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Patient-derived organotypic tumor spheroids, tumoroids, and organoids: advancing immunotherapy using state-of-the-art 3D tumor model systems

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Preclinical *ex vivo* models capable of probing patient-specific tumor–immune interactions are particularly attractive candidates for interrogating mechanisms of resistance, developing predictors of response as well as assessing next-generation immunotherapeutics. By maintaining features of a patient's own tumor microenvironment, such patient-derived *ex vivo* models are poised to meaningfully contribute to the functional assessment of individual tumors to provide a tailored approach to treatment. Among contemporary *ex vivo* models, patient-derived organotypic tumor spheroids (PDOTS) have emerged as a promising microfluidic-based platform that is well positioned to become a useful tool for precision medicine efforts. The advantages and limitations of PDOTS and related state-of-the-art patient-derived tumor models, as well as ongoing challenges facing the clinical implementation of patient-derived *ex vivo* tumor models, are reviewed.

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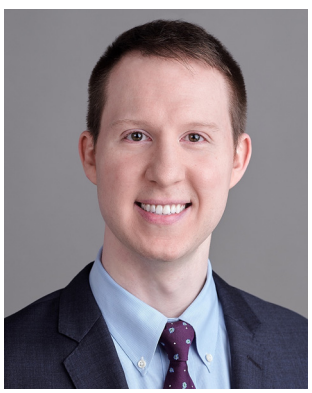
I. Introduction

The treatment of advanced cancer has undergone a dramatic transformation over the past decade with the advent of immune checkpoint blockade (ICB) targeting co-inhibitory receptors expressed on the cell surface of tumor-reactive T



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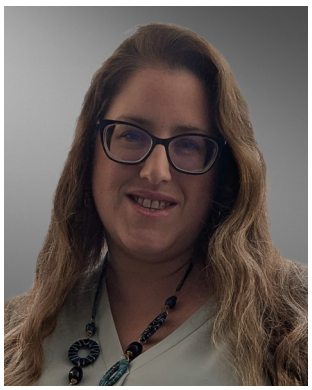
lymphocytes and other immune cells.[1] Patients with select tumor types exhibit high response rates to ICB, including melanoma, non-small cell lung cancer (NSCLC), and renal cell carcinoma (RCC).[2,3] Combination ICB with ipilimumab (CTLA-4) and nivolumab (PD-1) enhances response rates compared to single-agent ipilimumab or nivolumab in patients with metastatic melanoma with nearly half of patients in the combination ICB group still alive 10 years after starting treatment.[4] Despite the success of ICB in melanoma and other cancers, therapeutic resistance (or lack of sensitivity) remains a central challenge and is ultimately fatal for patients without suitable alternative therapies.[5]

The clinical success of ICB for a discrete subset of patients with advanced cancer has intensified efforts to develop preclinical model systems to inform use of current ICB therapies (targeting PD-1 and CTLA-4 immune checkpoints) and guide development of next-generation immunotherapeutics, including bispecific T cell engagers,[6,7] chimeric antigen receptor (CAR)-T cells,[8] tumor-infiltrating lymphocytes (TILs),[9,10] and therapeutic cancer vaccines,[11,12] amongst others. Cancer immunotherapy researchers face several challenges in their efforts to understand mechanisms of response and resistance to ICB, including (i) the need for preclinical models that translate to human immunity and (ii) the need for strategies to effectively and efficiently assess cancer immunotherapy combinations.[13] Furthermore, despite hundreds of clinical trials examining 'rational' combination strategies, these therapies remain "one size fits all" due to the lack of robust biomarkers to guide clinical decision-making or other biological insights to inform therapeutic selection. While certain biomarkers of ICB sensitivity have emerged including tumor mutational burden (TMB), immunohistochemical PD-L1 expression, DNA mismatch repair deficiency status, and microsatellite instability (MSI) status,[14] robust predictive biomarkers of ICB sensitivity (and resistance) are still lacking. With the expanding number of combination trials,[15] and failure of promising combination strategies in phase III clinical testing,[16,17] there has been renewed focus on the preclinical and early-phase clinical development of combination strategies. With over 1000 cancer immunotherapy combination trials under evaluation,[15] novel approaches are needed to identify mechanisms of response and resistance to ICB, identify and validate predictive signatures/biomarkers, evaluate next-generation therapies, and aid efforts to deprioritize ineffective strategies earlier in development to pave the way for more promising approaches.

Immune-competent murine tumor models remain the gold standard for most tumor immunology studies and are amenable to *in vivo*, *ex vivo*, and *in vitro* manipulation and iterative experimentation.[18] However, inbred murine tumor



Or-Yam Revach

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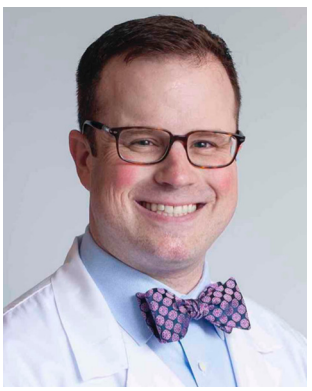

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models lack the patient-to-patient heterogeneity observed in human cancer and do not fully recapitulate all key features of human tumor immunity.[18–20] With the emergence of ICB as a key therapeutic modality for many cancer patients, there is growing appreciation of the numerous tumor microenvironmental factors capable of modulating therapeutic response. Hence, there is growing recognition that integration of patient-specific biological insights from preclinical models capable of recapitulating clinical responses in a laboratory setting would address major challenges facing cancer immunology researchers, the medical oncology community, and biopharma.

Given the need to understand patient-specific factors contributing to the heterogeneity of clinical response to ICB, three-dimensional patient tumor avatars (3D-PTA) derived from patient tumor tissue have emerged as promising model systems in which to study tumor–immune dynamics in a patient-specific manner.[21,22] Patient-derived organoids (PDOs), 3D constructs formed *via* culture and serial passaging of patient-derived tumors, have been utilized in a vast array of translational applications to date, including testing the effectiveness of chemotherapy, radiotherapy and small-molecule drugs, and correlating *ex vivo* therapeutic responses to clinical responses.[23] However, PDOs are comprised of tumor cells and typically lack immune components, which are lost during prolonged *in vitro* culture. To enable the study of immunotherapies, PDOs have been consequently "enhanced" or "reconstituted" by combining the tumor-only constructs with peripheral or tumor-infiltrated immune cells and have proven useful in studying ICB as well as adoptive cellular therapies.[24] More recently "native" 3D-PTAs have emerged as models focused on preserving the native architecture of the parent tumor, thereby maintaining the tumor-immune interactions of the original tumor, which are lost in reconstituted PDO models. These native 3D-PTA models include patient-derived tumor samples in air-liquid interface culture,[25] patient-derived tumor fragments (PDTFs),[26] and patient-derived organotypic tumor spheroids (PDOTS) in 3D microfluidic culture.[27,28] Evaluation of patient-derived samples that preserve the composition and organization of the tumor microenvironment (TME) may facilitate identification of novel predictive and/or prognostic biomarkers, accelerate identification of therapies to overcome ICB resistance, and advance translational research efforts to ultimately guide precision medicine efforts to tailor therapy decisions for individual patients.[29,30]

By utilizing samples of a patient's tumor, such models can capitalize on the native and unique features of an individual tumor and can begin to realize the potential of functional precision medicine, an emerging concept within cancer treatment which aims to maximize therapy effectiveness and minimize toxicity risk, by informing the use or discovery of more personalized treatment regimens.[31] Though progress has been made in preclinical model development, creating models that are both able to replicate the complexity of human tumor immunity and be used for these purposes remains an unmet need.[32] The approach to developing models for immunotherapy differs from previously employed methods to study tumor-targeted therapy, such as traditional chemotherapy, as immunotherapy relies on interactions among components of the TME, which in addition to tumor cells includes immune cells, stromal cells, non-cellular stromal components, and vasculature.[33] The inclusion of the human TME is critically important for probing immunotherapy as this type of therapy is fundamentally driven by tumor–immune interactions; success of immunotherapy has been linked not only to characteristics of the tumor cells, but also to features of the immune compartment.[33] For example, pathologic insight demonstrated that tumors containing a high degree of acute inflammation often responded more favorably to ICB compared to tumors featuring chronic inflammation.[34] Immune interactions also play heavily into the emergence of therapy resistance and features of immune cells such as their cell states (*e.g.*, dysfunctional/exhausted $CD8^+$ T cells) and the evolution of tumor–immune composition over a treatment course have been appreciated.[35] Notably, the incorporation of the human immune compartment has also been recognized as important for the study of non-malignant native tissue physiology and function, such as in studying autoimmunity, with recent efforts establishing truer models of human tissue, particularly for the gastrointestinal tract.[36,37]

In this review, we will discuss contemporary *ex vivo* patient-derived models and their utility in the context of investigating immunotherapeutics. We will highlight models that preserve key elements of the TME to faithfully recapitulate immunotherapy response (and resistance) with specific focus on PDOTS, a microfluidic-based "TME-on-a-chip" platform that leverages patient-derived tumor tissue to study tumor–immune dynamics and with which our group has extensive experience. Introduced in 2017, PDOTS have continued to show utility in preclinical evaluation of standard-of-care ICB and next-generation immunotherapeutics. PDOTS demonstrate advantages over other state-of-the-art models as a versatile platform which preserves the native structure and composition of the TME while allowing for culture times that enable both media profiling and dynamic visualization of tumor killing by immune cells without growth factor support. Recent accomplishments of PDOTS and similar platforms in aiding translational discovery in cancer biology are also highlighted.

II. Patient-derived organoids, reconstituted organoids, and xenografts

Patient-derived organoids

Early tumor models consisted of 2-dimensional monolayers or 3-dimensional constructs composed of only tumor cells which were largely derived from induced pluripotent stem

cells or human adult stem cells.[38–40] Hence, the term "organoid" when used in its purest sense refers to a monotypic group of tumor cells, exclusive of other components of the TME. These models replicated features of tumor cells and generated valuable insight into tumor-intrinsic factors of malignant growth and proliferation as they enabled the genetic perturbation of tumor cells as well as subsequent non-immune-based therapy testing.[41,42] The field of tumor organoids has since expanded to include PDOs or alternatively patient-derived tumor organoids (PDTOs) created from tumor biopsies which allowed for patient-specific investigation of tumor biology and targeted-therapies.[40,43] PDOs and PDTOs are typically formed *via* the long term culture of patient-derived tumor biopsies which result in the elimination of immune and stromal cells, and consequently an enrichment in tumor cells. PDOs can be cryopreserved and banked, permitting longer-term, iterative experimentation of patient-derived samples in certain contexts. PDOs have been successfully deployed for drug sensitivity testing using traditional cancer therapies including chemotherapy, radiotherapy, and small-molecule drugs, and have been recently reviewed.[23] For example, correlation between chemotherapy or radiotherapy treatment and patient responses to those treatments has been shown using PDOs alone or alongside *in vivo* PDO-based orthotopic mouse xenograft models.[44–46] More recently, PDOs have been offered as a platform for predicting the clinical efficacy of standard-of-care chemotherapies as demonstrated in a small cohort of treatment naive patients with metastatic colorectal cancer (n = 56), though the diagnostic turn-around time of the test was long, on the order of 7 or more weeks from biopsy to readout.[47] PDOs have been used to screen a cohort of samples from patients with sarcoma, a histologically heterogeneous tumor type, to correlate therapeutic sensitivity and resistance to clinical outcomes across non-immune based treatments using a mini-ring platform with a turn-around time of about 1 week.[48] A similar screening of small molecule therapeutics was performed against neurofibroma, a non-malignant tissue, using the mini-ring platform which was shown to retain some myeloid and stromal cell components of the original tumor.[49] However, much like their parent stem-cell derived constructs, PDOs exclude immune cells and other native components of the TME which has limited the study of immunotherapy, particularly for ICB.

Reconstituted patient-derived organoids

In the past decade, there has been heightened interest in the development of *ex vivo* patient-derived models which have recombined immune cells and tumor cells to better simulate the TME.[50] Reconstituted models,[51] sometimes referred to as enriched models,[52] involve the deliberate reassembly of pre-selected aspects of the TME *via* co-culture. Reconstituted models can be created by using a standard PDO and directly adding TME components, or by first dissociating a patient's tumor into single cells and recombining them with or without the addition of exogenous components. In this respect, though reconstituted models contain TME components, they do not preserve the native arrangement or proportions of tumor, immune, or stromal cells as featured in the original tumor. Reconstituted models are diverse not only in culture configuration but also in the types of immune and stromal cells introduced.[53,54] The inclusion of immune cells had enabled the investigation of tumor–immune interactions and immune-based therapeutics to study the effects of adoptive cell therapies, probe novel drug candidates for combination therapies, and nominate potentially useful biomarkers of therapeutic response *ex vivo*.[55]

Reconstituted models have been used to study interactions between tumor cells and a host of immune and stromal cells including fibroblasts,[56] macrophages,[57] dendritic cells,[58] NK cells,[59] B cells,[60] and T cells.[61] Among tumor-related efforts, the incorporation of T cells has been of particular interest. Reconstituted PDO models have been used for activating and expanding tumor-reactive T cells as well as investigating the effectiveness of TILs, engineered T cells, and CAR-T cells.[24,62–65] For example, Dijkstra *et al.* reported a protocol to expand tumor-reactive T cells using PDOs co-cultured with peripheral blood mononuclear cells (PBMCs) and demonstrated that the expanded tumor-reactive T cells could exhibit tumor killing against matched organoids.[24] More recently, PDOs from patients with recurrent glioblastoma were generated over the span of 2–3 weeks and were used as a target scaffold to demonstrate tumor cell death and tumor antigen reduction upon treatment with CAR-T cells targeting epidermal growth factor receptor (EGFR) and interleukin-13 receptor subunit 2.[65] These experiments were performed alongside an ongoing phase 1 clinical trial and hence *ex vivo* cytokine data were able to be compared with patient CSF data at matched timepoints. The authors showed that trends in markers of T cell activation (TNF-α, IFN-γ, IL-2) from both the PDO model and from patient CSF were similar across matching time-points, supporting the potential use of this model for preclinical purposes. Reconstituted approaches have also been used in "organoid-on-a-chip" models to study non-tumor related therapeutic consequences such as "off-tumor, on-target" effects of immunotherapies.[66]

In addition to adoptive cell therapies, reconstituted models have also enabled the study of ICB. PDOs from mismatch-repair proficient (pMMR) early stage colon cancers resistant to neoadjuvant ICB (n = 6) were co-cultured with both patient-matched PBMCs after neoadjuvant ICB and anti-PD-1 antibodies and showed that the absence of T cell responses *ex vivo* correlated with clinical non-response. An *ex vivo* T cell response was identified in 2 of 5 mismatch-repair deficient (dMMR) clinical responders and in 1 of 1 pMMR tumor with partial response.[67] Furthermore, in another study, melanoma samples reconstituted with immune cells from patient-matched lymph nodes or PBMCs and exposed to ICB demonstrated that *ex vivo* tumor killing

correlated with positive clinical response in 6 of 7 patients in a small cohort.[68]

In addition to standard co-culture techniques, microfluidic-based approaches have been used in the development of reconstituted models with the aim of generating effective preclinical platforms for therapeutic testing. Microfluidic devices previously have been used to study many aspects of cancer biology such as tumor growth, metastasis, angiogenesis, and drug screening.[69] Furthermore, microfluidic devices have gained significant clinical attention with one liquid biopsy-based platform FDA approved for predicting prognosis in patients with metastatic cancer, and several others in development.[70] Microfluidic culture enabled the careful positioning of PDOs which when exposed to NK cells with and without a trispecific killer cell engager (TriKE) demonstrated that the addition of TriKE enhanced tumor killing and caused upregulation of chemokines associated with NK cell migration.[71] In another example, Ding *et al.* employed droplet emulsion microfluidics to create micro-organospheres (MOSs) which encapsulate a mixture of largely single-cell components derived from digested patient tumor biopsies into Matrigel spheres (diameter 250–450 μm) which can be further cultured and used for testing.[72] As the patient's tumor is nearly completely digested into single cell components, native architecture between tumor, immune, and stromal components is lost in processing. Sensitivity of MOSs derived from patients with metastatic colorectal cancer to oxaliplatin (chemotherapy) correlated with clinical observations in 4 of 4 patients whose MOSs were sensitive to oxaliplatin and 3 of 4 patients whose MOSs were resistant. Drug sensitivity data from MOS testing was available relatively quickly within 14 days of initial patient biopsy. Furthermore, the platform was extended to investigate immunotherapy and demonstrated tumor killing *via* apoptosis assays in MOSs exposed to ICB, bispecific antibodies, PBMCs exposed to anti-PD-1 antibodies, and more recently CAR-T cell therapy.[72,73]

Patient-derived xenografts

Patient-derived xenografts (PDX) are *in vivo* models where explanted patient tumors are implanted into immune-deficient mice with the ultimate goal of evaluating drug sensitivity in a living organism. PDXs (like PDOs) tend to lose immune/stromal cells over time and are best suited to examine tumor-intrinsic drug targets. To facilitate *in vivo* testing of cancer immunotherapeutics specific for human immune and/or tumor targets, "humanized" mice have been developed that incorporate a human-like immune system, within limitations.[74,75] Notably, these models have been used in a number of immunotherapy applications including investigating ICB,[76–78] and autologous CAR-T cells[79] across a spectrum of tumor types. Though patient-derived *in vivo* models have been useful in studying cancer therapies, they suffer significant drawbacks, including limited engraftment rates, time and labor-intensive processing, and high cost of maintenance.[80–82] To enable potential clinical utility, models that incorporate the TME ideally should utilize available processing techniques, be implementable on a reasonable timeframe, and scalable. For these reasons, we focus our discussion on *ex vivo* patient-derived platforms which have the potential to be employed as effective preclinical assays for immunotherapy in the context of precision oncology.

III. Patient-derived native tumor models

Immune-reconstituted PDO models enable the evaluation of cancer immunotherapeutics, but lack the native organization, structure, and/or composition of the original tumor. However, a select number of models have utilized direct *ex vivo* culture of patient-derived tumors while preserving the native architecture and composition of the TME.[25–27,83] These "native" models (or avatars) rely on minimally processing surgically-resected patient biopsy samples to achieve tumor samples that can be used for immunotherapy testing, cytokine profiling, and therapeutic candidate selection.[51] Typically, gross surgically-resected tumors are mechanically minced, sectioned, or otherwise dissociated to preserve cell–cell and cell-stromal configurations and/or composition *ex vivo*. Notably, the term "tumoroid" which was first coined as a surrogate for a PDO,[23] has also more recently been applied to describe a patient-tumor model that preserves features of the native TME, which emphasizes the importance of understanding the details of each model when reviewing the literature.[84] Native models vary in their execution and aid in the study of tumor samples across a range of sizes, configurations, and culture conditions. These features not only dictate the length of time in which the tumor and/or immune components of a sample are viable but also inform the analysis techniques that can be used. These models have been shown to maintain immune cell subpopulations over reasonable time frames, depending on the platform and expected analysis, which is a critical consideration for capturing tumor-immune dynamics. Notably, native models have already begun to demonstrate preclinical utility as avatars composed of tumor fragments have proven useful in correlating ICB responses in small cohorts of patients across solid tumors.[26,85]

Air–liquid interface model

The model described by Li *et al.* and employed by Neal *et al.*, known as the air–liquid interface (ALI) model, utilized a "double dish" configuration to culture and test patient-derived tumor samples.[25,83,86] In this configuration, the minced tumor was resuspended in type I collagen hydrogel which was dispensed atop a preformed collagen layer within an inner dish. The base of the inner dish was composed of a polytetrafluoroethylene membrane. The inner dish was placed within a larger outer dish which held media and contacted the polytetrafluoroethylene membrane. The top

collagen-tumor layer was exposed to humidified air, while the bottom collagen layer was exposed to media (air–liquid).[25,83] Tumor particle sizes in this model were several hundred micrometers after mincing while the total volume of the cultured particles was generally less than 1 mm^3. The authors showed that the minced tumor contained a representative cohort of immune cells, including $CD8^+$ and $CD4^+$ T cells, B cells, macrophages, NK cells, and NK T cells, and further highlighted that the T cell receptor repertoire of the TILs were representative of the larger tumor.

The ALI model enabled the long term growth of patient-derived tumor components for weeks to months, though this often required media supplementation with cytokines and growth factors. Though long term culture was technically feasible, not all tumor compartments could be sustained over this period of time, as the authors showed that the viability of $CD3^+$ T cells decreased by 80% by day 7 of culture, despite optimization, though the T cell population could be incrementally restored with the addition of the T cell growth factor, interleukin-2 (IL-2). Thus, tumor–immune interactions were best studied at earlier time points (~1 week). The cultured tumor samples were suitable for standard immunohistochemical and immunofluorescence analysis, as well as flow cytometry and RNA sequencing for T cell profiling after dissociation to single cells. Using this model, the authors showed that addition of nivolumab (anti-PD-1) to ALI cultured tumors increased T cell activation with upregulation of *IFNG*, *PRF1*, or *GZMB* in 6 of 20 patient samples across three immunotherapy-sensitive tumor types (*i.e.*, NSCLC, clear cell RCC, and melanoma). The model also enabled bulk cytotoxicity analysis to be performed after ICB by staining the cells for apoptotic factors and determining the relative changes of cell subtypes compared to a control. Using this method, the authors demonstrated evidence of tumor killing in 2 of 10 patient-derived samples in a second cohort. The ALI culture method has been employed in a variety of other contexts such as modeling tumorigenesis in the context of tumor-stromal interactions,[86] TIL migration,[87] and studying the effect of personalized immunotherapy selection.[88]

Patient-derived tumor fragments

In contrast to the ALI model, the platform described by Voabil *et al.* utilized larger pieces of minced patient-derived tumor "fragments" on the order of 1–2 mm^3 which were directly embedded into an extracellular matrix (ECM).[26] In their setup, each patient-derived tumor fragment (PDTF) was placed on top of a preformed matrix layer in a 96-well plate over which a liquid collagen/Matrigel mixture was added and allowed to solidify before introducing media. Owing to the size of the tumor fragments, culture times were limited to a period of 3 days due to a reported decline in cell viability after this time point.[89] Using this model, the authors identified a cytokine signature (driven largely by CXCL1, IFNγ, and CXCL10) associated with ICB sensitivity by examining cytokine profiles in conditioned media following PD-1 blockade compared to control PDTFs in a cohort of 37 patient-derived tumors. Importantly, the *ex vivo* PDTF cytokine signature correlated with clinical response in 12 of 12 patients who subsequently received ICB. A validation cohort of 26 patient tumor samples confirmed these findings, highlighting the potential of this native tumor platform in developing predictive biomarkers of clinical response. Flow cytometry was used to categorize non-responsive tumors into several subgroups based on their relative abundance of immune cells and the authors paired this information with digital imaging and immunohistochemistry of tumor slices to further characterize tumors as immune-excluded or -rich. They paired this bulk spatial analysis with available clinical data to show that 6 of the 7 immune-excluded tumor samples did not show immune reactivation after ICB. In a follow-up study, the PDTF platform was used to show that a CD8-targeted IL-2 fusion molecule could revive a dysfunctional T cell pool after ICB within the tumor, demonstrating the model's utility in testing new therapies and contributing to fundamental biological insight.[90] Of note, other models using larger pieces of patient-derived tumors have been proposed as preclinical models correlating patient responses to chemotherapy; however, they have not yet been translated into clinical use.[91–93]

Tumor slice cultures

PDTFs retain key tumor-infiltrating immune and stromal elements, but are generated from different regions of the tumor. Organotypic tumor slice cultures (*i.e.*, precision-cut tumor slices) use larger pieces of tumor to maintain a high degree of native tumor architecture and preserve intrinsic tumor–immune interactions with the goal of capturing changes in the spatial organization of key features of tumor immunity.[94,95] Tumor slices typically have lateral dimensions on the order of millimeters and thicknesses of approximately 250–500 μm and are cultured in a variety of ways including free-floating, matrix-embedded, or membrane-supported configurations.[96] They have also been employed in microfluidic systems in an effort to move toward high-throughput analysis.[97,98] These models have extended the analysis of traditional pathologic immunohistochemistry owing to their advantage to perform functional and spatial analysis of patient-specific cellular components when introduced to immunotherapy. For example, Seo *et al.* utilized slices of pancreatic adenocarcinoma cultured between 2 to 6 days to show that combination treatment with PD-1 and CXCR4 blockade resulted in expansion of TIL and killing of tumor cells.[99] Live 3D confocal fluorescence microscopy was also used to visualize $CD8^+$ T cell migration into juxtatumoral compartments and subsequently follow the dynamic apoptosis of tumor cells.[99] Slice cultures have been used to map the extent of spread of oncolytic viruses in tumor samples of head and neck squamous cell carcinoma; T cells were shown to be present and remain functional for up

to 48 hours of culture.[100] Furthermore, they have also been used to study new cellular therapies. The addition of CEA-targeting CAR-T cells to tumor slice cultures of colon adenocarcinoma in the presence of an IL-10 blocking antibody demonstrated heightened tumor killing by the CEA-targeting CAR-T cells by showing an increase in apoptosis of tumor cells adjacent to the CAR-T cells, whereas no change was observed in tumor cells that were farther away.[101]

Despite their ease of processing and short-term turnaround, tumor slice cultures face challenges regarding viability, reproducibility (particularly for tumors with high degree of intratumoral heterogeneity), and limited use in high-throughput applications, though some examples exist.[96] As noted, though some slice cultures can be maintained for up to 1 week, the majority of slice cultures retain original cellular composition and morphology up to 72 h, similar to larger tumor sample models such as PDTFs. This time frame is useful in studying the molecular perturbation of the TME, such as media cytokine and chemokine profiling, though is not adequate to observe tumor killing. Furthermore, depending on the overall spatial heterogeneity of the tumor sample, tumor slices may not capture a representative assortment of cellular and stromal features across samples, which complicates their use in reliably testing multiple therapeutic configurations. One should note that this is a potential drawback of any model which employs larger tumor pieces and requires a large number of experimental conditions to be tested. Tumor slice cultures and PDTFs represent prominent examples of native tumor models which can be more broadly categorized as tumor "explants", given their size.[51,102] We note that other tumor explant models are being developed to study immunotherapy, particularly for media profiling and biomarker discovery given the short-term viability of these cultures.[103,104]

Patient-derived organotypic tumor spheroids

Murine- and patient-derived organotypic tumor spheroids (MDOTS/PDOTS) are multicellular aggregates that retain autologous lymphoid and myeloid populations and subpopulations and which have been shown to be responsive to *ex vivo* PD-1 blockade, combination therapies with ICB, and cellular therapies.[105,106] PDOTS combine microfluidic technology with explanted patient tumors which are partially digested into spheroids, filtered to achieve initial diameters of 40 to 100 μm, and suspended in a collagen matrix. The spheroid-collagen mixture is placed into the central chamber of a microfluidic device which is flanked along its loading region by two media channels which are exposed to the central channel by triangular posts. Once assembled, PDOTS can be cultured for ~7 days, and analyzed using fluorescence-based live-dead imaging, secreted cytokine profiling, light phase contrast microscopy, immunofluorescence microscopy, and time-lapse imaging. The microfluidic device used for MDOTS/PDOTS was developed by Prof. Roger Kamm (MIT) to study tumor cell migration and angiogenesis[107] and was adapted for the 3D culture of MDOTS/PDOTS for *ex vivo* profiling of cancer immunotherapeutics.[27,28] Though the PDOTS platform was first developed using microfluidic culture, other groups have demonstrated utility in culturing PDOTS-like tumor spheroids in a standard well-plate culture configuration as well.[108]

PDOTS, like other native tumor models, are distinct from traditional PDOs, which lack immune cells and require specific culture conditions, growth factors, and small molecule inhibitors to facilitate propagation of $LGR5^+$ epithelial stem cells.[109] It frequently takes several weeks to obtain sufficient material for phenotypic analysis of PDOs, whereas PDOTS are generated within hours of tumor procurement and processing for same-day drug sensitivity testing. While use of larger tumor fragments (*e.g.*, PDTFs and organotypic slice cultures) may offer certain advantages, tissue viability becomes compromised over time and reliable experimentation tends to be limited to evaluation of biological phenomena observable within 48–72 hours of culture. Given that *in vitro* and *ex vivo* immune-mediated cancer cell killing *via* endogenous lymphocytes, exogenous CAR-T cells or expanded TILs occurs over longer time intervals, the utility of such models may be limited. Furthermore, larger fragments rely heavily on bulk analysis methods such as histologic imaging, flow cytometry, or cytokine profiling of immune activation. The PDOTS microfluidic-based platform provides a promising alternative native tumor platform for the study of the TME in the context of immunotherapy.[27] Presently, PDOTS are used as a preclinical platform for evaluating existing and emerging immunotherapeutics and combinations thereof. Though *ex vivo* immunotherapy responses using PDOTS have not yet been validated to predict the clinical outcomes in patients, we highlight the current applications of PDOTS and outline an actionable pipeline for clinical correlation.

IV. Immunotherapy applications of PDOTS

Immune checkpoint blockade

Response and resistance to ICB in MDOTS/PDOTS. To establish feasibility and proof-of-concept that ICB response can be observed *ex vivo* using 3D microfluidic culture of organotypic tumor spheroids, MDOTS derived from syngeneic, immune-competent murine tumor models were examined and *ex vivo* response to PD-1 blockade was compared with established *in vivo* sensitivity profiles. MDOTS recapitulated response and resistance to PD-1 blockade *ex vivo* using established mouse tumor models with stereotyped sensitivity profiles. In ICB-sensitive models, initial loss of cell viability following ICB treatment was observed as early as day 3 and became maximal at day 5–6. Successful *ex vivo* PD-1 blockade required "native" tumor-infiltrating $CD8^+$ T cells and intact IFNγ signaling and mirrored *in vivo* response/resistance.[27,110] After establishing

proof-of-concept using MDOTS, *ex vivo* profiling of PDOTS derived from patients with advanced melanoma and other cancers was performed to assess pharmacodynamic response to PD-1 with/without CTLA-4 blockade. PDOTS profiling permitted evaluation of dynamically secreted cytokines and chemokines in addition to evaluation of PDOTS viability. Upregulation of homeostatic chemokines CXCL13 and CCL19 was observed, but almost exclusively in ICB-sensitive tumor types (*e.g.*, cutaneous melanoma, Merkel cell carcinoma). CXCL13 is highly expressed in tumor-reactive $CD8^+$ T cells which were subsequently associated with tertiary lymphoid structure formation.[111–113] and favorable response to ICB *ex vivo*.[114] Interestingly, CXCL13 is not expressed in tumor-infiltrating $CD8^+$ T cells in most established implantable syngeneic murine tumor models, further highlighting important distinctions between murine and human anti-tumor immune responses.

Defining T cell states associated with ICB response and resistance. Single cell RNA-sequencing (scRNA-seq) analysis of tumor-infiltrating immune cells revealed enrichment of a subset of $CD8^+$ T cells defined by increased expression the transcription factor *TCF7*, and by absence of T cell exhaustion associated surface markers CD39 (*ENTPD1*) and TIM3 (*HAVCR2*), with clinical response to ICB.[35] Similar observations have been made with murine tumor models[115] and in patients treated with TIL therapy.[116] Surface expression of CD39 and TIM3 readily discriminates between these two CD8 sub-populations, with $CD39^-$ $TIM3^-$ (double negative, DN) $CD8^+$ T cells enriched for memory/effector-like genes, while $CD39^+$ $TIM3^+$ (double positive, DP) $CD8^+$ T cells represented a terminally exhausted state. Using MDOTS from partially ICB-responsive CT26 tumors, it was shown that enrichment of memory/effector-like (DN) $CD8^+$ TILs enhanced response to *ex vivo* PD-1 blockade, whereas DP $CD8^+$ TILs not only failed to enhance response to PD-1 blockade, but appeared to blunt the activity of native $CD8^+$ T cells. Enrichment with equal numbers of DP and DN (DN : DP = 1) $CD8^+$ TILs failed to enhance, or inhibit, the response to PD-1 blockade, underscoring the importance of the ratio of these CD8 sub-populations in determining response to ICB.

Targeting immune persister cells. Cancer de-differentiation, often described as epithelial-to-mesenchymal transition (EMT), is characterized by loss of cellular differentiation gene programs and acquisition of de-differentiation gene programs and occasionally stem cell-like gene programs, has been implicated the development of aggressive biology (*e.g.*, invasion, metastasis).[117] Cancer de-differentiation has also been implicated in the development of resistance to cancer therapy, including targeted therapies[118–120] and immune-based therapies.[121,122] Using MDOTS derived from the ICB-responsive MC38 murine tumor model of colon cancer, Sehgal and colleagues identified immune persister cells (IPCs) that evaded $CD8^+$ T cell mediated killing following ICB (anti-PD-1) treatment.[123] Analysis of differentially expressed genes in bulk and single cell RNA-seq in MDOTS confirmed upregulation of genes involved in EMT, including Sca-1 (*Ly6a*), a gene associated with stem cell-like properties. Sca-1 expression on cancer cells was inducible following treatment with IFN or IL-6 and therapeutic targeting of IPCs with a cIAP1/2 inhibitor (LCL161) sensitized resistant tumors to ICB. These findings demonstrate feasibility of identifying and targeting IPCs in short-term *ex vivo* organotypic tumor spheroid cultures.

Combination therapy with ICB

To date, PDOTS have been applied as a model system for a variety of purposes within cancer immunology including interrogating drug candidates as combination therapy for ICB, enabling tumor–immune cytokine profiling, and testing next-generation immunotherapeutics. PDOTS have helped demonstrate the effectiveness of agents, which can overcome ICB resistance when used in combination with immune checkpoint inhibitors across a variety of tumor types. Foundational experiments first with MDOTS demonstrated that an investigational TANK-binding kinase 1 (TBK1) inhibitor in combination with PD-1 inhibition reduced tumor growth to a greater extent when compared to ICB monotherapy as demonstrated by live-*versus*-dead fluorescence imaging.[27] Subsequent studies expanding into patient-derived tumors and PDOTS confirmed these findings.[27,106] PDOTS have helped promote additional novel candidates for combination therapy that take advantage of unique mechanisms to overcome ICB resistance, including commercially available CDK4/6 inhibitors;[110] (*S*)-mepazine, an investigational MALT1 inhibitor;[124] and daratumumab, a clinically relevant anti-CD38 antibody.[125] Furthermore, PDOTS derived from patient tumors known to be immunotherapy-resistant responded favorably *ex vivo* to several of these combination therapies compared to single- or even double-agent ICB.[27,106,125] Though these studies rely predominantly on solid tumor biopsies, PDOTS have been formed using ascites in patients with high grade serous ovarian cancer, a tumor particularly resistant to immunotherapy. Ascites-derived PDOTS showed that epigenetic priming increased the effectiveness of ICB in these tumors; in addition to validating a unique combination regimen, this study demonstrated the versatility of the platform in accommodating tumor from liquid sources.[126]

PDOTS profiling has also been further used to examine effector mechanisms of immunotherapies. For example, addition of a TBK1 inhibitor to PD-1 blockade in immunotherapy-resistant tumors was shown to sensitize tumors to exogenously added TNF/IFNγ, implicating that TBK1 inhibitors can lower the cytotoxic threshold to pro-inflammatory cytokines.[27] Similarly, the positive effects of combined (*S*)-mepazine – a MALT1 protease inhibitor previously shown to promote IFNγ production in regulatory T cells (Tregs)[127] – and PD-1 blockade in PDOTS were reversed with the addition of anti-IFNγ antibodies confirming the drug's dependence on this downstream cytokine to kill tumor.[124] Analysis of the media from PDOTS experiments is

amenable to profiling elaborated cytokines and offers a method to nominate biomarkers of ICB effectiveness and further interrogate intracellular pathways. Bead-based cytokine profiling was used from media from PDOTS treated with CDK4/6 inhibitors and showed an increase in cytokines associated with a T helper type 1 (T_H1) cell response, a pattern which may serve as a proxy of therapy effectiveness. In another study, PDOTS from a subset of *KRAS*-mutant non-small cell lung cancer patient samples demonstrated marked response to IFNγ stimulation with enhanced production of multiple cytokines and CXCL10, a chemokine involved in the direct recruitment of Th1 cells.[128] Furthermore, cytokine analysis has been performed after perturbing critical components of intracellular pathways in PDOTS to uncover mechanisms of response.[106] Comparison of patterns of secreted cytokines elaborated from PDOTS after ICB correlated with the observed response and resistance patterns seen in patients receiving ICB.[27] In the case of melanoma, high signatures of IFNγ have been associated with favorable responses to ICB.[129] As the technology matures, such cytokine analysis may be able to be used as a predictive assay of clinical response.[27]

Cellular immunotherapies

CAR-T cell therapies are effective for subsets of patients with hematologic malignancies, although their use in solid tumors has been limited by a lack of tumor-specific targets, deleterious on-target off-tumor toxicity,[130] and the development of CAR-T cell dysfunction within the hostile TME. In recent years, PDOTS have been used as a pre-clinical model to evaluate next-generation CAR-based therapies and to examine tumor microenvironmental factors leading to treatment resistance and examination of novel therapeutic strategies to augment efficacy (or overcome resistance) to CAR-T cells and related cellular therapies.

Preclinical testing of CAR-T cells with an inducible caspase gene and targeting B7-H3, an immunoregulatory protein and tumor-associated antigen, effectively killed PDOTS composed of uveal melanoma liver metastases; findings of which were confirmed in immunodeficient and humanized murine models.[131] PDOTS were also used as a model system to demonstrate the use of low-affinity high-avidity CAR-T cells in targeting a tumor-associated antigen in models of renal cell carcinoma.[132] Knelson and colleagues examined the effectiveness of an investigational agonist of stimulator of interferon genes (STING) alone and in combination with engineered mesothelin-targeting CAR-natural killer (NK) cells using PDOTS derived from patients with malignant pleural mesothelioma, a tumor with particularly high level of STING expression.[133] Of note, the authors examined CAR-NK cells due to the known toxicity of STING agonists on T cells.[134] Combination treatment led to a sustained suppression of the pleural mesothelioma PDOTS growth over the span of 10 days, compared to treatment with STING agonist alone, in which the PDOTS experienced a growth rebound at the 10 day mark.

Highlighting the importance of the inclusion of the TME in developing therapies, PDOTS also enabled the testing of an experimental CAR-T cell therapy that capitalized on features of the tumor's stroma in models of pancreatic ductal adenocarcinoma (PDAC). CAFs are a component of the TME of PDAC that create a thick ECM which inhibits immune infiltration.[135] CAR-T cells were designed with a tumor-specific CAR and armed with the ability to secrete a T cell engaging antibody molecule (TEAM) targeting CAFs to facilitate immune-mediated killing of the tumor cells.[136] Using PDOTS, this dual-targeting CAR-TEAM was found to be superior to CAR-T cells that targeted either antigen alone, demonstrating the ability to examine different 'builds' of a given cellular therapy targeting discrete elements of the TME using patient-derived tumor models.[136]

Examining CAR-T cells using PDOTS has also provided insights into the impact of the TME on the development of CAR-T dysfunction. B7-H3.CAR-T cells were more recently shown to be active in PDOTS derived from patients across multiple cancer histologies, although treatment activity was variable.[105] While target antigen (B7-H3) expression partly accounted for diminished CAR-T cell efficacy in certain specimens, upregulation of co-inhibitory receptors (*e.g.*, PD-1, TIM-3, LAG-3) was observed during 3D microfluidic culture consistent with development of CAR-T cell dysfunction within 72 hours of PDOTS co-culture. PD-1 blockade was effective in improving CAR-T cell efficacy in PDOTS, although pharmacologic inhibition of TBK1 was similarly effective. TBK1 inhibition not only limited the development of CAR-T dysfunction, but also rendered cancer cells more sensitive to TNF/IFNγ-driven, inflammatory cell death. Together, these studies demonstrate the utility of examining CAR-T/NK cells and other cellular therapies using PDOTS to examine efficacy across a heterogeneous cohort of tumor histologies, examine the impact of the TME on the development of CAR-T cell dysfunction, and evaluate novel therapeutic strategies to overcome resistance to CAR-T cell therapy.

V. General considerations for model design

Extracellular matrix composition

Native ECM is composed of a fibrous mixture of collagens, fibronectin, laminins and glycoproteins.[137] In tumors, the ECM is often altered, becoming stiffer and denser due to the presence of tumor cells and cancer associated fibroblasts (CAFs) changing their secretion patterns of ECM components which can generate immune suppressive signals. There is increasing evidence that the ECM can regulate immune cells and their response to immunotherapy.[138–140] Those signals can be driven by ECM inhibitory receptors on immune cells, like the collagen receptor LAIR1 that was shown to be an inhibitory receptor on lymphocytes and in the form of physical barriers to drug delivery and immune

infiltration.[138,139,141] Thus, the composition and density of the ECM are important considerations in *ex vivo* model development. Tumor specimens are often embedded in ECM which generates the 3D environment and offers mechanical signals that imitate physiological conditions.[142] These tumor models typically employ a simplified version of ECM such as collagen type I that can be further modified with additional ECM components or use a more complex and variable ECM substrate, like Matrigel. Both options are commercially available, with Matrigel having a large inter-batch variation, and both are from animal origin, which introduces a non-human variable for clinical applications.[43] Gel density is another important consideration as higher density can deter immune responses and immune infiltration,[143] and lower density may make the model more permissive to cellular transport.

Several platforms take advantage of tumor-derived decellularized ECM. One example is presented in an *ex vivo* platform developed by Nagaraj *et al.* for the functional assessment of high-grade serous ovarian cancer (HGSC) tumors. In this model, a gel matrix produced from patient-derived tumor-free omentum (a common site of ovarian cancer metastasis) was shown to be a superior culture substrate for ovarian tumor cells when compared to more standard basement membrane extract.[144] In this study, the authors formed patient-derived cultures (PDCs) by enzymatically digesting ovarian tumors, filtering through a 70 μm filter, suspending the filtrate in the omental gel matrix and allowing drops of the mixture to solidify in the bottom of a 96-well plate. After the addition of pembrolizumab (anti-PD-1), the PDCs showed a reduction in the number of tumor cells by single-cell imaging techniques, and bulk flow cytometric analysis revealed an increase in $CD8^+$ T cell activation markers. The platform was further used by Launonen *et al.* to mechanistically show that myeloid-driven $CD8^+$ T cell exhaustion which occurred post-chemotherapy could be reversed with both pembrolizumab and tiragolumab, an investigational immune checkpoint inhibitor against TIGIT, a co-inhibitory molecule found on the surface of T cells.[145] Other groups have used decellularized porcine small intestine mucosa/submucosa and showed that they can support multiple endoderm-derived human organoids, from gastric, hepatic, or pancreatic origin.[146] As the Food and Drug Administration (FDA) has approved protocols to obtain hydrogels from decellularized tissues for several clinical applications, this approach could have promising clinical applications in the near future.[147]

Culture conditions: from well plates to microfluidics

As mentioned, culture conditions are intimately linked to model design. Traditional cancer cell lines, PDOs, and reconstituted PDO models have been developed using a variety of culture conditions including co-culture in well plates[44,148,149] and microfluidic configurations,[71,72] whereas current native tumor models are in part defined based on their culture conditions. For example, the ALI model uses a well-in-well configuration; PDTFs utilize 96-well plates, and PDOTS were established using microfluidic culture. Of these methods, microfluidic culture is a particularly attractive culture modality owing to its ability to manipulate and sample small volumes of fluids flowing in miniature channels with widths on the order of millimeters.[150,151] Microfluidic culture has already been used to model a host of biological phenomena including tumor growth, tumor migration and extravasation, immune cell recruitment and activation, and angiogenesis, among others.[69] The use of microfluidic culture systems offers several practical and theoretical advantages over traditional *in vitro* culture systems including opportunities for enhanced biological relevance, such as recreation of tissue-specific microenvironments (*e.g.*, organ-on-a-chip models) thereby providing insights into complex biological interactions and disease mechanisms in a physiologically relevant context. Additionally, microfluidic platforms provide the structural and biochemical cues necessary for cells and tissues to grow and interact in 3D, resembling their natural environment, and the low shear stress provided by these devices makes them particularly well-suited for sensitive cell types, such as primary cells obtained from tumor biopsies. Microfluidic culture systems also enable dynamic monitoring and manipulation of cellular responses and facilitate examination of multiple culture conditions or treatments making them ideal models for drug sensitivity or combination therapy testing.

VI. Challenges as opportunities for PDOTS looking forward

Tumor heterogeneity

Tumor heterogeneity refers to the uneven spatial and temporal distribution of tumor components as well as their molecular and genetic features. It is an active area of investigation in cancer modeling as it can contribute to treatment resistance.[152,153] Tumor heterogeneity can exist at many length scales: within a particular tumor (*i.e.*, intratumoral), across separate tumor sites within an individual patient (*i.e.*, intertumoral), or across patients with similar tumor types. Patient tumor biopsies may not capture the entire diversity of a patient's malignancy, in part due to the inability to sample a patient across every site, in the case of metastatic disease, and on a smaller scale, due to the inability to process the entirety of a biopsy sample, as pieces are reserved for additional genetic and molecular testing and histological examination. This challenge is not unique to PDOTS, and is universally encountered not only in other patient-derived tumor platforms, including organoids, tumoroids, and explants, but also exists in standard clinically-employed molecular diagnostic testing to look for actionable driver mutations.[154,155] A theoretical solution to this challenge is to increase the amount of tumor analyzed as a greater extent of tumor heterogeneity may be captured in

the analysis. However, practical trade-offs exist. First, tumor biopsies are precious samples in that there is a limited supply and they are difficult to acquire as they are often obtained surgically. Second, increasing the amount of tumor analyzed increases the processing burden and may limit the number of independent experiments able to be performed if a greater amount of tumor is required. Practically, models may benefit from continued biologic insight into the evolution of the molecular and genetic features of tumors to identify patterns or correlations in seemingly heterogeneous tumors in an effort to more faithfully extrapolate trends observed in a tumor sample to clinically relevant trends in the parent tumor. For example, PDTFs generated from tumors collected from patients with metastatic disease at multiple sites and treated with PD-1 blockade showed concordant immunologic responses in 8 of 11 patients analyzed.[26] Notably, our discussion has focused primarily on processing surgical solid tumor biopsies; however, non-surgical samples from fine-needle aspiration (FNA) have been used in PDOTS and in other platforms[156] though it is often challenging to obtain a cell yield of sufficient quality for profiling. Samples from core biopsies face similar challenges, though have been successfully used in other platforms (*e.g.*, MOSs).[157] Hence, limited tissue samples further raise questions as to the impact of potential heterogeneity on experimental results.

The size of the tumor sample used in patient-derived models directly influences the viability of the cellular components and, as discussed, may limit a model's generalizability across individual samples undergoing different experimental treatments. Tumor fragments and explants, for example, which have typical sizes from 1–2 mm^3, are much larger than spheroids and are only amenable to short-term culture on the order of several days due to waning viability. Furthermore, as larger pieces of tumor are sampled, individual experiments performed on larger isolated pieces of tumors may yield disparate results owing to intratumoral heterogeneity. For this reason, the results of experiments using larger tumor samples (*e.g.*, PDTFs) are typically averaged over multiple tumor samples in order to appropriately gauge treatment effect.[26] PDOTS offer an intermediate solution in both preserving viability of spheroids and creating a homogeneous mixture of organotypic tumor spheroids from the entire explanted tumor such that every device and treatment condition contain a representative assortment of cellular components, allowing for more reliable conditional testing of the patient's tumor. The degree to which tumor heterogeneity may limit the predictive potential of patient tumor-derived platforms is not known presently, though promising results correlating *ex vivo* responses with clinical outcomes *via* biomarkers of immune activation provide encouragement for ongoing investigation in this area.[26] We also note that though tumor heterogeneity is often viewed as a negative for patient-derived tumor modeling, the presence of heterogeneity within an accessible tumor biopsy does potentially allow for the identification of diverse therapeutic targets, molecular biomarkers or cellular responses, which may be not be appreciated in a more homogeneous tumor sample.[158]

Assessing dynamic immune-mediated tumor killing

In PDOTS, live-*versus*-dead cellular imaging is performed *via* differential nuclear staining using Hoechst and propidium iodide dyes where the effect of a given treatment on cell viability is determined by a decrease in cell viability and/or an increase in dead cells.[28] However, a limitation of this technique is that the nuclear dyes do not distinguish tumor cells from immune cells. This creates a challenge, in particular, for the study of cellular therapies as the addition of cellular components may require more accurate means to gauge effector cell expansion and tumor killing over time. To overcome these challenges, analysis of PDOTS will need to incorporate emerging multiplexed staining, imaging, and image analysis methodologies. Recent advances in 4D imaging have enabled the tracking of 3D organoid structures over time; however, such techniques are limited in the number of markers they can track. Multiplex imaging methods allow for the tracking of multiple cellular biomarkers over time and are particularly attractive in evaluating the complexities of tumor-immune interactions. Recently, advances in bioorthogonal click chemistry enabled multiplexed immunofluorescence on living cells and tissues.[159] Such approaches have already been applied to organoid models of glioblastoma to achieve cyclic imaging involving successive rounds of staining and fluorescence quenching while preserving the viability of the organoids.[160] This technique paired with PDOTS would allow for the dynamic monitoring of the immune response over the course of days and provide a more accurate method to assess tumor-immune killing.

Target identification and drug sensitivity testing

PDOTS and related 3D-PTA models enable use of tumor material to perform patient-specific drug sensitivity testing using existing and emerging drugs alone and in combination. The throughput of current methods and approaches precludes examination of more than a handful of treatment conditions and doses. Current barriers to high-throughput testing are the availability of tumor material and the throughput of PDOTS/3D-PTA testing itself. To date, nomination of potential drug targets for PDOTS profiling has included hypothesis-driven efforts, as well as unbiased discovery efforts using *in vivo* or *in vitro* CRISPR screening or single cell RNA-sequencing data followed by validation studies of promising drug candidates. Currently, PDOTS are typically deployed after target nomination and initial *in vitro* validation studies have helped define 1–2 drug concentrations to ensure maximum efficacy and minimum off-target toxicity.[106,110,123,124] PDOTS profiling can then be performed to test the drug alone or in combination with another drug (*e.g.*, anti-PD-1 antibody) in an effort to better

Table 1 Strengths and limitations of *ex vivo* patient-derived tumor platforms for immunotherapy applications

Platform	Strengths	Limitations	Highlighted immunotherapy applications
Traditional organoid models			
Reconstituted PDO/PDTO[a]	- Allows for direct addition of cellular components to existing PDOs or reconstitution from single cell suspensions - Addition of PBMCs enables study of ICB and other antibody- or small-molecule based combination immunotherapies - Allows for study of adoptive cell therapies	- Though models contains immune and/or stromal cells, they lack the native composition and architecture of TME present in the original tumor	- ICB[67,68] - Engineered T cells[62] - TILs[24,72,163] - Combination therapy with TILs[164] - Bispecific antibodies with TILs[72] and autologous T cells[165] - CAR-T cells[63,73] - Combination therapy with CAR-T cells[166]
Native tumor models			
ALI model	Early culture (~1 week): - Allows for study of native TME - Allows for cytokine analysis and tumor killing analysis of native tumor and immune components Late culture: - Model approaches that of conventional PDO/PTDO given loss of immune and stromal components - Amenable to augmentation with PBMCs or adoptive cell therapies	- Genetic engineering is challenging - Heterogeneous tumor pieces - Low throughput	- ICB[25]
Tumor slice culture	- Maintain native TME at millimeter length scale - Amenable to augmentation with PBMCs or adoptive cell therapies - Ideal for mapping cellular spatial relationships	- Genetic engineering is challenging - Very short culture times given sample viability due to large size of tumor sample - Heterogeneity across tumor slices - Low throughput	- ICB[99] - CAR-T[167]
PDTF	- Maintains native TME architecture at millimeter length scale - Allows for mainly cytokine analysis of native tumor and immune components - Amenable to augmentation with PBMCs or adoptive cell therapies	- Genetic engineering is challenging - Very short culture times due to sample viability due to large size - Heterogeneity across individual tumor fragments - Low throughput	- ICB[26] [a]Response scores calculated from media analysis of PDTFs and anti-PD1 correlated with clinical responses in a validation cohort
PDOTS	- Maintain native TME architecture at submillimeter length scale - Cultured up to 9 days in which immune and stromal components are preserved - Cellular material from tumor less than 40 μm and greater than 100 μm can be biobanked - Amenable to augmentation with PBMCs or adoptive cell therapies - Established in microfluidic devices	- Genetic engineering can be challenging - Employs enzymatic digestion - Low throughput	- ICB[27,126] - Combination therapy with ICB[27,106,110,124,125] - CAR-T[131,132] - Combination therapy with CAR-T[105] - CAR-T with T cell engaging antibody molecule interacting with stroma[136] - CAR-NK cell therapy[133] - Adenoviral-delivery of gene therapy[168]

Abbreviations: PDO = Patient-derived organoid; PDTO = Patient-derived tumor organoid; PBMCs = Peripheral blood mononuclear cells; ICB = Immune checkpoint blockade; TME = Tumor microenvironment; TIL = Tumor infiltrating lymphocytes; CAR-T cells = Chimeric antigen receptor T cells; ALI = Air–liquid interface; PDTF = Patient-derived tumor fragment; PDOTS = Patient-derived organotypic tumor spheroids. [a] Traditional PDOs and PDTOs generally require reconstitution with immune cells for the study of immunotherapy.

select the patient population most likely to benefit from the treatment/combination. We consider such efforts examining the distribution of responses across a cohort of PDOTS a "phase minus one clinical trial". *Post hoc* analyses can be done to identify potential pharmacodynamic markers of activity and biomarkers of response (or resistance) to treatment.[105,125] It is possible that such investigations will guide the design of clinical trials to identify the patients most likely to benefit, thereby decreasing the failure rate of early phase clinical trials.

Functional precision medicine

In addition to using PDOTS and related 3D-PTAs purely as an orthogonal preclinical tumor model to examine emerging cancer therapeutics, there is a clear value proposition for developing and optimizing these models for clinical decision making. However, it has yet to be determined whether sensitivity of PDOTS (and other related 3D-PTAs) to *ex vivo* ICB challenge is associated with clinical efficacy of ICB therapy. Currently, the vast majority of excisional biopsies obtained for PDOTS profiling are derived from patients who have progressed on front-line ICB treatment, often from a growing, symptomatic metastatic lesion. While these specimens from ICB-resistant patients are ideal for examining novel combination approaches to overcome ICB resistance,[106,125] they are not suitable for examining ICB sensitivity as they derive from patients insensitive or resistant to ICB therapy. To date, clinical opportunities for a surgical (excisional) biopsy prior to first-line immunotherapy have been limited, resulting in very few opportunities to compare pre-treatment *ex vivo* responsiveness of PDOTS with clinical

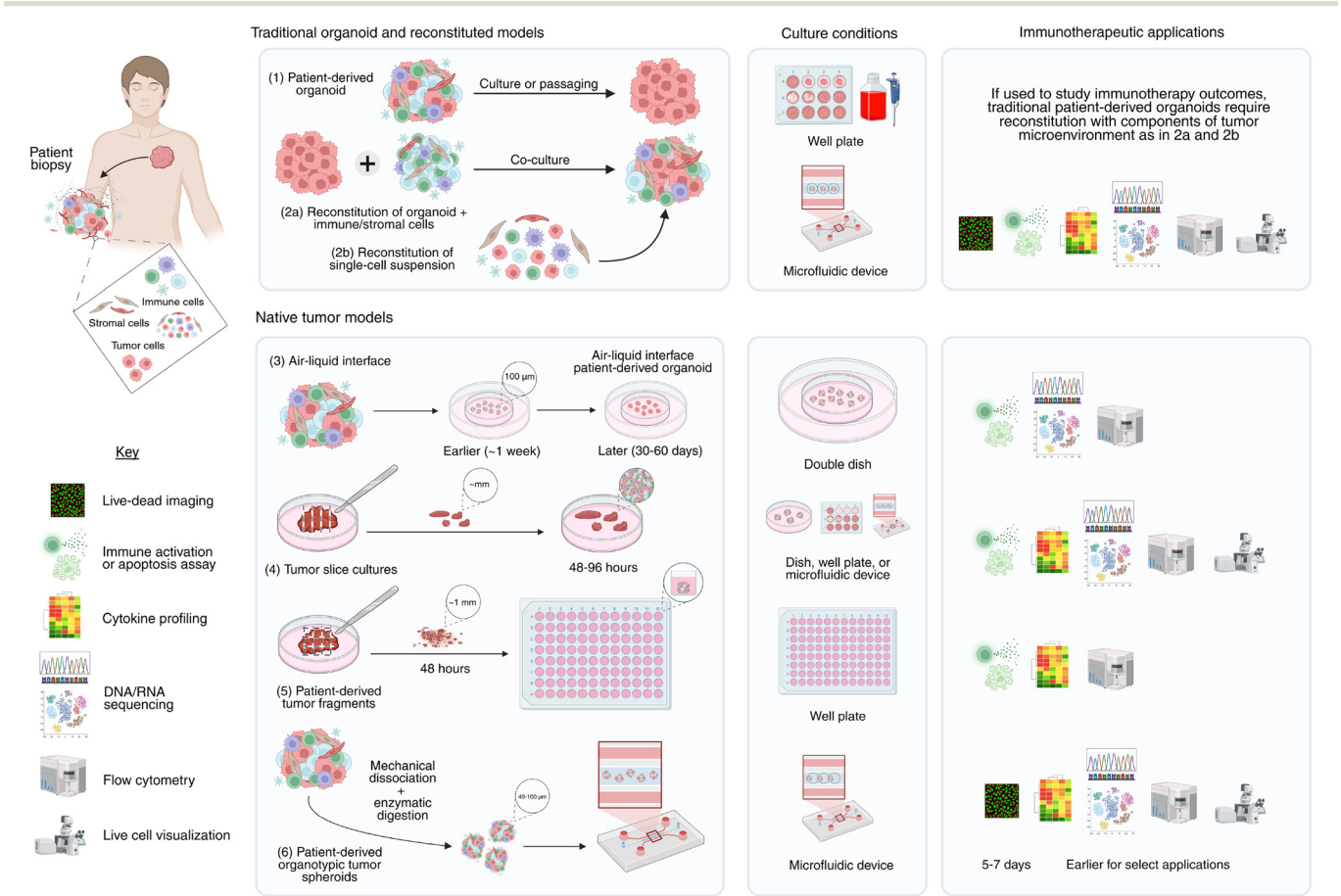

Fig. 1 Landscape of state-of-the-art patient-derived tumor model. LEGEND: patient-derived organoids (1) are generally formed *via* long-term culture of patient tumor resulting in loss of immune cells. Addition of TME components is possible by reconstituting patient-derived organoids with immune or stromal cells (2a) or by recombination of a single cell mixture of tumor components (2b) though these approaches do not preserve native tumor composition or architecture. The air–liquid interface model (3) preserves the TME at short culture times and requires growth factors to support an immune compartment beyond ~1 week. At long culture times, the air-liquid interface model approaches that of traditional patient-derived organoids owing to loss of immune and stromal cells. Tumor slice cultures (4) and patient-derived tumor fragments (5) are larger pieces of tumor that also retain TME components and can be analyzed over a period of days. Patient-derived organotypic tumor spheroids (6) are formed *via* physical mincing and partial enzymatic digestion of tumor to retain TME structure and components and can be maintained in microfluidic culture up to 1 week. *Ex vivo* patient-derived tumor models have employed a variety of analysis techniques including live-dead imaging, immune activation or apoptosis assays, cytokine profiling, DNA/RNA sequencing, flow cytometry, and live cell visualization. Figure created in **https://www.BioRender.com**.

response to front-line ICB. However, given the clinical success of neoadjuvant single[161] and dual[162] ICB for resectable stage III/IV melanoma, there is an emerging opportunity to conduct non-interventional, co-clinical trials to determine the relationship between clinical response and PDOTS sensitivity to ICB. Despite the success of combination ICB in melanoma, robust predictive biomarkers and signatures of response (and resistance) are still lacking.

Advantages of performing PDOTS analysis on biopsy specimens obtained immediately prior to administration of neoadjuvant ICB therapy include a high clinical response rate as comparator (especially with dual ICB), a more uniform treatment-naive patient population, an early disease stage with lower disease burden and therefore less tumor heterogeneity, and well-defined clinical endpoints (*e.g.*, pathologic response) against which PDOTS response can be compared. Further, for patients in which neoadjuvant ICB is ineffective, PDOTS profiling using existing and emerging therapies may offer insights into effective strategies to overcome ICB resistance. Combination therapies can be tested on subsequent biopsies and the patient can continue to be tracked for a longitudinal analysis of therapy effectiveness as well as the emergence of resistance mechanisms.

VII. Conclusions

Immunotherapy is now a cornerstone of cancer treatment for many cancer types offering durable disease control (and in some instances cures) for a discrete portion of patients with advanced solid tumors. However, many patients do not derive sustained benefit from available immunotherapies due to the emergence of resistance or toxicity. Furthermore, the heightened interest in leveraging the immune system against

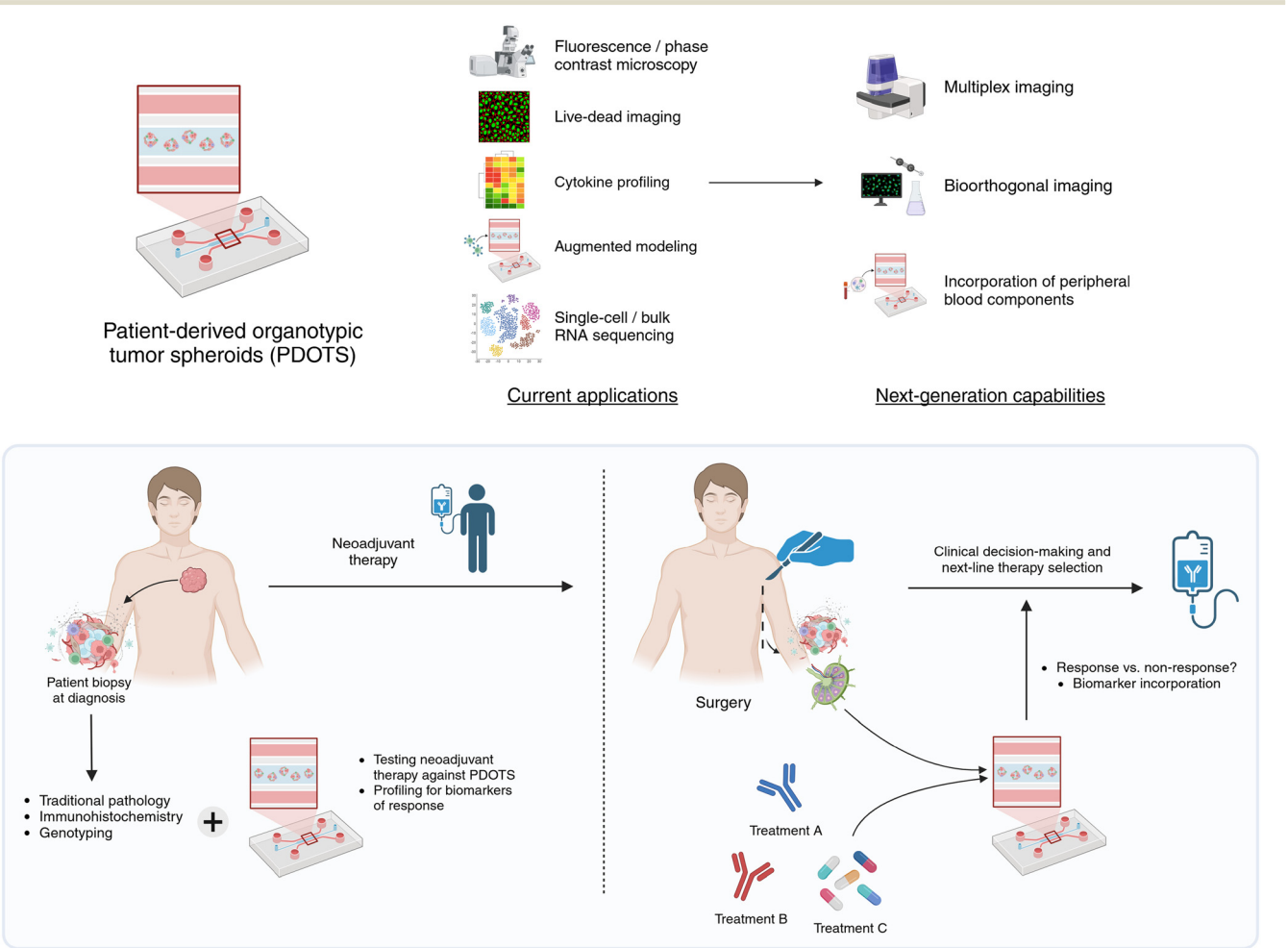

Fig. 2 Imaging and analysis capabilities of PDOTS and future clinical applications. LEGEND: (Top) Current and next-generation imaging and analysis capabilities of the PDOTS platform. (Bottom) Proposed clinical utility of PDOTS for functional precision medicine. At the time of diagnosis, in addition to traditional pathologic analysis and genotyping, PDOTS can be generated from the initial tumor biopsy and tested against neoadjuvant ICB treatment that the patient will receive. At the time of subsequent surgery, PDOTS response rates can be compared with pathologic response rates for platform validation and predictive biomarkers can be interrogated. PDOTS can also potentially be used in clinical decision-making to screen candidate therapies and apply biomarkers to determine the next best therapeutic option for a patient. Figure created in **https://www.BioRender.com**.

cancer has led to a growing number of immunotherapeutic options including bispecific T cell engagers,[6,7] CAR-T cells,[8] TILs,[9,10] and therapeutic cancer vaccines.[11,12] 3D-PTA models which preserve key aspects of the TME fulfill a critical role in this space as they are increasingly being utilized to perform drug sensitivity testing of approved and next-generation immunotherapeutics. These models enable dynamic evaluation of tumor-immune interactions using clinically relevant biospecimens and complement research efforts using established murine tumor models and *in vitro* co-culture systems with the ultimate goal of identifying mechanisms of drug response and resistance. This important capability of 3D-PTA models will be particularly useful in informing the design and execution of early phase clinical trials in hopes of realizing the promise of precision medicine by delivering the "right drug to the right patient at the right time".

While the use of PDOTS among other 3D-PTAs was highlighted in this review, our focus on PDOTS is largely a reflection of our group's experience with this model. As the first native tumor model platform to be developed,[27] the PDOTS platform has proven to be adaptable, versatile in its applications, and primed to accommodate next-generation analysis techniques to deepen our understanding of tumor–immune dynamics and advance the development and use of new immunotherapeutic approaches. However, each 3D-PTA model has its own advantages and limitations, and further enhancements and optimization will be needed to realize their promising potential in the area of functional precision cancer medicine (Table 1) (Fig. 1 and 2).

Data availability

No new data were created or analyzed in this study.

Author contributions

DJB: conceptualization, writing – original draft preparation, reviewing and editing, project administration; DXZ: conceptualization; writing – reviewing and editing, visualization; O-YR: conceptualization, writing – original draft preparation; AA: conceptualization, writing – original draft preparation; RWJ: conceptualization, writing – original draft preparation, reviewing and editing, supervision, project administration.

Conflicts of interest

R. W. J. is a member of the advisory board for and has a financial interest in Xsphera Biosciences Inc., a company focused on using *ex vivo* profiling technology to deliver functional, precision immune-oncology solutions for patients, providers, and drug development companies. R. W. J. has received honoraria from Incyte (invited speaker), G1 Therapeutics (advisory board), Bioxcel Therapeutics (invited speaker). R. W. J. has ownership interest in U.S. patents US20200399573A9 and US20210363595A1. R. W. J.'s interests were reviewed and are managed by Massachusetts General Hospital and Mass General Brigham in accordance with their conflict-of-interest policies.

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