múltiplo, microambiente medular, modelo 3D, modelo *ex vivo*, terapia personalizada

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List of Abbreviations

2D – Two-dimensional

3D – Three-dimensional

3DTEBM – Three-dimensional tissue-engineered bone marrow

3Rs – Three Rs (Replacement, Reduction, and Refinement)

APC – Antigen-presenting cell

BaM – Basement membrane

BCMA – B-cell maturation antigen

BM – Bone marrow

BMAT – Bone marrow adipose tissue

BMMC – Bone marrow mononuclear cell

BMSC – Bone marrow stromal cell

CD – Cluster of differentiation

CO_2 – Carbon dioxide

CRAB – hyperCalcemia, Renal failure, Anemia and Bone disease

CTLA – Cytotoxic T-Lymphocyte associated antigen

CXCR – C-X-C chemokine receptor

DAPI – 4',6 -Dimidino-2-phenylindole

DC – Dendritic cell

DMSO - Dimethyl sulfoxide

ECM – Extracellular matrix

EGF – Epidermal growth factor

EMMA – *Ex vivo* Mathematical Myeloma Advisor

EV – Extracellular vesicle

FBS – Fetal bovine serum

FGF – Fibroblast growth factor

GAG – Glicosaminoglycan

HA – Hyaluronic acid

HIF – Hypoxia inducible factor

HSC – Hematopoietic stem cell

HUVEC – Human umbilical vein endothelial cell

ICAM – Intercellular adhesion molecule

Ig – Immunoglobulin

IGF – Insulin-like growth factor

IHC – Immunohistochemistry

iHDAC – Histone deacetylase inhibitor

IL – Interleukin

IMiD – Immunomodulatory drug

iPSC – Induced pluripotent stem cell

mAb – Monoclonal antibody

MCP – Monocyte chemoattractant protein

MDE – Myeloma defining events

MDSC – Myeloid-derived suppressor cell

MGUS – Monoclonal gammopathy of undetermined significance

MIP – Macrophage inflammatory protein

MM – Multiple myeloma

M-MDSC – Monocytic-myeloid-derived suppressor cell

MMP – Matrix metalloproteinase

MSC – Mesenchymal stem cell

MVD – Microvessel density

NE – norepinephrine

NF-kB – Nuclear factor kappa B

NK – Natural Killer

ns – not statistically significant

P/S – Penicillin/Streptomycin

PBS – Phosphate buffered saline

PD – Programmed cell death protein

PDGF – Platelet derived growth factor

PD-L – Programmed cell death ligand

PDTX – Patient-derived tumor xenograft

PFA – Paraformaldehyde

PG – Proteoglycan

PI – Proteasome inhibitors

PIM – Proto-oncogene serine/threonine-protein kinase

PMN-MDSC – Polymorphonuclear-myeloid-derived suppressor cell

Pref – Preadipocyte factor

PrI – Propidium iodide

RANK - Receptor activator of NF-κB

RANKL – Receptor activator of NF-κB ligand

RBC – Red blood cell

RBCL – Red blood cell lysis

RCCS – Rotary cell-culture system

rEnd-rBM – Reconstructed Endosteum-Bone marrow

R-ISS – Revised International Staging System

RNA - Ribonucleic acid

ROCK – Rho kinase

ROS – Reactive oxygen species

rpm – rotations per minute

RT – Room temperature

SAM - Synergy Augmented Model

SDF – Stromal cell derived factor

SMM – Smoldering multiple myeloma

STAT – Signal transducer and activator of transcription

TAM – Tumor associated macrophage

TCR – T-cell receptor

TGF – Transforming growth factor

TNF – Tumor necrosis factor

Treg – Regulatory T cell

VCAM – Vascular adhesion molecule

VEGF – Vascular endothelial growth factor

VLA – Very late antigen

XPO – Exportin

β-AR – β-adrenergic receptor

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1. Introduction and Thesis Outline

According to the most updated Global Cancer Statistics, multiple myeloma (MM) is the third most common blood cancer worldwide (Sung et al., 2021). This challenging and progressive disease still remains incurable, with a 5-year survival rate of less than 50% (Rajkumar et al., 2014). Although substantial progress has been made in the last years, in both staging and treatment, most patients eventually relapse, with more than 90% of the relapsed patients developing drug resistance (de la Puente & Azab, 2016). MM treatment is challenged due to the heterogeneous nature of the disease, in terms of symptoms, phenotype and genomic aberrations. Therefore, there is still a need to find a better way of evaluating each patient characteristics and determining the precise best treatment approach for each case.

As the cells involved in this hematological malignancy are dependent on their microenvironment, the BM niche plays a critical role in disease progression, allowing immune evasion, proliferation, survival, migration and drug resistance (Lopes et al., 2021). Thus, developing a platform that allows for the MM cells to grow, maintaining their viability and integrity, while carrying out their functions and communicating with their surrounding cells is crucial, in order to advance in this area of research. Yet, the existing pre-clinical model systems still lack different key aspects for the evaluation of effective patient-specific clinical situations, leading to incongruities when compared to clinical outcomes. *In vitro* models are mainly two-dimensional and do not faithfully recapitulate tissue architecture, biochemical intrinsic properties, cell interactions, or the personal heterogeneity aspects of the disease (Knight & Przyborski, 2015). Mouse models also do not fully replicate the human tumor microenvironment, since murine and human immune systems vary significantly (Mestas & Hughes, 2004). Moreover, only 5% of the new anti-tumor molecules end up obtaining clinical approval (Sharma et al., 2011; Zhang et al., 2020), reinforcing the importance of unveiling molecular mechanisms associated with the disease and resistance development, and identifying therapies with less toxicity and meaningful efficacy.

Advances in cancer cell biology, biofabrication, and microengineering technologies have led to the development of more complex three-dimensional models. These allow (1) the culture of whole BM patient samples, creating a model that has each patient own cells, in a three-dimensional organization; (2) cells to establish and interact with each other; and (3) the presence of extracellular matrix, as in the *in vivo* MM niche environment. Moreover, 3D models allow the recreation of nutrient, oxygen and drug gradients, which will increase the ability of studying disease mechanisms and precisely predicting drug sensitivity/resistance. On top of that, with the increasing emphasis on animal welfare, having a culture model that can recreate the *in vivo* BM setting is appealing and needed, as it means that the use of laboratory animal models can be reduced (Workman et al., 2010).

Nonetheless, the translation of these models to clinical setting has been impaired by the disregarding for the incorporation of primary cells and/or tissues into culture, rather focusing on immortalized cell lines (Sarin et al., 2020). While this is, undoubtably, a necessary step to establish and validate new models, the necessity of including patient-derived samples is becoming more and more evident.

Despite the existing MM models, that already allow culture of whole BM samples with promising results, complexity is still a problem that hampers reproducibility and its use in clinical day to day practice. Therefore, our goal was to develop a model that would allow the culture of the whole BM patient sample, being complex enough to mimic the *in vivo* environment but still simple enough to establish and recreate. For that, we decided to create a three-dimensional *ex vivo* co-culture model of MM, in a Matrigel matrix, combining its ability to support a wide range of cell types in 3D culture (Lee et al., 2007; McCracken et al., 2011) and its simplicity and easy handling in technical terms.

The present work was developed at Champalimaud Foundation, in the Myeloma and Lymphoma Research Group, from November 2021 to August 2022. It includes the practical steps for the generation of a 3D *ex vivo* model of multiple myeloma, using cells from BM samples of MGUS and MM patients followed at Champalimaud Clinical Center, as well as both published peer-reviewed, and in submission papers, regarding the subject. This thesis is organized as follows:

- In Chapter 1, a brief overview of the problem addressed was presented.
- In Chapter 2, the current knowledge on MM and its microenvironment is depicted, as well as the achievements in the field of 3D MM models.
- In Chapters 3 and 4, the hypothesis and objectives are described, followed by the methodology used to develop this 3D *ex vivo* MM BM model and to analyze the obtained results.
- In Chapter 5, results are illustrated.
- Chapter 6 comprises the discussion regarding both the results and approaches to the faced problems.
- Chapter 7 concerns the future perspectives involving this project.

I am confident that the data obtained with this research contributes to a better insight on multiple myeloma characteristics, and it is a step forward in the development of a reliable and affordable 3D *ex vivo* MM BM model for studying this disease and developing tailored therapies, aiming at patients' longer survival and better well-being.

I hope you enjoy reading this thesis as much as I enjoyed working on this project.

2. State of the Art

2.1. Multiple Myeloma

Cancer is a major public health problem worldwide and one of the most predominant life-threatening diseases in the world. According to the World Health Organization, in 2020, more than 19 million cancer cases were diagnosed, and 10 million cancer related deaths occurred, globally (referred as website number [1], [2]). MM accounts for 1.8% of new cancer diagnoses and for 2.1% of all cancer deaths (Ackley et al., 2021). More specifically, it accounts for 10% of all hematological malignancies (Sung et al., 2021), being the third most commonly occurring blood cancer, after leukemia and non-Hodgkin lymphoma – Figure 1.

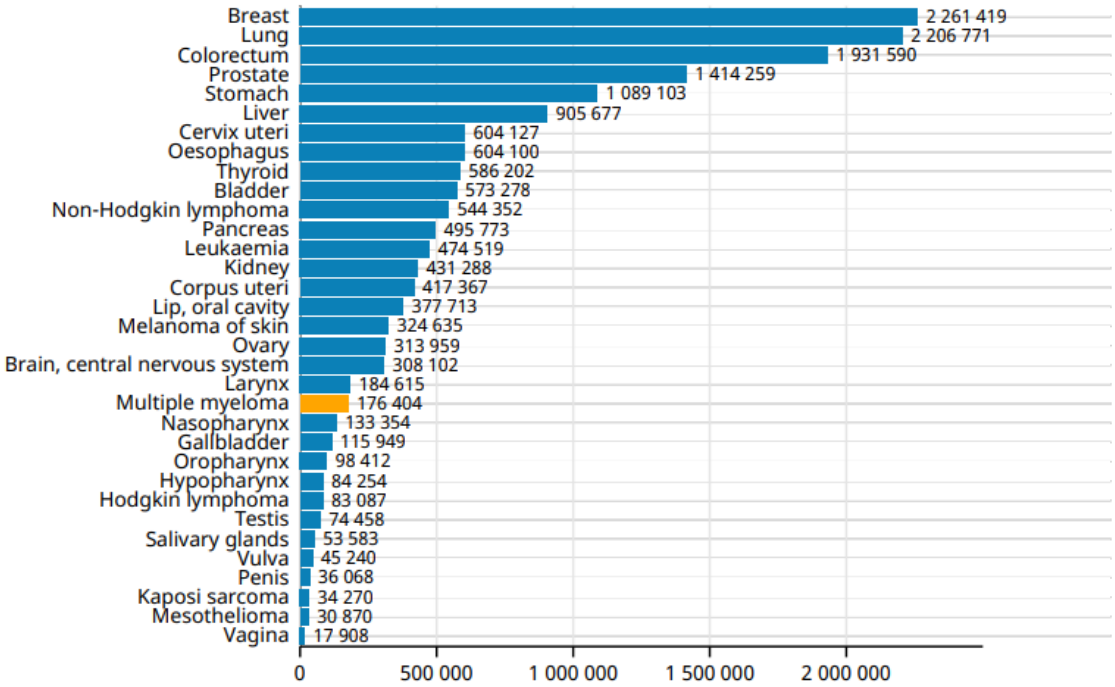


Figure 1. Number of new cancer cases in 2020, in both sexes and all ages, globally ascertained. Multiple myeloma is the third most common hematological malignancy. Adapted from International Agency for Research on Cancer Globocan: Multiple Myeloma (referred as website number [3]).

2.1.1. Epidemiology, Symptoms and Stratification

The median age of MM patients at diagnosis is of 65 years of age, with 37% of patients being younger than 65 (Kazandjian, 2016). Incidence of MM varies between most countries, but overall it has increased uniformly since 1990 (Cowan et al., 2018). MM is slightly more common in men than in women, and is twice as common in African Americans, compared with Caucasians (Rajkumar, 2022). In Portugal, 886 individuals were diagnosed with MM, in 2020, and 640 lost their lives to the disease (Sung et al., 2021). 5-year survival rate across Europe ranges from 23.1% to 46.7% (referred as website number [4]).

Despite the existing therapies, MM remains incurable, being characterized by periods of remission and relapse (Quiñoa-Salanova et al., 2019). Following a first-line of treatment with VRd regimen – proteasome inhibitor (Bortezomib, V), combined with an immunomodulatory drug (Lenalidomide, R) and dexamethasone (d), Durie et al. showed that 16% of all included patients reached complete response, 18% were non-responsive, and 20% relapsed in less than 18 months (Durie et al., 2017) .

MM is associated with significant morbidity due to its end-organ destruction (Hussein, 2007). Patients may experience anemia, renal insufficiency, pain, emotional distress and increased dependency on caregivers (Kumar et al., 2018; Quiñoa-Salanova et al., 2019). The costs associated with it are among the highest in health costs, being a burden to the patients, their families and society (Cook, 2008). In 2018, average yearly direct costs per patient with MM in Portugal amounted to €31,449 and total yearly direct costs were estimated at €61 million (Neves et al., 2021).

Hematological malignancies comprise a collection of heterogeneous conditions, all originating from cells of the BM and lymphatic system (Hamid et al., 2019). Resulting from multistep genetic and microenvironmental changes, MM comprises neoplastic clonal plasma cells, originated from the post-germinal lymphoid B-cell lineage (Palumbo et al., 2011). In normal conditions, plasma cells produce polyclonal immunoglobulins (Ig), crucial in the immune defense. B lymphocytes mature first in the BM and then in secondary lymph nodes, where antigens are presented to them (Hauser et al., 2003). MM occurs when terminally differentiated B lymphocytes start to proliferate in an uncontrolled manner, overproducing large amounts of monoclonal immunoglobulins (Anderson et al., 2011), also called M protein - IgG, IgM, IgA, and less frequently, IgE or IgD (Mangan, 2005) – Figure 2.

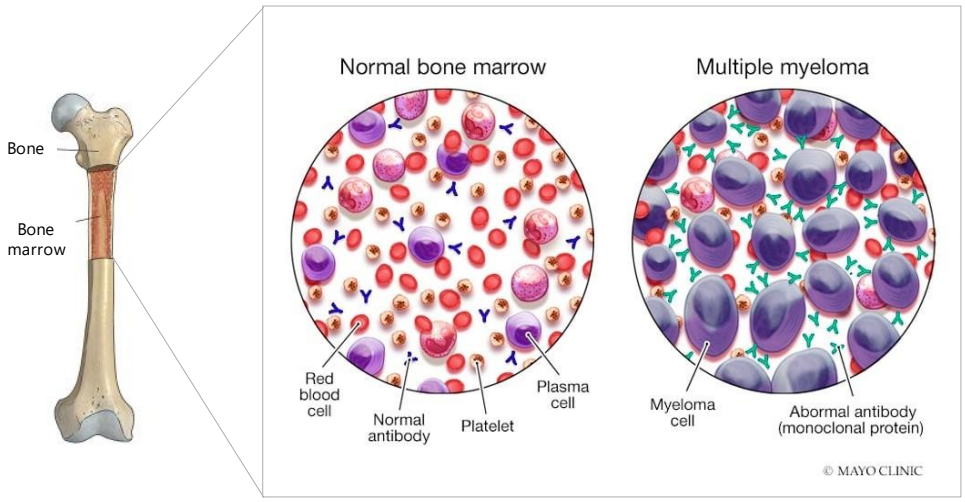


Figure 2. Microscopic view of the healthy BM (left) and of the BM in MM setting (right). Adapted from mSMART: A clear and simple guide for treating patients with multiple myeloma (referred as website number [5]).

MM arises from an asymptomatic premalignant stage, termed "monoclonal gammopathy of undetermined significance" (MGUS) (Anderson et al., 2011). According to the International Myeloma Working Group, MGUS is characterized by less than 10% of BM plasma cell, less than 30 g/L of serum M protein and absence of end-organ damage (Rajkumar et al., 2014) – Table 1. The prevalence of MGUS increases with age, with 3.2% of cases relating to white population over 50 years of age, and 5.3% of cases in people over 70 years of age (Joshua et al., 2019). Research has determined that almost all cases of MM evolve from MGUS, but the majority of the cases of MGUS do not progress to a malignant neoplasm (Landgren et al., 2009). However, some develop into Waldenstrom's macroglobulinemia, primary amyloidosis, or a lymphoproliferative disorder (Kyle et al., 2002). The risk of MGUS progression to MM is estimated to be of 0.5-1% per year, independently of age (Kazandjian, 2016).

In some patients, an intermediate stage can be clinically identified, being referred to as "smoldering multiple myeloma" (SMM). SMM patients are also asymptomatic, but already present BM plasma cell infiltration of more than 10% and/or serum M protein above 30 g/L – Table 1. The risk of progression from SMM to malignant disease (MM) in the first 5 years after diagnosis is much higher than in the case of MGUS, being of around 10% per year (Rajkumar et al., 2014).

MM is defined by BM plasma cell infiltration of more than 10%, combined with the detection of M protein in serum and/or urine, and the presence of one or more Myeloma Defining Events (MDE) (Rajkumar, 2022; Rajkumar et al., 2014) – Table 1. Specifically, MDE include end-organ damage – "CRAB" criteria (hyperCalcemia, Renal failure, Anemia, and Bone disease) and biomarkers such as BM plasma cell infiltration of 60% or higher; involved:uninvolved serum free light chain ratio over 100 mg/L; and more than one focal lesion (of 5mm or more) detected by magnetic resonance imaging (Rajkumar, 2022). Anemia is present in about 73% of patients at diagnosis and is generally related to BM plasma cell infiltration or renal dysfunction. About 80% of patients with newly diagnosed disease develop bone lesions. Renal failure occurs in 20% to 40% of patients, mainly because of damage from excess protein load, dehydration and hypercalcemia, being the latter usually less common. The risk of infection also increases in patients with active disease (Firth, 2019).

The recommended tests for the diagnosis of MM include laboratory examination, with a complete blood count, biochemical analysis, serum and urine protein electrophoresis with quantification of monoclonal protein, BM assessment for plasma cells quantification, and cytogenetic analysis by fluorescence *in situ* hybridization (Anderson et al., 2011). MM patients are currently classified by high risk and low risk, based of cytogenetic abnormalities. The Revised International Staging System (R-ISS) risk stratification model forms the foundation for this classification, attending to chromosomal abnormalities and serum β2-microglobulin and albumin levels, with implications in life expectancy (Palumbo et al., 2015) – Table 2. High risk (R-ISS III) features include the following cytogenetic alterations: t(14;16), t(14;20) and del(17p) - Table 2. 60% of patients are considered of intermediate-risk (R-ISS II), most likely comprising different risk of progression. Both amplifications and deletions in chromosome 1 have been associated with poor prognosis in newly diagnosed MM patients (D'Agostino et al., 2020; X. Hu et al., 2022). Recently, a new Risk Stratification Model (R2-ISS) has been proposed, including 1q copy number alterations (D'Agostino et al., 2020) that are not included in the R-ISS – Table 2. This way, R-ISS II patients can be better distributed into low-intermediate, intermediate-high and high risk groups (D'Agostino et al., 2020).

Although MM is still thought of as a single disease, it is, in reality a combination of several different abnormalities, making it very heterogeneous in symptoms, phenotype, genetic alterations and response to therapy (Rajkumar, 2019). In fact, the sequencing of the MM genome led to the identification of a wide range of molecular aberrations (Chapman et al., 2011; Walker et al., 2018). Additionally, all patients demonstrated multiple different subclones at diagnosis, that eventually have

different importance in disease progression (Bolli et al., 2018; Egan et al., 2012). Therefore, it is important to bear in mind that MM is not a linearly progressive disease and that although some clones may be suppressed by therapy, clones resistant to treatment can end up dominating (Keats et al., 2012). This may explain the still relatively poor response of MM to new therapeutic approaches.

Table 1. Diagnosis criteria for staging of multiple myeloma disease, according to the International Myeloma Working Group. Adapted from Rajkumar et al., 2014.

	STAGE OF DISEASE		
DIAGNOSTIC CRITERIA IMWG	**MGUS**	**SMM**	**MM**
Plasma Cells in the BM	<10%	10-60%	≥10%
Seric M protein	<30 g/L	≥ 30 g/L	Detectable in serum and/or urine
Myeloma Defining Events	Absent	Absent	Present (one or more)

Table 2. Revised International Staging System for multiple myeloma. Adapted from Palumbo et al., 2015 and D'Agostino et al., 2020.

	Revised International Staging System for Myeloma	
STAGE	**DEFINITION (all criteria must be met)**	**5-YEAR OVERALL SURVIVAL**
STAGE I	Serum albumin ≥ 35g/L	82%
	Serum β-2 microglobulin < 3.5mg/L	
	None of the high risk cytogenetics	
	Normal serum lactate dehydrogenase	
STAGE II	Not fitting Stage I or III	62%
STAGE III	Serum β-2 microglobulin ≥ 5.5mg/L	40%
	High risk cytogenetics - t(4;14); t(14;16); del(17p), or elevated serum lactate dehydrogenase. 1q copy number alterations (R2-ISS)	

2.1.2 Treating Multiple Myeloma

The history of MM patients is usually characterized by several periods of remission and relapse. Even after different lines of treatment, patients develop refractory disease, conducting to a clinical situation with lower survival rates (Dimopoulos et al., 2015).

Even though several Phase 2 and Phase 3 clinical trials have proven the efficacy of multiple recently approved novel agents, a substantial portion of the MM patients does not respond to current

therapies, or shows a short-term duration response (Shah et al., 2020). Moreover, these treatments can result in significant adverse effects and are not uniformly tolerated in all patients (Shah et al., 2020). Therefore, there is an unmet need of having MM treatment options with mechanisms of action that can lead to effective and long responses, avoid cell resistance, and be better tolerated by the patients.

To select the most fitting therapy option for each patient, it is crucial to understand patient-related and disease-related factors, as well as previous toxicities, if existent. Symptomatic patients, with active disease, must receive adequate treatment, while for asymptomatic patients the standard of care is not to treat and only follow clinical observation (Mateos et al., 2020). Nevertheless, ongoing trials are working on determining the ability of immunomodulatory drugs (IMiDs) and monoclonal antibodies (mAbs) to delay the progression from asymptomatic to symptomatic MM (Mateos et al., 2013; Terpos 2018).

From the 1960s until the early 2000s, melphalan chemotherapy in combination with steroids (dexamethasone or prednisone) was on the basis of MM treatment (Joshua et al., 2019). Currently, MM patients are usually treated with combinations of selected drugs, that have different mechanisms of action. The major classes of anti-MM agents include: alkylating agents (e.g., melphalan, cyclophosphamide); corticosteroids (e.g., dexamethasone, prednisone); IMiDs (e.g., thalidomide, lenalidomide, pomalidomide); proteasome inhibitors (PIs; e.g., bortezomib, carfilzomib, ixazomib); histone deacetylase inhibitors (iHDACs; e.g., vorinostat, panobinostat); mAbs (e.g., elotuzumab, isatuximab, daratumumab); and nuclear export inhibitors (exportin-1) (Cea et al., 2013; Pinto et al., 2020; Rajkumar et al., 2020).

Of interest, the incorporation of IMiDs and PIs in MM treatment regimens has proven to improve survival rates among patients (Gerecke et al., 2016; Rajkumar et al., 2005; Sonneveld et al., 2012). Briefly, the anti-myeloma activity of IMiDs and PIs results from the disruption of signaling pathways that support the growth, proliferation, and survival of MM cells. More specifically, IMiDs have both tumor and immune targeting effects, leading to apoptosis of MM cells, and an anti-tumor immune response, through the stimulation of T and natural killer (NK) cells, together with a decrease of suppressor cells and corresponding functions (Busch et al., 2014). PIs act by interfering with the degradation of proteins by the proteasome, and induction of the endoplasmic reticulum stress, stimulating different apoptotic pathways (Ito, 2020). PIs also inhibit angiogenesis factors, cytokine signaling, and cell adhesion in the microenvironment (Gandolfi et al., 2017). The introduction of mAb-based therapies targeting MM cells has been considered a great improvement in MM treatment, due to its clinical results, with high efficacy and low toxicity, even in patients with advanced disease (Richardson et al., 2011; Zanwar et al., 2020). The anti-CD38 mAbs, such as daratumumab, can be used in combination with IMiDs and PIs, since they have been described to sensitize MM cells to immune effector cells activity, via antibody-dependent cellular cytotoxicity and/or antibody-dependent cellular phagocytosis (Fedele et al., 2018).

The development of new effective targeted therapies has revealed to be a challenging task, due to the complexity of MM, that arises both from intra- and interpatient heterogeneity (Paulus et al., 2016). While interpatient heterogeneity is a result of the unique personal genetics and epigenetics, combined with dynamic factors such as age, environment, lifestyle, and medical history (Alizadeh et al., 2015), intra-patient heterogeneity is related to the heterogenous distribution of distinct tumor-cell subpopulations across body sites affected by disease (spatial heterogeneity), or temporal variations in the molecular composition of cancer cells (temporal heterogeneity) (Dagogo-Jack et al., 2018) – Figure 3.

In addition, tumor diversity can also be attributed to other equally important but not as explored fonts, including microenvironmental cues, such as the presence of non-neoplastic cells, niche-relevant soluble factors, and the altered extracellular matrix (ECM). In the next section, the important role of the MM BM microenvironment, including the cellular and non-cellular compartments, in healthy conditions and in MM setting, will be addressed.

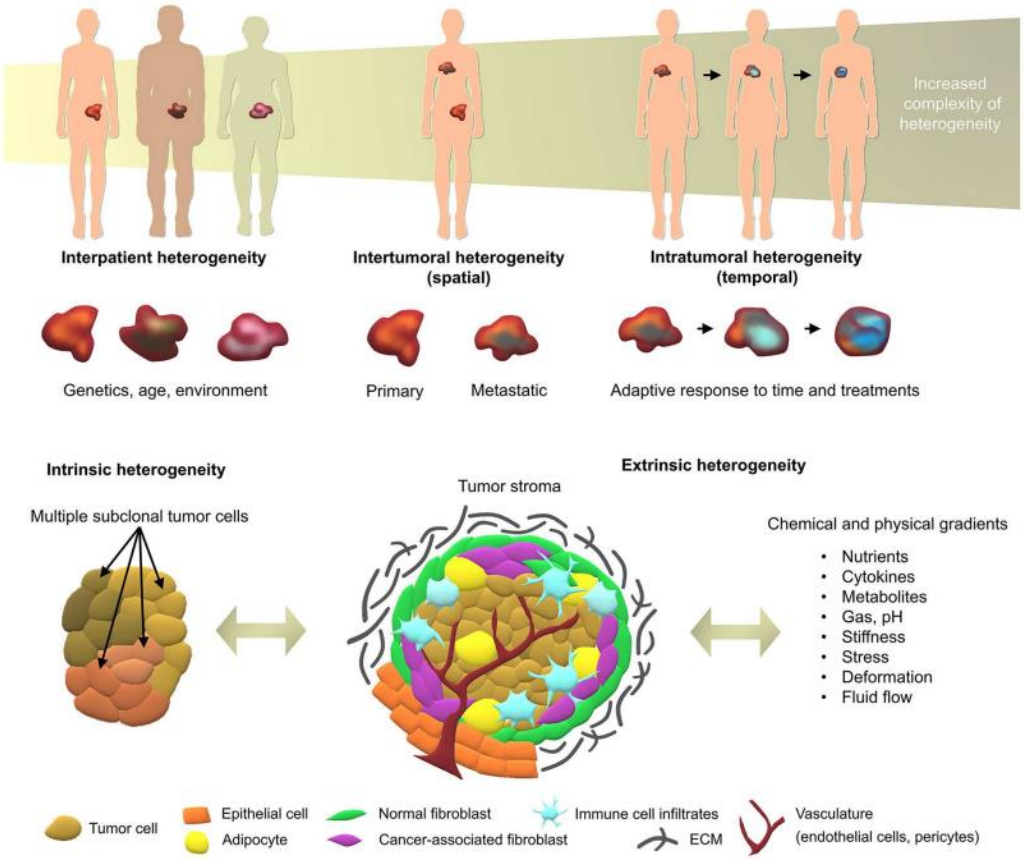


Figure 3. Interpatient *versus* intrapatient tumoral heterogeneity. Adapted from Bray et al., 2019.

2.2. The Bone Marrow Microenvironment in Multiple Myeloma

MM cells strongly depend on the BM microenvironment. This niche is characterized by having a high biological diversity, which can influence neoplastic cells, both directly and indirectly, through soluble cues, cell–cell contact and ECM remodeling. The BM microenvironment plays an important role in differentiation, migration, proliferation, survival, and drug resistance to therapeutic targets of the malignant plasma cells (Manier et al., 2012).

The BM niche is composed of a cellular compartment, with both hematopoietic cells, including red blood cells (RBCs), hematopoietic stem cells (HSCs) and immune cells (e.g., B and T lymphocytes, NK cells, dendritic cells (DCs) or macrophages), and non-hematopoietic cells, such as endothelial cells, stromal cells, osteoblasts and osteoclasts, adipocytes and neurons. It also includes a noncellular compartment, comprising the ECM (and adhesion molecules), and soluble factors (e.g., cytokines, chemokines and growth factors).

2.2.1. Cellular Compartment

2.2.1.1. Immune Cells

MM is characterized by progressive immune dysfunction and immune evasion by MM cells. Cell-mediated immune responses result from the interaction between antigen presenting cells (APC) and effector T cells, and are regulated by the dynamic balance between the activating and inhibitory signaling molecules and cytokines (Zanwar et al., 2020), discussed further ahead.

In MM patients, several immune populations are altered or impaired, negatively correlating with patient survival (Bryant et al., 2013). Some of these events include (1) a decrease on CD19+ B cells, with altered differentiation and antibody response (Zhang et al., 2017); (2) reduced polyclonal immunoglobulin (Rossi et al., 2013); (3) reduction in CD4+ T cells (helper T lymphocytes) and abnormal Th1/Th2 cells ratio (Gupta et al., 2011; Sharma et al., 2010), with (4) reduced Th1 cytokine production and overexpression of Th2 cytokines (Rodríguez-Otero et al., 2017); (5) impaired CD8+ T cell response (cytotoxic T lymphocytes) (Zelle-Rieser et al., 2016); and (6) dysfunction of the anti-tumor activity of NK cells (D'Souza et al., 2021).

Moreover, in MM context, there is also an upregulation of inhibitory molecules by immune T, B, and NK cells (Pardoll, 2012), namely the programmed cell death protein (PD)-1 (Paiva et al., 2015; Ray et al., 2015), or the cytotoxic tumor lymphocyte antigen (CTLA)-4. PD-1 and CTLA-4 represent important immune checkpoints and regulate the production of antigen-specific T cells, thus playing an important role in immune tolerance (Zou & Chen, 2008). However, when PD-1 binds to its ligands (e.g., PD-L1 or PD-L2), present on the surface of MM cells and APCs, it leads to the suppression of T cell activation and an effector anti-tumor immune response (Zanwar et al., 2020) – Figure 4. CTLA-4 competes with the CD28 on T cells to bind to CD80/CD86 on APCs, limiting the production of antigen-specific T cells (Zou and Chen 2008) – Figure 4. Both PD-1 and CTLA-4 are known to have an increased expression in the BM of MM patients (Braga et al., 2014). MM cells are known to express PD-L1 (Liu et al., 2007). As well, circulating T and NK cells in MM patients reveal increased expression of PD-1 (Rosenblatt et al., 2017).

DCs are highly specialized APCs with a critical role in activation of antigen-specific responses (Pratt et al., 2007). In MM, these cells are often dysfunctional and can release different soluble factors, including vascular endothelial growth factor (VEGF), IL (interleukin) -6 and stromal cell-derived factor (SDF) -1α, resultant from the interaction with MM cells, promoting tumor growth and survival (Ratta et al., 2002). Defects in DCs of MM patients have already been described by several groups (Brimnes et al., 2006; Brown et al., 2004; Brown et al., 2001; Ratta et al., 2002; Wang et al., 2006), showing that some immunological properties of these type of immune cells are compromised during MM establishment, with defective antigen presentation, which weakens effective anti-tumor immune responses (Cohen et al., 2020; Rodríguez-Otero et al., 2017).

Macrophages are phagocytic cells with a crucial role in pathogen elimination and tissue repair. These can be divided into M1 and M2, depending on the cues they receive from the microenvironment. M1 are pro-inflammatory macrophages and represent powerful anti-tumor effectors, to contradict the suppressive pro-tumoral microenvironment. M2 macrophages are anti-inflammatory and are involved in anti-tumor suppression (Kloc et al., 2019). During MM progression, M1 macrophages may start to lose their anti-tumor properties, resembling M2 properties, known as Tumor-Associated Macrophages (TAMs). In fact, various studies have reported that it is possible to correlate the higher expression of CD163 by macrophages in the BM with more aggressive disease and shorter survival of MM patients (Panchabhai et al., 2016; Suyanı et al., 2013).

Myeloid-derived suppressor cells (MDSCs), are a population of immature myeloid cells, that have been emerging as negative regulators in infection, autoimmune diseases, and cancer (Gabrilovich et al., 2009). Accounting for their morphological and phenotypic properties, these cells can be divided into polymorphonuclear MDSCs (PMN-MDSCs) and monocytic MDSCs (M-MDSCs) (Movahedi et al., 2008). MDSCs are known to accumulate in the BM of MM patients (Görgun et al., 2013; Ramachandran et al., 2013) and their levels correlate with the disease stage and prognosis (Favaloro et al., 2014; Vacca et al., 2003). Specifically, a study showed that high levels of PMN-MDSCs were associated with higher formation of blood vessels, while M-MDSCs were identified as osteoclast precursors, favoring disease progression (Binsfeld et al., 2016), which suggests that MDSCs sub-populations act in different ways in the BM milieu of MM, supporting tumor growth.

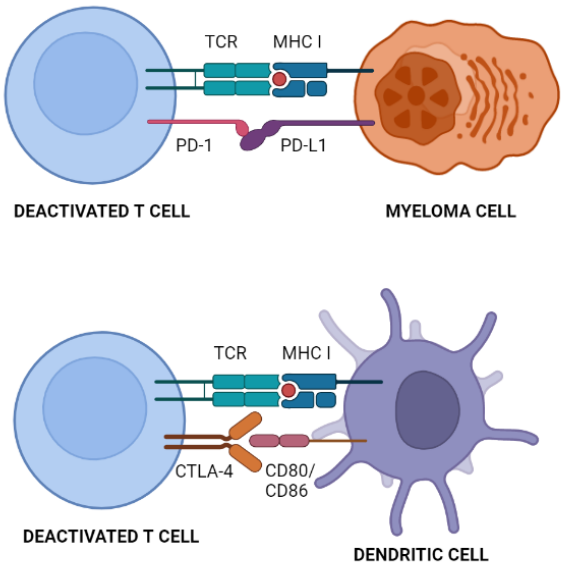


Figure 4. Mechanism of action of PD-1/PD-L1 (top) and CTLA-4/CD80 or CD86 (bottom). Created with BioRender.com

2.2.1.2. Non-Hematopoietic Cells

There are also non-immune populations in the MM BM niche that suffer deregulation, leading to drug resistance and MM escape from immune surveillance (Manier et al., 2012) – Figure 5.

Endothelial cells in MM possess an over-angiogenic phenotype that is able to directly sustain proliferation and invasion of MM cells, through secretion of several growth factors, including IL-6 (Vacca et al., 2003). This production results from the crosstalk between MM and endothelial cells, where the first secrete VEGF, stimulating the second to produce IL-6 (Dankbar et al., 2000). IL-6 also enhances the production of VEGF by MM, promoting a continuous loop where both types of cells proliferate and angiogenesis is increased (Kawano et al., 2015) – Figure 5. This is vital for tumor growth and progression, due to the providing of oxygen and nutrients (Kawano et al., 2015). Comparative gene expression profiling revealed 22 differentially expressed genes in MM endothelial cells, compared with MGUS endothelial cells (Ria et al., 2009). Interestingly, these genes were attributed with roles in regulation of not only angiogenesis, but also ECM formation and cell adhesion, bone remodeling and apoptosis, for example (Ria et al., 2009).

MM cells can adhere to the ECM and to bone marrow mononuclear cells (BMSCs), thanks to adhesion molecules, that include the inter-cellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 (Manier et al., 2012). These molecules are shown to be present in higher levels in MM BM cells than in normal ones, and the adhesion of tumor cells to BMSCs can activate several pathways, leading to the upregulation of proteins related to cell cycle and cell survival (Kawano et al., 2015). Specifically, the interaction between MM cells and BMSCs can trigger the nuclear factor kappa B (NF-κB) signaling pathway and the secretion of IL-6 by BMSCs (Hideshima et al., 2004).

NF-κB signaling is also involved in the imbalance of osteoblasts (bone formation) and osteoclasts (bone resorption) functions, known to cause osteolytic lesion in MM (Terpos et al., 2017). This imbalance can be mediated directly by MM cells, via the receptor activator of NF-κB ligand (RANKL) (Manier et al., 2012). RANKL is a member of the tumor necrosis factor (TNF) family and a transmembrane signaling receptor expressed by osteoclasts. MM cells can bind BMSCs within the BM, leading to increased RANKL expression. RANKL then binds to its receptor in osteoclast precursors, further promoting their differentiation through NF-κB signaling pathway (Roodman, 2009) – Figure 5. Additionally, osteoclasts secrete proangiogenic factors, such as osteopontin, that enhance angiogenesis (Tanaka et al., 2007). Other factors that can be implicated include the production of macrophage inflammatory protein (MIP)-1α, IL-3, IL-6 (Giuliani et al., 2012), IL-1β or TNF-α (Terpos et al., 2018).

Recently, adipocytes have also been emerging as players in MM progression, as researchers have shown that they may represent another critical regulator of MM cell survival, proliferation, and migration (Caers et al., 2007; Trotter et al., 2016). Some examples of the action of adipocytes include the releasing of pro-tumoral molecules, including the monocyte chemoattractant protein (MCP)-1, SDF-1, preadipocytes factor (Pref)-1, resistin, or leptin (Trotter et al., 2016) – Figure 5, and the production of the fatty acid binding protein 4, upregulating MM cell metabolism (Shu et al., 2021). BM pre-adipocytes and mature adipocytes, as well as leptin, have been identified as being increased in MM patients compared to healthy subjects (Liu et al., 2022; Trotter et al., 2016).

The BM is also innervated by the peripheral nervous system, receiving input from both sympathetic (adrenergic, response activation during stress conditions) and parasympathetic (cholinergic, state of calm functions) neurons (Maryanovich et al., 2018). In the BM, there are sensory fibers that transmit signals to the central nervous system (Maryanovich et al., 2018), and neuronal cues are known to control the circadian mobilization of HSCs into the bloodstream (Méndez-Ferrer et al., 2008, 2010).

HSCs function can be impaired with the age-associated decrease in number of sympathetic BM fibers and reduced β3-adrenergic receptor-signaling (Maryanovich et al., 2018), which in turn can be linked to cancer progression, namely in blood disorders (Hanoun et al., 2014). On the other hand, β-adrenergic signaling in the BM also induces IL-6 and VEGF overexpression, promoting MM progression (Vacca and Ribatti 2006) – Figure 5. The exact linking of neurons and MM is still unknown. Nonetheless, it has been shown that, in glioma, tumors can form new synapses with neurons, to promote growth and metastasis (Venkataramani et al., 2019). Of note, recent studies show that anti-adrenergic β-blockers reduce the risk of death in MM patients, suggesting that adrenergic signaling might be related to unfavorable outcomes (Cheng et al., 2021)

2.2.2. Non-Cellular Compartment

2.2.2.1. Soluble Factors

As previously mentioned, the adhesion of MM cells to hematopoietic and stromal cells induces the secretion of varied cytokines and growth factors in the BM microenvironment (Palumbo et al., 2011), which are crucial for regulating the progression of MM. Some of these cytokines are directly produced by the malignant cells, whereas others are produced by the stimulated cells of the BM microenvironment. Therefore, both autocrine and paracrine loops permit control of these mechanisms and should be further blocked.

Given the multiple events in which IL-6 is involved, this cytokine represents a key growth and survival factor in MM (Kawano et al., 1988). IL-6 is mainly produced by BMSCs, resulting in paracrine MM cell growth, and can also be produced by MM cells themselves, in an autocrine manner (Manier et al., 2012). Aberrant production of IL-1β by MM cells seems to be responsible for the paracrine production of BMSC-derived IL-6 (Manier et al., 2012). Elevated serum IL-6 levels are usually associated with a poor prognosis (Ludwig et al., 1991). IL-1β and IL-6 are described to act both as anti-tumoral and pro-tumoral in MM, depending on their context (Musolino et al., 2017).

IL-10 is a pro-tumoral cytokine proven to inhibit different immune related functions, such as macrophage activation, antigen presentation and other cytokine production (Wang et al., 2016). In newly diagnosed MM patients, the levels of serum IL-10 significantly correlated with poor prognosis, and clinical features such as elevated lactate dehydrogenase levels. It has been reported that IL-10 can be secreted by MM cells and regulatory T cells (Tregs), inducing both plasma cell proliferation and angiogenesis in MM (Alexandrakis et al., 2015). Another study showed that Tregs from MM patients expressed increased levels of IL-10 and transforming growth factor (TGF)-β, when compared to healthy controls, indicating increased immune suppressive function (Beyer et al., 2006). During hematopoiesis, the TGF-signaling pathway acts as a negative regulator of proliferation (Dong et al., 2006). However, in hematologic malignancies, including MM, mutations in TGF-β pathway lead to the development of mechanisms of resistance (Dong et al., 2006). TGF-β is secreted from both MM cells and BMSCs (Urashima et al., 1996) Higher serum levels of TGF-β correlate with lower levels of normal immunoglobulins in MM patients, and with increased IL-6 and VEGF secretion by BMSCs (Blobe et al., 2000; Kyrtsonis et al., 1998).

The balance between proangiogenic and antiangiogenic factors is lost in the MM BM niche, favoring the formation of new blood vessels, also known as neo-angiogenesis. Several factors are involved in the unravelling of these events, including VEGF, hypoxia-inducible factor (HIF)-1α, fibroblast growth factor (FGF), angiopoietin-1, platelet derived growth factors (PDGF), and epidermal

growth factor (EGF) (Romano et al., 2014). Concentration of these components is proven to be increased in the BM of MM patients and has been associated with MM progression and poor prognosis (Calcinotto et al., 2015; Vacca & Ribatti, 2006). Moreover, increased microvessel density (MVD) in the BM of MM patients was consistently increased, compared to MVD of patients with MGUS (S. Kumar et al., 2004).

The hypoxia inducible factor family members are key players in either normal cell homeostasis or tumorigenesis (Borsi et al., 2014). Particularly, HIF-1α has been recognized as the most important player in hypoxia response, in tumor survival and progression (Semenza, 2014; Vaupel, 2004). The regulation of this oxygen sensitive factor is made by post-translational modifications of the HIF-α subunit, including hydroxylation, acetylation and phosphorylation. Under normoxic conditions, the oxygen-dependent degradation domain is hydroxylated and ubiquitinated for proteasomal degradation. In hypoxic conditions, HIF accumulates (Borsi et al., 2014; Semenza, 2014). In MM, the rapid tumor growth creates a state of hypoxia in the BM microenvironment, leading to HIF-1α increased levels and stimulation of angiogenesis, in order to raise oxygen delivery to the tumor cells, and benefit tumor growth (Bhaskar et al., 2016).

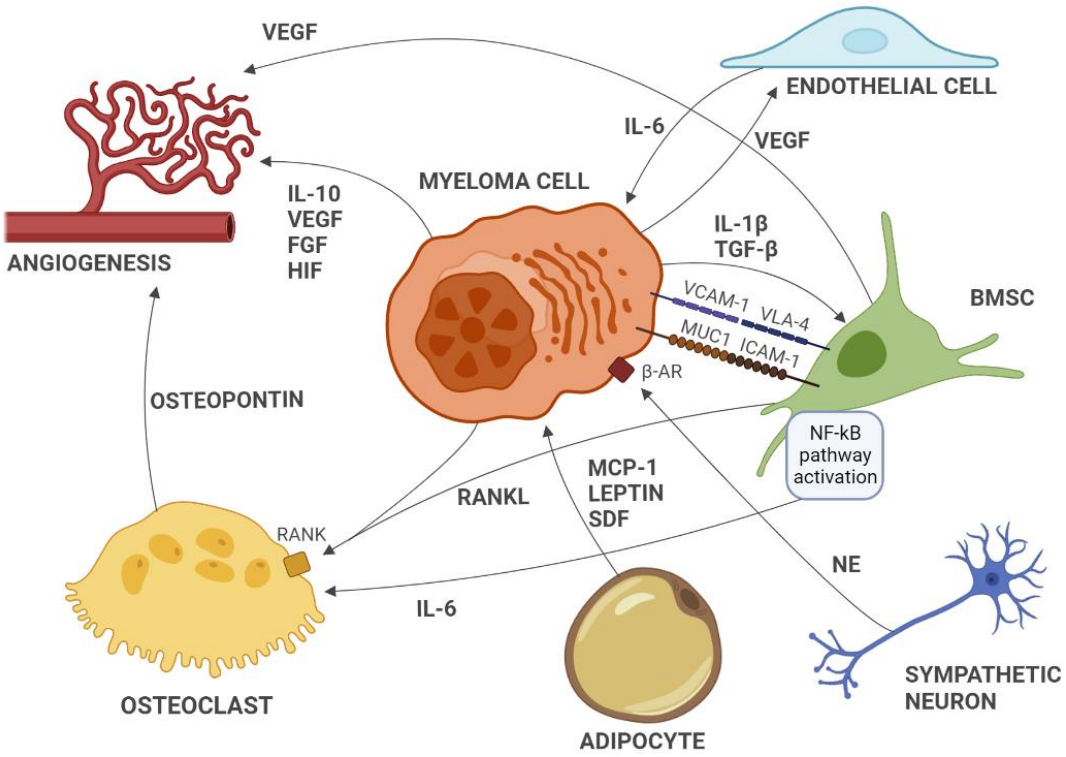


Figure 5. Crosstalk between multiple myeloma cells and non-immune cells in the BM microenvironment. Based on Palumbo et al., 2011. Created with BioRender.com
Abbreviations: FGF – Fibroblast growth factor; HIF - Hypoxia inducible factor; ICAM-1 – Intercellular adhesion molecule 1; IL- 1β – Interleukin 1β; IL-6 – Interleukin -6; IL-10 – Interleukin 10; MCP-1 – Monocyte chemoattractant protein 1; MUC1 – Mucin 1; NE – norepinephrine; SDF– Stromal cell derived factor; TGF-β – Transforming growth factor β; VCAM-1 – Vascular adhesion molecule 1; VEGF – Vascular endothelial growth factor; VLA-4 – Very late antigen 4; β-AR – β-adrenergic receptor

2.2.2.2. Extracellular Matrix

The ECM is composed by a complex network of macromolecules that can assemble into three-dimensional structures. The ECM is the scaffold upon which tissues are organized, and provides essential biochemical and biomechanical cues to regulate cell function (Pickup et al., 2014). The organization, composition and number of adhesion sites are the most common signals known to be processed by each cell, directing cell growth, survival, migration and differentiation, as well as modulating vascular development and immune function (Baker et al., 2012; Geiger et al., 2009; Orr et al., 2006).The ECM also regulates the availability of the soluble growth factors and cytokines and is responsible for regulating hydration and pH levels, controlling homeostasis within the microenvironment (Pickup et al., 2014). A distinct characteristic of the ECM regards its dynamic remodeling, tailoring of composition, and biomechanics, reflecting the physiological structure/function of each tissue (Egeblad et al., 2010).

Structurally, the ECM can be described as a combination of water, fibrous proteins (e.g., collagen, laminin, fibronectin, and elastin) and polysaccharides (e.g., proteoglycans (PGs) and glycosaminoglycans (GAGs)) (Morales et al., 2021) – Figure 6. Collagen is the main structural component of the matrix, and provides tensile strength, regulates cell adhesion, and triggers cell differentiation and survival cues (McKee et al., 2019; Yamada et al., 2019). Fibronectin and laminin fibers are involved in ECM assembly, acting as adhesive proteins, for the simultaneous binding to cell-surface receptors (e.g., integrins and syndecans) and other focal adhesion molecules, influencing cell proliferation, differentiation, and motility (Weber et al., 2011). Elastin fibers provide mechanical resistance and elasticity to tissues. PGs and GAGs fill the interstitial spaces and recruit water molecules, providing compressive strength and protecting properties to tissues. GAGs are also a reservoir of growth factors, such as TGF-β, EGF and PDGF, that trigger a wide range of processes, including cell proliferation, and differentiation for cell adhesion and motility (Kim et al., 2011).

In MM patients, the composition of the BM ECM is often altered and disorganized, correlating with disease progression (Asimakopoulos et al., 2017; Glavey et al., 2017; Slany et al., 2014). Interactions between MM cells and BM cells or ECM proteins, mediated through cell-surface receptors, increase tumor growth, survival, migration, and drug resistance (Palumbo et al., 2011). MM cells directly interact with the ECM via binding of syndecan-1 and very late antigen (VLA)-4 to collagen type I and fibronectin (Palumbo et al., 2011). These adhesive interactions result in upregulation of anti-apoptotic proteins and cell cycle dysregulation (Hideshima et al., 2004). In a proteome profiling study of primary BMSCs from healthy donors, MGUS and MM patients, researchers were able to identify a group of ECM proteins, receptors and modulating enzymes (e.g., laminin a4, lysyl-hydroxylase 2, integrin a5b5 and matrix metalloproteinase (MMP)-2) that were upregulated in a stepwise manner from MGUS to MM, suggesting that the ECM remodeling starts on the MGUS phase (Slany et al., 2014). ECM components such as integrins, have also been shown to play an important role in drug resistance (Damiano et al., 1999).

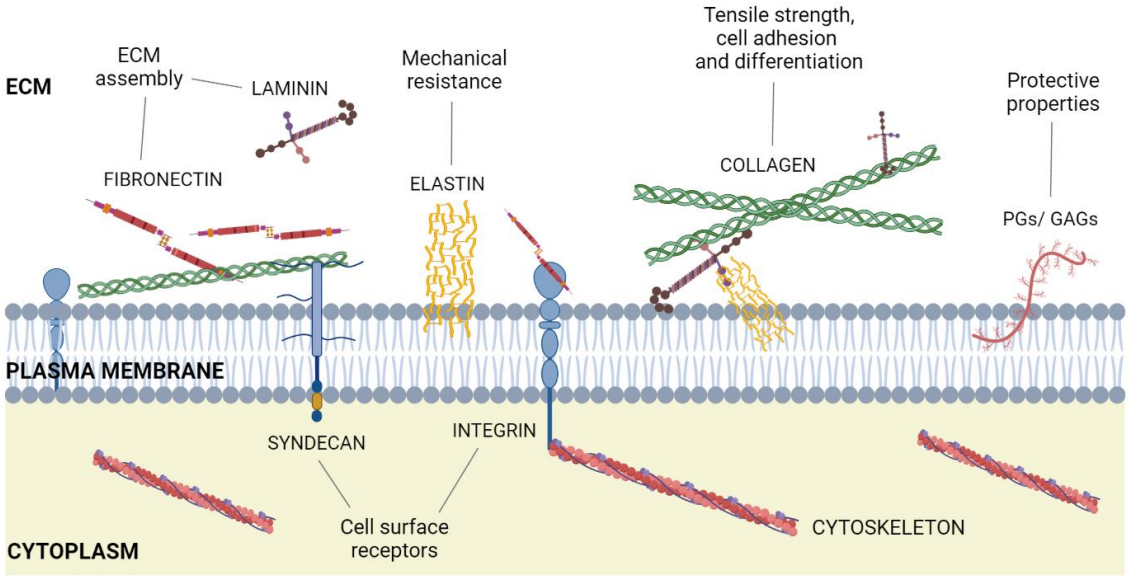


Figure 6. The extracellular matrix proteins and functions. Based on Morales et al., 2021. Created with BioRender.com

Even when primary cells can be grown and maintained, cellular, biochemical, and biophysical characteristics differ between conventional and microenvironment-mimicking cultures (Kapałczyńska et al., 2018). The development of appropriate *in vitro* models that allow reproduction of authentic cell phenotypes and functions is currently one of the challenges to be tackled in MM research. In the following section, the current state of the art on the field of three-dimensional MM BM modelling will be reviewed.

2.3. Currently Available Pre-Clinical Models for Multiple Myeloma

Biomedical research depends heavily on developing models that support the growth of cells for studying biological mechanisms or inter-cellular processes, either *in vitro* or *in vivo*.

Improvement in techniques and knowledge on cell culture have come a long way ever since the first cell line HeLa was established, in 1951 (Masters, 2002; Ravi et al., 2015). Scientists are now able to culture and maintain a wide range of cell types, for different purposes, including immortalized cancer cell lines, immune cells, and even primary human cells (Dronkers et al., 2018; Raulf, 2019). With the development of more and more therapeutics aiming at targeting cancer, it is vital to assess cell behavior and mechanisms of disease in a model that represents the best reality proxy. At the present, the most valuable systems used are conventional two-dimensional (2D) systems, 3D cultures and animal models. The quality and complexity of such models and how they reflect the behavior of cells in real tissues has true impact on the value of the data obtained – Figure 7.

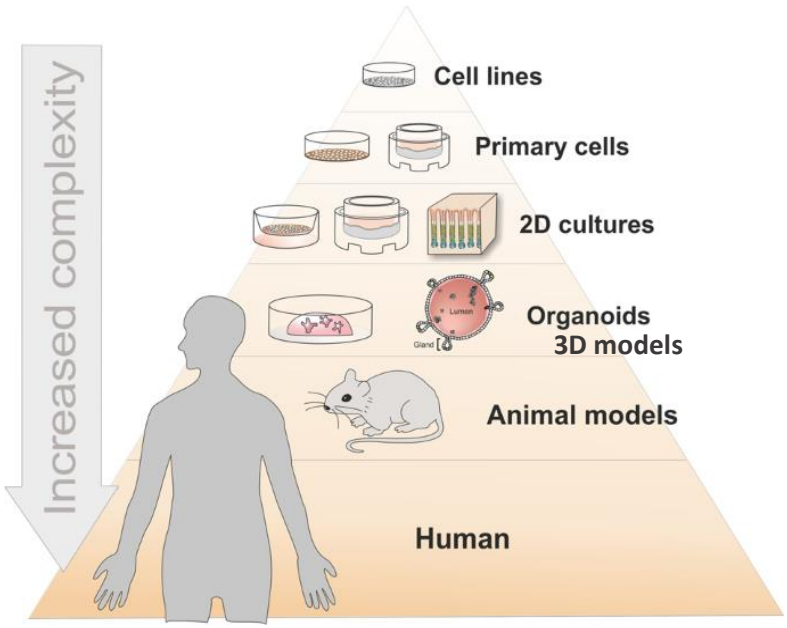


Figure 7. Available preclinical models and corresponding level of complexity. Adapted from Aguilar et al., 2021.

More recently, the scientific community has started to realize the divergence between the used *ex vivo* models and the intricate surrounding environment where cells function *in vivo*, with events such as force sensing, migration, differentiation and proliferation. The complex BM architecture, cellular and molecular composition, and cell interactions needs to be reproduced in adequate platforms to study cancer cells behavior.

Next, the most striking advantages and disadvantages of 2D culture and animal models, in specific, murine models, are presented. The need for more biomimetic systems is then discussed.

2.3.1. 2D Models and its Limitations

Conventional two-dimensional cell culture presents numerous benefits, such as low-cost, high throughput screening, standardization and reproducibility (Barbosa et al,. 2021; Fitzgerald et al., 2020). However, after isolation from their native tissue and transferring to 2D conditions, cell morphology often undergoes variations. In fact, studies have shown how several cell types suffered changes to their cytoskeleton, becoming progressively flatter, divided atypically and lost their differentiated phenotype, in rigid plastic cell surfaces (Fuchs et al., 2004; Knight & Przyborski, 2015; Petersen et al., 1992). The altered cell morphology can affect their functions, namely protein synthesis, gene expression and cell signaling (Knight & Przyborski, 2015). Moreover, because cells grow on planar culture, they can also lose their polarity, impacting cellular response to different events. For example, the apical-basal polarity that cells are forced to acquire has been shown to regulate the sensitivity of cells to apoptosis (Frantz et al., 2010). In this type of culture, there is also a lack of natural ECM proteins and sites for cellular adhesion, that allow specific cells to interact with their adjacent cells and the surroundings (Frantz et al., 2010; Kleinman et al., 2003; Riedl et al., 2017). Particularly, research has shown that the extracellular

environment is highly implicated on preserving the specificity and homeostasis of each tissue, through mechanic transduction and stimulation of cell signaling (Baker et al., 2012; Luca et al., 2013).

2D models are usually based on cell line monocultures, that have lost their BM dependence, therefore inaccurately representing tissue architecture and interaction between cells, and lacking the personal heterogeneity aspects of the disease (de la Puente & Azab, 2016; Fan et al., 2019). Of note, transcriptomic studies demonstrated that MM cell lines only reflect a limited segment of the whole profile of the tumor *in vivo*, making them unreliable preclinical models (Birgersdotter et al., 2005; Sarin et al., 2020). Moreover, as the same cell lines are commonly used in different institutes and are spread across multiple laboratories, they may undergo different selection processes due to differences in culture conditions and passage techniques (Liu et al., 2019).

Importantly, in monolayer culture, cells have unlimited access to the medium supplements, signaling molecules and oxygen, thus failing to reproduce nutrient and hypoxia gradients, as well as drug penetration (Santo et al., 2017). This limits the ability of precisely predicting drug sensitivity in 2D culture, with a significant impact on drug selection. According to literature, it is expected that a great deal of the drug candidates will lose efficacy in a 3D environment, when compared to the treated cells response in 2D (Wang et al., 2021). On the other hand, the opposite can also be true as, most likely, some molecular targets are only expressed under 3D conditions, resulting in higher efficacy of the tested drug. As a matter of fact, the last years declining drug success rates and increasing costs point to the utmost importance of finding alternative strategies in early drug discovery (Booij et al., 2019).

2.3.2. Animal Models and its Limitations

Animal models, namely murine, provide advantages over 2D models, as they harbor more tumor complexity and cancer cells develop surrounded by natural microenvironment.

Through the years, mouse models have revealed to be useful in the representation of several 3D features of the BM. While the 5TMM mice model has been a milestone for the understanding of MM pathogenesis (Radl et al., 1988), Yaccoby and Epstein developed a novel xenograft model, through the implantation of human fetal bone chips in SCID mice (SCIDhu). This widely known and used mouse model supports the development of human MM, forming a humanized BM microenvironment in great resemblance with the BM physiology (Yaccoby et al., 1998).

Because mouse models can maintain several characteristics of certain *in vivo* tumors, including histopathological features, gene expression profiles, and metastatic behavior (Balak et al., 2019; Derose et al., 2011; Zhao et al., 2012) they have been used for biomarker discovery, identification of resistance mechanisms and preclinical evaluation of treatment options.

Patient-derived tumor xenografts (PDTXs) can be generated by heterotopically (subcutaneous) or orthotopically (directly to the organ of interest) transplanting freshly derived patient material into immunodeficient mice (Siolas et al., 2013). However, they still fail to accurately recreate some human disease conditions. For example, immunodeficient mice impair the study of immune and cancer cells interactions in the microenvironment. Moreover, the immune system and microenvironment of immunocompetent mice varies significantly from the human one: differences in both innate and adaptive immunity include, among others, the balance of leukocyte and immunoglobulins subsets, B cell and T cell signaling pathway components, cytokines and cytokine receptors, Th1/Th2 differentiation, antigen-presenting function of endothelial cells, and chemokine and chemokine receptor expression (Mestas & Hughes, 2004). This problem could eventually be bypassed using humanized mice, reconstituting, this

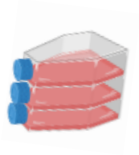
way, the human immune landscape, alongside cancer-stroma and cancer-matrix interactions (Shultz et al., 2012). Nonetheless, studies have pointed out problems with graft-*versus*-host disease. It has been shown that PDTXs may undergo mouse-specific tumor evolution, with dynamics of copy number alterations observed in primary tumors of 24 different cancer types, including breast, lung and bone, differing in mice (Ben-David et al., 2017). Interestingly, Meyer et al. developed PDTX models of human acute lymphoblastic leukemia and acute myeloid leukemia, and reported that the murine environment selected specific subclones, resulting in a number of different sub-models (Meyer & Debatin, 2011).

On top of that, PDTX establishment is a time and resource consuming approach, limiting its use in personalized medicine. The success of engraftment varies significantly, and time between implantation and growth of the tumor can go from approximately 1 month to several months, depending on the model (Dangles-Marie et al., 2007). For example, studies using NOD/SCID mice have shown that MM cells only engrafted at week 3 post-injection and had significant proliferation between weeks 5 and 7 (Fryer et al., 2013). The same can happen with immunocompetent murine models, such as the MOPC315.BM MM mouse model, that takes a median of 30 days for phenotype to manifest (Hofgaard et al., 2012). Considering the timeframe for drug testing, this means that the results may be obtained after the patient in question has suffered mutations or even developed metastasis, which would compromise the effectiveness of the selected treatment regimen and reduce predictive power. Strikingly, it has been found that a mere 4-week delay in treatment is linked to an increase in mortality among several common forms of cancer (e.g., bladder, breast, colon and lung) (Hanna et al., 2020). Among the reasons that can be on the basis of poor scientific validity and reproducibility of the *in vivo* studies in biomedical research, are also included the inadequate choice of animal model or the use of insufficiently characterized/validated ones (Barbosa et al., 2021; Cheon et al., 2011).

Finally, with the increasing emphasis on animal welfare, ethical issues also arise, with a growing concern on reducing and replacing laboratory animals for other models, according to the 3R's principles – replacement of animals with alternative methods, reduction in the numbers of animals used to achieve scientific objectives and refinement of methods to minimize animal suffering of research animals (Russel & Burch, 1960). By developing a 3D model that can capture the tumor microenvironment and its complexity, the use of laboratory animals can be drastically reduced. Although the absolute replacement of animal models from research is still difficult, several mechanistic studies and drug screening of different therapeutic combinations could be conducted with 3D models initially, so that only the best options are studied *in vivo* (Workman et al., 2010). This will improve animal wellbeing and minimize distress, according to ethical and regulatory laws. Species-specific 3D models can be used as stand-alone or part of a larger strategy, for successful precision medicine.

All of these aspects reinforce the need for a personalized, biologically relevant, complete and accurate preclinical MM model – Table 3. Over the last few years, there have been ongoing efforts in the development and increasing adoption of techniques that support cultured cells to acquire or maintain their natural morphology. Advances in cancer cell biology, biofabrication, and tissue-engineering have guided the development of more complex *in vitro* 3D structures, that can potentially be used as more exciting alternatives for drug testing and interpreting cancer mechanisms, considering the tumor microenvironment (Germain et al., 2022; Díaz et al., 2019). It is particularly important to understand how the composition of the surroundings of each cell influences its function. Ideally, MM models should parallel the biological, molecular, genetic, immunological, biophysical and biochemical properties of human pathology. The last decade and a half have brought to light several different options to culture MM cells in 3D, sparing animals, with specific advantages and limitations. In the next section, these models are summarized and reviewed, considering the chosen approach, cells cultured, techniques used and main goals with the development of each of the models.

Table 3. Comparison of the characteristics of currently available types of models for pre-clinical studies – *in vitro* 2D culture, 3D culture and animal models. Adapted from Heydari et al., 2021 and Ryan et al., 2016.

	TYPE OF MODEL		
	2D CULTURE	**3D CULTURE**	**ANIMAL MODELS**
CHARACTERISTICS			
Generation Time	Minutes to a few hours	Hours to a few days	Weeks to months
***In vivo* Resemblance**	Lack of tissue structural representation and cell-matrix interactions; altered morphology and gene expression	3D organization, cell-matrix interactions, possibility of creating differentiated niches	High biological similarity to humans
Environmental Cues	Absent	Present	Present
Access to Components	Unlimited access to oxygen, nutrients and signalling molecules gradients	Oxygen, nutrients and signalling molecules gradients	Oxygen, nutrients and signalling molecules gradients
Spatial Heterogeneity	Bad	Good	Good
High Throughput Drug Screening	Good	Good	Bad
Reproducibility	Simple, easy to reproduce and interpret	More complex and difficult to reproduce	More complex, need for well defined and characterized animal models
Maintenance	Easy to maintain	High maintenance	Special care required, ethical concerns
Costs	Low	Medium	High

2.4. Patient-derived Multiple Myeloma 3D Models for Personalized Medicine – paper in submission

The idea of 3D culture was first conceived in 1912, by Alexis Carrel, when this scientist cultured an explant from a chick embryo and maintain it over a period of 3 months (Carrel et al., 1912). Later, in attempts to create a cell culture environment that resembled the human body more closely, Hamburger and Salmon developed a model using a soft agar solution (Hamburger et al., 1977). Over the last decades, 3D culture methods have been widely used to study biology in multiple cancer types, giving light to new discoveries in the areas of metastasis, hypoxia, angiogenesis and drug screening (Cheema et al., 2007; Driehuis et al., 2020; Magdeldin et al., 2017; Wang et al., 2018). 3D cell culture models enable cultured cells to maintain their natural morphology and tumor architecture, with a proliferating zone, and a quiescent zone with limited oxygen, nutrient and growth factor distribution, that might influence drug response (Barbosa et al., 2021; Knight & Przyborski, 2015) – Figure 8.

The following section depicts 3D patient-derived MM BM models that are currently available and under development.

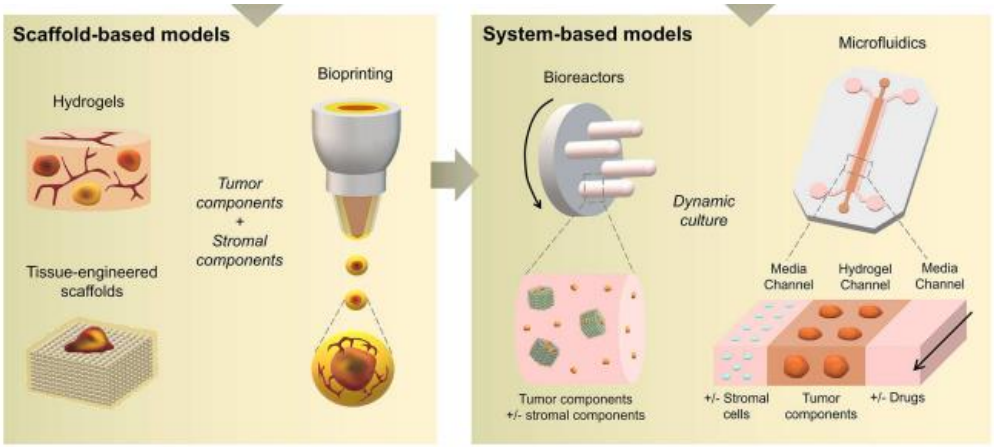


Figure 8. Existing static and dynamic methods for 3D culture. Adapted from Bray et al., 2019.

2.4.1. The Reconstructed Endosteum-Bone Marrow Model

In 2008, a key progress in MM 3D modelling was achieved by Kirshner et al., with the reconstruction of the human MM BM microenvironment in the "rEnd-rBM" model – Figure 9. 24-well plates were pre-treated with fibronectin and collagen type I, creating the reconstructed endosteum-marrow junction (rEnd) compartment. This layer was covered with patient BM mononuclear cells (BMMCs) suspended in a gel mixture of Matrigel and fibronectin, creating the recombinant BM (rBM) compartment (Kirshner et al., 2008). Cells were cultured in growth medium supplemented with the patient's own plasma and kept viable for up to 21 days. Curiously, plasma from healthy donors did not support MM cell growth, which reinforces the importance of patient specificity. When compared with the patient BM, the rBM environment showed similarities with the natural BM niche architecture. Moreover, this breakthrough enabled the proliferation of MM cells, including putative stem cells. This is a very relevant feature, as this cell subset seems to be one of the main responsible for drug resistance

and relapse (Matsui et al., 2008; Zhang et al., 2020). Interestingly, an increase in the total number of clonotypic cells was observed, and cells retained the same chromosomal abnormalities present *in vivo,* as observed after 15 days of culture. Obtaining the entire stromal compartment is a marked improvement of this model over standard culture methods. It allows the testing of several drugs with different targets, and the understanding of how these drugs act, not only on MM cells, but also on non-tumoral cells, facilitating the determination of the precise targets of each agent. Furthermore, the interaction with stroma may be enough by itself to influence molecular mechanisms related to drug sensitivity and resistance. Results also showed the inability of the system to support populations of CD34+ hematopoietic stem cells, CD20+ B cells, and CD138+ plasma cells without the stromal compartment of the BM (Parikh et al., 2014).

More recently, Kirshner established zPredicta, Inc., commercializing the Reconstructed Bone (r-Bone™) technology. BMMCs from 21 newly diagnosed or relapsed MM patients were set-up in the r-Bone™ and cultures were treated according to the clinical single agent or combination treatment regimen received by the patients. Subsequently, MM cell death was evaluated by flow cytometry. The system successfully predicted the patient clinical outcome in 90% of the cases (Kirshner et al., 2020). This technology has already been applied in various medical contexts, growing primary BM cells from healthy donors, and from patients with amyloidosis and various hematological malignancies (Kirshner et al., 2020).

Huang et al. adapted the rBM concept, with the goal of studying the activation of the signal transducer and activator of transcription (STAT) 3 pathway in MM, in 3D *versus* conventional 2D cultures. The STAT3 pathway was activated when cells were cultured in 3D and remained inactive in conventional 2D cultures, showing how some MM mechanisms are dependent on the 3D structure. Moreover, inhibition of the STAT3 pathway using the pharmacological selective inhibitor Stattic significantly decreased the viability of MM cells (Huang et al., 2021) and increased their susceptibility to bortezomib (Huang et al., 2018), providing insight on targeting STAT3 in MM treatment. Overall, the obtained results suggest that the 3D environment is crucial for recapitulation of ECM proteins and cytokines interactions with MM cells. This is valuable because MM is usually characterized by deregulated levels of both pro- and anti-inflammatory cytokines (Musolino et al., 2017). Adapting Huang's work, Caillot et al. tested whether the inhibition or overproduction of reactive oxygen species (ROS) could sensitize or re-sensitize MM cells to bortezomib, as they typically produce high levels of ROS (Lipchick et al., 2016). Upon modulation of redox balance, apoptosis and autophagy in MM cells was increased, suggesting that this may be useful in relapsed/refractory patients, partially reversing tumor microenvironment-mediated drug resistance (Caillot et al., 2020). Nonetheless, the inclusion of other components of the BM microenvironment would be of interest. Moreover, further characterization of the primary MM cells embedded is needed, as most assays were conducted using only MM cell lines (Caillot et al., 2020).

2.4.2 3D Matrigel Matrix Embedded Models

Using Matrigel and fibronectin to form a 3D matrix, Perez et al. studied MDSCs, which are commonly increased in MM niches (Yu et al., 2022). BM samples from MM patients were cultured, and granulocytic cell subsets were identified, by multidimensional flow cytometry, before and after exposure to daratumumab (Perez et al., 2020). Cultures were maintained for 10 days, and no differences were seen *in vitro* in the percentage of granulocytic subsets between timepoints of drug exposure. These

findings were also observed in samples from patients, collected before and after treatment with daratumumab, suggesting the model reproduced *in vivo* behavior.

Cucè et al. used a Matrigel spheroid model to evaluate apoptosis, cell cycle, and changes in cytokine production and release, both in MM cell lines and patient-derived primary MM cells. Cultures were exposed to increasing concentrations of trabectedin, both in 2D and 3D monoculture, or co-culture with monocytes from healthy donors, to understand the impact on the nucleotide excision repair pathway, described to be dysregulated in MM (Ali et al., 2022). Trabectedin was able to alter the pro-inflammatory cytokine network, reducing MCP-1, VEGF, and IL-10 secretion (Cucè et al., 2019). The authors verified that the presence of monocytes was of utmost importance to recreate the protective effect of the microenvironment on MM cells, since the 3D Matrigel-spheroid co-culture promoted MM cell viability and proliferation, in the presence of trabectedin.

Spheroids are of particular interest to cancer researchers because they can include different cell populations, and areas of both proliferating and quiescent cells, owing to limited oxygen and nutrient transport (Alemany-Ribes et al., 2014; Knight & Przyborski, 2015). Being scaffold-free, these models take advantage of the natural ability of the cell types to self-aggregate and secrete their own ECM over time (Barbosa et al., 2021). Nevertheless, spheroids have important limitations, because they grow as independent cellular aggregates and have reduced interactions with the extracellular setting (Hutmacher et al., 2009). Still, if combined with more recent techniques, such as microfluidics or 3D bioprinting, scientists could be able to develop more physiologically relevant 3D models of the disease.

Very recently, Khan et al. put their efforts into developing a 3D platform with the best homology possible to the native hematopoietic tissues, thus allowing the engraftment and growth of primary cells from patients with blood cancers, including MM. For that, differentiated human induced pluripotent stem cells (iPSCs) were embedded in a hydrogel with an optimized composition – Matrigel, and type I and type IV collagen, in order to support differentiation of the 3D BM perivascular niche (Khan et al., 2022). Cells committed to the vascular and hematopoietic lineages. Organoids were obtained, containing hematopoietic stem and progenitor cells, and stromal and myeloid cellular subtypes, with homology to human BM cellular subtypes, as confirmed by single-cell RNA sequencing. Moreover, cell-cell communication and a vascular network were observed. Interestingly, MM cells isolated from BM aspirates of MM patients engrafted into organoids presented a viability of more than 90% at day 10 of culture, whereas in 2D culture, they started to die after only 2 days. These results confirm that, in fact, patient-derived MM cells require a supportive niche to proliferate and stay viable in *ex vivo* conditions. This system addresses a major problem of most existing models, providing vascularization. However, it does not include key populations, such as lymphoid or osteoid cells. Moreover mesodermal, endothelial and hematopoietic lineages were obtained by differentiation of a human episomal cell line and not primary cells, which can lead to a suboptimal mimicking of the BM physiology.

Also using Matrigel as the scaffold, Braham et al. cultured primary MM cells with mesenchymal stromal cells and endothelial progenitor cells, to facilitate its survival and proliferation, over a 28-day period. Their aim was to investigate the use of a novel class of αβ-engineered T cells, called TEGs, to target and eliminate primary MM cells. These TEGs migrated inside the Matrigel matrix, finding and killing their targets, after 48 hours (Braham et al., 2018). The 3D model was far more effective when compared to a similar 2D approach. The same 3D model was later employed to test liposomal delivery of drugs (e.g., doxorubicin, bortezomib), and also to test the efficacy of γδ T-cell receptor (TCR) anti-CD3 bispecific molecules in redirecting T lymphocytes against MM cells, while leaving healthy tissues intact (Braham et al., 2018; Van Diest et al., 2021). The authors exploited this system to provide pre-clinical *in vitro* testing of different therapies on primary MM samples from 7 relapsed/refractory MM

patients (Braham et al., 2019). Interestingly, the responses of each donor cells to the given therapies were analyzed according to two different readout parameters – the percentage of dead MM cells or the number of live MM cells, resulting in different outcomes. This reflects the importance of selecting the best predictor of clinical response. Nevertheless, the model showed a very poor agreement between *in vitro* and clinical treatment responses to IMiDs. This is most likely due to the lack of representation of mechanisms of the *in vivo* disease within the BM microenvironment, including angiogenic and inflammatory processes, possibly because of the absence of a more complete representations of BM cellular subpopulations. The group also used a bioprinting method with bioactive and biocompatible calcium phosphate cement disks to create separate but interacting endosteal and perivascular subniches of the MM BM, representing an interesting option for studying myeloma-bone interactions. The 3D BM model with combined subniches significantly increased the proliferation of CD138+ MM cells (Braham et al., 2018). However, even though this is a more complete model, it still does not fully represent the whole BM microenvironment, as it lacks, for instance, immune system interactions.

2.4.3. Bioprinted BM MM Models

Cell bioprinting is an emerging approach for 3D cancer cell patterning, that simplifies the control of spatial and temporal distribution of cells in a biocompatible material (Augustine et al., 2021). In 2019, Rodriguez et al. developed a bioprinted MM organoid model using a hydrogel scaffold that combined fibronectin, denatured collagen (Gelin-S), a photo initiator and a crosslinker, with primary BM aspirates from MM patients. 3D bioprinters were programmed to extrude 200 000 cells per organoid, per well, in a 96-well plate. Organoids were maintained with a viability above 70% long enough to assess chemosensitivity after one week (Rodriguez et al., 2019). This was the first MM organoid model produced using high throughput bioprinting.

More recently, Wu et al. co-cultured MM cells with HS-5 stromal cells in a coaxial extrusion bioprinted construct, that consisted of a stiff mineral outer alginate layer and a soft hydrogel core. The authors showed that patient-derived MM cells were maintained with good cell viability for up to 7 days, whereas in a 2D environment were only maintained for up to 5 days (Wu et al., 2022). Interestingly, 2D cultured cells showed higher toxicity to bortezomib than in 3D cultures, once again pointing to the potential improvement in physiological relevancy of 3D platforms, compared to planar cultures.

Although promising, this model encompasses a low cellular diversity, hindering real mimicking of the BM cellular interactions. Common limitations of this method include slow printing speed and the challenge of developing nontoxic bioinks. Although this is still an underexplored approach in the field of MM, the microscale resolution, high precision in forming 3D constructs, and the ability to use multiple materials point to the potential of the technique, especially if using the right biomimetic compatible matrix and/or combined with techniques such as microfluidics (Davoodi et al., 2020; Ma et al., 2018), addressed further ahead in this section.

2.4.4. The 3D Tissue-Engineered BM Model

Contributing to another key advance, de la Puente et al. developed a 3D tissue-engineered BM (3DTEBM®), that consisted of a mixture of MM cells, endothelial cells, and stromal cells from the BM of MM patients – Figure 9. Fibrinogen naturally found in the human plasma and calcium chloride were added for promoting clotting and the crosslinking reaction. Tranexamic acid was added, to provide stability to the structure, for up to 7 days (de la Puente et al., 2015).

Proliferation rates of MM cells increased 250% when cocultured with stromal and endothelial cells, comparing to monoculture. Proliferation rates of MM cells in the 3DTEBM® were also compared with conventional 2D co-culture and commercially available 3D models, but these failed to sustain MM cells growth, showing the importance of recreating a 3D biomimetic environment. Of interest, the 3DTEBM® allowed the recreation of drug and oxygen gradients, as suggested by the upregulation of HIF-1α and proto-oncogene serine/threonine-protein kinase (PIM) hypoxia markers, downregulation of CD138 and overexpression of C-X-C chemokine receptor (CXCR) 4 in deeper areas of the scaffold. In addition, the drug uptake measured in cells demonstrated an inverse correlation with depth. Thus, this model seems to be able to recreate the conditions observed *in vivo*, with two distinct regions, where cells in proximity to vasculature are more proliferative and sensitive to therapy than those in the endosteal niche, closer to the bone. This is an important achievement, as it can influence prediction of resistance to the drugs delivered (Kawano et al., 2013; Kyle et al., 2004; Muz et al., 2017). Curiously, even though several listed models could have the potential for investigating the effect of these gradients, it seems that this is still underexplored.

Due to its impressive results in resembling the BM niche, the team further explored this model. For instance, they tested the potential of targeting CD47 for immunotherapy (Sun et al., 2020). Results showed that when CD47-expressing MM cells were co-cultured with macrophages and treated with an anti-CD47 mAb, a significant killing of MM cells was seen in the 3DTEBM®, but not in classic 2D cultures. Extensive motility of macrophages during the phagocytosis process was observed, thanks to the hydrogel-like structure of the 3DTEBM®, that simulates *in vivo* conditions, in opposition to the adherent nature of the 2D cultures. More recently, the 3DTEBM® model was used to assess different therapies on 19 patient samples and found an 89% predictable rate when comparing *in vitro* results to clinical outcomes (Alhallak et al., 2021). These remarkable drug efficacy findings obtained using the 3DTEBM® technology suggest that this is a highly promising approach for studying primary BM malignancies, and those of hematologic origin such as MM, or acute myeloid leukemia.

2.4.5. Hydrogel-based Models

Jakubikova et al. used PuraMatrix (BD™), a synthetic self-assembling peptide hydrogel (Gurski et al., 2010). BMMCs were obtained from BM aspirates of MM patients and the adherent fraction containing mesenchymal stem cells (MSCs) was expanded for several days before seeding, to promote a higher degree of similarity with the *in vivo* tissue (Jakubikova et al., 2016). Cultures were maintained for up to 21 days. A higher expression of both cytokines and ECM molecules known to promote *in vivo* stem cell expansion and MM survival was observed in the 3D MSC model. These included IL-6, IL-8, MCP-1, and VEGF; and collagen I, collagen IV, fibronectin, and laminin, respectively. Protein expression analysis comparing 3D *versus* 2D models revealed activation of osteogenesis and osteoclastogenic differentiation, as shown by the upregulation of matrix metallopeptidase-13, osteopontin, and matrix gla protein, and by the down regulation of calmodulin 1 and CXCR4. They also tested a wide range of novel and conventional anti-MM drugs. Particularly, using BM samples from MM patients, sensitivity results between 3D and 2D cultures showed that the drug response in the 3D co-culture model paralleled clinical resistance. Although promising, a higher number of patients is needed to validate these results. Authors report that prospective trials are being conducted to assess the model value in testing personalized targeted and immune therapy in MM (Jakubikova et al., 2016).

On a different approach, Waldschmidt et al. established a conical agarose 3D platform for the propagation of primary BMMCs *ex vivo*. First, MM cells growth in the system was compared to a 2D

monolayer culture. In 3D, MM cells showed a slower initial proliferation, but maintained a more stable growth after 12 days of culture (Waldschmidt et al., 2022). When comparing 3D monoculture and co-culture conditions, primary MM cells did not expand in 3D monoculture, requiring co-culture support by a human stromal cell line (e.g.: HS-5, MSP-1). Similarly to other groups, detailed cytokine characterization within the developed 3D model revealed a high expression of pro-angiogenic and pro-inflammatory cytokines, such as IL-6, IL-1β, IL-8, and TNF-α (de la Puente et al., 2015; Jakubikova et al., 2016). This secretion seemed to be stimulated by the interaction of MM cells with a stromal co-culture partner, providing the optimal conditions for tumoral cells proliferation and allowing maintenance of cells in culture for 21 days. In a study where newly diagnosed MM patient's cytokine profiling was made, high levels of IL-6, IL-8 and TNF-α were detected in patients but not in healthy controls. Taking these results into consideration, such cytokines seem to be appealing targets for treating MM (Gu et al., 2021). Drug sensitivity tests with bortezomib and auranofin also showed that less toxicity was induced under 3D *versus* 2D condition and in co- *versus* monoculture (Müller et al., 2016; Waldschmidt et al., 2022). Together, these results point to the important role of stromal co-culture for the *in vitro* modeling of drug resistance events and to correctly determine the efficacy of therapeutic compounds. From a technical point of view, this model shows some advantages, as it allows for clear cut, observation and monitoring by confocal microscopy, flow cytometry or western blot (Müller et al., 2017).

Hyaluronic acid (hyaluronan or HA) hydrogels also represent a reliable alternative with similar advantages. HA is an increasingly popular biologically derived matrix, due to its abundant presence in the natural ECM. HA is easier to chemically manipulate compared to other matrix molecules and it allows activation of a variety of pathways, including those affecting cell adhesion and motility, inflammation and drug sensitivity (Gurski et al., 2010). Although already being tested in several types of cancer cells, namely brain, breast and liver (Ahn et al., 2020; Turtoi et al., 2021; Wang et al., 2022; Xiao et al., 2018), few data is available in MM. In 2014, Narayanan et al. developed a 3D hyaluronic acid-based model and showed how matrix composition and stiffness can impact results obtained. In this study, HA based hydrogels were developed for encapsulating BMSCs and MM cells. The percentage of survival of MM cells grown on medium stiffness (≥ 90%) hydrogels was higher than low or high stiffness hydrogels (70-80%) (Narayanan et al., 2014). Proliferation was also higher in medium stiffness HA hydrogels, when compared to 2D or Matrigel cultures. As it is well accepted, during cancer, cells are subjected to a variety of mechanical changes in their microenvironment, resultant in part from ECM remodeling, with events such as increased crosslinking activity and deficient matrix degradation (Leiva et al., 2018). These events lead to increased matrix stiffness, that in turn activates transcription factors that promote tumor cells proliferation and growth (Ishihara & Haga, 2022). A study conducted with MGUS and MM patients showed that altered ECM proteins expression (e.g., osteopontin, periostin, entactin and fibulin) is associated with decreased overall survival (Sidhu et al., 2021). Therefore, proteins that are involved in normal matrix crosslinking and stiffness management may be an interesting target if found upregulated in MM.

2.4.6. Dynamic MM BM Models

Nonetheless, there are some characteristics that cannot be assessed in approaches of static culture nature, such as circulation of nutrients, waste removal and sheer forces. Thus, in 2013, Ferrarini et al. first adapted the bioreactor technology (Rotary cell-culture system (RCCS™), Synthecon Inc., Houston, TX, USA) to grow primary MM cells in coculture with primary allogeneic BMSCs and human umbilical vein endothelial cells (HUVECs). The RCCS™ allowed the creation of a dynamic model in

which cells are in a “free fall” form, favoring mass transfer of nutrients and low shear stress conditions (Ferrarini et al., 2013, 2017). On a first approach, this was designed to culture tissue explants from MM patients, with well-preserved tissue architecture and cell viability. Through histology, it was possible to observe that MM cells were kept viable within their microenvironment, as well as the vessels, that appeared disturbed in static culture. Moreover, assessment of β2-microglobulin levels in supernatants from bortezomib-treated samples and in patients sera after bortezomib-based therapy showed an overall agreement in the drug response *ex vivo* and *in vivo*. However, there were issues with tissue harvesting and reproducibility: (1) the obtained biological samples are very sensitive to manipulation, requiring careful handling to preserve integrity; (2) the number and size of the tissue fragments needs to be thoroughly determined to counterbalance the hydrodynamic forces generated in the culture chamber and maintain the free-fall condition; and (3) for different conditions tested in parallel, each culture vessel needs to have the exact amount of samples and comparable weight and volume (Ferrarini et al., 2017).

In a follow-up study, the same group focused on isolated primary MM cells (Belloni et al., 2018). These were co-cultured with allogeneic BMSCs and HUVECs, supporting the survival of MM cells for up to 7 days, in the bioreactor. Of note, genomic analysis was performed in one MM patient and compared to the bioreactor culture, showing that it paralleled the expansion of the clone that dominated *in vivo* (13q14.3 clone, typically associated with poor prognosis (Gerecke et al., 2016)). Interestingly, signaling pathways associated with tumor survival, proliferation, and drug resistance, were upregulated in the 3D bioreactor model, when compared with conventional 2D models. Overall, this construct allowed reproduction of several MM characteristics and tumor-stroma interactions. The use of allogeneic BMSCs in co-culture with the patient-derived MM cells can be considered a strength of this study, when compared to the models developed using stromal cell lines (e.g., HS-5 (Caillot et al., 2020; Müller et al., 2016; Wu et al., 2022)). However, one could wonder if using autologous BMSCs would not allow a better recapitulation of the patient BM niche specificity.

2.4.7. 3D Models of Myeloma-Bone Interactions

Despite carrying advantages, the rotary system model requires sophisticated devices (Mitteregger et al., 2000). A more affordable approach includes microfluidic devices. Microfluidics seems to be a promising technology for 3D cell culture as it brings the possibility of creating biomimetic structures in which biological processes can be recapitulated (Edmondson et al., 2014), including circulation, and cellular compartmentalization, in a controlled physiological environment (Gu et al., 2015; Kwapiszewska et al., 2014). In the last years, this micromanipulation technique has been employed in efforts to develop a system that allows the easy placing of cells and matrix into the culture chambers of the device and supports replication and characterization of tissues and tumor microenvironment, at a low-cost (Li et al., 2012).

Zhang et al. also focused on the continuous nourishment of the cells, attempting to replicate the body's circulation in an 8-chamber microfluidic device. Primary BMMCs were introduced in each chamber, after generation of an ossified tissue scaffold from a human osteoblastic cell line (line hFOB 1.19), resembling the endosteal surface, to mimic interactions between the two cellular populations (Zhang et al., 2014). Real-time monitoring confirmed that MM cells expanded after 21 days in culture and were drawn towards the osteoblast layer. The group also compared cell viability and proliferation of cells in their model to a 3D static culture, showing that perfusion in the endosteal niche is a key factor of the microenvironment and improves maintenance of long-term culture of MM cells (Zhang et al., 2015; Zhang et al., 2015). However, the authors showed concerns regarding the microfluidic device

material, polydimethylsiloxane, as this has been reported to leach hydrophobic components from cell culture media, including drugs, antibodies and growth factors, potentially affecting the system reproducibility, essential for validation steps and clinical testing (Zhang et al., 2015). Moreover, this model is based on a simple co-culture of MM cells with bone-derived cells. Because, essentially, a monolayer of osteoblasts is cultured on the surface of the microfluidic device, and then MM cells are pumped into the chambers, this methodology raises the question of if in fact a 3D scaffold is considered here. The addition of multiple patient-derived BM components would be of interest, to increase the quality and complexity of the model, paving the way for relevant studies of cell adhesion mediated drug resistance, for example.

In this context, Reagan et al. attempted to recreate the BM microenvironment by stimulating MSCs to undergo osteogenic differentiation inside porous silk scaffolds, prior to the inclusion of MM cells. Cell viability after bortezomib treatment was determined and imaged, showing drug resistance in their 3D culture when compared to hydrogel and 2D cultures, that did not contain differentiated niches (Reagan et al., 2014). These findings suggest that the inclusion of bone cells and recreation of a bone-like component are important factors for cell adhesion–mediated drug resistance. Additionally, the authors were able to identify a novel microRNA signature (miR-199a) associated with dysfunctional osteogenesis of MSCs in contact with MM cells, indicating that targeting it can contribute to develop new therapeutic approaches that can inhibit clonal proliferation of plasma cells within the BM and induce bone formation (Xu et al., 2013). 3D silk scaffolds from Reagan et al. have also been employed to develop the first 3D tissue-engineered BM adipose tissue (BMAT) model (Fairfield et al., 2019). MSCs were seeded in silk scaffolds and cultured with adipogenic media, driving differentiation of adipocytes. With this approach, Fairfield et al. were able to shed light on complex interactions between BMAT and MM cells, even if no patient-derived cells were used. Compared with 2D cultures, pathways of DNA replication, metabolic and proliferation events were upregulated in BM adipocytes, in this model (Fairfield et al., 2019). Of note, a study combining *in vitro* and *in vivo* methods showed that the BM of MM patients contained in fact an increased number of both preadipocytes and mature adipocytes, pointing to its potentiality as targets (Trotter et al., 2016).

2.4.8. Computational and Mathematical MM BM Models

Given the complexity inherent to the development of an adequate and complete 3D MM model, it is important to characterize the effects of all potential factors interacting with the embedded cells. For that, combining experimental and computational/mathematical modeling is advantageous, allowing for an interdisciplinary approach that considers cell populations and BM microenvironment interplay in disease development and progression.

In 2014, Khin et al. developed a pre-clinical platform to assess drug response in MM. Their work consisted of a two-phase project, combining *in vitro* microfluidics and *in silico* approaches, for studying the effect of bortezomib and melphalan (Khin et al., 2014). More recently, this mechanism was improved through the development of an *ex vivo* platform able to make a three-month prediction of clinical response, aiming to guide clinicians in their treatment decisions for each patient (Silva et al., 2017) – Figure 9. Dose response assays were performed in 3D cell culture, where primary MM cells were seeded in collagen type I with pre-established human bone marrow mesenchymal stem cells and patient-derived plasma supplemented medium, thus reconstructing a microenvironment with adequate ECM and growth factors. Their model gathered digital microscope snap live images every 30 minutes over 4 days, detecting live and dead cells through the presence or absence of membrane motion over

time. However, the described system was unable to quantify cellularity of adherent cancer cells (Silva et al., 2015). Data derived from these experiments was then used to parameterize mathematical models for clinical outcome simulation. This method is advantageous as it allows drug sensitivity testing in a non-destructive way, contrary to other protocols where, on top of cell number limitations, time points are also sparse. The *Ex vivo* Mathematical Myeloma Advisor (EMMA) makes use of a digital image analysis algorithm, mathematical models, and pharmacokinetic data to predict the effect of 31 drugs, alone or in combination, within 5 days after BM biopsy. This is a great improvement compared to previous approaches, as time for clinical decision making is so critical, and results coming from an ideal model ought to be provided as soon as possible. Another of the novelties of Silva's work was the model's ability to characterize tumor heterogeneity with different degrees of chemosensitivity to a given drug. This consideration is of utmost importance, as patients presenting heterogeneous tumors may exhibit some clones that are more resistant to therapy. Although the use of EMMA in the presence of combined drugs revealed some flaws, its use for single agents is strongly validated, with the group stating the use of the Synergy-Augmented Model (SAM) in the case of agents combination (Sudalagunta et al., 2020). In fact, this model correctly classified 50 out of 52 patients (96%) according to response or no response to treatment with both chemotherapeutic agents and immunomodulatory drugs. Based on EMMA model predictions, 60% of the 52 patients in this study received at least one drug with no clinical efficacy (Silva et al., 2017).

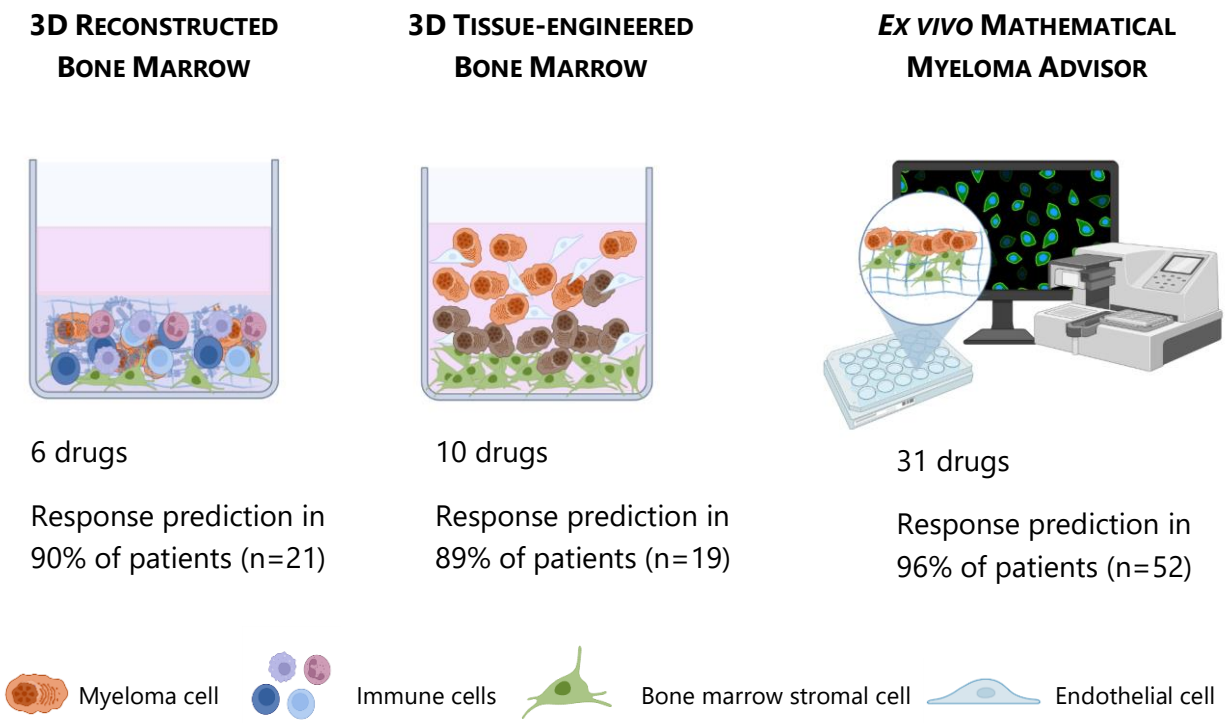


Figure 9. Patient-derived MM models able to predict clinical response.
3D Reconstructed Bone Marrow: Kirshner et al., 2008; Parikh et al., 2014; Kirshner et al., 2020. 3D Tissue-Engineered Bone Marrow: de la Puente et al., 2015; Alhallak et al., 2021. *Ex Vivo* Mathematical Myeloma Advisor: Silva et al., 2017.

While all these models have a 3D component and used primary MM cells in combination with other cell types found in the BM niche, some represent layered cell types in coculture and others a more heterogeneous mix, that self-organizes through time and better represents the *in vivo* configuration. As shown here, presently, there is no universal solution or technology that can satisfy the needs of all 3D cell culture types. Therefore, researchers are required to think critically and select the most appropriate model for their assay, depending on their questions and goals. Up next, an historical overview of Matrigel development and usage is given, including a more detailed characterization of its composition and advantages, as the model developed in this work was built over a Matrigel platform.

2.5. Matrigel as a Suitable Scaffold for MM 3D Culture

The ideal 3D biomimetic model should be able to provide the power of identifying key factors and modulators of tumor development, such as cell–cell, cell–matrix and cell-receptors interactions (Alemany-Ribes et al., 2014). Ideally, it should comprise the following aspects: good recapitulation of biochemical cues, adequate tissue stiffness, permeability and possibility of drug penetration, recreation of oxygen gradients, sites for cell adhesion, and allow cell differentiation and proliferation (Braham et al., 2018; Fairfield et al., 2019; Griffith et al., 2006; Kirshner et al., 2008; de la Puente et al., 2015; Reagan et al., 2014). In fact, most of these aspects depend, not exclusively but also, on the chosen scaffold. Studies performed by different groups have shown how culturing the same cells in different biomaterials leads to changes in the obtained results (Narayanan et al., 2014; Reagan et al., 2014). Hence, selecting the matrix to be used is a key point of the experimental process, and should not be disregarded. Although several new options have been emerging in the past years, the most commonly used scaffolds include hydrogels, which can be natural, synthetic, or a combination of both (Booij et al., 2019). In particular, scaffolds of natural origin, namely Matrigel (Benton et al., 2011), collagen (Szot et al., 2011; Wolf et al., 2009) and hyaluronic acid (Goodarzi et al., 2021) are still the gold standard in cancer research. These allow minimal processing and are naturally recognized and remodeled by the cells (Rosso et al., 2004). They are animal-derived basement membrane (BaM) extracts that possess ECM binding sites, ligands, cytokines and growth factors, allowing improvement of viability, while cells carry out their functions (Jain et al., 2022).

Matrigel is a gelatinous mixture of matrix proteins secreted by Engelbreth- Holm-Swarm mouse sarcoma cells, named after J. Engelbreth Holm of Denmark, who discovered it, and Richard Swarm, who maintained and characterized it (later named Matrigel by John Hassell). After murine tumor extraction, several ECM proteins are retained in a reconstituted basement membrane preparation, to provide both structural and biological support to encapsulated cells (Kleinman et al., 2005).

Basement membranes are a specialized type of ECM with a thin and dense sheet-like structure, that surrounds most animal tissues, lining the basal side of epithelial and endothelial tissues. Their self-assembled structure results from two independent polymeric networks, one of laminin and one of type IV collagen, that are believed to be linked by several additional ECM proteins, including entactin (nidogen) and heparan sulfate proteoglycan (perlecan) (Jayadev et al,. 2017). Functionally, this membrane is involved in providing physical and biochemical cues to the cells, shaping the tissue into its correct size and form. Importantly, multiple BaM components, including laminin, are known to be overexpressed by different cancer cells (De Arcangelis et al., 2001; Fujita et al., 2005; Vande Broek et al., 2001).

Thus, Matrigel was created because it contained all the common ECM molecules found in the BaM. Predominantly, it is constituted by laminin (~60%), collagen IV (~30%), and entactin (~8%). Besides these structural components, Matrigel also contains growth factors, including TGF-β, EGF, insulin-like growth factor (IGF)-1, FGF, and VEGF, and other components such as tissue plasminogen activator, heparan sulfate proteoglycan and residual matrix proteins that are mediators of cell growth, differentiation, and self-organization into 3D structures (referred as website number [6]) – Table 4.

Table 4. Components of Matrigel matrix. Adapted from Corning Matrigel Matrix Frequently Asked Questions (referred as website number [6]).

CATEGORY	COMPONENT	ABUNDANCE
STRUCTURAL COMPONENTS	Laminin	60%
	Collagen Type IV	30%
	Entactin	8%
	Perlecan	non-defined
GROWTH FACTORS	bFGF	< 0.1 pg/mL
	EGF	0.5-1.3 ng/mL
	IGF-1	11-24 ng/mL
	NGF	< 0.2 ng/mL
	PDGF	5-48 pg/mL
	TGF-β	1.7-4.7 ng/mL
	VEGF	5-7.5 ng/mL
OTHER COMPONENTS	Tissue plasminogen activator	Residual
	Fibronectin	Residual
	Vitronectin	Residual

Cells in or on this matrix associate in three dimensions and form structures similar to those in the tissue of origin (Kleinman et al., 2005). For example, both established cell lines and primary mammary epithelial cells are able to form ducts and gland-like structures (Li et al., 1987) and, in contrast, chondrocytes form cartilaginous nodules (Bradham et al., 1995). As well, both established cell lines and primary cells have shown evidence of differentiating within this matrix. For example, immortalized microvascular endothelial cells and HUVECs formed identical capillary-like structures (Kleinman et al., 2005).

Equally exciting, a study conducted with high and low metastatic human breast cancer cells showed that they grew at similar rates and exhibited similar morphologies, on plastic surfaces. However, when cultured on the basement membrane Matrigel matrix, the highly metastatic cells expanded, forming branched and invasive structures, while the low metastatic cells assembled into small aggregates (Benton et al., 2009). This shows that even though Matrigel is of animal origin, it does not significantly interfere in the functions of the cells that are embedded. As both APC and immune effector cells are originated from the same source, this would minimize immune activation. Moreover, Matrigel-based hydrogels are reported to be sensitive to matrix metalloproteinases-mediated remodeling, as required for tumor progression (Fakhari et al., 2022; Morales et al., 2021).

Regarding technical management, the polymerization of Matrigel is a simple process that is primarily triggered by the rising of temperature, between 22°C and 37°C, allowing bond formation and providing structural organization. Laminin self-assembles, and there is also crosslinking of laminin and collagen by endogenous nidogen (Takagi et al., 2003). At 4°C, the Matrigel matrix liquefies and becomes a solution (referred as website number [6]). Despite requiring environment control and rapid liquid handling to avoid premature polymerization, the workflow for dome formation with this matrix is simple and easy – Figure 10. This method involves the formation of droplets of cells resuspended in Matrigel, on a pre-incubated tissue culture-treated surface. After polymerization, medium is added to each well. For this type of protocol, factors such as the used plate type, plate preincubation and chilled pipette tips are vital.

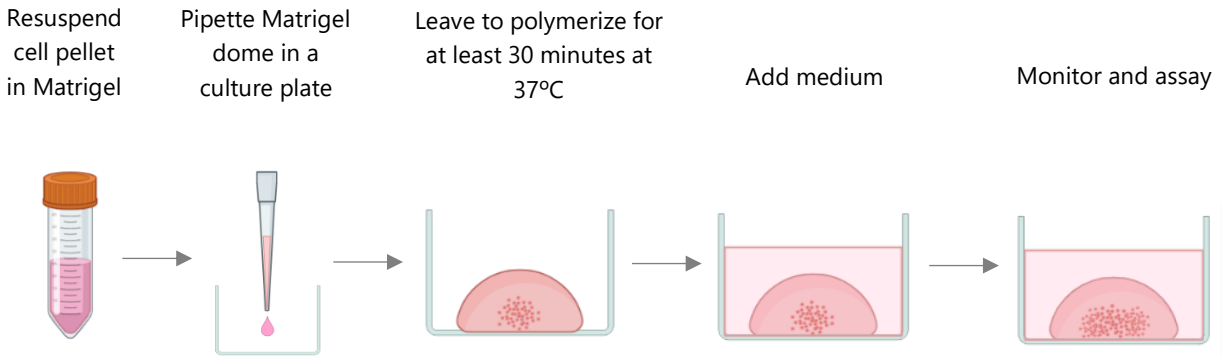


Figure 10. Matrigel 3D culture setting workflow. Created with BioRender.com

Although developed in the 80's, Matrigel is still widely used for studies on cell differentiation and angiogenesis, today. In fact, some models developed based on this protein mix represent widely used assays nowadays, such as the capillary-like tube formation for angiogenesis (Kubota et al., 1988). Later, Matrigel was also adopted in cancer organoid research and has provided a tumor-relevant environment for the establishment of many cancer subtypes, thus opening the possibility of modelling patient-specific cancer biology (Hocevar et al., 2021; Jiang et al., 2021; Laperrousaz et al., 2018).

For now, there is still a lack of standardized procedures for the creation of reliable platforms to be used in personalized medicine. A lot of the developed models are complex and recreating them is a major task for other groups. The ideal 3D MM model should be easy to achieve, reproducible, timesaving, and cost-effective, while still mimicking the tumor cells behavior and their crosstalk with the BM microenvironment.

In this project, Matrigel was the selection of choice for developing a 3D MM BM model, offering advantages in terms of simplicity, cost, and minimal labor. In the following chapters, our objectives and methodology used for achieving them will be described, followed by a chapter of results obtained and discussion of the present and future work.

3.Hypothesis and Objectives

Despite the latest improvements in clinical results and emergent new therapy options, the majority of MM patients still doesn't respond as expected and eventually relapse and become treatment resistant (de la Puente & Azab, 2016; Tandon et al., 2019; Yang & Lin, 2015)

To date, the common strategy for genetic assessment and immune profile of MM is the one-site BM biopsy, which may not reflect the whole setting of the disease in each individual (Carneiro et al., 2022; Lee et al., 2017) Therapies are selected based on patients' comorbidities and previous administered drugs, instead of taking into account each individual's tumor and its microenvironment characteristics as weighing factors in sensibility to treatment (Rajkumar et al., 2014).

In the last decade, 3D models have been emerging as a potential solution to bridge the gaps between 2D cultures and the *in vivo* setting. However, even though there are already some established MM models (de la Puente et al., 2015; Kirshner et al., 2008), several have yet only been tested with MM cell lines, or using only some specific populations of the BM sample. Even though a few already allow culture of whole BM samples and showed promising results, they are very complex and difficult to reproduce, and are still not used in clinical day to day practice.

Our hypothesis was that the establishment of a patient-derived 3D MM BM model using whole patient BM samples would allow the study of the pathophysiological alterations and interactions between cells in each patient's BM niche, enabling a better understanding of the mechanisms involved in disease progression.

Therefore, the main goal of this thesis was to develop a simple and reproducible patient-derived 3D *ex vivo* model that mirrored the cellular diversity of the BM and the architecture of the ECM in the MM setting. For that, freshly collected whole BM aspirates from MM patients were used, embedding cells in a Matrigel matrix.

The main objectives of this thesis were:

1- Generation of a human 3D *ex vivo* co-culture model of MM using whole patient BM samples

   a. Determination of the ideal culture conditions for maintaining cellular viability and integrity

   b. Characterization of cell populations and 3D organization

2- Validation of the 3D model to assess drug response

The comprehensive reconstruction of BM elements in 3D culture allows monitoring of drug impact on both malignant and healthy cells. From a translational point of view, this model holds the potential to be used in the clinic, for testing currently available or new anti-MM therapies, either as single agents or in combination, as a personalized platform. This may help predicting treatment effectiveness, toxicity and, more importantly, determining a carefully selected therapy for each MM patient, while avoiding animal use and following Russel and Burch's 3R's principles - Replacement, Reduction and Refinement (Workman et al., 2010). In the long run, this knowledge might be extended to other hematological malignancies.

4. Materials and Methods

4.1. Primary Cells

Primary cells were obtained from the BM aspirates of MGUS and MM patients followed at the Hematology Unit of Champalimaud Clinical Centre, Lisbon, Portugal. General informed consent form was approved by Champalimaud Foundation Ethics Committee and obtained for all patients (see section 4.10).

Inclusion criteria used were: (1) ≥18 years old; (2) perception of the study; (3) agreement in participating in the study with signed informed consent form; (4) MGUS or MM diagnosis (International Myeloma Working Group criteria (Rajkumar et al., 2014)). Exclusion criteria used were: (1) active auto-immune disease; (2) other active malignancy; (3) disagreement in participating; (4) no signature of the informed consent form.

For all MM patients, both demographic and clinical data (e.g., date of birth, gender, date of diagnosis, presence of extramedullary disease, MM risk score (International Staging System and Revised International Staging System), cytogenetic abnormalities, number and type of previous treatments, and treatment responses), were collected and kept in an internal clinical database.

All studies were in accordance with the Declaration of Helsinki.

4.2. MM Cell Lines

The MM cell lines MM.1S and RLD were a kind gift from Dr. Mercedes Garayoa Berrueta (Centro de Investigación del Cáncer, University of Salamanca, Salamanca, Spain).

All cells were cultured at 37ºC, 5% carbon dioxide (CO_2), in RPMI-1640 medium supplemented with 1% L-Glutamine (Corning®); 10% fetal bovine serum (FBS; Gibco); 1% penicillin/streptomycin (P/S; Sigma-Aldrich); 1% MEM non-essential amino acids (Sigma-Aldrich). Because MM cell lines are semi-adherent, detachment from the culture flasks for cell passage was done by mechanical disruption.

4.3. MM 3D Matrigel-based Culture

3D cultures were developed using whole patient-derived BM samples or MM cell lines, in Matrigel growth Factor Reduced Basement Membrane Matrix (Corning®). 24-well culture plates (24 well plate- Nunc™ Cell-Culture Treated Multidishes) were placed on incubator (37ºC, 5% CO_2) at least 1 hour before starting experiments. Pipette tips were placed at 4ºC, to allow Matrigel manipulation. As a control, 2D cultures under the same conditions were established. Viability of 2D cultures was assessed in the same timepoints as 3D culture, by Trypan Blue assay (Corning® 0.4% (w/v) in phosphate buffered saline). 2D cell cultures were detached by adding 500µL of TrypLE Express (Gibco, 1X) for 5 min, 37ºC, 5% CO_2, for BM patient samples, or mechanical disruption, for MM cell lines.

4.3.1. Patient-derived 3D Culture

3D patient-derived cultures were established using whole patient-derived BM samples, after red blood cell lysis (RBCL). Cell concentrations tested were of $0.25x10^6$, $0.5 x10^6$, $1x10^6$ and $2x10^6$ cells per 40µL of Matrigel.

BM sample was transferred to a 50 mL Falcon tube filled with RBCL buffer (10X, BD PharmLyse) and left to incubate on a roller for 15 minutes, at room temperature (RT), protected from light. Afterwards, cells were centrifuged for 5 minutes at 1500 rotations per minute (rpm). Supernatant was discarded and cells were resuspended in phosphate buffered saline (PBS; 1X, Corning®). Cells were again centrifuged for 5 minutes at 1500 rpm and then resuspended in PBS, for cell counting, with Trypan Blue assay.

The volume of cells required for the final concentration per well was determined, placed on individual Eppendorf tubes, and centrifuged for 5 minutes at 1500 rpm. Supernatant was carefully discarded, and cells were resuspended in 40µL of Matrigel, plated in the center of the well and left to incubate for 20 to 30 minutes, at 37ºC, 5% CO_2, allowing polymerization of Matrigel. Finally, 1 mL of RPMI-1640 medium (Corning®) supplemented with 1% L-Glutamine; 10% FBS (Gibco); 1% P/S (Sigma-Aldrich) and 1% MEM non-essential amino acids (Sigma-Aldrich) – complete medium (cRPMI), was added per well. 500µL of complete medium per well were changed every three days, at RT, carefully and as fast as possible to avoid disruption of the Matrigel.

For culture with 10% of patients own plasma supplemented complete medium, BM samples were centrifuged prior to RBCL, for 10 minutes at 3000 rpm, for plasma isolation.

For culture with Rho kinase (ROCK) inhibitor (Y-27632 dihydrochloride; Sigma-Aldrich), 2µL were added for each mL of medium or Matrigel, at day 0 of culture.

Cell viability was assessed for 14 days, by Trypan Blue assay. Matrigel was digested with Cell Recovery solution (Corning®) at a volume ≥2X that of the Matrigel matrix volume, for 1 hour at 4ºC.

4.3.2. MM Cell Line 3D Culture

MM cell line 3D cultures were established using MM.1S and RLD cell lines. Tested cell concentrations were of 10 000, 20 000, 40 000 and 50 000 cells per 40µL of Matrigel.

The volume of cells required for the final concentration per well was determined, placed on individual Eppendorf tubes, and centrifuged for 5 minutes at 1500 rpm. Supernatant was carefully discarded, and cells were resuspended in 40µL of Matrigel, plated and left to incubate for 20 to 30 minutes, at 37ºC, 5% CO_2, allowing polymerization of Matrigel. Finally, 1 mL of cRPMI medium (1% L-Glutamine; 10% FBS; 1% P/S and 1% MEM non-essential amino acids) was added per well. Medium was changed every three days, at RT.

Cell viability was assessed for 14 days, by Trypan Blue assay. Matrigel was digested with Cell Recovery solution at a volume ≥2X that of the Matrigel matrix volume, for 1 hour at 4ºC.

4.4. MM 3D JellaGel-based Culture

After BM sample processing, as previously described, $2x10^6$ cells were embedded in 40µL of JellaGel (Jellagen). Following manufacturer's instructions, for JellaGel polymerization before cell embedding, 100µL of crosslinker solution were added for every 1 mL of JellaGel solution. After 3 minutes, cell pellet was resuspended in 40µL of JellaGel and plated on 24-well plates. Plates with the matrix mix were left to polymerize for 15 minutes, at RT, followed by 30 minutes, at 37ºC. Afterwards, 1 mL of cRPMI medium (1% L-Glutamine; 10% FBS; 1% P/S and 1% MEM non-essential amino acids) was added per well.

For JellaGel dissociation, Liberase™ Research Grade (Sigma-Aldrich) was used, at a final concentration of 5mg/mL. 3D cultures with Liberase were left to incubate for 10 minutes, at 37ºC, and then cells were recovered.

4.5. Flow Cytometry Analysis

For analysis of the cell populations in the 3D model, patient-derived BM cells were used. After RBCL or Matrigel digestion (at day 0 and day 7 of culture, respectively), as previously described, cells were incubated with Fc-block Human TruStain FcX (BioLegend), for 10 minutes at RT. After washing, cells were surface stained for 30 minutes, at 4ºC, in the dark, with the following antibodies: CD45 (BV510; BioLegend), CD138 (BV421; BD Biosciences), CD19 (BV785; BioLegend), CD3 (PerCP-Cy5.5;), CD4 (BV711; BioLegend), CD8 (Alexa700; Exbio), CD11b (APC; BioLegend), CD105 (FITC; Exbio), CD31 (BV605; BioLegend) and CD140 (PE-Cy7; BioLegend) – Table 5. BM cells were then washed and incubated with Annexin V (PE; BD Biosciences) in Annexin Buffer (10X, eBioscience), for 30 minutes, at 4ºC, in the dark. After incubation, cells were washed, filtered and transferred into 5 mL tubes for acquisition.

To study the effect of the IMiD lenalidomide on MM cells viability in 3D culture, MM cell lines MM.1S and RLD, cultured in Matrigel were used. After Matrigel digestion, cells were incubated with Fc-block Human TruStain FcX (BioLegend), for 10 minutes at RT. After washing, cells were stained with Annexin V (PE; BD Biosciences) in Annexin Buffer (10X, eBioscience), for 30 minutes, at 4ºC, in the dark. After incubation, cells were washed and transferred into 5 mL tubes. 10 minutes before acquisition, 7-AAD was added to each sample tube – Table 5.

Calibration beads (Rainbow Calibration Beads; BioLegend) were used in all experiments, and compensations were performed using cells or compensation beads (UltraComp eBeads ™; Invitrogen), stained with single antibodies. Samples were acquired on LSRFortessaTM X-20 (BD Bioscience) and data was analyzed using FlowJo™ v10.8.0 Software (FlowJo, LLC; BD Bioscience). The defined gating strategies are described in Figures 1 and 2 of Supplementary Data.

Table 5. List of used flow cytometry antibodies.

ANTIBODY	FLUOROPHORE	CLONE	DILUTION	COMPANY
CD45	BV510	HI30	1:20	BioLegend
CD138	BV421	MI15	1:50	BD Biosciences
CD19	BV785	HIB19	1:20	BioLegend
CD3	PerCP-Cy5.5	SK7	1:30	BD Biosciences
CD4	BV711	SK3	1:20	BioLegend
CD8	Alexa700	MEM-31	1:20	Exbio
CD11b	APC	M1/70	1:200	BioLegend
CD105	FITC	MEM-226	1:20	Exbio
CD31	BV605	WM59	1:20	BioLegend
CD140	PE-Cy7	16A1	1:20	BioLegend
7-AAD	7-AAD		5 µL	BD Biosciences
Annexin V	PE		1:20	BD Biosciences

4.6. Microscopy and Image analysis

4.6.1. Bright-field Microscopy

Images of the 3D Matrigel model were acquired at days 0, 1, 3, 7, 10 and 14 of culture, using a Zeiss PrimoVert Compact Inverted microscope, equipped with a 10x/0,25Ph1 objective lens.

Data was analyzed with Zen 3.2 (ZEN lite; ZEISS) software.

4.6.2. Confocal Microscopy

Distribution of BM cell populations embedded in Matrigel scaffolds was assayed using confocal microcopy at days 1 and 7 of culture.

3D cultures were fixed in 1 mL of Paraformaldehyde (PFA) 2%, Glutaraldehyde 1% solution, for 45 minutes at 37ºC, 5% CO_2. Afterwards, samples were incubated with the following antibodies: 4',6 -Dimidino-2-phenylindole (DAPI; Sigma-Aldrich), for 30 minutes; CD45-BV650 (BioLegend) and CD38-PE (Miltenyi Biotec), for 1 hour; and Propidium Iodide (PrI; BD Biosciences), for 15 minutes – Table 6. Washing in between incubations was done with 1 mL PBS, for 10 minutes, 3 times.

Images were acquired using a Zeiss LSM 980 inverted confocal microscope equipped with a 10x/0,3 EC Plan-Neufluoar objective lens. All experiments were performed at 37ºC, and a Pecon stage incubator was used to maintain temperature control. The pinhole was set to 1 Airy Unit for each channel. Scan speed was set to 0.77 µs and an averaging of 4 was applied. Each frame consisted of a 1024 x 1024 pixel image, corresponding to 848,53 x 848,53 µm.

The cultures were excited at 353 nm (DAPI), 519 nm (PrI), 577 nm (CD38-PE), and 653 nm (CD45-BV650), and the range of detection wavelength was at 413-473 nm, 535-640 nm, 588-640 nm and 638-756 nm, for each channel respectively. Z-stack images of 400µm thickness were taken for the whole sample, and three Z-stack images of 133µm thickness were taken for bottom, middle and top sections of each sample, with an interval of 3µm between slices.

Data was analyzed using ZEN 3.4 (blue edition; ZEISS) software and ImageJ (Fiji; National Institutes of Health) software.

Table 6. List of used confocal markers.

MARKER	FLUOROPHORE	CLONE	DILUTION	COMPANY
DAPI			1:1000	Sigma-Aldrich
PrI			5µL	BD Biosciences
CD38	PE	IB6	1:50	Miltenyi Biotec
CD45	BV650	HI30	1:100	BioLegend

4.7. Immunohistochemical Analysis

For observation of the cells inside the Matrigel matrix, immunohistochemistry (IHC) techniques were used. At day 7 of culture, medium was removed and the 3D cultures were fixed in 1 mL of PFA 2%, Glutaraldehyde 1% solution, for 45 minutes, at 37ºC, 5% CO_2. Afterwards, the Matrigel domes were embedded in paraffin. Serial 4µm horizontal histological sections were obtained. Samples were stained with hematoxylin solution for further analysis in brightfield microscopy.

4.8. Cytological Analysis of the 3D Culture Model Supernatant

To identify the different cell populations escaping the Matrigel matrix, cytological analysis of cells exfoliated from the 3D MM model was performed in culture supernatant smears. Culture medium was collected after 7 days of culture and centrifuged for 5 minutes at 1500 rpm. The liquid fraction was discarded and the cellular sediment was placed onto a glass slide, smearing with the help of a coverslip. Slides were immediately air-dried, fixed with 100% methanol for 5 minutes and stained with Giemsa solution for 15 minutes.

Analysis was performed using a Leica Axioscope 5 microscope coupled to an Axiocam 208 color camera (ZEISS).

4.9. Drug Response in 3D Culture

For studying the effect of the 3D culture on MM cells response to drugs, 40 000 cells from sensitive and resistant to lenalidomide MM.1S and RLD cell lines, respectively, were cultured.

Lenalidomide (Revlimid® 25mg, Celgene) was dissolved in dimethyl sulfoxide (DMSO) to 1mg/mL. DMSO concentration was under the cell toxicity threshold (<0,048%), as confirmed by flow cytometry comparison of a control group of cells cultured with normal cRPMI medium *versus* a group of cells cultured with DMSO diluted in the cRPMI medium – Supplementary Figure 3. Stock solutions were diluted in sterile PBS to a final concentration of 487 ng/mL, according to the drug pharmacokinetic maximum serum concentration (Cmax), provided in the product monograph (referred as website number [7]).

When MM cells achieved maximum viability, the lenalidomide solution was added to the 3D culture. At 48h post-treatment, MM cells were retrieved from culture using Cell Recovery solution at a volume ≥2X that of the Matrigel matrix volume, for 1 hour, at 4ºC. Cell survival was analyzed by flow cytometry (see section 4.5.). Experiments were performed in triplicates.

4.10. Ethics approval/ Patients Informed Consent

BM samples were obtained from MGUS and MM patients followed at the Hematology Unit of Champalimaud Clinical Centre, who fulfilled the inclusion criteria (described in section 4.1) and signed the general informed consent form, approved by Champalimaud Foundation Ethics Committee, as adequate for this purpose – form enclosed on page 67.

The samples were obtained during routine medical appointments, required for diagnosis, treatment evaluation or disease follow-up. The patient's involvement in this study did not imply additional health risks or interventions. It did not influence treatment or required subsequent monitoring.

All clinical and personal information collected were stored and processed according to the Champalimaud Foundation Ethics Committee guidelines and international and national legislation, including the "Regulamento Geral de Proteção de Dados nº 2016/679 de 25 de maio de 2018" and the "Lei Portuguesa de Proteção de Dados (Lei 67/98, de 26 de outubro)".

4.11. Statistical Analysis

Statistical analysis was performed using GraphPad Prism (Dotmatics). Results were presented as mean ± standard deviation. A p value less than 0.05 was considered significant: * $p \leq 0.05$ and ** $p \leq 0.001$. If the p-value is not indicated, results presented were not statistically significant (ns).

Statistical significance was analyzed using non-parametric Wilcoxon signed-rank test for two paired groups, in the following experiments:

- determination of differences in the number of live cells inside Matrigel after 7 days;
- determination of differences in the total number of cells inside Matrigel at day 1 *versus* day 7, detected by confocal microscopy;

- determination of differences in the proportions of cell populations analyzed by flow cytometry in Matrigel 3D culture at day 7, compared to patient sample at day 0;
- determination of differences in the percentage of PrI+, CD45+ or CD38+ cells in Matrigel after 7 days of culture, detected by confocal microscopy.

Mann-Whitney U test was applied to determine a statistical significance in the presence of two independent groups, in the following experiments:

- impact of the presence of ROCK inhibitor on cell viability after 7 days in Matrigel 3D culture;
- impact of the presence of ROCK inhibitor on the cell populations in Matrigel, determined by flow cytometry, at day 7;
- determination of the differences in cell viability at day 7, of MGUS and MM samples in Matrigel 3D culture;
- determination of the differences in cell viability and cell number at day 7 in 3D culture with Matrigel *versus* JellaGel.

Non-parametric Friedman test was used to determine differences between more than two paired groups, in the following experiments:

- determination of the best BM cell seeding concentration in Matrigel 3D culture;
- determination of the best MM cell line (MM.1S and RLD) seeding concentration in Matrigel 3D culture.

Mixed-effects model and Two-way ANOVA were used to predict the influence of two variables on our continuous dependent variable.

Mixed effects analysis was used in the following experiments:

- impact of the presence or absence of patient-derived plasma in 3D and 2D culture for 7 days.

Two-way ANOVA was used in the following experiments:

- effect of the days and the regions of the model (bottom, middle and top) on the percentage and distribution of PrI+, CD38+ or CD45+ cells, determined by confocal microscopy after 7 days in 3D culture;
- effect of lenalidomide treatment in MM cell line cultures at 48h post-administration.

5. Results

5.1. 3D Model Development – Testing the Ideal Conditions for the 3D MM BM Culture

5.1.1. Red Blood Cells Presence

To optimize the conditions of co-culturing, the viability and structural stability of the 3D model in the presence or absence of RBCs was compared. Patient samples were incorporated with Matrigel, without processing, or after RBCL, according to the condition tested. When plating samples with RBCs included, Matrigel domes did not sustain its form, presenting a lose shape, and in some cases not even formed. On the other side, samples embedded in Matrigel after RBCL allowed the plating of a well-defined, round dome, that kept its shape through time. Moreover, samples with RBCs did not allow its observation on the microscope, due to their dense and opaque nature. Furthermore, cell viability was increased in the absence of RBCs, from 10% to 60%, even at day 1 post-plating – Figure 11. These results pointed to the clear necessity of removing RBCs from the patient BM sample before proceeding to the embedding in Matrigel and this condition was followed in the further tests.

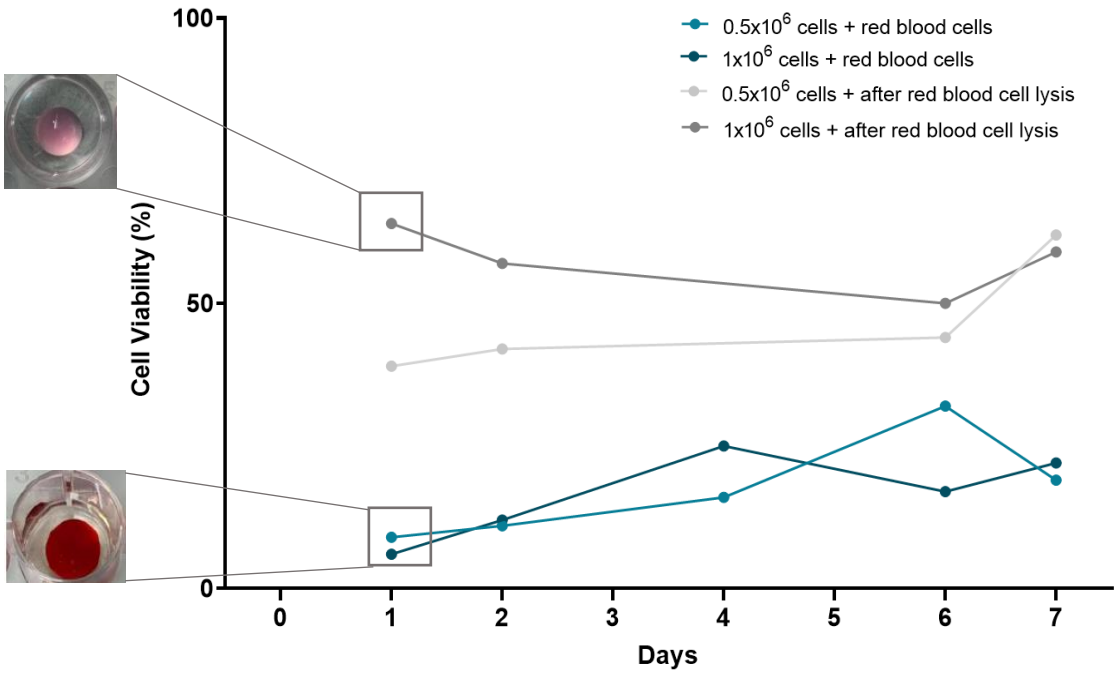


Figure 11. Cell viability in the presence and absence of red blood cells. 3D model with RBCs (blue and dark blue) *versus* 3D model without RBCs (grey and dark grey), at days 1 and 7 of culture. Patient-derived cells embedded in 40µL of Matrigel (n=1).

5.1.2. ROCK Inhibitor Effect

ROCK inhibitor is a selective inhibitor of the Rho-associated, coiled-coil containing protein kinase, commonly used in cell culture to kick-start cells (Braam et al., 2010; Claassen et al., 2009; Watanabe et al., 2007). To determine the impact of ROCK inhibitor on cell viability after 7 days in Matrigel 3D culture, cells were embedded in Matrigel with or without ROCK inhibitor, and cultured with complete medium with or without ROCK inhibitor, respectively. Viability was assessed on day 0, before embedding the cells in Matrigel, and on day 7 of 3D culture. Overall cell viability was significantly higher in the presence of ROCK inhibitor ($p \leq 0.05$) – Figure 12A. The impact of the presence of ROCK inhibitor on the specific cell populations in Matrigel, was the determined, using multiparametric flow cytometry. Results showed that there were no significant differences in cells percentages with and without ROCK inhibitor, after 7 days, except for endothelial cells, which were nearly 0% in the absence of ROCK inhibitor– Figure 12B (right). Because ROCK inhibitor can have an undesired impact on MM cells, in the context of disease setting (Federico et al., 2020), the results obtained confirmed that it was possible to culture the patient-derived cells without this molecule and this condition was followed in the further tests.

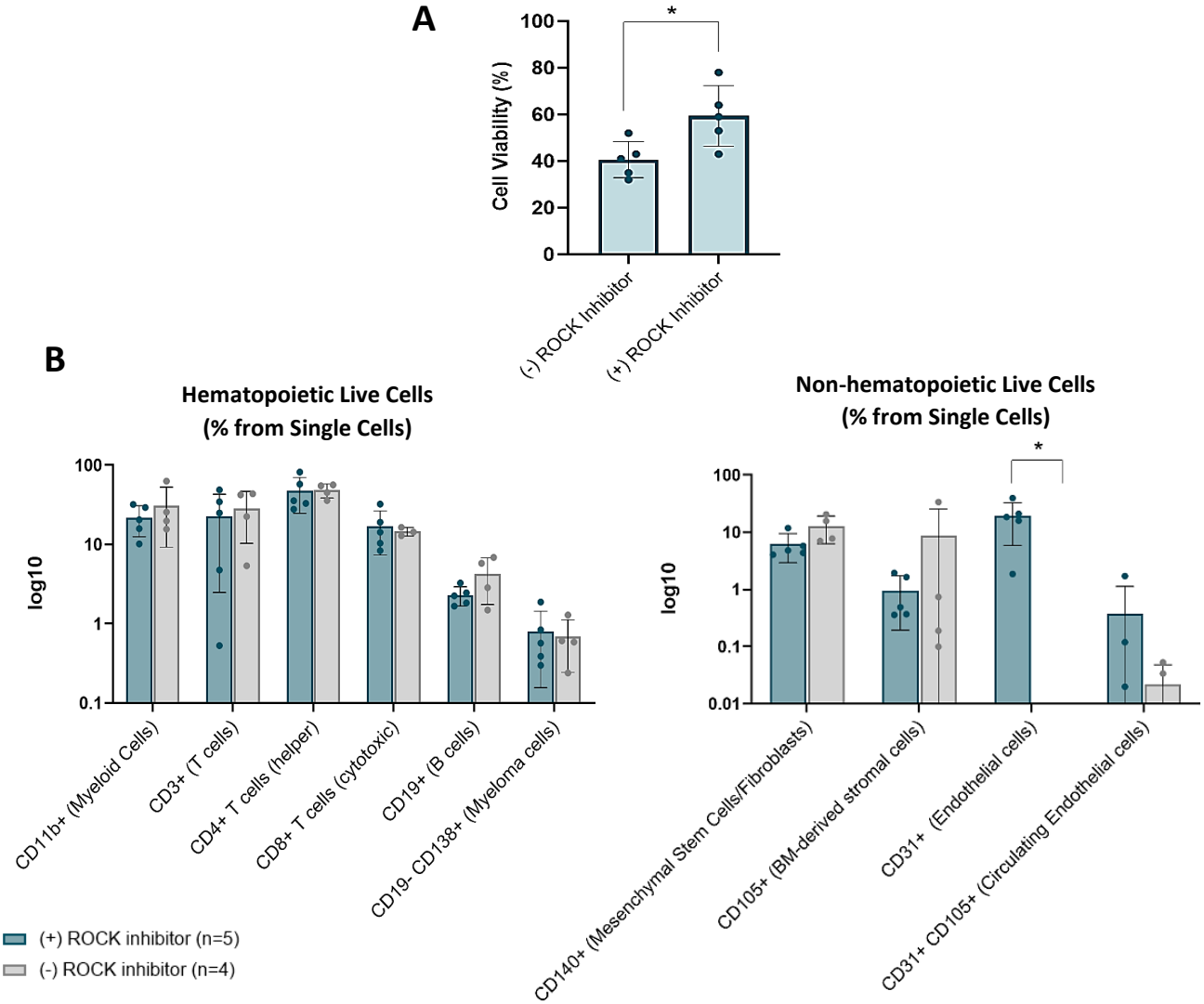


Figure 12. Effect of ROCK inhibitor on 3D culture. (A) Cell viability at day 7 of culture, with and without ROCK inhibitor. $2x10^6$ patient-derived BM cells without RBCs, embedded in 40µL of Matrigel (n=5). Mann-Whitney test (ns). (B) Percentage of hematopoietic (left) and non-hematopoietic (right) cell populations in 3D culture, after 7 days, in the presence (blue, n=5) or absence (grey, n=4) of ROCK inhibitor, determined by flow cytometry. Mann-Whitney test (*$p \leq 0.05$). A log-transformation base 10 was applied to better visualize the data.

5.1.3. Cell Seeding Concentration

After removing RBCs and ROCK inhibitor from the culturing conditions, 3D MM patient-derived cultures were established and a range of cell concentrations in 40µL of Matrigel were tested, to determine the ideal seeding concentration. Cell viability was assessed on days 0, 1, 3, 7, 10 and 14. $2x10^6$ was the concentration of cells that allowed the maintenance of a higher viability through time – Figure 13. The model was able to sustain a 60% cell viability for 14 days – Figure 14A. No differences were observed in cell viability between MM or MGUS samples – Figure 14B. Together, these results reflect the model ability to maintain a variety of cells.

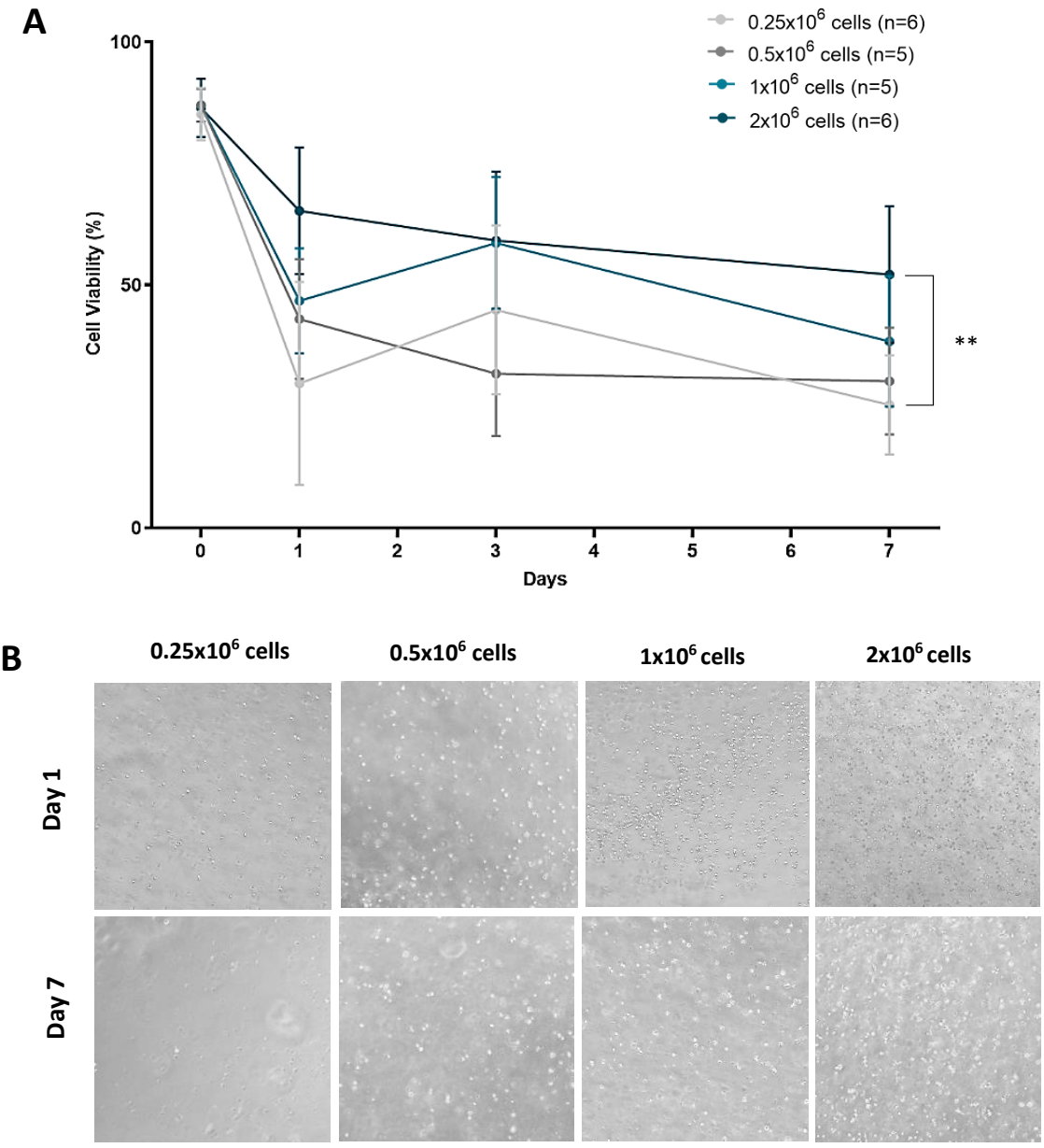


Figure 13. Cell viability of different cell seeding concentrations, after 7 days in culture. (A) Cell viability at days 1, 3 and 7 of culture. $0.25x10^6$ (light grey, n=6), $0.5x10^6$ (dark grey, n=5), $1x10^6$ (light blue, n=5) and $2x10^6$ (dark blue, n=6) patient-derived cells without RBCs in 40µL of Matrigel, without ROCK inhibitor. Friedman test (**$p \leq 0.001$). (B) Brightfield microscopy images of cells inside 3D Matrigel domes at days 1 and 7 of culture (10x objective).

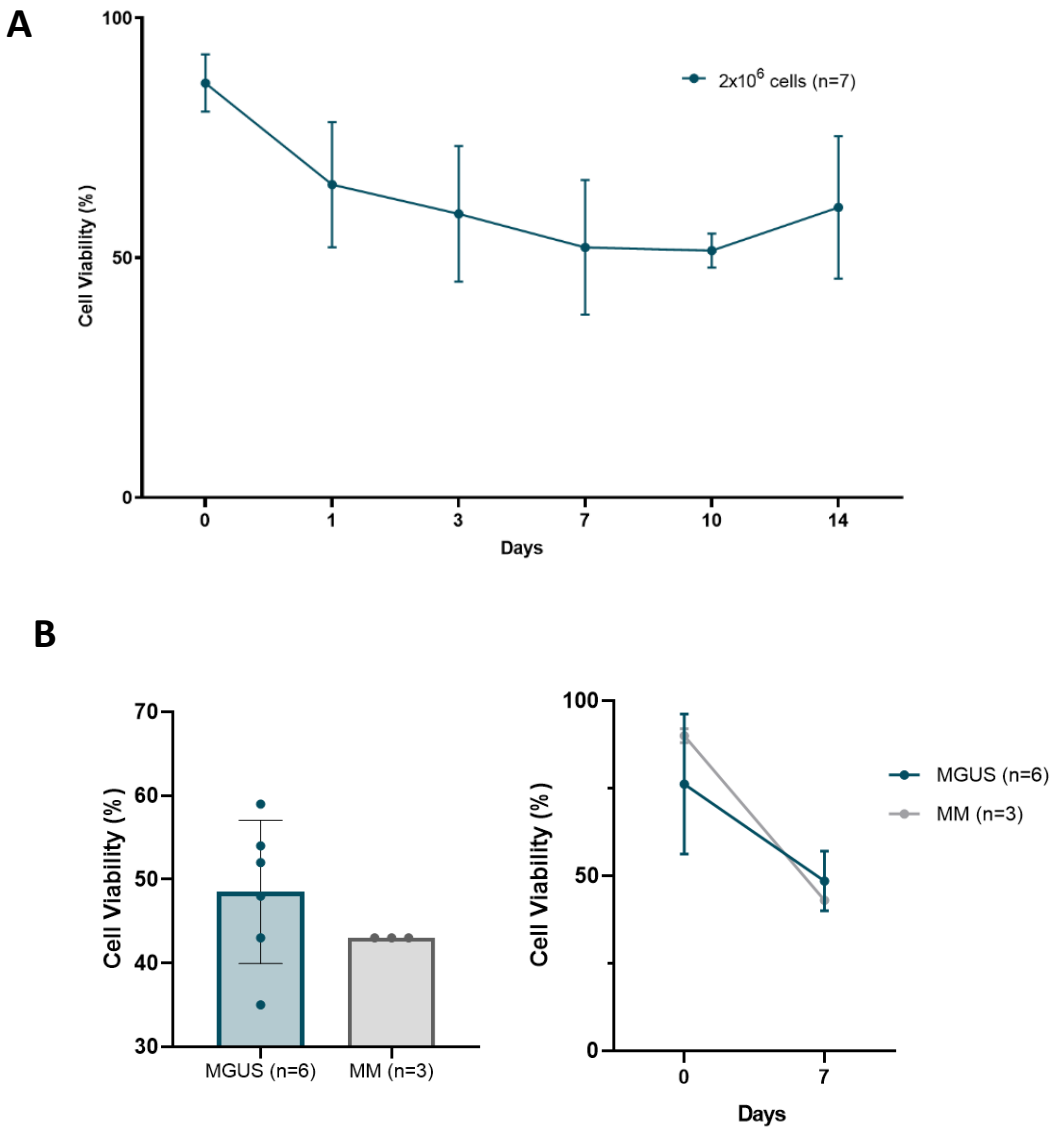


Figure 14. Cell viability after 7 and 14 days. (A) The 3D model is able to sustain cell viability for up to 14 days. Cell viability at days 1, 3, 7, 10 and 14 of culture. $2x10^6$ patient-derived cells without RBCs in 40µL of Matrigel, without ROCK inhibitor (n=2). (B) The 3D model is able to sustain cell viability of samples from different stages of disease. Cell viability of MGUS (blue, n=6) and MM samples (grey, n=3) after 7 days in culture. $2x10^6$ patient-derived cells without RBCs in 40µL of Matrigel, without ROCK inhibitor. Mann-Whitney test (ns).

5.1.4. Plasma Supplemented Medium Effect

To determine if cell viability could be improved by supplementing the culture media with the patient's corresponding plasma, BM samples were centrifuged prior to RBCL, for plasma isolation. Cells were cultured with normal complete medium or with 10% of patients own plasma supplemented complete medium. Cell viability after 7 days of culture was significantly lower in plasma supplemented medium samples ($p \leq 0.05$) – 40% *versus* 60% in the normal medium condition – Figure 15A. To exclude the possibility of the decrease in cell viability being due to plasma and Matrigel matrix protein

unfavourable interactions, 2D cultures with or without 10% of patients own plasma supplemented medium were also set-up. Results showed that cell viability in 2D culture was also lower in the presence of plasma in the culture medium – Figure 15B. These results suggest that combining patient-derived plasma with the culture medium can negatively impact the different cell populations present in the model.

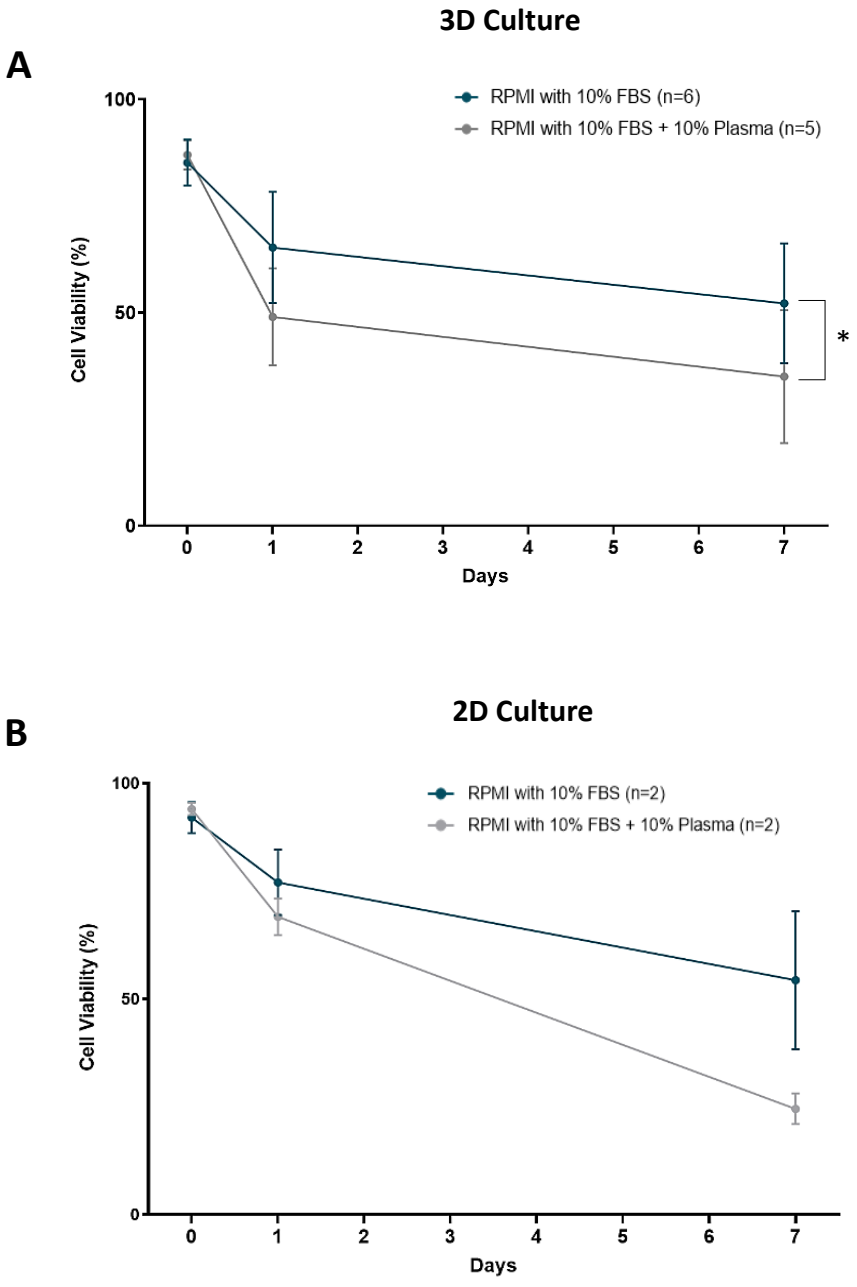


Figure 15. Effect of the presence or absence of patient-derived plasma supplemented medium in 3D and 2D culture, after 7 days. (A) 3D culture of $2x10^6$ patient-derived cells without RBCs in 40µL of Matrigel, without ROCK inhibitor, cultured with normal complete medium (blue, n=6) or with patient-derived plasma supplemented complete medium (grey, n=5). Mixed effects analysis (*$p \leq 0.05$). (B) 2D culture of $2x10^6$ patient-derived cells without RBCs and ROCK inhibitor, cultured with normal complete medium (blue, n=2) or with patient-derived plasma supplemented complete medium (grey, n=2). Mixed effects analysis (ns).

5.2. 3D Model Characterization

5.2.1. Are cells escaping the matrix? Which cells?

During the optimization tests, a reduction in the number of cells inside the Matrigel matrix was noticed after 7 days of culture – Figure 16A. To further investigate the phenomenon, bright field pictures were taken throughout the period of culture, and it was possible to observe the cells accumulating in the borders of the matrix, even after only one day of culture – Figure 16B. Cells started to get out of the Matrigel matrix as time progressed, and the number of cells that remained inside the model after 7 days was significantly decreased ($p \leq 0.05$) – Figure 16C.

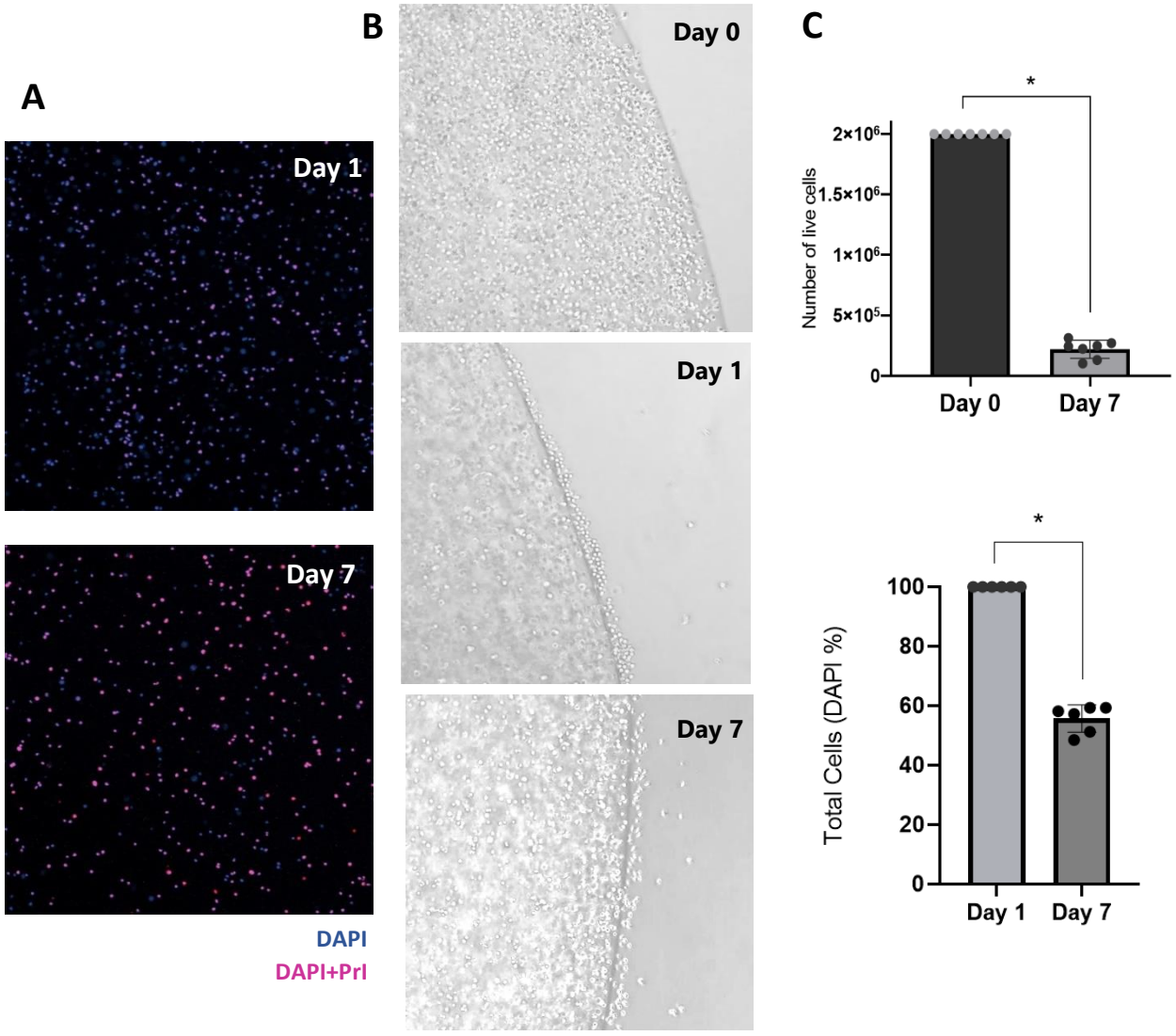


Figure 16. Decrease in the number of cells inside Matrigel after 7 days in 3D culture. (A) Maximum intensity projection of confocal microscopy z-stack images of cells inside the Matrigel matrix at day 1 *versus* day 7 (10x objective). Blue – DAPI; Red – PrI; Pink – DAPI + PrI (dead cells). (B) Brightfield microscopy images of cells escaping the Matrigel matrix at days 0, 1 and 7 (10x objective). (C) Number of live cells inside Matrigel after 7 days in culture (top, n=7) and number of total cells at day 1 *versus* day 7 of culture, determined by confocal microscopy (bottom, DAPI stained cells, n=5). Wilcoxon test (*$p \leq 0.05$).

In order to understand if this was specific to one population of cells, or random, cell culture medium was collected after 7 days, centrifuged, and cell pellet was used for a smear – Figure 17. The results showed a variety of cells and their precursors, of both hematopoietic and non-hematopoietic nature, including lymphocytes, adipocytes, macrophages and stromal cells. Therefore, this suggests that the migration events were not specific to a determined type of cells but rather a generalized event.

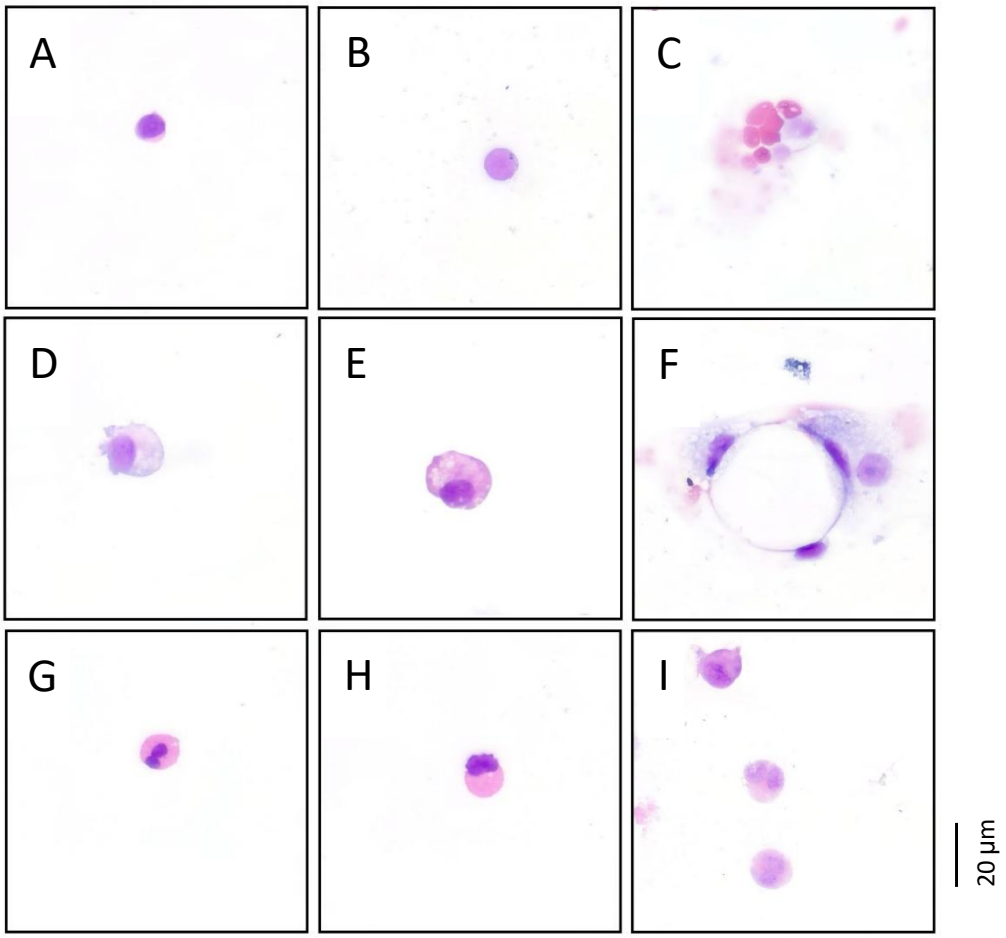


Figure 17. Representative microphotographs of patient-derived cells in smears from supernatants of 3D culture at day 7. (A), (B) lymphocytes; (C) RBCs; (D), (E) macrophages; (G), (H), (I) granulocytes; (F) adipocyte. n=2. Giemsa staining (40x amplification).

5.2.1.1. Matrigel *versus* Collagen Matrix

Due to the existence of cells escaping the Matrigel matrix, an alternative matrix option was explored, composed of collagen (type 0), the main structural component of human ECM. After BM sample processing, as previously described, $2x10^6$ cells were embedded in the same volume of JellaGel or Matrigel. Cell viability was determined on day 0 before plating, and on days 1 and 7 of culture. Results showed that, even though an initial higher viability was noticed on JellaGel samples, as cells in Matrigel slowly started to become stable and improve viability, cells in JellaGel suffered a decline – Figure 18A. Moreover, when assessing the number of cells in culture, our 3D model in Matrigel showed a better capability of maintaining cells inside the dome – Figure 18B. Overall, it was possible to observe a similar behavior of cells in both types of matrix – Figure 18C, and similar viability curves.

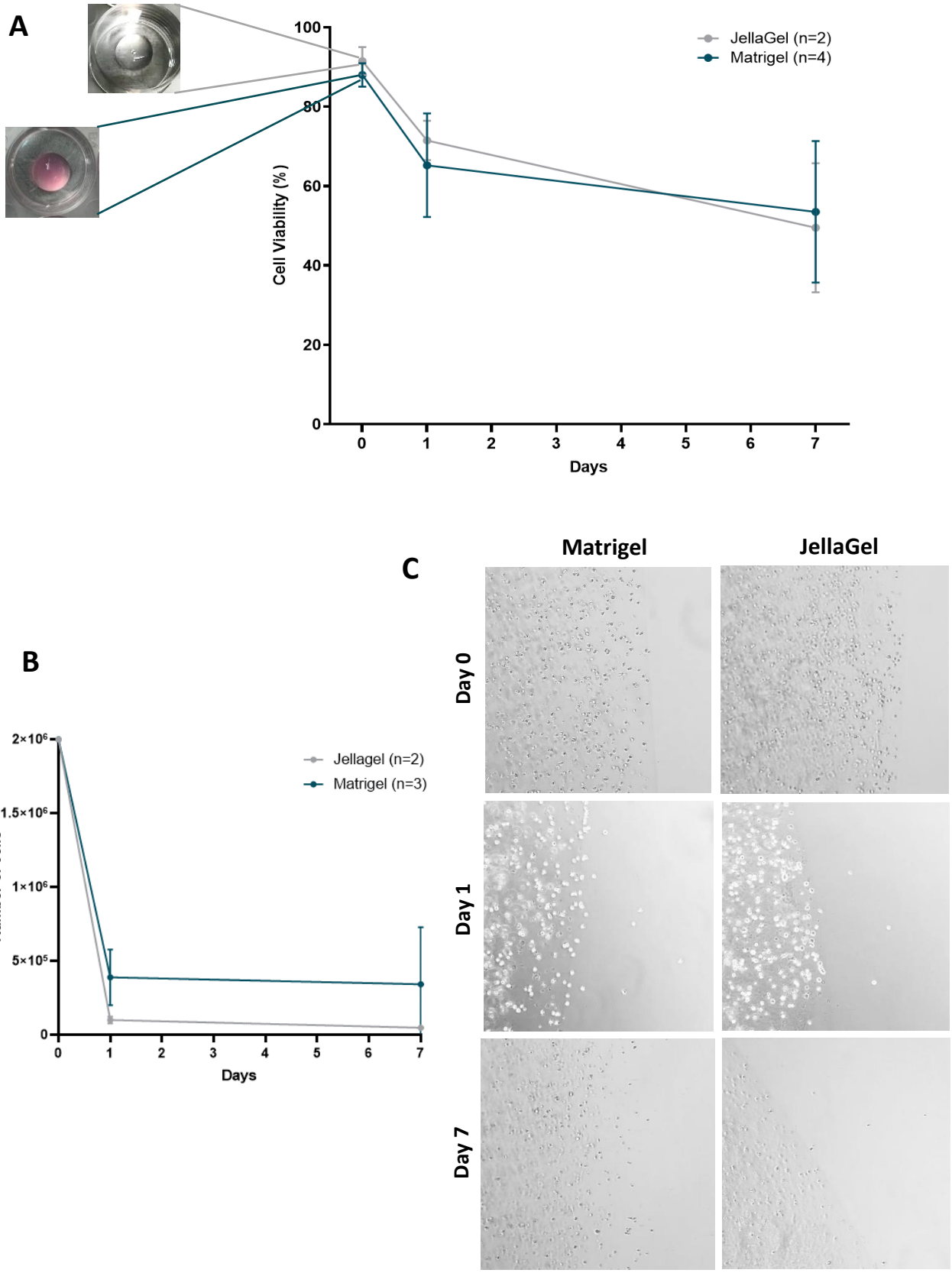


Figure 18. Matrigel *versus* JellaGel 3D culture. $2x10^6$ patient-derived cells seeded in 40µL of Matrigel or JellaGel. (A) Cell viability after 7 days of culture, in Matrigel (blue, n=4) *versus* JellaGel (grey, n=2). Mann-Whitney test (ns). (B) Number of cells inside the Matrigel (blue, n=3) or JellaGel (grey, n=2) at days 1 and 7 of culture. Mann-Whitney test (ns). (C) Brightfield microscopy images of $2x10^6$ patient-derived cells seeded in 40µL of Matrigel (left) or JellaGel (right) at days 0, 1 and 7 of culture (10x objective).

5.2.2. Which cell populations are present in the 3D model?

To determine the different cell populations present in the 3D culture, as well as their proportions, BM cells were analyzed through multiparametric flow cytometry at day 0, before cell embedding in Matrigel, and after Matrigel digestion, at day 7. After 7 days in culture, it was possible to observe a similar representativity of all the cell populations, including both hematopoietic CD4+, CD8+, CD11b+, CD19-CD138+ cells, and non-hematopoietic CD105+CD31+ and CD140+ cells, when compared to the corresponding patient BM sample – Figure 19A. This indicates a good recapitulation of the initial BM sample composition. To further investigate the content of the 3D model, cells inside Matrigel were stained with PrI, CD45 and CD38, and cells were detected by confocal microscopy, showing a similar percentage of the cells inside Matrigel after 7 days, when compared to day 1 of culture – Figure 19B. Together, these results suggest that our model may be providing a benefic environment for different types of cell populations.

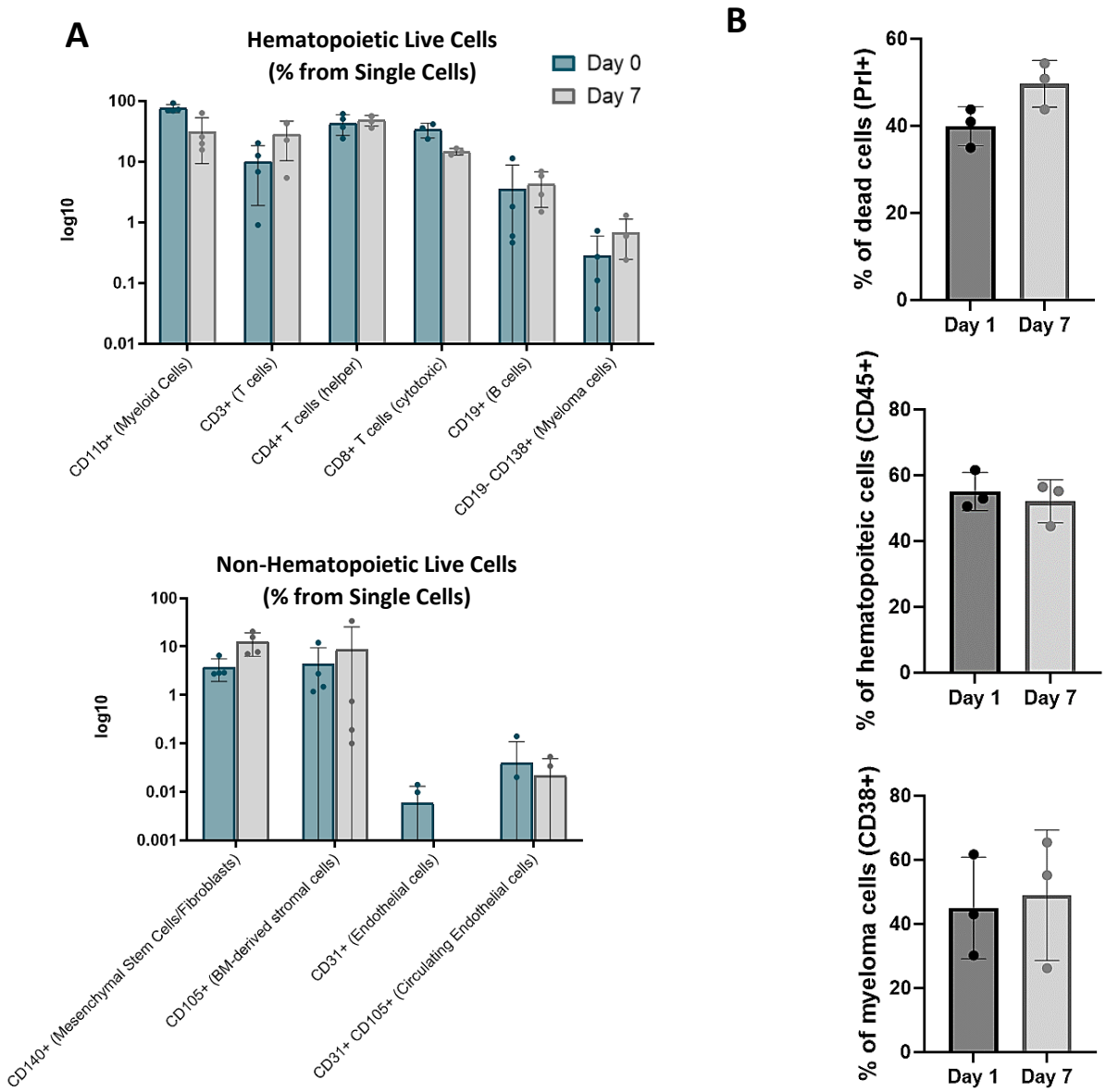


Figure 19. Representation of the different cell populations in the 3D model after 7 days of culture. (A) Percentage of cell populations present in 3D culture, at day 7 (grey) *versus* BM sample composition, at day 0 (blue), determined by flow cytometry (n=4). Wilcoxon test (ns). A log-transformation base 10 was applied to better visualize the data. (B) Percentage of dead (top, PrI+), hematopoietic (middle, CD45+), and MM (bottom, CD38+) cells present in the 3D model at days 1 (dark grey) and 7 (light grey), determined by confocal microscopy (n=3), Wilcoxon test (ns).

5.2.3. How are cells organized in the 3D structure?

Aiming at a better characterization of our model, the distribution of the BM cell populations embedded in Matrigel scaffolds was studied, using confocal microcopy, at days 1 and 7. Cells inside Matrigel were stained with PrI, CD45 and CD38, and confocal microscopy images of 3 regions (bottom, middle and top) of the scaffold were acquired. While dead cells seemed to localize preferentially on the top layer of the matrix, at day 7, when compared to day 1 – Figure 20A and D, no other preferential localization was identified. Both CD45+ and CD38+ cells showed a homogenous distribution through the scaffold – Figure 20B, C and E.

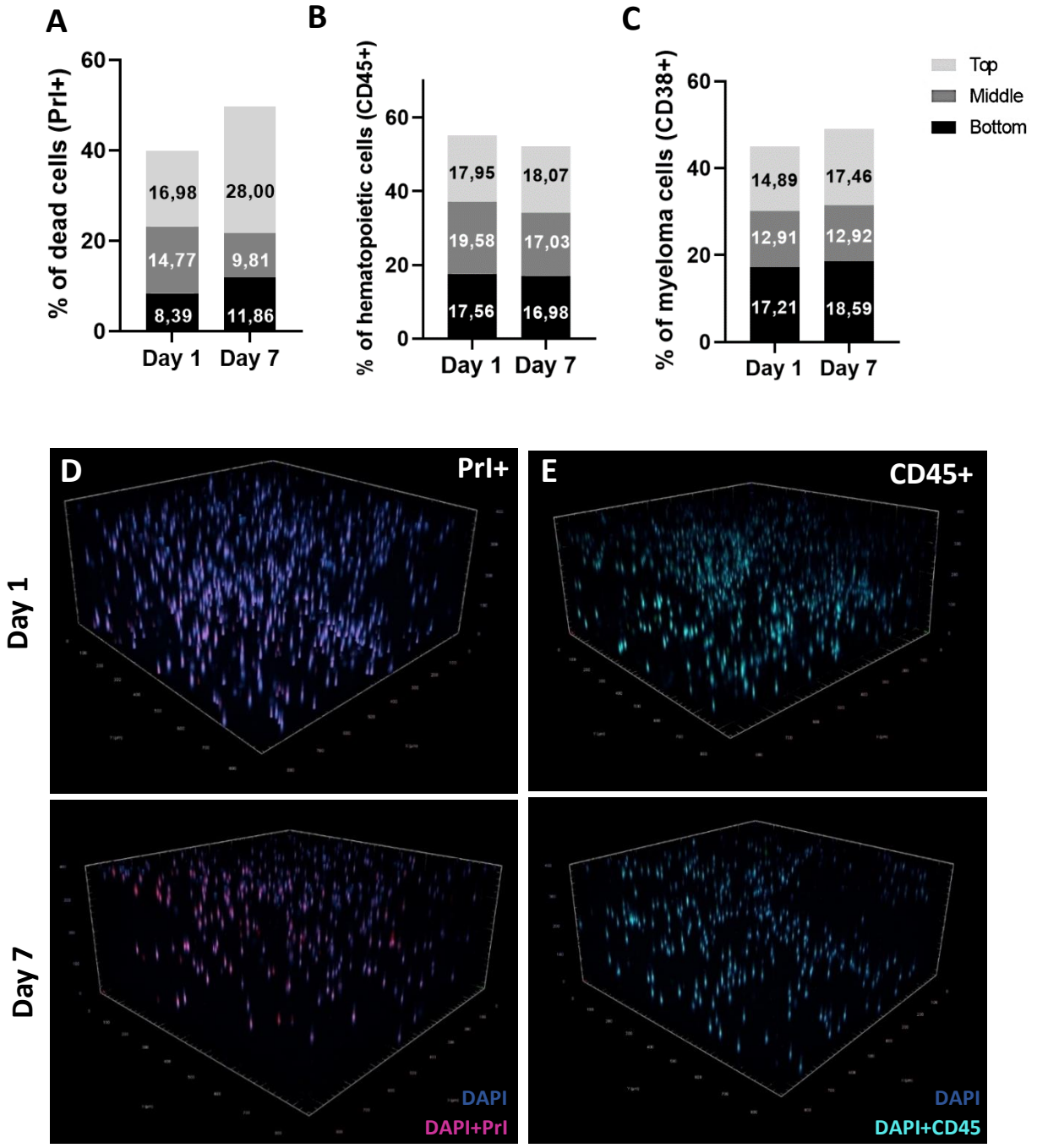


Figure 20. Distribution of different cell populations from patient BM samples within the 3D model. (A) percentage of dead cells at day 1 and 7; (B) percentage of hematopoietic cells at day 1 and 7; and (C) percentage of myeloma cells at day 1 and 7 (n=3, Two-Way ANOVA (ns)). (D) Live/dead. Blue – DAPI; Pink – DAPI + PrI (dead cells) and (E) hematopoietic cells (CD45+). Blue – DAPI; Light Blue – DAPI + CD45+. Determined by confocal microscopy (10x objective).

5.2.4. Does the 3D model allow the assessment of drug responses?

To study the effect of the 3D culture on MM cells response to therapy, a lenalidomide resistant MM cell line, RLD, and a lenalidomide sensitive MM cell line, MM.1S, were used, in monoculture. When MM cells achieved maximum viability, above 80% – Supplementary Figure 4, lenalidomide was added to culture, as a single agent, for 48h. Bright field microscopy images revealed that both cell lines responded as would be expected: RLD cells treated with lenalidomide showed resistance to treatment, migrating and proliferating, as well as cell forming cell aggregates (clusters) in culture, the same way it is observed under normal culturing conditions – Figure 21 (top). MM.1S cells showed sensitivity to lenalidomide and cells did not proliferate or form clusters, maintaining a single cell morphology, contrary to the cells in the control group – Figure 21 (bottom).

At 48h post-treatment, MM cells were retrieved from culture and cell survival was analyzed by flow cytometry. The percentage of live and dead RLD cells treated with lenalidomide was like the ones in the control group, with around 70% of live cells *versus* 20% of dead cells (from single cells) – Figure 22 (top). On the contrary, the percentage of live lenalidomide-treated MM.1S cells was much lower, compared to the corresponding control group, with around 50% live cells in control group *versus* 20% in lenalidomide culture (from single cells) – Figure 22 (bottom). Both cell lines responses to lenalidomide treatment agreed with their original cell line characteristics of drug sensitivity or resistance. Thus, these results suggest that our 3D model allows the study of cells response to drugs in culture.

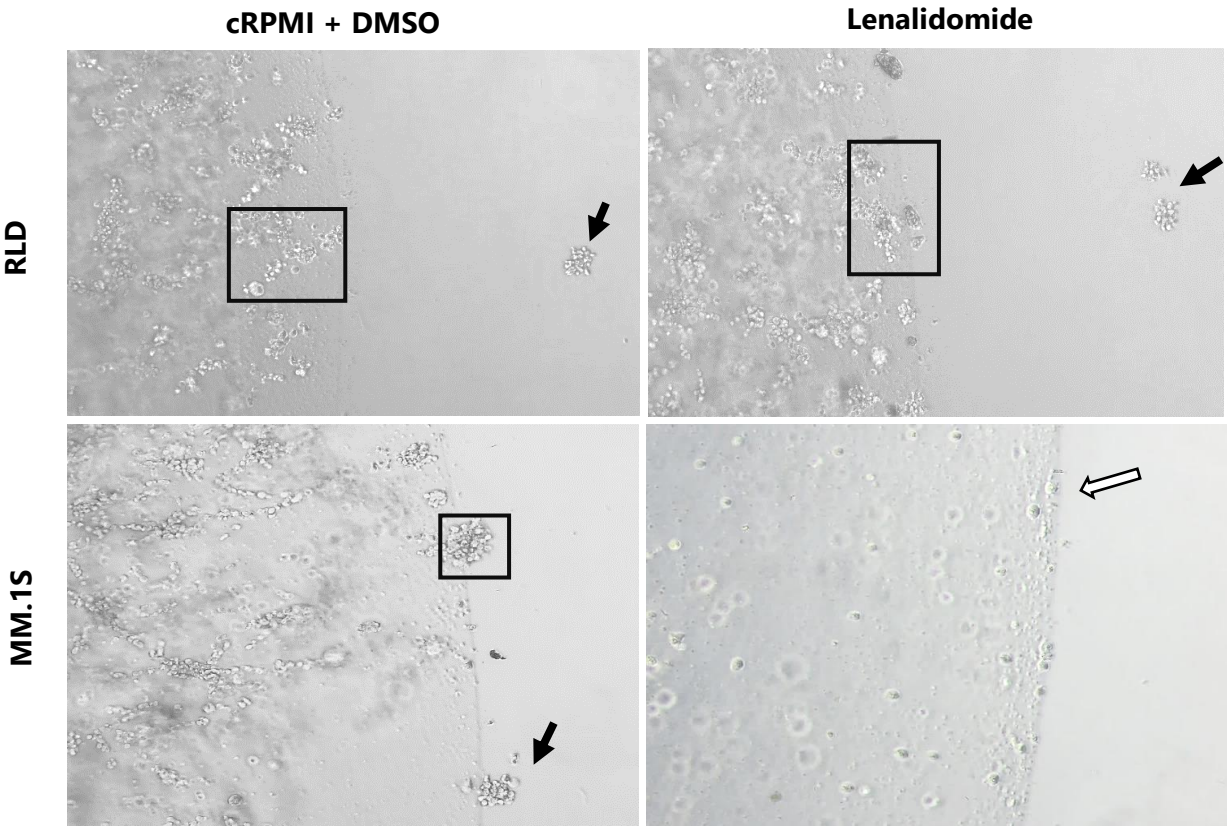


Figure 21. Effect of lenalidomide treatment on RLD and MM.1S cells activity. Both cell lines responses to lenalidomide treatment agreed with their original cell line characteristics of drug sensitivity or resistance. Brightfield microscopy images (10x objective) of 3D cultures with 40 000 RLD or MM.1S cells, control group (right) *versus* 48h lenalidomide treatment (left). Square: cell clusters; Black arrows: migrating cells; White arrow: single cells, not migrating.

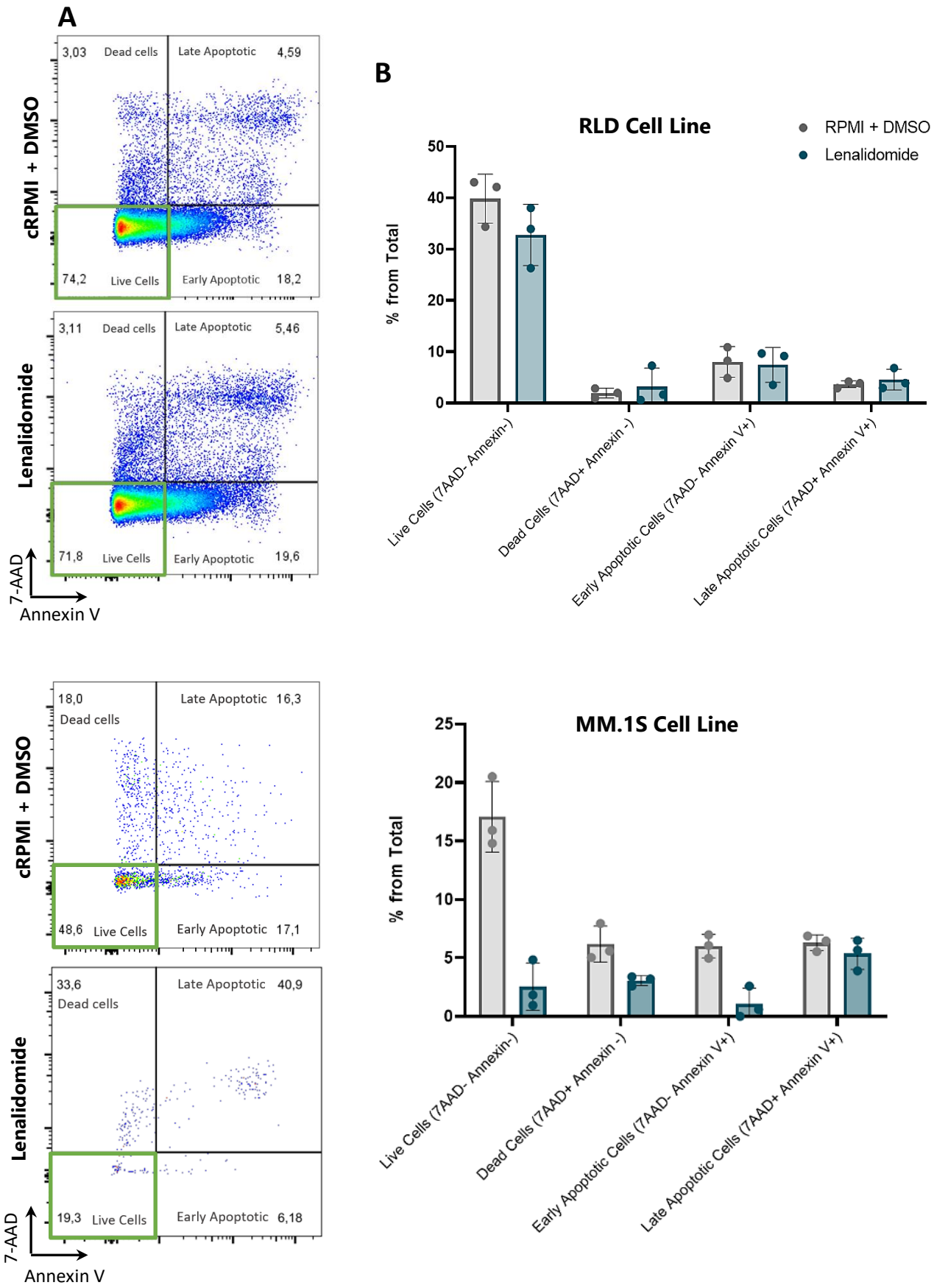


Figure 22. Cellular viability of RLD and MM.1S cell lines after lenalidomide treatment. (A) Representative dot plots of RLD (top, lenalidomide resistant) and MM.1S (bottom, lenalidomide sensitive) live cells in control group (cRPMI + DMSO) *versus* 48h lenalidomide-treatment. (B) Percentage of live (7AAD-, Annexin V-), dead (7AAD+, Annexin V-) and apoptotic (7AAD-, Annexin V+/ 7AAD+, Annexin V+) cells, in control group (grey) *versus* 48h lenalidomide-treatment (blue), determined by flow cytometry. RLD (n=3, 3 independent experiments. Two-Way ANOVA (ns)) and MM.1S (n=3, 1 experiment) cell lines in 40µL Matrigel, without ROCK inhibitor.

6. Discussion

As previously mentioned, MM cells are heavily dependent on their BM microenvironment, in the context of disease and MM progression (Ho et al., 2020). In co-cultures, it is possible to find different cell types that are being grown together in the same environment. This type of culture was described in the 70s as a system that allows the study of communication amongst cells, divided into three types: cell-cell, cell-microenvironment and paracrine signaling by soluble factors (Lawrence et al., 1978). Co-culture systems allow scientists to observe interactions that resemble *in vivo* conditions, and this way attribute different functions to different cell types involved in determined events or mechanisms. In fact, studies show that both target and accessory cells thrive in co-culture conditions (Bian et al., 2011; de la Puente et al., 2015). In the last year, several groups have developed studies in their MM models, highlighting the importance of co-culture. Khan et al. showed that MM cells seeded in 2D monoculture were less than 20% viable, as soon as 48h hours after seeding, whereas MM cells cultured with mesenchymal, endothelial and hematopoietic cells were able to expand and maintain a >90% viability for at least 10 days (Khan et al., 2022). Waldschmidt et al. and Wu et al. proved that co-culture with stromal cells enhanced MM proliferation compared to monocultures (Waldschmidt et al., 2022; Wu et al., 2022). Moreover, bortezomib and auranofin induced less cytotoxicity in co-culture *versus* monoculture (Waldschmidt et al., 2022). This information points to a crucial role of the supportive niche for primary blood cancer cells. Also, the effects of normal BM stroma (using, for example, HS-5 cell line) on MM progression was different to the MM-derived stroma environment (Adamo et al., 2020), suggesting that patient-derived microenvironment cell populations are required, in order to recreate the malignant environment accurately. Therefore, we aimed to develop a complete patient-derived MM BM 3D model, that included all cell populations present in the provided BM sample. That work is presented and discussed in this thesis.

In this chapter, firstly, the results obtained are discussed (sections 6.1. to 6.3.). Then, the challenges encountered during the development of this model are explained. Limitations of the model are also pointed out (section 6.4.). Finally, the future goals and next steps in further characterizing and validating our model are described (section 6.5.).

6.1. The 3D MM BM model does not require red blood cells, ROCK inhibitor, or plasma supplemented medium for optimal culture conditions

To avoid as much manipulation as possible, and maintain a faithful representation of the original sample, in a first attempt, the whole BM sample was incorporated into the Matrigel matrix, including RBCs. However, when plated, Matrigel domes did not form or if so, shape was not uniform or sustained for long. Moreover, microscopic analysis was not possible, as RBCs densely populated the matrix, impeding identification of any other type of cells. Most importantly, overall cell viability in the 3D model was below 15%, becoming clear that it was not possible to include RBCs in the model. To have a better starting point for our experiments, before embedding in Matrigel, a lysing buffer solution was added to the samples, lysing RBCs.

Next, we sought to determine the effect of ROCK inhibitor on overall cell viability, as well as on cellular sub-populations, assessed by flow cytometry. Rho-kinase is a downstream effector of the small guanosine triphosphatase protein. This kinase is described in multiple cellular events, including cell contraction, motility, polarity, morphology, and proliferation (Amano et al., 2010). By inhibiting ROCK, maintenance, self-renewal, reprogramming and differentiation of cells can be achieved, in

culture (Hu et al., 2015). ROCK inhibitor was reported to decrease human embryonic stem cells dissociation-induced apoptosis (Watanabe et al., 2007), improve survival of both cardiomyocyte and non-cardiomyocyte cell populations (Braam et al., 2010), and enhance cell recovery of iPSCs after cell sorting (Emre et al., 2010) and cryopreservation (Claassen et al., 2009). Thus, ROCK inhibition can be used as a way to revive cells. It is therefore a target of interest in various disorders, including ophthalmic (Saha et al., 2022), neurological (Niftullayev and Lamarche-Vane, 2019), and autoimmune (Pernis et al., 2016). Also, ROCK protein expression was found to be elevated in tumor settings (Liu et al., 2011; Yi et al., 2018) and has already been associated with tumor cell migration, metastasis and ECM remodeling (Morgan-Fisher et al., 2013). In MM, researchers found that SDF1-CXCR4 mediated MM cell adhesion to ECM fibronectin, was disrupted by inhibiting the Rho/ROCK pathway (Azab et al., 2009). Following this work, the team found that by combining ROCK inhibitor and bortezomib in liposomes, there was an improvement in efficacy of drug delivery, resulting in the abrogation of the MM–BM microenvironment interaction (Federico et al., 2020).

Based on this, knowledge, we decided to test and understand if ROCK inhibitor could be excluded from our culture conditions. Results showed that, even though overall cell viability was higher in the presence of ROCK inhibitor, when analyzing the different cell populations of the patient BM sample in culture, after 7 days, there were no significant differences in cells percentages with and without ROCK inhibitor. An exception was noticed in the case of endothelial cells, that were found to be close to 0% in the absence of ROCK inhibitor in complete culture medium and Matrigel matrix. Studies conducted on endothelial cells and progenitor endothelial cells have shown that ROCK inhibitor contributes to the successful differentiation and expansion of endothelial cells, and proliferation of adherent endothelial progenitor cells (Joo et al., 2012; O et al., 2011). For example, in a study with coronary artery disease patients, ROCK inhibitor contributed to improvement of endothelial function (Nohria et al., 2006). This leads us to believe that ROCK inhibitor is, in fact, essential for endothelial cells survival. Even so, and given the possible interference that this inhibitor has on MM cells, ROCK inhibitor was not used in this 3D model development.

After removing RBCs and ROCK inhibitor from the culturing conditions, the model was established using whole MM BM samples and the best cell seeding concentration was determined. Importantly, the model was able to sustain a stable cell viability for 14 days. Nevertheless, given the aims of our experiments, 7 days are enough for assays, and that is what was used as our timeframe. No differences were observed in the viability of cells from MM *versus* MGUS samples, indicating that the model can be used to sustain cells originated from different disease settings.

Finally, to try to enrich our culture conditions, emulating a supportive microenvironment, and increasing cell viability, plasma was isolated from MM patient BM samples, prior to processing and embedding in Matrigel. Surprisingly, viability of cells cultured with 10% patient-derived plasma supplemented medium showed a significant decrease, compared to normal complete medium cultures. The obtained results do not go in line with the literature, where several groups successfully established their MM models using patients own plasma as part of their culture medium composition (Huang et al., 2018; Kirshner et al., 2008; Perez et al., 2020; Silva et al., 2017; Zhang et al., 2015). Most likely, these results are due to a specific interaction unchained by the soluble factors present in the patient plasma. However, further studies are necessary to be able to explain this result, which is further addressed in the "Unanswered Questions and Next Steps" section. Moreover, even though overall cell viability was decreased, it is important to notice that flow cytometric analysis was not performed to assess cellular populations in this case, and therefore it is impossible to conclude which cell populations suffered with the supplemented plasma medium. Furthermore, plasma inhibitory signals were not studied in the patients plasma (addressed below).

6.2. The cells inside Matrigel matrix are enough to recreate the *in vivo* MM BM setting

The number of cells inside Matrigel after 7 days in culture was significantly lower, compared to the $2x10^6$ cells embedded on day 0, which was attributed to the migration capacity of cells through the Matrigel membrane and some cellular death during the culture time. To study the cellular escaping phenomena, we examined which cell types were escaping. Microscopic analysis of smears of the culture supernatant cellular content indicated that the event was common to all cellular sub-populations. Still, flow cytometry analysis of the culture supernatant would be of interest to obtain more accurate information regarding the specific types of cells that leave the Matrigel dome. Even though this phenomenon is not ideal, it is not surprising, as Matrigel is porous and allows exchanges with the medium. The cell seeding concentration does not play a factor here, as even in much lower concentrations the phenomenon was still observed – Supplementary Figure 5. Because Matrigel is derived from an *in vivo* mammal source, cells are able to digest and restructure the matrix (Cuddihy et al., 2013), tunnelling through the hydrogel. In particular, cancer cells overexpress ECM proteolytic degrading enzymes, such as MMPs and hyaluronidases, compared to normal cells (Nguyen-Ngoc et al., 2012). In fact, Matrigel is widely used in invasion and angiogenesis studies (Khoo et al., 2011). The first described method for the *in vitro* invasion chemo-assay goes back to the 80s, where researchers used Matrigel-coated Boyden chambers (Albini et al., 1987). Since then, several groups have used and adapted this matrix to analyze drug effects on cancer cells invasive capacity. Most recently, in 2021, Aslan et al. described a novel technique to test the invasion and migration ability of cells in a 3D platform *in vitro*, over 4 to 6 days, using a similar methodology to the one in here described for developing our model (Aslan et al., 2021).

Nonetheless, after 7 days in culture, it was possible to observe a similar representativity of any of the cell populations, both hematopoietic and non-hematopoietic, when compared to the corresponding BM patient sample, collected at day 0. This proves that the cells that stay inside the Matrigel dome are enough to allow a good recapitulation of the *in vivo* MM BM setting and to conduct desired experiments. Confocal microscopy of the 3D model at day 1 and 7 also confirmed that even though the total number of cells inside the 3D structure decreased after 7 days, there are no differences in the percentage of specific subpopulations of cells in culture throughout time, including MM cells. Moreover, compared to JellaGel, a collagen matrix, Matrigel showed a higher capacity of retaining cells within the formed dome. Collagen represents 30% of total protein mass in mammals, and is a fundamental structural component of the ECM (Gelse et al., 2003). JellaGel is derived from *Rhizoztoma pulmo* jellyfish and can be defined as Collagen Type 0 due to its homogeneity to the mammal collagen types I, II, III, V, and IX (Flaig et al., 2020). Stated to be biochemically simple, allows flexibility across multiple applications, with low interference with cells. This marine-derived collagen can mimic tissue architecture in 3D sponges and was able to support proliferation of ovarian cancer and expression of epithelial to mesenchymal transition markers (Paradiso et al., 2019). Still, after 7 days, cells cultured in Matrigel presented a higher viability than the ones cultured in JellaGel, indicating a more favorable general environment within our scaffold.

6.3. The 3D MM BM model allows the study of drug responses to anti-myeloma agents

Lenalidomide is an IMiD widely used in MM treatment, that inhibits the production of pro-tumoral cytokines and angiogenesis and stimulates T cell activity. It has been found to be both more potent and safer than thalidomide, the first IMiD used in MM treatment (Chang et al., 2014). We tested administrating lenalidomide to MM cell line monocultures, as a single agent, to assess the suitability of our model for drug testing. Both MM.1S (sensitive to lenalidomide) and RLD (resistant to lenalidomide) maintained their cell line characteristics, responding as expected, with sensitive cells dying and resistant cells maintaining viability and proliferation. This shows that our model can be used for studying therapeutic approaches and targets. Nonetheless, further studies using patient-derived cells and co-culture conditions are required.

In summary, we report a new, straightforward, and reproducible 3D *ex vivo* system, for culture of primary human BM cells in preclinical context. Generally, Matrigel is used for solid tumor organoids (Barker et al., 2010; Sachs et al., 2019; Wang et al., 2018). The proposed use of this matrix, to incorporate all cells from a hematological tumor, and without combining it with other types of ECM, as previously seen, is both innovative and simpler. This system is versatile and can be applied to different study goals. This model can maintain both cell lines and primary cells, in monoculture and co-culture, including hematopoietic and non-hematopoietic cell populations, allowing the study of cell interactions. This Matrigel-based scaffold is inexpensive, less time consuming than other 3D MM models, and more ethically viable than MM animal models. We have shown that our system allows assessment of cell viability, morphology, migration, surface markers expression and therapeutic targets. This model brings researchers a step closer to have a right tool to test the right drug to each patient, at any time.

6.4. Challenges and Limitations

While 3D cell cultures provide a more accurate representation of the natural environment of cells, this type of models also comprises difficulties.

One of the most important challenges faced during the development of our model, was to define the best approach for preservation of cellular viability and integrity. Primary cells are often difficult to maintain *ex vivo*, as they easily become senescent and have a limited lifespan (Kapałczyńska et al., 2018). Ideally, the culture should be established using freshly harvested samples through biopsies. The obtained sample usually requires processing, either for isolation of specific types of cells or for removal of others, according to the study goal, which can introduce additional variations in sample composition and, consequently, in tissue architecture and microenvironment. The complexity of our model was also increased using co-cultures, requiring optimization of cell needs and cell media composition, including cytokines, growth factors and patient-derived plasma, to allow the proper growth of all cell types in terms of biological and disease relevance. Culture medium renewal was also a weighing factor, as the procedure must be done carefully, to avoid disruption of the scaffold. Additionally, the amount and frequency of medium changing also required optimization, since changing it more frequently than needed, and/or all at once, might remove factors secreted by the cells, that are most likely essential for their maintenance and can interfere with physiological behavior and overall viability of the system. For example, and also in agreement with the protocol we developed, Kirshner et al. advise to only change half of the total medium volume per time (Parikh et al., 2014). Some of the most critical steps for

ensuring the growth of all the BM cells comprised in our model are: (1) the obtaining of a good BM sample, in which BM cells present an initial viability of over 85%; (2) being able to successfully remove RBCs, without compromising the other cell populations; (3) ensuring that the resuspension of the cell pellet in Matrigel is homogenous and done as fast as possible, avoiding matrix polymerization before plating; and (4) taking care of the scaffold through culture period, keeping manipulation and handling to the bare minimum, until the timepoints of our experiments are reached. As mentioned earlier, cells' ability of digesting and restructuring the Matrigel matrix can be useful, as it allows the rearranging of the cells and establishment of interactions as if they were in their natural environment, but it can also be a drawback, if the 3D structure is disrupted.

Analysis of our 3D samples by flow cytometry can also be challenging, as samples must be of cell suspension, requiring dissociation by single or combined enzymes (e.g., trypsin, hyaluronidase, collagenase, dispase, or cell recovery solution, in our case). This can affect cells integrity and viability. In addition, spatial distribution is lost. Spelat's model introduces an interesting alternative, as their worm-like micelle gel can be converted into free-flowing spheres simply by incubating the culture in a 4ºC solution (Spelat et al., 2020), therefore avoiding enzymatic degradation, as required for many commercially available protein based matrix gels (Simon et al., 2015).

In terms of studying the tumoral environment, the biggest current gap in modeling cancer stroma is the disregard for the inclusion of immune cell populations, which can often be tricky, due to the existing heterogeneity. While this system aimed to overcome the lack of representativity of all the different cellular populations present in the BM and involved in MM establishment, including the immune ones, no bone cells were included, for example. To better mirror the BM physiology, both this and the inclusion of blood flow could be considered, although building complexity would be significantly increased, losing the simplicity advantage of our model.

Another major challenge regards the high tumor heterogeneity, not only interpatient, with analyses revealing a high level of genomic heterogeneity across patients (Lohr et al., 2014), but also temporal and spatial intra-patient heterogeneity (Bahlis, 2012; Rasche et al., 2017). Thus, the whole setting of a patient disease may not be represented simply by the portion of biologic material collected in the on-site biopsy (N. Lee et al., 2017). Moreover, antitumoral drugs might work out differently in cells in different stages, impacting the guidelines for treatment. Time can also be a hurdle, since the model needs to be grown and validated before any screening assays can be performed.

6.5. Unanswered Questions and Next Steps

6.5.1. Expression of Cytokines in Patient-derived Plasma and Culture Supernatant

As previously mentioned, contrary to the expected outcome, cell viability of our culture in the presence of patient-derived plasma not only did not increase, but significantly decreased. Our main hypothesis is that there are specific immunomodulators present in the plasma that can inhibit cell proliferation and impact viability. Therefore, performing an assay to examine what activator and inhibitory factors are up and down regulated is of utmost importance, as well as determining what cell populations are being affected. Interestingly, Zhang et al. performed a study where cell proliferation and viability were assessed in two different plasma concentrations in the culture medium: 5% and 10%. Flow cytometry analysis revealed that while 10% plasma supplemented medium induced a higher expansion of MM cells, 5% plasma supplemented medium resulted in a higher expansion of BMMCs

(Zhang et al., 2015). This suggests that the overall decrease in survival of our cells could be a result of cell death or proliferation inhibition of specific subpopulations.

Some of the main interest cytokines to be studied include IL-1β, IL-6, IL-10 and TNF-α, due to (1) their altered expression in MM patients and role in MM progression (Alexandrakis et al., 2015; Musolino et al., 2017); and (2) previous detected secretion in other groups' 3D MM models, both by MM and microenvironment cells (de la Puente et al., 2015; Jakubikova et al., 2016; Narayanan et al., 2014; Waldschmidt et al., 2022; Zdzisińska et al., 2009). Particularly, this can be achieved by multiplex-ELISA assay (Lehmann et al., 2017). The expression of cytokines in the plasma obtained from the patient BM sample, could be assessed individually and compared to the culture supernatant of both plasma-supplemented and non-plasma supplemented cultures, thus providing information that could contribute to understand the underlying mechanisms in the reduction of cell viability. This experiment would be an additional validation point of our model, if cytokine expression, and possibly distribution, in the 3D culture matches the one observed in the original sample plasma.

6.5.2. Immune Profile of the Populations within the 3D Model

Further research is required to assess the role of less characterized BM cellular components, including the immune profile of the immune populations already identified to be present in our model. This would allow us to understand if the immune suppression typically associated with the disease is maintained in our culture. As well, analysis of therapy targets generally expressed by MM cells is of interest, providing another validation point. Namely, the expression of the B cell maturation antigen (BCMA) and of PD-L1 could be assessed. BCMA is normally expressed by mature B lymphocytes and its overexpression is associated with MM, supporting BCMA value as a therapeutic target (Shah et al., 2020). Moreover, BCMA can be used as a biomarker for MM, showing correlation with clinical outcomes, even in traditionally difficult-to-monitor patients (Visram et al., 2021). As previously mentioned, PD-L1 is expressed on the surface of MM cells, and increased expression is found in newly diagnosed and relapsed MM patients, compared with MGUS and healthy donors. The relevance of targeting the PD-1/PD-L1 axis in MM has already been demonstrated in pre-clinical models (Dhodapkar et al., 2015; Hallett et al., 2011), with current ongoing clinical trials (e.g., NCT05338775).

Comparing the levels of expression of these two targets at day 0 *versus* day 7 of 3D culture could contribute to further characterization of our model. If the expression in our model matches the initial values, the potential of our system for studying therapeutic targets and link them to events of drug resistance and disease progression is increased.

6.5.3. Cellular 3D Organization and 3D Structure

To further characterize our model and define it as a BM resembling system, additional cell populations should be studied in terms of spatial distribution in the matrix, after 7 days in culture. This information could be obtained both by confocal microscopy, as already initiated, and by IHC analysis, where a larger range of markers and cell subsets can be identified, including T, B or myeloid cells. Nonetheless, it is important to know that IHC does not allow a well-defined spatial notion and it is not as accurate as confocal microscopy.

Another relevant characteristic to be assessed is the ability of the cells incorporated in our model to form networks within the Matrigel matrix. Braham et al. were able to identify stromal-endothelial cells networks (by using phalloidin) in their 3D Matrigel based model, proving that Matrigel facilitates cell-cell interactions and allows supporting cells growth (Braham et al., 2018).

6.5.4. Identification of Proliferating Cells, and Proliferative and Hypoxic Regions

A key event to be addressed is the ability of cells to proliferate in our model. This could be assessed by combining flow cytometry and confocal or IHC techniques. This way, information regarding not only the existence of proliferating cells, but also which populations are proliferating, and if this is homogenous or restricted to a limited area of the scaffold, could be obtained. Although we did not conduct these studies with patient-derived cells, due to the scarce number of MM patient samples, we used the MM.1S cell line. MM.1S cells embedded in Matrigel showed increasing growth and cell aggregates formation, usually observed when cells are proliferating, in normal 2D culture – supplementary Figure 6A. Moreover, histological analysis of these cells embedded in Matrigel allowed the detection of cells undergoing mitosis, after only 3 days in culture – Supplementary Figure 6B. This proves that the matrix does not interfere with the proliferative properties of MM cells, leading us to believe that the same happens in the patient-derived 3D culture.

Moreover, if different levels of proliferation can be linked to different regions of the model, expression of hypoxia markers can also be of interest. For example, de la Puente et al. proved that their model recapitulated oxygen gradients, where the top areas of the culture showed lower expression of HIF1α, and higher expression of Ki67 and CD138, and a higher expression of HIF1α and lower expression of Ki67 and CD138, the further down in the system. This is similar to the *in vivo* BM niche, where oxygen availability is decreased with increasing distance to blood vessels (de la Puente et al., 2015).

6.5.5. Drug Tests Validation

Even though preliminary studies using MM cell lines treated with lenalidomide showed satisfactory results, additional work is necessary, using patient samples, in retrospective studies. For that we will need to compare the impact of a specific treatment regimen on our patient-derived 3D model to the actual known patient response in the clinic. Specifically, MM patient cells that were shown to be sensitive to a certain treatment, are expected to die in the 3D model. On the other hand, MM cells that survive in our 3D culture system should correspond to a patient resistant to therapy. If proven effective, clinical sample size can be expanded, as well as drugs tested, including other IMiDs, PIs, mAbs, and other strategies targeting the tumor microenvironment. In a subsequent step, prospective studies could be conducted.

7. Future Perspectives

The importance of having a biomimetic model of the *in vivo* setting of MM has been growing considerably in the last years and is not going unnoticed by the scientific community. As efforts are increasing to bring research closer to real life conditions, it seems more and more clear that the successful creation of a 3D platform for studying mechanisms of disease establishment and progression requires a model that can recapitulate both the tumor biology and pathophysiology, and its microenvironment. Due to the very well-known patient heterogeneity and intra- and inter-tumoral heterogeneity, combining the incorporation of patient samples with the development of an *ex vivo* structure that allows self-organization of cells, in a 3D multi-culture model, should bring advantages to the field, reducing dependency on animal models and improving translational research.

Notwithstanding the obvious potential of 3D models, there is still a long way to go. Indeed, it is important to find the right balance between the necessity of having a perfectly reliable and physiological model of MM and the necessity of simplifying it enough so that it can be reproduced and seen as an appealing and practical option for research use.

In here, we proposed an easy way of culturing whole BM samples from MM patients in an ECM protein mixture, obtaining a three-dimensional structure where cells can grow and interconnect. With this work, we aimed to overcome the lack of representativity of all the different cellular populations present in the BM and involved in MM establishment, observed in other existing MM BM models. The ability of this system to sustain viability of patient-derived cells in co-culture for 14 days, including MM and BM microenvironment cells, that are known to be particularly difficult to maintain *in vivo*, shows that our model has the potential to be used in relevant studies regarding MM.

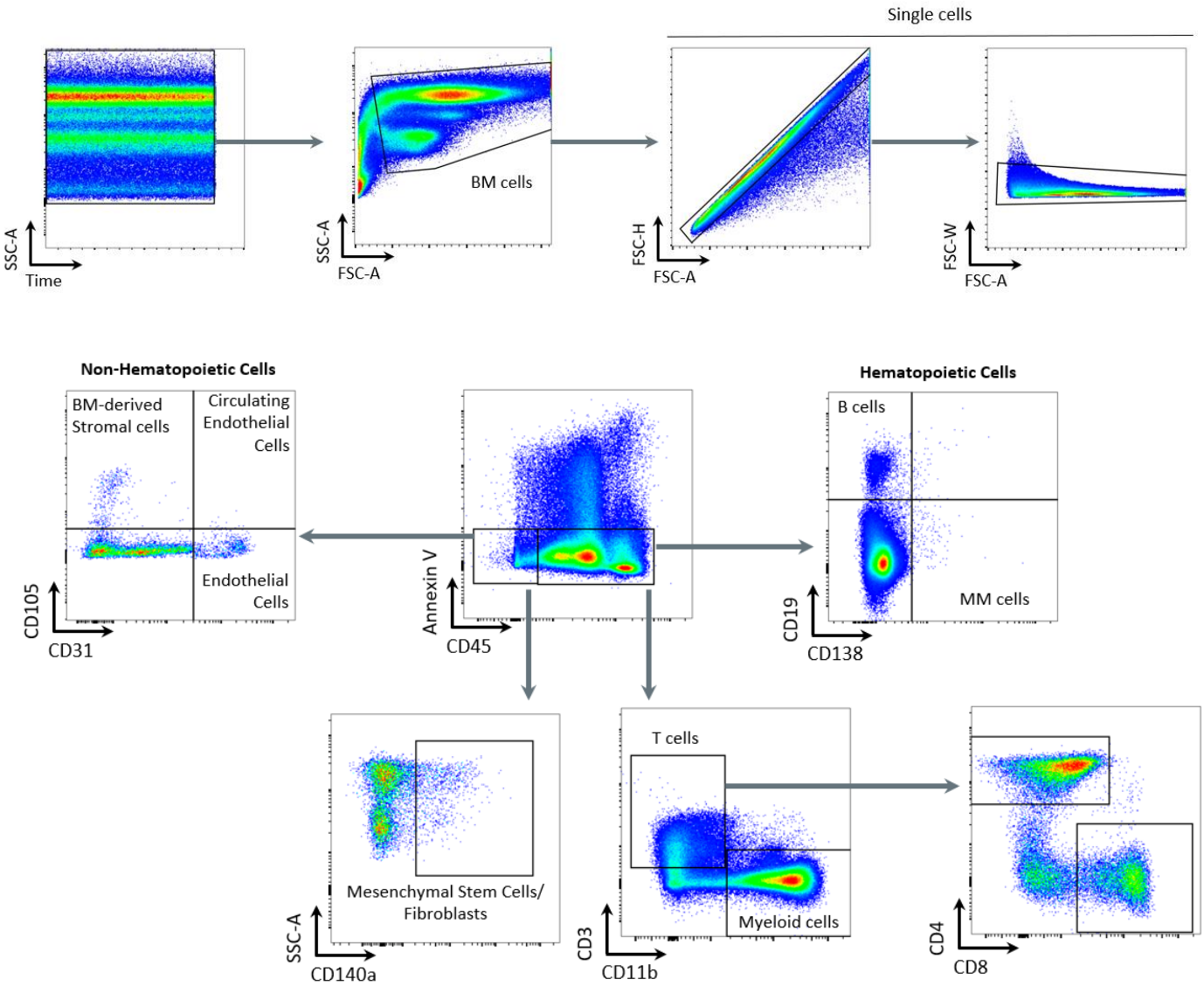
In the future, and as previously mentioned in section 6.5 of this thesis, additional studies will be needed to further characterize the populations present in our model and their role in disease progression. Likewise, a better understanding of the 3D structure is necessary, namely regarding the existence of drug and oxygen gradients. Importantly, drug tests using BM samples from patients both sensitive and resistant to different treatment approaches are essential to validate our model.

Despite its limitations, the presented model represents a new and simpler system for replicating disease mechanisms *ex vivo*, with low cost and time expense. Due to its easy handling, this is a tool with potential to be used in routinely research, and relevant in clinical context, allowing for personalized medicine and aiding in decision making regarding treatment course.

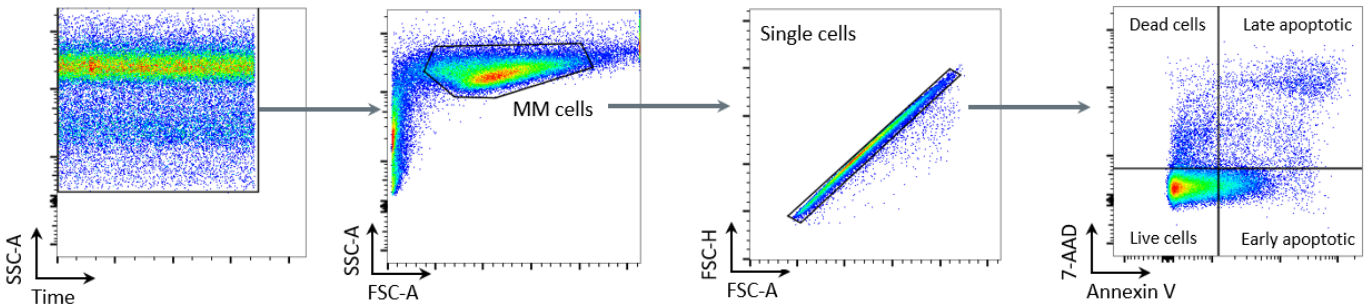
The development of 3D models has been revolutionary in other disease settings, including brain, lung, intestine, and kidney modelling. The same way, we believe that this model can contribute to the questioning of disease mechanisms in MM and target identification, potentiating the success rate of therapeutic approaches.

Adapting to each patient specific characteristics and necessities, in the long run, our simple 3D model can be improved to support a wider range of BM and hematological diseases and cell types, paving the way for obtaining a tissue-relevant system.

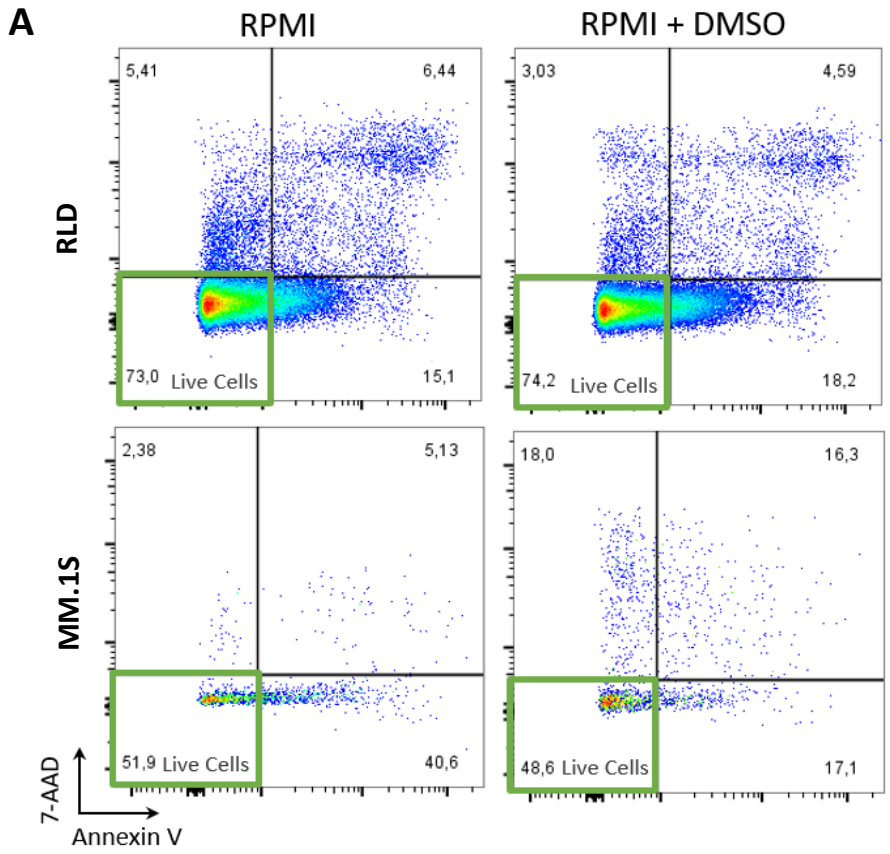
Supplementary Data

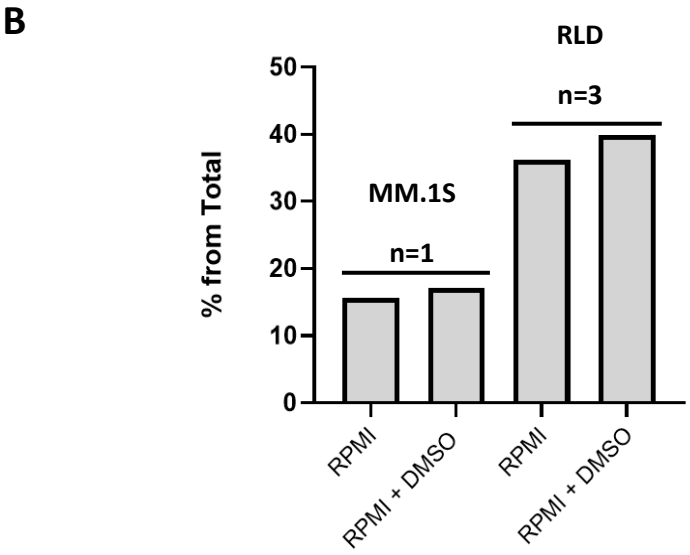


Supplementary Figure 1. Gating strategy used on BM samples from MM patients for determining the cell populations at day 0, and after 7 days in 3D culture.

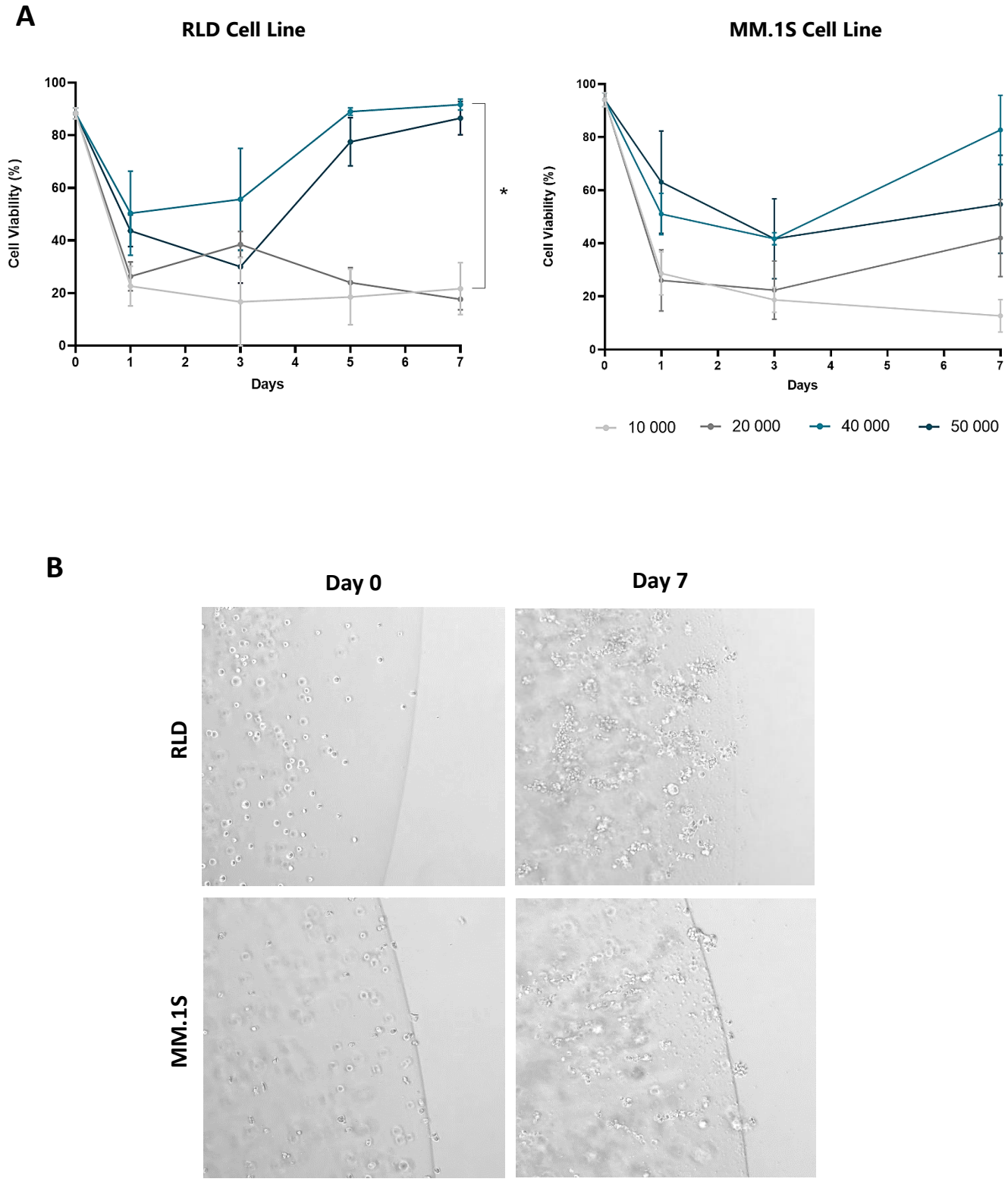


Supplementary Figure 2. Gating strategy used to determine live and dead cells at 48h post-treatment with Lenalidomide, in MM.1S and RLD cell lines in 3D culture.

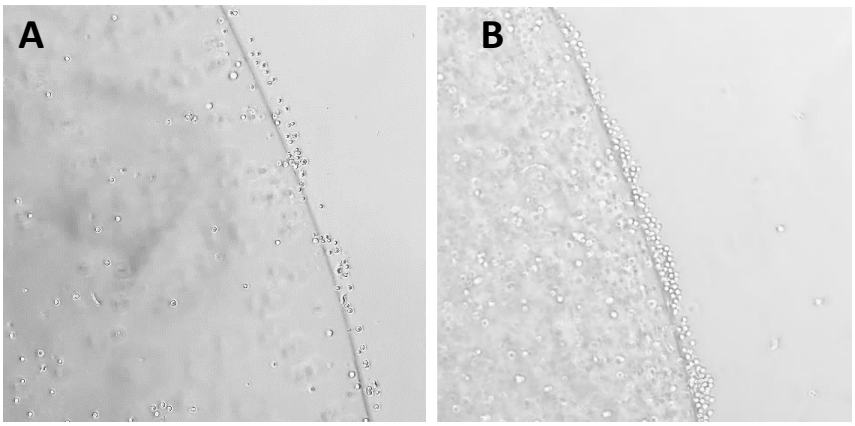




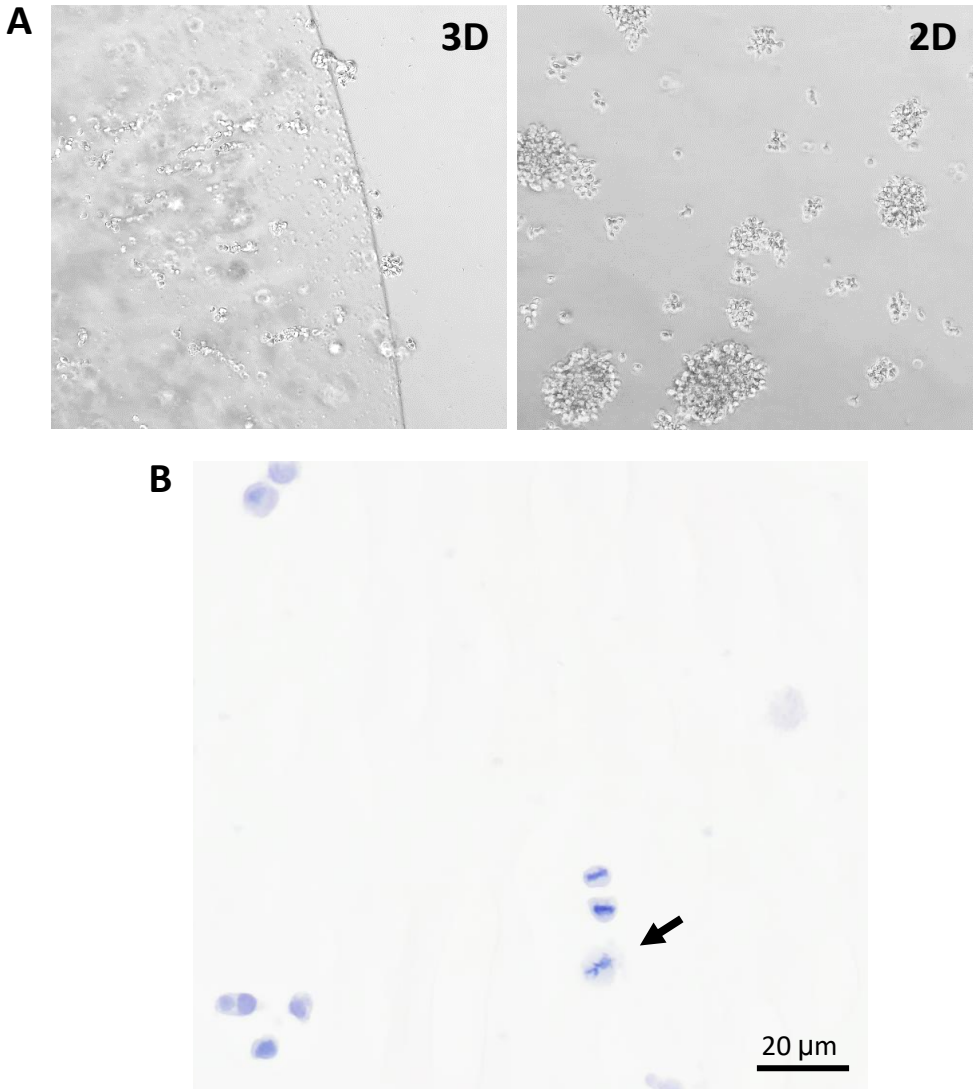
Supplementary Figure 3. Effect of DMSO used for Lenalidomide solubilization on MM.1S and RLD cell lines viability. The percentage of DMSO added was below the toxicity range (<0,048%) and did not affect cell viability. (A) Representative dot plots of live and dead cells in normal culture conditions (control condition, RPMI) *versus* culture with DMSO (RPMI + DMSO). (B) percentage of live cells in normal culture conditions (control condition, RPMI) *versus* culture with DMSO (RPMI + DMSO).



Supplementary Figure 4. Ideal cell seeding concentration for RLD and MM.1S cell lines in 3D Matrigel culture. (A) Cell viability of 10 000 (light grey), 20 000 (dark grey), 40 000 (light blue) and 50 000 (dark blue) cells in 40µL Matrigel, without ROCK inhibitor. RLD cell line (left, n=4), Friedman test (* $p \leq 0.05$) and MM.1S cell line, (right, n=3), Friedman test (ns). (B) Brightfield microscopy pictures (10x objective) of RLD (top) and MM.1S (bottom) cells at day 0 (left) *versus* day 7 (right) of culture. 50 000 cells embedded in 40µL of Matrigel without ROCK inhibitor.



Supplementary Figure 6. Cells leaving the Matrigel matrix is not related to cell concentration. (A) $0.25x10^6$ *versus* (B) $2x10^6$ patient-derived MM cells cultured in 40µL of Matrigel, at day 1 of culture. Brightfield microscopy pictures (10x objective).



Supplementary Figure 5. MM.1S cells proliferating in the Matrigel 3D scaffold. (A) 50 000 MM.1S cells in 3D *versus* 2D conditions, forming cell aggregates, at day 7 of culture. Brightfield microscopy pictures (10x objective). (B) 4µm horizontal section of the model on day 3, where mitotic MM.1S cells can be identified (black arrow, hematoxylin staining). Brightfield microscopy images (40x amplification). 40 000 cell cultured in 40µL of Matrigel, without ROCK inhibitor.

General informed consent form approved by Champalimaud Foundation Ethics Committee

Centro Clínico Champalimaud

Fundação Champalimaud

CONSENTIMENTO INFORMADO PARA INVESTIGAÇÃO CIENTÍFICA
DECLARAÇÃO

O consentimento informado, esclarecido e livre, manifesta o princípio do respeito pela pessoa e pretende garantir a participação do doente nas decisões sobre os seus cuidados de saúde. Se no decurso do ato médico proposto for feita a recolha de alguma amostra biológica para diagnóstico ou tratamento, da qual resulte material já não necessário na condução do seu caso, é também solicitada a autorização para a sua utilização exclusivamente para fins científicos. Assegurando a utilização de acordo com as normas éticas vigentes e a confidencialidade, só terão acesso ao seu nome a equipa clínica e de investigação que o(a) segue. É igualmente solicitada, sempre que perante procedimentos de visualização endoscópica ou de cirurgia, a recolha de imagens internas para fins educacionais desde que devidamente anonimizadas.

Por favor, leia com atenção todo o conteúdo deste documento, que será assinado pelo(a) seu(sua) médico(a) e por si. Verifique se todas as informações estão corretas e não hesite em solicitar mais informações se não estiver completamente esclarecido(a). Se não tiver dúvidas e entender que está tudo em conformidade, por favor assine este documento.

Identificação do(a) doente

N.º CCC: **Nome:**

Sexo: **Data de Nascimento:** **Idade:**

Declaração do(a) médico(a)

Declaro que expliquei à pessoa acima indicada, de forma adequada e inteligível, o propósito da utilização de dados demográficos e clínicos, devidamente anonimizados, do material biológico remanescente ou imagens recolhidas durante exames diagnósticos, para fins científicos ou pedagógicos sem que daí decorra qualquer risco adicional para o(a) doente.

Nome: *N.º cédula profissional ou vinheta:*

Assinatura: *Data:*

Declaração do(a) próprio(a) ou seu(sua) representante legal

Autorizo a utilização de dados demográficos e clínicos devidamente anonimizados para fins científicos e/ou educacionais.

Autorizo a utilização de material biológico já não necessário para diagnóstico ou tratamento, exclusivamente para fins científicos e/ou educacionais.

Autorizo também a recolha de imagens internas para fins científicos e/ou educacionais.

Assinatura do(a) próprio(a) ou representante legal: *Data:*

Se for assinado pelo(a) representante legal indicar:

Tipo de representação / Grau de parentesco: _____

Morada: _____

Documento de identificação e validade: _____

Telefone: _____ *Email:* _____

Este documento tem duas vias, ficando o original no Centro Clínico Champalimaud e sendo o duplicado entregue ao(à) próprio(a) ou ao(à) seu(sua) representante legal.

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