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SPOTLIGHT ON:

Nonclinical tools update: are they improving in their capabilities of predicting clinical response?

Guest Editor: Angus Sinclair

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SPOTLIGHT

FOREWORD

To a mouse

Róisin McGuigan

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FOREWORD

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This September, *Immuno-Oncology Insights* spotlights a key issue for successful translation of promising therapeutic modalities: the pre- and nonclinical toolbox currently available for I–O applications. As translation between the bench and the clinic continues to be a major challenge, and substantial issues with recapitulating human biology in mouse models

remain, our contributors review the current options available, and explore emerging approaches that may help to bridge the translational gap more successfully in future.

First up, Yufei Wang *et al.* review preclinical models – including cell lines, 3D cultures, and mouse models – to support the development of novel I–O therapeutics. Next, we speak to Michelle Morrow, who shares insights on the preclinical tools that are underpinning the continued evolution of the bispecific antibody field.

Also in this issue, Aleksander Skardal discusses advances in biomaterials, organoid, and organ-on-a-chip technologies, and his work towards generating immune-competence in patient-derived tumor organoids. In her article, Stephanie Casey considers the translational mouse models available for I–O applications and the decision between syngeneic and humanized mouse models. She also shares an illuminating quote:

"A theory has only the alternative of being right or wrong. A model has a third possibility: it may be right, but irrelevant."

Much like in the I–O space as a whole, challenges remain in the nonclinical tools space – but innovation continues to drive progress towards more useful (and relevant) models to aid in answering some of the field's most pressing questions.

AFFILIATION

Róisin McGuigan
Editor
Immuno-Oncology Insights

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IMMUNO-ONCOLOGY INSIGHTS

NONCLINICAL TOOLS UPDATE: ARE THEY IMPROVING IN THEIR CAPABILITIES OF PREDICTING CLINICAL RESPONSES?

SPOTLIGHT

EXPERT INSIGHT

Preclinical models for development of immune–oncology therapies

Yufei Wang, Sarah E Shelton, Gabriella Kastrunes, David A Barbie, Gordon J Freeman, Wayne A Marasco

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

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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

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

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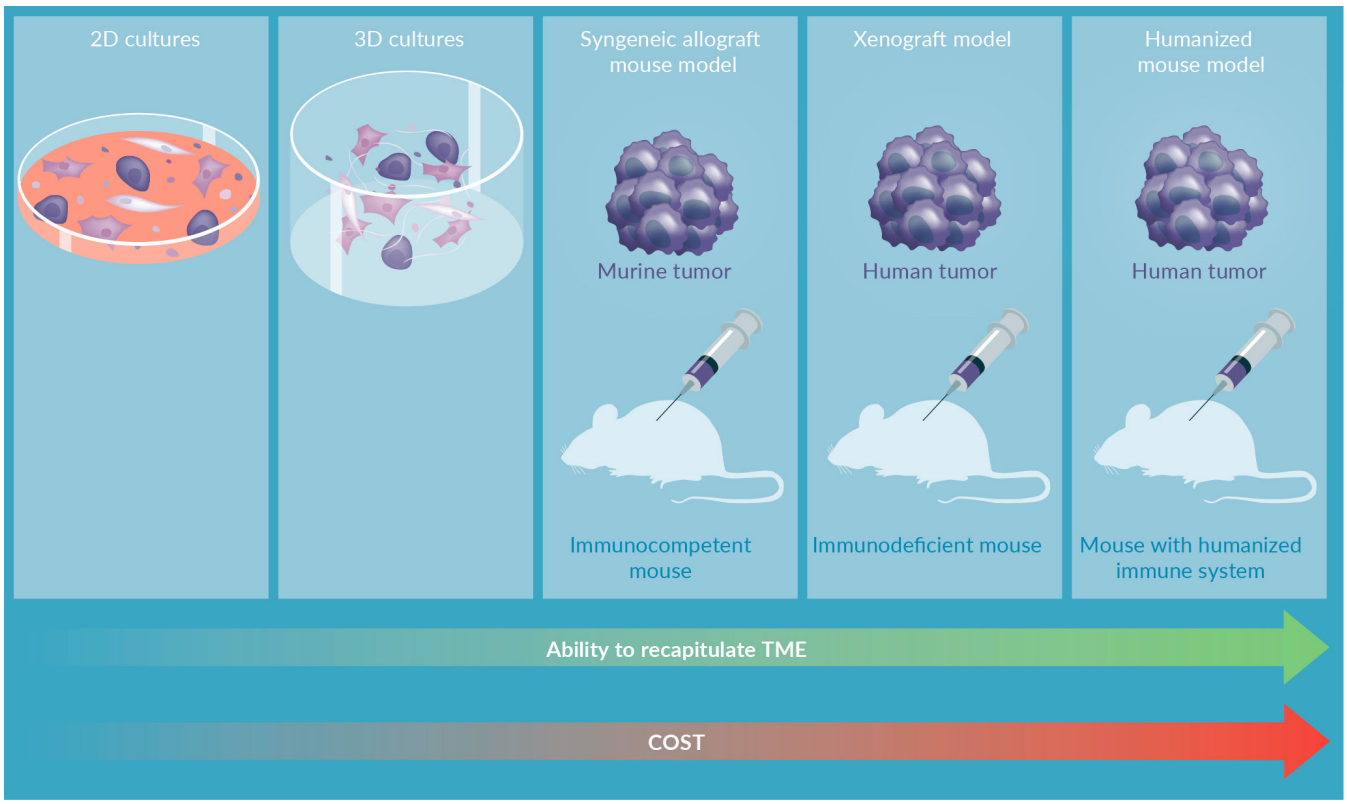
FIGURE 1

Preclinical immuno-oncology models.



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

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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

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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

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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

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

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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/HeSnJSmn 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

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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.

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AFFILIATIONS

Yufei Wang
Department of Cancer Immunology and Virology,
Dana-Farber Cancer Institute,
Boston, MA
and
Harvard Medical School,
Boston, MA 02115, USA

Sarah E Shelton
Department of Medical Oncology,
Dana-Farber Cancer Institute,
Boston, MA 02215, USA
and
Department of Biological Engineering,
Massachusetts Institute of Technology,
Cambridge, MA 02139, USA

Gabriella Kastrunes
Department of Cancer Immunology and Virology,
Dana-Farber Cancer Institute,
Boston, MA

David A Barbie
Harvard Medical School,
Boston, MA 02115, USA
and
Department of Medical Oncology,
Dana-Farber Cancer Institute,
Boston, MA 02215, USA

Gordon J Freeman
Harvard Medical School,
Boston, MA 02115, USA
and
Department of Cancer Immunology and Virology,
Dana-Farber Cancer Institute,
Boston, MA

Wayne A Marasco
Department of Cancer Immunology and Virology,
Dana-Farber Cancer Institute,
Boston, MA
and
Harvard Medical School,
Boston, MA 02115, USA

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IMMUNO-ONCOLOGY INSIGHTS

NONCLINICAL TOOLS UPDATE: ARE THEY IMPROVING IN THEIR CAPABILITIES OF PREDICTING CLINICAL RESPONSES?

SPOTLIGHT

INTERVIEW

Bispecifics come of age: how preclinical tools are helping drive translation to the clinic

Roisin McGuigan, Editor, *Immuno-Oncology Insights*, **speaks to Michelle Morrow,** PhD, SVP, Biology & Translational Sciences, F-star Therapeutics

MICHELLE MORROW PhD, is Senior Vice President of Biology & Translational Sciences at F-star Therapeutics, overseeing the company's biology and translational sciences teams' research activities ranging from discovery through to clinical phase programs. Major achievements during this time include progressing FS118, FS120 and FS222 from candidate selection into the clinic. She also plays a key role as a Project Leader for FS118, a LAG-3/PD-L1 bispecific antibody in Phase 2 clinical trials. Michelle joined F-star from MedImmune, where she was a Project and Team Leader from 2007 to 2017, and she successfully established an immuno–oncology preclinical modelling group, supporting projects across the MedImmune and AstraZeneca portfolio. She led research project teams for several novel therapies within the Oncology portfolio, including FDA-approved ImfinziTM (durvalumab) and MEDI5752 (PD-1/CTLA-4 bispecific). Michelle earned a PhD in Immunology at the University of Cambridge and completed her post-doctoral studies in leukemia research at the Institute of Child Health, London.

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Michelle Morrow of F-star Therapeutics discusses the company's unique approach to creating bispecifics, the evolution of the bispecifics field, and new and emerging nonclinical tools for I-O applications.

Q What led you to immuno-oncology, and to your current role?

MM: I have always had a curiosity about science, since I was a child. My dad worked as a science teacher and my mum as a pharmacist. I worked with my mum in the pharmacy on weekends and was curious about why people were taking medicines and how they worked.

I decided to follow a scientific path and following my undergraduate degree in Molecular Biology, completed a PhD in immunology at the University of Cambridge, and then post-doctoral research into childhood leukemia at Great Ormond Street Hospital, part of UCL. I wanted to do something more for patients and to move closer to finding new ways of treating patients with cancer. In 2007, I moved to Cambridge Antibody Technology, which evolved to be MedImmune, and is now part of the AstraZeneca organization.

I joined the team at Cambridge Antibody Technology, working on I-O projects. My role was to develop model systems and assays to support the emerging I-O. I set up a research group to establish *in vivo* models for I-O. I progressed to discovery project leader on the PD-L1 antibody project, which went on to become Imfinzi®, an FDA-approved drug. I was there for 10 years and had a great experience working on many different programs.

Then the opportunity came along to join F-star, and I jumped at that. I'm fascinated by bispecifics, and it was a fantastic opportunity to join a biotechnology company that was truly pioneering this type of drug development. I took on the role of establishing the biology team to build the capability to take molecules from discovery through to the clinic.

It's been a really busy 5 years. We have progressed three drug candidates from discovery phase through to clinical-stage programs. In my current role as head of the F-star Biology and Translational Sciences team, I support research into new bispecific opportunities, as well as driving forward the clinical-stage programs with biology expertise and pharmacology data. I also work as a project leader and have followed the FS118 program all the way through – this is our PD-L1/LAG-3 bispecific, now in Phase 2 studies. It has been rewarding to be a part of the team to build the basic science and the tools to develop a drug, then to move it from concept through to the clinic, and into the patient setting.

Q What is different about F-star's approach to bispecifics?

MM: The F-star approach to making tetravalent bispecifics is simple, and that is the beauty of it.

We generate bispecifics that look like natural antibodies by engineering additional binding sites into the existing structure. We do this by making a small number (around 10–15) of amino acid substitutions in the Fc region of the antibody. This creates two mirrored binding sites in the Fc regions right at the bottom of the antibody, creating a binding domain called an Fcab.

Combined into this antibody we now have four binding sites, two on the fragment antigen-binding (Fab) end and two on the Fcab end. We do not add any domains or linkers, and being able to get four binding sites into a normal antibody structure is what makes it different.

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We can make this in the same way we make a normal monoclonal antibody, and we believe that it will be safe for the patient because the immunogenicity liability is lower.

When we are making bispecifics, we are always looking for something that a bispecific can achieve that a combination of two antibodies cannot. Our tetravalent bispecific structure allows us to create a unique pharmacology where we can crosslink two different types of cells and bring them together for a novel function. We can cluster receptors via the avid binding we get from the two binding sites. We can have conditional activity as well, which is often very important when trying to direct an immune response in a tumor without the unwanted side effects of activation in the periphery.

> "We generate bispecifics that look like natural antibodies by engineering additional binding sites into the existing structure."

Q What therapies are currently in your pipeline?

MM: We have four clinical-stage programs in our pipeline, all in the I-O space, driving forward our mission to transform the lives of patients with cancer using immunotherapy.

FS118 is the project I lead and our most advanced program. It is targeting two validated cancer targets: LAG-3 and PD-L1. LAG-3 is an exciting target. The first LAG-3 inhibitor has recently been approved for the treatment of melanoma and there is also potential in other tumor types. We are testing FS118 in the clinic in patients who have not received checkpoint inhibitors in both non-small cell lung cancer and diffuse large B-cell lymphoma, and also in patients who have acquired resistance to checkpoint inhibitors in a Phase 2 trial in head and neck cancer.

Our next program is FS222, which is a T-cell redirector targeting CD137 and PD-L1. There is a lot of interest, and huge promise, in the CD137 space. We have demonstrated unique benefits of our platform in terms of the additional activity in the tumor. The data we generated show 100% tumor clearance in mice, so we believe this program has the potential to be transformational for the treatment of patients with cancer.

Our third bispecific is FS120, which is a dual agonist of both OX40 and CD137. This is a different way of activating CD137, through activating multiple immune effector mechanisms, and has a very broad applicability across several tumor types as an activator of anti-tumor immunity.

Finally, we have a second-generation STING agonist, SB 11285, that is intravenously administered and rapidly taken up by cells. We are dose escalating this both as a monotherapy and in combination with atezolizumab.

We also have a discovery engine to generate more bispecific antibodies, which we hope will bring more projects through in the future.

Our focus this year is on delivering on our ongoing clinical studies and we are well positioned to announce data readouts across all four of these programs. It is always satisfying when you see the data. And of course, seeing the benefit for patients is ultimately what we want to do.

Q How has the bispecific field evolved in recent years?

MM: There has been a huge revelation in how we think about treating patients with cancer. Immunotherapy in itself has been a massive revolution. We have had checkpoint PD-1 inhibitors on the market for less than a decade, which is hard to believe as they are now considered a mainstay of cancer treatment. Bispecifics are coming of age, and we are seeing numerous technologies out there. All of them are subtly different in the way that they target cells and are dependent on the structure of the molecule, the valency, the affinity, and how those molecules are uniquely designed.

As we understand more about how the tumor environment works, we can build on what we have seen to select the right patients. We are also thinking about resistance mechanisms, and how to avoid and overcome resistance with checkpoint inhibitors. The aim is to rescue patients for whom checkpoint inhibitors have failed using some of the targets we are looking at to sensitize patients and increase response.

Future progress will likely come from modulating existing targets in new ways using new technologies, such as bispecific antibodies. I am also very interested in thinking about how we can use existing, proven therapies in a combination setting. How do we build on therapies that have been out there for a long time, such as chemotherapy? How can we use those to potentiate the activity of our drugs by killing cells and releasing antigen, that we can then drive an immune response to?

Q What nonclinical tools have you found most beneficial for your own applications when moving from the preclinical to clinical stage?

MM: I-O research requires a suite of assays and models to piece together a jigsaw of how your molecule is working. There is no one model that will tell you everything.

With bispecific antibodies, it is at least twice as complicated because you have both this novel biology and the impact of engaging two targets. At F-star, we utilize *in vitro* assays using human cells so that we can profile the effects and easily understand the potency of these molecules. We also screen molecules and select the ones that have the desired properties.

Once we have a shortlist of lead candidates, we then start *in vivo* modeling. It is not as straightforward in I-O as with tumor-targeted oncology, because you are primarily targeting the host immune system in your mouse model, not the tumor. We use mice that have an intact immune system into which we transplant a tumor. These are not human cancer cells, so they do not harbor the same genetic mutations. Due to the immune reaction the mouse has to the tumor, these models are an acute model of inflammation, which is different from human cancer in which a tumor may have been developing for years, if not decades. This means the immune context could be quite different with respect to

> "Even in tumor types that are considered I-O sensitive, a large proportion of patients do not benefit from these therapies. It is a crowded field, but there is still potential for so much more to be done."

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human cancer, where the patient has an exhausted immune system, rather than a new immune response we would get in the mouse models.

However, despite some limitations, *in vivo* modelling has been hugely important in the discovery of transformational therapies, including the work done with PD-1 and CTLA-4 that led to Nobel Prize-winning research. I think of these experiments as immunology experiments rather than cancer experiments. I am always thinking about how the tumor interacts with the immune system in this context. The mouse tumors do have some features that mirror human cancer, such as the same cell types and hallmarks of immune suppression, so you can study how those two things interact very well. What is still unclear is how we predict efficacy and how a shrinking tumor in a mouse translates into a human. This is possibly the missing piece we don't understand.

Mechanistically, the models are also very good, though their limitations must be understood. In addition to understanding what they can tell you, you equally must understand what they can't tell you. This provides the ability to interpret your data appropriately, to manipulate the model to ask certain scientific questions, and then use the data to build biomarker strategy, support your translational hypothesis, and ensure you have a clinical development strategy with the greatest chance of success. They are simple models, but you can ask complex questions of them.

Q What new and emerging nonclinical tools are you most excited by? What developments would you most like to see?

MM: For many years, there has been a search to find preclinical models that predict efficacy. The ultimate goal is to be able to run a preclinical study and know whether it is going to work in patients. The mouse tumor models we have now do not always predict efficacy in humans, but they do guide our thinking about how to design our clinical trials and how to interpret data from the clinic.

There are some interesting emerging technologies that allow growth of human cancer cells *ex vivo*; particularly looking at fragments of cancer cells or organ explants, using material that is taken from patients during surgery. These are more disease relevant, and you are more likely to see mechanisms that mirror what you get in the patient in terms of activation of tumor-infiltrating lymphocytes (TILs) and overcoming suppression. However, they are still limited in terms of measuring tumor killing, and again become more of a mechanistic model. *Ex vivo* models may also be better at studying that exhaustive tumor microenvironment in patients who have failed checkpoint therapy.

When you combine these models with cutting-edge technology such as spatial transcriptomics and proteomics, and then try and understand what is in the tumor microenvironment, this is going to help us better understand how to tailor the therapies to the right patients. In many cases, we have drugs that do work but we do not necessarily know the right patients to deliver them to. These technologies will be very important in helping to understand that in the future.

Q If companies target earlier line treatments with I-O agents, how does that change the R&D approach? And how can companies compete in an increasingly crowded field?

MM: It is important to recognize that despite the fact there is a lot of progress and research, there are still large populations of patients who simply do not have a standard therapy that works for them. Even in tumor types that are considered I-O sensitive, a large proportion of patients do not benefit from these therapies. It is a crowded field, but there is still potential for so much more to be done.

From a research perspective, it is important to understand the clinical landscape when we start to design new drugs. When we are doing discovery, we should be thinking about providing a solution to a clinical problem rather than just making cool molecules that can activate cells. Understanding the niches and gaps and the biology of those settings is important.

Having a differentiated approach is important. That's where F-star comes in, with a tetravalent bispecific platform. We are trying to bring in novel mechanisms that will target the tumor and drive that new biology. We have seen this with our agonist programs in which we get conditional activity potentially at the tumor site or where the target is expressed. With FS118, we are able to drive shedding of LAG-3 from the tumor as well as blocking the pathway. Looking for those sweet spots in your technology, and where you can match those with unmet patient need, is how we identify opportunities for our compounds. Having a clinical strategy grounded in science is very important.

Q What will be your own chief goals and priorities over the next year?

MM: It is an important year for F-star, and we anticipate having data readouts for all four of our programs. Translating what we have discovered in the lab into the clinic is going to be very exciting. We continue to develop our clinical plans and think about advancing our programs. We need to increase understanding of the science of our molecules and the science of the disease, whilst also looking for opportunities where patients are underserved by current therapies.

We are also working to select the next molecules from our platform, to see where we can unveil some exciting biology. The bar continues to be raised, and we need to be thinking in increasingly novel and innovative ways to overcome the impact the tumor has on the immune system.

Both F-star and the wider immuno-oncology community have a great opportunity to design immune therapies to transform patients' lives, and we hope that this will be a great year to see our work come to fruition in the clinic.

AFFILIATION

Michelle Morrow, PhD
Senior Vice President, Biology & Translational Sciences at F-star Therapeutics

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NONCLINICAL TOOLS UPDATE: ARE THEY IMPROVING IN THEIR CAPABILITIES OF PREDICTING CLINICAL RESPONSES?

SPOTLIGHT

VIEWPOINT

Harnessing components of the immune system in bioengineered 3D tumor models

Aleksander Skardal, Ohio State University

"...in our *in vitro/ex vivo* bioengineered 3D tumor model systems, we have new technologies that could make significant differences in the quality of clinical care in the coming years, and as soon as now."

VIEWPOINT

Immuno-Oncology Insights 2022; 3(8), 373–377

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Cancer research has been limited by the lack of model systems that accurately recapitulate tumor physiology seen in human cancer patients. For decades, the most common of these model systems were simplistic two-dimensional cell cultures – often using tumor cell lines – and mouse models. Of course, today we know very well that no tissues in the human body look like 2D cell cultures – that is, cells in a monolayer on a rigid plastic surface. Furthermore, we understand the immense differences between murine and human biology. While both 2D and murine models have provided important insight into and advances in cancer biology, we can do better.

With the advancement of biomaterials, bio fabrication, microfluidics, and tissue engineering technologies, researchers now have a wide variety of 3D human-based models that can be deployed in cancer research studies [1,2]. These include simpler cancer cell spheroids, more complex organoid cultures comprised of single or multiple cell populations encapsulated in extracellular matrix hydrogels, and microfluidic device-based tumor-on-a-chip platforms that combine 3D tumor architectures with fluid flow. Over the past decade or so, we have made tremendous progress in making these humanized tumor models more viable, accurate, and consistent, enabling use in applications such as disease modeling, drug development, and even personalized medicine [3]. Importantly, a number of labs, including our own, have demonstrated that one can employ tissue engineering and bio fabrication technologies to generate tumor models from patient-derived tumor biospecimens [4-11]. Data derived from these patient-specific tumor models shows that if created using the correct methods and materials, they accurately recapitulate phenotypes and genomic profiles of the originating tumors, and thus can serve as powerful diagnostic tools. While wide deployment of 3D tumor models to drive clinical decisions is limited, this is a goal that we believe will see realization within the next several years.

While screening of chemotherapies and targeted therapies has been relatively straightforward in platforms such as organoids and tumor-on-a-chip systems, [6,8–13] only in the last several years have we seen evidence of organoid and tumor-on-chip models successfully integrating aspects of the immune system, [14,15] thus enabling treatment screening studies beyond chemo- and radiotherapy such as immune checkpoint blockade (ICB) and cell therapies. However, given the clinical and research-based focus on determining efficacy of existing immunotherapies and developing new ones, there has been a crucial need for immune-competent models. It should be noted that the term 'immune-competent' may take very different forms and scopes across bioengineered *in vitro* models. More often than not, the level of immune competence is rather limited, but determined by a specific research question being asked or an objective being sought.

Our efforts toward generating immune-competence in patient-derived tumor organoids (PTOs) began as finding a solution to the problem: How does one integrate an immune system into a PTO that might be able to respond to an ICB therapy? Certainly, there may be immune cells present in a clinical tumor biospecimen, such as tumor infiltrated lymphocytes (TIL), but this may not be the case in all biospecimens [16,17]. One can also isolate lymphocytes from peripheral blood, but T cells from these populations are often not necessarily tumor-reactive [18]. Our solution was to resect not only tumor tissue from the patient during debulking surgery, but also a nearby lymph node. Lymph nodes are quite remarkable in that they contain cells that represent approximately 80% of all immune cell types. These include T cells, and antigen-presenting cells such as B cells and dendritic cells – the very combination of immune cells needed to mount a T cell-mediated response to tumor cells [19]. By introducing this heterogeneous population of lymph node-derived cells into our melanoma PTOs – thereby forming immune-enhanced PTOs (iPTOs) – we were able to successfully

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generate tumor cell killing by T cells under ICB therapeutics such as pembrolizumab, nivolumab, and ipilimumab [15,20]. It should be noted that as in human patients, we also observed organoid sets from some clinical biospecimens that were still resistant to the therapies. We have now demonstrated the efficacy of our approach in additional cancers such as appendiceal cancer, sarcomas, and Merkel cell carcinoma [21–25]. Notably, our organoids were able to identify a new therapeutic intervention in a melanoma patient that wasn't previously being considered [15].

Combinations of organoids with organ-on-a-chip microfluidic systems now enable the design and deployment of more realistic multi-site and multi-tissue type models connected by fluid flow [26–28]. We have demonstrated that we can model phenomena such as tumor metastasis in our 'metastasis-on-a-chip' platform, in which we can track metastasizing tumor cells from an initial tumor organoid site to one or more downstream tissue organoid sites [29,30]. In the context of immune-oncology, we can utilize platforms akin to this not necessarily for modeling metastasis, but rather to model and assess T and natural killer cell homing to tumors using human-based systems, rather than animals. Moreover, organ-on-a-chip and tumor-on-a-chip microfluidic device platforms offer unprecedented direct access to, and observation of what cells are doing within these systems. While we cannot place most animal tumor models under a microscope and retrieve usable data, we can with tumor-on-a-chip platforms. We can engineer them to be transparent and compatible with any microscope or alternative imaging or data capture systems. We can even engineer biosensors into the chips that report additional environmental parameters in real-time [28,31].

These advances in biomaterials, organoid, and organ-on-a-chip technologies have resulted in a powerful toolbox of methodologies with which to better model human cancer biology. We as a field have only begun to tap into the potential of these tools when it comes to integration and assessment of the role of the immune system in cancer progression and therapies through the use of tumor organoid and tumor-on-a-chip platforms. While cancer continues to be one of the most difficult and significant medical hurdles in our society, in our *in vitro/ex vivo* bioengineered 3D tumor model systems, we have new technologies that could make significant differences in the quality of clinical care in the coming years, and as soon as now.

BIOGRAPHY

ALEKSANDER SKARDAL is an Assistant Professor in the Department of Biomedical Engineering at the Ohio State University (OSU). He is also a member of the James Cancer Hospital and Solove Research institute at the Ohio State University Comprehensive Cancer Center, the Center for Cancer Engineering, and is the co-founder and co-director of OSU's Organoid Technology Program.His research focuses on using modular and customizable ECM-based biomaterials and 3D bio fabrication techniques to create tissue and tumor model systems such as organoids and organ-on-a-chip platforms for drug and toxicology testing, disease modeling, and personalized medicine. Dr Skardal received his BSc in Biomedical Engineering at Johns Hopkins University and his PhD in Bioengineering at the University of Utah, focusing on the development of extracellular matrix (ECM)-derived hydrogels for 3D bioprinting. This work yielded several of the very first published papers describing the development of 'bioinks' for 3D bioprinting. Dr Skardal did his postdoctoral work at the Wake Forest Institute for Regenerative Medicine (WFIRM), applying ECM biomaterials technologies for applications in stem cell biology, wound healing, organoids for drug screening, bioprinting, and in vitro tumor modeling. As an assistant professor at WFIRM, Dr Skardal further developed a toolkit of ECM biomaterials as higher quality cell-supportive bioinks for bioprinting, built multi-tissue type multi-organoid body-on-a-chip systems, and created a tumor organoid/tumor-on-a-chip research program that employs human patient-specific tumor biospecimens to bio fabricate personalized tumor models for precision oncology applications. These efforts continue at OSU today.Dr Skardal's research efforts have been supported by awards from

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AFFILIATION

Aleksander Skardal
Department of Biomedical Engineering,
The Ohio State University,
140 W. 19th Ave, Columbus, OH, 43210,
USA
and
The Ohio State University and Arthur G. James Comprehensive Cancer Center,
460 W. 10th Ave, Columbus, OH, 43210,
USA
and
Center for Cancer Engineering,
The Ohio State University,
460 W. 10th Ave, Columbus, OH, 43210,
USA

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IMMUNO-ONCOLOGY INSIGHTS

NONCLINICAL TOOLS UPDATE: ARE THEY IMPROVING IN THEIR CAPABILITIES OF PREDICTING CLINICAL RESPONSES?

SPOTLIGHT

COMMENTARY

Translational mouse models for immuno-oncology: from syngeneic to humanized models

Stephanie C Casey

"A theory has only the alternative of being right or wrong. A model has a third possibility: it may be right, but irrelevant." [1]

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INTRODUCTION

As the field of immuno-oncology continues to advance, researchers have a multitude of options when selecting preclinical models to test biological questions, evaluate novel molecules, and generate preclinical datasets. While tumor cell-intrinsic biology is often interrogated with genetically engineered mouse models that express driver oncogenes or loss of tumor suppressor genes and target validation work is performed in human xenografts in immunocompromised mice, immuno-oncology biology is interrogated in a multitude of mouse models all uniquely poised to answer different questions [2]. Consequently,

the mouse model used for immuno-oncology programs should be selected based on the biology of the question at hand, and a multitude of models may even be deployed for the same target or program. This article considers the decision tree of syngeneic vs humanized mouse models; although out of the scope of this review, the history of genetically engineered mouse models for preclinical and translational oncology research is reviewed extensively elsewhere [3–10].

SYNGENEIC MODELS

Syngeneic tumor models are derived from tumors that spontaneously arose in a wild type mouse, arose in a genetically engineered mouse model as driven by programmed genetic events, or developed after administration of a cancer-causing agent [11]; these tumor cell lines are then grown in host mice of the same background. Most of these models have a rapid growth rate, allowing for a timely answer to new experimental questions and high-throughput data collection [11]. However, the rapid growth rate of most syngeneic tumor cell lines may not allow enough of an experimental 'runway' to see the biological effect of a therapeutic manifest before tumor volume endpoints are met. In syngeneic models, the whole system (host, tumor, microenvironment) is fully mouse; while a fully functional immune system may be of particular interest for certain experimental questions, it also carries the implication that for large molecule drug discovery work, both human (for the clinic) and mouse (for the lab) molecules must be generated if the clinical candidate does not cross-react to rodents. Mechanism of action questions must thus be answered with a mouse tool molecule or surrogate or in tumors that are engineered to express a human target. For small molecule immuno-oncology drug discovery, cross reactivity between preclinical species and human should be prioritized such that other datasets (e.g., safety, toxicology) are performed with the clinical candidate whenever possible. In instances where mouse surrogates are not available or applicable, models must be tailored to allow meaningful use of human targeted reagents. For models in which evaluation of a human molecule is desired, a variety of humanized mouse models may be customized to the biology at hand to ensure a relevant and decision-enabling dataset is generated.

HUMANIZED MODELS

Scientists have been using immunocompromised mice to interrogate cancer since the 1960s. The nude mouse was first described in 1966 [12] and was further characterized to be missing a functional thymus in 1968 [13]. It was first used for tumor transplantation in 1969 [14], and its genetics were further clarified in 1994 [15]. The burgeoning field grew in the decades to come as researchers sub lethally irradiated severe combined immunodeficient Prkdc^{scid} (SCID) mice and infused human peripheral blood lymphocytes [16], and then researchers sub lethally irradiated SCID mice on the non-obese diabetic background (termed NOD/SCID) and infused in CD34^{+} human fetal liver, cord blood, or bone marrow cells, which gave rise to expected progenitors [17]. As work with immunocompromised mice grew and developed over the years, scientists devoted time into generating 'humanized' mouse models. In different facets of *in vivo* oncology work, 'humanized' mice may refer to mice genetically engineered to express human genes (e.g., knock-ins), or it may refer to immunodeficient mice that have received engraftment of human hematopoietic cells or lymphocytes. In this article, it will refer to the latter. For preclinical oncology work, these humanized models generally receive human cells, are implanted with a human tumor, and are then treated with a therapeutic that recognizes a human target.

Development of humanized mice was further bolstered by the development of the NOD/LtSz-scid IL2R gamma null (NSG) mouse [18,19], which can easily support peripheral blood mononuclear cell (PBMC)

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engraftment, but which improves the transfer of human hematopoietic stem cells (HSCs) as compared with its predecessors [18–20]. NSG mice that receive recipient HSCs develop 6-fold higher percentages of $CD45^+$ lymphocytes in their bone marrow as compared with NOD/SCID mice, leading to robust numbers of B cells, NK cells, and a myeloid compartment [19]. The advantage with using HSC transfer over PBMC is that the emerging human lymphocytes undergo negative selection in the mouse and consequently are tolerant of the murine host, which allows for lineage and developmental studies. Other than PBMCs and HSCs, other human immune cell types may be transferred to immunocompromised hosts. Importantly, in some models involving cellular therapy (tumor-infiltrating lymphocytes [TILS], chimeric antigen receptor T cells [CAR-Ts] T cell receptor engineered T cells [TCR-T cells], engineered immune cells, etc.), the 'humanized' aspect of the mouse model is also the therapeutic; these types of models were set into action with work characterizing human anti-CD19 CAR-T cells in NSG mice xenografted with Nalm-6 leukemia [21].

The NSG mouse served as a springboard for other mice such as the NOG rag gamma (NRG), which is less sensitive to preconditioning irradiation [22], and the NSG-SGM3, which combines the best attributes of the NSG with transgenes known to improve expansion and retention of human myeloid cells [23,24]. NOD/Shi-scid/IL-2Rγnull (NOGs) expressing IL-6s are being explored for their ability to better enhance monocyte and macrophage engraftment and differentiation [25]. NSGs and NOGs expressing human IL-15 are being used to better recapitulate the natural killer cell compartment [26,27]; these mice have demonstrated higher levels of functional human $CD56^+$ NK cells following the transfer of HSCs. Researchers have also begun exploring an NSG that expresses human major histocompatibility complex (MHC), an NSG lacking MHC, and an NSG lacking beta-2 microglobulin to reduce graph vs host effects [28–30]. Investigators may select their desired mouse model based on the desired characteristics of their biology, such as necessity of a more robustly reconstituted myeloid compartment, a longer runway before the onset of graft versus host disease (GvHD), or ability to withstand vigorous preconditioning. Taken together, the above-mentioned strains and models are mere examples of how the field has progressed, and they represent just a modest snapshot of the exciting developments in humanized mouse biology. As this field continues to grow, scientists may now tailor their mouse model to their biology or program and use specific mouse models to ask specific biological questions.

DEPLOYING HUMANIZED MODELS FOR PRECLINICAL STUDIES

Our own internal work at Amgen utilizing humanized mouse models has been tailored over the years to the biology of the target and the biology of the molecule to test specific hypotheses. To evaluate a BiTE® (bispecific T cell engager) molecule targeting CD19, an ad mix NOD/SCID mouse model was employed, wherein the PBMCs and tumor cells were mixed just prior to implantation at selected effector to target ratios. The BiTE® molecule was administered at various time points after administration of PBMCs and tumor cells [31]. In this study, the presence of the PBMCs alone had a modest impact on tumor growth, and the addition of the BiTE® molecule reduced tumor burden and increased survival as compared with control. Humanized SCID and NOD/SCID mice bearing admix, established, or disseminated tumors have also been used to evaluate a BiTE® molecule targeting BCMA for the treatment of multiple myeloma [32]. All these models revealed dose-dependent effects of BiTE® molecule treatment on the tumors. BiTE® molecules have also been evaluated in preclinical models of acute myeloid leukemia (AML). To evaluate a BiTE® molecule targeting the AML target

Flt3 in a disseminated model, female NOD/SCID mice were sub lethally irradiated and injected intravenously with AML cells; animals next received human $CD3^+$ T cells and were then treated with a BiTE® molecule at increasing doses. Treatment with BiTE® molecule extended survival in a dose-dependent fashion [33].

Lastly, we have employed other humanized models to evaluate additional therapeutics in the context of human immune effectors. To test whether an anti-PD-1 mAb x IL-21 fusion could extend survival through a tumor-specific T cell mechanism, we developed a system in which mice were implanted with human melanoma cells (SKMEL-30-Luc) engineered to express a model antigen (a peptide antigen derived from CMV) and these mice were then treated with either a human-mouse chimeric PD-1 mAb with a variable domain recognizing human PD-1 and a constant Fc-region from mouse IgG1 or a fusion protein consisting of the same parent PD-1 monoclonal antibody and a monomeric variant of human IL-21 R9E:R76A. The 'humanization' in this instance was the transfer of human CMV-specific CTLs on the same day of the human tumor cell line implantation, and therapeutic agents were administered when tumors were established. In this model, the tumor-specific T cells did not control tumor growth and the addition of an anti-PD-1 mAb did not have an impact on tumor growth, but the administration of an anti-PD-1 mAb x IL-21 fusion did inhibit tumor growth and improve overall survival [34]. Thus, given the complexities of building a mouse molecule and the low likelihood that mutations in the mouse IL-21 would result in the same degree of biological attenuation, a humanized model was the appropriate preclinical setting in which to test this fusion protein.

CONCLUDING REMARKS

In conclusion, scientists have a multitude of preclinical mouse models in their toolkit to interrogate translational questions, ranging from genetically engineered mouse models to syngeneic models to humanized models. Syngeneic models are excellent workhorses due to their fully intact immune system, but they generally require mouse surrogate molecules or at least some alteration of the human clinical candidate if it does not cross-react with the corresponding mouse targets. Most syngeneic models are fast-growing, allowing for a high throughout evaluation of test agents, but may not offer a long enough runway to see biological effects of interest. Meanwhile, immunocompromised mice bearing human tumor cells and reconstituted with human immune cells allow for functional characterization of novel therapeutics that engage human targets and allow for investigation of the clinical candidate in a human tumor. Refinements in humanized mouse models over the years have allowed for more precise characterizations of immune cells of interest and the testing of novel immuno-oncology agents in the preclinical setting [35]. Immunocompromised mice are now available armed with transgenes for human growth factors that may better support the creation of an experimental cellular niche of interest, although with the caveat that the transgenes are not currently regulatable. Humanized models face the limitations of potential GvHD onset as well as a limited immune repertoire; their tumor microenvironment may not always recapitulate an immune-replete tumor microenvironment. A preclinical model is only relevant if the target and cell population being targeted, as well as the molecular and cellular partners with which they interact, are accurately reflected in the model. Researchers seeking to characterize human-engaging molecules must select or design humanized mouse models that recapitulate enough of their molecular and cellular biology such that the model is not irrelevant to the question at hand in the clinic. Thus, as more sophisticated humanized mouse models emerge, investigators should be able to execute preclinical experiments that will better translate to the clinic and better predict clinical success.

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BIOGRAPHY

STEPHANIE C CASEY obtained her BSc in Microbiology, Immunology, and Molecular Genetics from UCLA and her PhD from UC Irvine in cancer biology. She performed her postdoctoral work at Stanford, where she studied the role of oncogenes in the modulation of the anti-tumor immune response using genetically engineered mouse models. At Amgen, her group works to understand *in vivo* biology of emerging targets, characterize the pharmacodynamic and efficacy responses to new molecules, and create new syngeneic and humanized models. Her research interests include both discovery and translational oncology research, spanning from tumor-intrinsic biology to immuno-oncology and the tumor microenvironment.

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AFFILIATION

Stephanie C Casey, PhD
Amgen Research,
Department of Oncology,
Amgen, South San Francisco, CA 94080, USA

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