UNIVERSITA' VITA-SALUTE

SAN RAFFAELE

CORSO DI DOTTORATO DI RICERCA
INTERNAZIONALE IN
MEDICINA MOLECOLARE

CURRICULUM IN
Immunologia e Oncologia
di Base e Applicate

3D CULTURE AS A MODEL TO STUDY MALIGNANT MESOTHELIOMA TUMOR MICROENVIRONMENT

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Tesi di DOTTORATO di RICERCA di

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Ciclo di dottorato XXXVI

SSD BIO/11

Anno Accademico 2020/2021

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1. *Imaging flow cytometry was performed in collaboration with Desiree Zamboni, Imaging Facility of San Raffaele Hospital, Milan, Italy.*

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3. Pneumothorax, *MOSR14, MOSR15 biopsies were provided by Prof. Angelo Carretta, Ospedale San Raffaele and Vita-Salute San Raffaele University, while biopsy 956 was obtained from the Alessandria Biobank thanks to Dott.ssa Roberta Libener.*

4. *PDX mice were generated by Francesca Caprioglio, Chromatin Dynamics Unit, Vita-Salute San Raffaele University, Milan Italy*

5. *Real-time PCR in figure was performed by Alessandro Mesaglio, Chromatin Dynamics Unit, Vita-Salute San Raffaele University, Milan Italy*

6. *FACS analysis on figure 21 was performed by Francesca Caprioglio, Chromatin Dynamics Unit, Vita-Salute San Raffaele University, Milan Italy*

7. scRNA-seq data analysis was performed by Dr. Emanuele Monteleone, Chromatin Dynamics Unit, *Vita-Salute San Raffaele University*, Milan Italy

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ABSTRACT

Malignant Pleural Mesothelioma is a very aggressive cancer arising from pleural mesothelial cells exposed to asbestos. MPM poses a significant clinical challenge with an average survival of 8-12 months post-diagnosis. MM is preceded by prolonged asbestos-driven pleural inflammation. Monocytes are recruited by several chemokines and interleukins, produced by tumor cells and infiltrating lymphocytes. The latter promote differentiation of tumour associated macrophages (up to 30% of cells in MM) towards an M2 phenotype, which is a negative prognostic factor in MM.

Three-dimensional organoid cultures, a robust in vitro system, have shown success in mimicking tumor pathophysiology for various cancers. However, MM lacks reliable organoid models. This thesis details the establishment of patient-derived MM 3D cultures and explores their interaction with immune cells, specifically macrophages, crucial in tumor microenvironment dynamics. 3-Dimensional cultures were established from different histological and clinical stages as preclinical models for therapeutic drug screening.

One part of my study reveals successful generation and characterization of MM organoids, highlighting challenges in long-term propagation. Organoids exhibit key MM markers, providing a promising tool for in vitro studies.

Another part introduces spheroids as a simpler model for studying immune-tumor interactions. A comprehensive analysis of macrophage polarization in the 3D system demonstrates dynamic shifts in M1-like and M2-like phenotypes over time.

Further, the study explores the gene expression profiles of infiltrating macrophages in MPM spheroids. Despite technical challenges, valuable insights into M1-like and M2-like populations, their migration, and phagocytosis activities emerge. The dynamic alteration in phenotypic status over time emphasizes the complexity of TAM involvement in the immune response.

The final part investigates the impact of macrophages on drug response in a 3D system. BoxA treatment demonstrates its potential in reducing tumor growth and overcoming pro-tumoral effects of M2-like macrophages. This model offers a platform for drug screening and personalized precision medicine.

Overall, my study underscores the utility of 3D models in replicating TME complexities in MM. Organoids and spheroids emerge as valuable tools for drug testing, personalized medicine, and understanding immune-tumor interactions.

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ACRONIMS AND ABBREVIATIONS

AUC: Area Under the Curve

ASA: Acetylsalicylic Acid

BAP1: BRCA1-Associated Protein 1

BAPE: Benign Asbestos Pleural Effusion

BLM: Bloom Syndrome RecQ Like Helicase

BRCA1: Breast Cancer 1

CAFs: Cancer-Associated Fibroblasts

CAR: Chimeric Antigen Receptor

CCA: Carcinoembryonic Antigen

CCL2: Chemokine (C-C motif) ligand 2

CD47: Cluster of Differentiation 47

CDK2NA: Cyclin-Dependent Kinase Inhibitor 2A

CEA: Carcinoembryonic Antigen

CCR2: Chemokine (C-C motif) receptor 2

CO_2: Carbon Dioxide

CRISPR/Cas9: Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein 9

CTLA-4: Cytotoxic T-Lymphocyte Associated Protein 4

CXCR4: C-X-C chemokine Receptor type 4

DAMPs: Damage-Associated Molecular Patterns

DC: Dendritic Cells

DNA: Deoxyribonucleic Acid

ECM: Extracellular Matrix

EGF: Epidermal Growth Factor

EGS: Emergency General Surgery

EMA: European Medicines Agency

F4/80: Specific Antigen for Macrophagese

FGF: Fibroblast Growth Factor

FGFR: Fibroblast Growth Factor Receptor

GFP: Green Fluorescent Protein

GM-CSF: Granulocyte-Macrophage Colony-Stimulating Factor

HCMI: Human Cancer Models Initiative

H&E: Hematoxylin and Eosin

HMGB1: High Mobility Group Box 1

ICD: Immunogenic Cell Death

ICI: Immune Checkpoint Inhibitor

IFN-γ: Interferone gamma

IGF-1/IGF1R: Insulin-like Growth Factor 1/Insulin-like Growth Factor 1 Receptor

IL-1α: Interleukin-1 alfa

IL-1β: Interleukin-1 beta

IL-4: Interleukin-4

IL-6: Interleukin-6

IL-8: Interleukin-8

IL-10: Interleukin-10

IL-13: Interleukin-13

IL-18: Interleukin-18

IL-34: Interleukin-34

MAPKs: Mitogen-Activated Protein Kinases

MAPS: Mesothelioma Avastin Cisplatin Pemetrexed Study

M-CSF: Macrophage Colony-Stimulating Factor

MDSCs: Myeloid-Derived Suppressor Cells

MMPs: Matrix Metalloproteinases

MM: Malignant Mesothelioma

MTAP: Methylthioadenosine Phosphorylase

NLRP3: NOD-like receptor family pyrin domain-containing 3

NF-κB: Nuclear Factor kappa B

Nf2: Neurofibromin 2

NSG: NOD scid gamma mouse

OS: Overall Survival

PDX: Patient-Derived Xenograft

PD-1: Programmed Cell Death Protein 1

PDGF: Platelet-Derived Growth Factor

PD-L1: Programmed Death-Ligand 1

PRC2: Polycomb Repressive Complex 2

PTEN: Phosphatase and tensin homolog

RAGE: Receptor for Advanced Glycosylation End products

ROC: Receiver Operating Characteristic

ROS: Reactive Oxygen Species

sRAGE: Soluble RAGE

scRNA-Seq: Single-Cell RNA Sequencing

SMRP: Serum Mesothelin-Related Protein

SNVs: Single Nucleotide Variants

SV40: Simian Virus 40

TAA: Tumor-Associated Antigen

TAMs: Tumor-Associated Macrophages

TGF-β: Transforming Growth Factor β

TCGA: The Cancer Genome Atlas

TLR: Toll-Like Receptor

TME: Tumor Microenvironment

TNF-α: Tumor Necrosis Factor α

TRAIL: Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand

Tp53: Tumor protein 53

TTF1: Thyroid Transcription Factor 1

USFDA: United States Food and Drug Administration

VEGF: Vascular Endothelial Growth Factor

WT-1: Wilms Tumor 1

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Introduction

Mesothelioma

Malignant mesothelioma (MM) is a rare, aggressive neoplastic lesion originating from mesothelial cells lining body cavities like the pleura (lung and chest cavity) and peritoneum (abdomen), it can also be found in the pericardium, tunica vaginalis testis, and ovarian epithelium. This tumor is mainly a result of exposure to carcinogenic mineral fibers, particularly asbestos and erionite, but factors like Simian Virus 40, radiation, and other similar fibers also contribute such as carbon nanotubes. Notably, 50-80% of pleural MM in men and 20-30% in women are attributable to asbestos exposure.

This exposure can cause various pulmonary conditions, both benign—such as asbestosis, pleural plaques, and benign asbestos pleural effusion (BAPE)—and malignant, including MM and bronchogenic carcinoma (Husain et al, 2013).

Moreover, the combination of asbestos exposure and smoking significantly amplifies the risk of lung cancer by ten to a hundred times compared to non-exposed individuals. However, no evidence suggests that smoking increases the risk of developing mesothelioma.

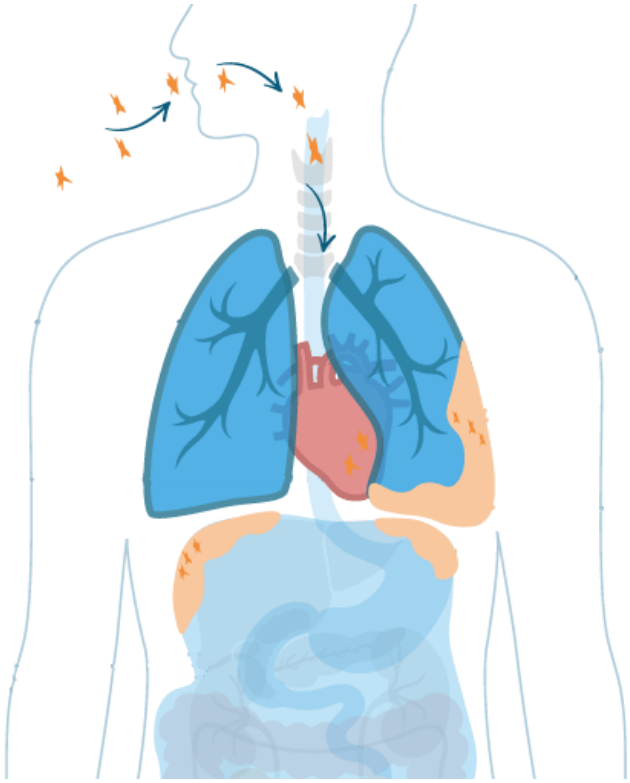


Figure 1. MM is mainly caused by asbestos exposure. Asbestos is inhaled and migrates into the lung and pleura lining the lung. The asbestos triggers inflammation and lead to pleural plaques

Asbestos fibers, once inhaled, can infiltrate the pleural tissue, inducing chronic inflammation. This inflammatory response, combined with chronic asbestosis and pleural fibrosis, contributes to MM onset. However, diagnosing MM is no easy task. There's a substantial latency period between asbestos exposure and MM diagnosis, averaging 48 years for men and 53 for women. The intensity and duration of exposure are key variables in mineral fiber-induced diseases, with individual susceptibility influenced by the intensity of exposure, genetic factors, exposure to other agents, and the specific mineral fiber type (Galateau-Salle et al, 2016).

One important genetic factor linked to MM is the loss of the oncosuppressor BAP1 due to mutations or deletions, which hampers DNA damage response and promotes cell survival, making BAP1 testing crucial for MM diagnosis.

Histologically, MM has three primary subtypes: epithelioid, sarcomatoid, and biphasic, each with varied characteristics and prognoses. The epithelioid subtype is the most common (found in up to 80% of patients), display oval to polygonal malignant mesothelial cells, and boasts the best prognosis. Sarcomatoid MM, in contrast, is the most aggressive, characterized by the proliferation of spindle cells. The biphasic subtype embodies characteristics of both previous forms, and its prognosis depends on the balance of the two types present. Most MM cases are found in the visceral pleura, with the rest in the parietal pleura (Galateau-Salle et al, 2016).

Immunohistochemical approaches play a pivotal role in distinguishing epithelioid MM from metastatic lung adenocarcinoma, typically employing a diagnostic panel that encompasses mesothelial markers exhibiting positivity (for instance, Calretinin, Cytokeratin 5/6, Wilms Tumour-1) alongside markers delineating adenocarcinoma negativity (such as TTF1, CEA, Ber-Ep4) (Husain et al, 2013).

Treatment options for MM patients have historically been limited.

While traditional treatments like chemotherapy, radiotherapy, and surgery have been largely palliative, recent advancements with immune checkpoint inhibitors have shown some promise, extending median survival by about four months. However, challenges remain, given the immunosuppressive nature of MM, which hampers treatment efficacy, necessitating continued research and refinement of this therapeutic approach.

Globally, MM incidence is on the rise, with peaks anticipated between 2015 and 2030. Despite efforts to curtail asbestos exposure since the 1970s, MM incidence remains steady in countries like the US.

In the context of mesothelioma incidence within specific Italian regions, over 50% of the detected cases are recorded among residents in Lombardy, Piedmont, Liguria, and Emilia-Romagna, accounting for 56.7% of the total. The primary anatomical site of onset is the pleura, MM representing 93.2% of cases, with the peritoneum being implicated in 6.3%. The disease is exceedingly rare before the age of 45, constituting only 1.4% of the total cases registered. The median age at diagnosis is 70 years, with no significant gender disparities observed. The gender ratio, calculated as the number of male cases per female case (M/F), is 2.6. Moreover, 72% of the archived cases are male, with the percentage of women accounting for 27.2% in pleural mesotheliomas (FIGURE 2).

It is anticipated that the maximum number of MM incidents will occur between 2020 and 2024, with an estimated 7,000 cases. Following this peak, a gradual decline is projected. However, around 26,000 MM cases are still predicted to arise in Italy over the subsequent two decades, from 2020 to 2039 (Oddone *et al*, 2020).

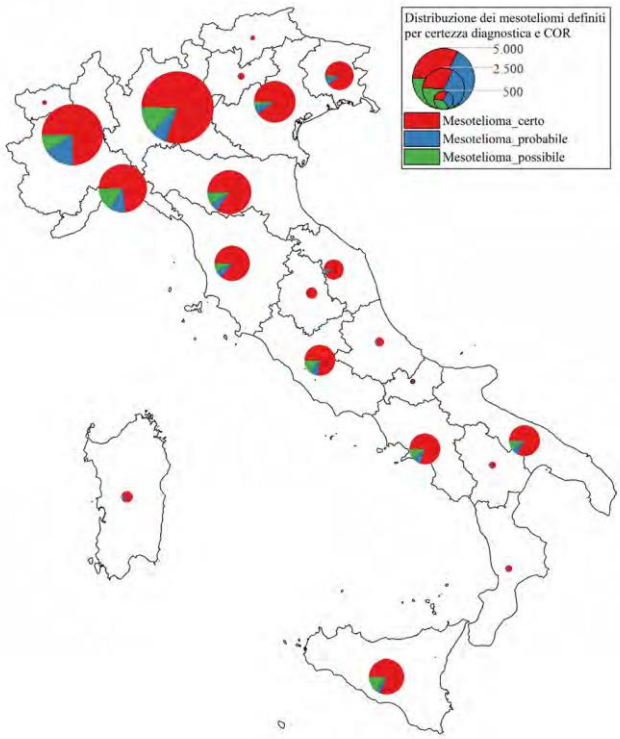


Figure 2. Number of mesothelioma cases reported to ReNaM by level of diagnostic certainty and region of residence at diagnosis (Italy, 1993 - 2018, N = 31,572) (ReNaMe settimo rapporto 2021).

In summary, MM, a malignant tumor primarily associated with asbestos exposure, affects the lining of the lungs and abdomen. With its stealthy onset and often late diagnosis, the disease poses significant challenges for treatment and prognosis.

Continued research and advanced therapeutic interventions, like immune checkpoint inhibitors, offer a beacon of hope for those affected. As global awareness about the dangers of asbestos grows, efforts are intensifying to reduce exposure and, hopefully, the incidence of MM.

Mesothelioma: Pathogenesis

Mesothelial cells form the lining of various body cavities and are among the most undifferentiated cells in the human body, capable of morphing into either epithelial-like or fibroblast-like cells. These cells, the adult counterpart of the mesoderm, can be identified through their unique ultramicroscopic characteristics and immunophenotype. They possess high levels of wild-type p53, which is significantly detectable via immunostaining. Mesothelial cells are the origin of MM, aggressive tumors resistant to chemotherapy with a median survival rate of one year post-diagnosis. The term "mesothelioma" often requires the "malignant" qualifier as it is sometimes inaccurately used to describe rare, mostly benign tumors unrelated to MM. The term "mesothelioma" in this context refers to malignant tumors often linked to asbestos exposure, SV40 infection, and genetic abnormalities (Khan et al, 2023; Carbone et al, 2002).

Asbestos

Asbestos refers to a family of naturally occurring, inorganic, fibrous silicates found in various types of rocks, possessing a distinct chemical composition and crystal structure. The name "asbestos" is derived from Greek, indicating the mineral's notable features of being unquenchable or indestructible. These minerals have been historically used and valued for their thermochemical and electrical resistance, high tensile strength, and flexibility, which allowed them to be woven into textiles and incorporated into a wide range of materials for industrial applications.

Asbestos minerals are primarily categorized into two groups: serpentine (represented by chrysotile) and amphibole (consisting of crocidolite, amosite, tremolite, anthophyllite, and actinolite). Each group may also encompass related non-fibrous forms with similar or identical chemical compositions, which are not harmful to humans.

The fibrous structure of asbestos is due to the parallel growth of fine and elongated crystals. While asbestos forms are less common, deposits are found in various rocks,

including banded ironstones, alpine-type ultramafic rocks, stratiform ultramafic inclusions, and serpentinized limestone (Sporn, 2011).

Despite their utility, continuous asbestos exposure led to the emergence of malignancies among workers from the 1940s to the 1970s, prompting a ban by the EPA in 1989.
In Italy, asbestos saw extensive use in the 20th century, with the notable Eternit factory operating from 1907 to 1985 in Casale Monferrato. From 1946 to 1986, the factory recorded significant worker deaths due to lung cancer, asbestosis, and pleural malignancies. Additionally, sites with a history of direct asbestos usage, including Balanghero, Broni, Genova, Taranto and various shipyards and harbors, witnessed a surge in mesothelioma cases. Italy prohibited asbestos use in 1992, followed by the European Union in 1999 (Sporn, 2011).

SV40

Simian virus 40 (SV40) is a DNA monkey virus linked to MM, primarily transmitted to humans through contaminated polio vaccines produced between 1955 and 1978. Approximately 32 million people in the United States were injected with vaccines containing infectious SV40.
The SV40 genome, consisting of 5243 base pairs, is divided into early and late regions.
SV40 is known for producing two oncogenic proteins: Large T and small t antigens. The Large T antigen (Tag) has been found to bind and inhibit crucial tumor suppressor proteins, playing a role in MM carcinogenesis. This Tag not only inactivates tumor suppressor activities but, when combined with other proteins, it forms a complex that promotes cell growth by activating the IGF-1/IGF1R pathway. The small t antigen affects cellular processes by inhibiting a protein involved in dephosphorylation, possibly activating signaling pathways that induce cell growth and transformation (Sporn, 2011).

SV40's actions vary in different cells: it replicates in monkey cells causing cell lysis; doesn't replicate but leads to tumor development in rodent cells; and in semipermissive human cells, it can both replicate and cause cellular transformation. Human mesothelial cells (HM) are particularly susceptible to SV40 but can inhibit Tag-mediated SV40 replication due to high levels of wild-type p53. However, SV40 can persist in these cells, with Tag accumulation leading to a much higher rate of malignant transformation compared to other human cell types infected with SV40.

Furthermore, studies have shown that SV40 and asbestos might act as co-carcinogens. It has been demonstrated that the combination of asbestos and SV40 substantially increased the incidence of MM in animal models, compared to exposure to either factor alone. The combination of the two also enhanced cellular processes related to tumor development and invasion. This co-carcinogenic effect suggests that even lower levels of asbestos exposure might be dangerous for individuals who have been exposed to SV40, raising concerns about the true safety of certain levels of asbestos exposure (Yang et al, 2008).

Carbon nanotube

Carbon nanotubes (CNTs), when manufactured, share similarities with asbestos due to their fibrous structure and long-lasting properties, potentially presenting a similar inhalation risk.

In this scenario, studies in rodens shown that lung inflammation caused by CNTs inhalation is marked by the emergence of epithelioid granulomas and a swift initiation of intense fibrosis, which is equally or more severe than the reactions to quartz or asbestos fibers. This result is mechanistically linked to the stimulation of IL-1β discharge in macrophages. This discharge then prompts the production of PDGF and TGF-β in the alveolar epithelial cells, eventually causing the epithelial-mesenchymal transition (EMT) (Huang et al, 2020).

The pathogenicity of both CNTs and asbestos fibers is not solely dependent on their basic chemical composition but is also determined by several other factors: (i) the diameter or width of the materials, influencing deep lung deposition, clearance interference, and access to the pleural area; (ii) the length of the fibers or tubes, affecting macrophage processing, clearance, and the potential to block pleural stomata; (iii) durability, leading to biopersistence in the lungs. Additionally, the mechanical bending stiffness of CNTs is crucial in defining their shape, whether needlelike or tangled. Other surface characteristics, including functional groups, coatings, defects, metal impurities, and the extent of reactive surface area, can also intensify the degree of injury (Huang et al, 2020).

Genetic alteration

Malignant Mesothelioma is predominantly propelled by pervasive somatic copy-number alterations on a genomic scale. Such variations predominantly include the subtraction of specific tumor suppressor genes like the BRCA1-associated protein 1 (BAP1), localized at 3p21, and CDKN2A, positioned at 9p21, whereas oncogenic gain-of-function modifications are sporadic phenomena. The loss of function pertaining to

these genes is frequently represented through genomic structural variants, conspicuously through chromothripsis.

BAP1, a definitive tumor suppressor gene, operates intricately within cellular mechanisms, administrating control over gene expression through the regulation of histone H2A activities. Its influential participation extends to the orchestration of apoptosis, along with the manipulation of DNA replication and repair processes. BAP1's mutation prevails as a recurrent anomaly in MM, its modifications, including somatic mutations and deletions, manifest conspicuously in approximately 55% of documented cases. Specific mutations of BAP1 conspicuously delineate the epithelioid MM, setting it apart from other subordinate subtypes. In a heterozygous state, BAP1 mutations unveil themselves both in germline and somatic scenarios, following an autosomal dominant pattern of inheritance. Individuals inheriting these mutated genes exhibit an escalated susceptibility to MM development, further intensified post asbestos exposure, and show a predisposition towards melanoma, clear-cell renal cell carcinoma, and cholangiocarcinoma (Perrino et al, 2023).

Research elucidates that individuals bearing BAP1 germline mutations exhibit a significantly extended survival trajectory following chemo-immunotherapy, a revelation uncovered in the PrE0505 trial which evaluated the efficacy of durvalumab coupled with chemotherapy in a primary MM setting. In addition, pathogenic truncating variants (PTVs) were found in PALB2, BRCA1, FANCI, ATM, SLX4, BRCA2, FANCC, FANCF, PMS1, and XPC. These genes play roles in DNA repair pathways, primarily in homologous recombination repair (Betti et al., 2017).

MM also prominently features the 9p21 deletion as a frequent copy-number mutation, encapsulating genes like CDK2NA and its neighbouring gene, MTAP. This genomic alteration has been synonymously linked with an aggravated prognosis and an intrinsic resistance towards immune checkpoint therapies. Comprehensive genomic analyses unfold that the 9p21 deletion aligns itself with a desolate tumor microenvironment, characterized by a depreciated infiltration of T, B, and NK cells, and a subdued immune cellular activation coupled with a diminished PD-L1 expression and an enhanced immunosuppressive signaling (Perrino et al, 2023).

A consummate understanding of this mechanism is pivotal, especially when considering its prevalence in nearly 50% of TCGA MM samples, substantiating its

significant role in delineating the resistance pattern against Immune Checkpoint Inhibitors (ICIs) in MM. Concurrently, an expansive genomic analysis heralds the loss of CDKN2A on 9p21.3 as being intricately associated with a concomitant deletion of the proximally located Type I Interferon (IFN) genes, elucidating a potential pathway facilitating immune evasion in MM cells (Perrino et al, 2023).

Physiopathology

In addressing the comprehensive physiopathological aspects of mesothelioma, I will refer to an insightful review published on *Frontiers Oncology* by Sahu et al in 2023 that elucidates its multifaceted nature. Specifically, I will draw upon essential passages from the same review.

Asbestos fibers are well-established carcinogens implicated in the pathogenesis of MM, an association substantiated by the fact that more than 80% of MM patients have encountered asbestos In Italy, specifically, approximately 2.5% of all deaths from MM occurred in individuals aged 50 or younger, and these deaths could likely be attributed to asbestos exposure during childhood (Fazzo et al, 2020). The pathogenic mechanisms of asbestos begin when inhaled fibers provoke a sequence of detrimental cellular events. These fibers, longer than 5 μm and rich in iron (II) ions (Fe^{2+}), possess a particular proclivity to penetrate lung epithelium and lodge in the pleura, leading to persistent inflammation, DNA damage, and mesothelial hyperplasia. The iron content facilitates the Fenton reaction, a specific oxidative process that results in the production of reactive oxygen species (ROS) and hydroxyl radicals, promoting oxidative stress and subsequent DNA damage (Sahu et al, 2023a).

Inhaled fibers instigate a chronic cycle of tissue damage and repair, marked by recurring inflammation. These fibers disrupt normal mitotic processes, inducing chromosomal aberrations and aneuploidy in mesothelial cells. Additionally, the fibers act as a nidus for macrophage accumulation, which, upon asbestos exposure, release Tumor Necrosis Factor (TNF)-α. Mesothelial cells possess TNF-α receptors and can also produce TNF-α, creating an autocrine and paracrine loop that stimulates the NF-κB signaling pathway. This pathway promotes cellular proliferation, enabling mesothelial cells with genetic aberrations to survive and proliferate, increasing the likelihood of malignant transformation.

Despite the high incidence of asbestos exposure, only about 5% of exposed individuals develop MM, highlighting the involvement of dose-dependent and genetic

factors, such as NF2 and/or LATS2 mutations that activate the transcriptional coactivator YAP, in disease manifestation. The cellular and molecular events underpinning MM development are complex, and empirical in vivo studies reveal that MM requires a specific exposure threshold to manifest. However, this threshold remains undefined in human subjects, suggesting that even minimal exposure can potentially lead to MM.

Beyond the inflammatory response, asbestos fibers induce the activation of several kinase pathways, including the mitogen-activated protein kinases (MAPKs) and extracellular signal-regulated kinases 1 and 2, which result in the expression of early response protooncogenes. Additionally, the phagocytosis of asbestos fibers leads to oxidative radicals that contribute to DNA damage and mutations.

The relationship between asbestos exposure and mesothelioma is further complicated by the discovery of numerous growth factors and cytokines, including Transforming Growth Factor β (TGF-β), Platelet-Derived Growth Factor (PDGF), Vascular Endothelial Growth Factor (VEGF), and Insulin-like Growth Factor (IGF). These factors, alongside cytokines like TNF-α, IL-6, and IL-8, play critical roles in the promotion of cancer cell growth, mitosis regulation in MM cells, and the encouragement of tumor proliferation and migration.

In the cellular microenvironment, the release of High Mobility Group Box 1 (HMGB1), a DNA-binding chaperone, is a consequence of asbestos-induced cell death, which acts as a damage-associated molecular pattern (DAMP). HMGB1 is crucial in recruiting macrophages and sustaining chronic inflammation, and it also plays a role in regulating autophagy, a cell survival mechanism under stress conditions. This is highlighted by studies showing that HMGB1's translocation influences asbestos-induced autophagy, potentially aiding in the survival of cells with asbestos-related DNA damage.

Mesotheliomas often demonstrate polyclonality due to the 'field effect' of asbestos, with genomic studies revealing mutations in genes such as CDKN2A, NF2, TP53, LATS2, and SETD2.

Overall, the mechanisms by which asbestos fibers initiate MM are multifaceted and include the direct interference with cell division, the activation of proto-oncogenes, the stimulation of chronic inflammation, and the promotion of a milieu conducive to genetic damage and cell survival, culminating in a high-risk environment for the development of mesothelioma in susceptible individuals (Sahu et al, 2023a).

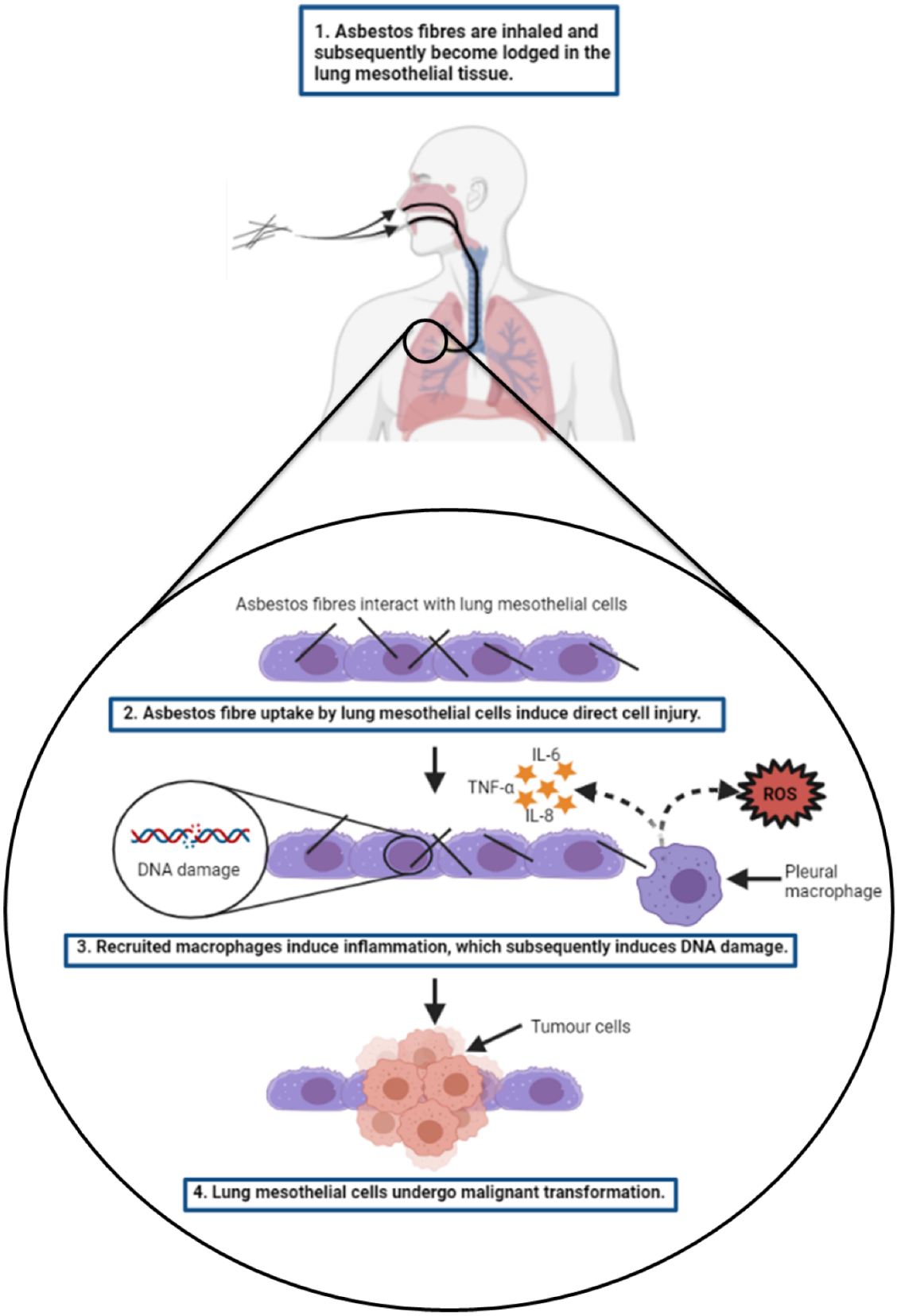


Figure 3. Overview of asbestos-induced mesothelial cell injury leading to the development of Malignant Mesothelioma (Johnson et al, 2021).

Biomarkers

Recurrent Mesothelioma Biomarkers

In recent scientific explorations, cancer biomarkers have manifested as pivotal elements in augmenting the efficacy of cancer management strategies, particularly in the realm of mesothelioma. Diagnosis and treatment decisions should be made in conjunction with other clinical and imaging findings, as well as pathological examination of tissue samples. The IHC have an indispensable role in diagnosis of MM where tumor architecture is difficult or impossible to identify.

A combination of different mesothelial markers:

Cytokeratins are intermediate filaments found within epithelial and mesothelial cells. The CK5/6 pair is predominantly present in keratinizing and non-keratinizing squamous epithelium, as well as in the basal-myoepithelial cell layer. Epithelioid or biphasic malignant mesothelioma, typically exhibit CK5/6 expression while sarcomatoid mesothelioma shows lower CK5/6 expression. CK5/6 has a reported sensitivities ranging from 63% to 100% (Shield et al. 2008).

Calretinin is a 29 kDa calcium-binding protein that is expressed in central and peripheral neural tissue. It has long been considered a reliable marker for establishing mesothelial lineage. Immunoreactivity for calretinin was represented by strong nuclear and diffuse cytoplasmic staining of the neoplastic cells is a consistent feature of the epithelial subtypes (Doglioni, 1997).

Wilms Tumor 1 (WT1) gene was originally identified as a tumor suppressor gene and it is known to regulate various cellular processes, including cell proliferation, differentiation, and apoptosis (programmed cell death). It is detected in the nucleus of tumor cells of mesothelioma (Natatsuka et al. 2006).

Podoplanin, a small type I integral membrane mucin-type sialoglycoprotein (frequently termed "D2-40" as a clone) is a sensitive marker of epithelioid mesothelial lesions, showing membranous staining. It is more specific for mesothelioma (Ymaki et al. 2023).

As previous reported BAP1 is a nuclear ubiquitin hydrolase located at the epicenter of 3p21.1 It is involved in controls of different activity, including DNA repair, cell proliferation, and cell cycle. When BAP1 has a biallelic mutations lead to BAP1 loss

detected by IHC. The loss of BAP1 is presente in most cases of biphasic and sarcomatoid mesothelioma. However, monoallelic mutation does not cause BAP1 loss (Kinoshita et al. 2018).

MTAP, positioned adjacent to the telomere end of the p16 gene, contains genetic information for an enzyme known as 5'-deoxy-5'-methylthioadenosine phosphorylase. This enzyme holds significance in the processes of polyamine metabolism as well as the recovery of adenine and methionine. MTAP expression is widespread across normal human tissues. However, it has been observed to be absent in numerous tumor categories, such as mesothelioma, often coinciding with the deletion of the p16 gene (Kinoshita et al. 2018).
Was reported that the combination of MTAP IHC and BAP1 IHC confirm the MM diagnosis with a good sensitivity of 76.5%, which was higher than that of BAP1 IHC alone (60.8%) (Kinoshita et al. 2018).

Mesothelin, a cellular adhesion glycoprotein, manifests a pronounced over-expression in the cellular pathology of MM, and concurrently in pancreatic and ovarian carcinomas. Concentrations of mesothelin within the serum, also conceptualized as Serum Mesothelin-Related Protein (SMRP), exhibit elevated trajectories in individuals diagnosed with MM relative to a control group characterized by asbestos exposure (Creaney et al, 2008).
Scientific contributions have delineated the sensitivity metrics for MM diagnosis to oscillate between 56% and 77%, with a specificity benchmark established at 95% (Creaney et al, 2014).

Osteopontin (OPN) is a notable glycoprotein that is abundantly present in several cancers, playing a role in cellular communication. Despite its substantial presence, continual monitoring of OPN levels has not proven particularly beneficial. Studies suggest that elevated levels of OPN at the beginning stages of the disease may be indicative of a challenging prognosis.
Pass et al delineated the manifestation of augmented levels of serum Osteopontin in a cohort consisting of 76 individuals diagnosed with MM, in comparison to a control group comprising individuals subjected to asbestos exposure without consequent MM manifestation. The diagnostic sensitivity associated with Osteopontin as a biomarker was ascertained to be 78%, accompanied by a specificity of 86% (Pass et al, 2005; Hollevoet et al, 2012).

Fibulin-3, a glycoprotein encoded by the EFEMP1 gene, is predominantly localized in the extracellular matrix and is composed of six EGF-like modules that exhibit calcium-binding properties, along with a fibulin-type module. It is ubiquitously expressed in various human tissues, including cartilage, bone, and skin, playing a pivotal role in maintaining the structural integrity of connective tissues' basement membranes. Its molecular functionality includes binding to elastin precursors, contributing substantively to the architectural assembly of elastic fibers intertwined with collagen (Creaney et al, 2014).

Furthermore, a surge in Fibulin-3 expression has been discerned in some aggressive cancer forms, driving oncogenic processes such as tumor angiogenesis through potential activation of intricate signalling pathways, such as EGFR.

Evaluative studies have posited Fibulin-3 as a potential biomarker in malignant pathologies like pleural mesothelioma, marked by elevated Fibulin-3 levels in afflicted individuals relative to healthy controls. In a comprehensive synthesis of the extant literature, robust empirical evidence has been observed underpinning the diagnostic efficacy of Fibulin-3 in MM patients (Kaya *et al*, 2015).

HMGB1

HMGB1, a universally conserved nuclear protein, is ubiquitously observed across various cellular types, affirming its fundamental biological significance (Yang *et al*, 2020a). This protein coexists with its analogous counterparts, HMGB2 and HMGB3, thereby constituting the essential HMGB family which predominantly resides in the chromosomal framework, undertaking vital nuclear responsibilities (Yang et al, 2020b).

Primarily localized within the nucleus, HMGB1 exhibits a noteworthy versatility in its spatial presence. Instances of cellular necrosis manifest the passive translocation of HMGB1 to the cytoplasm, while certain immune cells, notably macrophages, portray an active extranuclear secretion of the protein (Yang *et al*, 2010). This extracellular presence of HMGB1 initiates inflammatory pathways, prominently through interaction with specific receptors such as the Receptor for Advanced Glycosylation End products (RAGE) (Hori *et al*, 1995) and Toll-Like Receptors (TLR2 and TLR4) (Park *et al*, 2004), in conjunction with the C-X-C chemokine Receptor type 4 (CXCR4) (Schiraldi *et al*, 2012).

HMGB1's architecture embodies a remarkable conservation, marked by the presence of two essential DNA-binding domains, namely BoxA and BoxB, complemented by a

negatively charged C-terminal domain (Yang et al, 2013). This strategic structural configuration facilitates its nuclear localization, substantiated by the existence of nuclear localization sequences (NLS) within the protein's domain structure. Remarkably, cellular adversities such as damage or necrotic occurrences instigate a passive release of HMGB1 into the extracellular milieu, a process intensified by hyperacetylation events influencing the NLS sequences (Lu *et al*, 2014).

In its nuclear habitat, HMGB1 orchestrates a plethora of molecular interactions, including binding to the minor groove of double-stranded DNA, culminating in the modulation of the helical DNA structure (S Müller P Scaffidi et al.). This molecular adaptability allows HMGB1 to facilitate interactions essential for cellular processes such as DNA repair mechanisms and nucleoprotein complex formations, ensuring cellular genomic integrity and regulatory compliance (Agresti & Bianchi, 2003; Yuan *et al*, 2004).

Externally, HMGB1 delineates a multifaceted role, prominently as a Damage-Associated Molecular Pattern (DAMP) entity. This implicates HMGB1 in the orchestration of immune responses, facilitating leukocyte chemoattraction and acting as a pivotal mediator of pro-inflammatory pathways. Its functional diversity is manifested through its redox potential, particularly influencing cysteines at specific positions, thereby mediating a spectrum of activities ranging from inflammatory mediation to chemoattractant functionalities (Venereau et al, 2012).

HMGB1's extracellular journey exhibits a profound influence on cellular signaling pathways, manipulating cellular responses through a spectrum of mechanisms including autocrine, paracrine, and endocrine modalities. Its interaction with receptors such as RAGE accentuates its involvement in a multitude of disorders, ranging from septic conditions to oncological, metabolic, and neurological anomalies. Notably, the strategic inhibition of the HMGB1-RAGE signaling axis emerges as a potential therapeutic paradigm in mitigating HMGB1-mediated inflammatory cascades (Kang *et al*, 2010; Yang *et al*, 2012).

In the broader spectrum of immune modulation, HMGB1 interacts with an array of Toll-like receptors (TLRs), potentiating the host's immune arsenal against infectious challenges. Specific TLR interactions, such as those involving TLR2, TLR4, and TLR9, underscore HMGB1's role in the activation and regulation of crucial immune cells and pathways, thereby corroborating its pivotal role in immune surveillance and regulation. Additionally, HMGB1 in its fully reduced state exhibits a propitious interaction with CXCL12, synergistically engaging with the CXCR4 receptor,

epitomizing its versatile influence on cellular signaling landscapes (Qiu *et al*, 2014; Schiraldi *et al*, 2012).

HMGB1 in cancer and mesothelioma

In the realm of oncology, HMGB1 has been discerned to manifest at elevated levels across a spectrum of solid neoplasms, encompassing those of the colon, lungs, breasts, ovaries, prostate, and mesothelioma (Kang *et al*, 2013). HMGB1 exhibits a dichotomous functionality: it propels cell survival, augmenting tumor evolution and advancement, while simultaneously operating as a tumoral inhibitor through the facilitation of cellular apoptosis and the modulation of autophagy processes.

Upon its liberation from infiltrating leukocytes or the neoplastic cells themselves, HMGB1 fosters a pro-tumoral inflammatory milieu, igniting proinflammatory signaling conduits such as the NF-kB and inflammasome pathways (Yang *et al*, 2010). These pathways further catalyze the expulsion of proinflammatory cytokines, thereby amplifying the inflammatory resonance through the perpetuation of a positive feedback loop mediated by RAGE and TLR4 signaling mechanisms (Jube *et al*, 2012; Gebhardt *et al*, 2008). Furthermore, the HMGB1 interaction with RAGE, within the confines of the activated NF-kB pathway, holds the potential to invigorate tumoral angiogenesis by precipitating the expression of pivotal proangiogenic growth facilitators such as VEGF (Van Beijnum *et al*, 2013).

Tumoral progression is punctuated by cellular invasiveness, which begets metastasis. HMGB1 fosters metastatic potentials through its interaction with RAGE; inhibitory interventions targeting the RAGE-HMGB1 nexus can thus subdue tumoral expansion and metastatic manifestations (Taguchi et al, 2000). HMGB1 also orchestrates the energy metabolism of the tumor, enhancing ATP synthesis and cellular proliferation within pancreatic neoplasms (Kang et al, 2014). Additionally, it exerts inhibitory pressures on anti-neoplastic immunity, inducing apoptotic processes in macrophage-derived dendritic cells and augmenting the potential of tumor-associated regulatory T cells to produce IL-10, thereby attenuating CD8+ T cell-dependent responses (Liu et al, 2011c).

In an intracellular context, HMGB1 operates as an antineoplastic agent by forging direct interactions with Rb via the LXCXE motif, culminating in G1 phase arrest, the induction of apoptosis, and the mitigation of tumorigenesis, particularly within breast

cancers (Jiao *et al*, 2007). HMGB1 is intrinsic to genomic stability maintenance, safeguarding telomeres which are quintessential for chromosomal integrity, shielding them against recombination and degradation (Giavara et al, 2005). A deficiency of HMGB1 precipitates telomere attrition, promoting genomic instability and consequently fostering tumorigenesis (Polanská et al, 2012).

As a regulatory architect of autophagy, HMGB1 facilitates the inhibition of inflammasome activation by propagating its degradation or diminishing ROS production (Tang *et al*, 2010). The absence of HMGB1 engenders a deficiency in autophagy, catalyzing genomic instability and inflammation, thereby potentiating tumorigenesis. In the orchestration of immunogenic cell death (ICD) within neoplastic cells, HMGB1 emerges as a pivotal entity. ICD, a manifestation of immunogenic apoptosis, is incited by specific chemotherapeutic agents and radiotherapy, characterized by the externalization of damage-associated molecular patterns (DAMPs) (Apetoh *et al*, 2007).

These DAMPs underpin the maturation, antigen absorption, and presentation of dendritic cells (DC), functioning as potent immunological adjuncts for the activation of the cytotoxic T lymphocyte response. Within this framework, HMGB1, liberated from apoptotic cells, engages with TLR4, promoting the anticancer immune responses associated with ICD during chemotherapy (Apetoh et al, 2007). The modulation of HMGB1 dynamics, particularly within the context of apoptosis and necrosis, remains instrumental in optimizing therapeutic outcomes in both chemotherapy and immunotherapy paradigms (Luo *et al*, 2010).

Supplementary research revelations have unveiled the heightened expression and secretion patterns of HMGB1 within malignant mesothelioma cells, thereby delineating an autocrine regulatory circuit essential for the sustenance of their malignant phenotypic expressions. These discoveries underscore the significant participatory role of HMGB1 in navigating the developmental and progressive pathways of mesothelioma, as evidenced within preclinical investigational realms (Jube *et al*, 2012).

Moreover, clinical evaluations, particularly in patients manifesting peritoneal and pleural mesothelioma, have elucidated elevated serum concentrations of HMGB1 in comparison to control demographics. Such observations amplify the potential applicability of HMGB1 as a diagnostic and prognostic biomolecular marker,

elucidating distinctive insights into the malignant manifestations of mesothelioma (Hollevoet *et al*, 2012).

HMGB1 as target

In therapeutic contexts, the inhibition of HMGB1 presents a promising avenue. Numerous agents targeting HMGB1 have been meticulously examined, revealing encouraging outcomes both in vitro and in animal models.

These agents encompass a range of mechanisms and have been studied in various experimental settings. Notably, soluble RAGE (sRAGE) functions as an interceptive molecule, effectively hindering RAGE signaling. This has been demonstrated in animal tumor models, where sRAGE has successfully disrupted the HMGB1-RAGE signaling axis (Liu et al, 2011a).

Intriguingly, platinating agents, specifically cisplatin and oxaliplatin, possess a unique ability to confine HMGB1 within the nucleus. This is achieved through the induction of conformational changes in the DNA double helix, to which HMGB1 exhibits a high affinity (Ohndorf et al, 1999).

Ethyl pyruvate, recognized as the inaugural HMGB1 inhibitor in animal sepsis models, achieves its effect through the inhibition of the NF-κB pathway (Liang *et al*, 2009). This compound has also shown promise in inhibiting liver tumor growth. Furthermore, compounds like glycyrrhizin and quercetin, which either bind directly to HMGB1 or inhibit the PI3K pathway, have been noted to enhance the efficacy of anticancer agents in various tumor models (Liu *et al*, 2011b).

Additionally, strategies such as the use of HMGB1 neutralizing antibodies and BoxA protein have been employed to impede the actions of extracellular HMGB1, which is significant in tumor therapy.

In accordance with available data, it has been documented that the utilization of monoclonal antibodies or BoxA directed against HMGB1 contributes to an extension in the survival rate of mice subjected to xenografts with human multiple myeloma cells. This observed effect is attributed to the interference imposed upon tumor cell proliferation, elucidating a potential avenue for therapeutic intervention in the context of MM (Yang *et al*, 2015a). We are recent published that BoxA triggers the the exposure of the "eat me" signal ecto-calreticulin and the internalization of the "don't

eat me" signal CD47 promoting the a direct antiproliferative effect on MM tumor cells, and promotes tumor cell phagocytosis by macrophages. Moreover, BoxA induces tumor rejection and immunization in mice inoculated with mesothelioma and colon carcinoma cells (Mezzapelle et al, 2021).

Furthermore, conventional medications like aspirin (acetylsalicylic acid, ASA) and its metabolite, salicylic acid (SA), have emerged as novel modulators of HMGB1, showcasing potential in decelerating the evolution and progression of MM (Yang *et al*, 2015a).

An exploration into various anti-inflammatory compounds reveals potential therapeutic strategies against MM. Substances like flaxseed lignan demonstrate capabilities in attenuating acute asbestos-induced inflammation, marking them as prospective agents in chemopreventive strategies against MM. Additionally, contemporary investigations have unveiled a plethora of potential therapeutic targets such as the IL-4/IL-4R axis and anti-IL-6, elucidating new horizons in the therapeutic approach towards MM (Mutti et al, 2018).

Despite these promising developments, there is a pressing need for further research to fully evaluate the potential of these therapies, particularly their applicability and effectiveness in clinical practice. As the understanding of HMGB1's role in cancer progresses, these agents represent a promising frontier in the development of novel cancer therapies.

Current treatment

In addressing the comprehensive treatment aspects of mesothelioma, I will refer to an review published in *Frontiers in Oncology* by Kuryk et al. in 2022, that elucidates different therapies. Specifically, I will draw upon essential passages from the same review.

Mesothelioma treatment can involve individual methods such as chemotherapy, or a multimodal approach that combines radiotherapy and targeted therapy. Early detection and intervention are crucial for the successful surgical management of mesothelioma (Sahu et al, 2023a).

Supportive Care in Mesothelioma

Effective palliative strategies are instrumental in alleviating mesothelioma's physical discomfort and distress, as emphasized by global respiratory and thoracic societies. This approach, distinct from end-of-life hospice care, concentrates on enhancing patients' overall wellbeing, offering multifaceted support—physical, mental, and spiritual. When integrated with therapies such as chemotherapy and immunotherapy, palliative care plays a pivotal role in improving patients' life quality, especially those in advanced disease stages or with significant health deficits (Sahu et al, 2023a).

Surgical Intervention in Mesothelioma

The appropriateness of surgical measures in mesothelioma management is subject to deliberation, being primarily recommended for those identified in the disease's nascent stages with a satisfactory health status. The decision to pursue surgery hinges on a constellation of factors like the disease's progression, patient vitality, and histological variables. Surgical endeavors, particularly within a palliative framework, are employed predominantly for moderating symptoms and managing tumor size, predominantly when the disease's identification occurs in preliminary stages, reflecting its restrained utility in scenarios of protracted disease progression (Rice, 2011)

Some common surgical procedures used in the treatment of mesothelioma include (Rice, 2011):

- Extrapleural pneumonectomy (EPP): This surgery involves removing the affected lung, part of the diaphragm, the lining of the heart (pericardium), and the affected tissue lining the chest cavity (pleura). It's typically considered for patients with early-stage mesothelioma and good overall health.
- Pleurectomy/Decortication (P/D): This surgery involves removing the pleura lining the chest cavity and any visible tumor masses while sparing the lung. It's often considered for patients with early-stage mesothelioma who are not candidates for EPP.
- Cytoreductive surgery (CRS): This surgery aims to remove as much of the visible tumor as possible, often followed by heated chemotherapy (HIPEC) directly to the affected area to kill any remaining cancer cells.
- Pleurodesis: This procedure involves using chemicals or medications to create scar tissue between the layers of the pleura, preventing the buildup of fluid in

the chest cavity (pleural effusion). While not a curative treatment for mesothelioma, it can help alleviate symptoms.

Following the primary treatment for MM, many patients might need more chemotherapy sessions. Sadly, current clinical data doesn't provide clear guidance on whether patients can resume antifolate therapy after pausing platinum-pemetrexed-based treatment (Kuryk et al, 2022).

Radiotherapy

Common radiotherapy approaches struggle with MM's pervasive spread along the pleura and surrounding vital organs, leading to unsatisfactory therapeutic outcomes and significant adverse effects such as pneumonitis and myocarditis.

Technological advancements, particularly in three-dimensional imaging, have somewhat improved the precision and impact of radiotherapy. These developments enable the management of larger treatment volumes more effectively and have broadened the therapeutic window. Despite these advances, conventional radiotherapy has primarily been utilized for palliative purposes, focusing on symptom relief, especially pain management, given the extensive and complex nature of mesotheliom (Kuryk et al.2022).

Recent trials and studies, such as the SYSTEMS2 trial, have shifted focus towards exploring the efficacy of intensity-modulated radiotherapy (IMRT) in managing symptoms and improving pain control for MM patients. Studies have shown promising results, with patients undergoing post-surgery hemithoracic radiation experiencing a median survival of around 13.5 months, although approximately 10% of patients faced serious lung-related complications (Gupta et al. 2005).

Additionally, prophylactic radiotherapy is being considered to minimize the risk of tumor seeding post-invasive diagnostic techniques and surgery, supported by studies showing reduced risks associated with its use.

Trials like the SMART trial have introduced novel approaches such as short-course hemithoracic IMRT, aiming for enhanced outcomes in early-stage, node-negative mesothelioma. However, the conclusions from these trials are not definitive, warranting further exploration and research. Nonetheless, certain pivotal trials have demonstrated improved outcomes with radical hemithoracic radiotherapy approaches, offering insight into the potential evolution of MM management

strategies and the integration of emerging technologies such as proton therapy to minimize adjacent tissue toxicities (Kuryk et al.2022).

In summation, while there is a continuous evolution in the exploration of multimodal treatment paradigms, including surgery and hemithoracic radiotherapy, conclusive evidence remains limited. The integration of new therapeutic agents and technological advancements, in conjunction with systemic therapies, could potentially enhance the efficacy of multimodal treatment approaches in managing MM, within the realms of clinical trials and palliative care settings.

Advanced Therapies

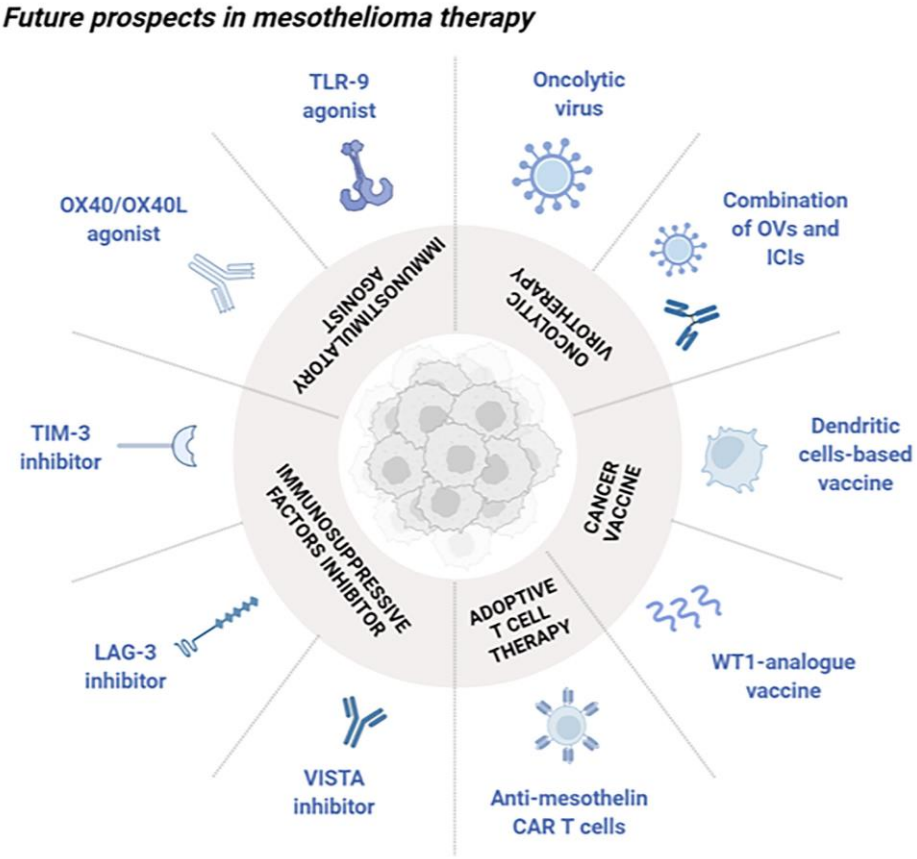


Figure 4. Illustrative overview of prospects in immunotherapeutic interventions for mesothelioma (Kuryk et al, 2022).

Anti-Angiogenic Drugs

Angiogenesis, the process of new blood vessel formation, is fundamental in the growth and development of MM, as it facilitates the delivery of essential nutrients and oxygen to cancerous cells. MM cells have been found to produce angiogenesis-promoting growth factors, notably VEGF (Vascular Endothelial Growth Factor), which further encourages the creation of autocrine loops. The VEGF pathway has been identified as a key therapeutic target due to its pivotal role in angiogenesis, supported

by the observed elevated levels of serum VEGF in MM patients, which is associated with a poor prognosis.

Bevacizumab, an anti-VEGF molecule, has been one of the most extensively studied and utilized drugs to combat angiogenesis. The FDA has approved it for use alongside chemotherapy. Studies reveal that its combination with other drugs such as pemetrexed has shown a synergistic effect in mitigating cell growth and improving survival rates in MM cases. Clinical trials have been undertaken to assess the effectiveness of incorporating bevacizumab into chemotherapy regimes. While some trials did not show significant improvements in progression-free survival, the Mesothelioma Avastin Cisplatin Pemetrexed Study (MAPS) reported positive outcomes, showcasing an increase in overall survival and progression-free survival rates.

Another therapeutic agent explored is Ramucirumab, which specifically targets VEGFR-2, manifesting in decreased tumor growth and proliferation. Its combination with chemotherapy, according to research, has been seen to enhance the efficacy of the treatment in animal models (Kuryk et al, 2022).

Immunotherapy

Immunotherapy, a treatment that stimulates the immune system against cancer, offers a different angle from traditional therapies that directly target cancerous cells. Considering the correlation between lymphocyte infiltration and improved prognosis, immunotherapy might be promising for MM patients. The focus is on reversing the tumor's immune-suppressive environment.

Immunotherapy, including the use of immune checkpoint inhibitors, is a pivotal approach in mesothelioma treatment, stemming from the understanding that mesothelioma induces a rather feeble immune response. Researchers have delved into various strategies to bolster this response. Traditional techniques, such as the intrapleural administration of cytokines, face challenges due to the cytokines' brief half-life, demanding continuous or frequent administration. For instance, the consistent administration of intrapleural interferon-gamma has shown a 56% response rate in initial stage diseases, while continuous infusion of interleukin-2 has resulted in partial responses and an average survival period of 16 months with manageable side effects such as fever (Kuryk et al, 2022).

Studies indicate that interferons could hinder the proliferation of mesothelioma cells and augment the effectiveness of cisplatin, a chemotherapy drug. A clinical trial involving the combined use of cisplatin, doxorubicin, and interferon alpha-2 has shown promising outcomes with a 29% overall response rate but was also associated with significant myelosuppression (Kuryk et al, 2022).

ICIs, specifically the combination of nivolumab and ipilimumab, have shown promise as a foundational treatment strategy for inoperable MM. A pivotal clinical trial, CheckMate743, provided significant data that influenced the FDA's endorsement of this combined therapeutic approach. Within this substantial study, 605 participants were selectively assigned to receive either a novel immunotherapeutic regimen or traditional chemotherapy agents like pemetrexed and cisplatin (Kuryk et al, 2022).

The outcomes presented a remarkable improvement in overall survival among those administered immunotherapy, demonstrating its potential effectiveness over traditional platinum-based chemotherapy. Although recipients of immunotherapy faced a higher incidence of adverse effects, these were generally manageable with interventions like steroids and supportive treatments, ensuring patient safety and tolerability (Kuryk et al, 2022).

Exploring the applicability of ICIs as subsequent lines of defense in treating MM has also been a focal area of research. Trials like the DETERMINE study, which focused on the anti-CTLA-4 antibody tremelimumab, did not yield substantial improvement in survival rates, indicating limited efficacy as a secondary treatment strategy.
In contrast, the KEYNOTE-028 study, cantered around the anti-PD-1 antibody pembrolizumab, demonstrated more favourable outcomes, with some patients showcasing partial responses and disease stability, aligning with an acceptable safety profile (Kuryk et al, 2022).

Multiple studies are actively exploring the potential of diverse ICI-based treatments in various therapeutic sequences, aiming to unveil optimized combinations and applications that could bolster the efficacy of MM treatment regimens. Continuous research efforts are underway to decipher the full spectrum of benefits that ICIs can offer across different stages and strategies in MM management (Kuryk et al, 2022).

Virotherapy

Utilizing viruses to infect cancer cells can spark an immune response against the malignancy. These viruses offer therapeutic benefits by modifying the infected cells at the genetic level. Various oncolytic viruses have been studied, with the most frequently used being replication-deficient ADV.

This method leverages genetically engineered viruses, possessing the ability to replicate within and eradicate cancer cells, while concurrently amplifying the immune system's responsiveness to the tumor microenvironment. One notable oncolytic adenovirus is ONCOS-102, characterized by a chimeric serotype 5/3, which fosters enhanced interactions with respective adenovirus receptors. ONCOS-102 is specifically modified, exhibiting a 24 base pair deletion in the E1A region, which facilitates selective replication within neoplastic cells.

Uniquely, ONCOS-102 stands as the sole oncolytic adenovirus presently under clinical exploration for its applicability in mesothelioma treatment. The viral genome of ONCOS-102 is further fortified with a transgene encoding GM-CSF, an element capable of eliciting immunostimulatory repercussions.

In a structured phase I clinical assessment (NCT02879669), the administration of ONCOS-102 was meticulously evaluated for its safety and immunological efficacy. This involved the juxtaposition of intrapleurally administered ONCOS-102 in combination with conventional therapeutic agents, pemetrexed and cisplatin, against the exclusive administration of the traditional agents. A pivotal observation within this study was the amplified intratumoral presence of cytotoxic T cells amongst individuals within the experimental ensemble, a phenomenon absent in the control faction. This therapeutic approach further instigated a discernible transition of macrophages from an M2 to an M1 phenotype, heralding the induction of immune stimulation.

Noteworthy, also, is the observed upregulation of PD-L1 expression in a significant portion of participants subjected to viral therapy, thereby illuminating the prospective integration of oncolytic viral strategies with immune checkpoint inhibitors, aiming to harness a synergistic therapeutic impact (Kuryk et al, 2022).

Adoptive T-cell Treatment

Chimeric antigen receptors (CARs) are sophisticatedly engineered entities resembling T-cell receptors, with an advanced design allowing for an exquisite, precise targeting and engagement with tumor-associated antigens (TAAs). These receptors exhibit versatility by binding directly to various cellular surface

constituents, such as proteins, lipids, and carbohydrates, transcending the reliance on Major Histocompatibility Complex (MHC) antigen presentation. Structurally, CARs consist of intracellular domains primarily characterized by the CD3ζ signaling domain, augmented with co-stimulatory domains to bolster sustained T-cell activation and viability.

Scientific strategies are employed to optimize the therapeutic exploitation of CARs, focusing particularly on amplifying the population of T cells proficient in establishing interactions with tumor cells. In the context of Malignant Mesothelioma (MM), investigational emphasis has been placed on target antigens such as mesothelin and fibroblast activation protein (FAP), currently under rigorous clinical examination for their therapeutic viability and specific overexpression in tumoral and stromal cells respectively (Kuryk et al, 2022).

In the dynamic landscape of CAR-T cell research, mesothelin emerges as a crucial antigen undergoing extensive scientific scrutiny in numerous clinical trials. These investigations aim to delineate the therapeutic prowess of these re-engineered T cells against malignancies. The intrapleural delivery of CAR T cells, specifically attuned to mesothelin, has been documented to be clinically tolerable in patients afflicted with pleural malignancies, exhibiting negligible toxicity towards non-tumoral tissues expressing mesothelin (Klampatsa et al, 2020).

Furthermore, clinical strategies involving the confluence of mesothelin-specific CAR T cells with PD-1 inhibitory blockade have demonstrated promising potential in instituting durable therapeutic responses against solid tumor manifestations. In enhancement of the CAR T-cell's therapeutic perseverance and functional efficacy, combinations with agents such as pembrolizumab have been explored. Preliminary clinical observations indicate that patients with MM manifest a median survival rate of approximately 23.9 months subsequent to the administration of CAR T cells in conjunction with pembrolizumab, reflecting a one-year overall survival probability of 83% (Adusumilli *et al*, 2021).

Cancer Vaccines

Cancer vaccines aim to leverage the unique capabilities of certain cells, such as dendritic cells (DCs), to enhance immune responses against malignancies.

In the context of clinical applications, mRNA vaccines emerge as a paramount selection for addressing tumor-specific antigens (TSAs). Comprehensive

investigations encompassing both preclinical models and clinical trials have illuminated the efficacy of mRNA vaccines encoding TSAs, showcasing robust anti-tumor responses across diverse malignancies. Noteworthy among these are melanoma, gastrointestinal cancer, colorectal cancer, pancreatic adenocarcinoma, and hepatocellular carcinoma. The amalgamation of preclinical insights and clinical evidence underscores the compelling potential of mRNA vaccines in eliciting potent anti-tumor activity, thereby charting a promising course in the therapeutic landscape for these various tumor types (Pardi et al, 2020).

Clinical trials are ongoing to understand the practical applicability and effectiveness of these vaccines in disease management.

VISTA Inhibitor

The emergence of VISTA (V-domain Ig suppressor of T cell activation) as a promising immunotherapy target in multiple myeloma (MM) underscores its significance in modulating immune responses within cancerous environments. Muller et al. (2020) highlight VISTA's prevalent expression in tumor-associated inflammatory cells in MM, suggesting its potential as a target for anti-VISTA antibody treatments.

Hmeljak et al. (2018) conducted a detailed genomic analysis revealing elevated VISTA mRNA levels in MM compared to other neoplasms, particularly accentuated in the epithelioid subtype. This subtype also showed a correlation between higher VISTA expression and improved overall survival. Immunohistochemical examination further elucidated VISTA's presence in infiltrating immune cells and epithelioid MM cells, indicating a potential role in sustaining antigen-presenting cell properties.

VISTA functions as a negative regulator of T cell proliferation and activation, primarily on myeloid cells like tumor-associated macrophages (TAMs). Analogous to PD-L1, VISTA possesses the capability to facilitate the differentiation of naïve T cells into FoxP3+ regulatory T cells, and exerts its inhibitory influence on T cells, functioning both as a receptor on T cells and as a ligand on antigen-presenting cells (Lines et al, 2014).

Given these findings, there is growing interest in exploring VISTA as a predictive biomarker for responsiveness to immune checkpoint inhibitors (ICIs) in MM. Clinical trials investigating anti-VISTA therapies are expanding their scope, recognizing the potential of targeting VISTA in MM immunotherapy.

Tumor microenvironment

The neoplastic microenvironment, commonly referred to as the Tumor Microenvironment (TME), is comprised of a heterogeneous assortment of cellular entities inclusive of neoplastic cells, an array of immune constituents, both innate and adaptive, as well as a multitude of non-immune cells. Additionally, this environment is characterized by the secretion of a diverse array of molecules. Recent scientific discourse has acknowledged the parallels between oncogenic pathologies and evolutionary phenomena, emphasizing the continuous, dynamic, and bidirectional interplay that occurs between malignant cellular structures and their surrounding TME (Merlo *et al*, 2006).

The TME encapsulates a plethora of non-malignant cellular types that envelop the tumor mass. Noteworthy among these are the fibroblasts, endothelial cells, along with cells that constitute the innate and adaptive immune system. The TME further comprises extracellular components, notably the extracellular matrix (ECM), extracellular vesicles, and an array of soluble entities such as metabolites, chemokines, cytokines, and growth factors.

The literature is replete with evidence that illustrates the TME's role in fostering tumorigenesis through various mechanisms. These include the facilitation of proliferative signal secretion, engendering resistance to apoptotic processes, enabling neoangiogenesis, catalyzing invasion and metastatic activities, inciting pro-tumoral inflammation, and orchestrating evasion from immune surveillance (Xiao & Yu, 2021).

Prognostic outcomes for oncological patients are intricately linked to the characteristics of the TME. A correlation has been established where immunopermissive TMEs bear a more favorable prognosis in contrast to their immunosuppressive counterparts, underscoring their pivotal role in cancer progression and the determination of patient outcomes (Cornelissen *et al*, 2014).

Current research endeavors are directed towards manipulating the TME to assume an anti-tumoral, immunopermissive phenotype. Within the milieu of MM, TAMs and cancer-associated fibroblasts (CAFs) represent the predominant infiltrative cell populations. These cells, upon receiving signals from the tumor and its microenvironment, adopt properties that are supportive of tumor growth and

immunosuppression, thus influencing the progression of the malignancy (Nicolini *et al*, 2020).

The TME's propensity for immunosuppression is further accentuated by the presence of dendritic cells (DCs) and T regulatory cells (Tregs), which attenuate the activation of antitumoral CD8+ T cells. Additionally, multiple studies have elucidated the prognostic significance of immune cell presence within the mesothelioma microenvironment. A high density of activated CD8+ T cells correlates with improved prognosis and outcomes. In contrast, a preponderance of TAMs within the MM TME is associated with adverse prognoses and reduced survival rates (Hinshaw & Shevde, 2019).

Despite the high representation of pro-inflammatory mediators such as IL-1β, IL-6, and HMGB1 within the MM TME, immunosuppressive characteristics predominate. These include antigen presentation by antigen-presenting cells (APCs) like DCs and myeloid-derived suppressor cells (MDSCs), which suppress adaptive immune responses to the tumor. TAMs contribute to this immunosuppressive milieu by secreting cytokines such as IL-10, further inhibiting the response of T cells and natural killer (NK) cells against the tumor. Hypoxia within the TME, mediated through HIF1α, leads to an upregulation of programmed death ligand 1 (PD-L1), augmenting T cell exhaustion rates. The secretion of growth factors by TAMs and CAFs, including TGFβ, VEGF, and PDGF, is linked to enhanced tumoral growth and neoangiogenesis (Ruffell *et al*, 2014; Chu *et al*, 2019; Cersosimo *et al*, 2021).

Consequently, the TME in MM presents significant challenges in diagnostic, prognostic, and therapeutic contexts due to its inter-patient heterogeneity and complexity. The development of immunotherapeutic strategies is particularly arduous as the overall immune response within the TME is mitigated. The diverse cellular components within the TME not only partake in immunosuppression but also in the secretion of factors that stimulate tumor growth, concurrently forming a protective shield against immunotherapies and cytotoxic treatments, thereby promoting the uninterrupted proliferation of the tumor.

Role of Macrophages in tumor

In the intricate process of embryogenesis, macrophages emerge from progenitor cells initially developing from the yolk sac's primitive ectoderm. This is succeeded by

hematopoietic activity in the fetal liver, which spawns circulating monocytes. Coinciding with the genesis of bones postnatally, the hematopoietic baton is passed from the fetal liver to the bone marrow, establishing it as the progenitor of circulating monocytes and, by extension, the majority of tissue-resident macrophages (Wynn et al, 2013).

Within the realm of oncology, these macrophages represent pivotal components of the innate immune system, holding the capability to engulf neoplastic cells and present their antigens to the adaptive immune system, thus spearheading antitumor immunity. Consequently, there is burgeoning interest in harnessing phagocytosis for cancer immunotherapy (Shapouri-Moghaddam et al, 2018; Mantovani et al, 2017). Owing to their inherent plasticity, macrophages exhibit the ability to polarize into a spectrum of phenotypes, contingent on the surrounding stimuli. Typically, activated macrophages are dichotomized into two phenotypes: M1 macrophages, indicative of classical activation known to stimulate inflammatory responses against pathogens and tumor cells, and M2 macrophages, which are alternatively activated and tend to exhibit immunosuppressive characteristics conducive to tissue repair and tumor progression. The former secretes pro-inflammatory cytokines such as IL-12, TNFα, CXCL12, and INF-γ, while the latter releases anti-inflammatory mediators including IL-10, IL-13, and IL-4 (Gordon & Plüddemann, 2019; Mantovani et al, 2004; Biswas, 2015).

TAMs predominantly exhibit an M2 phenotype, fostering an immunosuppressive milieu that facilitates tumorigenesis and progression (Martinez & Gordon, 2014). Macrophages, as integral constituents of the mononuclear phagocyte system, which includes both tissue-resident macrophages and circulating monocytes, are mobilized to sites of inflammation or tissue damage, such as tumors. They derive from common myeloid progenitors that are stimulated to differentiate fully into macrophages by macrophage colony-stimulating factor (M-CSF or CSF-1) (Murray et al, 2014). In contrast, resident macrophages are of embryonic origin, having migrated to peripheral tissues in early life (Epelman *et al*, 2014).

A salient characteristic of macrophages is their adaptability to local stimuli, exhibiting a pronounced phenotypic and functional plasticity contingent on the microenvironmental context (Shapouri-Moghaddam et al, 2018). In their role as immune effectors, macrophages can mount a pro-inflammatory defense against pathogens and are capable of cytotoxicity against tumor cells. Conversely, they can assume anti-

inflammatory roles, instrumental in tissue healing, remodeling, and paradoxically, in promoting tumor growth through the establishment of an immunosuppressive environment. This adaptive capability is referred to as polarization (Mantovani et al, 2013).

To fulfil these disparate roles, macrophages undergo a state of polarization, distinguished by a differential gene expression profile, surface molecule pattern, and cytokine production. When activated, macrophages can polarize towards an M1 or M2 state; these represent extremes of a continuum, with the potential for intermediate states reflecting their substantial plasticity and context-dependent heterogeneity (Mantovani et al, 2002).

M1 polarization occurs in response to pro-inflammatory signals such as LPS and IFNγ, whereas the M2 state is elicited by anti-inflammatory cytokines like IL-4 and IL-13. The equilibrium of M1/M2 polarization is critical in regulating the progression and resolution of inflammation. In the initial phase of inflammation, M1 macrophages release TNF-α, IL-1β, and ROS. However, persistent inflammation and ROS can inflict tissue damage, necessitating the intervention of M2 macrophages, which secrete IL-10 and TGFβ to dampen the inflammatory response and contribute to tissue repair, remodeling, angiogenesis, and the re-establishment of homeostasis (Viola et al, 2019).

The secretomes of M1 and M2 states, along with other distinctive features such as gene expression, surface molecule expression, and metabolic processes, can be employed to differentiate between these polarized states. For instance, CD86 is a prevalent surface marker for M1, while M2 macrophages are identified by markers such as CD68, CD163, and CD206, varying with their subset, anatomical location, and specific function (Hickman *et al*, 2023).

Metabolic pathways within macrophages also undergo significant alterations. M1 macrophages predominantly rely on anaerobic glycolysis for ATP production, whereas M2 macrophages prefer oxidative phosphorylation (OXPHOS). A notable metabolic divergence in these polarization states involves the arginine catabolic pathways: M1 macrophages process arginine to citrulline and nitric oxide (NO) through nitric oxide synthase (iNOS), crucial for microbial killing and inflammatory responses, while M2 macrophages utilize arginase 1 (ARG1) to convert arginine to ornithine, thus promoting tissue repair. Moreover, ARG1 activity can inhibit T-cell proliferation and

cytokine production by competing with iNOS for substrate arginine, contributing to an anti-inflammatory phenotype (Biswas, 2015; Kelly & O'Neill, 2015; Zhang et al, 2019).

Polarization also triggers transcriptional shifts in response to pro-inflammatory or anti-inflammatory stimuli, with M1 and M2 macrophages upregulating different genes. These transcriptional changes are vital for their pro-tumoral or anti-tumoral actions, and analyzing these transcriptional signatures allows for an in-depth characterization of macrophage states post-stimulation. Epigenetic studies have further illuminated that these states exhibit distinct chromatin configurations and histone modifications (Atri et al, 2018; Wang et al, 2014; Kerneur et al, 2022).

These diverse attributes endow macrophages with a vast array of functions. Combined with their plasticity, they are adept at modulating immune responses during microbial infections, tissue damage, and cancer. Their pivotal role in resolving inflammation is underscored by the understanding that aberrant macrophage activation can exacerbate pathophysiological processes.

Macrophages in Mesothelioma

Macrophages constitute a diverse array of immune cells known for their role in the phagocytosis and eradication of exogenous antigens. The inability of macrophages to successfully phagocytose asbestos fibers is posited as a contributing factor to the oncogenic transformation seen in mesothelial cells. When macrophages are incapable of fiber degradation, they emit oxidative species and pro-inflammatory cytokines, thus fostering an inflammatory milieu and activating signaling cascades within tumor cells that confer an adaptive advantage against asbestos-induced damage (Cersosimo et al, 2021).

A pivotal inflammatory mediator implicated in the transformation of mesothelial cells is the HMGB1. This cytokine is secreted by mesothelial cells in response to asbestos contact, which in turn mobilizes and stimulates macrophages. HMGB1 also plays a role in diminishing the phagocytic efficacy of macrophages and triggers the secretion of tumor necrosis factor-alpha (TNF-α), which shields mesothelial cells from apoptotic stimuli and perpetuates the inflammatory response (Yang *et al,* 2015b).

In the investigation conducted, as reported that Hmgb1ΔpMeso, whose mesothelial cells cannot produce HMGB1, shown a reduced inflammatory response to asbestos. Remarkably, the mesothelial cells of Hmgb1ΔpMeso exhibited a noteworthy decline in the expression and secretion levels of TNFα.

Furthermore, an analysis of the tissue microenvironment in regions with asbestos deposits revealed a noteworthy shift towards an augmented fraction of M1-polarized macrophages in contrast to M2 macrophages (Suarez et al, 2023).

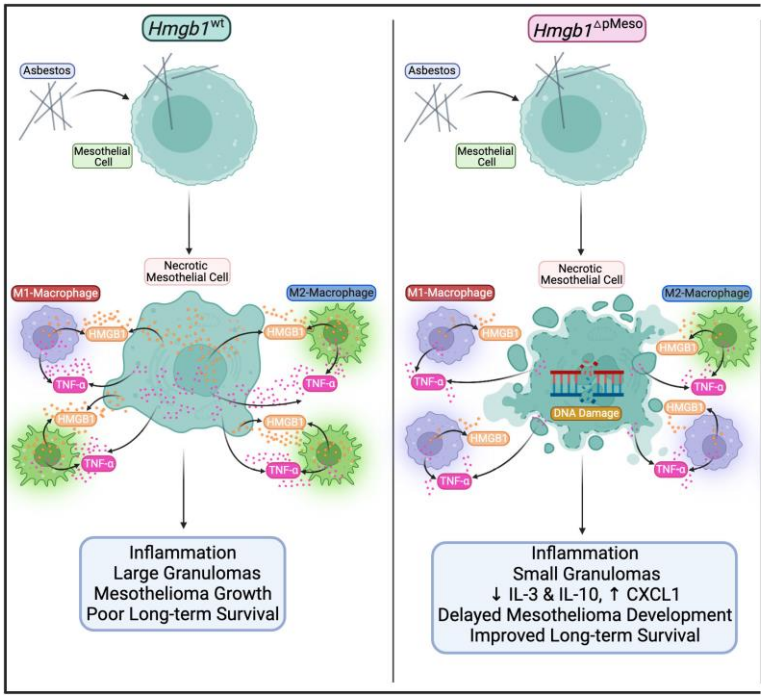


Figure 5. Schematic representing the major findings in the different HMGB1-cKO mouse models. Adapted from Suarez et al., 2023, PNAS.

Interaction of HMGB1 with specific receptors on macrophages leads to the activation of the NLRP3 inflammasome, resulting in the release of interleukins IL-1β, IL-18, IL-1α, and HMGB1 itself, thereby sustaining a persistent inflammatory feedback loop (Boyles *et al*, 2015). This inflammatory context is conducive to the proliferation of mesothelioma cells and the subsequent emergence of neoplastic progeny.

Furthermore, mesothelioma tissue has been documented to express elevated levels of the "don't eat me" signal CD47, which aids tumor cells in evading immune surveillance, including macrophage-mediated phagocytosis (Schürch *et al*, 2018). Macrophages are known for their phenotypic plasticity, adapting to environmental cues to assume various forms, including the classical or pro-inflammatory M1 and the alternative or anti-inflammatory M2 phenotypes.

In the tumor milieu, TAMs tend to adopt an M2-like phenotype, contributing to a tumor-promoting environment through the secretion of growth factors and enzymes that support angiogenesis, immune suppression, and metastatic spread. In the specific context of mesothelioma, TAMs predominate within the immune cell population, and their abundance correlates with a dire prognosis (Cornelissen *et al*, 2014; Chu *et al*, 2019).

Experimental models of mesothelioma have revealed an expansion of $CD206^+$ M2-like macrophages (Colin *et al*, 2018). The presence of these cells is often driven by the tumor's secretion of the chemoattractant protein CCL2, which lures monocytes to the neoplastic site. The CCL2/CCR2 axis is known to mediate interactions between TAMs and tumor cells, and its activation has been associated with cancer metastasis and progression (Chen *et al*, 2018).

Furthermore, there is evidence that mesothelioma cells can induce M2-like polarization, stimulate the release of pro-inflammatory cytokines, and encourage an immunosuppressive milieu. Pleural effusions from patients with mesothelioma have shown high levels of immunomodulatory cytokines, including TGF-β, IL-10, and M-CSF, and an increased infiltration of macrophages with an M2-like phenotype has been observed in both pleural and peritoneal mesothelioma (Chu *et al*, 2019).

These TAMs also engage in crosstalk with mesothelioma cells through the IL-1β/IL-1R signaling pathway, and activation of this route in tumor cells correlates with the development of a cancer stem cell-like phenotype (Kadariya *et al*, 2016). The presence of M2-like TAMs has been associated with heightened tumor cell proliferation and a reduced efficacy of chemotherapeutic agents.

Emerging research indicates that TAM-secreted molecules like IL-6, IL-10, and IL-34 contribute to the emergence of chemotherapy- and radiotherapy-resistant tumor phenotypes. In the pleural effusions of mesothelioma patients, the presence of the CSF-1R ligands, M-CSF, and IL-34 has been linked with shorter survival times, with only M-CSF correlating with M2-like marker expression, suggesting a distinctive role for IL-34 in the tumor microenvironment (Blondy *et al*, 2020).

Recently, chemoresistant phenotypes of CSF-1R+ mesothelioma cells have been supported by the co-expression of IL-34 and M-CSF, as evidenced in primary cultures

and cell lines. Inhibition of CSF-1R has been shown to potentially reinvigorate the CD8+ T cell anti-tumor response and improve the efficacy of PD-L1 inhibitors in mesothelioma treatment (Sluijter et al, 2014).

Furthermore, in vivo effectiveness of the monoclonal antibody anti-CSF-1R (RG7155) has been demonstrated by the reduced presence of CD68+/CD163+ TAMs in mesothelioma samples, underscoring the potential of targeting IL-34, M-CSF, and CSF-1R as a therapeutic approach against both mesothelioma cells and pro-tumor macrophages (Ries *et al*, 2014).

In their role as immunomodulators, TAMs also protect tumor cells from immune attack. Research has highlighted their immunosuppressive effects, particularly through mechanisms that impair CD4/CD8-T cell proliferation. The prostaglandin PGE2, found in high levels in the pleural effusions of patients, has been suggested to facilitate the promotion of a suppressive macrophage profile (Pace *et al*, 2007).

Reduction of mesothelioma growth and metastasis has been linked to targeting M2-like TAMs. Additionally, zoledronic acid has been observed to impede M2-macrophage differentiation and their accumulation in mesothelioma, offering further avenues for therapeutic intervention (Kaneko et al, 2018).

While it's clear that TAMs can proliferate within tumors and may serve as an important marker of malignancy, the mechanisms governing TAM self-renewal remain an active area of research and a promising therapeutic target. Despite the scarcity of molecular insights into TAM regulation in mesothelioma, and the limited development of targeted therapies, the central role of TAMs in resistance to treatment and disease progression highlights the potential of strategies aimed at modulating these cells, either in isolation or in combination with other therapeutic approaches, in cancer treatment.

In vitro Mesothelioma models

MM is characterized by an inherent resistance to conventional therapeutic modalities and the global incidence of the disease is on an upward trajectory. The endeavor to scientifically and clinically appraise this malignancy is hampered by a dearth of adequate cell lines and animal models that accurately mirror the condition, further exacerbated by the limited size of patient groups undergoing treatment at distinct institutions. It is pivotal to establish representative cell lines and

corresponding animal models, as these are instrumental in enhancing diagnostic and therapeutic techniques while also probing into the intrinsic biological underpinnings of MM (Shamseddin et al, 2021a).

Clinical trials targeting MM have largely returned unfavorable outcomes. Such results may be indicative of inherent phatways resistant to classical anti-cancer drug within MM tumors (Yap *et al*, 2017; Bronte *et al*, 2016). Moreover, the absence of biomarkers for gauging the efficacy of targeted therapies has hindered the segmentation of patient groups, which, in turn, might lead to the oversight of positive clinical responses within smaller subsets of patients.

The quest for novel therapeutic interventions for MM necessitates a more comprehensive and varied suite of preclinical models that faithfully reflect the genomic and histopathological heterogeneity of the patient populace and can accurately simulate drug responses. To this end, substantial efforts have been channeled into the development of two-dimensional (2D) cell lines derived from primary MM tumors and pleural effusions (Rintoul *et al*, 2016; Chernova *et al*, 2016). More intricate three-dimensional (3D) in vitro models, along with several murine in vivo models, have been introduced.

Clinical trials, often informed by data from rudimentary preclinical models, have failed to yield benefits for patients, indicating that current models may not adequately mimic human physiology or that there is a need for a wider array of models to capture the disease's diversity. High-caliber preclinical models are indispensable for pioneering new treatments against this deadly cancer. Each model—cell lines, spheroids, and animal models—has its distinct advantages and constraints, with no single model encapsulating all desirable characteristics. Consequently, the choice of model must be meticulously aligned with the specific objectives of a study.

Advancements in 3D cell model technology, specifically in the realm of organoids, have effectively tackled numerous inherent limitations present in preclinical models (Sato et al, 2011). Organoids, cultured in a 3D matrix that emulates the basement membrane, are capable of prolonged propagation in defined media, rendering them suitable for biobanking and thus a readily available resource. Protocols for organoid derivation from a multitude of epithelial tissues and cancers have been established, with significant success rates. These organoids not only mirror the genomic and

histopathological aspects of the original tumors but also the subclonal structures within them (Jeong *et al*, 2022). They are susceptible to various experimental approaches, such as drug sensitivity assays, and have demonstrated the capability to reflect actual patient responses in co-clinical trials and prospective clinical studies.

Moreover, techniques have been honed to allow organoids to be co-cultured with other cell types present in the tumor microenvironment, which heightens the complexity and fidelity of the organoid culture system (Tsai et al, 2018; Neal et al, 2018; Dijkstra et al, 2018).

Projects such as the Human Cancer Models Initiative strive to generate and document a variety of cancer organoids that capture the heterogeneity present in the patient population, thereby fostering the discovery of new therapeutic strategies across various cancer types (https://www.cancer.gov/ccg/research/functional-genomics/hcmi). Yet, the application of organoid technology to MM remains unrealized. The generation of MM-specific organoids stands as a beacon of hope for advancing our understanding and treatment of this formidable disease.

Two-Dimensional culture of Human Mesothelioma cells

The following section was written with reference to Shamseddin et al, 2021a.

Numerous cell lines derived from human MM have been successfully established from both tumor tissues and pleural effusions. These cell lines represent MM subtype histopathologically and genetically display genetic abnormalities found in mesothelioma tumors that include the inactivation of genes such as NF2, CDKN2A, and BAP1 (Kukuyan *et al*, 2019a). Despite this, a comprehensive comparison between these cell lines and their original tumors, particularly regarding their genomic and histopathological characteristics, remains largely unexplored. To date, only one study has undertaken such a comparison, revealing a high degree of concordance in genomic alterations between the tumor and its corresponding early passage cell lines. Notably, certain single nucleotide variants (SNVs) were exclusively identified in the cell lines, which may either signify the expansion of a rare tumor clone or the acquisition of new SNVs during in vitro culture (Kim et al, 2017).

In a significant endeavor to enhance the diagnostic capabilities of mesothelioma, researchers undertook a comprehensive genome-wide analysis. This analysis employed a substantial panel consisting of 61 MM cell lines derived from pleural

effusions (Gueugnon *et al*, 2011). The findings of this study underscore the predominant expression of specific genes, namely COL3A1, SLPI, ITLN1, and CCL2, in MM cells as opposed to lung adenocarcinomas (Gueugnon *et al*, 2011). Of particular note is the perpetual passaging capability of most malignant cell lines, resulting in genomic instability marked by occurrences such as polyploidy, copy number alterations or formation of novel aberrations in long-term cultured MM models (Relan *et al*, 2013). Additionally, the phenomenon of kataegis, characterized by the clustering of numerous single nucleotide substitutions in a specific locus, was identified in MM cell cultures but not in primary tumor tissues (Ströbel *et al*, 2014).

Recent investigations have revealed a significant distinction in the transcriptome of newly derived primary mesothelioma cells compared to established MM cell lines (Oey *et al*, 2019). Moreover, it was observed that the drug response of primary MM cells could be influenced by prolonged culturing in 2D conditions and undergo changes that result in the loss of several characteristics inherent to the original tumor (Szulkin *et al*, 2014a). This alteration in cellular properties raises questions about the fidelity of long-term cultured MM cell lines in accurately representing the intricacies of mesothelioma for research purposes.
Moreover, cell lines only partially represent the subclonal diversity of primary tumors. Instances have been reported where different subclones dominate in MM tumors and the derived cell lines (Schulten et al, 2007). Indeed as reported two distinct MM cell cultures derived from the same patient exhibit diverse gene expressions and chromosomal aberrations (Usami *et al*, 2006). Thus, cell lines may not fully capture the complete heterogeneity present in patient tumors.

In the initial stages of exploration transcriptomic analyses, aid in identifying potential diagnostic markers and treatment approaches. A notable study involving primary MM cultures, established in the 1990s, identified a variety of molecular subgroups derived from patient with different diagnosis. These cells shown a different mutation profiles and deregulated of EMT pathways and transforming growth factor-β signaling. Moreover, some lines presented alteration in expression of genes like PPL, UPK3B, and TFP1 (De Reynies *et al*, 2014).

The use of MM cell lines has played a crucial role in drug sensitivity assays, revealing variations in individual responses to drugs and underlining the importance of personalised medicine (Szulkin et al, 2014b).

The integration of drug sensitivity assays with gene expression data has identified a key role for the FGF signalling pathway (Cerruti *et al*, 2017).

Indeed, MM cells treated with FGF-targeted drugs exhibit heightened sensitivity following FGFR inhibition and the death receptor agonist TRAIL, which in turn is significantly linked to the loss of BAP1 (Kalyan Kolluri *et al*). Encouraging responses were observed in a phase Ib clinical trial, where an FGF ligand trap was combined with pemetrexed/cisplatin chemotherapy. However, thorough validation is imperative before considering BAP1 loss as a definitive biomarker for responsiveness to FGF/FGFR inhibitors or TRAIL (Quispel-Janssen *et al*, 2018; van Brummelen *et al*, 2020).

In summary, patient-derived mesothelioma cell lines provide a simplified model for studying MM biology and therapeutic sensitivity. However, their utility in guiding personalized medicine is limited by several factors: the tendency of MM cells in phenotypic changes after different passaging in 2D condition; their inability to fully replicate tumor complexity; and the absence of tumor microenvironment with immune and stromal cells. This is one of the primary reasons why we are transitioning from two-dimensional technology to three-dimensional, a shift that will be elucidated in the following paragraphs.

Three-Dimensional culture

Over the past decade, there has been a burgeoning interest in the utilization of three-dimensional (3D) cell cultures to investigate physiological and pathological processes in vitro. Unlike conventional monolayer cell cultures, 3D cultures offer a more nuanced representation of in vivo biological processes due to their ability to reproduce three-dimensional structures (Lee *et al*, 2007). This spatial configuration, absent in 2D monolayer cell cultures, can better functional knowledge of the complex intra- and intercellular signaling circuits underlying communication between the different cell types populating a tumor tissue (Thoma et al, 2014a).

As elucidated by Kimlin et al., the adoption of 3D growth for immortalized established cell lines or primary cell cultures is considered a more stringent and representative model (Kimlin *et al*, 2013). The distinctive features of 3D cell cultures, such as cell-cell interactions, hypoxia, drug penetration, response and resistance, and the

production/deposition of extracellular matrix, emulate several in vivo aspects of tumors (Wartenberg et al, 2003; Baker & Chen, 2012).

The study of cancer cell dynamics in a 3D context not only allows for the recapitulation of the architecture of living tissue but also facilitates a more profound investigation into the pathobiology of human cancers (Surina *et al*, 2023; Minchinton & Tannock, 2006). Moreover, in vitro 3D cultures can bridge the gap between in vitro and in vivo models (Yamada & Cukierman, 2007); facilitating and accelerating the in drug screening as an intermediary step before engaging in animal protocols (Friedrich et al, 2009; Jaganathan et al, 2014).

Most recent types of 3D culture models have been developed liquid-based and scaffold-based technology that used synthetic or naturally-derived polymers to mimic extracellular matrix condition and providing a supportive environment for cell growth (Thoma *et al*, 2014b).

Spheroids

Tumor spheroids have emerged as a predominant and adaptable scaffold-free approach for 3D cell culture. Whether originating through self-assembly or induced growth from individual cell suspensions, these spheroids faithfully mimic the intricate environment of tissues and organs. They effectively capture the interplay among cells by fostering the development of desmosomes and dermal junctions. The ability to obtain spheroids of varying dimensions, particularly large spheroids with distinct proliferating, quiescent, and necrotic zones, adds to their utility in mimicking the cellular heterogeneity of solid in vivo tumors (Cristaldi et al, 2020).

Compared to traditional 2D cultures, cancer spheroids offer several advantages, including enhanced cell-cell and cell-extracellular matrix interactions, increased resistance to chemotherapeutic agents, and the ability to model gradients of oxygen, nutrients, and signaling molecules present in solid tumors. These features better recapitulate the in vivo tumor microenvironment, thereby improving the predictive power of preclinical models and reducing the translational gap between bench and bedside (Harane et al. 2023).

Functionally, spheroids serve a myriad of purposes, with applications extending across various research domains. In cancer research, their structural fidelity allows

for a more accurate representation of the dynamics observed in solid tumors (Nayak *et al*, 2023).

Cancer spheroids find diverse applications in cancer research and drug development. Spheroids are recognized for their deposition of extracellular matrix components, mimicking the compaction of solid tumors (Costa et al, 2016). This feature proves invaluable for distribution studies of anticancer compounds, evaluating their penetration into the tumor mass (Shi et al, 2022; Gendre et al, 2021). One key application is in elucidating the mechanisms underlying drug resistance and sensitivity. By exposing spheroids to chemotherapeutic agents, researchers can assess drug penetration, metabolism, and efficacy within a more physiologically relevant context. Additionally, cancer spheroids facilitate the screening of novel anticancer agents and targeted therapies, enabling the identification of compounds with improved efficacy and reduced toxicity profiles (Harane et al. 2023).

Furthermore, spheroids play a crucial role in studying the interactions between tumor cells and immune cells. Co-cultures of tumor spheroids with immune cells offer a realistic representation of the complex interactions within the TME. These co-cultures encompass diverse interactions, ranging from spheroid cell killing to crosstalk between different cell types, providing insights into their phenotypic effects (Singh & Gautam, 2023; Sargenti et al, 2020).

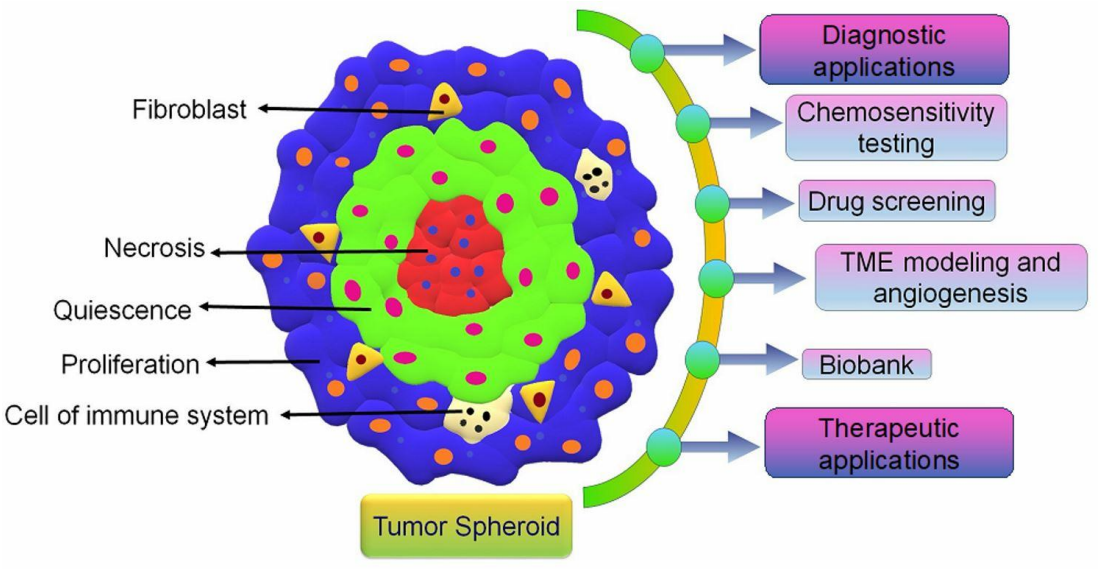


Figure 6. Illustration of co-cultured spheroid structure and their application (Nayak et al, 2023a).

Recent interest has gravitated towards microfluidics-based approaches for spheroid culture. This innovative method enables the in vitro study of dynamics akin to those found in solid tumors when nourished by vasculature. Microfluidics-based approaches offer a platform for investigating immune chemotaxis and provide a more faithful representation of in vivo tumor cell metabolism (Sontheimer-Phelps et al, 2019).

In mesothelioma, utilizing both commercially available cell lines and primary patient-derived cells, scientists have successfully generated MM and lung tumor spheroids, providing a platform for testing the efficacy of various treatments.

These spheroids, characterized by their 3D structure, closely resemble the architecture and behavior of tumors in vivo. Unlike traditional 2D cell cultures where cells grow uniformly on flat surfaces, spheroids exhibit distinct regions akin to the in vivo tumor environment. Cells at the periphery of the spheroids represent actively proliferating tumor cells, while those in the center tend to become quiescent or undergo cell death through apoptosis or necrosis (Gendre et al. 2021).

One of the key observations made during the validation of these spheroid models is the difference in cytotoxic drug response between cells cultured in 2D and those in 3D. In particular, researchers noted that mesothelioma cells grown in spheroids displayed enhanced resistance to cytotoxic drugs compared to their counterparts cultured in traditional monolayer cultures (Huaikai et al.2022).

In addition, co-culture of MM spheroids with immunity component was performed, for example reduced CXCL8 transcripts, and IL-8 release reduced recruitment of neutrophils in deficient-CDKN2A mesothelioma (Boumya et al. 2023).

In summary, spheroid culture systems offer a fast, versatile and comprehensive framework for a wide range of studies applicable to various experimental fields. Their ability to closely replicate in vivo healthy and diseased tissues opens avenues for diverse analyses, marking them as an invaluable tool in contemporary research endeavors.

Organoids

Today, we stand at the forefront with a groundbreaking project – the evolution of 3D-culture with cells derived from patient tumor. This union promises to redefine the way we study and understand the intricacies of healthy and tumoral organ development and function.

Organoids represent a groundbreaking advancement in pre-clinical cancer models, addressing the challenges faced in drug development, particularly in the field of oncology. The escalating costs of novel anti-cancer drugs, attributed to the heightened complexity of clinical trials and regulatory prerequisites, underscore the pressing need for innovative approaches. Notably, the success rate of anti-cancer drugs traversing from phase 1 clinical testing to market approval remains notably lower compared to drugs targeting other diseases (Michael Hay et al, 2014).

A pivotal hindrance in the drug development pipeline is the limited translatability of pre-clinical cancer models to actual patient scenarios. This bottleneck not only impedes overall drug development but also hinders the progress of companion diagnostics aimed at identifying patient subgroups suitable for treatment with molecularly targeted agents. Addressing this challenge, 3D tumor organoid cultures emerge as a novel pre-clinical model system in oncology, allowing for the ex vivo propagation of tumors from individual patients (Michael Hay et al, 2014). This innovative approach holds promise in streamlining the drug discovery process.

The genesis of organoid cultures stems from the discovery that healthy mouse intestinal stem cells could be successfully propagated in vitro using a specific combination of factors (Weeber et al, 2017). This discovery paved the way for the long-term culture of primary colorectal cancer cells and the development of similar protocols for various tissues, including the pancreas, stomach, prostate, and liver (Nadauld *et al*, 2014; Drost *et al*, 2015).

The ability to establish organoid cultures from diverse tumor types marks a significant breakthrough, offering potential avenues for more successful drug development and the realization of precision medicine. Well-characterized patient-derived organoids have been cultivated, providing a valuable resource for studying cancer biology and drug responses. This transformative approach has already contributed to enhancing our fundamental understanding of cancer and holds promise for its application in improving drug development and clinical practices.

In the realm of in vitro cell culture models, 2D models have long prevailed but are now recognized for their limitations. The lack of native tissue-specific characteristics in conventional cell cultures, attributed to the absence of the surrounding extracellular matrix (ECM), specific biochemical cues, and environmental stimuli, has

led to an imperative shift toward advanced in vitro cell culture platforms (Clevers, 2016).

This paradigm shift has given rise to 3D cellular models, with organoid cultures gaining prominence due to their ability to faithfully replicate the physiological features of native organs or tissues (Gómez-Álvarez et al, 2023). The term "organoid" has been widely employed, characterized as structures resembling organs and manifesting the inherent traits of their original tissue but grow under specific conditions in vitro. While consensus on the definition varies, organoids are generally understood as 3D cellular structures derived from a heterogeneous population of cells that compose the patient-organ biopsies embedded in an extracellular scaffold (Rossi et al, 2018). This microenvironment allows cells to self-organize into structures that closely mimic the native tissue's structure, function, and characteristics, presenting notable benefits compared to conventional 2D cell cultures. These advantages encompass the retention of cell–cell and cell–extracellular matrix (ECM) interactions, the sustenance of genotypic and phenotypic features, the portrayal of cellular heterogeneity, and a markedly prolonged lifespan (Mu et al, 2023).

Organoids can be further classified based on morphology and cellular material, presenting a versatile platform for modeling various health and disease conditions (Zhao *et al*, 2022). About epithelial tumor organoids, it becomes evident that the unchecked proliferation of normal cells has a consequential impact on the trajectory of tumor cell growth. This phenomenon introduces a potential confounding variable in experimental outcomes due to the limited purity of tumor samples. Therefore, the meticulous identification of organoid morphology emerges as a pivotal aspect in this context (Yang & Yu, 2023).

Normal organoids originating from epithelial tissue exhibit a characteristic single-layered epithelium and a cyst-like structure. It is noteworthy, however, that the addition of supplementary cytokines can influence the morphology of these normal organoids. Conversely, tumor organoids derived from epithelial cancers faithfully mirror the structural intricacies of their respective original tumor tissues. These tumor organoids manifest diverse architectural patterns, encompassing glandular formations, solid structures, and poorly cohesive arrangements, as visually represented in figure 7 (Yang & Yu, 2023).

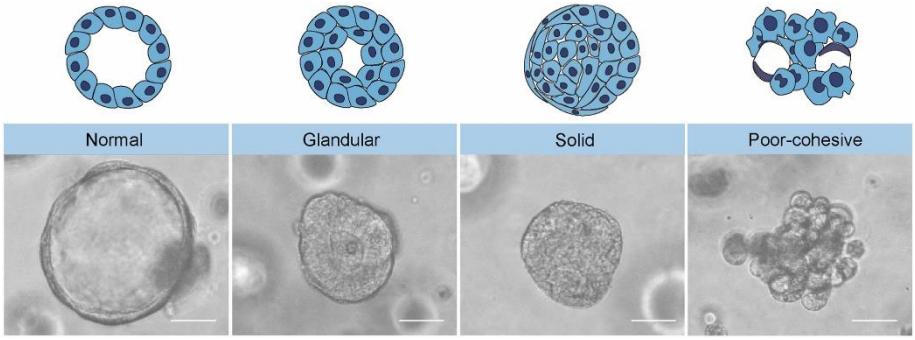


Figure 7. Representative images of different structures of organoids (Yang & Yu, 2023).

Furthermore, well- or moderately differentiated adenocarcinoma typically presents with discernible glandular structures. In contrast, poorly differentiated adenocarcinoma tends to adopt a growth pattern characterized by solid clusters. The undifferentiated adenocarcinoma, on the other hand, exhibits a distinctive growth pattern, manifesting either as isolated cells or poorly cohesive clusters that adopt a grape-like configuration. Notably, in this state, there is a discernible loss of apical-basal polarity, accentuating the complexity of the adenocarcinoma's developmental trajectory (Yang & Yu, 2023).

The success of organoid cultures relies on essential components, including extracellular scaffolds, cellular material from different sources, and a carefully tailored culture medium. Hydrogels, particularly those of natural or synthetic origin, serve as crucial extracellular scaffolds, providing mechanical and biochemical cues for organoid growth. The culture medium, customized to meet unique tissue-specific demands, plays a pivotal role in organoid establishment, development, maintenance, functionality, and responses (Urbischek *et al*, 2019).

The robustness of organoid cultures is underscored by their extended lifespan, facilitating the possibility of biobanking for future research endeavors.

General application of 3D structures

3D culture strategies have ushered in a new era of advancements in tissue biobanking, disease modeling, drug discovery, and personalized medicine. The integration of organoids into these fields has yielded significant progress, as outlined in the following sections.

Biobanking

The concept of biobanking, characterized as the systematic gathering of human biological specimens for research porpose, addresses the requirement for enduring and replicable reservoirs of human tissue (Ren et al, 2022). Existing challenges, such as limited availability and inter-individual variability of conventional samples, are mitigated by the utilization of organoids (Lancaster & Huch, 2019). Organoids, distinguished by their inherent self-renewal capabilities and adeptness in preserving tissue-specific characteristics, offer an ideal solution for creating biobanks (Clevers, 2016b). Initiatives like the Human Cancer Models Initiative (HCMI) exemplify global efforts to establish comprehensive biobanks, providing standardized samples and fostering collaborative research (https://ocg.cancer.gov/programs/hcmi).

Disease Modeling

3D culture serve as a powerful platform for modeling various pathological conditions, bridging the gap between in vitro and in vivo models. This approach is particularly valuable for studying infectious diseases, genetic diseases, and cancer.

Differentiated organoids from various tissues facilitate the investigation of infectious agents, offering insights into host–pathogen interactions. Respiratory and intestinal organoids, for example, have been employed to model infections caused by viruses, aiding in the understanding of viral replication and potential antiviral targets (Finkbeiner et al, 2012; Ettayebi et al, 2016).

The advent of gene-editing technologies like CRISPR/Cas9 enables the modeling of genetic diseases in organoid cultures. Specific mutations, such as those associated with cystic fibrosis, can be introduced, allowing for the study of disease pathology and testing potential therapeutic approaches (Dekkers *et al*, 2013). Organoids derived directly from patient tumor samples preserve genetic and phenotypic heterogeneity, enabling the study of specific tumor subpopulations (Vlachogiannis et al,2019).

Solid tumors consist not only of cancer cells but also various stromal components like fibroblasts, immune cells, and endothelial cells. Despite not being malignant, these cells play crucial roles in tumor progression, angiogenesis, and drug resistance through intricate interactions with cancer cells. These interactions involve activation of pathways related to DNA repair, inflammation, and invasion. 3D tumor spheroids, particularly heterotypic ones comprising both cancer and stromal cells, serve as valuable models to study tumor complexity and drug resistance mechanisms. By co-culturing different cell types, researchers mimic the cellular diversity of tumors and the resistance conferred by tumor-stromal interactions. These spheroids facilitate the

recreation of in vivo tumor signaling networks through direct cell-cell interactions and the release of signaling molecules. Fibroblasts, being abundant in the TME, are pivotal in tumor development, metastasis, and therapy response. Consequently, heterotypic spheroids combining tumor cells with CAFs are extensively used in drug discovery efforts. Varying stromal to cancer cell ratios in spheroids helps replicate the tissue composition found in actual tumors (Zanoni et al, 2020).

Drug Discovery and Personalized Medicine

Human 3D models present advanced alternatives to traditional 2D cell cultures for drug discovery and toxicity screening. Physiologically accurate responses to treatments, expedited patient-specific drug testing, and reduced reliance on animal experimentation characterize the efficiency and ethical considerations of drug discovery in 3D cultures (Harane et al. 2023, Xu et al, 2018).

The transition towards personalized medicine is enabled by the translational integration of 3D models into clinical applications. Patient-derived 3D models play a pivotal role in anticipating personalized responses to pharmaceutical interventions, thereby enabling the implementation of precise and targeted therapeutic approaches (Harane et al. 2023).

Organoids, by mimicking tumor heterogeneity serve as suitable experimental models for drug sensitivity screening in traditional treatments. However, their limitations become evident when attempting to capture the intricate crosstalk between cancer cells and various components of the TME, including fibroblasts, endothelial cells, immune cells, and microorganisms (Yang & Yu, 2023). It is crucial to recognize that stromal elements play an active role in cancer biology by releasing chemokines, growth factors, enzymes, and extracellular vesicles (Wu et al, 2021). These components contribute significantly to the overall dynamics of the TME.

Cancer spheroids have profound implications for personalized medicine and precision oncology. By integrating patient-derived cells into spheroid models, researchers can predict individual responses to therapy and identify personalized treatment strategies. Moreover, cancer spheroids serve as platforms for evaluating the efficacy of emerging therapeutic modalities, such as immunotherapy and nanoparticle-based drug delivery systems, in a more clinically relevant setting (Shamir et al. 2014, Edmondson et al. 2014, Bialkowska et al. 2020).

In order to address this limitation and enhance the representativeness of experimental models, researchers are exploring the co-culture of 3D co-culture with lymphocytes. This approach provides a valuable platform for assessing the effectiveness of immunotherapy. By incorporating immune cells into the 3D model, scientists aim to better simulate the complex interactions that occur within the tumor microenvironment, shedding light on the potential efficacy of immunotherapeutic interventions. Incorporating immune cells into organoid systems provides insights into the impact of immune cells on tumor organoids, promoting the discovery of novel immunotherapeutic targets and personalized treatments.

In conclusion, 3D models have revolutionized tissue engineering, offering faithful reproduction of biological characteristics. From biobanking to disease modeling, drug discovery, and personalized medicine, organoids have become indispensable tools in advancing our understanding of various pathological conditions and developing effective, personalized treatments for patients.

Organoid and spheroid culture models fulfil distinct and complementary purposes, exhibiting disparities in tumor cell origins, methodologies of cultivation, and the duration required for formation.

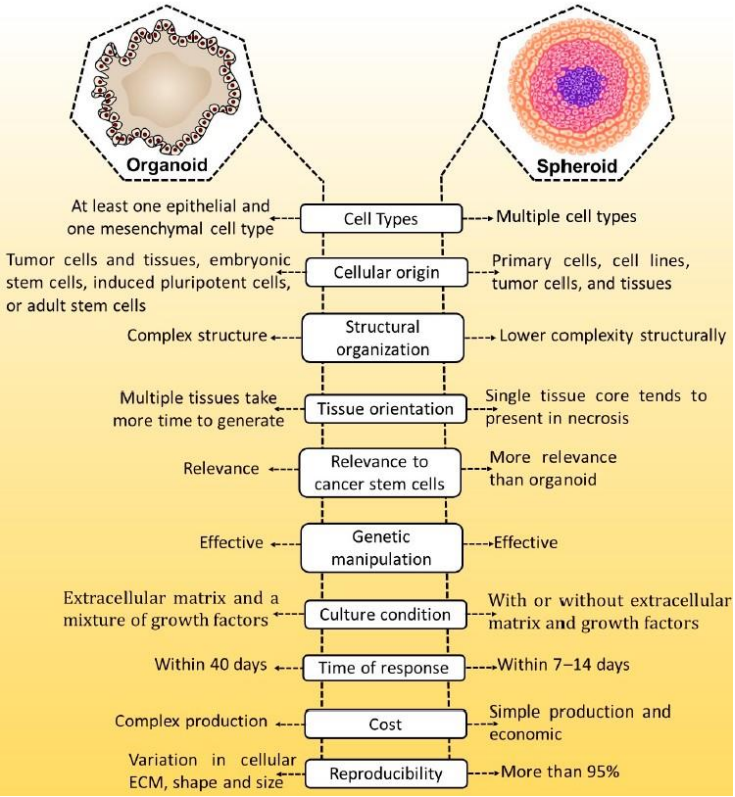


Figure 8. Schematic comparison of organoid with spheroids models (Nayak et al, 2023b).

Mesothelioma Mouse Models

The following section was written with reference to Shamseddin et al, 2021a.

Genetically Engineered Mouse

Genetically modified mouse models have been instrumental in mesothelioma research, created via Adeno-Cre virus injections to delete Nf2, p53, and Ink4a/Arf mesothelial genes. These conditional knockout (CKO) mice exhibit diverse phenotypes based on gene combinations. Notably, Nf2;Ink4a/Arf CKO mice showed heightened pleural MM compared to Nf2;p53 CKO mice. Triple knockout mice (Nf2;p53;Ink4a) displayed highly malignant forms with reduced median survival (~11 weeks), contrasting with other CKO mice (~19-31 weeks), highlighting Ink4a's aggressive nature (Jongsma et al, 2008).

Murine MM immunohistochemical analysis revealed epithelioid phenotypes in Nf2;p53 and Nf2;Ink4a/Arf mice but not in Nf2;p53;Ink4a mice, primarily showing sarcomatoid phenotypes, differing from human MM (Jongsma et al, 2008). Asbestos-exposed CKO mice, notably Nf2;p53;Ink4a/Arf, better replicated the human epithelioid subtype, indicating species-specific differences or distinct oncogenic mechanisms (Farahmand et al, 2023).

Asbestos-Induced Murine Models and Alternative Agents

Asbestos exposure, a well-known causative factor for MM in humans, has also been used to induce MM in murine models. Most of these models involve intraperitoneal injections of asbestos, leading to the development of peritoneal mesothelioma. Intriguingly, Bap1, Nf2, Ink4a/Arf, and Tp53 inactivation in these models resulted in a heightened incidence and accelerated progression of mesothelioma compared to wild-type mice (Shamseddin et al, 2021b).

Another significant development is the use of carbon nanotubes (CNTs), which mimic some properties of asbestos, in mesothelioma research. The introduction of multiwalled CNTs into rats and long-fiber CNTs into mice's pleural cavities has led to the development of pleural mesothelioma in a significant percentage of these animals. This model is valuable for studying the molecular events during the latency period, though its long latency makes it less suitable for drug testing (Suzui et al, 2016; Chernova et al, 2017).

Graft Models in Mesothelioma Research

Graft models, particularly xenografts involving human MM cell lines injected into mice, have been widely used to study mesothelioma (Kalra *et al,* 2015). However, these cell lines often lack the intratumour heterogeneity characteristic of human MM. To address this, some studies have generated xenografts directly from human MM tissues or pleural fluid, maintaining more of the original tumour's characteristics. Despite this progress, the models predominantly represent more aggressive forms of MM and are less effective for studying epithelioid subtypes. Furthermore, these models' utility in immunotherapy testing is limited due to the lack of an immune system in the host mice (Shamseddin *et al*, 2021b).

Syngeneic murine models, on the other hand, involve implanting murine mesothelioma cells into immunocompetent hosts (Mezzapelle *et al,* 2016). While these models better represent the immune interactions in mesothelioma, they too are limited by the lack of intratumour and interpatient heterogeneity and the adaptation of cell lines to in vitro conditions.

In summary, genetically engineered mouse models have provided significant insights into the pathogenesis and potential treatment strategies for MM. However, limitations such as species-specific responses, the predominance of non-epithelioid subtypes in some models, and the lack of heterogeneity observed in human MM underscore the complexity and challenges in accurately replicating human mesothelioma in animal models.

Predict-Meso Project

PREDICT-Meso represents an international consortium of researchers spanning the United Kingdom and various global regions, dedicated to investigating mesothelioma—a relentless, incurable cancer primarily affecting the pulmonary lining and attributed to asbestos exposure. Characterized by decades of pleural inflammation preceding its manifestation, mesothelioma presents a unique temporal window for precision prediction and early intervention, a focal point the network aims to explore comprehensively. The collaborative efforts of these research teams aspire to enhance the comprehension of mesothelioma's progression from its nascent stages, subsequently translating this knowledge into more efficacious diagnostic and therapeutic modalities for afflicted individuals.

Secured with a funding allocation of £5 million over a duration of 6 years, PREDICT-Meso is a beneficiary of the Accelerator Awards. These awards, established through a collaborative initiative involving Cancer Research UK (CRUK), Fondazione AIRC (AIRC), and Fundación Científica de la Asociacion Española Contra el Cáncer (FC AECC), seek to amplify their impact by fostering shared priorities. The financing facilitated by the Accelerator Awards underscores a commitment to advancing the understanding and management of mesothelioma through strategic research initiatives.

The PREDICT-Meso project is structured around five meticulously designed work-packages, each spearheaded by accomplished mesothelioma researchers from across Europe. These work-packages are strategically tailored to acquire and leverage human tissues and data, with the overarching goal of innovating novel therapies and early detection tests specific to mesothelioma. The collaborative and multidisciplinary approach championed by PREDICT-Meso embodies the essence of team science, fostering synergy among experts to unlock groundbreaking insights into unresolved questions surrounding cancer.

In the pursuit of advancing pre-clinical platforms for secondary drug screening and subsequent target-drug validation, my PhD project is a part of Working Package 3 and 4. I will generate and validate new *in vitro* PRE-CLINICAL MODELS (cell lines and organoids) in collaboration with MacFarlane and Psallidas from Cambridge University. These organoids will encompass mesothelial cells, immune cells, and fibroblasts, constituting an enhanced model for drug screening.

Aim of the work

In recent years, there has been a growing interest in the utilization of 3D cell cultures for the examination of physiological and pathological phenomena in vitro.

While 2D cultures and murine models serve their purpose, 3D structures accurately replicate the intricate biology and complex architecture observed in primary cancers. The ongoing advancements and refinement in 3D culture technology not only facilitate the generation of diverse microenvironments but also enable the modulation of the tumor microenvironment (TME). This, in turn, opens up new avenues for investigating tumor immunology, thereby charting a novel course in this realm of research (Chu et al, 2019).

Cancer organoid culture systems have successfully been established for various cancer types, including colorectal, gastrointestinal, pancreatic, prostate, liver, and brain cancers, also for mesothelioma. Notably, these cancer organoids faithfully retain both histological and mutational characteristics akin to their corresponding tumors, rendering them instrumental for drug screening purposes (Li et al, 2020; Artegiani & Clevers, 2018; Chéné et al, 2016; Fang et al, 2024).

In addition, tumor spheroids present a viable alternative to high-throughput assays, serving as a complementary tool to murine models. They proficiently emulate the drug response observed in primary human tumors, proving valuable in the comprehensive exploration of tumor physiology. This spans investigations into metabolic and chemical gradients, as well as intricate cell-cell and cell-matrix interactions.
To date, 3D technologies are already applied to mesothelioma and attempts are being made to reproduce this type of tumor in vitro.
The Aim of this thesis is to generate and validate new Malignant Mesothelioma in vitro pre-clinical 3D models using biopsies from patients.

In this project I propose the: 1) Establishment and characterization of Malignant Mesothelioma 3D-culture model organoids and spheroids derived from murine and human MM cell line; 2) Replacement of TAM component with co-culture and characterization Mesothelioma cells and macrophages crosstalk; 3) Test BoxA as new therapeutic molecules on this 3D mesothelioma platform.

Results

Murine mesothelioma cell lines and generation of a 3D system

Generation of MM monoculture spheroids and co-culture with Bone Marrow-Derived Macrophages

Accumulation of pleural fluid is a common observation in diffuse Malignant Mesothelioma. Cytological analysis often detects free spheroid aggregates of malignant cells in the pleural fluid, raising questions about the resistance of non-adherent tumor cells to anchorage-induced apoptosis (anoikis). This resistance may contribute to the development of new tumor foci in the pleural cavity, strongly linked to malignancy (Daubriac et al, 2009).

MM progresses locally by invading the pleural space and interlobular fissures, forming plaques and nodules on the visceral and parietal pleura. The presence of free MM cell aggregates in pleural fluid suggests their ability to survive in this microenvironment, highlighting the intricate interplay between tumor cells and their surroundings (Whitaker, 2000).

To investigate the mesothelioma microenvironments in vitro, we developed 3D tumor spheroids. We used the AB1 mesothelioma cell line, which was originally isolated from female BALB/c mice injected i.p. with asbestos fibers and has similar phenotypes to cells of human mesothelioma; it was also used to grow MM tumors in mice (Mezzapelle *et al*, 2016).

Monoculture 3D MM spheroids (mesospheres) of AB1 cells that stable express GFP were successfully formed using a hanging drop or ultra-low attachment plate technique from 1,000 cells according to techniques in several studies (Gendre et al, 2021; Yakavets et al, 2020). After 48 hours of incubation, we found the formation of a round spheroid with smooth edges that appeared to be uniform with a diameter of approximately 128 µm (FIGURE 9A). We seeded 1 spheroid per well into 24-well low adhesion plates. Spheroids were then cultured at 37°C (5% CO2) and their morphology and size were monitored every day for 9-10 days by brightfield and fluorescence microscopy. The area of the spheroids increased over time indicative of a proliferation phase from day 3 to day 9. The kinetics of spheroid growth is shown in figure 9B.

Fig 9

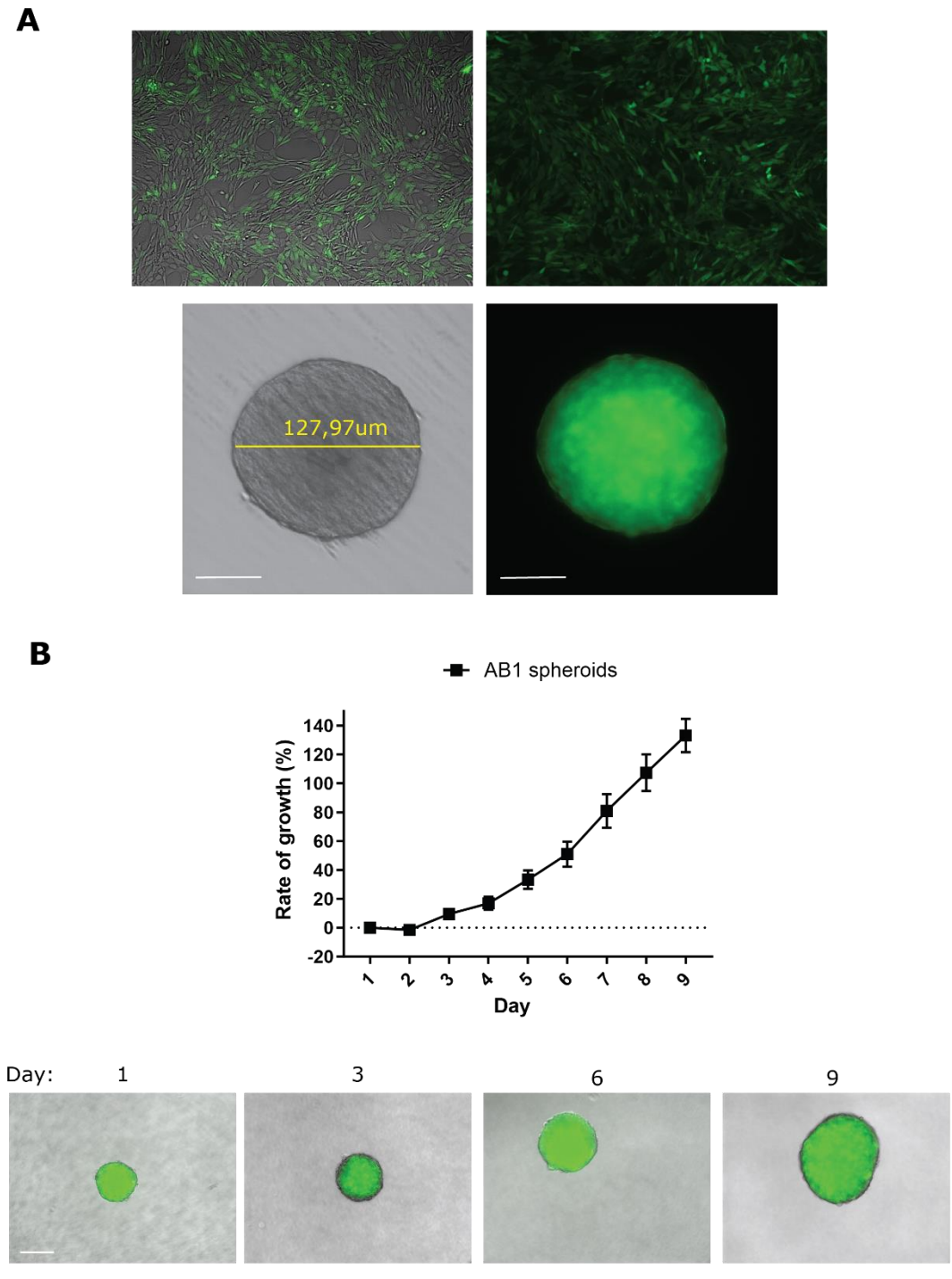


Figure 9. (A) (Upper part) Brightfield images of the AB1-GFP cell line. (Bottom part) Brightfield and fluorescent images of AB1-GFP spheroids. Scale bar= 100 µm (B) Growth curve of MM spheres. The graph illustrates the temporal increase in the growth rate of mesospheres, as evident from the data acquisition. Images were acquired by Axio microscope and area was calculated using ImageJ software.

Bone Marrow-Derived Macrophages Infiltration

Macrophages (Mϕs) play a pivotal role in the initiation of MM after exposure to asbestos fibers (Lievense et al, 2013). Furthermore, myeloid cells constitute a substantial portion of the immune infiltrate observed in MM patients' biopsies, actively contributing to the progression of MM by fostering tumor growth (Lievense et al, 2016).

To investigate the repercussions of the interplay between Mϕs and MM cells, we established a three-dimensional co-culture system wherein mesospheres were co-cultivated with bone marrow-derived macrophages (BMDMs).

Macrophages were stained with Celltracker© Alexa-647 dye to determine their distribution in the co-culture spheroids. Optical imaging 24h post-seeding of BMDMs revealed the formation of reproducible 3D heterotypic spheroids with uniformly distributed BMDMs, as demonstrated by fluorescence microscopy of whole spheroids. In particular, BMDMs invaded the periphery and infiltrated from the outer layer to the center of the mesosphere as shown in z-stack images (FIGURE 10A). The mesosphere co-culture with macrophages was analysed by IHC by staining with F4/80, a general macrophage marker (FIGURE 10B). The staining revealed a uniform tumor cell distribution with a compact core of extracellular matrix and infiltrating BMDMs.

Fig 10

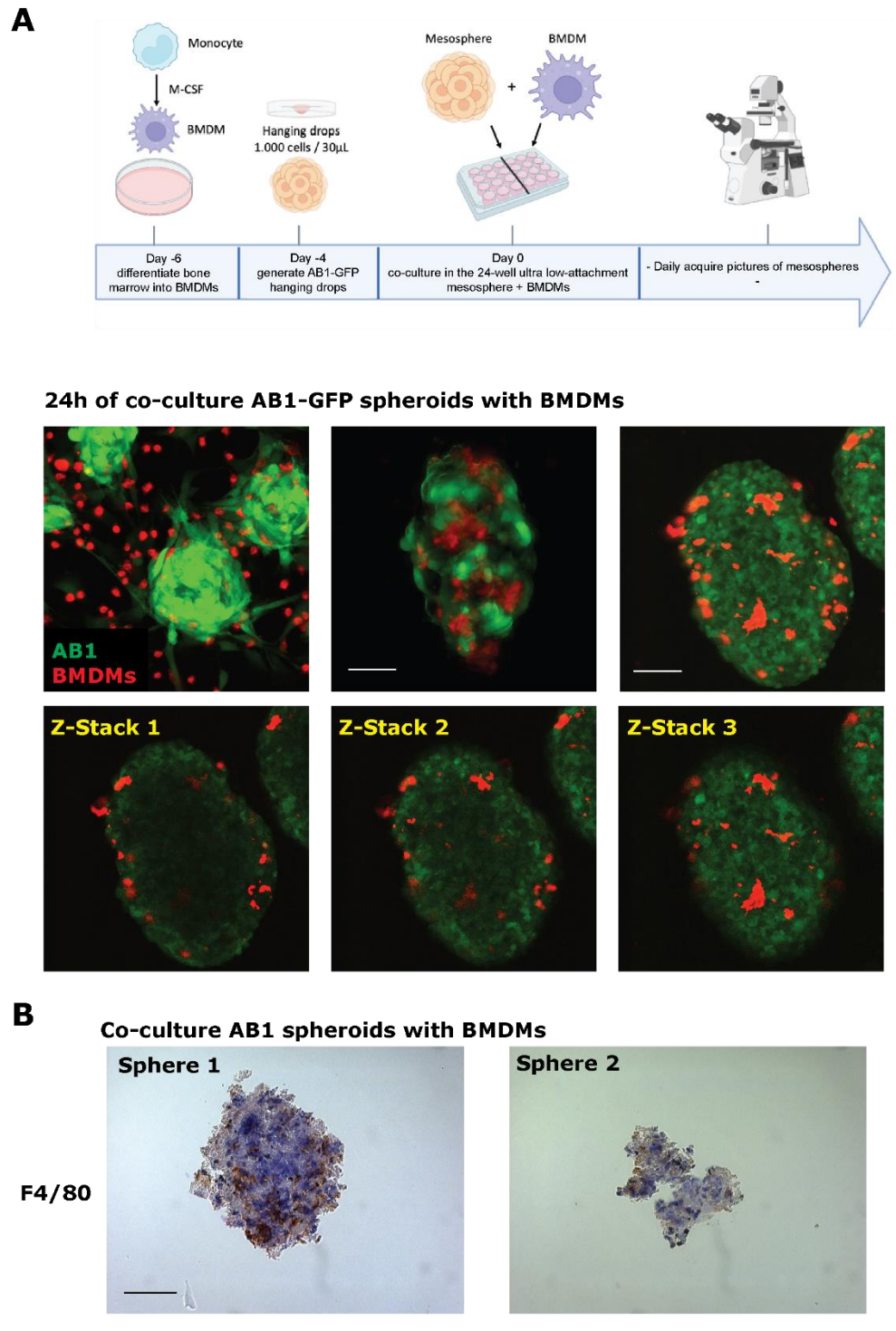


Figure 10. (A) BMDMs able to infiltrate inside the mesosphere. Representative confocal images of a mesosphere (green, AB1-GFP cells) with infiltrating BMDMs (red, macrophages stained with CellTracker© Alexa-647). First line of images represents spheroids from three different experiments, second line represents z-stack of one sphere. Scale bar 50µm (B) IHC

of mesospheres with infiltrated macrophages marked with F4/80. Images represents two replicates from one experiment. Scale bar 50μm

To complete the analysis of spheroids composition, flow cytometry showed that after 24h of co-culture, irrespective of macrophage phenotype, spheroids were composed mainly of cancer cells together with ~34% of F4/80 positive BMDMs (FIGURE 11). Temporal analysis of infiltrating BMDMs was performed by flow cytometry quantification of F4/80-positive cells in the dissociated spheroids. Macrophages persisted within spheroids for about 9 days. Generally, infiltration was highest after 2 days and then declined slowly about 10%, with an increasing of cell death component, suggesting that the BMDMs infiltrate spheroids within the first days of incubation and decrease in macrophage frequency might relate to cell death and simultaneous of the growth of tumor cells that compose spheroids over the observational time (FIGURE 11).

Fig 11

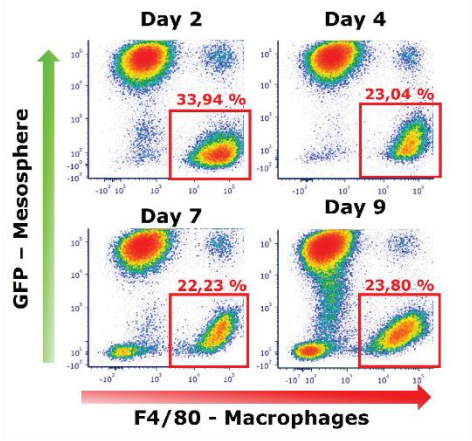


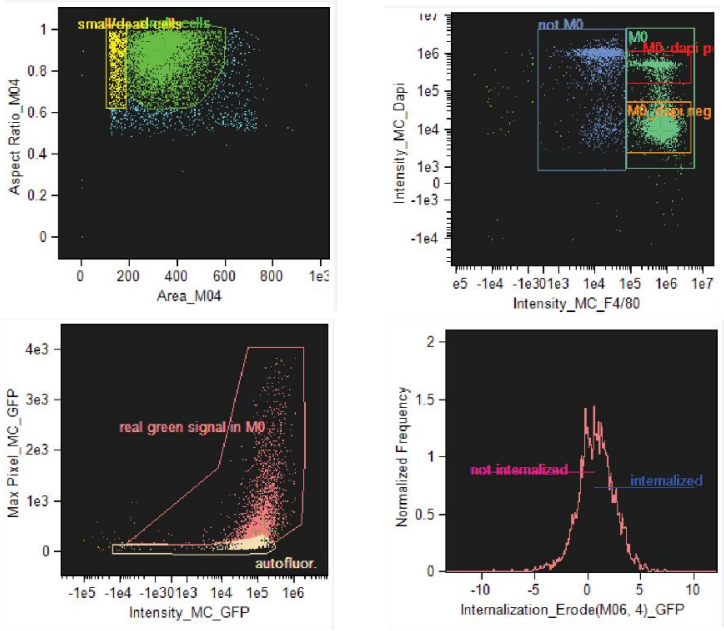
Figure 11. Flow cytometry analysis of dissociated spheroids (GFP+) co-cultured with BMDMs. Alexa 647-labeled F4/80 was utilized to identify infiltrated Mϕs. Spheroids+ MϕSs were dissociated at various time points after co-culture.

Of note, at all days of co-culture a population of cells appeared that were GFP/F4-80 double positive (FIGURE 11). A key mechanism by which TAMs can affect cancer progression is by phagocytosing tumor cells (Lecoultre *et al*, 2020). After processing phagocytosed cells, TAMs can behave as antigen-presenting cells, modulating the adaptive immune response towards the tumor (Lecoultre et al, 2020). To investigate the engulfment of GFP-expressing mesospheres by BMDMs in co-culture, we employed

fluorescence imaging coupled to flow cytometry (IFC). Following a 4-day co-culture period, BMDMs and mesospheres were harvested and stained with an anti-F4/80 (APC-780) antibody (FIGURE 12A). Subsequently, we conducted fluorescence flow cytometry sorting and single-cell image acquisition. Individual cell images exhibiting a double positive signal for GFP and F4/80 were analysed using an algorithm to assess internalization. The algorithm assigned a score greater than 0 when the GFP signal was detected within the cell and a score lower than 0 when the signal was observed outside the cell. Our findings revealed that 39%±0.5% of BMDMs in the mesospheres displayed GFP signal localized inside their plasma membrane and therefore had phagocytosed cancer cells or fragments thereof (FIGURE 12B)

Fig 12

A



B

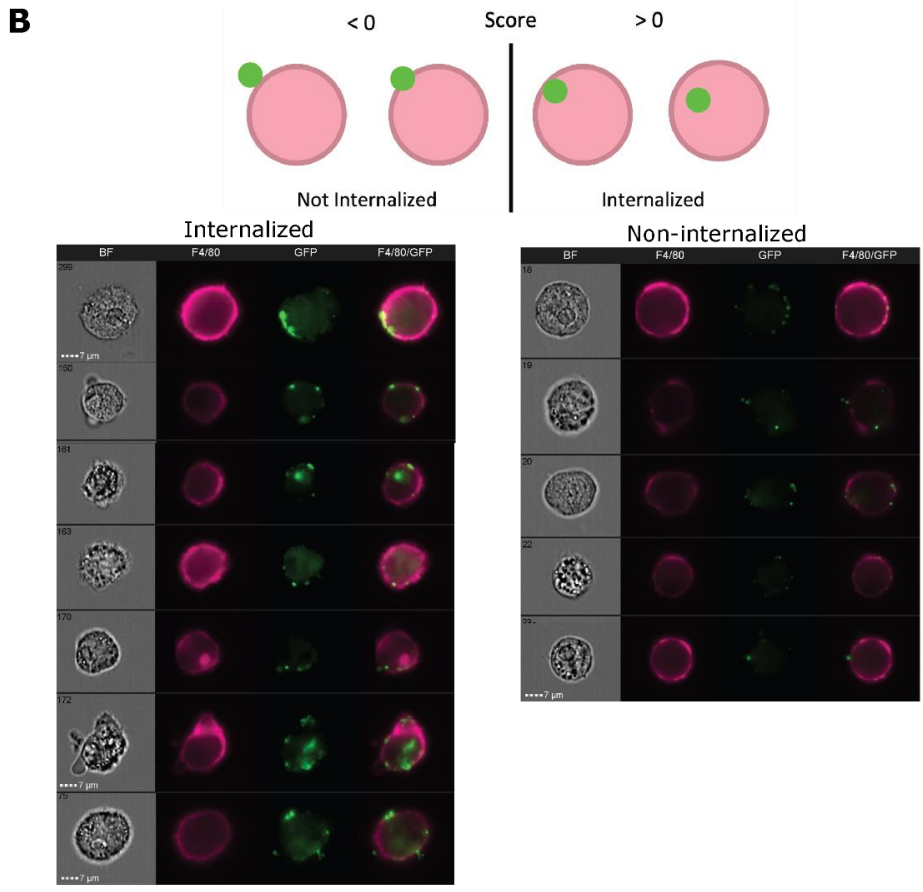


Figure 12. (A) Flow cytometry analysis of dissociated spheroids co-cultured with BMDMs at day 4. (B) (Upper part) Schematic representation of the internalization algorithm and its scoring. In pink, a stained macrophage; the green dot represents GFP signal. Normalized frequency of the internalization algorithm scoring for double positive GFP+ and F4/80+ cells. Two gates are depicted after applying the internalization algorithm: pink gate includes non-internalized events; blue gate includes internalized events. (Bottom part) Images of IFC overview of F4/80+ cells, GFP signal and merge with internalized event (panel left) and non-internalized events (panel right).

We also proved that co-culture is possible also using tumor and macrophage cells that are respectively derived from different mouse strains (FIGURE 13).
These results show that macrophages infiltrate mesospheres and phagocytose mesothelioma cells, and that in regard to this aspect our model reproduces the pathophysiological system.

Fig 13

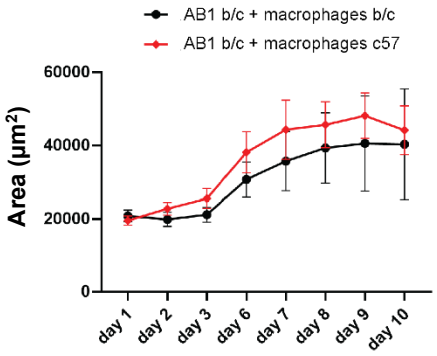


Figure 13. Growth curve of MM Balb/c spheres co-cultured with BMDMs derived from Balb/C mice (black line) and C57BL/6 mice (red line).

Study of macrophage polarization in 3D MM spheroid models

In the TME, Mϕs are known to support tumor growth when polarized towards an M2-like (pro-tumoral) phenotype. Conversely, M1-like Mϕs act against the tumor (Cendrowicz et al, 2021). Single-cell RNAseq data has identified differentially expressed genes in Mϕs polarized towards M1 or M2 (Jablonski et al, 2015).

As reported that elevated concentrations of M2 macrophages expressing CD163 and CD206 proteins were correlated with poorer overall survival (Laberiano-Fernandez et al, 2023).

To determine which polarization status BMDMs acquire when in culture with MM cells, we co-cultured unstimulated BMDMs (M0) with mesospheres. We used as a control BMDMs polarized in vitro to M1, or M2 with cytokines.

After 4 days of co-culture, to characterize the polarization status of infiltrated BMDMs, mesospheres were removed from the culture plates and lysed, RNA was extracted, and gene expression analysis was performed by rt-PCR. We typically use specific marker genes associated with each subtype as references for characterization. For example, for M1 macrophages, commonly used marker genes include iNOS, IL-1β, and TNF-α, among others. For M2 macrophages, markers may include CD206, Arginase 1 (Arg1), and IL-10, among others. For this experiment we used only as genes indicative of M1 polarization typo iNOS and Fpr2. These genes exhibited

increased expression during M1 polarization but decreased expression during M2 polarization. Conversely, for M2 polarization, we used CD206 as marker gene with elevated expression in the M2 state and reduced expression in the M1 polarization state (Jablonski et al, 2015).

The expression levels of marker genes suggest that a 4-day co-culture with MM cells drives M0 BMDMs towards the M2 status rather than the M1 (FIGURE 14A), recapitulating what has been observed in vivo with TAMs (Laberiano-Fernandez et al, 2023).

These results were supported by the expression pattern of CD206 and CD86 at the protein level by FACS analysis. Using as markers F4/80 and CD11b first, we observed that BMDM M0 after 4 days of co-culture tended to shift toward M2 phenotype (FIGURE 14B).

In conclusion, the co-culture of Mϕ BMDMs with MM cells over a 4-day period promotes their polarization towards the M2 phenotype, as evidenced by gene expression analysis and FACS analysis of CD206 markers, mirroring the pro-tumoral characteristics observed in the MM tumor microenvironment with TAMs.

Fig 14

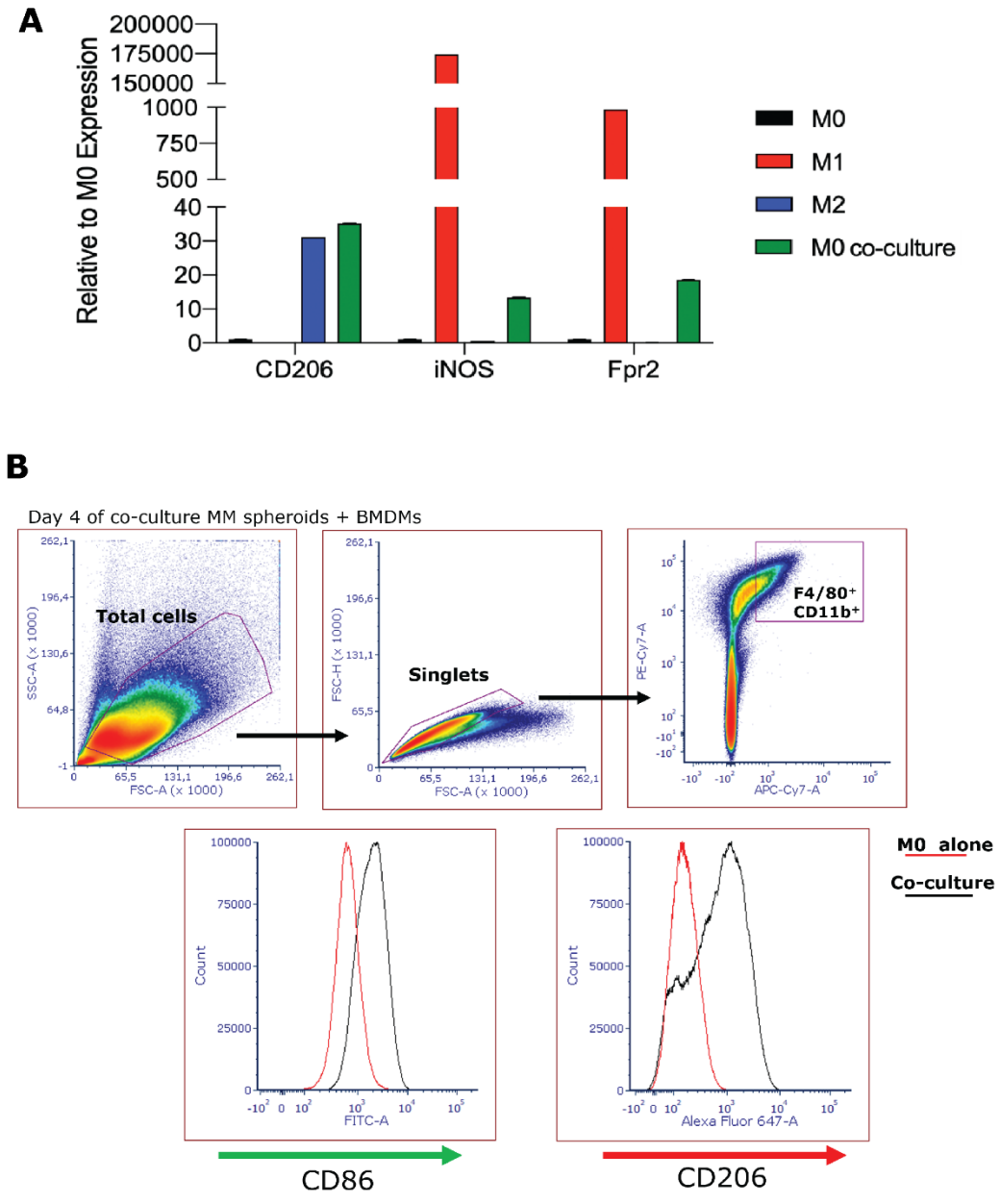


Figure 14. (A) BMDMs co-cultured with mesospheres polarize towards an M2-like phenotype. Gene expression of CD206, iNOS and Fpr2 in M0, M1, M2 and M0 co-cultured with mesospheres. Gene expression profile of Mϕs co-cultured with mesosphere is observed to be more similar to M2-like than to M1-like. (B) Flow cytometry analysis of CD206 marker in M0 Mϕs (red line) and in sphere-infiltrating MϕSs (black line). The analysis was performed in gated F4/80+ CD11b+ cells.

Single-Cell RNA Sequencing (scRNA-Seq) characterization of infiltrating BMDMs

Further characterization of infiltrated BMDMs over time was performed by scRNA-Seq to characterize gene expression at single cell level and identify possible subpopulations.

We performed Multiplex-based single-cell transcriptomics at 4 timepoints. We used 4 specimens as reference (spheroids of AB1, M0 BMDMs, M1 BMDMs, M2 BMDMs) on day 0, and we analyzed the co-culture of spheroids with BMDMs for the other three timepoints (day 3, day 6 and day 8).

To reduce the number of samples for SCT analysis, we tagged each sample with a specific Cell Multiplexing Oligo (CMO), a molecular tag, before pooling and performing single-cell RNA-seq. However, we had a technical problem with the number of cells not assigned to any particular CMO.

Despite the technical problems in the identification of samples, we still were able to extract valuable information from the experiment. One dataset contained vital and high-quality cells from the 4 samples at day 0; here, we identified tumoral AB1 cells by the expression of GFP and Mϕ based on their specific cell markers. We clearly distinguished 3 populations of AB1 cells expressing GFP, M1-like BMDMs expressing CXCL9 and M2-like BMDMs expressing CD206/MRC1, while M0 cells appear contiguous or partially overlapping with M2-like BMDMs (FIGURE 15A).

Using the same strategy, we then examined co-culture cell populations from the 4 timepoints. We observed that some cells belonging to the Mϕ cluster do express GFP, suggesting that they phagocytosed mesothelial cells and still contained GFP transcripts after phagocytotic events (FIGURE 15B). This is in line with the FACS results shown in figure 11A-B. Of note, we observed that BMDMs expressing MRC1, show a distinct M2-like phenotype but never contain GFP transcripts (FIGURE 15B). This may mean that at least one population of M2 cells displays a decreased phagocytic activity, relative to M1-like macrophages whose functions include phagocytosis of non-self-cells.

Fig 15

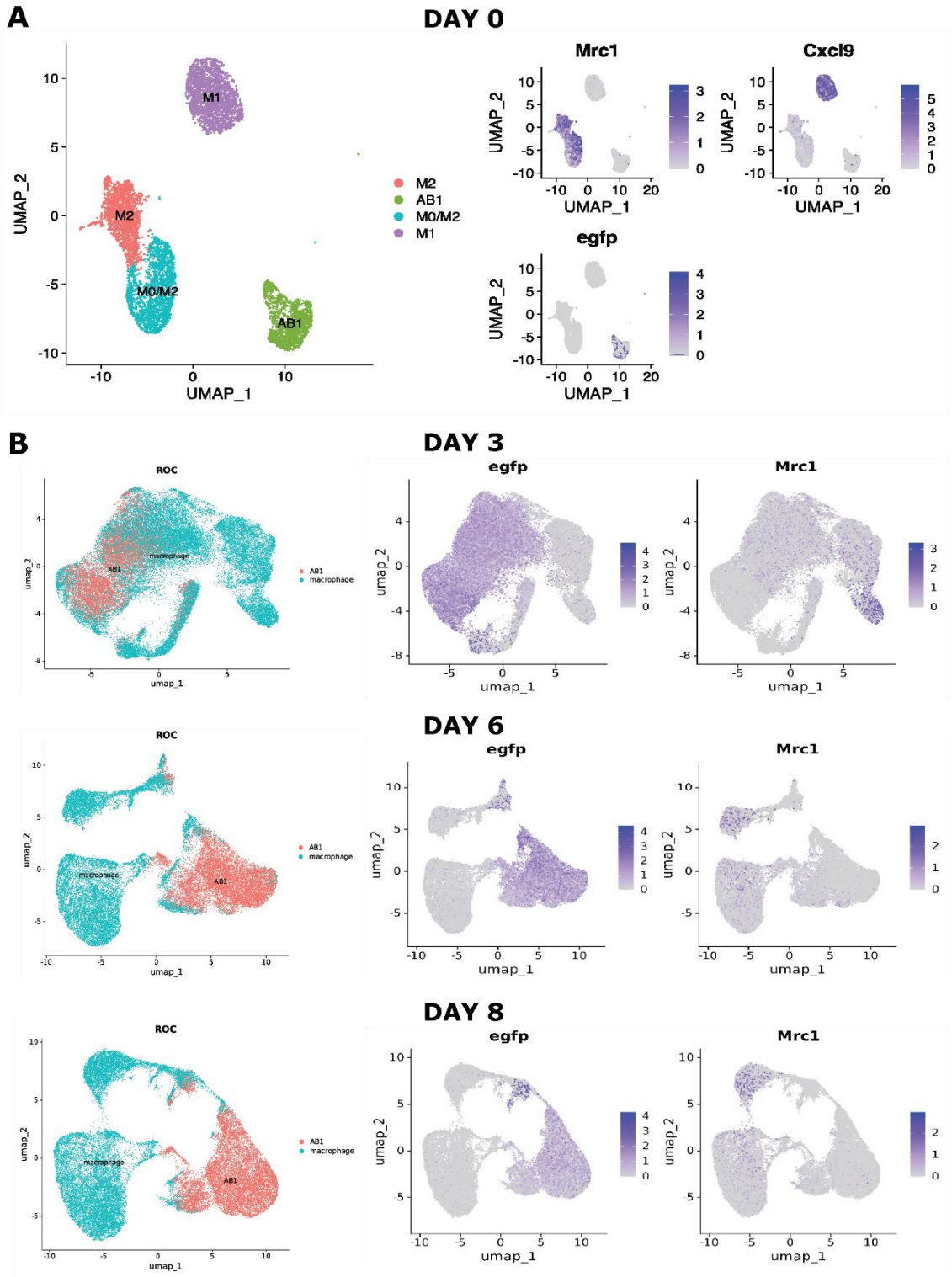


Figure 15. (A) (Left) UMAP visualization of Day 0 clustering AB1,M0,M1,M2 populations. (Right) UMAP colored based on GFP, CXCL9 and MRC1 expression. (B) UMAP visualization of Day 3, Day 6, Day 8 clustering into macrophages and AB1 cell populations, and colored based on GFP and MRC1 expression.

On days 3, 6 and 8 we identified clusters of GFP^+ BMDMs with upregulated genes enriched with fold change >0.5 in Cell migration pathways (Cell motility, Plasma membrane, Extracellular matrix organization, Digestion extracellular space, Locomotion migration, Banded collagen fibrillin, MAPK cascade, Signaling receptor activity, Animal organ morphogenesis, FIGURE 16 A-B-C and 17). Indeed, the signaling pathways triggered by phagocytic receptors have been widely recognized for their impact on the cytoskeleton (Mylvaganam et al, 2021).

Fig 16

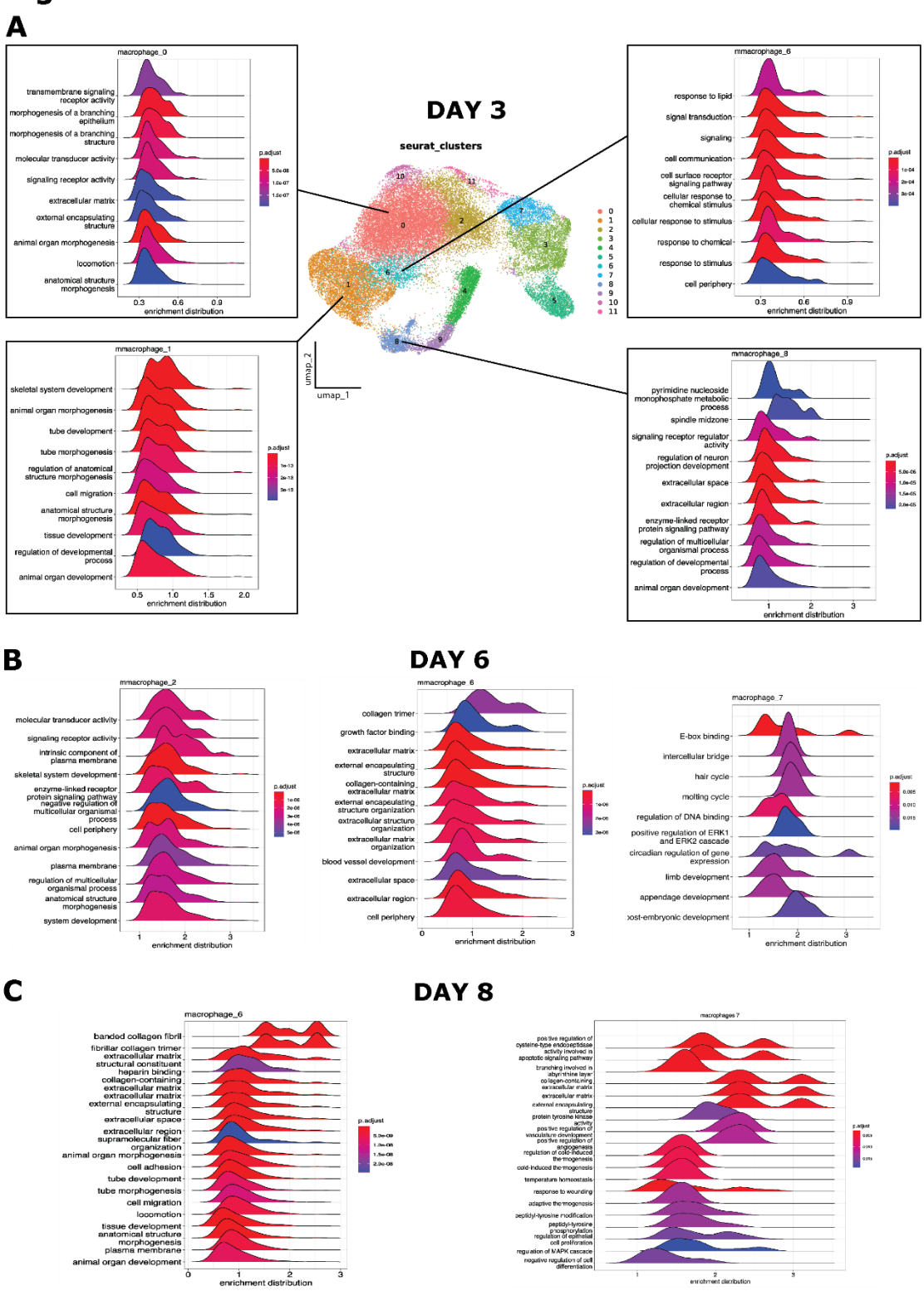


Figure 16. (A) UMAP visualization of Day 3 clustering into 11 different population. GO showing the top pathways expressed in cell population with high and evident gene expression profile. (B) GO of Day 6 and Day 8 showing the top pathways expressed in cell population with high and evident gene expression profile.

Fig 17

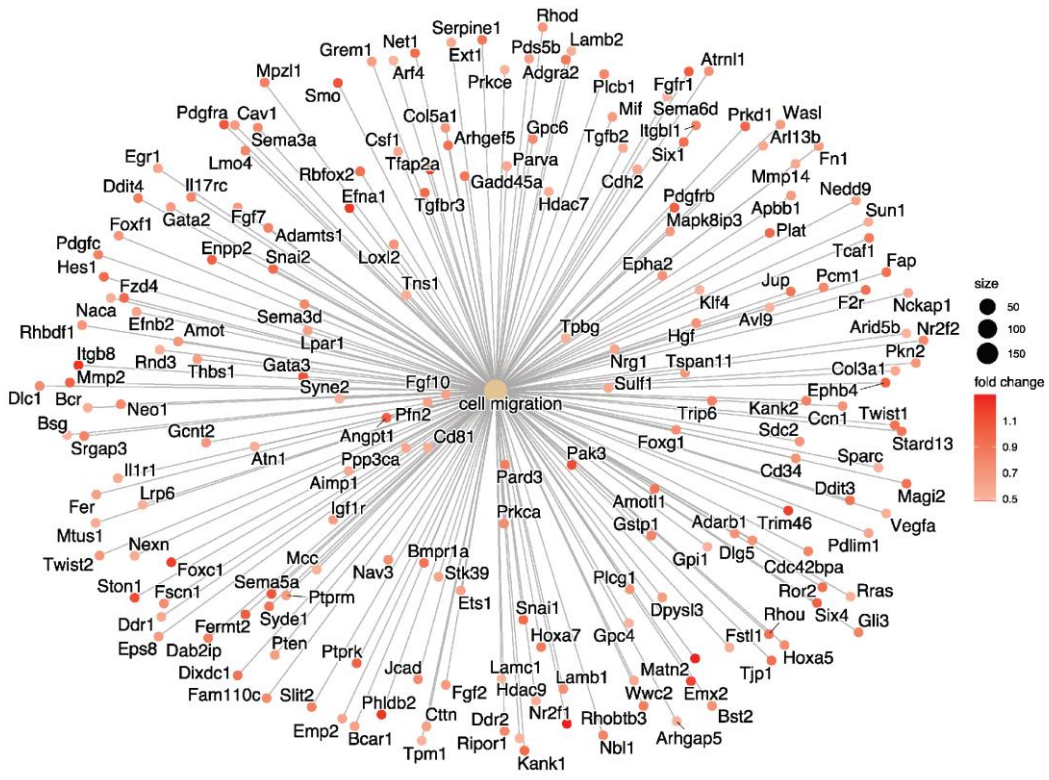


Figure 17. CNET plot illustrating the linkage of differentially expressed genes (positive markers) of cBMDMs that phagocytosed AB1-GFP cells in comparison to GFP-negative BMDMs, and the indicated Gene Ontology term (cell migration). Genes are color-coded based on the fold change increase, and the size corresponds to the number of differentially expressed genes associated with the respective Ontology term.

These results suggest that TAMs, in addition to exhibiting high levels of activity in migration and phagocytosis, tend to shift their status over time. This transition not only aligns with their inherent role as innate immune cells, but aligns with an polarization induced by the tumor itself to promote tumor growth, and with our data on macrophages-infiltrated spheroids.

In brief, human mesospheres infiltrated by macrophages recapitulate much of the interplay between TAMs and tumor cells observed in vivo. In addition, the dynamic behavior of TAMs in response to tumor signals can be better resolved in time, since we control the start of their interplay by deciding the time of infiltration of macrophages. Of note, these results have been obtained from a partially flawed experiment, which we hope to improve as soon as the technical glitches have been resolved.

BoxA reduces the infiltrated macrophage's pro-tumoral effect

To evaluate if this co-culture system recapitulates mesothelioma TME with infiltrated TAM; I co-cultured mesospheres with either M0 unstimulated, M1 stimulated with LPS and IFN-γ, M2 stimulated with IL-13 and IL-4. The culture of AB1 spheroids without macrophages is not extremely necessary because immortalized tumor cells growth indefinitely without any external influence and not reproduce what happen in physiological condition as showed by untreated condition of first graph in figure 18B.

The results shown that mesospheres co-cultured with M2 polarized macrophages showed substantial and sustained growth for 10 days, while in the same time span, M1 polarized macrophages grew significantly less respect the condition with unstimulated M0 macrophages (FIGURE 18A).

Our laboratory published last year on a new potential therapeutic molecule, BoxA, which is a truncated form of HMGB1 with anti-inflammatory and therapeutics properties on a mouse MM model. Specifically, BoxA promotes tumor cell phagocytosis by macrophages by surface reduction and internalization of CD47 (a don't-eat-me signal) (Mezzapelle et al, 2021).

We observed a significant reduction in the size of spheroid treated with BoxA over 9 days (FIGURE 18B). Daily assessments of spheroid area in co-cultures involving unstimulated M0 and M1-type shown any effects of BoxA on the % growth of mesosphere (FIGURE 18C-D). While co-culture with M2-type macrophages condition showed that BoxA significant efficacy in decreasing the growth rate, abrogating their pro-tumoral effect. Notably, spheroids co-cultured with M2 macrophages treated with BoxA we observed a drastically drop of growth in the first 3 days (FIGURE 18E).

Gene expression analysis showed that in co-cultures of mesosphere with M0 macrophages the BoxA treatment did not impact on CD206 and iNOS gene expression levels, while we observed a significant downregulation of iNOS in co-culture with M1 macrophages and a significant upregulation in co-culture with M2 macrophages. The expression of CD206 was stable (FIGURE 18F).

These findings suggest that BoxA reduces the protumoral effects of M2 macrophages.

Fig 18

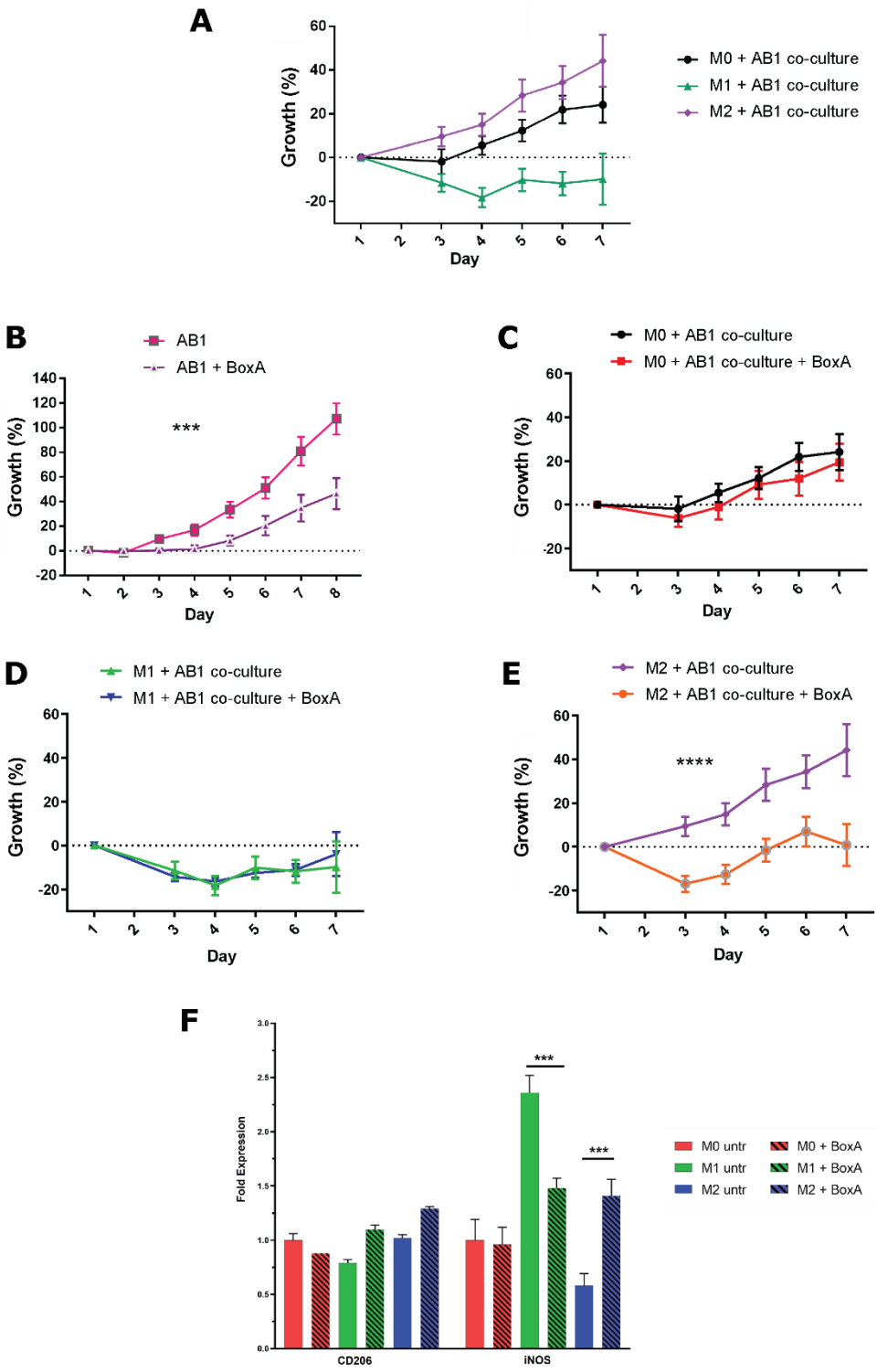


Figure 18. (A) Growth curve relative to day 1 of MM spheres cultured with or without differently polarized MϕSs. Images were acquired by Axio microscope and area was calculated using ImageJ software. (B-C-D-E) growth rate of spheroids co-cultured with M0, M1 BMDMs untreated and treated with BoxA Stat: n=3 experiments with 8-12 sphere each condition, Two-way ANOVA. SE. Significancy for time and treatment (F) Gene expression of CD206 and iNOS

in M0, M1, M2 polarized-BMDMs co-cultured with mesosphere untreated and after administration of BoxA. Stat: n=3, T-test, *** : p<0.001, SD

Human 3D tumor models

We have translated our insights into human using human mesothelioma biopsies. We used the samples to generate organoids, and human primary cell lines for the subsequent establishment of spheroid cultures. Given the inherent constraint posed by the limited annual availability of biopsy specimens, we addressed this challenge by initiating the generation of patient-derived xenograft (PDX) from mesothelioma biopsies. PDX facilitated the expansion of our biological material.

Patient-derived xenografts

NSG mice received fresh or frozen tumor tissues from patients with MM to establish PDX models. Tumor were obtained from San Raffaele Hospital and Alessandria Biobank.

In our observations, the subcutaneous implantation of tumors into the flanks of mice resulted in the development of detectable masses (approximately 1 cm) within a timeframe ranging from 12 to 16 weeks. A comprehensive examination by a pathologist confirmed that the explanted masses maintained histological and immuno-phenotypic features identical to the original tumor, as illustrated in figure 19. Specifically, explanted masses exhibited positivity for Calretinin, CK5/6, and WT-1 and partially negativity for BAP1, confirming their derivation from mesothelioma original tumor.

Fig 19

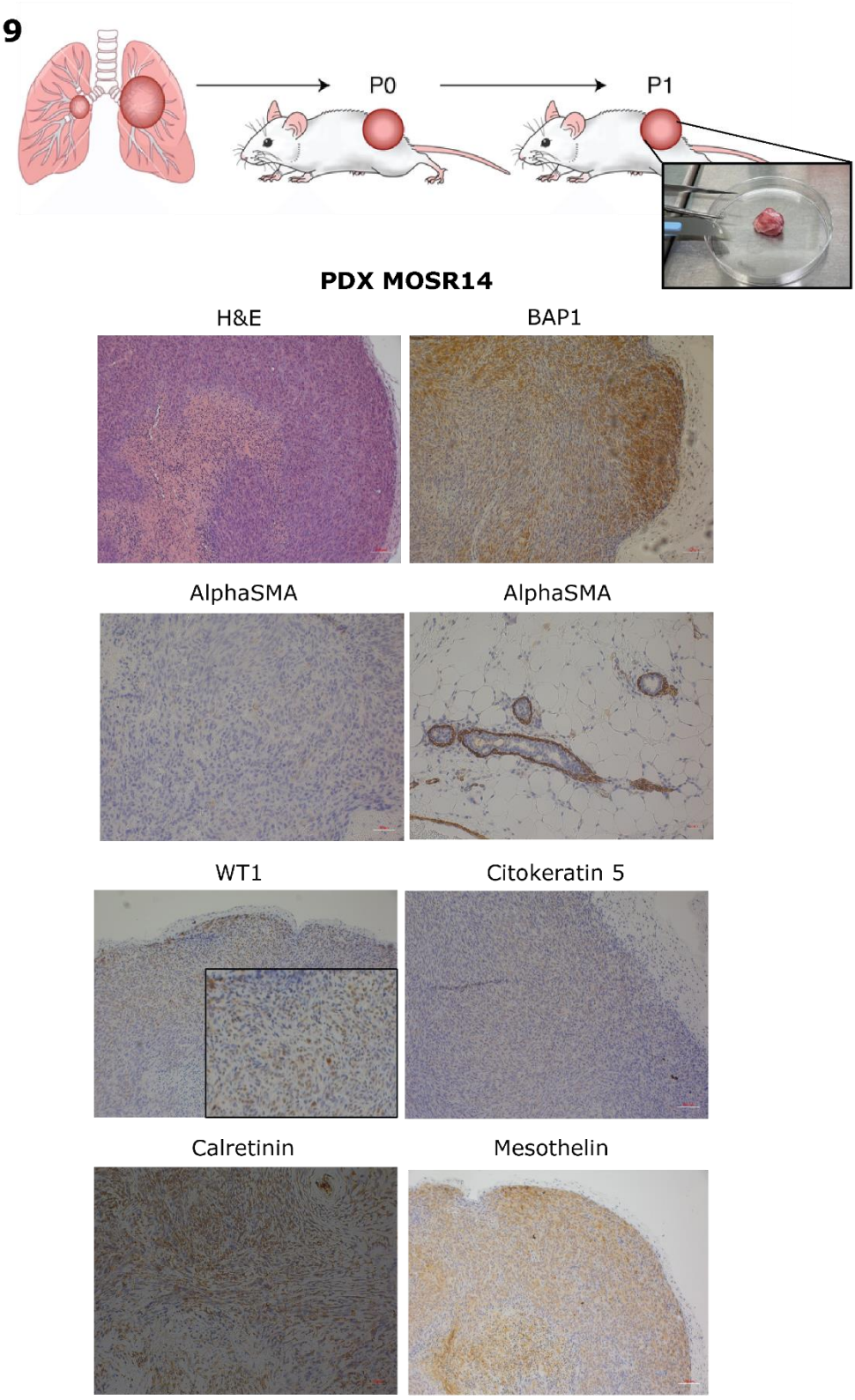


Figure 19. Schematic presentation of Patient Derived Xenograft model of MM and one representative mass extracted. Immunohistochemical characterization of the explanted tumor mass from NSG mouse using marker BAP1, Wilms Tumor 1, Calretinin, CK5, α-SMA, Mesothelin.

Establishment of primary mesothelioma cell lines

Numerous human MM cell lines have been derived from both tumor tissues and pleural effusions, with varying rates of success (Chernova *et al*, 2016; Shamseddin *et al*, 2021b). These cell lines encompass a spectrum of MM histopathological subtypes, and a substantial portion of them carry the genetic aberrations frequently identified in MM tumors (Shamseddin et al, 2021b).

To generate human MM primary cell line we used solid pleural tumor from MM patients: two fresh biopsies MOSR14 (ephitelioid) and MOSR15 (biphasic); from one frozen biopsy stored in biobank 956 (ephitelioid); from one liquid pleural efflux G02 (biphasic), and from one pleural inflammation (pneumothorax).

The human MM cell line was successfully established and maintained through more than 10 passages in vitro, displaying adherent growth with no floating cells even when confluence was reached. The cells exhibited a polygonal morphology, as illustrated in figure 20. Cultured cells preserve the podoplanin marker and express CD47 (FIGURE 21A-B).

In parallel, we also generated primary MM cells derived from PDX MOSR14; however, this culture also contains mouse-derived cells (FIGURE 21C).

Fig 20

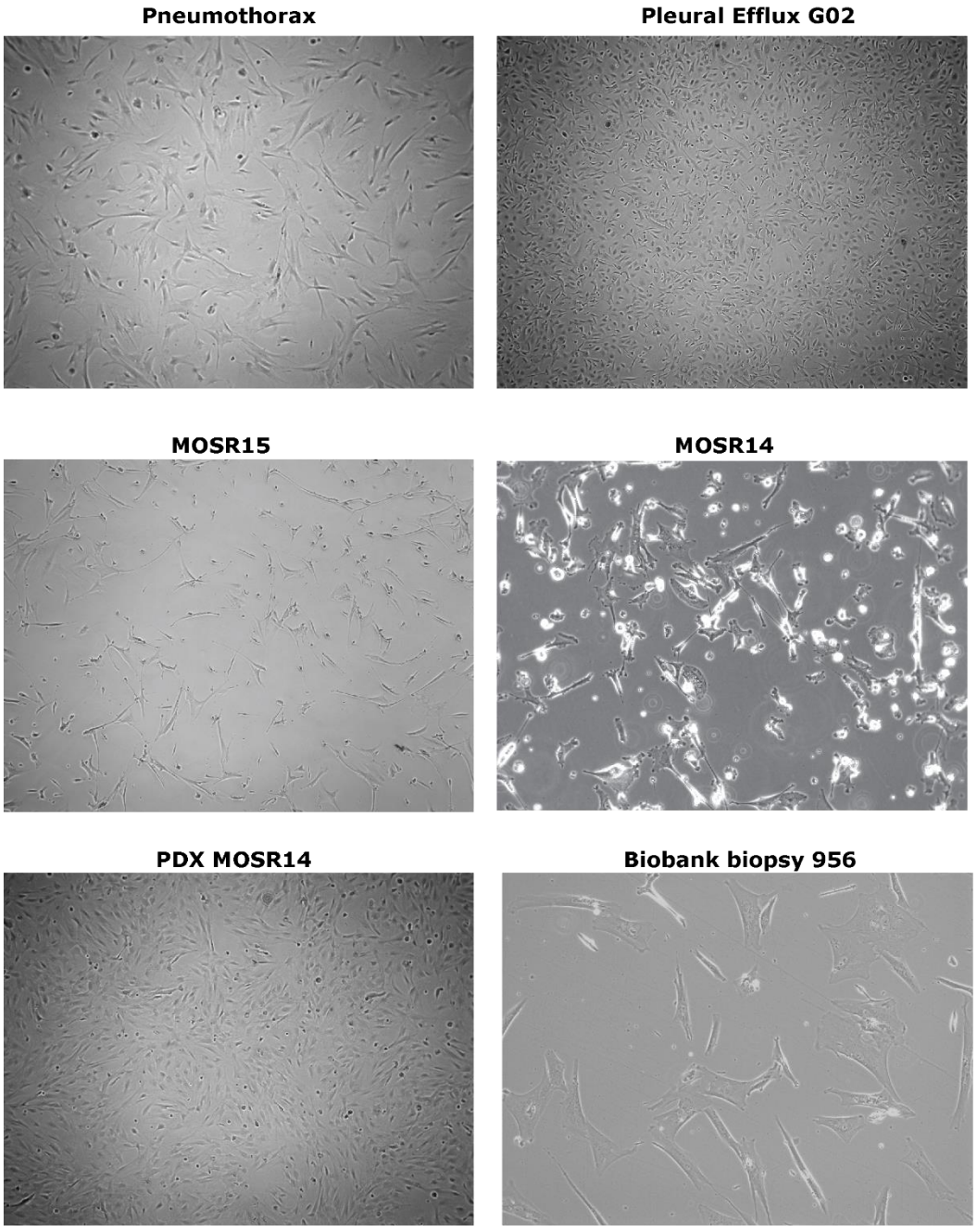
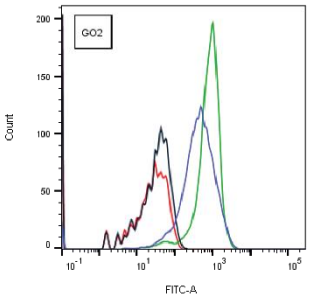


Figure 20. Phase-contrast micrographs of MM cells in tissue culture. MOSR14 and 956 cells mainly composed of polygonal epithelioid cell types, while MOSR15 and G02 composed of mixed cell type can be seen.

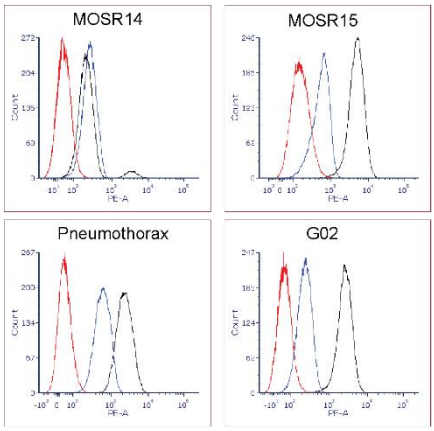
Fig 21

A



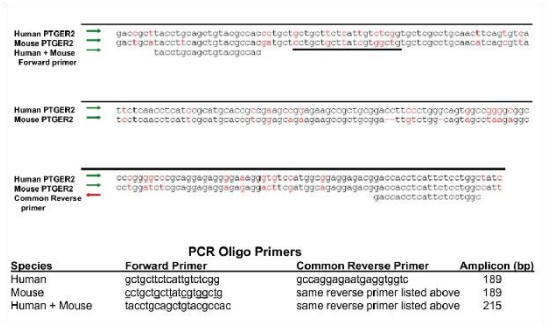
Red: unstain,
Black: secondary Ab control,
Blue: Ab anti-mesothelin,
Green: Ab anti-podoplanin

B



Red: unstain,
Blue: isotype,
Black: Ab anti-CD47

C



PCR Oligo Primers

Species	Forward Primer	Common Reverse Primer	Amplicon (bp)
Human	gctgcttctcattgtctcgg	gccaggagaatgaggtggtc	189
Mouse	cctgctgctatcgtggctg	same reverse primer listed above	189
Human + Mouse	tacctgcagctgtacgccac	same reverse primer listed above	215

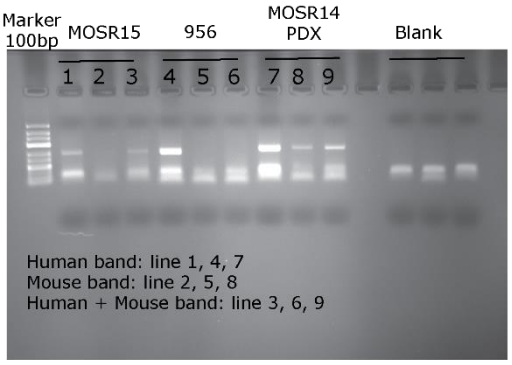


Figure 21. (A) Flow Cytometry analysis of Podoplanin and Mesothelin in G02 pleural efflux cells. Unstained cells (red), secondary antibody control (black), ab anti-mesothelin (blue), ab anti-podoplanin (green). The G02 cells line and result were shared by Marion Macfarlane Lab from University of Cambridge. (B) CD47 surface exposure. Independent cultures of G02, MOSR14, MOSR15, Pneumothorax cells. Unstained cells (red), control isotype (blue), ab anti-CD47 (black). (C) (left part) "Relevant species alignment using the PTGER2 gene and the PCR primers. a) The human and mouse PTGER2 DNA sequences are aligned, with red letters signifying non-conserved base-pairs. Human+Mouse PCR primers are listed underneath, and a black bar is below the qPCR probe sequence. The reverse-compliment Common Reverse primer sequence is shown to illustrate alignment on the DNA strand shown. b) Qualitative PCR primers are listed, as well as the expected size of the amplified sequence. All sequences listed 5' to 3'. Mouse genome specific base pairs are underlined in the mouse forward primer and mouse probe sequences." Image and description from Alcoser et al, 2011 (right part) Notably, the xenograft samples subjected to serial passages manifest the concurrent presence of both human and mouse DNA.

Generation of human MM spheroids and co-culture with PBMCs

The human MM cell line demonstrated the capacity to grow under non-adherent conditions, forming floating spherical structures. Phase-contrast microscopy revealed the distinctive morphology of human mesospheres for MOSR15 and pneumothorax but pleural efflux G02 and MOSR14 presented a less compact structure that we term "aggregates" (FIGURE 22). Following the collection of tumor spheroids, they were fixed, sectioned, stained with haematoxylin and eosin (H&E) and subjected to IHC using the mesothelioma antibody panel. Figure 22 illustrates the positive staining for Vimentin and WT-1, Calretinin, and mesothelin in human mesospheres, confirming their mesothelial origin in the non-adherent conditions and low positivity for the fibroblast marker α-SMA.

Similar to what we did for murine mesospheres, we performed a co-culture of human mesosphere with human Mϕs. To introduce myeloid immune cells in our spheroid cancer models, donor-derived $CD14^+$ monocytes were differentiated to macrophages by adding human MCSF and were infiltrated into 2-day-old mesospheres.

We focused on elucidating whether the mesosphere induces the polarization of infiltrating monocytes into M2-like macrophages, a phenomenon frequently observed in vivo. We evaluated polarization with markers such as CD86 and CD206. Gene expression shows that the pneumothorax cell line strongly activates Mϕs inducing generally increased gene expression and their polarization to M1 and also to M2. On the other hand, MOSR14 MM spheroids polarized infiltrating monocytes to M2-like macrophages with high CD206 and low CD86 expression, while mesospheres consisting of G02 cells from pleural efflux did not induce any particular polarization status (FIGURE 22).

Fig 22

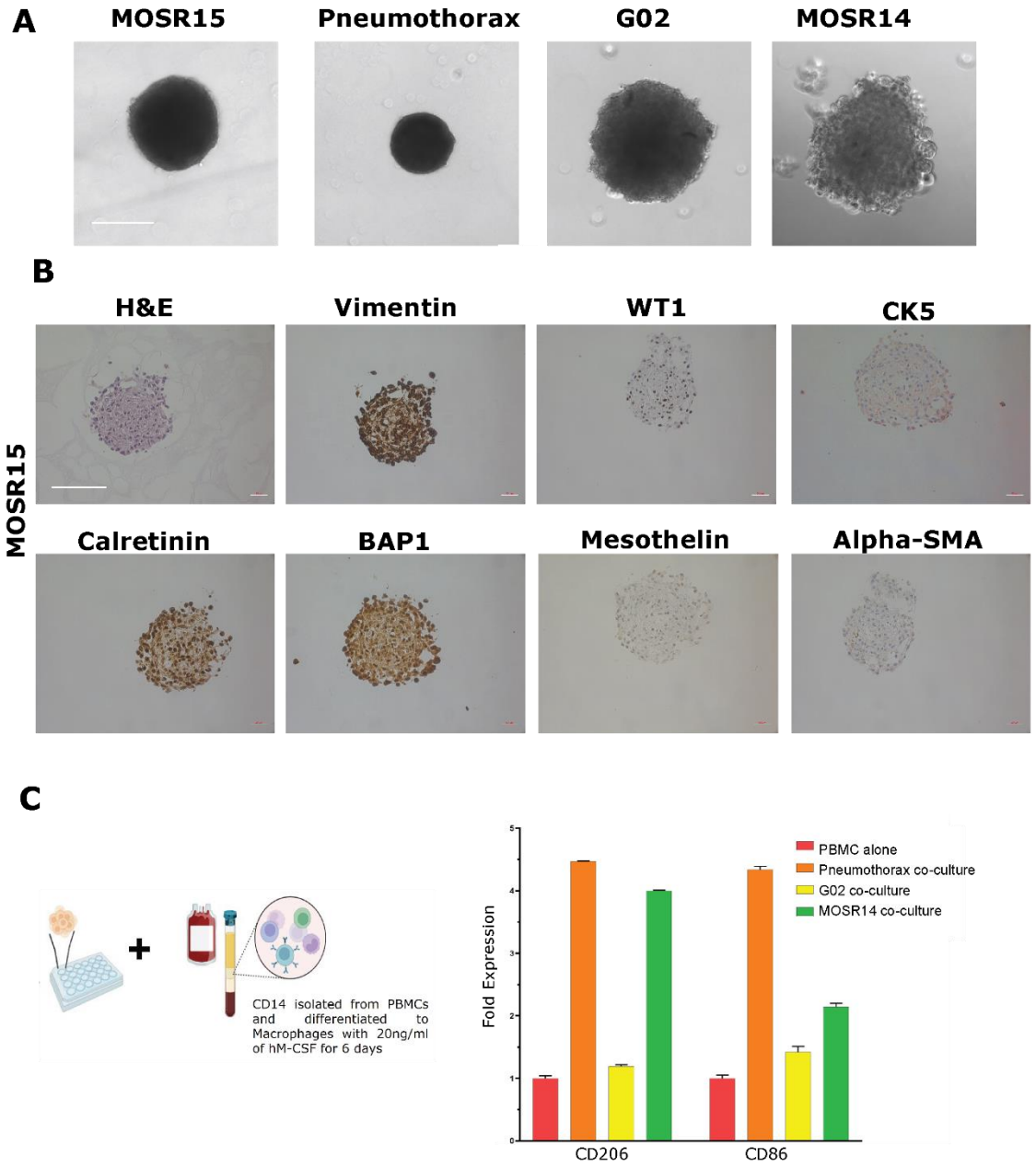


Figure 22. Phase-contrast micrographs of MM spheres in culture. The MOSR15, Pneumothorax, and Go2 spheres seems to be rounded, compact, but also irregular. While MOSR14 appear as aggregates. Scale bar= 100 µm (B) Representative acquisition and IHC staining images of 3D spheroid aggregates derived from primary human mesothelioma cell line. Markers using human antibody for: Alpha-Smooth Muscle Actin (α-SMA), BAP1, Calretinin, Wilms Tumor 1, Cytokeratin 5, Vimentin, Mesothelin. Scale bar 100 µm (C) Relative gene expression of CD206 and CD86 in co-culture of Pneumothorax, G02 and MOSR14 sphere whit PBMCs differentiated macrophages.

Establishment of 3D structures with human MM cell line and organoids from human MM biopsies

The human primary cell lines were resuspended in the Cultrex© matrix and seeded as drops in 48-well plates. All samples successfully formed globular structures after culturing for 3–20 days initially measuring approximately 100 µm in diameter and eventually expanding to an impressive 250 µm in about 17 days (FIGURE 23A); the 3D structures could be passaged and continued to grow after several passages.
In-depth analysis using H&E staining shows that these structures develop a dense extracellular matrix (ECM) core (FIGURE 23B).

Simultaneously, we embarked on the creation of a human MM organoid, utilizing fresh biopsies obtained from patients at San Raffaele Hospital. The emergence of human mesothelioma organoids became apparent just seven days after seeding (FIGURE 23C). Notably, these organoids exhibited the potential for successive generations — second, third, and fourth— opening new avenues for exploration.

Upon further examination through H&E staining, the organoids displayed a distinctive inside-outside polarity and an intricate internal matrix, as vividly illustrated in figure 23D. Significantly, the positive expression of mesothelioma markers, such as Vimentin, WT-1, and mesothelin, and negativity for BAP1 were robustly confirmed, solidifying the authenticity and relevance of our organoid model in capturing key aspects of human mesothelioma biology.

In conclusion, this study marks the first comprehensive exploration into the complicate field of mesothelioma, translating insights from human mesothelioma biopsies to the establishment of patient-derived xenograft models, primary cell lines, spheroids, and organoids.

Fig 23

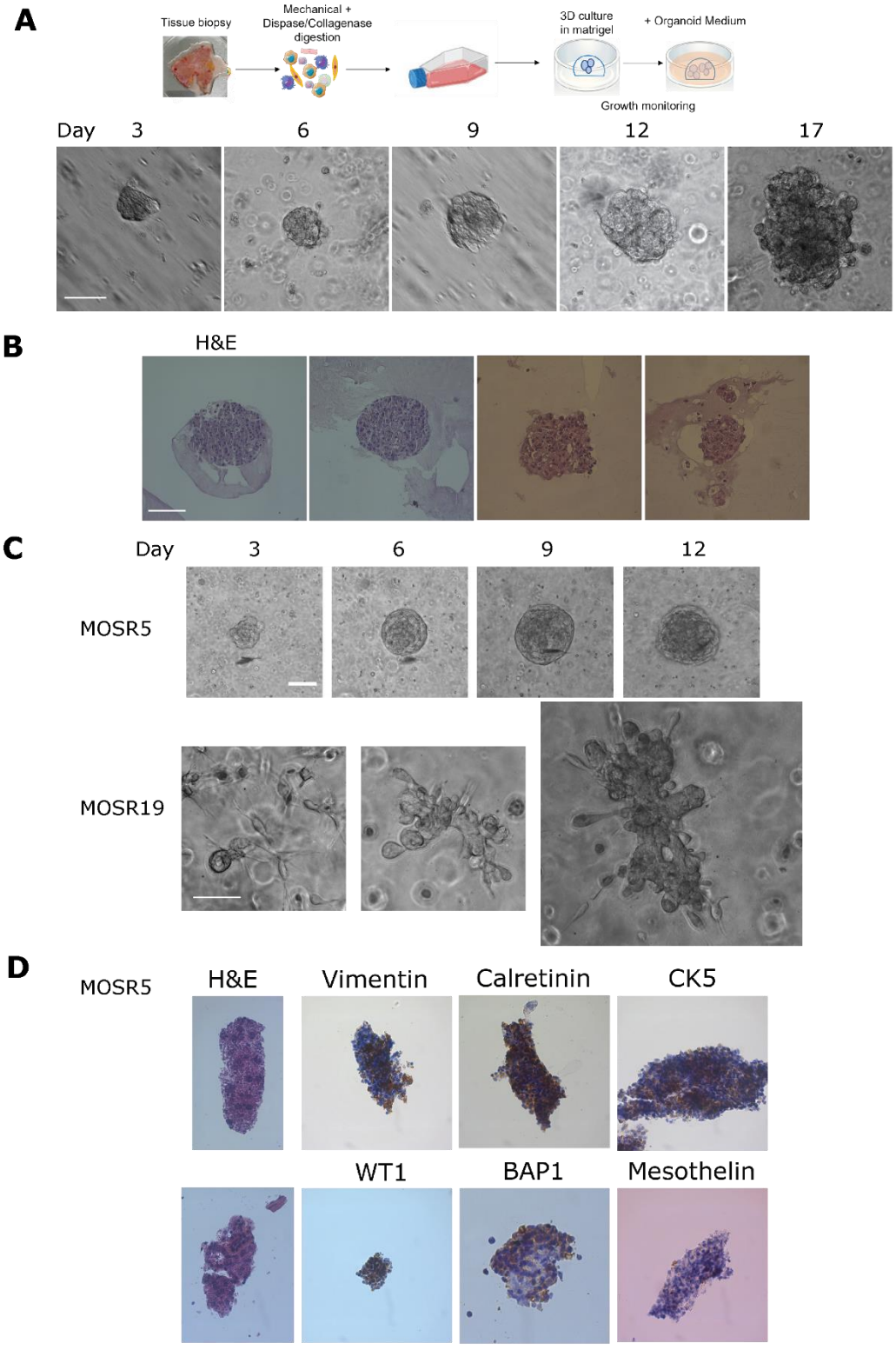


Figure 23. (A) Representative phase-contrast images following growth of MM Human 3D structure generated from primary cells derived from patient biopsies and cultured in Cultrex©. Scale bar= 100 µm (B) H&E staining of Human MM 3D structures. Scale bar= 100 µm (C) Phase-contrast images following the Organoids growth. Primary organoid generated from patient MOSR5 and MOSR19. Scale bar=100 µm (D) IHC characterization of Human Organoids.

Discussion

Malignant Mesothelioma is currently an incurable cancer that develops in most patients many decades after inhalation of asbestos dust. Few treatment options exist and most patients die within 8-12 months after diagnosis (Galateau-Salle et al, 2026). Clinical trials of cytotoxic drugs, targeted agents and immune checkpoint inhibitors have disappointed (Kuryk et al, 2022). This poses a major challenge for effective drug development and a new approach is urgently required. Landmark longitudinal studies in mice have the potential to more effectively identify the key drivers of MM evolution and thus more effective direct new drug development, but this requires longer time (Shamseddin et al, 2021a).

Therefore, establishing new in vitro models for MM is crucial for accelerate diagnostic and therapeutic approaches and for delving into the fundamental aspects of the disease.

In the last decade, a powerful in vitro model system has emerged for the expansion and exploration of both healthy and tumor cells, namely three-dimensional "organoid" cultures. Organoids function as diminutive tissues faithfully reproducing both the architectural and growth attributes of their source tissue. Various laboratories have effectively produced a range of organoids, encompassing models derived from cancers such as prostate, lung and pancreatic cancers (Gao et al. 2022, Barbosa et al, 2022).

Recently, a culture method for human peritoneal mesothelioma organoids was developed using tumor tissue from patients. Pathological examination and Whole Genome Sequencing (WGS) demonstrated that these organoids faithfully replicated both the histological features and genomic diversity observed in the original tumors (Fang et al. 2024).

Also, the lab of prof.re Marco Bianchi developed patient-derived MM organoids and lung mesothelial organoids. They obtained data from single-cell transcriptomics of donor MM biopsies and their related organoids. MM organoids contain descendants of the original tumor cells derived from biopsies but very few immune cells, in particular macrophages, survive in organoids. We don’t know if they die or are lost during organoid passing (unpublished data).

Given that macrophages in the TME play an indispensable role in cancer progression, the original aim of my thesis is to re-supplement human MM organoids with human macrophages, in order to investigate the crosstalk between tumor cells and the immune microenvironment and to test possible therapeutic molecules.

Generating MM organoids and maintaining them in culture was not easy, and human MM samples are resources not always available. During the first year of my PhD I obtained, with significant effort, a collaboration with the Alessandria Biobank. We tried to use frozen biopsies from the biobank but the growth of organoids was unsuccessful (data not shown). Also, after pandemic SARS-COVID-2 period we obtained and used some fresh biopsies from San Raffaele Hospital and I was able to get a few organoids.
In light of these considerations, we looked for another type of preclinical models that faithfully replicate the in vivo milieu of tumors in a simpler way, specifically spheroids. Therefore, this thesis is about development of two 3D technologies, with patient-derived organoids being more complex and sophisticated, while spheroids have a much simpler accessibility and usability.

The impact of the COVID-19 pandemic on research

The emergence of the novel coronavirus disease, COVID-19, attributed to the pathogen severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was initially detected as an unidentified illness in Wuhan, China, in December 2019. It was subsequently identified on the 7th of January, 2020. Since the epidemic's inception, the SARS-CoV-2 pathogen has been responsible for infecting more than 250 million individuals globally, leading to severe acute respiratory syndromes and resulting in over 5 million fatalities (McNally et al, 2021).

In the face of the escalating global health crisis presented by the COVID-19 pandemic, immediate and decisive policy responses were imperative to curb the spread of the novel coronavirus. This urgency precipitated the adoption of various non-pharmaceutical measures aimed at mitigating transmission. Such measures encompassed a concerted effort to minimize interpersonal contact, safeguard those deemed at heightened risk, enact closures of educational entities, suspend public events, mandate the shutdown of dining establishments, curtail working hours for many, facilitate remote working arrangements for employees, and impose travel restrictions. The institution of these unprecedented social distancing policies at the dawn of 2020 compelled academic institutions worldwide to adapt, particularly in the completion of practical courses and related activities. The pandemic exerted a substantial impact on the research domain, presenting numerous obstacles for clinical and surgical trainees alike. Directly stemming from lockdown edicts and the consequent shuttering of most university-based research infrastructures, research initiatives and clinical trials not directly related to COVID-19 experienced a

suspension of activities (Sathian *et al*, 2020). This redirection of research focus toward COVID-19-related topics became an inevitable shift during the pandemic.

The repercussions of COVID-19 on clinical research were instantaneous and are likely to be enduring. A multitude of clinical trials encountered a standstill, barring those investigations directly about COVID-19. The commencement of new study participant enrollment was also halted, chiefly due to the associated risks of propagating the COVID-19 virus. One of the principal effects experienced within the surgical sphere was the observable decrement in the totality of surgical procedures.

An evaluative and analytical study was undertaken to scrutinize the impact that the lockdown, prompted by the COVID-19 outbreak, exerted on emergency general surgery (EGS) within the Milan region at the pandemic's incipience. A questionnaire was disseminated across 14 distinct medical facilities within the Milan catchment to gauge the fluctuation in EGS procedures. Each institution collated and compared the number of EGS procedures carried out during an identical time interval spanning 2019 and 2020. The gathered data disclosed a 19% reduction in patient numbers during the pandemic year of 2020 relative to the previous year. This decrement was statistically significant solely in the context of abdominal wall surgeries (Kurihara et al, 2021).

The cessation of operations at Universities, research, and hospital entities has precipitated a situation where scientists might find themselves unable to execute crucial experimental procedures, which are pivotal for the finalization and submission of their scholarly contributions.

Organoid and spheroid 3D models to investigate tumor-immune cell interactions.

A part of this thesis (the first from a temporal point of view) is focused on the generation of patient-derived MM organoids. We established a protocol for generating organoids from mesothelioma biopsies, and their growth was monitored. We successfully generated primary organoids; these organoids can be passaged 3 or 4 times, but the establishment of immortal or quasi-immortal mesothelioma "organoid lines" remains elusive.

Additionally, morphological and immunophenotypic characterizations were performed. Hematoxylin and Eosin staining indicated a defined structure in mesothelioma organoids with inside/outside polarity and an internal matrix, a structure that is lost when organoids are dissociated and potentially contributes to

their challenging propagation. Immunohistochemistry demonstrated positivity for the MPM markers used for diagnosis; positivity is maintained upon passaging.
Given the current absence of MM organoids, we consider our MM organoids to be a significant breakthrough. Ongoing experiments are being conducted to correlate the transcriptomic profiles of organoids and their corresponding biopsies, providing insights into whether organoids faithfully recapitulate tumor biology and heterogeneity.
Significantly, in the context of therapeutic interventions, tumor organoids can play a pivotal role as an essential complement to sequence-based treatment modalities. Organoids derived from patients can be subjected to direct in vitro testing, enabling the evaluation of their sensitivity to particular drugs or combinations thereof. This approach provides valuable insights for oncologists, facilitating the customization of treatment strategies tailored to the unique characteristics of individual patients.

I also developed spheroid 3D models as a system to study the interaction between tumor and immune cells in MM tumors. To this end, we generated setup the 3D cultures from both murine tumors and MM patient-derived cell lines.
Co-culture systems are another application in which 3D models could offer a great opportunity to reflect the "in vivo" situation. Accordingly, in the last years, several groups focused their interest on exploiting the 3D model to study the crosstalk between cancer cells and the components of the TME, with particular attention to stromal and immune cells (Mu et al, 2023). Significantly, an equilibrium was achieved between tumor and immune components, affording the prospect of monitoring molecular aspects and cell evolution in vitro, all things that prove challenging using extant 2D culture systems.

How cancer progresses and responds to therapy is related to intrinsic characteristics of cancer cells, but also to the TME. The TME is composed of tissue-resident cells and recruited immune cells. Many clinical and experimental data indicate that TAMs, present abundantly in most tumor types, have a major regulatory role in promoting tumor progression.
The predominant TME constituent of numerous tissues consists of resident macrophages, frequently assuming the responsibility of orchestrating tissue homeostasis and manifesting distinct intrinsic transcriptional programs. In the context of malignancies, infiltrating macrophages are recruited from the periphery in response to signals such as CCL2, CCL9, CSF-1, VEGF, and TGF-b (Lin et al, 2019). The

features of these macrophages in situ are modulated by signals originating from the healthy organ or the tumor.

Mϕs are very plastic cells that respond in a versatile way to the different microenvironmental cues. In a simplified description, MOs can polarize towards an activated "M1" phenotype, or an activated "M2" phenotype (Pan et al, 2020). M1 macrophages are pro-inflammatory because they respond to signals such as bacterial products or IFN-γ and attract and activate cells of the adaptive immune system. A salient characteristic of M1 is its ability to express nitric oxide synthase (iNOS), reactive oxygen species (ROS) and the cytokine IL-12. Additionally, M1 functions encompass engulfing and eliminating target cells.

Conversely, M2 macrophages make a pro-tumoral contribution because they express an abundance of scavenger receptors, correlating with elevated expression levels of IL-10, IL-1β, VEGF, and matrix metalloproteinases (MMP). M2 undertake tasks such as debris removal, promotion of angiogenesis, tissue reconstruction, and injury repairments, as well as fostering tumorigenesis and tumor development.

We thus wondered if we could replicate these behaviors with our 3D culture system. TAMs have been associated with an M2 polarization state, correlated with an unfavorable prognosis and a TME immune-suppressive for cancer immunotherapies. Our focus was on elucidating the polarization state adopted by Mϕs when co-cultured with MM cells. Through direct co-cultivation of M0s with mesospheres, we demonstrated that MM cells can instigate an M2-like gene expression response signature in Mϕs. This observation implies that the interactions between MM cells and M0s lead to their polarization towards a pro-tumoral M2 state.

Furthermore, our observations indicate an increase in mesosphere growth when co-cultivated with M2-like macrophages, whereas the presence of M1-like monocytes exerted inhibitory effects on mesosphere growth. We propose that the co-culture of mesospheres with macrophages can effectively replicate what is happening in the context of MM. This model could be potentially used for drug screening, particularly for compounds or approaches targeting the interactions between Mϕs and MM cells.

Transcriptomic landscape in Mesothelioma spheroids: a single-cell analysis of TAMs phenotypic shift, phagocytosis and migration

In support of discussed results above, this study provides a preliminary characterization at single-cell level of infiltrating BMDMs in MM spheroids for 8 days. Unfortunately, we are constrained to discuss only the data derived from an experiment that encountered technical issues. Despite these limitations, certain

pertinent and valuable insights have surfaced. These findings will be subject to further exploration in ongoing experiments.

To define the starting point, we first established the transcriptional landscape of AB1-GFP spheroids without BMDMs and of single populations of BMDMs polarized to M0, M1 and M2. The BMDMs that we classified as M0 continuous with the cluster of M2-like BMDM, which was clearly separated from the clusters of the M1-like BMBM.

Notably, the BMDMs contained also a GFP transcript derived from phagocytosed AB1-GFP cells.

Interestingly, we observed that BMDMs that highly express the MRC1 gene, and therefore have a M2-like phenotype, clustered separate from the BMDM GFP^+ cluster. This suggests that M2-like macrophages display a decreased phagocytic activity, while M1-like macrophages have intrinsic functions to trap and phagocytose foreign and tumor cells.

The high plasticity of macrophages makes it challenging to pinpoint genes with high transcriptional expression. However, the gene MRC1 stands out, as the mesothelioma tumor itself promotes macrophage polarization towards the M2 phenotype, supporting its own growth and inhibiting the immune system.

Numerous questions persist regarding how the tumor communicates with macrophages and how macrophages, in turn, communicate with other immune cells, such as presenting antigens post-phagocytosis. Additionally, the extent to which M1 and M2 differ in functional properties within the tumor microenvironment remains uncertain. Does the spatial localization of macrophages, especially M2, significantly contribute to tumor progression?

Another important observation is that all BMDM clusters on the different days showed a similar pattern of cell migration signatures. We speculate that macrophages first migrated from outside the spheroid surface to the inside of spheroids, upregulating the cell migration genes. Moreover, the same cytoskeletal components (and their transcripts) might be necessary for engulfing cells during phagocytosis. To confirm this hypothesis, we will try to inhibit cytoskeleton rearrangements and quantify phagocytotic events.

In parallel macrophages were stimulated by tumor cells: from day 3 we observed an increase in expression of pathways involving matrix metalloproteinases, promotion of angiogenesis, tissue reconstruction, and injury repairments, all functions typical of M2 polarized macrophages.

We therefore conclude that the tumor-infiltrating macrophages not only manifest elevated levels of functionality in terms of migration and phagocytosis but also undergo a temporal shift in their phenotypic status. This shift implies a dynamic

alteration in their functional characteristics over time. We speculate that the migratory and phagocytic activities observed in TAMs correlate to their crucial role in the tumor microenvironment, including possibly antigen presentation.

3D MM model reveal the therapeutic potential of BoxA in modulating macrophage response/effect

We also investigated in our 3D system if the interaction between tumor cells and tumor microenvironment can be targeted by drugs. Recombinant BoxA, truncated HMGB1 protein, is a therapeutic HMGB1 antagonist in many experimental models of tumor, sterile, and infectious tissue inflammation (Venturini et al, 2022; Andersson & Tracey, 2011). MM spheroids treated with BoxA showed a reduction in growth rate and a small reduction in the presence of M0 naïve macrophages and with M1-like M0s. This may correlate with BoxA inhibits tumor cell growth and CD47 internalization, leading to tumor cell phagocytosis by macrophages (Mezzapelle et al, 2021).

The most surprising result is that we observed a shrinking of spheroids in the presence of M2-like M0s during treatment with BoxA. This suggest that BoxA contributes to abolishing the pro-tumoral effects of TAMs. As published that BoxA binds to Receptor for Advanced Glycation End-products (RAGE) and competes with full-length HMGB1 binding to RAGE provides an attractive explanation for its mode of action in the prevention of RAGE-mediated endocytosis of HMGB1-partner molecule complexes and subsequent immune activation and modulation (Fan et al, 2020). RAGE is equally expressed in both macrophage phenotypes and consequently RAGE activation by HMGB1 promotes pro-tumoral activities of M2 macrophages (Rojas et al, 2016).

Although we noticed a significant decrease in growth within the initial 3 days, this is likely attributed to gradient penetration effects. BoxA primarily impacts cells in the outer layers, while growth persists in the inner regions. Spheroids inherently exhibit a gradient of nutrients and drugs from the periphery to the core, potentially impeding drug access to the inner core due to diffusion barriers posed by cellular layers. This phenomenon can significantly influence the efficacy of treatments.The upregulation of iNOS gene in M2 populations after treatment of BoxA may be explained by the activation of NFkb pathways that are involved in modulating macrophages' gene transcription during their activation (Mussbacher et al, 2023; Arias-Salvatierra et al, 2011; Jia et al, 2013). In addition, investigations focusing on tumor-cell-derived factors reveal that TAMs' NF-kB activation occurs in a calcium-dependent manner, inducing an M2-like pro-tumoral, anti-inflammatory phenotype in macrophages (Ryan et al, 2014; Zhang

et al, 2022). The inhibition of NF-kB pathways using BoxA by RAGE or Toll-like receptor 4 (TLR4) can be a possible explanation.
TLR4 is also involved in promoting pro-IL-1β production through HMGB1 interaction (Maroso et al, 2010). Secreted IL-1β interacts with IL-1R on MM cells via a paracrine mechanism which acquires a cancer stem cell-like phenotype promoting spontaneous spheroid formation (Horio et al, 2020). Blocking TLR4, BoxA might be able to ameliorate the outcome of MM patients by interfering in pro-tumoral contributions of M2-like M0s and reducing tumor growth.
To confirm whether BoxA has the potential to modulate macrophage responses and inhibit mesothelioma tumor growth, it is necessary to use a negative control for the next experiment, such as BoxB, which does not interact with BoxA receptors.
In the future this model could be used as a drug screening platform for compounds, or strategies that alter M0s-MM cell interactions could be tested. Not only murine or commercial MM cell lines but also primary tumor cells obtained from bioptic specimens of patients can be used in this system, allowing the assembling of personalized precision medicine.

In recent years, the spotlight has shifted towards the imperative need for robust preclinical models that faithfully replicate the intricate facets of the TME. This necessity is driven by the urgency to discover and evaluate novel cancer therapeutics effectively. In response to this demand, 3D spheroids and organoids have ascended in prominence as invaluable tools in the scientific arsenal.

Conclusion

This thesis underscores the role of innovative preclinical models, particularly 3D culture, in unraveling the complexities of Malignant Mesothelioma and devising novel therapeutic strategies. The scientific hypothesis explored the dynamics of tumor-immune cell interactions, with a specific focus on macrophages within the tumor microenvironment. The findings elucidated the pivotal influence of macrophage polarization on tumor progression and response to therapy.

Despite encountering technical challenges and resource constraints, the thesis made significant strides in establishing patient-derived MM organoids, which faithfully recapitulated the histological diversity of original tumors. Through different techniques, the thesis demonstrated the potential of spheroids in reproduce the crosstalk between tumor cells and the immune microenvironment, particularly the polarization of macrophages towards a pro-tumoral M2 phenotype.

Furthermore, our observations demonstrate that macrophages infiltrated MM spheroids, exhibiting sustained phagocytosis activity and the upregulation of pathways associated with migration.

Moreover, the thesis explored the therapeutic potential of targeting macrophage responses in MM using recombinant BoxA, which showed promising results in inhibiting tumor growth and modulating macrophage behavior. This approach highlights the importance of understanding and manipulating the TME for developing effective anti-cancer therapies.

Despite encountering setbacks such as the COVID-19 pandemic disrupting research activities, the thesis underscores the resilience and adaptability of scientific endeavors in the face of challenges. Looking ahead, the established 3D models hold immense promise as platforms for drug screening and personalized precision medicine, offering new avenues for combating MM and advancing cancer research. Through continued exploration and innovation, these models pave the way towards a deeper understanding of tumor biology and the development of targeted therapies for MM and beyond.

Materials and methods

Cell lines

AB1 mouse mesothelioma cells (Cell Bank Australia) were cultured at 37°C under 5% CO2 in RPMI 1640 (Life Technologies) supplemented with 5% v/v fetal bovine serum (Life Technologies), respectively, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin.

GFP-expressing AB1-GFP cells were previously described (Mezzapelle *et al*, 2016, 2021)

All cell lines were passaged for no longer than 10 passages after thawing. Cell lines were routinely tested for mycoplasma contamination by PCR.

RNA extraction and real-time PCR analysis

Total RNA was extracted from cells and tumors using NucleoSpin RNA (Macherey-Nagel) and treated with DNase I. The amount of total RNA was determined using a NanoDrop Spectrophotometer (Thermo Fisher Scientific). Next, 1 µg of total RNA was reverse-transcribed using Superscript IV Vilo Reverse Transcriptase (Thermo Fisher Scientific) following the manufacturer's protocol. PCR analysis was performed using Sybr Green Master Mix (Roche Diagnostics).

Expression of mouse and human macrophage marker genes was evaluated using the following primers

Mouse genes:

- CD206

Fwd: 5'- ATG CCA AGT GGG AAA ATC TG - 3'

Rev: 5'- TGT AGC AGT GGC CTG CAT AG - 3'

- iNOS

Fwd: 5' - AGC CAA GCC CTC ACC TAC TT - 3'

Rev: 5' - TCT CTG CCT ATC CGT CTC GT - 3'

- Egr2

Fwd: 5' - CTA CCC GGT GGA AGA CCT C - 3'

Rev: 3' - AAT GTT GAT CAT GCC ATC TCC - 3'

- F4/80

Fwd: 5' - CCC CAG TGT CCT TAC AGA GTG - 3'

Rev: 5' - GTG CCC AGA GTG GAT GTC T - 3'

- Fpr2

Fwd: 5' - TCT ACC ATC TCC AGA GTT CTG TGG - 3'

Rev: 5' - TTA CAT CTA CCA CAA TGT GAA CTA - 3'

- β-actin (used to normalize the results)

Fwd: 5'- AGA CGG GGT CAC CCA CAC TGT GCC CAT CTA - 3'

Rev: 5’- CTA GAA GCA CTT GCG GTG CAC GAT GGA GGG - 3’

Human genes:

- MRC1

Fwd: 5’ – AGC CAA CAC CAG CTC CTC AAG A – 3’

Rev: 5’ – CAA AAC GCT CGC GCA TTG TCC A – 3’

- iNOS

Fwd: 5’ – GCT CTA CAC CTC CAA TGT GAC C – 3’

Rev: 5’ – CTG CCG AGA TTT GAG CCT CAT G – 3’

- CD11b (used to normalize the results)

Fwd: 5’ – GGA ACG CCA TTG TCT GCT TTC G – 3’

Rev: 5’ – ATG CTG AGG TCA TCC TGG CAG A – 3’

Generation of macrophages and polarization to M1 and M2

Bone marrow cells were isolated from the femurs of 8-week-old male BALB/c mice. Macrophages were obtained by culturing bone marrow cells in DMEM containing 10% (v/v) FCS, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, 1x non-essential amino acids, 1x sodium pyruvate, 1% (v/v) β-mercaptoethanol supplemented with macrophage colony-stimulating factor (CSF-1, 20 ng/ml) for 6 days. On day 4, the medium was replaced with fresh M-CSF, and on day 6, adherent bone marrow-derived macrophages (BMDMs) were harvested.

After 3 days (day 7 of BMDMs differentiation) macrophages were treated for 24 hours with the above-mentioned DMEM, supplemented with 100 ng/mL of LPS and 40 ng/mL of IFN-γ for M1 polarization, or with 40 ng/mL of IL-4 and 40 ng/mL of IL-13 for M2 polarization.

Generation of AB1-GFP spheroids

AB1-GFP spheroids were generated by hanging drop or by forced floating techniques. Complete DMEM medium was used to generate 30 µL hanging drops containing 1000 cells and left incubating for 4 days, allowing cells to aggregate until spheroids were fully formed.

Forced floating generation of spheroids was achieved by plating 5.0 x 10^6 AB1-GFP cells in 10 mL of DMEM complete medium in a 10 cm non-adherent bacterial cell culture dish for 4 days. The lack of adherence to the culture plate forced the cells to float and assemble into spheroids.

Spheroids co-culture

BMDMs were dethatched after differentiation using Versene (0.5 μM EDTA in PBS), counted, and resuspended in a complete DMEM medium. A suspension of $30*10^3$ BMDMs/500μL was aliquoted in each well of an ultra-low adhesion 24-well plate (Corning product #3473). One hanging drop was then transferred to each well. Co-cultures were carried on for 10 days and 100 μL of fresh medium was added daily to each well. Forced floating co-cultures were performed by removing the culture medium from fully differentiated BMDMs in bacterial Petri dishes and replacing it with the spheroid suspension generated from forced floating.

Spheroids were imaged daily by fluorescent microscopy. Green color thresholding was applied by ImageJ software to measure the area of AB1-GFP spheroids.

Histology

All samples were processed by the Mouse Clinic Histological Facility of the San Raffaele Hospital.

Spheroids and organoids were fixed in formalin for 16h, while Tumor masses were explanted from each sacrificed mouse, fixed in zinc-formalin for 24h, processed with Leica TP1020, embedded in paraffin, and sectioned. Briefly, the sections (3 μm) were deparaffinized in xylene and rehydrated in graded alcohol.

For mouse samples:

Immunohistochemical staining was performed using the following antibodies: anti-F4/80 (clone A3-1 Bio-Rad). Slides were counterstained with hematoxylin and mounted.

For human samples:

Immunohistochemical staining was performed using the following antibodies:

rabbit monoclonal antibody against calretinin (abcam, ab92341, dilution 1:100), vimentin (EPR3776, abcam, ab92547, dilution 1:200), WT-1 (abcam, ab216646, dilution 1:100), mesothelin (abcam, ab93620 dilution 1:50), mouse monoclonal cytokeratin 5/6 (D5/16 B4 abcam, ab 17133) and rabbit polyclonal BAP-1 (abcam, ab199396).

Cytofluorimetric analysis of spheroid invasion

Spheroids incubated with BMDM as described above, were starved from a dish and washed with PBS to remove free-floating BMDMs. AB1-GFP spheroids co-cultured with BMDMs were dissociated to single cells using TrypLE Express (Gibco) and incubated for 5-10 minutes at 37°C on a shaker. BMDMs were stained with mouse antibody: APC-780 anti-F4/80 (Invitrogen clone: BM8), PE-Cy7 anti-CD11b (BD Pharmigen

Clone: M1/70), FITC anti-CD86 (Biolegent clone: GL-1), Alexa-647 anti-CD206 (Biolegend clone: C068C2); live cells were negative for Zombie Violet™ dye staining (BioLegend). For supplementary we used human antibody PE anti-CD47 (BD Pharmigen).

Imaging flow cytometry

Acquisition of single-cell images was performed with the flow cytometer imager Merck Amnis Imagestream 100 (Merck-Millipore). AB1-GFP spheroids co-cultured with BMDMs were dissociated to single cells using TrypLE Express (Gibco). BMDMs were stained with an APC-780-conjugated anti-F4/80 antibody (Invitrogen clone: BM8); live cells were negative for Zombie Violet™ dye staining (BioLegend). Images were processed and analysed with IDEAS software by Desiree Zamboni, operator of Alembic Microscopy and Imaging Facility of San Raffaele Hospital.

The internalization features is define as ratio of the intensity inside the cell to the intensity of the entire cell. The higher the score, the greater the intensity inside the cell. All pixel are background-subtracted. The user must create a mask to define the inside of the cell for this feature. The feature is invariant to the cell size and can accommodate concentrate bright regions and small dimensions spots. The ratio is mapped to a log scale to increase the dynamic range to value between (-inf,inf). Internalized cells typically have positive scores while cells with little internalization have negative score. Cells with scores around 0 have a mix of internalization and membrane intensity.

Chromium Next GEM Single Cell 3' v3.1: Cell Multiplexing

Following Protocol 1 Overview : Cell Multiplexing Oligo Labeling of 10X Genomics manual (https://www.10xgenomics.com/support/single-cell-gene-expression/documentation/steps/sample-prep/cell-multiplexing-oligo-labeling-for-single-cell-rna-sequencing-protocols).

SCT was conducted on the 10x platform by COSR Facility of San Raffaele Hospital. scRNA-seq libraries were pooled and sequenced on the NOVAseq instrument (Illumina). The sequencing setup included an 8-base index read, a 28-base Read 1 containing 10X cell-identifying barcodes (CBs) and unique molecular identifiers (UMIs), and a 100-base Read 2 containing the transcript sequence.

Raw sequencing data underwent processing using the cellranger pipeline (v6.3; https://www.10xgenomics.com/support/software/cell-ranger). This process generated digital gene expression matrices, which were then analyzed using the 'Seurat' package in R. Cells were filtered to allow a maximum percentage of

mitochondrial gene expression of 5%. For Day 0 only, cell doublets were estimated using functions from the DoubletFinder package (https://github.com/chris-mcginnis-ucsf/DoubletFinder) and filtered accordingly.

Cells at Day 0 were utilized to analyze M0, polarized macrophages, and AB1 cell populations. The expression of EGFP, combined with cell clustering, was employed to distinguish AB1 from macrophage cell populations. Firstly, positive markers expressed in AB1 EGFP-positive cells, compared to macrophages (FDR <= 0.05 and log2FC >= 2), were identified.

Subsequently, for each cell, we calculated the ssGSEA (single-sample Gene Set Enrichment Analysis, https://github.com/broadinstitute/ssGSEA2.0) score based on AB1 positive markers. A binary classifier was then constructed by selecting a cutoff for the ssGSEA score using Receiving Operator Curve (ROC) analysis. This classifier was applied to time-series data to distinguish AB1 and macrophages in co-cultures.

Clusters of macrophages displaying either EGFP or some AB1 markers positivity underwent differential expression analysis using the 'FindAllMarkers' function of the 'Seurat' package, with the option 'min.pct = 0.1, only.pos = TRUE', and the default parameters for the rest of the analysis.

Markers identified in the last step were further analyzed using EnrichR to perform gene ontology analysis.

MM samples

The use of all human samples was approved by the ethical committee of IRCCS Ospedale San Raffaele. All patients provided informed consent before sample acquisition.

The tumor biospecimens were delivered within one hour of removal to the lab for cell processing or storage in liquid nitrogen. Once received, the sample was washed in phosphate-buffered saline (PBS) with 2% penicillin-streptomycin and then processed for the generation of primary cells or organoids.

Human primary cell lines

Tumor specimen was cut into small pieces using sterile sharp blades. Then, the fragments were incubated with enzyme collagenase/hyaluronidase + 1.25 U/ml dispase for about 10 minutes at 37°C on a shaker. After that mashed through a 100 µm cell strainer with the plunger of a syringe, taking care to thoroughly rinse the strainer with culture medium to minimize cell loss. The cell suspension was collected into 50 ml- sterile falcon, and the further cell disaggregation is stimulated by pipetting cell suspension up and down at different times using a 10 ml pipette. The

disaggregated cells of were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum and 1 × penicillin/Streptomycin (Gibco BRL Life Technologies), 1X Glutamax (Gibco). The cells were incubated at 37°C in 5% CO_2 with a balance of air at 37°C. After 3-4 days, the medium was replaced to remove nonadherent cells; thereafter the medium was replaced twice weekly and cells were passaged when they reached confluency in a monolayer. The established cell line was stored in liquid nitrogen.

Human tumor-sphere formation

Human mesospheres were formed using either the hanging drop method or the BIOFLOAT™ 96-Well Plate, an ultra-low attachment plate. In the hanging drop method, a total of 30 µL of complete DMEM medium was utilized to create hanging drops, each containing 1000 cells. These drops were then left to incubate for 4 days, allowing the cells to aggregate until fully formed spheroids were observed.
For the BIOFLOAT method, cell seeding ranged from 2000 to 5000 cells per well, with a total volume of 100 µL. Spheroids were observed to form either 24 hours or 48 hours after seeding.

Generation of human mesothelioma 3D structure

The human primary cells generated from donor patient biopsies, previously described, and cultured in flask T75 were collected and pelleted. The cells were resuspended in 1-2ml of Cultrex and plated on 50ul drop per well in a 48-well plate and incubated in the incubator (5% CO2, at 37 °C) for 20- 30 min to allow the solidification of the Cultrex. The culture media used was DMEM/F12 (Euroclone) supplemented with Glutamax 1X (Gibco), Pen/Strep., 50 ng/ml human EGF (Peprotech). We monitored the structure's growth every 3 days with an inverted microscope (Axio Observer.Z1, Zeiss) and changed the medium every 2 days post-seeding. We changed Cultrex after 3 weeks of organoid culture.

Generation of Patient-Derived Xenograft from mesothelioma biopsies

The MM specimens were used for performing direct xenograft establishment into Immunodeficient mice NSG (NOD/SCID Il2rg – / –). Immunodeficient mice NSG aged 6 to 8 weeks should be housed under specific pathogen-free conditions to prevent sickness and infectious outbreaks. Tumor fragments (0.7-1.0 cm) were implanted subcutaneously, in the flank of mouse, in a heterotopic site that permit us to monitor accurately and measure the tumor size. The tumor implantation will be

performed as described in the protocol (Kim et al, 2009).After tumor implantation, mice should be monitored daily for signs of illness and surgical wounds assessed for infection. To maintain and expand tumors derived from a specific xenograft 'line', we routinely propagate heterotopic tumors.

Direct transfer of human tumors into immunodeficient mice requires an institutional review board as well as Institutional Animal Care and Use Committee (IACUC) approval and must be conducted following institutional and national regulations.

Generation of Mesothelioma Organoids

Fresh mesothelioma biopsies were collected, immediately frozen in FCS 10% DMSO and stored in liquid nitrogen. Biopsies were then thawed and minced using scalpels into small pieces (1-5 mm3). The small tissue fragments were collected and mashed into a cell strainer of 70 µm until cell aggregates were obtained. If necessary, red blood cells were removed using a red blood lysis buffer (eBioscence). After washing/filtering, cell aggregates generated by mechanical disaggregation were embedded in Cultrex© Reduced Growth Factor Basement Membrane Extract, Type 2, Pathclear (# 3533-010-02 | R&D Systems, Bio-Techne) and plated into plate 24-well (Corning Costar). The culture plate with just-seeded cell aggregates enclosed in Cultrex was placed in the incubator (5% CO2, at 37 °C) for 20- 30 min to allow the solidification of the Cultrex. We refresh the medium with 10 µM Y-27632 dihydrochloride every 2–3 days. To make up the medium, combine B27 supplement (#17504-044, Life technologies, Gibco), N2 supplement (##17502, Life technologies), 100 ng/ml of Noggin (#120-10 C, Peprotech), 50 ng/ml of rhEGF (#AF-100-15, Peprotech), 10 ng/ml of recombinant human FGF-basic (#100-18B, Peprotech), FGF-10 (Peprotech), FGF-7 (Peprotech), R-Spondin (Bio-Techne), and Wnt conditioned medium 50%. Top it up to the desired volume with adDMEM/F12 (#12634-034, Life technologies) supplemented with HEPES (# 15630-056, Life technologies), L-Glutammine, Pen/Strep.,10 µM Y-27632 dihydrochloride. We monitored the organoid growth by inverted microscope (Axio Observer.Z1, Zeiss) every 2 days post-seeding. We changed Cultrex after 3 weeks of organoid culture. The organoids were subcultured by using a harvesting solution of Trevigen (#3700-100-01, Cultrex). The final cell aggregates pellet obtained were resuspended in matrix Cultrex. We replaced the medium every 2–3 days of culture.

Statistics

In all experiments each sample contained at least one technical duplicate; all experiments were performed two to three times. Statistical analyses were performed with GraphPad Prism software, version 8.4.1 (GraphPad Software, Inc.).

For the growth curve of Mesosphere cultured alone or in co-culture was performed a RM two-way ANOVA comparison matched values are stacked into a subcolums, on 3-4 different experiment composed by 8-12 single spheroid daily monitored each condition. For q-PCR was performed a t-test comparison.

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Milano, 21/03/2024

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