IJPPR
INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH
An official Publication of Human Journals
ISSN 2349-7203
HUMAN

Human Journals
Review Article
October 2023 Vol.:28, Issue:3

New In Vitro Methods for Cancer Research Using 3D Tumour Models

IJPPR
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An official Publication of Human Journals
ISSN 2349-7203
HUMAN

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Submitted: 18 September 2023
Accepted: 23 September 2023
Published: 30 October 2023

Keywords: Cancer Research, 3D Tumour Models

ABSTRACT

Each year, 10 million people die from cancer, a multifactorial disease. It is challenging to create single targeted approaches due to the intra- and inter-heterogeneity of malignant tumours. But it seems that they are frequently less suitable than the three-dimensional (3D) cell culture approach for simulating the biological behaviour of tumour cells, particularly the mechanisms causing therapeutic escape and drug resistance. The last 20 years have seen the development of immunotherapy as a cutting-edge and effective cancer treatment method. Preclinical tests for novel medicines are frequently unreliable, predictive indicators are frequently still poorly characterised, and mechanisms of action are sometimes still uncertain. Models used in vivo during research and conventional bidimensional culture systems may not accurately reflect the analysis of tumor/immune system interaction in this context, it still plays a crucial role. To connect in IVIVC systems, tumour cell cultures in three dimensions have been produced. Interesting characteristics of the interaction between tumour cells and cells from the innate and adaptive immune systems may also be reflected by 3D preparations. Here we evaluate in vitro models of cancer/immune cell interaction and suggest that new technologies may aid in the development of novel therapies, the identification of biologicals with potential clinical relevance, and the identification of patients who may benefit from immunotherapy.

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Introduction

According to the World Health Organisation (2011), cancer was one of the leading causes of mortality globally in 2007, accounting for around 7.9 million fatalities. A solid cancer is a tumour mass that is hypoxic at the centre and heavier than the surrounding tissue, such as those found in the breast, colon, stomach, lung, and liver. Cancer is marked at the molecular level by the cancer cells' escape from normal growth regulation and their acquisition of an invasive potential. Along with this, a supporting stroma that has a larger vasculature is formed. It takes beneficial chemical interactions between cancer cells and the tumour stroma for malignancies to spread and invade. These come together to form a solid tumour-specific clinically observable invasive tumour mass [1,2]. Epithelial cancer cells that form cancer foci are the primary cause of most malignancies (carcinomas). They engage in dynamic interactions with the extracellular matrix (ECM), which is a source of glycosylated proteins, and the stroma surrounding them, which is made up primarily of fibroblasts and endothelial cells (ECs, which line blood vessels). For growth and development, epithelial-stromal interactions are essential. For instance, Calmels et al. (1995) found that without a blood supply, the cancer mass cannot expand over 200 m in circumference [3,4]. Hypoxia-inducible factor (HIF1) is increased in cancer cells when there is a lack of oxygen. The angiogenic factor cascade proteins, such as vascular endothelial growth factor (VEGF), whose transcription is regulated in a temporally different manner, are afterwards promoted by this. In order to create new capillaries and provide the cancer cells that are multiplying with enough nutrients and oxygen, the latter draws ECs towards the cancer cells. The hypoxic centre of the cancer mass feeds the cycle of growth and vascularization. Inflammatory cells (macrophages, neutrophils, and mast cells) are also present in the tumour stroma. Since they produce pro-angiogenic factors (like VEGF and basic fibroblast growth factor [bFGF]) on demand at specific locations, these promote tumour aggressiveness through stimulation of angiogenesis (Pollard 2004).

Integrins, an ECM protein involved in cell-cell and cell-matrix interactions, are frequently the driving force behind cancer cell invasion. Through cascades like the focal adhesion kinase signalling pathway or the mitogen-activated protein kinase pathway, which regulate the invasive and metastatic progression of malignant cells, they play a crucial role in metastasis (Hemler et al. 1996; Dedhar and Hannigan 1996) [2,3,9].

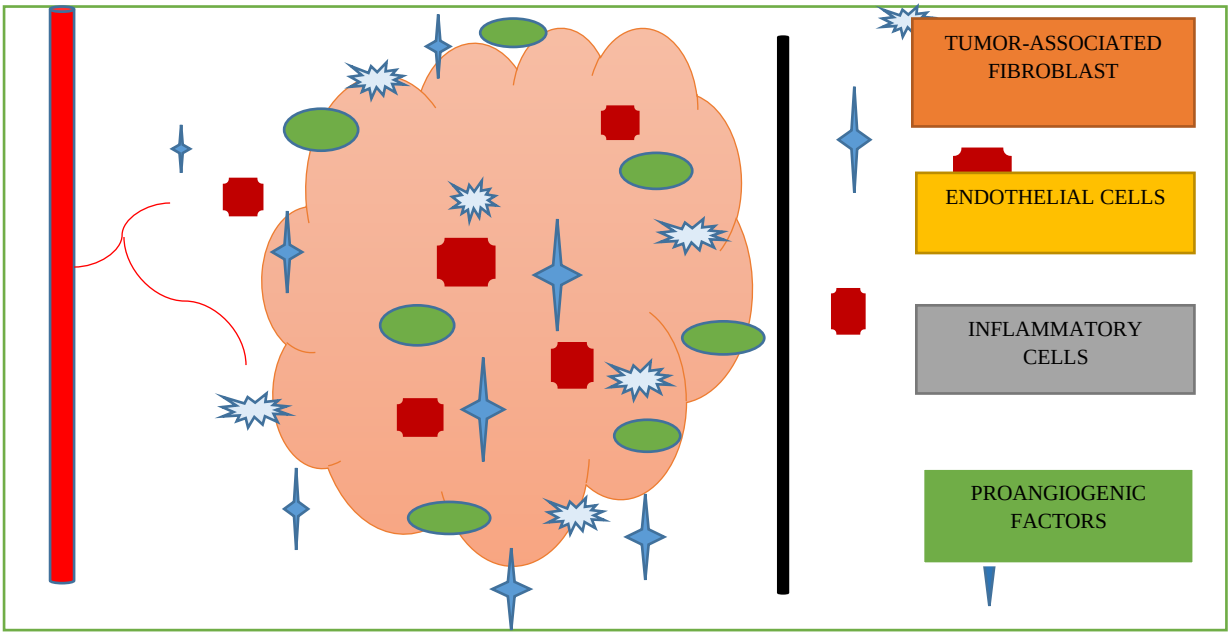

Fig.1. exhibiting the main supportive non-cancer stromal cells, key protein families, and tumour bulk with stroma. No specific cancer cells are displayed.

In controlling tumour growth and progression, the relationship between cancer cells and the immune system is crucial. Indeed, the ability to resist immune-mediated destruction and tumour-promoting inflammation are true cancer hallmarks. Studies on clinical samples have powerfully validated findings from experiments, and examination of innate and adaptive immune system infiltration of human tumours has revealed highly significant predictive connections. Most significantly, immunotherapies are now commonly used to treat individuals with malignancies with various histological origins [10,11]. For approximately 20 years, several monoclonal antibodies (mAbs) have been employed frequently in the management of cancer [12]. They were frequently created to stop the binding of growth factor-induced receptors expressed by tumour cells that promote their proliferation. However, immune cell-mediated processes like their clinical effectiveness are frequently shown to be mediated via phagocytosis and antibody-dependent cell cytotoxicity. Therapeutic mAbs may, in fact, play a crucial role in the cytotoxicity of target cells caused by lymphocytes or myeloid cells that contain activating Fc receptors, depending on their affinity and isotype. Whether it is preferable to kill target cells or only prevent them from interacting with certain ligands without killing them is a key question in mAb-mediated immunotherapy, especially in light of cutting-edge medicines that recognise markers released by immune cells. In the latter scenario, it would be advised to employ mAbs that bind inhibitory Fc receptors. Isotype

is becoming increasingly important for the success or failure of reagents recognising the same target molecule in light of the present uncertainty surrounding the mechanisms of action of some therapeutic mAbs. On the basis of this backdrop, chemicals with varying affinities and capacities to bind the Fc receptors expressed by effector cells are continuously being created [11,13]. Furthermore, advanced clinical experimentation is currently being conducted on bispecific mAbs that specifically target defined effector functions in tumour cells [14]. The most significant development is the successful testing and application of therapeutic mAbs that recognise immunological checkpoints in clinical settings over the past ten years. Their creation is justified by the theory that they are intended to prevent the physiological inhibition of adaptive T cell responses caused by interaction between antigen-specific T cells' activation markers and their ligands, which are expressed by cancer cells or antigen-presenting cells, respectively. Numerous cancers have shown benefit from releasing the brakes on antitumor responses. However, the mechanisms of action are still unclear, and markers that accurately predict clinical responsiveness have yet to be found. Anti-CD47 mAbs have also been employed to encourage macrophage phagocytosis of tumour cells along similar lines [15, 16].

Additionally, in the last 20 years, adoptive cancer immunotherapies have been created. They are founded on giving autologous cells to patients after in vitro expansion and culture. Current adoptive therapies typically rely on the use of T cells from patients transduced with genes encoding conventional or enhanced-avidity HLA-restricted T-cell receptors recognising tumor-associated antigens, or chimeric HLA-unrestricted antigen receptors (CAR) recognising surface molecules expressed in high levels by cancer cells. Clinical trials are currently being conducted that also focus on solid malignancies, despite the fact that these technologies are mostly employed to treat haematological malignancies [12, 17].

As a result of these discoveries, a sizable number of novel biologicals and methods for cancer immunotherapy are being created and tested in clinical studies. [18]. There is currently an unheard-of explosion of information and applications in this field of study, which necessitates the development of preclinical assays and the identification of individuals who may benefit from treatment [20].

3D Models

Connections between individual cells and the extracellular matrix cannot be fully understood using culture in two dimensions that are based on the development and proliferation of a

monolayer of cells. With the help of 3D culture models, cells become polarised between a basal and an apical pole, which results in changes to proteins and the genome. When a tumour is present, the extracellular matrix and the tissue microenvironment are both changed. This manifests as a change in the distribution of nutrients, oxygen, and metabolites as well as in cell growth and communication. All of the 2D models, however, fall short of accurately simulating in vivo interactions because they cannot simultaneously evaluate each of these crucial characteristics. Compared to 2D cultures, greater immuno-modulatory properties than 2D models, such as melanoma-derived spheroids. , proliferative, and activating properties [21].

Immune checkpoint molecules express differently in vivo compared to 2D culture models. This makes it crucial to adopt 3D cell culture models that more faithfully represent the TME. Spheroids and organoids are two terms used to describe 3D cell culture models. Technical developments allowed for the complexification of cocultures in both of these models, improving the TME representation [22,20]. Tumors-on-ship and bioprinting do, in fact, combine technology and 3D culture to mimic fluid dynamics or tumour cell architecture. However, the semantic distinction between spheroid and organoids is hazy and appears to be based on the author's personal preference [23].

To preserve the tissue architecture and tissue variety when undigested or lysed tissue is grown in an extracellular matrix, tumour organoids should be utilised. As a result, we categorised 3D models into a gradient of complexity, with cellular composition at its core, as seen in **Figure 2.**

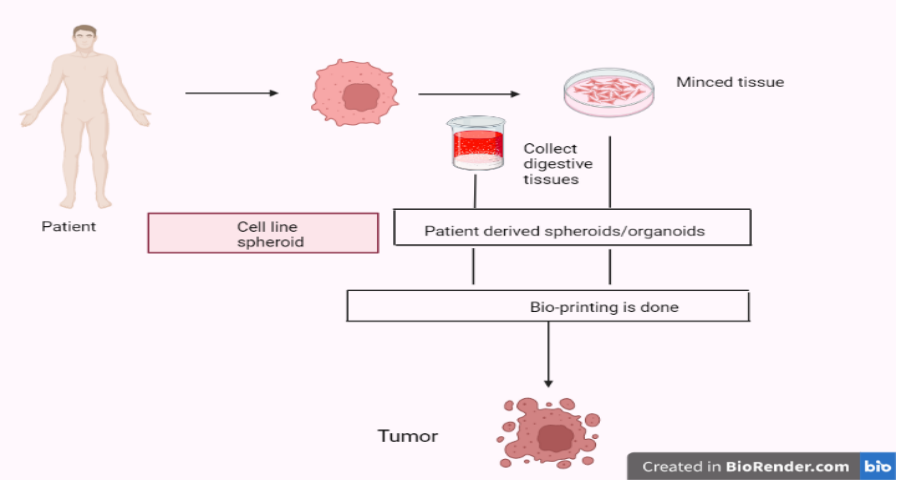

FIGURE 2. 3D culture models are represented based on how complicated they are. Spheroids made from a single cell line to extremely complex models made from patient tissue or tumours improved with a microfluidic chip are all examples of 3D culture models. Tumour biopsy and 2D culture are employed as complexity references. Cell line-derived models and patient-derived models can be distinguished in 3D cultures. Tissue mincing, or tissue mincing and enzymatic digestion, are both necessary for patient-derived 3D models before the culture. Notably, bioprinting can be used to create the majority of models that call for cell type-dependent structures in multiple cell types and can be used directly on microfluidic chips.

The Current State of Modelling Human Tumor-Immune System Interactions

Experimental animal models used in vivo have provided important information that has been used to produce therapeutic mAbs and novel immunotherapy techniques. The availability of enough quantities of newly isolated tumour or immune cells as well as autologous immune/tumour cell systems has proven to be a challenge in vitro research using human cells, which have been more challenging. In addition, while having a comparable histological origin, developing established tumour cell lines from clinical specimens is still a difficult task, and the inherent variability of human tumours should not be overlooked [23,24]. However, conventional in vitro models have consistently shown to be of the utmost significance in human immunology, and particularly in tumour immunology. Standard bidimensional cultures have made it possible to grow tumour-infiltrating lymphocytes, create T cell clones that specifically target tumours, and keep track of how well therapeutic antitumor vaccinations are working.

51Cr release assays have served as the gold standard for identifying human tumour-associated antigens [25]. For the evaluation of adaptive T-cell responses, flow-cytometry

techniques based on the detection of cells expressing T-cell receptors that recognise antigenic peptides restricted by defined HLA determinants, such as multimers, are frequently supplemented by the analysis of intracellular cytokine expression upon antigenic triggering. [26,27]. In conjunction with these methods, so-called Elispot tests are routinely used to pinpoint individual cells that, in.

Why Novel Tumour Immune System Interaction Models Are Vital?

According to in vitro research, macrophages and T and NK cells can effectively elicit antitumor actions under specific test settings. The clinical success of immunological checkpoints targeted therapy, however, also indirectly suggests that cytotoxic tumour infiltrating T cells are commonly dysfunctional in vivo [28]. Additionally, immune-histochemical research suggests that solid tumours most frequently lack NK cell infiltration that can be seen. More importantly, macrophage infiltration of solid tumours is typically linked to a poor prognosis, except for a few cases, such as colorectal cancer (CRC) [29,30].

Immune cell functional profiles that differ between in vivo and in vitro have sparked an investigation into the factors that lead to. Alternatively activated macrophages, regulatory Hypoxia and adenosine receptor triggering, as well as the production of ligands for immunological checkpoints, have all been demonstrated to be immunosuppressive mechanisms at work in the tumour microenvironment [31,32]. The responsiveness of lymphocytes may be significantly impacted by oxygen levels, according to earlier research. More recently, a significant number of studies seem to suggest that hypoxia and specific metabolic conditions present in tumour tissues may act as a unifying background for a number of previously noted immunosuppressive mechanisms and significantly reduce the potential efficacy of anticancer immune responses. Hypoxia has been found to promote immunological tolerance by attracting Tregs. It is intriguing that metabolic changes in tumour tissues have been linked to PD-1 immune checkpoint expression [33]. The battle for glucose between tumour cells and T-cell receptor-triggered, antigen-specific T cells, both of which are characterised by aerobic glycolysis, seems to be a significant issue. Additionally, increased glycolysis has been linked to pro-tumor M2 macrophage activation, and hypoxia has been linked to the emergence of myeloid-derived suppressor cells within the tumour microenvironment **(Figure 4).**

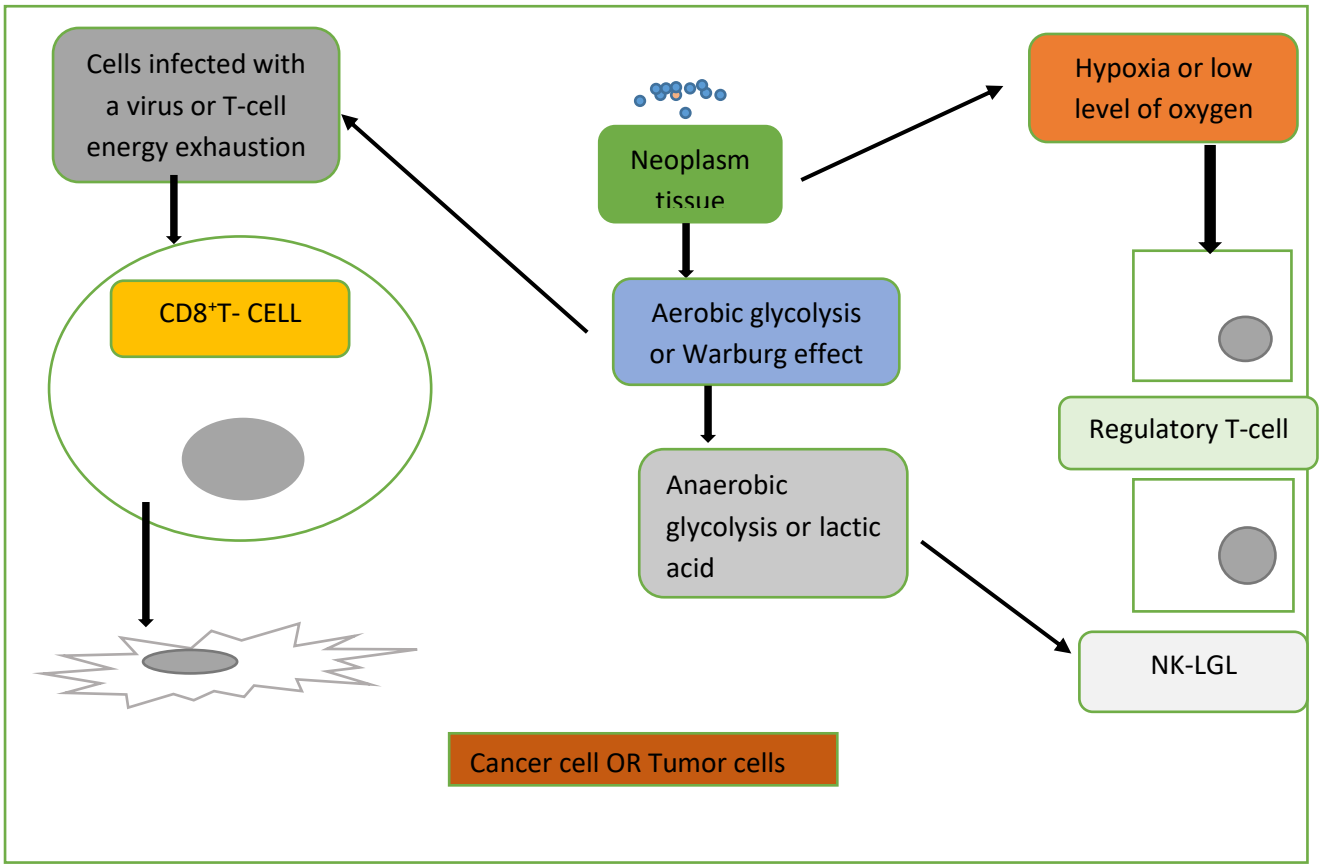

FIGURE 3. Hypoxia and aerobic glycolysis, among other metabolic characteristics, are present in the in vivo tumour microenvironment, where they cause competition between tumour and immune cells for glucose and other resources and the formation of lactic acid.

Methods of 2D vs. 3D Cell Culture

Table.1. 2D versus 3D Cell Culture Methods

Characteristics	2D	3D
Cell morphology	Loss of epithelial cell polarity and altered cell shape, which is typically flat and elongated.	Cells continue to proliferate in 3D aggregates while maintaining their native structure and polarisation.
Gene expression	Typically, genes associated with cell adhesion, proliferation, and survival are altered	Representation of gene expression patterns accurately
Cell proliferation and differentiation	Poor cell differentiation and excessively high	Cells are well differentiated; proliferation is realistic

	proliferation are both present in cells.	depending on 3D matrix interactions
Cell interactions	Deprived of cell-cell and cell–ECM interactions, no cell niches are created	Cell junctions are common and allow cell communication
Tumoral heterogeneity	Basic; all cells receive the same amount of nutrients; inaccurate replication of the TME	Better approximation and representation of the TME; nutrients are not equally supplied
Response to stimuli	Inaccurate representation of mechanical and biochemical cues	Cells grow in a 3D environment and receive stimuli from all directions that properly represent in vivo stimuli
Reproducibility	Highly replicable	Difficult to replicate some conditions
Analysis and quantification	Easy interpretation of results; better long-term cultures	Difficult to analyze data, especially with multiple cell types or when in spheroid/organoid conformation
Cost	Cheaper for large-scale studies	More complex and expensive techniques

Three-dimensional modelling of interactions between the immune system and human tumours

In an effort to address the high attrition rate in the creation of novel anticancer drugs, numerous tridimensional culture models have been developed in the past. Identifying the metabolic processes, gene expression patterns, and sensitivity to treatment resistance of cancer cells. These results have motivated the development of innovative high-throughput drug screening tools, which are now being applied in pharmacological research. Early research involved creating multicellular spheroids by preventing tumour cells from adhering to plastic cell culture surfaces. Later technologies based on microfluidics, scaffolds, and hanging drops were developed with success[23,34].

Spheroid size control has made it possible to create structures with controlled levels of hypoxia, and perfused bioreactors have proven effective at producing tissue-like structures from well-established human tumour cell lines [35]. In this context, it is also remarkable that human cancer cells with the ability to initiate tumours, also known as tumour-initiating cells (TIC) or cancer stem cells (CSC), are often characterised by the capacity to produce spheres

that are able to slowly replicate with asymmetric divisions in tumours with a variety of histological origins, such as the colon, breast, and CNS.

Models with increased complexity are constantly being created with the goal of incorporating more tumour microenvironment elements that have been shown to be important for clinical outcomes and the emergence of treatment resistance. The scientific community is also becoming more and more interested in the physical characteristics of tumour tissues and the potential for recreating them accurately in vitro. Microfluidics models have been created in particular to address drug sensitivity and cancer cell dispersion, tumour lymphatic vessel interaction, and homing of tumour cells to specific metastatic niches. It's interesting to note that the earliest 3D culture models were initially created to study the immune response to solid tumour allografts [34, 36].

In light of the foregoing, it is surprising that only a small number of research have examined how 3D culture of tumour cells affects those cells' susceptibility to the actions of lymphocyte effectors [37]. Early research revealed that the breakdown of this architecture was a crucial prerequisite for the full elicitation of antitumor cytotoxicity and that cytokine-activated lymphocytes had difficulty targeting tumour cells cultivated in three dimensions. The effector activities of T cells are significantly compromised when target cells are arranged in 3D structures, as we and others have recently discovered [38].

Numerous mechanisms have been put forth. Ineffective antigen presentation, according to Dangles-Marie et al., may be caused by tumour target cells' reduced expression of the heat shock protein-70 [39]. Following culture in spheroids, we found that cells from well-established melanoma cell lines may down-regulate expression of HLA and melanoma differentiation antigens. It's interesting to note that clinical melanoma specimens with hypoxic areas showed lower expression of the Melan-A/MART-1 differentiation antigen [32,34].

On the other hand, lactic acid production increases in 3D-cultured cells compared to 2D-cultured cells. Notably, the levels of lactic acid produced under these circumstances are sufficient to significantly reduce the activation of antigen-specific cytotoxic T lymphocyte (CTL) clones' effector functions, establishing a crucial connection between the typical metabolic abnormalities of tumour cells and T cell functional impairment.

Natural killer (NK) cell cytotoxicity against targets cultivated in tridimensional structures has also been described, as has the study of NK lymphocyte infiltration in scaffold-free and 3D Matrigel-based models. Increased HLA-E expression by tumour cells has been specifically linked to tumour cells' resistance to NK lymphocyte-mediated cytotoxicity in 3D glioma models. It has been demonstrated that NK and Treg contact with breast cancer cells in 3D leads to an increase in the creation of inflammatory cells that are pro-tumor significant and CCL4-attractive. There aren't many studies looking into B-cell tumour-cell interaction in 3D designs, despite the fact that they might be important in the cancer microenvironment. The analysis of tumor/lymphocyte interaction has most recently also been proposed using models based on microfluidic technology [35].

Lymphocytes have been the subject of several works on tridimensional modelling. But human cancers also frequently contain macrophages and other myeloid cells. By cytokine treatment, monocyte/macrophage lineage cells from murine and, M1 macrophages with anticancer potential or M2 macrophages can be polarised into human tissues. Which have a pro-angiogenic functional profile and have been proven to be more tumor-supportive [36]. It is crucial to keep in mind that the M1/M2 polarisation concept is a convenient oversimplification of a process that is actually best described as a continuum. However, it has been demonstrated that tridimensional tumour spheroids can significantly alter the differentiation and functional profiles of monocytes and macrophages [34,39].

It has been demonstrated that coculture of squamous cell carcinoma cells with human and murine macrophages in 3D architectures, in the presence or absence of fibroblasts, promotes the polarisation of these cells towards an M2 functional profile and induces metalloproteases (MMP) production, favouring tumour invasiveness as related to increased extracellular matrix degradation. Similar findings were also made in trials using cell lines from bladder, breast, thyroid, and hepatic cancers. In each of these instances, changes in the chemokine secretome in 3D cultures with tumour cells and macrophages, whether fibroblasts were present or not, were consistently seen. Using microfluidic devices, it has been demonstrated that M2 macrophages preferentially bind to NSCLC cells grown in aggregates, hence promoting their migration and EMT. In this work, macrophages cultivated under various settings that may have been connected to intermediate polarisation stages were evaluated side by side. TNF and TGF-1 released by macrophages have most recently also been found to promote tumour cell motility in a 3D extracellular matrix [36,37].

Importantly, however, it has been demonstrated in 3D cultures, including microfluidic models, that the lactic acid produced by tumour cells inhibits the antigen presentation and differentiation capacity of DCs (Figure 5). According to these findings, tridimensional models could be utilised to analyse, under controlled circumstances, the interactions that occur in vivo between tumour cells and cells of the monocyte/macrophage/dendritic cell lineages (Table 2).

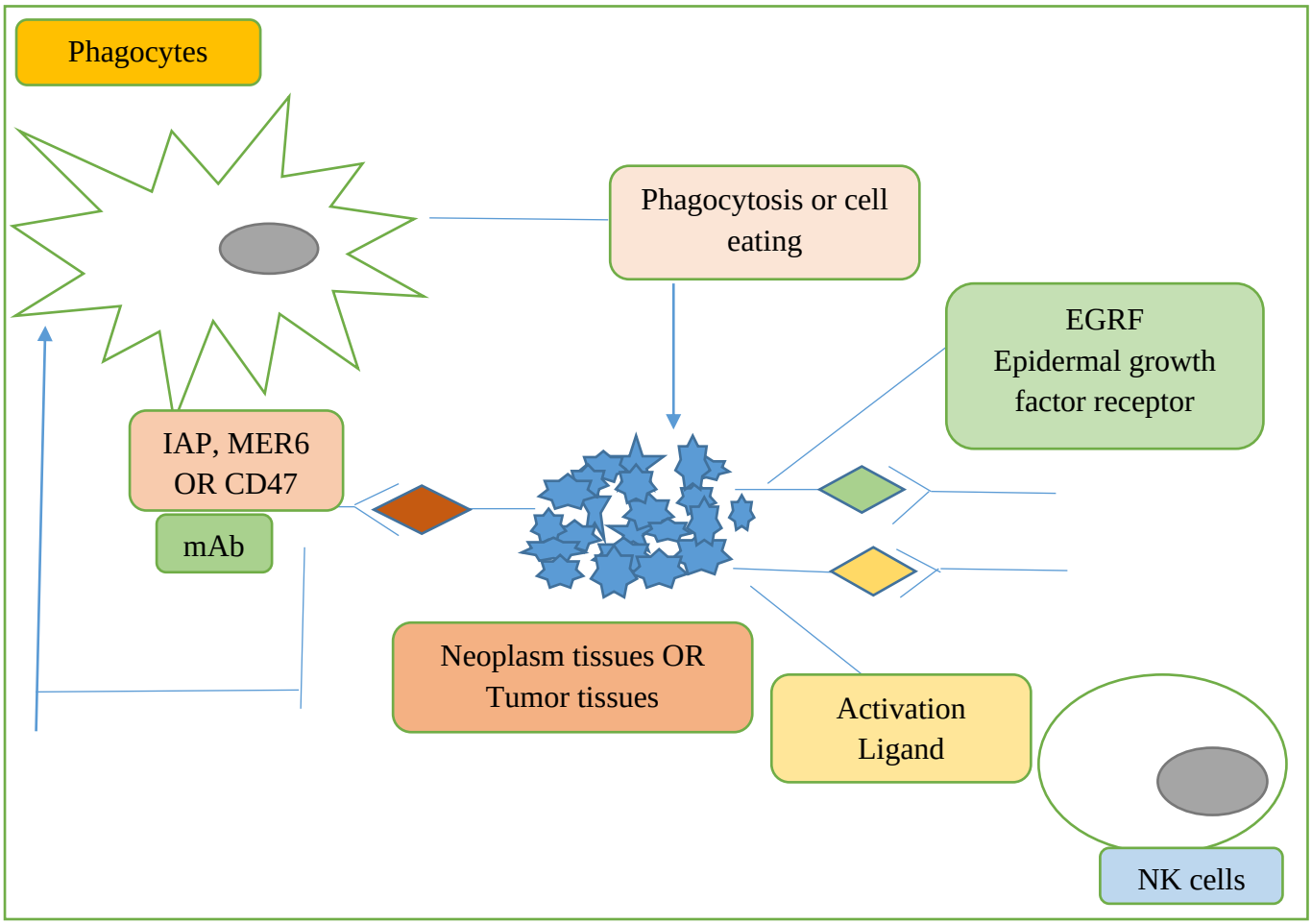

FIG. 4. Immune cell effector actions target tumour cell spheroids. Verification of the impact of tridimensional culture on a range of immune cell functions has been done using tumour cell spheroids produced by various techniques. T-cell clones that recognise antigens linked to melanoma have been cocultured with the cancer cells (panel A). Similar testing has been done on CAR-transduced cells for adoptive therapies. Additionally evaluated were the roles of cells from the monocyte/macrophage lineage in phagocytosis and antigen presentation. Furthermore, using target cells cultured as spheroids, NK cells have been studied in relation to antibody-dependent cell cytotoxicity. (panel B).

Table.2.

	3D CULTURE SYSTEM
ASSAYS OF CYTOTOXIC T LYMPHOCYTE ACTIVITY	Spheroids Engineered tumour models
CD107 ASSAY	Spheroids
DENDRITIC CELLS/ MONOCYTES	Spheroids
–CANCER INTERACTION CELLS	Microfluidic devices
ANTIBODY DEPENDENT CELL-MEDIATED, THERAPEUTIC MABS	Spheroids
DRUG EXAMS IN ENGINEERED TME	Spheroids, microfluidic devices, and bioreactors are examples of in vitro-created tissue models.

TME: tumor microenvironment.

It's interesting to note that recent research has also found that neutrophil polarisation shares functional characteristics with macrophages. However, the consequences of polarisation during incubation with 3D-cultured cancer cells have not yet been examined. , perhaps as a result of challenges inherent in a granulocyte culture, and more research in this area is necessary.

Conclusion:

In vitro models will never be able to capture the immense complexity of cancer progression in vivo, which is all too clear. However, they might offer the chance to put cutting-edge therapies and fundamental science hypotheses to the test under very strict controls. Due to the significant advancements of the last two decades, there have been an unprecedented number of preclinical and clinical studies on tumour immunobiology and immunotherapy. Innovative in vitro technologies must be developed in order to evaluate the efficacy of novel treatments. Surprisingly, it will be necessary to test these treatments' potential toxicity. In this field, on-target/off-target reactivity, tumour lysis syndromes, and cytokine release do pose serious

issues that call for the development of suitable in vitro models. The investigation of tumour genomes and the tumour microenvironment, on the other hand, is challenging the present tumour classification and staging criteria, which are typically used to select patients for conventional therapy procedures. An additional motivator for the development of novel culture technologies may be the growing desire for personalised treatments. The in vitro modelling of tumour immune-system interactions has a promising future, as may be easily predicted given.

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