Organoid Models of Tumor Immunology

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Abstract

Cellular interactions in the tumor microenvironment (TME) significantly govern cancer progression and drug response. The efficacy of clinical immunotherapies has fostered an exponential interest in the tumor immune microenvironment, which in turn has engendered a pressing need for robust experimental systems modeling patient-specific tumor-immune interactions. Traditional 2D *in vitro* tumor immunotherapy models have reconstituted immortalized cancer cell lines with immune components, often from peripheral blood. However, newly developed 3D *in vitro* organoid culture methods now allow the routine culture of primary human tumor biopsies and increasingly incorporate immune components. Here, we present a viewpoint on recent advances, and propose translational applications of tumor organoids for immuno-oncology research, immunotherapy modeling, and precision medicine.

Organoid Culture Systems for Modeling the Tumor Immune Microenvironment

Tumors comprise both neoplastic cells and a diversity of non-neoplastic host components, termed the **tumor microenvironment** (**TME**; see Glossary), which fosters carcinogenesis, tumor progression, and metastases of malignant cells. The complex TME includes mesenchymal-derived cells (pericytes and fibroblasts), resident or infiltrating vascular structure (endothelium), and an immune cellular network (innate and adaptive immune cells). These immune cells, including lymphocytes (T and B cells), natural killer (NK) cells, macrophages, dendritic cells (DCs), eosinophils, mast cells, and myeloid-derived suppressor cells (MDSCs), populate the tumor and can be derived from local tissue-resident populations, or infiltrate from secondary lymphoid organs (draining lymph nodes, spleen, Peyer's patches, and mucosal tissues). The immune TME is regulated by the balance between cellular and humoral components and diverse inflammatory responses to support the growth of neoplasms into an advanced tumor biomass [1–3].

The crucial functions of immunity during tumorigenesis have been historically illustrated by well-established cancer predisposition during inflammatory and immunodeficiency states [4,5]. Recently, the potential of cancer immunomodulation was unequivocally demonstrated by the transformative anticancer efficacies of both cellular and pharmacological

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immunotherapies. Such immunotherapies systemically augment the immune surveillance of the body and/or locally modulate the tumor immune microenvironment. These immunotherapies include tumor antigen-targeted monoclonal antibodies, vaccines, **pattern recognition receptor (PRR)**-targeted therapies, and other nonspecific small molecules [interleukins (ILs), interferons, and colony-stimulating factors], which have all been used in clinical settings [6,7]. Breakthrough immunotherapy approaches that have revolutionized conventional cancer therapy are: (i) **immune checkpoint inhibitors** (ICIs) [8–10], such as therapeutic monoclonal antibodies against programmed cell death-1 (PD-1)/PD-L1 (programmed cell death-1 ligand) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) to unleash cytotoxic T cell effector functions; and (ii) adoptive T cell therapies (ACT), including **chimeric antigen receptor (CAR)**- and T cell receptor (TCR)-T cells, as well as bulk **tumor-infiltrating lymphocyte (TIL)** therapy [11–13].

For decades, cancer research has utilized *in vitro* 2D cell cultures, and *in vivo* xenografts or genetically engineered animal models. The former enables viral transduction, pharmacological intervention, and multiplexed drug screening, while the latter provides the dynamic context of tumor tissue structure and vasculature. However, both conventional *in vitro* and *in vivo* models insufficiently model the complex immunobiology of native human tumors. 2D cultures can be co-cultured with different types of exogenously added heterogeneous cells to simulate cell-cell communication in tumors [14–16]. Addition of **peripheral blood mononuclear cells (PBMCs)** can be used for exploration of immunotherapeutic agents [17]. Furthermore, such reconstituted cells are typically not from the endogenous intratumoral stroma, and adherent monolayer cancer cells do not replicate 3D morphological structures. Furthermore, oncogene and tumor suppressor biology may be less accurate in 2D versus 3D culture [14,18]. Humanized immuno-oncology models are generated by the engraftment of patient-derived xenografts (PDXs) into immunodeficient mice bearing human immune cells, but cost, time, throughput, and complete immunocompatibility, remain challenges [19,20].

The recent advent of *in vitro* human organoid culture embodies a new approach to studying tumor immunobiology. As originally defined, organoids are 3D *in vitro* cultures of normal tissues with multiple cell lineages, including stem cells and differentiated cells, and tissue architecture *in vitro* [21–23]. However, organoid technology has been rapidly adapted to cancer modeling [24]. On the one hand, a forward genetic strategy can be used in which organoids from wild-type tissues or induced pluripotent stem cells (iPSCs) are engineered to bear oncogene or tumor suppressor mutations [25–29]. On the other hand, organoid methods can now robustly propagate human tumor biopsies *in vitro* (**patient-derived organoids, PDOs**). The large-scale application of 3D PDO culture has transformed *in vitro* cancer biology, allowing the establishment of large tumor biobanks capturing the histological and mutational diversity of human cancers [30–32]. Furthermore, current PDOs represent relatively early passage material, as opposed to long-passaged 2D cancer cell lines that, through continued genomic instability, may no longer represent the genetics of their original tumors [33,34]. Here, we discuss various organoid culture strategies in which tumor cells are grown with native or reconstituted TME immune components (Figure 1, Key Figure and Table 1). We also propose applications of tumor organoids recapitulating the immune TME

for: (i) investigating cancer immunobiology; (ii) testing cancer immunotherapeutics; and (iii) developing novel approaches for personalized medicine (Figure 2).

Submerged Matrigel Culture

A commonly used organoid method is to culture dissociated tumor cells in a dome or flat gel of 3D Matrigel, underneath tissue culture medium. In this 'submerged Matrigel' procedure, various growth factors and/or pathway inhibitors are supplemented depending on tissue type [27,34,35]. Exact culture conditions are customized for specific tumor histologies, but often include additives, such as Wnt3a, R-spondin, epidermal growth factor (EGF), and bone morphogenetic (BMP) inhibitor Noggin, which allow the stem cells to undergo self-renewal and differentiation (e.g., in intestinal organoids) [21]. These supplemented culture media have also been used for **air-liquid interface (ALI)** culture (see later) [36]. Submerged Matrigel PDOs facilitate cancer disease modeling and drug screening by recapitulating not only the genetic and phenotypic diversity of original tumors, but also by potentially modeling functional patient responses to clinical treatment [30,31,37–41]. Of note, typical submerged Matrigel PDOs exclusively enrich epithelial cancer cells but fail to retain stromal components [21]. Thus, TME modeling in this culture system requires a combinational co-culture of exogenously added immune cell types, as described later.

Microfluidic 3D Culture

Spheroid-based organotypic cultures within collagen gels in 3D microfluidic culture devices have been adapted to culture murine- or patient-derived tumors [42]. Tumor spheroids are allowed to grow in a 3D gel in the center region of the device with media from the media channels running parallel to, and located on either side of, the central region. Murine- and patient-derived organotypic tumor spheroids (MDOTS/PDOTS) from syngeneic immunocompetent murine models and patient tumor specimens, such as melanoma and Merkel cell carcinoma, can be cultured and evaluated for 1–2 weeks [43–45]. Flow cytometric immune cell profiling showed that MDOTS and PDOTS retain autologous lymphocytes (B cell and T cell) and myeloid populations [monocyte, DC, MDSC, and tumor-associated macrophages (TAM)] as well as tumor cells [43].

Air-Liquid Interface Culture—In this method, tumor organoids grown from minced primary tissue fragments are embedded in a collagen gel in an inner transwell dish. Culture medium in an outer dish diffuses via the permeable transwell into the inner dish and the top of collagen layer is exposed to air via an ALI, allowing cells access to a sufficient oxygen supply [23,25,46]. The culture of tissue fragments allows PDO generation as cancer cells *en bloc* alongside endogenous native stromal and immune components without reconstitution, which is distinct from submerged Matrigel culture.

Initially, ALI organoids from diverse normal tissues, including small intestine, colon, stomach, and pancreas, were shown to incorporate both epithelial and mesenchymal components [23,25]. Subsequently, the ALI organoid method was developed to culture PDOs from human biopsies, such as melanoma, renal cell carcinoma (RCC), and non-small cell lung cancer (NSCLC), and mouse tumors in syngeneic immunocompetent mice [36]. ALI PDOs preserve not only the genetic alterations of the original tumor, but also the

complex cellular composition and architecture of the TME. Indeed, both tumor parenchyma and stroma are retained, including fibroblasts and a variety of endogenous infiltrating immune cell populations [36].

Reconstitution Approaches for Studying the Immune TME in Organoids

Since conventional submerged Matrigel organoids contain epithelial cells exclusively, any investigation of the TME in this system necessitates addition of exogenous stromal cell types. Such reconstitution has been utilized to supplement PDO cultures with cancer-associated fibroblasts (CAFs) [35,47–49]. Accordingly, human ductal adenocarcinoma (PDAC) organoids co-cultured with CAFs revealed that CAF-secreted Wnt drives organoid growth in Wnt-nonproducing PDAC subtypes [35]. In addition, co-culture of pancreatic stellate cells (PSCs) with PDAC organoids produces desmoplastic stroma and reveals two distinct CAF subtypes from PSCs: IL-6-expressing inflammatory CAFs activated by paracrine secreted factors from tumor cells, and high **α-smooth muscle actin (αSMA)**-expressing myofibroblast-like CAFs that interact with tumor cells [48]. The co-culture of organoids and CAFs has identified tumor-secreted ligands, IL-1 and transforming growth factor (TGF)β, which promote distinct inflammatory and myofibroblast CAF subtypes, respectively [49].

Diverse immune cell reconstitution of submerged Matrigel organoids has also been performed. Addition of mouse mammary adenocarcinoma-derived $CD4^+$ T cells with and without associated macrophages (TAMs) to mammary epithelial organoids from the MMTV-PyMT mouse model demonstrated that $CD4^+$ T lymphocytes enhanced organoid disruption and invasive behavior by directly activating a protumorigenic TAM phenotype via $CD4^+$ T cell-secreted IL-4 [50]. Co-culture of patient-matched CAFs and **peripheral blood lymphocytes (PBLs)** with PDAC organoids demonstrated myofibroblast-like CAF activation and lymphocyte infiltration into Matrigel, migrating toward the tumor organoids [47]. A more complex setup involved the co-culture of **cytotoxic T lymphocytes (CTLs)** with DCs primed by tumor antigen released from mouse gastric tumor organoids; here, stimulated-CTLs killed gastric tumor organoids in the presence of anti-PD-L1 antibody, suggesting that the reconstitution of multiple immune cells would allow the study of tumor-immune and immune-immune cell interactions [51]. Additional reconstitution models of tumor organoid co-cultures with autologous PBLs to generate tumor-reactive T cells [52] or DCs in a *Helicobacter pylori* infection model [53,54] are discussed later in detail.

Holistic Approaches for Studying the Immune TME in Organoids

In contrast to adding exogenous immune components to epithelial-only organoids, holistic approaches culture tumor epithelium together with stroma endogenous immune cells as a cohesive unit without reconstitution. On the one hand, in a microfluidic culture, organotypic tumor spheroids (spheroids are 40–100 μm in diameter) are cultured as MDOTS/PDOTS with collagen in microfluidic devices for 5–9 days, preserving tumor cells and endogenous immune cells, such as lymphocyte and myeloid populations, without reconstitution [43]. This method allows the study of endogenous immune-tumor interactions or analysis of T cell infiltration into tumor spheroids by adding T cells, such as Jurkat cells, into the media

[55]. On the other hand, in ALI organoid culture, large regions of tumors can be grown in their native state and thus also faithfully preserve a diversity of endogenous immune cells, including T cells [T helper (T_h), cytotoxic (T_c), regulatory (T_{reg}), and **exhausted (T_{ex})**], B cells, NK cells, and macrophages, as identified by single cell sequencing [36]. Notably, ALI PDOs can also preserve the TCR repertoire of the original fresh tumor, and expanded T cell clonotypes can correspond to 'exhausted' T cell phenotypes, similar to what has been observed in human fresh tumors, such as clear cell RCC [36].

ALI PDO cultures can be grown from a diversity of tumor sites, including colon, lung, pancreas, colon, and kidney, and, at least over the short-term (30 days), can accurately recapitulate the histology and mutational burden of the original malignancies [36]. By contrast to the tumor epithelium, which can be serially passaged and cryopreserved, the immune components of ALI PDOs decline over time and, despite IL-2 supplementation, do not persist beyond ~2 months [36]. Furthermore, preservation of the vasculature that carries immune cells could improve modeling of the physiological immune cell circulation, but perfusion would likely remain a challenge (see Outstanding Questions). Nevertheless, the ALI organoid approach affords a holistic strategy to *in vitro* immune TME modeling that can explore complex crosstalk between multiple distinct cellular populations.

Applying Organoids to Cancer Immunotherapy Research

Recapitulating the Immune Checkpoint Inhibitor Response

ICIs targeting PD1/PD-L1 and CTLA-4 have demonstrated clinical responses in diverse advanced cancers, including melanoma [9,56,57], cutaneous squamous cell cancer [58], NSCLC [8,59], RCC [60], head and neck cancer [61], and mismatch repair-deficient tumors, regardless of histology [62]. Organoid biobanks have been established from multiple types of cancer, including colon [30,34,40,63,64], rectum, stomach [31,65], pancreas [66], liver [67,68], breast [32], ovary [69,70], and prostate [71], with distribution through entities such as the Human Cancer Models Initiative (HCMI)[i]. While these epithelial-only PDOs are widely available, their lack of immune components hinders immunotherapy assessment, such as the response to ICIs.

As one solution, immune checkpoint treatment has been performed on epithelial-only submerged Matrigel organoids reconstituted with exogenous immune components [72,73]. Notably, co-culture of patient derived-tumor-only organoids with autologous TILs after expansion of organoids and TILs separately, enabled TIL migration toward PDOs through Matrigel, and tumor cell cytotoxicity, suggesting the use of this co-culture system to assess cytotoxic TIL function [72]. The potential application for immunotherapy screening was suggested by co-culture of patient-derived tumor spheroids with autologous TILs treated with immunomodulatory antibodies targeting MICA/B and NKG2A antigens in colorectal cancer (CRC) [73]. We posit that, on the one hand, such reconstitution approaches can enable reproducible investigation by long-term expansion of the epithelial component; however, the addition of single immune cell types may not fully recapitulate the complex interplay between different immune populations following immunomodulatory drug treatment, either singly or in combination.

On the other hand, holistic culture systems including microfluidic and ALI strategies can be used to functionally model ICI. In 3D microfluidic cultures, MDOTS/PDOTS generated from mouse and human tumors can recapitulate the therapeutic sensitivity and resistance *in vivo* to PD-1 blockade for short-term cultures through the assessment of TIL cytotoxicity against tumor cells by tumor live/dead staining (e.g., PD1-sensitive **MC38** and **GL261** tumors, PD1-intermediate-sensitive **CT26**, PD-1- resistant **B16F10**, human melanoma, and Merkel cell carcinoma) [43]. In ALI cultures, ALI organoids grown from mouse tumors inoculated into syngeneic immunocompetent mice (B16-SIY, MC38, and A20) manifest $CD8^+$ CTL activation and tumor killing in response to anti-PD-1/PD-L1 antibodies, accompanied by antigen-specific clonal $CD8^+$ T cell expansion, where T cell functions were assessed by flow cytometry-based immune phenotyping and tumor live/dead staining [74]. Similarly, PDOs from diverse human cancer biopsies, such as NSCLC, melanoma, and RCC, exhibited $CD8^+$ T cell expansion, activation, and subsequent tumor cell killing after 1 week of anti-PD-1 antibody treatment [74].

Extending the Therapeutic Reach of Immunotherapy

The numerous promising immunotherapy clinical outcomes to date have been counterbalanced by both intrinsic and acquired resistance [75,76]. Furthermore, several tumor histologies appear refractory to checkpoint inhibition. Organoids represent a potential *in vitro* approach to: (i) optimizing the efficacy of existing immunotherapies; and (ii) functionally assessing novel approaches.

Clinical immunotherapy trials increasingly explore combinatorial treatments, leveraging the distinct biology of multiple immune checkpoints (e.g., PD-1, CTLA-4, LAG-3, TIM-3, and VISTA) [10] and immunosuppressive cellular populations, such as T_{regs} [77] and MDSCs [78]. Moreover, immunotherapy is further added to modalities such as chemotherapy [79], radiotherapy [80], or antiangiogenic therapy [81]. Addition of molecularly targeted therapy to ICI is possible, as with MEK or BRAF inhibitors in melanoma [82]. Combining ICI with blockade of locally acting paracrine pathways within the TME, such as TGFβ [83], or chemokine receptors, such as CCR4 [84], and CXCR4 [85], is underway. Other potential targets for immunotherapy combinations include tumor cell activation of JAK-STAT [86], MAPK [87], Wnt/β-catenin [88], and NF-κB pathways [89], which can promote the release of immunomodulatory cytokines and chemokines.

In vitro culture systems are further being deployed to explore novel mechanisms, therapeutic combinations, and putative biomarkers relevant to ICI response and resistance. For example, cytokine profiling of MDOTS identified particularly high concentrations of Ccl2 in a mouse CT26 tumor of partial sensitivity to PD1 blockade [43]. Blockade of TBK1/TKKε innate immune signaling kinases inhibited immunosuppressive cytokine production by CT26 tumor cells and enhanced cytotoxicity of the tumor in combination with PD-1 blockade [43]. Similar cytokine profiling of PDOTS from anti-PD1-responsive human cancers, such as melanoma, thyroid cancer, and Merkel cell carcinoma, identified acute production of cytokines and chemokines, such as CC-chemokine ligand 19 (CCL19) and CXV chemokine ligand 13 (CXCL13), after anti-PD1 treatment; induction of CCL19/CXCL13 was consistent with response in paired clinical samples with melanoma after PD-1 inhibition [43]. In

another study, small-molecule screening identified cyclin-dependent kinase (CDK) 4/6 inhibitors as compounds enhancing T cell activation in PD-1-overexpressing Jurkat T cells. Combination CDK4/6 inhibition and PD-1 blockade significantly induced tumor cell death *in vitro* in MC38 murine-derived MDOTS, as evidenced from tumor live/dead staining as well as from T cell-mediated tumor growth inhibition *in vivo* in syngeneic MC38 and CT26 mouse models [45]. Such studies highlight the potential of mechanistic *in vitro* immunotherapy drug studies, and could be extended to immune reconstitution of submerged Matrigel organoids or to holistic ALI organoids.

Application of PDOs for Adoptive Cell Immunotherapy—ACT immunotherapy represents a viable alternative to ICI and utilizes genetically engineered T cells with CARs or high-affinity TCRs recognizing tumor-enriched antigens, or alternatively, bulk autologous TILs. These strategies utilize *ex vivo* expansion of antitumor lymphocytes followed by reinfusion into patients [12]. Organoids are increasingly finding application for ACT research, such as in CAR-T cell therapy development. While CAR-T cells targeting CD19 exhibit impressive activity in hematological malignancies, such as B cell lymphoma [90] and acute lymphoblastic leukemia [91], solid tumor efficacy has been elusive [12]. PDOs have now been utilized to model tumor antigen-specific cytotoxicity of CAR-NK92 targeting EGFRvIII or FRIZZLED on CRC organoids [92]. This organoid-immune cell co-culture system could be used to assess CAR-mediated tumor-specific cytotoxicity in normal and tumor organoids [92]. Epithelial-only submerged Matrigel organoids, while lacking immune components, can serve as an antigen source enabling the selection of tumor-reactive lymphocytes. For instance, the generation of tumor-reactive CD8+ T cells by co-cultures of CRC or NSCLC PDOs with autologous PBMCs, in medium supplemented with IL-2, anti-CD28, and anti-PD1, can expand such lymphocytes with MHC-dependent cytotoxicity against autologous tumors, but not normal cells [52]. More complex organoid systems incorporating immune elements such as microfluidic or ALI methods might be similarly used for mechanistic or translational ACT studies, although further robust investigations are warranted.

Precision Medicine and Predicting the Immunotherapy Response

Conventional Matrigel PDOs can represent a promising platform for evaluating the functional responses of patients with cancer to chemotherapeutic drugs [37,39,93] and combined chemoradiation [37,39]. However, significant caveats include the need for reproduction in validation cohorts, the effects of tumor heterogeneity, the need for rapid real-time analysis, and, ultimately, the correlation against patient response and overall survival. Nevertheless, the application of PDOs to predict individualized responses to conventional treatment modalities is an active area of investigation.

The identification of biomarkers portending successful patient responses to immunotherapy has been problematic. Several biomarkers aid stratification of patient responses to anti-PD-1/PD-L1 checkpoint inhibition, including PD-L1 expression [94], CD8+ TIL density [95], mismatch repair deficiency (MMR), **high microsatellite instability (MSI-H)** [62], tumor mutation burden (TMB) [62], **tumor neoantigen** expression [96], and TCR clonality

[97], as well as immune gene signatures [98]. However, the selection of appropriate patient populations to enrich clinical checkpoint inhibitor response rates remains challenging.

Organoid modeling of patient-specific immunotherapy responses suffers from the same limitations as the prediction of chemotherapy and radiotherapy, but with the additional challenges of requiring both tumor and immune cell compartments. Here, holistic systems may find particular utility because they contain the immune TME. The evaluation of cytokine profiling and T cell cytotoxicity against tumor cells for 1 week in microfluidic culture PDOTS-retaining tumor cells and autologous immune components might provide the potential to predict or assess a patient's response to ICI treatment [43]. Alternatively, ALI organoids co-preserving tumor epithelium alongside diverse endogenous immune elements might model responses to immune checkpoint inhibition by evaluating T cell functions through flow cytometry, fluorescence staining, and tumor killing [72]. These and other predictive approaches will require prospective validation and correlation with clinical outcomes, but represent a substantial opportunity for clinical translation via identification of cohorts optimally responsive to immunotherapies.

Modeling Cancer-Associated Inflammation in Tumor Organoids

Chronic inflammation, accounting for 15–20% of cancer deaths, can promote both tumorigenesis and treatment resistance [4]. Most commonly, these inflammatory diseases can include inflammatory bowel disease (IBD) in CRC, hepatitis B or C virus (HBV/HCV) infection in hepatocellular carcinoma (HCC), *H. pylori*-induced gastritis, and Barrett's esophagus [99]. Oncogenic viruses, such as Epstein-Barr Virus (EBV) and human papilloma virus (HPV), not only encode transforming proteins, but can also elicit inflammation [99,100]. The substantial TME reciprocal crosstalk between neoplastic and inflammatory cells incorporates dynamic components of the adaptive, humoral, and innate immune systems, to cooperatively regulate cancer-associated inflammation and tumorigenesis [4,101]. Thus, organoid models might facilitate fundamental insights into such cancer-associated inflammation, providing opportunities for disease prevention and treatment.

Numerous infection models relevant to cancer utilize organoids, such as HBV [102,103] and HCV [104] infection of liver organoids and *H. pylori* infection of gastric organoids [105–107]. Microinjection of *H. pylori* into gastric organoids induced strong inflammatory responses, including the release of IL-8 [105]. The reciprocal interactions between epithelial cells and carcinogenic pathogens is illustrated by gastric organoids producing urease, a chemoattractant to *H. pylori*, after which the bacteria then deliver the bacterially encoded CagA transforming protein to the gastric epithelium [107]. Additionally, the prolonged exposure of carcinogenic *Escherichia coli* carrying the pathogenicity island *pks* to human intestinal organoids can elicit oncogenic mutations [108].

Such infection systems can be extended to organoid co-cultures with immune cells. Upon co-culture of human gastric organoids and DCs isolated from PBMC, *H. pylori* microinjection into gastric organoids increased DC recruitment into gastric organoids in a gastric epithelium secreted chemokine-dependent manner, followed by DC phagocytosis of *H. pylori* applied to the organoid lumen; this was evidenced from differential fluorescence

expression, labeling each cell and *H. pylori*, allowing the visualization of their interactions [53]. *H. pylori* also induced PD-L1 expression on gastric epithelium mediated by sonic hedgehog (Shh) signaling, and the inhibition of PD-L1-induced organoid cell death in a co-culture of CTLs, DCs, and human gastric organoids. This suggested that *H. pylori*-induced PD-L1 expression is protective against cytotoxic activity [54]. In addition to immune reconstitutive approaches, holistic microfluidic or ALI organoid strategies might also find application. Such organoid models containing triads of immune cells, tumor cells, and pathogens have the potential to elaborate a carcinogenic interplay.

Concluding Remarks

Recent advances in 3D organoid cultures of human malignant cells, engineered either from primary wild-type tissues or directly from tumor biopsies, are rapidly transforming the landscape of *in vitro* cancer experimentation and clinical translation. Intrinsic to the application of organoids to the study of tumor immunology is the need to incorporate immune components alongside tumor epithelium, as currently addressed by reconstitutive and holistic approaches. Optimally, the immune TME within organoids would contain an entire diversity of immune cells, including T, B, NK, and myeloid cells and other innate immune cells, and might conceivably incorporate both tumor-infiltrating and peripheral immune cell populations. The additional overlay of infectious and carcinogenic pathogens certainly adds further complexity (see Outstanding Questions). Co-culture of tumor organoids with peripheral immune cells from PBMCs or lymph nodes might also allow modeling of the cancer-immunity cycle, including T cell priming/activation, trafficking/infiltration of T cells into tumors, and recognition/killing of tumor cells by T cells. Introducing pathogens or commensal microbiota into tumor organoids with immune cells could ostensibly recapitulate cancer-associated inflammation and carcinogenesis through pathogen, epithelial-immune cell interactions, enabling an assessment of immunomodulatory outcomes and immunotherapeutic responses. Furthermore, these culture systems may enable investigations of innate immunity in promoting T cell effector function through inclusion of relevant cell types, such as DCs, monocytes, macrophages, mast cells, and NK cells, and further allow interrogation of toll-like receptor (TLR), NOD-like receptor (NLR), and stimulator of interferon genes (STING) agonists.

By analogy to biobanks of epithelial-only organoids, long-term cryopreservable PDO cultures containing immune components might allow experimental standardization of models, which otherwise are '$n = 1$' models that differ between laboratories. Substantial challenges remain in the long-term culture and preservation of immune cells within organoids; alternatively, matched tumor epithelium and immune cell components could be banked in parallel and then reconstituted. Additional supplements, such as IL-2, anti-CD3, and anti-CD28 antibodies, might facilitate the long-term maintenance of immune cells, but recapitulation of antitumor responses would certainly require robust validation. Primary organoid cultures of peripheral immune organoids, such as lymph nodes, might offer opportunities for investigating the crosstalk between immune cells, tumor cells, and local TME-specific events (see Outstanding Questions).

An important translational challenge for immune organoid research is represented by modeling sensitivity and resistance to immunotherapies, spanning checkpoint inhibition, novel pathways, and ACT strategies. In addition to elucidating underlying resistance pathways, organoids might facilitate therapeutic efforts, such as *in vitro* screening and optimizing pharmacological and cellular immunotherapies; and might also allow real-time determination of patient sensitivity to single or combinatorial treatments, if short-term responses in culture can indeed accurately reflect clinical responses and long-term outcomes (see Outstanding Questions). Ultimately, one can anticipate that current and future organoid methodologies will substantially further immuno-oncology basic science and translation, forging a path towards realizing the already considerable promise of human tumor immunotherapy.

Acknowledgments

Glossary

Air-liquid interface (ALI)
culture method by which cells are grown in a collagen matrix where the top of gel is exposed to the air

B16F10
murine melanoma cell line

Chimeric antigen receptor (CAR)
genetically engineered T cell receptors that recognize tumor antigens

CT26
murine colon carcinoma cell line

Cytotoxic T lymphocyte (CTL)
effector T lymphocytes that kill antigen-expressing target cells

Exhausted T cell
dysfunctional T cells characterized by loss of effector function, expression of multiple inhibitory receptors, and altered transcriptional network due to chronic antigen exposure, such as viral infection and cancer

GL261
murine glioma cell line

High microsatellite instability (MSI-H)
high frequency of mutations within microsatellites that are short, repeated sequences of DNA

Immune checkpoint inhibitor (ICI)

inhibitors, often monoclonal antibody based, that neutralize inhibitory immune checkpoints, allowing immune cells to recognize and attack cancer

MC38

murine colon adenocarcinoma cell line

Patient-derived organoids (PDOs)

3D cultures of patient's tumor cells grown in matrix that retain the architecture, genetic, and phenotypic changes of the primary tumor

Pattern recognition receptors (PRRs)

receptors capable of recognizing molecules such as pathogens; have crucial roles in innate immunity

Peripheral blood lymphocytes (PBLs)

blood cells comprising T, B, NK, and NKT cells. Depending on the purpose, specific lymphocytes, such as CD3$^+$ T cells, are isolated and used as PBLs

Peripheral blood mononuclear cells (PBMCs)

unfractionated circulating blood cells comprising lymphocytes (T, B, NK, and NKT cells), monocytes, and DCs

Tumor-infiltrating lymphocytes (TILs)

lymphocytes that infiltrate tumors

Tumor microenvironment (TME)

complex cellular milieu surrounding tumor epithelium, including diverse supporting cell types, such as activated fibroblasts, blood vessels, infiltrating immune cells, and extracellular matrix

Tumor neoantigen

tumor-specific antigens generated by somatic mutations

α-Smooth muscle actin (αSMA)

often used as a myofibroblast marker

Resources

i. https://ocg.cancer.gov/programs/hcmi/hcmi-organization

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Highlights

Diverse *in vitro* culture methods allow modeling of tumor immunity.

'Tumor-only' 3D patient-derived organoid (PDO) systems can be reconstituted with exogenously added immune components.

Holistic air-liquid interface (ALI) tumor organoid cultures and microfluidic cultures can recapitulate the tumor microenvironment (TME) by preserving endogenous stromal components including diverse immune cells (B, T, and natural killer cells, and macrophages) without reconstitution.

Tumor-infiltrating lymphocytes in the native TME of ALI PDOs or microfluidic PDO tumor spheroids can model programmed cell death-1 (PD-1)-dependent immune checkpoint inhibition and tumor cytotoxicity.

Regardless of methodology, *in vitro* culture models of the immune TME allow exploration of tumor immunology and novel immunotherapeutic targets.

Outstanding Questions

How can one apply organoids to understanding basic mechanisms of action of immunotherapies?

How can increased immune cellular complexity be both incorporated into, and maintained in, organoid cultures?

Can culture duration be extended, to the degree of establishing organoid biobanks containing tumor cells and immune components for model standardization across laboratories?

How can organoids be best deployed for the development of current and novel immunotherapies?

Can organoids accurately assess clinical responsiveness to pharmacological or cellular immunotherapies, and can this be performed in real-time?

Key Figure

Tumor Organoid Culture Systems Modeling the Tumor Immune Microenvironment
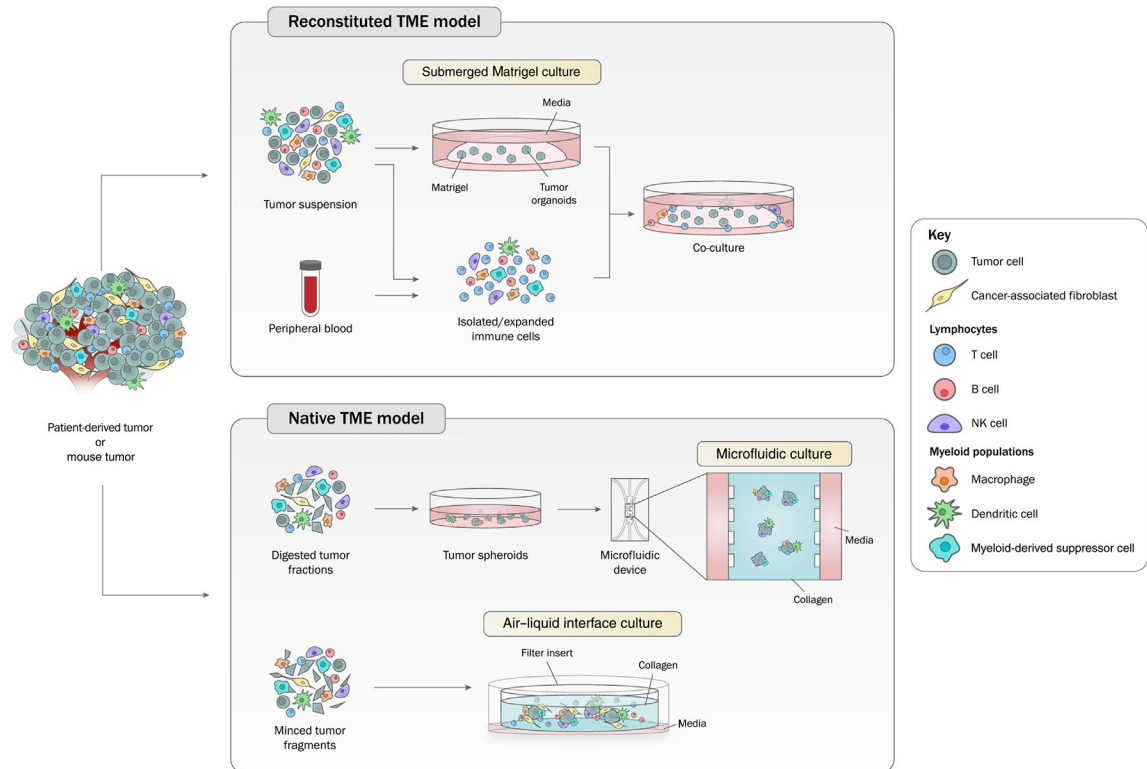
Figure 1.

The tumor immune microenvironment can be generated in organoids by two types of approaches. In reconstituted models, organoids containing exclusively tumor cells, often from physically and enzymatically dissociated tissues, are cultured in extracellular matrix domes (e.g., Matrigel or BME-2) and submerged beneath tissue culture medium. Exogenous immune cells, such as those from autologous peripheral blood or tumor, are isolated and subsequently co-cultured with grown organoids. In holistic native TME models, the intrinsic immune microenvironment of tumor specimens is preserved along with tumor cells without reconstitution. Tumor spheroids from digested tumor tissues can be mixed with collagen and injected into microfluidic culture devices. Alternatively, in air-liquid interface (ALI) culture, minced primary tissue fragments containing both tumor cells and immune components are embedded in collagen gels within an inner transwell dish. The top of the collagen gel is exposed to air, allowing cells access to a sufficient oxygen supply. Abbreviation: NK, natural killer.
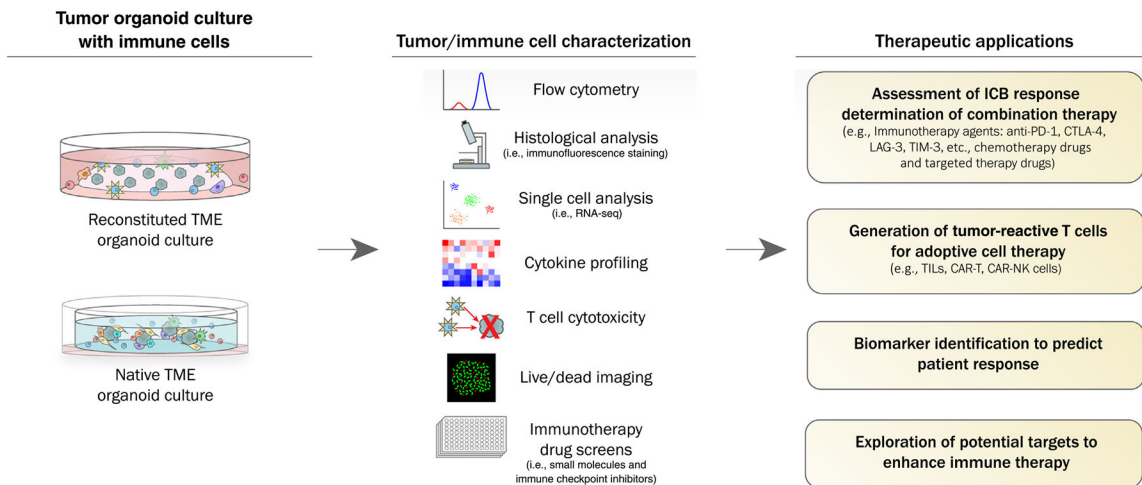
Figure 2.
Patient-Derived Organoids (PDOs) for Personalized Cancer Immunotherapy. The native immune tumor microenvironment (TME) can be modeled using PDO air-liquid interface (ALI) organoids or PDO tumor (PDOT) microfluidic devices; alternatively, the TME can be reconstituted by adding purified immune populations to submerged tumor epithelial organoids. Multiple downstream applications include defining interactions between tumor cells and immune cells, development of immunotherapies, biomarker research, and prediction of individualized patient responses. Abbreviations: CAR, chimeric antigen receptor; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; ICB, immune checkpoint blockade; NK, natural killer; PD-1, programmed cell death-1; TIL, tumor-infiltrating lymphocyte.

Table 1.

Overview of *Ex Vivo* Tumor Organoid Culture Systems Modeling the Tumor Immune Microenvironment

Feature	Method		
	Submerged Matrigel culture	**Microfluidic 3D culture**	**ALI culture**
Source	**Patient-derived and mouse-derived tumor specimens**		
Tissue processing before culture	Tissues are dissociated physically and enzymatically (e.g., collagenase, dispase, and trypsin)	Tissues are dissociated physically and enzymatically (collagenase); samples are passed over filters to collect 40–100 μm-sized spheroid fractions, subsequently maintained in ultra-low-attachment plates	Tissues are physically minced into fragments
Matrix	Matrigel	Collagen	Collagen
Culture instrument	Any size of plate or dish	3D microfluidic culture device	Diverse cell culture inserts and dishes or plates, including multiwells
Plating procedure	Cell-Matrigel mixture is plated; medium is added over Matrigel	Spheroid-collagen mixture is injected into central gel region of device; medium is added into media channels on both sides	Minced tumor tissue fragments are embedded in collagen and plated on bottom collagen layer; medium is added into an outer dish; top of collagen layer is exposed to air
Cell types of components retained in culture	Tumor cells exclusively; difficult to maintain stromal components long-term	Tumor cells, tumor-infiltrating lymphoid and myeloid cells, including DCs, MDSCs, and TAMs; determined by flow cytometry	Tumor cells, native immune cells (T and B cells, myeloid cells, macrophages, and NK cells) and stromal fibroblasts; determined by flow cytometry, single cell RNA-seq, and immunofluorescence
Culture period	Long-term culture to maintain and expand tumor organoids; short-term reconstitutive co-culture with different types of immune cell	Short-term culture; long-term culture is not reported	Tumor cells can propagate long-term; immune cells and fibroblasts in both human and mouse organoids decline over a 1–2-month period
Advantages	Easy to enrich and expand tumor organoids; can recapitulate genetic and morphological alterations of original tumor; potential recapitulation of clinical responses to chemotherapy and/or radiation	Requires small number of cells and small amount of medium and reagents to test; preserves multiple different types of cell in TME; enables study of tumor-immune interactions	Recapitulates genetic and morphological alterations of original tumor; preserves diverse immune cells and fibroblasts in TME; enables study of tumor–immune interactions
Limitations	Lack of native immune and stromal components; exogenously added TME only	Size limitation; requires specialized equipment; restricted to native tumor-infiltrating immune cells; does not reflect recruitment of circulating immune cells into tumor	Creation of uniformly sized organoids; restricted to native tumor-infiltrating immune cells; does not reflect recruitment of circulating immune cells into tumor
Co-culture system to reconstitute immune TME	Organoids can be co-cultured with PBMCs, primary leukocytes, TAMs, and DCs that are added to medium	Immune cells (Jurkat cells) can be added in medium to assess T cell infiltration into organotypic tumor spheroids; immune TME of primary tissue is faithfully reconstituted	Immune TME of primary tissue is faithfully reconstituted
Potential of immune cells in culture	Co-culture of autologous PDOs and PBMCs enriches tumor-reactive T cells, which can be used to assess efficiency of T-cell mediated cytotoxicity; enables assessment of tumor organoid killing by co-culture with TILs and CAR cells	Recapitulates response to anti-PD-1 antibody; useful culture system to test therapeutic combinations to enhance response to PD-1 response; secreted cytokine profiling	Recapitulate functional T cell activation and tumor-killing responses to anti-PD-1 and anti-PD-L1 antibodies; TCR repertoire highly conserved between TILs of original tumor and ALI PDOs
Refs	[37–40,50,52,53,72,92]	[43,44,55]	[36,46]