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Experimental animal modeling for immuno-oncology

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ABSTRACT

Immuno-oncology (I/O) research has intensified significantly in recent years due to the breakthrough development and the regulatory approval of several immune checkpoint inhibitors, leading to the rapid expansion of the new discovery of novel I/O therapies, new checkpoint inhibitors and beyond. However, many I/O questions remain unanswered, including why only certain subsets of patients respond to these treatments, who the responders would be, and how to expand patient response (the conversion of non-responders or maximizing response in partial responders). All of these require relevant I/O experimental systems, particularly relevant preclinical animal models. Compared to other oncology drug discovery, *e.g.* cytotoxic and targeted drugs, a lack of relevant animal models is a major obstacle in I/O drug discovery, and an urgent and unmet need. Despite the obvious importance, and the fact that much I/O research has been performed using many different animal models, there are few comprehensive and introductory reviews on this topic. This article attempts to review the efforts in development of a variety of such models, as well as their applications and limitations for readers new to the field, particularly those in the pharmaceutical industry.

Contents

Abbreviations: CAR-T, chimeric antigen receptor-T cell; CTL, cytotoxic T lymphocyte; CTLA-4, cytotoxic T lymphocyte antigen 4; FACS, fluorescence-activated cell sorting; GEMM, genetically engineered mouse model; GvHD, graft-versus-host disease; HLA, human leukocyte antigen; HPC, hematopoietic progenitor cell; HSC, hematopoietic stem cell; ICD, immunogenic cell death; IHC, immunohistochemistry; IL, interleukin; I/O, immuno-oncology; KI, knock-in; MDSC, myeloid derived suppressor cell; MOA, mechanism of action; NK, natural killer; PBMC, peripheral blood mononuclear cell; PD, pharmacodynamics; PD-1, programmed cell death protein 1; PD-L1, programmed cell death 1 ligand 1; PDX, patient-derived xenograft; POC, proof of concept; SCID, severe combined immunodeficiency; SOC, standard of care; TIL, tumor infiltrating lymphocyte; TME, tumor microenvironment.

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1. Introduction

Despite currently available oncology therapies, including surgery, radiation, chemotherapy and targeted therapies, the majority of certain malignancies are still incurable and unmanageable, perhaps until now, with new immuno-oncology (I/O) therapies appearing on the horizon (Ascierto, Melero, & Ascierto, 2015; Postow, Callahan, & Wolchok, 2012). The discovery of T-cell immuno-inhibitory pathways has uncovered powerful mechanisms by which tumors evade the immune system. These mechanisms are frequently referred to as immune checkpoints or co-inhibitory pathways (Sanmamed, Pastor, et al., 2015; Schreiber, Old, & Smyth, 2011). Targeting immune checkpoints, *e.g.* programmed cell death protein 1 (PD-1), programmed cell death 1 ligand 1 (PD-L1) and cytotoxic T lymphocyte antigen 4 (CTLA-4), has achieved benefits in multiple cancers by blocking immuno-inhibitory signals and enabling patients to produce an effective anti-tumor response. In 2011, the FDA approved a therapeutic antibody that blocks CTLA-4 for the treatment of melanoma, ipilimumab (YERVOY®/BMS), followed by the approval of pembrolizumab (KEYTRUDA®/Merck), the first approved therapeutic antibody targeting PD-1 in 2014 (Hamid et al., 2013; Hodi et al., 2010; "TCGA Research Network: http://cancergenome.nih.gov/,"). Several other therapeutic agents targeting CTLA-4, PD-1 and other immune checkpoints are currently in development; combination treatments with PD-1 and CTLA-4 blocking antibodies have also significantly increased objective response rates in melanoma and are currently in Phase III trials in multiple tumor types (Postow et al., 2015). With rapid clinical development, checkpoint inhibitors have now been approved in several more cancers, including NSCLC, HNSCC, RCC, and classical Hodgkin lymphoma (Ansell et al., 2015; Brahmer et al., 2012; Herbst et al., 2014; Hodi et al., 2010; Powles et al., 2014; Topalian et al., 2012), with the list increasing over time. Thanks to these breakthroughs, certain patients (those without other prospects of long term survival) may now greatly benefit from these new treatments, resulting in long term survival with manageable conditions. These treatments may also represent the coming of age of immunotherapy for cancers, and are now attracting unprecedented intense research which is rapidly changing the landscape of cancer treatments (Pardoll & Drake, 2012; Pardoll, 2012). The significant advantages of cancer immunotherapy include improved safety margin, *e.g.* as seen for checkpoint inhibitors in general (Topalian et al., 2012), and prolonged effect due to immune memory, therefore preventing relapse and metastasis in many cases. It is worth noting certain immunotherapies, *e.g.* chimeric antigen receptor-T cell (CAR-T) therapy can sometimes cause severe toxicity in patients.

2. The need for I/O animal models

Notwithstanding the considerable success of the current checkpoint inhibitors and the great promise of new immunotherapies, many important questions remain to be addressed (Pitt, Vetizou, Daillere, et al., 2016), including: 1) why only subsets of patients respond; 2) what determines response, the host factors (*e.g.* immuno-state of patients including the microenvironment (TME) of the tumors), and/or tumor specific factors (*e.g.* neo-antigens); 3) how to expand patient populations so more patients can benefit (*e.g.* effective combination therapy); and 4) how to discover and validate new I/O targets and agents, including new checkpoint targets/inhibitors. Major hurdles in addressing these key medical questions are the lack of adequate preclinical animal models capable of mimicking patient conditions and predicting responders (and non-responders) to I/O therapies. The ideal preclinical platform needs to be predictive of preliminary safety assessment and efficacy, be reproducible, and have clinical applicability.

Currently traditional therapies, including more recent targeted therapy, have been focused on the nature of tumors, and the most commonly-used experimental cancer models are human xenograft tumors grown in immunocompromised mice (*e.g.* athymic nude mice, and SCID mice), derived from either *in vitro* immortalized cancer cells (cell line derived xenografts) or patient tumors (patient-derived xenografts, or PDXs) (Tentler et al., 2012; Yang et al., 2013). However, both tumors and hosts are critical in today's I/O treatments, and thus the nature of the immunodeficiency of these models renders them generally inadequate for many I/O investigations. At present, preclinical efficacy/safety assessments of immunotherapies, on the other hand, are largely based on the evaluation of surrogate anti-mouse target antibodies using mouse syngeneic or genetically engineered tumor models, with the assumption that the mouse tumor and immunity mimics that of humans (Payne & Crooks, 2007; Takao & Miyakawa, 2015). However, this strategy is limited by that it can only test surrogate molecules that target the mouse immune system/tumors, where inherent differences between the two species occur (Mestas & Hughes, 2004; von Herrath & Nepom, 2005). Recent failures of MAGE-A3 (GSK) and tecemotide (Merck) in late stage clinical trials outline the urgent need for a model system that includes both adequate human tumors and immune cells to achieve a comprehensive understanding of human tumor immunobiology, which is necessary for the development of new immunotherapies. This article attempts to review the advancements of I/O animal modeling (see Table 1 for available I/O models of human and mouse origin).

3. Commonly used mouse strains in cancer models

Most commonly used experimental cancer models are tumors grown in mice, either human tumor xenografts or mouse tumor homografts. Although human xenografts in immuno-compromised mice have been much more widely used in traditional preclinical cancer pharmacology investigations, mouse tumor homografts in immuno-competent syngeneic mice are commonly used in today's I/O research. There are many mouse strains that have been broadly used to support tumor grafts: immunodeficient mice can support human tumor xenografts, and immuno-competent mice can support mouse tumor homografts. For the principle of reductionist experiments, laboratory inbred strains of mice were commonly used for their consistent biological properties and biocompatibility (non-rejection of homografts) (see below). The most commonly used inbred imunocompetent mice are C57BL/6 and BALB/c strains, or those derived from them.

Table 1
I/O animal models of both human and mouse origin.

Platform	Tumor	Immunity	Target	Therapy[b]	Property	Unique utility
Xenograft tumor	Hu	Defective, Mu	Human/Mu[a]	Hu/Mu[a]	Cell line derived, PDX (primary tumor, relevant path.)	Cell therapy (*e.g.* CAR-T), non-T I/O, *etc.*
Syngeneic tumor	Mu	Mu	Mu	Mu	Cell line derived	MOA, surrogate POC
GEMM tumor	Mu	Mu	Mu	Mu	Spontaneous, and primary, relevant path.	Target therapy combo, preventative vaccine
Homograft primary tumor	Mu	Mu	Mu	Mu	"Mouse PDX", primary, relevant path.	Target therapy combo
HuGEMM™	Mu	Mu	Hu	Hu	Partially humanized (target)	Human therapy
Xenograft/humanized	Hu	Hu	Hu	Hu	huPBMC, huHSC, relevant immunity	Human therapy

Note: huHSC: $CD34^+$ cells are usually derived from fetal liver, cord blood or PBMC; POC: proof of concept.
[a] Target immune system.
[b] Assuming species-specific.

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In supporting xenotransplantation, mouse strains with immuno-deficiency are particularly important (Sivan et al., 2015). Improvements in human tumor and leukemia engraftment protocols (An et al., 2016) have correlated with the increasing defectiveness of innate immune activity in mice (Table S1). Generally, the more immuno-deficient the mouse host, the higher the xenograft take rate (% xenografts which grow following injection of a defined number of cells) and growth rate (xenograft tumor volume increase over a defined period of time), as expected. Athymic nude mice, defective in the thymus therefore in T-cells, still have many other immune functions, *e.g.* full-natural killer (NK) cell functions, and can support many xenotransplantations, including many PDXs (Chen et al., 2015; Guo et al., 2016; Yang et al., 2013; Yang, Xu, et al., 2016). The remaining innate immunity can also be used for non-T I/O investigations. Subsequently, with increasing immunodeficiency, C.B-17-Prkdcscid (SCID, severe combined immunodeficiency) mice, lacking both T-(TCR)/B-cell receptor (BCR) rearrangement, thus defective in both T and B cell functions, were developed (Pitt, Andre, et al., 2016); followed by modified SCID called NOD/SCID (Pfirschke et al., 2016) with even greater deficiency in macrophage function, complement-dependent hemolytic activity and NK activity (Aspeslagh et al., 2016; Stewart et al., 1996). Mutations in the IL (interleukin)-2 receptor gamma chain (IL-2rg^{null}) have generated the commonly used NOG (CIEA) and NSG™ (The Jackson Laboratory) strains in NOD/SCID backgrounds, with the added elimination of innate immunity of both macrophages and NK cells. These latest strains of immunodeficient mice can be engrafted with many types of primary human tumors and leukemias, demonstrating significantly improved engraftment rate (Ito et al., 2002). Additionally, these strains are also able to robustly support the engraftment and maturation of human hematopoietic progenitor/stem cells, resulting in mice with a humanized immune system (Kenney, Shultz, Greiner, & Brehm, 2016; Pitt, Vetizou, Waldschmitt, et al., 2016; Shultz, Brehm, Garcia-Martinez, & Greiner, 2012; Shultz et al., 2014; Zitvogel, Pitt, Daillere, Smyth, & Kroemer, 2016). Table S1 summarizes some of the commonly used immunodeficient mouse strains for xeno-implantations, either tumor or immuno systems.

4. Cancer models for I/O studies

4.1. Human xenografts in immuno-deficient (and/or partially competent) mice

As mentioned above, the most widely used cancer animal models are xenografts of human origin in immuno-deficient mice, namely, those derived from human cancer cell lines (Adams et al., 2005; Kelland, 2004; Kerbel, 2003; "TCGA Research Network: http://cancergenome.nih.gov/"), sometimes called cell line derived xenografts, and PDXs (Guo et al., 2016; Tentler et al., 2012; Yang et al., 2013). However, for these human tumors to grow without rejection, the host mice cannot have full immunity, particularly those functions related to histocompatibility played by T-cells. It is worth noting that such xenografts in the non- or partially immuno-competent hosts can still be utilized for assessing certain I/O therapeutics in which exogenous immunity can directly be introduced (*e.g.* passive immunization, antibody drug conjugates (ADCs), certain cell therapies, and CAR-T), and/or the remaining partial immunity (mostly innate immunity) in the host (*e.g.* nude mice) can be utilized as therapies (*e.g.* host NK functions). For example, many xenografts grow in athymic nude mice with partial immunity, except for T-cell functions. The advantage of these models is that they can evaluate many human-specific biological therapeutics. Among the human xenografts, PDXs have several advantages as cancer models for mirroring corresponding patient diseases in histo- (Akashi et al., 2013) (see also Fig. S1) and molecular pathology both from anecdotal observations and also a big data perspective, which have been described and reviewed previously (Ding et al., 2010; Gao et al., 2015; Guo et al., 2016; Tentler et al., 2012; Walter et al., 2013; Yang et al., 2013; Zhang et al., 2013), as more predictive models of clinical outcome (Corcoran et al., 2015), and reflectiveness of patient diversity as large collections have been established and fully annotated, with pathology diagnosis, genomic profiling (Gao et al., 2015; Guo et al., 2016), patient treatment information, and human leukocyte antigen or HLA typing (Bladt, Friese-Hamim, Ihling, Wilm, & Blaukat, 2014; Jiang et al., 2015; Yang et al., 2013; Zhang et al., 2013).

4.2. GEMM cancer models

Over nearly 3 decades, genetically engineered mouse models (GEMMs), based on germline genetic alterations of key oncogenic pathways, have enabled numerous insightful mechanistic findings on tumor onset, progression, and metastasis (Talmadge, Singh, Fidler, & Raz, 2007). GEMM tumors driven by loss-of-function of tumor suppressors and/or gain-of-function of oncogenes (Brinster et al., 1984; Guy, Cardiff, & Muller, 1992), spontaneously arise and progress in mice accompanied by profound immune suppression and escape from immune surveillance, recapitulating key features of *de novo* human tumorigenesis. This is drastically different from transplant models which involve engraftment of fully progressed tumors to the naïve host, inevitability resulting in an acute immune response that is irrelevant to the natural course of disease. Through utilizing GEMMs, many have demonstrated how various oncogenic drivers in different tumor types, directly or indirectly impact tumor antigens, induce immune editing and immune tolerance, and create favorable microenvironments for the proliferation of tumor cells. For example, in lung adenocarcinomas, EGFR and KRAS are two of the most prevalent oncogenic drivers in human patients, which lead to histopathologically indistinguishable, yet molecularly distinct tumors in mice. These tumors also respond to treatment, including checkpoint inhibitors, very differently. Kwok-Kin Wong and colleagues (Akbay et al., 2013) created GEMMs with EGFR (Regales et al., 2009) or KRAS mutations (Chen et al., 2012). They found that mutant EGFR can significantly induce PD-L1 expression on tumor cells, leading to checkpoint blockade of T-cells. These tumors also produce cytokines *e.g.* IL-6 and progranulin, and recruit regulatory T-cells (T_{reg}) and suppressive macrophages to induce further immune suppression. They have been shown to respond favorably to PD-1 treatment. However, KRAS driven tumors usually have low PD-L1 levels and are insensitive to PD-1 antibodies. Alternatively, in a lung SCC (squamous cell carcinoma) model, PD-L1 expression on the tumor cells is led by loss of LKB1 and PTEN (Xu et al., 2014). In addition, inactivation of these tumor suppressors also resulted in enrichment of tumor-associated neutrophils, a distinct mechanism of immune escape in contrast to adenocarcinoma. These findings have highlighted the value of GEMMs in interrogating the interaction between a tumor cell and its microenvironment (TME), and understanding the mechanisms of action of anti-tumor immune responses. Another important utility of GEMM is that the spontaneous tumorigenesis is reflective of natural human tumorigenesis, and therefore preventive and therapeutic interventions can be modeled in animals. For example, a treatment schedule can be implemented properly and compared in animal models per the clinical perspective, *e.g.* adjuvant or neoadjuvant treatments (Liu et al., 2016), and ultimately guide clinical testing.

A major criticism of traditional GEMMs is that they were created by introducing germline mutations, through transgenes or targeted knockout (KO)/knock-in (KI), which resulted in genetic alterations in all of the cells of the animal. This is certainly not the case in patients and sometimes makes it difficult to correctly interpret the data, especially given that the perturbed genes/pathways are often also critical to immune cell proliferation and function. The development of conditional GEMMs provided more sophisticated models to control tumor onset and progression in an inducible, and cell lineage-/tissue-specific manner. More recently, technology advancements in RNA interference (RNAi), gene editing (*i.e.* CRISPR-Cas9), embryonic stem cell (ESC) culture, and viral delivery, have allowed us to build GEMMs with on-and-

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off, or even fine-tuning, of gene dosage, simultaneously or sequentially combining multiple disruptions of oncogenes/tumor suppressors in the targeted tissues/cells in grown animals, mimicking what truly occurs in human cancers. More importantly, the timeline required for generating these non-germline GEMMs has been significantly shortened.Livshits and Lowe (2013) provide a detailed review of the various types of new GEMMs as accurate, flexible and powerful platforms for I/O animal modeling.

In summary, GEMMs have advantages, including: 1) they are primary tumors grown with human tumor characteristics of histopathology, TME (Pitt, Marabelle, et al., 2016; Smyth, Ngiow, Ribas, & Teng, 2016), stem-cell and differentiations, *etc.*); 2) decades of research in this area have created large numbers of GEMM mouse tumors covering different cancer types; 3) certain important druggable disease pathways have been purposely engineered in mice to mimic specific human diseases, these particular pathways have been intensively studied and pharmaceutically targeted, which can be especially useful when testing combinations of I/O and specific pathway inhibitors, where available conventional syngeneic models cannot be used in general; 4) most importantly, they grow in a full immuno-competent environment, thus particularly equipped for I/O research; and 5) GEMM can be utilized to interrogate the complete process of cancer progression, including carcinogenesis initiation, and testing immuno-prevention strategies (prophylactic treatment, or preventative vaccines) (Liu et al., 2016). Conversely, GEMMs have not been generally widely used for pharmacological or immuno-oncology studies because the tumors arise non-synchronically and non-uniformly (disease heterogeneity, including type), which creates difficulties in conducting studies, including dose and data interpretations.

4.3. Other spontaneous mouse tumors

Some strains of mice have high tumor incidence, particularly at an advanced age, providing yet another source of mouse tumors. Carcinogens can also induce a range of cancers in animals, including many recapitulating general mechanisms of human disease (Talmadge et al., 2007). These models are also useful for I/O. However, in some cases, carcinogen-induced tumors carry artificially high numbers of neoantigens that may not be relevant to human disease.

4.4. Mouse syngeneic tumor models (homografts of syngeneic mouse tumor cell lines)

Mouse homograft tumors, also known as syngeneic models, have long been used as a surrogate transplanted tumor platform (as opposed to human xenograft tumors) for *in vivo* pharmacological studies (Bjorndahl et al., 2005; Gilliland & Griffin, 2002; Talmadge et al., 2007). However, although several dozens of syngeneic tumor cell lines of various tumor types have been established and validated for preclinical *in vivo* use in the past, most of them are not attractive to mainstream drug discovery due to several limitations: 1) most of these mouse tumor cell lines were generated from carcinogen-induced models, carrying complex and unstable genetic alterations, which do not closely resemble human tumors; 2) most of the lines grow rapidly *in vivo*, thus allowing only short dosing durations for studies before tumor volumes reach IACUC limits, both of which do not mimic human patient situations; 3) they are mouse tumors with mouse targets (Pasche, Wulhfard, et al., 2012); 4) very few cancer types and cell lines are available for testing a diversity of cancers, and only a few have been found to be responsive to current I/O treatments, *e.g.* MC38, CT26, H22 and EMT-6 (Table S2 lists some of the most commonly used syngeneic models). While there are hundreds of human cell line derived xenografts, and even more PDX models, which can be used for *in vivo* testing, it seems to leave no reason why mouse tumor cell lines should be used. However, the tide has turned in recent years fueled by the needs of preclinical models for I/O. The reason has simply been that syngeneic models are the only viable model system which can be easily setup in immuno-competent hosts and allow the mechanism of action (MOA) and efficacy evaluation of I/O treatments (Allard, Allard, & Stagg, 2016; Allard, Pommey, Smyth, & Stagg, 2013; Nagaraj et al., 2012; Pasche, Wulhfard, et al., 2012; Sharabi et al., 2015), as compared to all human xenografts. On the other hand, the implanted models also have the advantage of synchronized growth suitable for drug administration, as compared to the unsynchronized growth of primary tumors seen in GEMM. An additional advantage of traditional syngeneic cell line derived models *vs*. primary mouse tumors is that they can be readily engineered (Gilliland & Griffin, 2002), including MC38-OVA (Allard et al., 2013) and B16-OVA (Quetglas et al., 2015) for improved immunogenicity.

Despite their limitations, there is no doubt that syngeneic cell lines derived from mouse tumor models are the most commonly explored models for preclinical investigation. Most checkpoint inhibitors were first confirmed in syngeneic models as proof of concept (POC), *e.g.* PD-1 antibody with MC38 tumors. Now, many syngeneic models (>20 known) have been extensively profiled genomically, immunologically and for I/O agent efficacy by various laboratories, including ours (Figs. S2, S3). This assessment is both at baseline (pretreatment, as predictive factors) and for pharmacodynamics (Fig. S3) in terms of response to multiple immune checkpoint inhibitors for benchmarking new single agent and combination therapies. Other information, including genomic profiling data and baseline tumor infiltrating lymphocyte (TIL) profiling data are also made available to facilitate the easy selection of suitable models for efficacy evaluation. Syngeneic models have become, and will probably stay, the work horse for drug industry I/O *in vivo* studies for some time to come.

4.5. Homografts of primary mouse tumors in syngeneic mice

Homografts of spontaneous murine tumors, or “a mouse version of PDX”, are primary tumors in nature and never manipulated *in vitro*, mirroring original mouse tumor histopathology and genetic profiles (Fig. S4). They recapitulate their original disease significantly better than traditional *in vitro* immortalized cell line derived syngeneic models (Talmadge et al., 2007). Similar to original mouse cancers, they keep heterogeneous histopathology: different differentiation phenotypes and rich microenvironments, and they are cancer stem cell (TIC) driven diseases. Since they are never adapted for *in vitro* growth, tumors manifest completely different cell biology properties from mouse cell line based-syngeneic models: difficult to culture *in vitro* — requiring special hormones/growth factors and associating with massive apoptosis/growth arrest even for short term culture. The homografts do not have the irrelevant genetic shifts needed for robust *in vitro* growth. By analogy to PDX (Tentler et al., 2012), these models are expected to have improved predictive power as preclinical cancer models and enable discovery of predictive biomarkers for targeted therapeutics. As for cell line derived syngeneic models, homografts also have complete mouse immuno-competency and are suitable for surrogate cancer immunotherapy research, but with significant advantages over GEMMs or other spontaneous mouse tumors in operational simplicity for pharmacology research, without relying on labor intensive imaging capacity, in disease diversity, and study robustness and consistency. The sources of these homografts can be any spontaneous mouse tumors, including those from GEMMs (Guy et al., 1992), spontaneous tumors due to aging, and tumors from those induced by chemical carcinogens (Ngiow, Loi, et al., 2016). There is a particular advantage for the homografts derived from a specific GEMM where a specific human disease-mimicking mechanism is implemented. These models can be readily applied to study human diseases and targeted intervention, particularly in an immuno-competent environment, and also combination therapies with immuno-oncology therapies.

Considering large collections of GEMM cancers have been developed over the past decades, it would be particularly meaningful to build a

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library of homografts of primary mouse tumors covering a great diversity of cancer diseases (Table S3) to support immunotherapy research. Similar to many PDX libraries, the established homograft library can also feature the full homograft genetic and pathological annotations, including next generation sequencing (NGS) along with characteristic histopathology, TME (*i.e.*, immune profile) (Pitt, Marabelle, et al., 2016), growth information and standard of care data (SOC), as well as response to treatment, including immunotherapies. All of this information can be managed by an accessible database. The library will include, but not be limited to the tumors shown in Table S3. These homograft models can also enable "population-study" styled trials similar to PDX based mouse clinical trials (Chen et al., 2015; Zhang et al., 2013). Recent studies have linked the determinants of tumor response to both tumor-intrinsic factors, *e.g.* tumor neo-antigen/neo-antigen load (Pilipow et al., 2015), and it is worth noting that our preliminary data demonstrated that these type of primary tumors have similar neo-antigen load to those seen in syngeneic cell lines.

4.6. Chimeric GEMM with human targets for human biological therapy assessment

The mouse tumor models described above, with full mouse immunity, although used widely for testing mouse surrogate therapies *e.g.* anti-mouse PD-1 antibodies aiming at POC and investigating MOAs of I/O treatments, have two major concerns. First, murine tumors and immunity may be different from those of patients; second, human-specific therapeutics cannot be evaluated. Our laboratories and others (Lute et al., 2005; Peggs, Quezada, Chambers, Korman, & Allison, 2009) have developed and tested several novel chimeric mouse tumor models via the introduction of human targets that are recognized by human therapeutics (Concept see Fig. S5, Panels A and B). This therefore enables the evaluation of specific human biological therapies *in vivo* in mice with a fully functional murine immunity and mouse tumors. For example, the human PD-1, and CTLA-4 (Lute et al., 2005; Peggs et al., 2009) genes have been knocked-in C57H Black/6 mice to support evaluation of antibody immunotherapies recognizing human PD-1 and CTLA-4 *in vivo*, respectively. In some cases, human targets have also been knocked-in to mouse tumor cells, *e.g.* the human PD-L1 gene, in order to evaluate human therapeutics targeting human PD-L1. Certain other mouse genes have also been humanized for different I/O applications. For instance, human Fcγ receptor KI was used to enable passive immunization (antibody target tumor antigen) (Bournazos, DiLillo, & Ravetch, 2014); chimeric MHC-II transgenic mice are capable of mounting a human DR-restricted immune response (Woods et al., 1994); mice with human MHC-I (HLA-A2) and TCR KI can mount highly specific T-cell response to human antigens (Obenaus et al., 2015), *etc.*

4.7. Mouse models with human immunity and human tumor engraftments

Although rodents are considered close to primates/human, they have significant differences in tumorigenesis and immunological mechanisms (Pitt, Andre, et al., 2016; Pitt, Marabelle, et al., 2016; Yang, Yamazaki, et al., 2016). All of the above experimental models of mouse immunity/mouse tumors, even those with chimeric human targets (Lute et al., 2005), suffer from limitations that they may not be accurately reflecting human immunity and human malignances. Therefore, development of models of human tumors in mice with competent human immunity has become an urgent need. The reconstitution of human immunity in immuno-deficient mice via transplanting functional human immunity is an important approach (Agliano et al., 2008; Gradilone, Spadaro, Gianni, Agliano, & Gazzaniga, 2008). Such reconstitution started nearly 30 years ago after SCID mouse strains became available, and was also triggered by the need to support human virus infection in animal models, *e.g.* human immuno-deficiency virus (HIV) (Pitt, Vetizou, Daillere, et al., 2016; Vacchelli, Ma, Baracco, Zitvogel, & Kroemer, 2016), human T-cell leukemia virus (HTLV) (Feuer et al., 1996) and Epstein-Barr virus (EBV) (Ma et al., 2011) or to test gene therapy protocols in human hematopoietic lineages (Buque et al., 2016; Pitt, Vetizou, Waldschmitt, et al., 2016). Reconstitution of human immunity is usually be accomplished by one of two methods: 1) engraftment of adult immune cells, *e.g.* peripheral blood mononuclear cells (PBMC) (Gazzaniga, Silvestri, Gradilone, & Agliano, 2008; Gradilone et al., 2008); or 2) engraftment of hematopoietic stem cells (HSCs) into juvenile severe immuno-deficient mice via a variety of methods, including engrafting human fetal liver tissue fragments with rich hematopoietic stem cells in the kidney capsule ($SCID_{hu}$) (Agliano et al., 2008; Pitt, Vetizou, Daillere, et al., 2016), or simple injection of human $CD34^+$ cells (enriched for HSCs) (Buque et al., 2016; Pitt, Vetizou, Waldschmitt, et al., 2016), either from fetal liver or umbilical cord blood (also an important source of HSCs), into juvenile or newborn mice (Buque et al., 2016; Pitt, Vetizou, Waldschmitt, et al., 2016). Graft-versus-host disease (GvHD), an attack on the recipient by donor T cells during allogenic transplantation with unmatched HLA (human) or H2 (mouse) (Iribarren et al., 2016) can become an issue. GvHD is particularly strong for the first scenario with onset around 2–3 weeks post transplantation and subsequently becoming mortal (3–4 weeks). It is possible to test some immunological functions *in vivo* before the onset of GvHD, as the transplanted cells may transiently reconstitute certain aspects of human immunity. On the other hand, in general HSC transplantation into NSG mice causes only minor GvHD, usually without obvious symptoms, and does not affect the subsequent engraftment of tumors, and later immuno-oncology testing.

The creation of a highly immunodeficient mouse (NOG/NSG) capable of engrafting human hematopoietic progenitor cells ($CD34^+$ HPCs) and supporting the development and function of multiple aspects of human immunity is a critical step in this process (Hu-M™, Hu-NOGs, HuNSG, HuMice®) (Ishikawa et al., 2005; Ito et al., 2002; Shultz et al., 2005; Traggiai et al., 2004; Watanabe et al., 2009). These mice can display human T-cells ($CD4^+$, $CD8^+$), B cells ($CD19^+$), as well as lower levels of human NK cells ($CD56^+$) and macrophages ($CD14^+$). These mice can also sustain enhanced differentiation and maturation (possibly via mouse thymus) of human $CD4^+$ (T_h1 cells) and $CD8^+$ T cytotoxic T-cells (CTLs) for up to 32 weeks post engraftment. It has been speculated and proposed that the injected $CD34^+$ cells may themselves create a chimeric thymic microenvironment that may allow for some "normal" human T-cell maturation to occur within the humanized mouse (Ishikawa et al., 2005; Traggiai et al., 2004) (Fig. S6). There have been reports of functional immunity observed, including both T-cell response, delayed type hypersensitivity (DTH) and B-cell response (IgM) (Rajesh et al., 2010). In addition, NSGs (SMG3) with triple KI of hIL-3, hGM-CSF and hSCF-KITL (The Jackson Laboratory), or NOGs with double KI (hIL-3, hGM-CSF) (CIEA) are considered second generation immuno-deficient mice to support human hematopoietic reconstitution, particularly myeloid lineages. However, many aspects of the utility of these new strains for robust human immunity reconstitution have yet to be investigated (Nicolini, Cashman, Hogge, Humphries, & Eaves, 2004; Rongvaux et al., 2011, 2014; Willinger, Rongvaux, Strowig, et al., 2011; Willinger, Rongvaux, Takizawa, et al., 2011).

Importantly, these humanized mice also support the growth of human tumors, *e.g.* PDX, thus enabling the possibility of supporting I/O research and assessing I/O therapies in the context of human tumor/human immunity. However, there are two key challenges which have yet to be addressed adequately: first, whether functional human immunity in humanized mice can mimic human immunity, at least in the context of I/O investigations; and secondly, whether the interactions between the immune system and tumor xenografts represents a specific anti-tumor immune response, an allogeneic response or a combination of both responses (Morton et al., 2015; Wege et al., 2011, 2014).

As the blockade of immune checkpoints becomes a highly promising therapeutic avenue for cancer treatment, a preclinical murine model which closely recapitulates the complexity of a human PDX TME (Pitt, Marabelle, et al., 2016) and genetic profile (while concurrently allowing

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interactions with a human immune system,) would represent a significant achievement (Tentler et al., 2012). In comparison to preclinical testing in inbred mice, human genetic diversity can dilute efficacy or uncover previously unobserved off-target toxicities of immunotherapies as illustrated by the clinical experience of targeting the T-cell co-stimulatory receptor, CD28 (Suntharalingam et al., 2006). The development and validation of a PDX model with a functional human immune component are essential for the evaluation of new immunotherapies prior to their testing in humans in order to validate their mechanisms of action, efficacy, and preliminary safety assessment.

These humanized mice are found to be able to engraft a wide range of heterologous human PDX tumors (Chijiwa et al., 2015; Einarsdottir et al., 2014; Kobayashi et al., 2012). Recent reports have demonstrated that co-transplantation of human $CD34^+$ HPCs and breast cancer cells into NSG mice results in an immune response, including T-cell activation and the production of tumor specific antibodies (Wege et al., 2011, 2014). Another recent study showed that human $CD34^+$ cells in humanized mice recapitulates the TME as a result of $CD34^+$ cell maturation into stromal cells, promotion of lymphangiogenesis and prevention of 'genetic drift' of the original tumor, as gauged by the genetic expression profile of the PDX in humanized mice (Morton et al., 2015). It is unclear whether immune activation observed in these studies is a result of a specific anti-tumor immune response, xeno-recognition or a combination of both responses against non-HLA matched human tumors. Humanized mice are also predictive of immune related adverse responses to human immunotherapies providing a model to gauge efficacy and preclinical safety (Vudattu et al., 2014). There is a tremendous commercial demand for a predictive *in vivo* immunotherapy model using HLA matched PDXs and $CD34^+$ donor cells to generate a PDX-humanized mouse model to evaluate the efficacy, pharmacokinetic profile, and toxicity of antibody-based anticancer therapies.

Acknowledging the limitations and challenges faced by the commonly used engraftment approach, with respect to allogeneic and donor variability, characterization and delineation of T-cell response, will progressively create a platform which is more predictive of the safety and efficacy of a new generation of immune modulatory drugs. Characterization of even a limited tumor-specific human immune response in PD-1 antibody treated PDX/humanized mice, in conjunction with tumor regression, would be a significant milestone towards validation of the humanized mouse platform. Any adverse immune related events can be monitored and tumor regression, in conjunction with immunological markers of T-cell specific anti-tumor activity, would be necessary to demonstrate that the model recapitulates immunological features as observed in patients. Synergistic anti-tumor activity in combination therapy studies would demonstrate the utility and value of such models in testing the safety and efficacy of immune checkpoint inhibitors and would also provide foundational data for next-generation humanized mouse models expressing human HLA genes.

Although this model has great importance and shows great promise, there are also extreme challenges in developing an acceptable humanized mouse model for oncology research due to a lack of scientific understanding and limitations in resources, both funding and HSC source material. This has resulted in few published reports despite great efforts in this area over recent years. Fetal liver is a rich source of HSCs, but there are concerns over the ethics and regulatory issues of its use. Cord blood is another commonly used HSC source, but has a very limited number of HSCs. A renewable source of HSCs could obviously be of particular importance; however, there is no such technology. Recent advancements in the *ex vivo* expansion of $CD34^+$ HPCs could also potentially lead to the development of autologous PDX/Hu-NOG platforms using $CD34^+$ donor cells from PBMCs of patients (Bird et al., 2014). With the extreme importance of humanization technology in I/O, several non-profit organizations, as well as commercial ones, are investing in developing such models and providing services (*e.g.* The Jackson Laboratory, Crown Bioscience, Champions Oncology, HuMurine, Nanjing Galaxy, and OncoDesign).

Apparent alternatives to reconstitution based on HSC or HPC, PBMCs have also been used to reconstitute immunocompromised mice (see above). These mice will transiently host both implanted human tumors and human immune cells, *e.g.* adult T-cells, and can mount "anti-tumor activity" likely via allo-/xenogeneic reactions, or GvHD. The transit short window for I/O assessment has been commonly used to evaluate checkpoint inhibitors, bi-valent antibodies, NK function modulation, *etc.*

5. Factors considered in preclinical evaluation of I/O therapeutics

5.1. Treatment initiation and endpoints

When evaluating an immuno-oncology treatment, the most important endpoint would apparently be the typical tumor response/survival, similar to the endpoints commonly used in general cancer pharmacology (Yang et al., 2013; Zhang et al., 2013). However, several factors need to be considered when planning I/O studies. First, a survey of preclinical I/O studies, excluding adoptive cell transfers or passive immunization, seems to show that only small tumors demonstrate good responses, with only slowed or delayed tumor growth (Wen, Thisted, Rowley, & Schreiber, 2012) and not regression observed for large and staged tumors, as commonly seen for traditional small molecule therapies, *e.g.* chemotherapies and targeted therapies. Recently, more potent immunotherapies, *e.g.* checkpoint inhibitors, particularly in combination with either another I/O or other therapies, may however have improved response of larger staged tumors to a certain degree. Nevertheless, it is advised to consider first treating small tumors with an investigational agent, before moving on to large staged tumors, to ensure the possibility to observe drug effects.

Since the ideal consequence of an I/O treatment is its long term anti-tumor effect due to anti-tumoral immune memory, or the "cure", many preclinical studies require additional regrowth of treated tumors (or challenges) by re-implanting tumors of the same or different (control) syngeneic models to access the specific anti-tumoral immunity. With available spontaneous tumor models, as described above, one can also use different dosing schedules to mimic human treatments (*e.g.* adjuvant treatment) (Liu et al., 2016) ultimately for translation into the clinic.

5.2. Intrastudy varibility

Another important factor to take into consideration is the high variability among different mice within the same treatment group (as compared to a traditional pharmacology study) even when inbred mice and clonal tumors are used. This seemingly inherent nature of immunotherapy likely results from the varying immuno-states of each individual mouse that impacts tumor response. Therefore, study design needs to consider using more animals per group and using adequate statistic analysis. Because of this, "spider plots" have become a common practice for I/O efficacy readout (Fig. S2), in addition to the average tumor growth inhibition curves commonly seen in non-I/O cancer pharmacology studies.

5.3. Subcutaneous vs. orthotopic tumor engraftment

Although orthotopically implanted tumor models can potentially be used in I/O studies providing enhanced translatability, there may be particular technical challenges, more so than in other types of cancer pharmacology. These challenges can be reflected as an amplification of the challenges mentioned above, including initial dosing tumor volume, high variability, and limitations in measuring tumor volume (*e.g.* imaging). Marked syngeneic cell line derived homografts with luciferase or GFP transgenes could be a solution to help measure tumor growth or growth inhibition in orthotopic models. Other advanced imaging systems, such as micro-CT and -PET, could be alternative options.

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5.4. Pathogen environment and mice from different vendors

Significant variability in drug response to I/O treatment has been observed among mice that come from different vendors, even for the same inbred strain. It is recognized that gut microbiota that vary greatly among different vendors can significantly impact I/O therapies, including T-cell and macrophage mechanisms (Margolin, 2011b; Pitt, Vetizou, Waldschmitt, et al., 2016; Sivan et al., 2015; Vacchelli, Aranda, Obrist, et al., 2014). It is therefore advisable to pay close attention to mouse vendors, the types of microbiota in their mice and be consistent in using the selected mice. Recent studies have also demonstrated that the immune status of laboratory mice living in abnormally hygienic SPF barrier facilities are rather different from those of adult humans, although similar to immune naïve infants (Beura et al., 2016). One striking difference is the lack of effector-differentiated and mucosally distributed memory T-cells, specifically $CD8^+$ tissue-resident memory T-cells (TRM-cell), in the laboratory strain mice and also naïve human newborns, which may result in loss of translation for observations from laboratory mice to humans. However, free-living barn populations of feral mice and pet store mice with diverse microbial experience are similar to adult humans in this regard. Particularly, the co-housing of these mice with the laboratory rodents enables conversion of the immune status to closer to that found in adult humans. Using such converted laboratory strains in immuno-oncology research could be another choice for better translation.

5.5. Determination of dosing

The dose regimen is critical to the efficacy and toxicity of any pharmaceutical, in clinical or preclinical settings, including I/O treatments. I/O dosing is likely very different from, and much more complex than, small molecule drugs. However, understanding preclinical dosing in animals can still provide insights into clinical dosing. In addition to efficacy or toxicity readouts, certain pharmacokinetic (PK) and pharmacodynamic (PD, see below) parameters can also help understand optimal dosing. Receptor occupancy assays using fluorescence-activated cell sorting (FACS) or immunohistochemistry (IHC) can also help to investigate whether a drug (*e.g.* PD-1 antibody) has reached the intended target (*e.g.* PD-1).

5.6. Selection of the models

By mechanisms which remain unknown, different tumor models, *e.g.* syngeneic homografts, vary greatly in response to the same and/or different I/O agents, from no activity at all to complete response. Therefore, one model with no effect does not mean no effect in another model. In other words, it is always important to screen for a panel of available models to answer certain I/O questions. In addition, for I/O research, one must always be aware of host factors, in contrast to focusing more on the tumor only for non-I/O treatment, in selecting animal models, *e.g.* species specificity of agents, GvHD, autologous vs. allogenicity, as well as mouse suppliers, even for the same inbred mice.

With all of the factors described above together, *e.g.* tumors, environment, strains, *etc.* data have shown that I/O therapies are also intensely "personal", requiring precision diagnosis and treatment in order to achieve response, just like any other cancer treatment. However, it is even more important for I/O therapies since environmental parameters (host conditions) likely play an even more critical role than for traditional treatments, including age, sex, weight, diet, and hygiene (Klevorn & Teague, 2016). Therefore, it is also highly recommended to use multiple models in your preclinical plan in order to address the diversity of different models. Meanwhile, predictive biomarkers are also vitally important in guiding precision I/O treatments in both the preclinical and clinical settings.

6. Biomarkers used in preclinical I/O therapy evaluation

An important distinction between I/O and traditional cancer pharmacology studies is the need to monitor immunological parameters as predictive and/or prognostic biomarkers, usually in the form of pre-treatment immunological baselines (Ascierto et al., 2016) or PD change caused by treatment, compared with baseline. These biomarkers are not only on tumors but also on the hosts (different immune cells in hematological organs *e.g.* blood, spleen, lymph node or bone marrow), and not only on tumor cells but also on the TME (particularly those infiltrating immune cells). Selection of desired biomarkers are study case-specific per objective. If insufficient tumor tissue is available, draining lymph nodes may be analyzed instead. In general, it may be inadequate to collect termination samples from the efficacy study for PD analysis, as there may be insufficient samples or the samples may not be reflective of the true PD effect. Instead, sampling may occur between 24 and 72 hours post the 1st treatment, per specific marker of interest. In addition, optimal tumor size for PD/baseline should be in a range between 200 and 500 mm^3, to ensure that there is sufficient sample for analysis, but the sample is not too large to be penetrated by treatment and immune cells.

The two fundamental methods of biomarker analysis are flow-based cell phenotyping (surface/intracellular) and morphology-based (pathology-based) IHC (Dunstan, Wharton, Quigley, & Lowe, 2011; Mahoney, Sun, et al., 2015), also indicative of immune cell (pathological feature) tissue localization (Teng, Ngiow, Ribas, & Smyth, 2015) and subcellular localization (e.g. PD-L1) (Mahoney, Sun, et al., 2015). While fresh single cell suspensions generated from digested tissues are usually required for flow analysis, FFPE, or formaldehyde-fixed and paraffin-embedded, tissues are usually used for IHC assay. Frequently examined markers include those for different subsets of T-cells, macrophages, NK cells (*e.g.* CD45 for total leukocytes, CD19 for B-lymphocytes, CD3 for total T-cells, CD4 for helper T-cells, CD8 for cytotoxic T cells (Apetoh et al., 2015), FoxP3 for T_{reg} cells, CD335 for mouse NK cells, Iba 1 for macrophages, GR-1 (Ly6c + Ly6g) and CD11b for mouse (separating M-MDSC and G-MDSC) or CD33/CD11b/$HLA\text{-}DR_{lo/-}$ for human myeloid derived suppressor cells (MDSC)(Gabrilovich & Nagaraj, 2009; Gabrilovich, Ostrand-Rosenberg, & Bronte, 2012), F/480 for mouse macrophage, CD11c for dendritic cells and L/D for live gating. It is particularly important to realize that the functional lineages of immune cells and markers can be quite different between human and mouse, which is critical in the interpretation of assay results (Payne & Crooks, 2007). ELISA based methods can be used for liquid/liquidified sample analysis of certain protein biomarkers (*e.g.* cytokines).

Two particularly important classes of immune cells played key roles in I/O mechanisms. Per T-cell-centric view, a tumor evades anti-immunity via induction of immune suppression mediated by T_{reg} cells, which causes a reduction of activation/mobilization of effector T-cells within the tumor (TIL), *e.g.* $CD8^+$ cytotoxic T-cells in the TME (Apetoh et al., 2015; Im et al., 2016; Pitt, Marabelle, et al., 2016). On the other hand, MDSC which are immature myeloid cells with an immunosuppressive nature are expanded in cancer patients/models, and are assumed to contribute to immunosuppression, therefore promoting tumor progression. MDSC in the TME, also called tumor associated macrophages (TAM), belonging to the M2 (alternative activated) class can be monitored by CD11b/GR-1 in mice (Buque et al., 2016; Franklin et al., 2014; Gabrilovich & Nagaraj, 2009; Gabrilovich et al., 2012; Parker, Beury, & Ostrand-Rosenberg, 2015). The mechanisms of action of MDSC could include induction of T_{reg}, suppression of infiltrating T- (Nagaraj et al., 2012) and NK cells, and the induction of production of suppressive cytokines (*e.g.* IL10 and TGFb), nitric oxide (NO), reactive oxygen species (ROS) and arginase-1, indolamine 2, 3-dioxygenase (IDO)(Li, Han, Guo, Zhang, & Cao, 2009; Liu et al., 2009; Ostrand-Rosenberg, 2010).

These cells can be monitored by IHC/FACS using marker-specific antibodies. In particular, IHC can also reveal the exact localization of the infiltrating immune cells, either T-cells or macrophages. The PD

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response could reveal the effect on these biomarkers, along with correlative efficacy. Since syngeneic homografts are the workhorse of current I/O drug discovery, many of them have been PD benchmarked for such mentioned immune cell markers during common checkpoint inhibitor treatments, usually served as controls or as to facilitate combination therapies. In general, the good efficacy is associated with the reduction of infiltrating T_{reg} and/or MDSC, accompanied by increased $CD8^+$ TILs.

On the other hand, the pretreatment profile of these cells can be indicative of the host's immune status, thus the prognosis or I/O treatment response. T-cell panel markers and MDSC markers (with correlated markers for T_{reg}, infiltrate T-cell/NK) could potentially reveal information predictive of prognosis and treatment responses. There has been a hypothesis proposed based on this biomarker analysis that there are four types of tumor and TME as per the presence or absence of TILs and PD-L1 expression (Teng et al., 2015): including:

- type I ($PD\text{-}L1^+/TILs^+$: immune resistant)
- type II ($PD\text{-}L1^-/TIL^-$: immune ignorance)
- type III ($PD\text{-}L1^+/TIL^-$: intrinsic induction)
- type IV ($PD\text{-}L1^-/TIL^+$: other suppressor(s)).

It is possible that human melanoma has high type I/II; NSCLC has higher type III than melanoma, while pancreatic cancers are lower on both PD-L1 and TILs per human data, maybe due to factors related to the origin of tissues and oncogenic drivers (D'Incecco et al., 2015). This simplified cancer classification suggests that, if confirmed, checkpoint inhibitor sensitivity depends on both cancer intrinsic parameters (*e.g.* PD-L1 expression) and host factors (TME), and it may be useful to guide clinical patient stratifications. It also sets a stage for further in-depth analysis of checkpoint drug resistance, which can be potentially achieved through animal modeling. For instance, more detailed studies in animal models could explore the threshold of PD-L1 expression or $CD8^+$ TILs, *etc.* (Ngiow, Young, et al., 2015). In addition, more specialized, detailed and refined analysis of immune cell subsets, or sets of phenotype markers, *e.g.* T-cell lineages (*e.g.* $CD8^+$ CTL, T_{reg}, T_{h1} or T_{h2}), macrophage subsets (M1/M2, SM, etc.), NK or neutrophil subsets (antitumor N1, protumor N2) will be needed (depending on the mechanisms of interest) when conventional PD panels may not be sufficient. PD-L1 has been found to be an essential component for response to anti-PD-1 therapy (Fusi et al., 2015); however, many PD-L1 positive patients or tumor models do not actually respond to PD-1 treatment. Therefore, PD-L1 is not a very good simple predictive biomarker. Recent studies in both the clinical and preclinical settings have revealed that certain biomarkers in the local TME seem to regulate the response to checkpoint inhibitors (Ascierto et al., 2016; Buque et al., 2016; Lund, Medler, et al., 2016; Lund, Wagner, et al., 2016). Certain specific subsets of $CD8^+$ T-cells could be particularly important in PD-1 therapy (Im et al., 2016). Development of methods to monitor these special biomarkers, either inside or outside of tumors in animal models, could be a prerequisite biomarker strategy for future clinical applications. It is worth noting that conventional IHC and FACS may be insufficient or inadequate for such analysis on occasion, especially when many markers need to be simutaneously stained, there are only limited samples available and when the cells of interest are extremely rare. In these cases, multi-color FACS (up to 21 colors) or even mass-spectrometry based flow technology (*e.g.* CytoTOF) could be particularly enabling, and certain rare cell detection technology could also prove helpful. Furthermore, recent data seem to show that aberrant PD-L1 expression via 3′-UTR disruption and their correlation to checkpoint inhibitor sensitivity in several cancers, which can be revealed by genomic analysis of the tumor samples as well (Kataoka et al., 2016).

7. Types of immunotherapies and their preclinical evaluation

Immunotherapies are a broad array of biological treatments that have been proposed, developed and tested over several decades (Galluzzi et al., 2014). Although mostly still at the experimental stage, a number of these agents have finally gained regulatory approval, demonstrating great potential in patients, across a range of cancer types. One important distinction of I/O therapy from traditional treatments is that in addition to targeting the tumor cell itself, many I/O therapies target the host environment, particularly the TME (Smyth et al., 2016). These therapies can be categorized into the following types, many of which have already undergone preclinical evaluation in animal models.

7.1. Passive immunization

Using therapeutic antibodies against tumor-associated antigens (TAAs) or modulating the host immune system has been shown to prolong survival of patients with certain solid tumors and leukemias, particularly when combined with conventional treatments. Many of these antibodies have been broadly tested in preclinical models, e.g. EGFR, CD19, and CD47 tested in PDXs (Chao et al., 2011; Chen et al., 2015; Zhang et al., 2013).

7.2. Active immunizations, or therapeutic vaccines

Therapeutic cancer vaccines (Dranoff, 2012; Lin et al., 1996; Sioud, 2009), or active immunization against TAAs (Dakappagari et al., 2005), is another important I/O treatment approach that has been tested both preclinically and clinically, with some agents demonstrating efficacy. In particular, combination with checkpoint inhibitors would likely achieve practical efficacy which could benefit patients (Soares et al., 2015). Most preclinical evaluation of therapeutic vaccines has made use of mouse tumor models.

7.3. Targeting immune checkpoint inhibitors and co-stimulators

Amongst all of the newly developing immunotherapies, monoclonal antibodies targeting immune regulation checkpoints (Aranda, Vacchelli, Eggermont, et al., 2014; Sharma & Allison, 2015) have particularly demonstrated impressive clinical benefits *e.g.* agents targeting CTLA-4 (Dranoff, 2005), PD-1 and PD-L1 (Tang et al., 2016), OX40 (Aspeslagh et al., 2016; Ngiow, Young, et al., 2016), CD137 (Sanchez-Paulete et al., 2016; Sanmamed, Pastor, et al., 2015; Weigelin et al., 2015), and IDO (Vacchelli, Aranda, Eggermont, Sautes-Fridman, et al., 2014). This had led not only to significant medical advancements (Sanmamed, Pastor, et al., 2015), but has also created great potential commercial success for the pharmaceutical industry. Many of these agents have been greatly explored using preclinical I/O models (Allard et al., 2013).

7.4. Cytokine therapies

Cytokine therapies aim to enhance anti-tumor immunity by providing adequate pro-inflammatory cytokines to restore normal immune cell function, and subsequently eliminate malignant cells (Ardolino et al., 2014). Immunostimulatory cytokines including IL-2 (Klevorn et al., 2016), IL-7, IL-12, IL-15, IL-18, IL-21, and IFNβ, TNFα, GM-CSF, *etc.*, have been shown to be efficacious to a certain extent in the treatment of cancer (Dranoff, 2004; Lee & Margolin, 2011; Pasche, Wulhfard, et al., 2012; Vacchelli, Aranda, Obrist, et al., 2014). Cytokine therapies can achieve antitumor immunity via different mechanisms. For example, cytokines can restore MHC class I expression and enable presentation of neo-antigens to cytotoxic T-cells, therefore enhancing the function of infiltrating CTLs (Mittal, Gubin, Schreiber, & Smyth, 2014). NK cells can mount strong effects against MHC I-low tumor cells, but the prolonged exposure causes NK to become anergic, losing the ability to fight against these tumors. Cytokine therapy in animal models seems

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to reveal that it effectively restores NK activity against malignant tumors (Ardolino et al., 2014). Standalone IL-2 can support tumor reactive T-cells, but with high toxicity. However, in mouse models, the toxicity is dramatically reduced if complexed with an antibody with tumor targeting capability (Klevorn et al., 2016). IL-15 is known to be a stimulus for two major immune effectors of NK and CD8 T-cells, to exert antitumor immunity (Klebanoff et al., 2005; Pilipow et al., 2015; Schenkel et al., 2016; Steel, Waldmann, & Morris, 2012). IL-12, a potent proinflammatory cytokine with antitumor activity, and its fusion product have also demonstrated great antitumor activity in preclinical syngeneic models, particularly in combination with chemotherapy (Pasche, Wulhfard, et al., 2012).

7.5. Cell therapies

Immune cell based therapies are also becoming an important new I/O therapy modality (Rosenberg & Restifo, 2015), including those based on NK (Marcus et al., 2014), activated dendritic cells (DC) and tumor specific T-cells (Aranda, Vacchelli, Obrist, et al., 2014; Feldman, Assadipour, Kriley, Goff, & Rosenberg, 2015), engineered NK and T-cells (Margolin, 2011a), *e.g.* CAR-T (Kalos, 2012; Kalos et al., 2011; Maude et al., 2014; Porter, Kalos, et al., 2011; Porter, Levine, et al., 2011). These types of therapies can usually be readily evaluated using standard PDX models in the preclinical setting.

7.6. Virotherapy

We previously described that viral therapy based on oncolytic viruses is largely an I/O therapy, or to be more precise, a personalized vaccine (Li, Liu, & Wong-Staal, 2008; Pol et al., 2016). Its therapeutic power is now much more highly recognized, not only due to the recent regulatory approval of several such treatments in China and the West, but also their enhanced power when combined with checkpoint inhibitors. It has also been found that very little antitumor effects are observed if NSG mice (which completely lack immunity) are used in the preclinical setting. Commonly used models to evaluate virotherapy are syngeneic mouse tumor models (Li et al., 2008; Parato, Senger, Forsyth, & Bell, 2005; Quetglas et al., 2015).

7.7. Combination therapies

With the success of checkpoint inhibitors in a subset of cancer patients, it is only natural to try and expand their use to broader patient populations, and to increase their effectiveness through combination with other immunomodulators and/or existing therapies. For example, some SOC treatments such as radiotherapy (RT) (Demaria & Formenti, 2013; Formenti & Demaria, 2013) or chemotherapeutic agents and oncolytic viruses (Li et al., 2008), have been highlighted as potential inducers of immunogenic cell death (ICD) (Vacchelli, Aranda, Eggermont, Galon, et al., 2014). This process involves changes in the composition of the cancer cell surface, which activates dendritic cells consequently activating a T-cell response. Therefore, combination strategies of ICDs with immunotherapy could provide opportunities to harness the immune system to extend survival, even among metastatic and heavily pretreated cancer patients. However, each combination type will likely have their own benefits and limitations, making preclinical pharmacology modeling particular meaningful before moving on to clinical testing.

7.7.1. Combinations of different I/O therapies

As described above, different immunomodalities have distinct mechanisms of action as well as different toxicity profiles, which enables diverse combinations between different immunotherapies. Many reviews have discussed different types of immunotherapy combinations and their potential benefits and limitations (Mahoney, Rennert, et al., 2015; Melero et al., 2015). For instance, checkpoint therapies either combined together (against two different checkpoint inhibitors) (Ngiow, Young, et al., 2016; Sanmamed, Rodriguez, et al., 2015), or with virotherapy (Quetglas et al., 2015) or adenosine A2A receptor blockade (Young et al., 2014) have been observed to yield synergy in preclincal models. In some cases, a novel new generation of immunotherapy has been created from two different modalities, *e.g.* a fusion molecule of a checkpoint inhibitor combined with an immuno-promoting cytokine (Pasche, Frey, et al., 2012; Pasche & Neri, 2012; Pasche, Wulhfard, et al., 2012). Preclinical confirmation of the MOA of each type of combination strategy will continue to be particularly important for downstream development (Allard et al., 2013).

7.7.2. Combining with other therapies

7.7.2.1. Combination with radiation therapy. Radiotherapy is a common standard treatment used across nearly all cancer types. Recent advances in stereotactic and Image-Guided Micro-Irradiation™ (IGMI) have resulted in an increase in tumor specific targeting, with a corresponding reduction in associated side effects. The resultant use of high-dose, reduced fraction dosing regimens (hypofractionation) has resulted in improved clinical response. Cases have been reported in which the combination of a checkpoint inhibitor with radiotherapy has resulted in synergy of the treatments in the clinic (Demaria & Formenti, 2013; Formenti & Demaria, 2013; Mansfield, Park, & Dong, 2015; Ngiow, McArthur, et al., 2015; Postow, Callahan, Barker, et al., 2012). IGMI has also been applied to radiation therapy modeling in the preclinical setting, to more accurately reflect the clinical exposure of patients to irradiation, as well as to investigate ICD induction and synergy with checkpoint inhibitors. Numerous preclinical investigations have also studied combination therapy with radiation using mouse tumor models and surrogate I/O agents, gaining insights on the synergy and MOAs of these combinations (Deng et al., 2014; Kalbasi, June, Haas, & Vapiwala, 2013; Park et al., 2015; Sharabi et al., 2015; Sharon, Polley, Bernstein, & Ahmed, 2014).

7.7.2.2. Combination with chemotherapies. Similarly, I/O therapy can readily be combined with SOC ICD-inducing chemotherapy (Pfirschke et al., 2016; Vacchelli, Aranda, Eggermont, Galon, et al., 2014). In the preclinical setting, mouse tumor models using mouse surrogate I/O agents are commonly used for combination therapy evaluation.

7.7.2.3. Combination with targeted therapies. In principle, it should be relatively straightforward to perform combination studies between surrogate I/O and targeted agents in syngeneic mouse tumor models, with observed infiltratingimmune cells and synergistic anti-tumor activities (Ngiow, Meeth, et al., 2016; Yang, Yamazaki, et al., 2016); however, there are few such mouse tumor models available. Certain GEMM models with specific engineered driver mutations, or homografts derived from these GEMM tumors, could be used for this type of combination study in the future.

8. Summary

Due to a lack of human immunity, human tumor xenografts (the most commonly used cancer models) are not highly suitable for I/O research, unless there is no requirement for T-cell function. Mouse tumor models of murine immunity, particularly syngeneic cell lines, primary mouse tumors (or derived), are the current workhorse in such research including I/O target discovery and validation, and they are also the tools commonly used to evaluate surrogate biologic therapies and various treatment strategies including combination approaches. These models, however, cannot be used to evaluate human biologics directly, such as antibody therapeutics. Chimeric mouse tumor models of murine immunity featuring human drug targets knocked-in can be an alternative for assessing human specific therapeutics. Another important limitation of mouse tumor models is that mouse tumors and murine immunity may have differences from their human counterparts, therefore impacting

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on their predictive power in assessing clinical situations. PDX in humanized mice could be the future of I/O modeling, although many obstacles still need to be overcome before they can be meaningfully utilized. While this article is in submission, a number of other oncoimmune animal model review articles have been published, many with different emphases (Klevorn & Teague, 2016; Ngiow, Loi, et al., 2016; Smyth et al., 2016; Zitvogel et al., 2016). While this article attempts to be an introductory and comprehensive review of the field for readers new to this area, we also cite the mentioned new and specialized reviews for readers who are interested in more details on more specific topics.

Conflict of interest statement

QXL, XSO and XYA are employees of Crown Bioscience; GF is a co-founder and employee of HuMurine Technology, Inc.

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Appendix A. Supplementary data

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