The next level of 3D tumour models: immunocompetence

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Teaser

This article reviews the state-of-art regarding 3D models used to study interactions between cancer cells, stroma and immune cells, including but not limited to monocyte-derived macrophages and lymphocytes.

Abstract

The complexity of the tumour microenvironment encompasses interactions between cancer and stromal cells. Moving from two-dimensional (2D) cell culture methods into three-dimensional (3D) models enables us to more accurately investigate those interactions. Current 3D cancer models focus on cancer spheroid interaction with stromal cells, such as fibroblasts. However, over recent years, the cancer immune environment has been shown to play a major role in tumour progression. This review summarises the state-of-art on immunocompetent 3D cancer models that, in addition to cancer cells, also incorporate immune cells, including monocytes, cancer-associated macrophages, dendritic cells, neutrophils and lymphocytes.

Background

It is clearly established that the progression of malignant tumours (solid cancers) relies both on cell-cell interactions, through intertwined communications between cancer cells, cancer stem cells and stromal cells, and on cell-matrix interactions [1]. The complexity of tumour microenvironments has been recapitulated in recent years with the use of advanced three-dimensional (3D) approaches to cell culture that provide a means to study such interactions. The benefits of 3D culture over standard two-dimensional (2D) systems and animal models has been reviewed previously [2]. Since then 3D models have improved greatly to mimic specific microenvironmental cues within a solid tumour. Examples include incorporation of various extracellular matrix (ECM) proteins or artificial scaffolds to mimic matrix stiffness and composition, addition of different types of supporting cells and engineering an angiogenic environment [3-5].

Complex models are also the ideal platform to study the role of the immune system in malignancy. The ability of cancer cells to avoid the immune system is a hallmark of cancer and immunoediting may even support tumour progression [1]. Xenografts that incorporate the immune system have been developed but are expensive, may pose ethical dilemmas, and may not adequately recapitulate the events that occur in humans. On the contrary, 3D cultures may represent an optimal model to study this element of cancer. Microarray analysis of mesothelioma spheroids and monolayers showed that formation of 3D cultures resulted in 112 upregulated and 30 downregulated probe sets. The primary function of the upregulated genes was immune response, wound response, lymphocyte stimulation, and response to cytokine stimulation, while the downregulated genes were responsible for apoptosis [6], indicating that 3D models can provide improved methods for studying those responses.

The clinical success of immune checkpoint inhibitors, such as agents acting on the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed cell death protein 1 (PD-1) and programmed cell death ligand 1 (PD-L1) pathways [7], and the growing research on other immunotherapies create the need to better understand the immune interactions in cancer and to investigate and design platforms suited for drug development. This review will cover recent research efforts in developing complex 3D tumour models that incorporate immune cells and that can further elucidate the role of cancer immunoediting and immune interactions. The different immune cell populations covered in this review have been outlined in Table 1 together with their main characteristics.

Table 1 Immune cells and their function in cancer.

Legend: APC – antigen presenting cell; CCL – CC chemokine or β-chemokine ligand; CD – cluster of differentiation; CXCL - CXC chemokine or α-chemokine ligand; CXCR – CXC chemokine or α-chemokine receptor; DC – dendritic cell; GM-CSF - Granulocyte macrophage colony-stimulating factor; IDO - Indoleamine 2,3-dioxygenase; IFN – interferon; IL – interleukin; iNOS – inducible nitric oxide synthase; MDSC – myeloid-derived suppressor cell; MHC – major histocompatibility complex; MMP – matrix metalloproteinase; NK – natural killer; ROS – reactive oxidative species; TAM – tumour associated macrophages; Tfh – tumour-infiltrated follicular helper cell; TGF – transforming growth factor; Th – helper T cell; TNF – tumour necrosis factor; Treg – Regulatory T cell; VEGF – vascular endothelial growth factor.

Cell type	Subtype		Secretome		Function
Monocyte [8]	Classical ($CD14^{++}CD16^{-}$)		IL-8, IL-2, IFN-γ, IL-12, TNF-α	Impaired by cancer cells: Decrease in IL-2, IFN-γ, IL-12, TNF-α Increase in IL-10	Dual tumour effect Recruited to the tumour site Present tumour-associated antigens and activate antitumor T cell response Can promote extravasation and tumour metastasis
	Non-classical ($CD14^{+}CD16^{+}$)		TNF-α, IL-2, IFN-γ, IL-12		
	Macrophage [9]	TAM with M1 characteristics	TNF-α, IL-1β, IFN-γ, IL-12		Antitumour effect Pro-inflammatory, anti-tumor immune response, production of cytotoxic factors, phagocytosis, immune-editing
		TAM with M2 characteristics	VEGF, MMPs, IL-10		Protumour effect Immunosuppressive, low antigen presenting capability, low cytotoxic function, high tissue remodelling activity, angiogenesis, promoting metastasis
	Dendritic Cells [10]	Myeloid (mDCs, classical)	IFN-γ, TNF-α, IL-12 Impaired by cancer cells: Impaired release of IFN-γ, TNF-α, IL-12, Enhanced IL-10 secretion		Antitumour effect Present tumour-specific antigens Activate antitumor T cell response Protumour effect silence immunity and induce tolerance, depletion of T cells
		Plasmacytoid (pDCs)	IFN-α Impaired by cancer cells: IDO, IL-6, decreased IFN-α		Antitumour effect CD8+ T cell activation Protumour effect suppress CD8+ T cells, promote differentiation of Tregs

Myeloid-derived suppressor cells (MDSCs) [10]	Monocytic and granulocytic subpopulations			Arginase I, iNOS, ROS, IDO	Protumour effect Immunosuppressive, induce T cell tolerance, suppress proliferation of CD4+ and CD8+ T cells, induce the development of Tregs
Neutrophils [11]				IL-8, CCL2, CCL3, IL-6 Increased expression: CD54, CCR5, CCR7, CXCR3, CXCR4 Decreased expression: CD62L, CXCR1, CXCR2, CD16	Antitumour effect Increase T cell IFN-γ production and activation, amplify T cell proliferation
Innate lymphoid cells [12]	**Natural killer (NK) cells**			IFNγ	Antitumour cytotoxic effect
Lymphocytes	**T cells [12-17]**	γδ T cells		IFNγ, IL-17, IL-8, TNF and GM-CSF	Dual tumour effect Mostly have a direct cytotoxic effect but can have pro-tumour effect when in a highly inflammatory milieu
		NK T cells		IFNγ	Antitumour cytotoxic effect with direct and indirect cell killing via NK cells
		CD4+ T cells	Th1	IL-2, TNFα, and IFNγ	Antitumour effect in conjunction with cytotoxic CD8+ T cells, promote macrophage cytotoxic activity and upregulate antigen processing and expression of MHC I and II molecules in professional APCs
			Th2	IL-4, -5, -6, -10, and -13	Protumour effect Suppression of immune response to tumour by inducing T-cell anergy and loss of T-cell-mediated cytotoxicity, enhancing humoral immunity, and regulating the tumour-promoting activities of macrophages
			Th17	IL-17A, IL-17F, IL-21, IL-22, IL-26, CCL20	Dual tumour effect Suppress tumour progression through enhanced antitumor

					immunity, or promote tumour progression through an increase in inflammatory angiogenesis
			Tfh cell	CXCL13	Antitumour effect by recruiting B cells to sites of inflammation
			Tregs	IL-10, IL-35, TGF-β, galectins	Pro-tumour effect by dampening cytotoxic CD8+ T cell activity
		CD8+ T cells			Antitumour cytotoxic effect by binding to antigen presented by MHC class I. The major anti-cancer effector cells.
		Memory effector T cell			Antitumour effect: can be reprogrammed into cytotoxic CD8+ T cells
	B cells [18]	Regulatory B cells		IL-10, TNF-α, TGF-β, IL-21, IL-33, IL-35	Dual tumour effect Anti-tumour responses may be enhanced or suppressed depending on the regulatory B cell subsets recruited to the tumour site
		Plasma cell			Antitumour cytotoxic activity via tumour-specific complexes

Monocytes, macrophages, and dendritic cells

Cells from both the innate and the adaptive immune responses are present in malignant tumours (Figure 1). In this context cells acquire distinctive characteristics that differentiate them from resident immune cells at other tissues.

Monocytes can differentiate into two functionally distinct subtypes of macrophages: classic, anti-inflammatory macrophages, known as M1; and M2 macrophages, that promote matrix remodelling, angiogenesis, and favour tumour progression [19].

Cancer-associated macrophages are found in solid tumours [20], and are associated with poor prognosis [21] and drug resistance [22]. Spheroid culture (using the hanging drop technique, on low-attachment plates, or on artificial (agar) or ECM proteins (such as collagen, basement membrane extract) has been used to study the effects of monocytes/macrophages on cancer cells and the microenvironment (Figure 2).

An early study using cancer spheroids of a human rectal cancer cell line (HRT-18) formed on agarose-coated wells (Figure 2d) showed that macrophages adhere to the surface of spheroids within 24 hours, can infiltrate them, and cause a disintegration of cancer spheroids without associated cytotoxicity after 5 days of co-culture. Additionally, cancer spheroids transferred to a collagen type I layer showed enhanced migration in the presence of anti-inflammatory macrophages when compared to pro-inflammatory or resident macrophages [23]. The early results mimic the *in vivo* phenotype of resident cancer-associated macrophages and their ability to promote tumour progression. Agarose-coated wells were also used to investigate monocyte recruitment from blood into fibroblasts spheroids. In this scenario, only tumour-associated fibroblast spheroids were infiltrated by monocytes, while normal fibroblast spheroids were poorly invaded [24]. Additionally, monocyte migration kinetics were faster for tumour-associated fibroblast spheroids than for breast cancer spheroids [25], raising the question of whether immune cells are attracted by cancer cells or stromal cells. Nevertheless, in a study of squamous cell carcinoma of the head and neck (SCCHN), there was no difference in peripheral blood mononuclear cell (PBMC) infiltration between SCCHN spheroids and SCCHN-fibroblasts spheroids. Increasing PBMC number increased their concentration on the outer surface of spheroids, but not infiltration. Infiltration of SCCHN spheroids was, however, enhanced after blocking epithelial growth factor receptor (EGFR) expression [26]. Further studies on breast cancer spheroids have helped elucidate the mechanisms behind macrophage infiltration. Macrophages have been shown to invade breast cancer spheroids (SUM159PT cells, oestrogen receptor (ER) negative, and progesterone receptor (PR) negative anaplastic breast carcinoma) up to the necrotic core using mechanisms that rely on the heterogeneity and viscoelasticity of the tumour matrix, as well as on the action of matrix metalloproteinases (MMPs). Also, the breast cancer spheroids were able to invade fibrillary collagen type I, but lost their invasiveness in Matrigel (mouse ECM extract). Addition of macrophages (within the spheroids or added to Matrigel) allowed the breast spheroids to invade into the matrix, but this did not enhance cancer invasiveness in collagen [27]. These results indicate that matrix composition also affects the cross-talk between macrophages and cancer cells. Infiltration of monocytes and macrophages into breast cancer spheroids (T47D cells, breast ductal carcinoma) has been shown to be dependent on ribonucleic acid (RNA)-binding protein tristetraprolin (TTP), which regulates immune responses. TTP is downregulated in tumourogenesis and metastasis. In this 3D model TTP knockdown increased the rate of monocyte infiltration, with cells mainly distributed in the outer spheroids areas. Concurrent studies in an *in vivo* mouse model showed complementary data [28], suggesting that a 3D spheroid culture can be successfully used to study cancer immune responses.

Cancer cell characteristics also appear to influence monocyte infiltration into breast cancer spheroids. Using different breast cancer cell lines, the highest infiltration was observed with Hs578T spheroids (ER negative carcinoma), intermediate with T47D spheroids and poor with BT549 (ductal carcinoma), MCF7 (ER positive adenocarcinoma), and BT474 (ductal carcinoma)

spheroids [25], indicating that 3D cultures can replicate cancer with different molecular characteristics or at different points of the disease's natural history. The cross-talk between monocytes and cancer cells appears to contribute to bad prognosis in inflammatory cancers by increasing proteases involved in cell migration, such as urokinase plasminogen activator (uPA) and MMPs [29]. In a spheroid model, the cross-talk was shown to be dependent on the aggressiveness of the cell line. Two different breast cancer cell line (MCF-7 and MDA-MB-231) spheroids were cultured on top of a solidified layer of Matrigel, both alone and co-cultured with monocytes. On one hand, the presence of monocytes reduced the expression of tumour malignancy markers (MMP9, uPA, cyclooxygenase (COX)-2, osteopontin (OPN)) in less aggressive breast cancer spheroids (MCF-7), suggesting a tumour-suppressive effect. On the other hand, co-culture of monocytes with highly aggressive breast cancer spheroids (MDA-MB-231) increased expression of MMP1, and decreased expression of MMP2 and MMP9, resulting in cancer cells and monocytes showing greater migration through the 3D matrix [30], suggesting a tumour-favouring effect.

Additionally, cancer-associated macrophages appear to be influenced by other stromal cells and to actively contribute to cancer progression. Both cancer-associated fibroblasts and cancer cells contribute to the differentiation of M2 macrophages via secretion of soluble factors, such as interleukin (IL)-6 and stromal-derived growth factor-1 (SDF-1) [31]. M2 macrophages were shown to promote invasion via paracrine signalling (conditioned medium from primary tumour-associated macrophages isolated from blood) in breast cancer spheroids (SUM159PT) cultured in Cultrex® Basement Membrane Extract [32] and via direct cell-cell interactions during co-culture of mouse breast cancer spheroids with bone-marrow derived macrophages in collagen type I [33].

Spheroid cultures were also studied using the Algimatrix™ 3D culture system, a porous alginate cell culture platform, to assess macrophage effect on fibroblasts and cancer spheroids (Figure 2c). Co-culture of mouse breast cancer cells with mouse fibroblasts had a negative impact on cancer spheroid formation, whilst a co-culture with mouse macrophages inhibited their growth capacity in 3D. Only a triple culture of macrophages, fibroblasts, and breast cancer cells resulted in an increase in the amount of spheroids formed, indicating the need of interaction between the three cell populations for cancer growth [34]. In contrast, a study on organotypic co-culture of air-exposed murine squamous cell carcinoma cells grown as a layer on top of murine dermal fibroblasts in rat-tail collagen type I (Figure 3a) showed that the addition of macrophages to the dermal collagen compartment led to polarisation into the M2 phenotype by both fibroblasts and cancer cells. The presence of M2 macrophages induced squamous cell carcinoma cell invasion into type I collagen by disrupting the basement membrane. Increased collagenolytic activity via MMP-2 and MMP-9 was observed. Authors showed similar effects when using human skin squamous cell carcinoma cells, human primary dermal fibroblasts and human monocyte-derived macrophages [35].

Macrophages have been studied as a delivery system for nanotherapeutics. Glioma spheroids were co-cultured with gold nanoshells-containing macrophages in ultra-low attachment (Figure 2b) plates for photothermal (PTT) and photodynamic (PDT) therapy investigations. PTT treatment caused growth inhibition in glioma spheroids with gold nanoshells containing macrophages, while it had no effect in monoculture or co-culture with unloaded macrophages. PDT treatment showed similar results but a lower need of radiant exposure, indicating it can be more efficient than PTT [36]. This model was further confirmed *in vivo*, where mice with glioma spheroids were injected with gold nanoshells containing macrophages and PTT treatment resulted in eradication of cancer cells [37].

Microfluidic systems are 3D models with high-throughput capacity, and the ability to process multiple assays in an automated manner. Incorporation of tissue engineering has increased the complexity of these systems, enabling the design of mimetic environments according to organ or tissue type [38]. The interaction of melanoma cells (B16.F10) with immune cells was evaluated using such a model: using wild type immune cells and interferon regulatory factor (IRF)-8 knockout immune cells, it was shown that immune cells require IRF-8 to interact with cancer cells and limit their invasiveness. This is in concordance with the findings in an *in vivo* mouse model that showed that (IRF)-8 is necessary for immunosurveillance [39,40]. In another approach, tumour vasculature was added to the 3D microfluidic cancer model (Figure 3d). Here, the tumour channel was populated with human breast carcinoma (MDA231) cells and the endothelial channel with endothelial cells (HUVEC). The two channels were interconnected via 3D ECM hydrogel and cancer cells invaded into the 3D ECM, while endothelial cells formed layer on the top of the ECM. When macrophages (murine RAW264.7) were added to the tumour channel, tumour cells had higher rates of intravasation into the endothelial cell layer due to macrophage-induced permeability. These macrophages had variable expression of M1 and M2-specific markers, and secreted tumour necrosis factor (TNF)-α, a pro-inflammatory cytokine released by M1 macrophages, showing that a heterogeneous macrophage population can also lead to a malignant tumour phenotype [41].

Apart from macrophages, the monocytic lineage can also give rise to other immune cell types. Dendritic cells are antigen-presenting cells that can be derived from haematopoietic bone marrow progenitor cells, or from monocytes. Upon activation, dendritic cells promote immune response. 3D models have also been used to study the interaction between these cells and cancer cells. Dendritic cells derived from peripheral blood-derived monocytes were used in glioma (U87 and U251 cell lines) spheroids models cultured in agar-coated flasks to study migration of dendritic cells following aminolevulinic acid (ALA)/PDT treatment. After treatment, immature dendritic cells were attracted to the glioma spheroids, while control spheroids showed no or weak attraction. ALA/PDT treatment also resulted in significant uptake of tumour material by dendritic cells and their maturation, which can result in an immune response rather than immunological tolerance [42]. This correlates with the known role of dendritic cells in immune response, suggesting biomimicry between the model and clinical response.

Neutrophils

Like monocytes, neutrophils are phagocytic cells derived from myeloid progenitors found in tumours; still their role in cancer growth has not been studied extensively *in vitro*. Neutrophils have been shown to infiltrate cervical cancer spheroids (HeLa cell line) and lung cancer spheroids (A549 cell line) formed in agarose-coated wells. The relationship between monocyte and neutrophil infiltration is complex and inter-related. Studies have shown that when spheroids were infiltrated by $CD14^+$ monocytes, there was a subsequent decrease in neutrophil infiltration. On the contrary, prior neutrophil infiltration had no effect on subsequent monocyte infiltration. Similarly, macrophage-depleted tumours in mice showed higher infiltration with neutrophils [43]. The infiltration of neutrophils into lung cancer spheroids was mediated by chemokine (C-X-C motif) ligand (CXCL) and led to increase in the spheroid growth rate indicating that neutrophils secrete factors that affect tumour cell proliferation, possibly including proangiogenic molecules such as vascular endothelial growth factor (VEGF). Similarly, *in vivo* a chemokine (C-X-C motif) receptor (CXCR) $2^{-}/^{-}$ mouse model showed decreased neutrophil infiltration into tumours, which was associated with slower tumour growth [44].

Lymphocytes

Lymphocytes originate from common lymphoid progenitors and can be divided into three major groups of cells: T-lymphocytes, B-lymphocytes, and natural killer (NK) cells. The role of lymphocytes in immune response is widespread, from direct cytotoxicity to regulation of response of other immune cells. Likewise, other immune cells also regulate lymphocytic activity.

Like tumour-associated macrophages, myeloid-derived suppressor cells (MDSC) have the capacity to suppress both the adaptive immune response mediated by $CD4^+$ and $CD8^+$ T cells and the cytotoxic activities of NK cells. Additionally, MDSCs stimulate tumour growth by promoting cellular stemness, angiogenesis, and metastatic spread of tumour [45]. Th1 lymphocytes repress or stimulate the activity of other immune cells and a Th1-enriched microenvironment can reduce the MDSC population and attenuate their suppressive activity. This has been shown to reduce the size of breast cancer cell line spheres (SK-BR-3 and MDA-MB-231) cultured in Matrigel [46]. In further studies, the same authors used a 3D co-culture of pancreatic cancer cells (MiaPaCa-2 and MiaM) and PBMC-derived $CD3^+$ T cells treated with a chemical heteroconjugation of anti-CD3 and anti-EGFR antibodies. Here, treatment lead to attenuation of the suppressive powers of the MDSC population and increase in cancer cell cytotoxicity, especially in Th1 rich environments [47].

Matrigel has been used to develop a 3D co-culture model of breast cancer cells (MCF-7 and MDA-MB-231) with regulatory T (T_{REG}) lymphocytes ($CD4^+CD25^+$), cells associated with induction of tumour tolerance and suppression of anti-tumour T and NK cell cytotoxic responses, and NK cells ($CD56^+/NKp46^+$). In this study, NK cells were able to disrupt MCF-7

cell masses regardless of T_{REG} presence; while under T_{REG} presence alone the cell clusters remained unchanged. NK cells also disrupted MDA-MB-231 cell networks regardless of T_{REG} presence, while in the co-culture with just T_{REG} and cancer cells distinct stellate networks were noted [48].

Using a 3D glioma model, NK cells showed lower toxicity to cells grown in 3D than on cells grown in monolayer. Here, the U251 glioma cell line cultured in the Rotary Cell Culture System formed spheroids containing on average 4000 cells after 6 days (dimension 400 to 500 µm). Of note, the cells grown in 3D were also more likely to generate a tumour when implanted in nude mice reconstituted with human NK cells [49]. This suggests that in the 3D model, by unknown mechanisms, cells were provided protection against cytotoxic action.

Using a 3D porous chitosan-alginate (CA) scaffold (Figure 3c) prostate cancer spheroids attracted $CD45^+$ cells to their surface within 2 days, with $CD45^+$ cells infiltration occurring after 6 days of culture. Some populations of these $CD45^+$ cells also expressed CD8 (a marker present on T cells, NK cells and rarely on monocytes and neutrophils) and CD57 (present on NK cells), indicating heterogeneous lymphocyte population. In this study, the CA scaffold showed advantages over Matrigel in a prolonged culture and enabled recovering cells for further analysis using flow cytometry [50]. CA scaffolds were further used to evaluate T cell interactions with murine mammary carcinoma cells. T cell binding and infiltration of cancer spheroids was shown to be facilitated by cancer cell expression of chemokine (C-C motif) ligand (CCL)21, responsible for directing immune effector cell migration, and interferon (IFN)-γ, a molecule that activates effector adaptive immune cells [51]. Additionally, the presence of fibroblasts in the cancer/T cell spheroids resulted in increased immune suppression as shown by a decrease in the expression of TNF-α by T cells [52], indicating the need for complex stromas when studying cancer-immune cell interaction in 3D models.

Using spheroid cultures in low attachment plates allowed for the investigation of subpopulations of cancer stem cells in cervical cancer and head and neck carcinoma cell lines and their susceptibility to cytotoxic T lymphocyte-mediated immune responses. These cancer spheroids had higher expression of stem cell markers (ALDH, SOX2, Nanog and Oct3/4) than their monoculture equivalents and were lysed by as few as 100 cytotoxic T lymphocytes. Lysis appeared to be mediated by MHC class I-restricted cytolysis and was enhanced by IFN-γ treatment, and occurred at lower level when compared to monolayers [53]. These results indicate that spheroid cultures provide more heterogeneous cell populations, as well as protective mechanism against cytotoxic action, both of which affect immune response. This is turn suggests that 3D models may be more clinically relevant and biomimetic than cell monolayers.

Finally, the role of cytokine-induced killer cells (CIK), immune effector cells with T-cell and NK cell-like phenotype, has been studied in models of gastric cancer. The 3D engineered model used (Figure 3b) as a matrix for BGC823 stomach adenocarcinoma cell line, which formed multiple spheroids within the collagen matrix. When CIK were added to the cell culture

medium of the 3D model, CIK were able to infiltrate the collagen matrix, migrate towards tumour spheroids, and surround them, leading to tumour cell death [54]. This mimicking of immune cell action shows the potency of 3D in vitro models.

Conclusion

The interaction between the various components of the immune system, cancer and stromal cells is essential for tumour establishment and progression. 3D models have helped to elucidate some of these interactions. Some studies have shown a parallel between results in 3D models and *in vivo* models, which is reassuring and encourages further use of these platforms. Nevertheless, substantial development in this area is still needed. Most data were derived using cell lines, which have been argued to have a very distinct behaviour when compared to patient-derived cells, the control is often provided by animal models, a system known to be flawed in terms of studying immune interactions, and different 3D models have been used, limiting comparisons across results.

Building/engineering immunocompetent 3D cancer systems that can mimic human immune response is challenging. Nevertheless, these systems can provide increased knowledge on the role of the immune system in cancer as well as serve as less expensive, more reproducible models which can be humanised compared to animal models and can be used for drug/therapeutic development.

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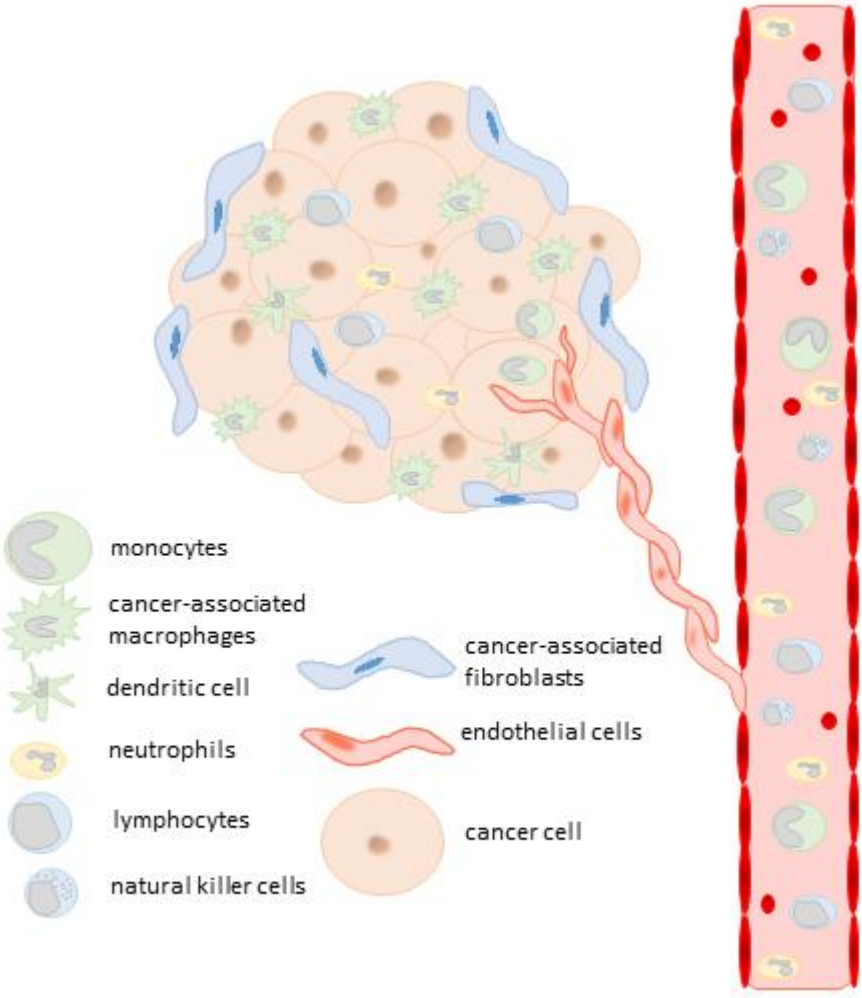

Figure 1 Tumour microenvironment is populated by cells of the innate and adaptive immune system that can enhance tumourigenesis and tumour progression. Cancer-associated fibroblasts in the tumour stroma, together with cancer cells, attract myeloid cells, such as monocytes, which differentiate into cancer-associated macrophages and dendritic cells, and neutrophils; and lymphoid cells, including lymphocytes and natural killer cells.

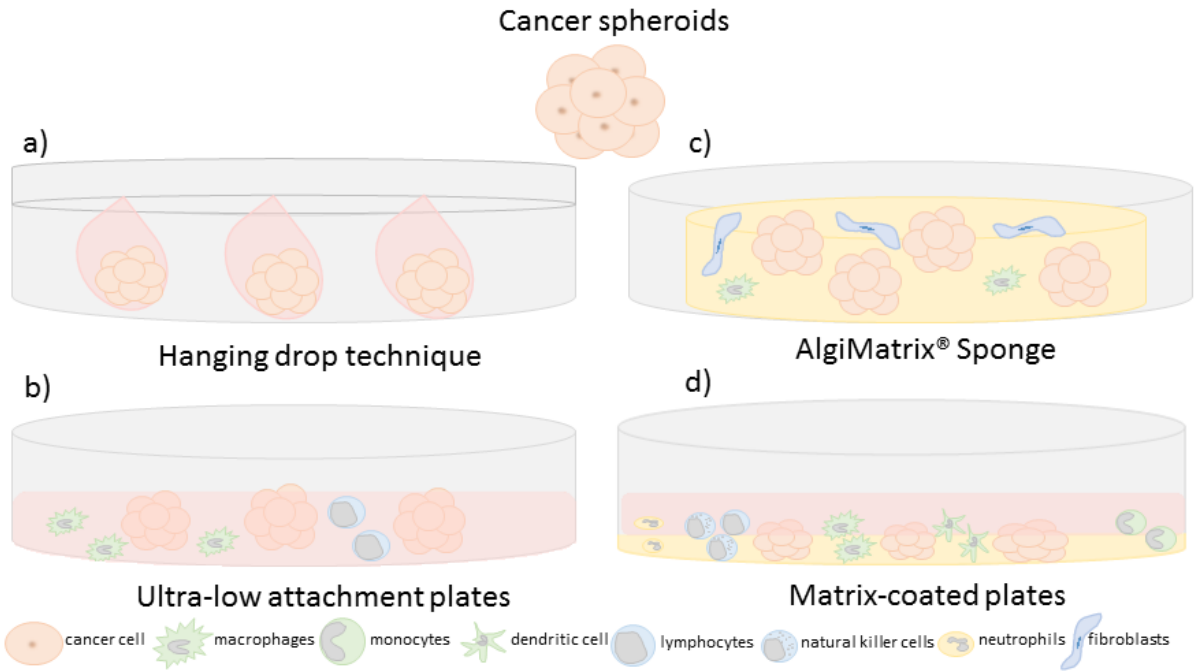

Figure 2 Cancer cells can be cultured as spheroids, giving them a 3D structure. Cancer spheroids can be formed using a hanging drop technique (a), ultra-low attachment plates (b) or in a 3D matrix, such as AlgiMatrix (c) or matrix-coated plates (d). These culture methods can be combined to first generate spheroids (a,b) followed by culture in a 3D matrix (c,d).

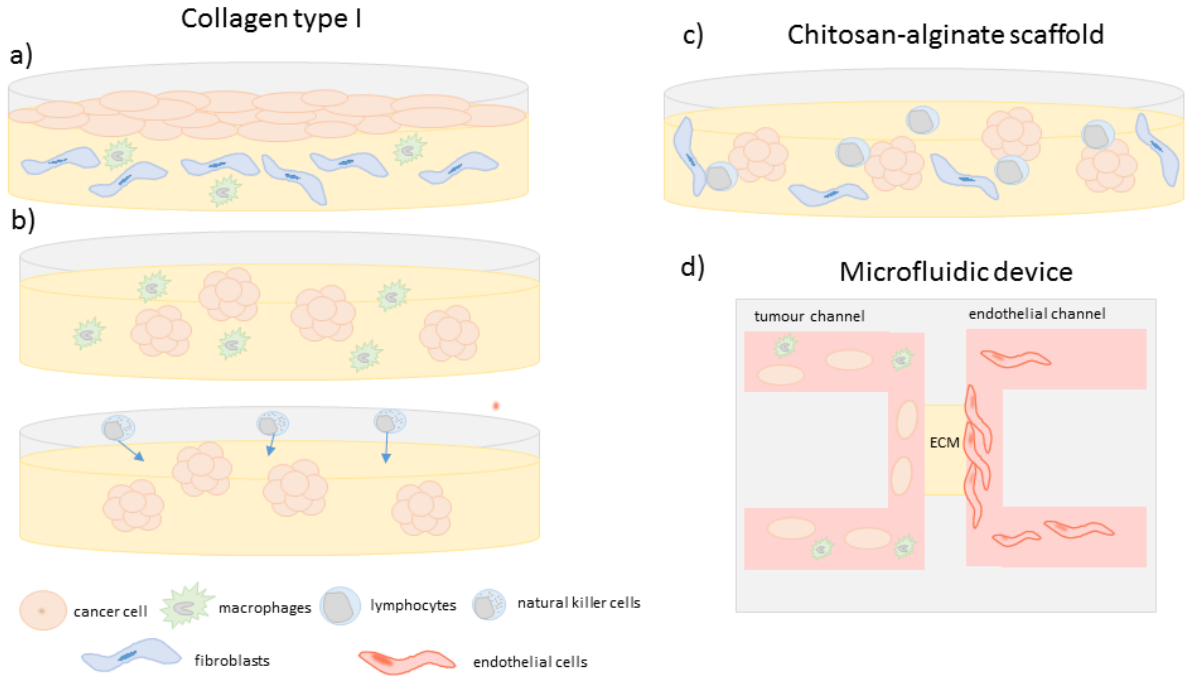

Figure 3 Tissue engineered approaches use natural and artificial scaffolds to provide cells with a 3D extracellular matrix. Natural scaffolds include collagen type I hydrogel, where cancer cells are cultured on top of gel populated with stromal cells (a), or within the collagen matrix allowing for spheroid formation (b). Artificial scaffolds include chitosan-alginate where cancer cells are seeded within the scaffold (c). Extracellular matrix can be also added to a microfluidic device containing channels for cancer and stromal cells (d).