HHS Public Access

Author manuscript

Immuno-oncol Insights. Author manuscript; available in PMC 2023 May 01.

Published in final edited form as:

Immuno-oncol Insights. 2022 ; 3(8): 379–398. doi:10.18609/ioi.2022.41.

Preclinical models for development of immune–oncology therapies

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Abstract

Immunotherapy has demonstrated great success in clinical treatment, especially for cancer care. Here we review preclinical models, including cell lines, three dimensional (3D) cultures, and mouse models to support the need for tools enabling the development of novel immune–oncology (I–O) therapies. While *in vitro* studies have the advantage of being relatively simpler, faster, and higher throughput than *in vivo* models, they must be designed carefully to recapitulate the biological conditions that influence drug efficacy. The growing prevalence of 3D *in vitro* and *ex vivo* models has enabled screening and mechanistic studies in more complex, tissue-like environments containing multiple interacting cell types. On the other hand, syngeneic mouse models have been instrumental in the historical development of immunotherapies and remain an important tool in drug development, despite lacking fidelity to certain aspects of human physiology and pathology. Xenograft and humanized mouse models address some of these challenges, yet present limitations of their own. Successful development and translation of new I–O therapies will likely require thoughtful combination of several of these preclinical models, and

Contributions: Wang Y and Shelton S contributed equally. All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.

we aim to help research and development scientists utilize the appropriate tools and technologies to facilitate rapid transition from preclinical evaluation to clinical trials.

INTRODUCTION

The field of immune–oncology (I–O) has transformed the care for cancer patients. In the late 19th century, William B. Coley, the father of immunotherapy, first attempted to harness the power of the immune system using 'Coley's toxin' for treating cancer patients. This cocktail of live and inactivated bacteria achieved some durable complete remissions in a series of malignancies, including sarcoma, lymphoma, and testicular carcinoma [1]. In the 1980s, Rosenberg *et al.* demonstrated that administration of high dose cytokine IL-2 could lead to durable, complete, and apparently curative regressions in some patients with metastatic melanoma and renal cancer [2,3]. Inspired by Paul Ehrlich's 'magic bullets' concept, in 1997 rituximab became the first approved monoclonal antibody (mAb) for the treatment of lymphoma [4,5]. The discovery of cytotoxic T-lymphocyte associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1) and the antibody drugs targeting them, immune checkpoint inhibitors (ICIs), propelled the I–O field into the current era [6–10]. On the other hand, chimeric antigen receptor T (CAR-T) cell therapy rewires patient immune cells to target tumor antigens independent of major histocompatibility complex (MHC) and there are six CAR-T products that have been approved by the US food and drug administration (FDA) [11–17]. The first pediatric patient in the world to receive CAR-T cell therapy has been tumor free for 10 years.

Through years of breakthroughs, as well as challenges and struggles, I–O therapies have been embraced by the oncology community due to their great clinical success. In this review article, we highlight emerging preclinical models for I–O therapy development [Table 1, Figure 1] and describe their ability to recapitulate the tumor microenvironment (TME), inclusion of extracellular matrix (ECM), discuss specific applications in drug development, and compare the advantages and limitations of current models.

2D cultures

Choosing a suitable cell line is critical for producing models reflective of tumor biology with appropriate antigenicity and driver mutations. Mutational statuses of cell lines used in I–O research should reflect tumor biology For example, the von Hippel–Lindau (VHL) gene is mutated in 90% of sporadic clear cell renal cell carcinoma (ccRCC) cases [18]. Other common mutations found in ccRCC include tumor suppressor genes, such as PBRM1, BAP1, SETD2 [19]. More than 20 cell lines are frequently used in renal cell carcinoma (RCC) research, including ACHN (uncertain RCC histotype), A-498 (used as a model of ccRCC and widely in cancer research), 786-O (used as a model of ccRCC), and SK-RC cell lines (obtained from ccRCC metastases) [20]. ACHN mRNA lacks mutations in VHL and hypoxia-inducible factor (HIF)-1α, 786-O bears mutated VHL, and SK-RC cell lines express either HIF-2α only or both HIF-1α and HIF-2α [20]. In order to use models most reflective of natural tumor biology, immunohistochemistry (IHC), gene sequencing, and histology analysis of tumors can provide insight into RCC subtypes to enhance the translational potential of experiments using 2D cultures [20].

Cell line choice is not only determined based on gene mutation status, but also on different antigen expression. For example, hormone receptor status plays an important role in determining a suitable model for breast cancer research. Estrogen receptor (ER), progesterone receptor (PR), and amplification of human epidermal growth factor receptor 2 (HER2) status provide information on tumor biology and therapeutic response, necessitating choosing cell lines for 2D culture that reflect tumor subtypes [21,22]. In addition, many of the long-established cell lines frequently used in research are derived from metastases, rather than primary tumors, which is not representative of varying stages of tumor progression [23]. Dai and colleagues categorized breast cancer cell lines into subtypes luminal A, luminal B, $HER2_+$, triple negative breast cancer (TNBC) A (TNA), and TNBC B (TNB), from least to most aggressive, to better reflect differences in receptor statuses [21]. Luminal A cell lines, including MCF-7, BT-483, CAMA-1, HCC-1428, HCC-712, and IBEP-2, are ER_+, $HER2_-$, and have varying PR statuses [21]. Luminal B cell lines, including BSMZ, BT474, IBEP1, and IBEP3, are ER_+, $HER2_+$, and have varying PR statuses [21]. $HER2_+$ cell lines, including 21MT2, HCC1008, HH315, and SKBR3 are ER_-, $HER2_+$, and typically PR_- [21]. TNA cell lines, including DU4475, EMG3, HCC1937, MDAMB436, and MDAMB468, and TNB cell lines, including Hs578T, MDAMB157, MDAMB231, and SUM149PT are ER_-, PR_-, and $HER2_-$ [21].

One limitation of 2D culture is that standard incubators mimic atmospheric oxygen concentrations. These conditions are not reflective of the lower oxygen tension, termed hypoxia, or the insufficient delivery of oxygen to cells that is commonly found in solid tumors [24]. This important aspect of cell physiology can be achieved in 2D culture by using hypoxia mimetic agents to increase HIF-1α availability [24]. For example, CoCl2 is a commonly used hypoxia mimetic that competes with $Fe2^+$ ions, inhibiting HIF-prolyl hydroxylases (PHDs) activity, which prevents the degradation of HIF-1α and thereby mimics hypoxia [24]. Adherent cell lines, even under ‘normoxic’ atmospheric concentrations, can experience hypoxia or anoxia, as oxygen exchange may only occur via diffusion from cell culture media, with oxygen availability and consumption rates periodically changing in response to one another [24]. Short-term, cyclic, ‘intermittent hypoxia’ (IH) experienced by cells *in vivo* may be mimicked *in vitro* through the use of flow-through systems supplying precise concentrations of oxygen through solenoid valves, growing adherent cells in a perfusion-based system of tube-like channels through which media is supplied, or using bioreactors with peristaltic pumps to periodically flow media with desired oxygen conditions [24]. Furthermore, cell culture media formulations also do not replicate the concentration of nutrients, amino acids, and electrolytes found in human plasma. The two most commonly used media formulations, Dulbecco’s modified Eagle’s medium and Roswell Park Memorial Institute-1640 (RPMI-1640), contain significantly higher glucose concentrations than physiologic and varying amounts of electrolytes [25]. Typically, cell culture media is supplemented with serum, often from fetal calves, to supply growth factors and other essential components lacking in the basal medium. However, serum is known to vary between batches, and there are now many efforts designed to reduce or eliminate the need for serum in cell culture. Among these reduced-serum or serum-free approaches, there are recently developed media designed to mimic human serum or plasma, with adjusted amino acid formulations. The balance of nutrients, metabolites,

amino acids, electrolytes, vitamins, and trace elements inevitably impact cell metabolism and gene expression, and adoption of more physiologic media may improve the likeness of cell culture to *in vivo* conditions [26,27].

Another challenge for 2D studies of cancer research is the heterogeneity of the TME, which in addition to cancer cells, include endothelial cells, epithelial cells, immune cells, and cancer-associated fibroblasts (CAFs), which are not replicated using cancer cell lines [28]. CAFs found in the stroma of human cancers provide signaling and remodeling functions, and typically exhibit upregulated ECM production and remodeling (e.g. collagen) and secretion of soluble pro-tumor cytokines and growth factors [28]. Recent scRNAseq studies have revealed that the heterogeneity of these CAFs in the TME may derive from the variety of spatial subgroups found in normal fibroblasts [28], and further work will be required to characterize the cross-talk between CAFs and other cell types in the TME [28]. These goals cannot be accomplished through the use of 2D culture alone but will require the use of co-cultured cells (in trans wells, for example) or 3D organoid culture to mimic the TME.

In addition to the variety of cell types that make up the TME, CAFs function to produce and assemble the complex composition of the tumor ECM through the production of fibrous proteins, proteoglycans, glycosaminoglycans, and glycoproteins, which contribute signaling and support for tumor growth and migration [28] and can also impede immune cell movement and activation. The composition of the ECM and resulting crosslinking of the tumor stroma impacts drug penetration, with CAFs playing an important role of remodeling the ECM through the production of lysyloxidase (LOX) family and MMP enzymes [28]. LOX oxidases catalyze the crosslinking of collagen and ELN in the ECM, increasing tumor stroma stiffness [28]. LOX oxidases are overexpressed in CAFs, with LOXL2 expression in gastric CAFs having been associated with invasive potential [28,29]. Inhibition of LOXL2 and LOX in breast cancer has resulted in reduction of tumors, angiogenesis, and metastasis [30].

ECM proteins can also function as ligands, binding integrin receptors on cell membranes [28]. Interaction with the rigid ECM can lead to integrin molecule dimerization, activating the focal adhesion cascade [28]. Further, ECM rigidity can trigger SRC-YAP-MYL9/MYL2, leading to maintenance of the CAF phenotype with CAF function reinforcing ECM stiffness, promoting an environment that facilitates improved tumor cell invasion [28,30].

Targeting ECM proteins, therefore, is an attractive method for generating an environment that is more permissive to the delivery of anti-cancer therapies. Generating models that are reflective of the crosstalk between cell types and CAF-ECM protein interactions cannot be accomplished through 2D culture alone. However, the use of cell lines is beneficial as they are able to provide a relatively high number of cells for experiments, compared to primary cultures and animal models increasing the speed at which research can be conducted [20]. Further, 2D cultures are an unlimited self-replicating source [23]. An important drawback of the use of cell lines, however, is the inability of these simplified models to exhibit crosstalk between cells and interactions with the tumor microenvironment [20]. This limitation can be overcome through the use of 3D cultures or co-cultures [20].

Another key benefit of using established cancer cell lines is experimental consistency and repeatability between labs. Short tandem repeat (STR) DNA profiling can be used to identify human cell lines to ensure the absence of cross-contamination or misidentification thereby improving the accuracy of assays [31]. STR DNA profiling uses DNA hypervariable regions, consisting of variable number tandem repeat (VNTR) units, for identification of a unique DNA 'fingerprint,' through the analysis of 1–6 bp core sequences of STR microsatellite regions [31]. The eight core STR loci used for identification include D5S818, D13S317, D7S820, D16S539, vWA, Th01, TPOX, and CSF1PO [31]. Cell lines are authenticated if the STR profile is a greater than 80% match with the tissue from which it originates. A match of 56% or more is considered unrelated, and values between 56 and 80% require further analysis [31]. In this way, independent research groups are capable of repeating and validating published studies using the same cell lines.

3D organoids

The ultimate promise of organoid technology is to improve the accuracy and predictive value of I–O research. Organoids represent a compromise between the simplicity and straightforwardness of traditional cell culture and the more complex and physiological conditions provided by *in vivo* experiments. While both of these methodological approaches will remain components of any research and discovery efforts, organoids have begun to take a larger role in basic and translational research programs. Whether these organoids are generated from differentiated stem cells to resemble specific tissue types, assembled from cell aggregates to form tumor spheroids, or are collected from patient samples for *ex vivo* organoid studies, they possess several advantages for I–O studies.

First, the most obvious feature of organoids and spheroids that distinguish them from traditional cell culture is their 3D structure. While this difference might seem subtle or arbitrary, cellular organization and culture substrates can have significant impacts on cell phenotypes in ways that influence tumor growth and immunity Through the years, many research groups have reported how conversion from 2D to 3D culture format changed cell phenotypes, with distinct gene signatures that are required to support 3D tumor growth identified by a recent CRISPR screen study [32].

When a cancer cell line is aggregated into tumor spheroids, they adhere to each other and form connections more similar to *in vivo* architecture, including the generation of ECM. Cancer cells produce more ECM in 3D than in 2D, and the 3D format may also alter the ratios between different ECM proteins, including collagens (I, III, IV, V), fibronectin, and laminin [33–36]. ECM organization can also evolve in 3D, in ways that cannot be modeled by simple 2D monolayers of cells [37], with these changes in the ECM likely to alter the density and stiffness of the tumor spheroid. Tumor mechanical properties such as stiffness may dramatically impact response to immunotherapies because lymphocytes are supremely mechanosensitive cells and respond to the mechanical conditions of both the microenvironment and of their target cells.

Natural killer (NK) and T lymphocytes are mechanosensitive as a consequence of their mechanisms of cytotoxicity. The immunological synapse (IS) of a T cell consists of the joining of the T cell receptor (TCR) on the effector cell and the peptide-MHC on the

target cell or antigen presenting cell [38]. T cell cytotoxicity is correlated to the force generated at the IS, largely through the efficiency of perforin delivery. Increasing the membrane tension of the target cell enhances the speed and efficiency of pore formation and perforin-mediated killing [39]. Stiff environments, such as tissue culture plastic surfaces, enhance T cell cytotoxicity due to increased membrane tension in monolayers of cancer cells [39–41]. Similarly, NK cells employ perforin-mediated cytotoxicity, and they have also demonstrated more rapid killing in higher density collagen gels [42]. Furthermore, T cells rely on stiffness cues to regulate their proliferation, migration, and activation, and T cell expansion methodology has been improved by optimizing the stiffness imposed by microparticles carrying activating antibodies [43,44].

Beyond the influence on cytotoxic efficiency of tumor infiltrating lymphocytes, stiffness can modulate immune checkpoint molecule expression in the spheroids, with higher stiffness upregulating the expression of programmed death ligand 1 (PD-L1) in breast cancer spheroids [45]. Simply culturing tumor spheroids in 3D has been shown to alter PD-L1 expression heterogeneously by tissue type. PD-L1 increased as a result of spheroid culture in colorectal cancer, renal cell carcinoma, and breast cancer cell lines, but was unchanged in gastric adenocarcinoma [45–48]. Therefore, models that faithfully recapitulate the mechanical properties of the native tissue are important to ensure realistic levels of lymphocyte cytotoxicity occur as would be seen *in vivo*.

In addition to PD-L1, additional phenotypic shifts occur in cancer cell lines cultured in 3D vs 2D monolayers, including changes in several cell surface molecules important to drug delivery and I–O studies. Studies examining NK or T cell killing in cancer spheroids have noted reduced activation and killing in 3D compared to 2D controls. Reduced T cell cytotoxicity was attributed, in part, to reduced expression of MHC-class I molecules by 3D spheroids [49], and these spheroids were less susceptible to cytokine-induced upregulation of MHC-class I [50]. On the other hand, HLA-E, an inhibitory ligand towards NK cells, was upregulated in cancer cells cultured in 3D [42,51]. Spheroids may also lose expression of death receptors required for apoptosis mediated by TNF-α -related apoptosis inducing ligand (TRAIL), through the upregulation of cyclooxygenase-2 and prostaglandin E2 (COX-2/PGE2) pathways [52]. 3D spheroids are also likely to increase expression of an efflux pump known as P-glycoprotein (P-gp), a recognized cause of multidrug resistance [53]. P-gp upregulation has been attributed to metabolic changes that occur in spheroids such as reactive oxygen species and activation of the HIF-1α pathway [54,55].

Hypoxia can be achieved in traditional cell culture using specialized equipment, but spatial gradients in oxygen tension occur naturally in spheroids due to the balance between diffusion and consumption. One study that measured the oxygen pressure in tumor spheroids found an average oxygen diffusion distance of 232 ± 22 μm [56]. Therefore, spheroids large enough to exhaust oxygen diffusion limits will develop concentric regions of oxygenation: from the well-oxygenated and proliferative outer shell, through a hypoxic transitional zone, and to a central anoxic, necrotic core [57,58]. Tumor spheroids have been observed to activate the HIF-1α pathway in cell lines that do not express it in 2D (e.g. HeLa, MCF-7) [59,60]. Hypoxia subsequently reduced the migration, infiltration and cytotoxicity of T cells in microfluidic models [50,61]. Like oxygen, nutrients must also diffuse sufficient distances

to reach distal cells in 3D organoids. A study of NK cell activation established a nutrient gradient in a microfluidic device and found that in the distal, nutrient-deprived region, NK cells became less proliferative and less responsive to cytokines, while at the same time, more pro-inflammatory [62].

Solid tumors have long been known to shift their metabolism to favor aerobic glycolysis, a phenomenon known as the Warburg effect [63]. Cancer spheroids exhibit increased expression of the glucose transporter 1 (GLUT-1) and lactate dehydrogenase, the enzyme responsible for lactate production [64,65]. As expected, levels of lactic acid and lactate have been found to be higher in 3D spheroids than in 2D, impairing T cell function [49,66]. Acidification of the TME reduces lymphocyte efficacy in a number of ways including impaired cytotoxicity, reduced cytokine production, increased immunoinhibitory activity of the VISTA pathway [67], diminished expression of T cell receptors and CD25/IL-2Rα, and decreased activation of signal transducer and activator of transcription 5 and extracellular signal-regulated kinase [68–70].

Therefore, establishing 3D tumor geometries that allow realistic gradients of oxygen, nutrients, and pH will influence the results of I–O studies based on the altered response of lymphocytes to these conditions.

Patient-derived organoids

Tumor organoids derived from fresh patient tissue (patient-derived organotypic tumor spheroids or PDOTS), yield even more similarities to *in vivo* human tumors than organoids generated from cancer cell lines. PDOTS maintain the molecular characteristics of the native tumor sample, preserve intra-tumoral heterogeneity that does not exist in cell line models, and can retain the original stroma and immune cell populations, depending on the method of generation [71–73]. Sources for PDOTS include surgical resections, biopsies (both core-needle and fine-needle aspiration), or pleural effusion, and the PDOTS generated can be expanded, passaged, and cryopreserved [74–77]. Typically, mechanical and/or enzymatic digestion are used to break down tissue before straining to isolate small spheroids or single cells. Methods that fully dissociate samples into single cells then re-form spheroids by culturing in ultra-low attachment multi-well plates [78]. Several groups isolate small spheroids (<100 μm) using incomplete digestion of patient-derived tissue, which ensures that PDOTS generated in this way retain intact stroma from the native tumor, as well as a representative population of immune cells, including a matching repertoire of T cell receptors as the original tumor [73,79].

PDOTS may be immediately used in experiments or expanded using air-liquid interface or submerged hydrogel techniques [80,81]. With growing adoption of patient-derived organoid models, more groups have begun to use them for drug screening and validating that the response in PDOTS correlates to the response of the patient from which the tumor fragments were isolated [71,82]. Studies that obtain PDOTS from patients in clinical trials can compare the response rate observed in organoids to the patient response (generally using RECIST criteria or progression-free survival as metrics) using quantifications of spheroid size changes or viability [71]. While some such studies have only small numbers of samples, they often report clear concordance between organoid and patient responses to targeted

therapies [83]. Larger studies have compared the molecular features of the native tumor to the PDOTS and found no significant differences between the genotype and phenotype of the tumor and PDOTS [84,85]. Furthermore, for immunotherapy testing, matched T cells can be obtained from peripheral blood mononuclear cells (PBMC) or from tumor infiltrating lymphocytes (TILs), and these can be added to PDOTS culture to assess spheroid infiltration and cytotoxicity by lymphocytes [73,86–88]. Further studies are required to determine the information gained from adding PBMC-derived immune cells vs. retaining the native immune population for immunotherapy efficacy. For example, PDOTS with intact stroma and immune cells were found to have a highly immunosuppressive environment [84]. PDOTS from colorectal cancer had high levels of myeloid-derived suppressor cells and low levels of effector lymphocytes such as NK cells and $CD8_+$ T cells.

The exploration and development of patient-derived organoids presents the opportunity to use them for ‘personalized medicine’ or ‘precision clinical trials’ [72]. Obtaining tissue for PDOTS isolation at the start of a new trial will allow researchers to correlate the *ex vivo* response of PDOTS to the clinical response of each patient, which could increase the speed of determining drug response in the future, since organoid drug screening studies typically last for days to weeks rather than the weeks to months necessary to determine clinical responses. Such trials have reported good correlation between organoids and the clinic, with one study reporting 100% sensitivity and 93% specificity when testing immune checkpoint blockade in melanoma [89]. Beyond I–O therapy, many groups have used patient-derived organoid models for drug screening. This means that testing PDOTS should be able to identify ineffective therapies and point clinicians toward drugs more likely to be effective in an individual patient, such as a recent study involving breast cancer in which an organoid drug screen was used to identify the most effective drug for a patient experiencing early metastatic relapse [90]. Treating the patient with the drug identified resulted in disease-free progression 3-times longer than any other drug. Other studies have screened large drug libraries against PDOTS and validated the results with xenograft mouse models [91] or with correlation to clinical outcomes for chemotherapies currently in clinical use [92]. However, limitations still exist, and not all studies report high specificity, such as a trial in colorectal cancer that found that interferon γ (IFN-γ) production by T cells in PDOTS did not correlate well with patient response to immunotherapy [93]. This discordance between *ex vivo* and *in vivo* response may not be due to inherent differences in tumor phenotype, but rather due to the aspects of the microenvironment missing from PDOTS studies. For example, immune cell trafficking (adhesion to vasculature, extravasation, and migration to tumors) remains a significant barrier to mounting a productive immune response to tumors, even with the administration of immune checkpoint blockade therapies. Therefore, studies that combine microvascular models, patient-derived organoids, and circulating immune cells will be required to recapitulate the full TME and additional barriers to response produced by the stroma [94–96].

Limitations of tumor organoid methods

While tumor organoid models offer several advantages that will ensure their continued use for I–O studies, there are a number of limitations as well. First, organoid models are more complex than traditional 2D cultures, and thus will require additional training

and resources, and potentially have reduced throughput. Cell line organoids generated in ultra-low attachment (ULA) plates do not require significantly more expertise that monolayer culture, but many other methods described here require more complex plating such as the air-liquid interface or submerged hydrogel methods for expansion of PDOTS, or microfluidic devices with compartments for tumor spheroids, stroma (vasculature, CAFs, etc.), immune cells, cell culture medium, etc. Many biological labs do not have equipment or expertise needed for soft-lithography fabrication of microfluidic devices. This limitation can be overcome by purchasing commercial microfluidic devices on the market, but at greater cost than tissue culture plastics and without the ability to customize device designs to suit specific needs.

Additionally, 3D organoid culture introduces additional variables that are not present in traditional cell culture, especially the choice of hydrogel for organoid embedding. Care must be taken to standardize and characterize these hydrogels. There is a growing desire to develop synthetic gels and culture conditions to eliminate these sources of uncertainty and variability [71]. Since lymphocytes are highly sensitive to mechanical cues, subtle changes in matrix density, stiffness, or composition could produce differences in therapeutic response that will be difficult to attribute to a single cause without thorough understanding of the role the microenvironment plays in lymphocyte behavior. However, this is also a key benefit of using micro physiological systems for basic science studies of interactions between tumor, stroma, and immune cells.

While many tumor spheroid models exist and have been described here, there are also increasingly sophisticated tissue-specific organoid models of normal tissue being developed. However, few groups have combined normal and tumor organoids [97]. Future cancer organoid models could integrate tumor spheroids with healthy organoids from the same tissue, which would enable us to model additional aspects of tumor growth and development such as invasion and metastasis. Similarly, micro physiological models of the immune system, such as lymph node on-a-chip, have been developed but not combined with tumor organoids, so there are opportunities to model features of lymphocyte maturation and proliferation that these platforms enable [98,99].

Finally, since the behavior of $CD8_+$ effector T cells is critical to response to ICIs, multicellular organoid models must address mismatched human leukocyte antigen (HLA) types and the graft vs host response that can result from combining cells from multiple donors. Though syngeneic mouse cells circumvent this limitation and can be used in organoid platforms, the need for human models remains [100–103]. An alternative is to use HLA-matched cells, such as the combination of HLA-A*0201 melanoma and MART-1 specific, HLA-A*0201 restricted T cells [49], or engineered MHC-non-restricted CAR-T or TCR T cells [50,61]. For patient-derived models, T cells can be isolated from the same patient and re-introduced into the organoid model [86,87]. However, these approaches may not work for all pre-clinical immune-oncology studies and new approaches such as knockout of MHC molecules on cell types required to generate the microenvironmental architecture could be employed [104,105].

Mouse models

In the early-stage development of immunotherapies, researchers heavily depend on the *in vitro* models which lack of systemic immunity to provide response from endogenous immune cells. Using mouse models to assess immunotherapy efficacy provides researchers a means to inquiry the relationship between tumor cells and immune cells, as well as assess efficacy and safety of immunotherapies in presence of systemic immunity. Here, we summarize multiple mouse models for preclinical research, including syngeneic mouse model, tumor bearing immunodeficient mouse model, and humanized mouse model.

Syngeneic mouse models

The syngeneic mouse model is able to mimic the pathological transformation process of oncogenesis from normal cells into malignant cells [106], and can be categorized into three classes, subcutaneous tumor cell line, orthotopic tumor cell line and genetically engineered orthotopic tumor development. Kirsten rat sarcoma virus gene mutations are presented in approximately 25% of lung adenocarcinoma and are associated with a worse prognosis [107,108]. Tumor cells derived from Kraslox-stop-lox(lsl)-G12D/+; p53flox/flox (KP) inversion induced Joined neoantigen (NINJA) mice expressed neoantigens, were immunogenic and able to response to ICIs, including anti-PD1 and anti-CTLA4 mAbs [109,110]. In addition to NINJA, Cre-Lox system enables mammalian genome modification *in vivo*, carrying out deletions, insertions, translocations and inversions at specific tissues via tamoxifen induced Cre recombinase activation [111,112]. For example, ccRCC is characterized by inactivation of the VHL gene. The dysfunction of VHL leads to HIF hyperactivation, resulting in overexpression of many downstream genes involved in angiogenesis, metabolism, and cell-cycle regulation including which represent important therapy targets for patients with ccRCC [113,114]. A tamoxifen inducible ccRCC mouse model generated by renal epithelial cells with specific deletion from Vhl, Trp53, and Rb1 is able to mimic the cancer pathological process from proximal tubule epithelial cells and share similar transcriptional signatures with human ccRCC [115,116]. Overall, the cell lines have natural number of neoantigens and the spontaneous developed tumor has fewer neoantigens.

Immunodeficient mouse models

Immunodeficient mice were designed to overcome the rejection of human cancer cells as well as human immune cells mediated by the mouse adaptive and innate immune responses, and serve as powerful tools to assess I–O therapies [117]. For example, the fork head box N1 (Foxn1null) mutation, commonly known as nude, lacks a thymus and therefore is deficient in T cells but has functional B cells and NK cells [118,119]. Knocking out the recombination activating gene 1 (Rag1) [120], recombination activating gene 2 (Rag2) [121], protein kinase DNA-activated catalytic polypeptide (Prkdc) genes [122] that are essential for variable (V), diversity (D), and joining (J) rearrangements, results in murine T and/or B cell deficiency Depletion of interleukin 2 receptor subunit gamma (IL2rg) [123] or β2-microglobulin (B2m) [124] genes that are required in interleukin signaling and NK development, leads to the absence or functional impairment of murine NK cells in non-obese diabetic (NOD) mouse model [125]. Combinations of these genetic strategies have been applied to develop the popular immunodeficient mouse strains, such as NOD/Prkdcscid

(NOD/SCID) [124], NOD/SCID IL2rg−/− (NSG or NOG) [126,127], and Balb/c Rag1−/− IL2rg−/− (BRG) that have all been used in human oncology studies [117].

To choose an appropriate immunodeficient mouse model for a specific project, a number of factors should be taken into consideration, including gene background, endogenous immune cell components, leakiness (B and T cell development), lifespan, and husbandry [128]. The table 2 summarizes the immune cell components (T cells, B cells, NK cells) in several commonly used immunodeficient mouse models. Leakiness refers to the tendency of some mouse strains to develop functional B and T cells as the mice age. In general, leakiness is higher in mice with the C57BL/6J and BALB/cByJ backgrounds, lower in the ones with C3H/HeSn-JSmn background [129]. Due to the severe immunodeficiency, Rag1null and Pkrdcscid mice have specific husbandry requirements including that they should be housed in specific pathogen-free (SPF) environments. In addition, due to lack of efficient DNA repair, the Prkdcscid mice are radiation sensitive [130] and therefore cannot be as intensively irradiated as other immunodeficient models before being engrafted.

Cell-derived xenograft (CDX) models and patient-derived xenograft (PDX) models

CDX [118] and PDX [136] models developed in immunodeficient mice are widely used in cancer studies. A cell-derived COLO205 colorectal cancer cell xenograft mouse model is able to assess the synergistic effect of combination therapy of anti-death receptor 5 antibody TRA-8 and SN-38, an active metabolite of antitumor agent irinotecan (CPT-11) [137]. Orthotopic, tumor-bearing, mouse models provide more relevant development environments compared to an ectopic model in evaluation of I–O therapies, such as antibody therapies [138] and CAR-T cell therapies [139–141], and could have a better predictive value of disease [142–145].

PDX established directly from patient tumor tissue, conserves patient tumor signatures as well as the complex interplay between cancer cells and TME and has a better prediction for response and prognosis [146]. It has been reported that PDX share remarkable similarity in response rates compared to respective clinical trials [147], and serve as a critical tool in personalized medicine [148,149]. The patient-derived colorectal cancer models can retain intratumoral clonal heterogeneity and chromosomal instability and can be used for prediction of the response to an anti-epidermal growth factor receptor (EGFR) antibody, cetuximab, in patients [150,151]. The RCC models maintain the ability to evaluate tumor angiogenesis, retain genetic and histological characteristics [152], and accurately represent their respective original patient tumors [153]. In 2016, US National Cancer Institute (NCI) decided to retire the NCI-60 (a panel of 60 human cancer cell lines), and preferentially use PDX models derived from patient clinical samples and tagged with their clinical information for drug screening because the TME in PDX mimics human tumor better [154].

Humanized mouse models

The application of CDX and PDX models remarkably facilitates human cancer research and antitumor drug development. However, recent studies have demonstrated that the absence of human immunity in these models severely compromise their value in translational research and the development of novel I–O therapies [106,155]. The construction of humanized

animal models through transplanting human tissues (such as bone marrow-liver-thymus, aka BLT), PBMCs (such as Hu-PBL-SCID) or hematopoietic stem cells (HSCs) (such as SRC-Hu) into immunodeficient mice has allowed for the development a rudimentary level of innate and adaptive human immunity in small animals [156].

In hu-PBL-SCID mice, the human T cells are highly engrafted and expanded and the mice developed severe graft-versus-host disease (GVHD) [157]. Using PBMC-engrafted NSG and SGM3 mice, Ye et al. were able to capture alloreactivity in the form of cytokine release syndrome (CRS) from individual human PBMC donors [158]. Thus, hu-PBL-SCID mouse models serve as a rapid, sensitive, and reproducible platform to screen novel therapeutics for CRS, and provides a potential translational bridge for the study and prediction of CRS *in vivo* [159]. HSC-derived humanized mouse models derived from $CD34_+$ progenitor cells are used to evaluate I–O therapies, such as anti-PD-1 mAb [160] and study antitumor effect in a physiologically relevant immune environment [161]. The humanization efficiency is determined by the mouse species, the CD45 cell resource, as well as the age of the mouse recipient [162]. The NSG-SGM3 strain is a particularly good mouse model for humanization to assess immunotherapies and to study the TME [117,163], as it expresses human stem cell factor, GM-CSF, and IL-3 transgenes, supporting HSCs engraftment and the development of myeloid cells *in vivo* [164–166, 167]. It has been reported that transferring cord blood or fetal liver derived HSCs results in a higher engraftment of human CD45 cells compared to engrafting the bone marrow or mobilized peripheral blood derived HSCs [168,169]. In general, newborn recipients exhibited a better reconstitution of human CD45 cells compared to adult recipients [167,170,171].

Due to the lack of human thymus in HSCs derived humanized mice, the T cell are educated in mouse thymus, leading to poor human thymopoiesis [160] and deficient HLA dependent antigen specific immune responses [172]. The Thy/HSC [173] and BLT [174] models can overcome this limitation, providing robust human thymopoiesis and generating HLA-restricted antigen specific human T cell reactions. However, this model is limited by the accessibility of fetal tissues and local policy regulation [106]. On the other hand, Chang et al. matured DCs to present tumor antigens to prime T cells *in vitro*, to assess cytotoxicity of CCR4 targeted mAb *in vivo*. Those tumor primed T (TP-T) cells had an increased IFN-γ expression reacting to the same tumor cells compared to unprimed T cells from the same donor *in vitro* and exhibited superior tumor control in combination with anti-CCR4 mAb in an ovarian cancer bearing mouse model [175].

CONCLUSION

Here, we summarize the applications of 2D culture, 3D cultures, and mouse models in I–O in order provide insights for research scientists trying to choose appropriate models in different phases of therapy development and to speed up the process of translating preclinical research to clinical trials. Selecting appropriate models will be critical to achieve robust results that enable accurate identification of effective and ineffective drugs and the successful clinical translation of new technologies. Therefore, researchers must carefully consider which features the TME are of key importance for testing a new therapeutic. Convincing I–O researchers to consider this additional layer of methodological scrutiny

and fostering greater understanding of the relative strengths and weaknesses of each of these preclinical drug screening methods will benefit the field as a whole by improving the predictive power of preclinical studies.

Funding declaration:

Wang Y received financial support for the research, authorship and/or publication of this article by the Wong Family Award. Wang Y is also a NIH-funded author, grant number NIH P50 CA101942. Shelton S recieved financial support for the research, authorship and/or publication of this article by National Institute of Health (NIH) fellowship made to MIT (K00CA212227). Freeman G received financial support for the research, authorship and/or publication of this article by NIH P50 CA101942. Marasco WA received financial support for the research, authorship and/or publication of this article by the Assistant Secretary of Defense for Health Affairs endorsed by the Department of Defense, through the FY21 Translational Research Partnership Award (W81X-WH-21-1-0442) and FY21 Idea Development Award (W81XWH-21-1-0482) to W.A.M. Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the Department of Defense.

Disclosure and potential conflicts of interest:

Barbie D discloses they are the co-founder of Xsphera Biosciences Freeman G discloses he has patents on PD-L1/PD-1 pathway at Roche, Merck MSD, Bristol Myers Squibb, Merck KGA, Boehringer-Ingelheim, AstraZeneca, Dako, Leica, Mayo Clinic, Eli Lilly, Novartis. He also has a patent at patents on TIM-3 pathway at Novartis. Also has consulting fees at Roche, Bristol-Myers-Squibb, Triursus, iTeos, NextPoint, IgM, Jubilant, Trillium, GV20, IOME and Geode. At Invaria he is on the board of directors. He also has stock options at Nextpoint, Triursus, Xios, iTeos, IgM, Trillium, Invaria, GV20, Geode.

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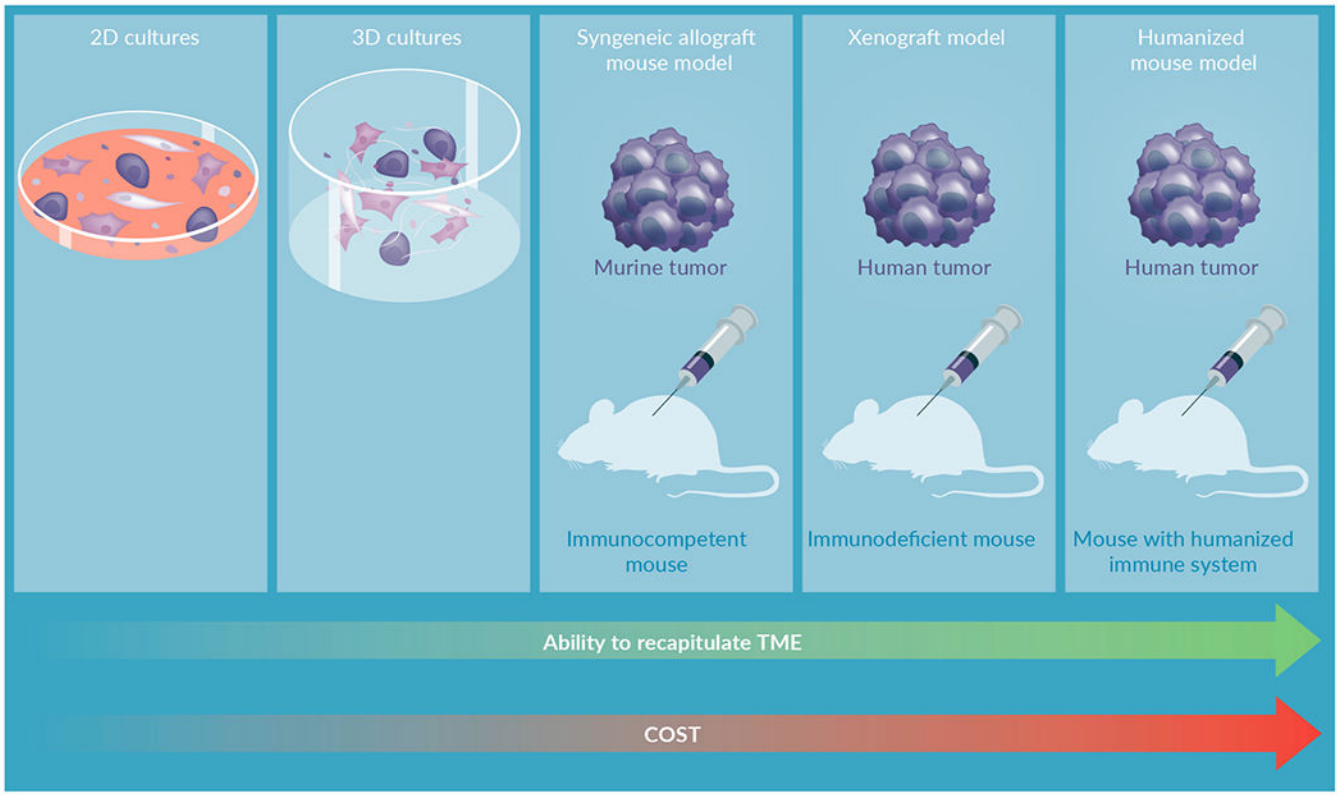

FIGURE 1.
Preclinical immuno-oncology models.

TABLE 1

Preclinical immuno-oncology models.

	Applications	Advantages	Limitations
2D cultures	► Drug screening ► *In vitro* evaluation	► Easy access ► Fast readout ► Low cost	► Can be poorly predictive
3D cultures	► Drug screening ► *Ex vivo* evaluation ► Study of TME and ECM	► Relatively easy to prepare ► Can recapitulate TME and ECM ► Histological fidelity to original tumor	► Lack of inter-organ communication
Syngeneic mouse models	► *In vivo* efficacy and safety assessment ► Study of disease development	► Can engineer specific genes	► Failure to accurately mimic human disease phenotypes
Xenograft mouse models	► *In vivo* efficacy and safety assessment	► Includes cell-derived xenograft (CDX) and patient-derived xenograft (PDX) ► PDX can recapitulate patient tumor signatures	► Costly
Humanized mouse models	► *In vivo* efficacy and safety assessment ► Study of TME	► Can recapitulate human TME	► Costly

TABLE 2

Immunodeficient mouse strains for human cancer study.

Name	Strain	T cells	B cells	NK cells
Nude [118]	Foxn1null	No	Yes	Yes
Scid [131]	B6.CB17-Prkdcscid/SzJ	No	No	Yes
BRG [117]	BALB/c.Rag2−/− IL-2Rg−/−c	No	No	No
NOD-scid [132]	NOD.CB17-Prkdcscid/J	No	No	Function impaired
NOD/SCID [124]	B2mnull NOD.Cg-B2mtm1UncPrkdcscid/SzJ	No	No	Function loss
NSG [126]	NOD.Cg-PrkdcscidIL2rgtm1Wjl/SzJ	No	No	No
NOG [127]	NOD.Cg-PrkdcscidIL2rgtm1Sug/JicTac	No	No	No
BRGS [133]	BALB/c.Rag2−/−IL-2Rg−/− c NOD.sirpa	No	No	No
hSIRPa-BRG [134]	BALB/c.Rag2−/−IL-2Rg−/− c human.sirpa	No	No	No
MISTRG [135]	C;129S4-Rag2tm1.1FlvCsf1tm1(CSF1)FlvCsf2/Il3tm1.1(CSF2,IL3)Flv Thpotm1.1(TPO)FlvIl2rgtm1.1FlvTg(SIRPA)1Flv/J	No	No	No