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Preclinical ex vivo and in vivo models to study immunotherapy agents and their combinations as predictive tools toward the clinic

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ABSTRACT

Almost every successful anticancer treatment has been preceded by preclinical scientific breakthroughs that encouraged clinical development. However, therapeutic strategies showing promising preclinical results often fail to confirm activity in clinical trials, particularly in immunotherapy. There are well-known inherent interspecies differences between human and rodent immunobiology. Moreover, human cancers progressively develop in nature over long periods, while preclinical models are deployed under controlled laboratory conditions. This translates into a suboptimal recapitulation of key features of human cancer, such as the marked interindividual differences, intercellular heterogeneity, and the immunoediting effects of chronic immunosurveillance. This review summarizes the current evidence of preclinical experimental models and research tools for cancer immunotherapy applications, with a focus on the incorporation of human sample-based methodologies, both ex vivo and in vivo using humanized mouse models. Methods to exploit highly valuable human specimens in preclinical research are called to bridge the gap between discovery observations in conventional mouse models and efficacy/safety tests in clinical trials. Novel immunotherapy agents and their combinations can be prioritized based on their effects on in vitro patient-derived tumor culture modalities or on as-perfect-as-feasible humanized mouse models bearing human tumor and immune cells. The ultimate goal is to reliably test immunotherapy interventions and reduce eventual clinical failures by means of preclinically prioritizing the best approaches.

INTRODUCTION: STATE-OF-THE-ART OF CANCER IMMUNOTHERAPY

Preclinical research in cell cultures and rodent models is key to establish which therapeutic strategies show anticancer activity before they are tested in early first-in-human (FIH) clinical trials. In fact, the development of currently approved immune checkpoint inhibitors (ICIs) is a successful example of drugs that showed positive preclinical efficacy results that were later confirmed in patients with immunogenic tumors.[1–3] However, many other strategies have failed clinical success in spite of having shown promising results in preclinical models.[4–9]

An approach to refine our understanding of human cancer immunobiology is incorporating human tumors to preclinical research models. This allows for studying samples that better resemble the characteristics of human tumors that may presumably explain the gap between observations in conventional preclinical models and clinical trials.

There are four main characteristics of human cancer biology that conventional preclinical models hardly recapitulate. First, human cancer is highly heterogeneous between individuals due to a whole range of pathogenic processes that cause malignancies in each person. Second, human carcinogenesis is a multistep process from premalignant lesions to overt metastatic disease, which can take years and drives intercellular heterogeneity of transformed cells, a true cornerstone of cancer resistance to treatment.[10 11] Third, carcinogenesis occurs in the context of a constant interaction between (pre)malignant cells with the immune system of the host since the beginning of oncogenesis.[12–14] Fourth, the existing phylogenetic divergence between human and murine immune systems is often relevant to cancer immunotherapy approaches.[15]

In the following sections, we will critically summarize the limitations and virtues of conventional preclinical models as well as the methodologies and potential advantages of incorporating patient-derived

samples and humanized models in the various steps of preclinical drug development.

Cell culture models

Two-dimensional cultures

Two-dimensional (2D) cultures continue to be the most widely used method to test the interaction between tumor cells and a variety of immune cells due to material availability and simplicity. For human sample interrogation, 2D cultures have been extensively used, particularly for evaluating ex vivo interactions between patient-derived immune cells (predominantly T lymphocytes and natural killer (NK) cells) and tumor cells. Of note, 2D culture methods have been successfully used to explore specific cell-to-cell interactions such as T-cell receptor (TCR)-dependent tumor specificity of lymphocytes in co-cultures with tumor-cell suspensions[16–20] or neoantigen-loaded antigen-presenting cells (APCs).[17–19 21 22] Therefore, these methods are suitable for addressing predefined experimental questions involving a limited number of cell types.

Nevertheless, 2D cultures face a number of limitations. The cell types involved are predefined and normally limited to a few immune cell types. Also, plastic-bound monolayer cultures and single-cell suspensions lack the complex three-dimensional (3D) configuration of solid tumors in vivo, which may influence immune cell reactivity. Lastly, experiments with patient-derived freshly harvested tumors and autologous immune cells are often limited by material availability and consequently require tumor and immune cell-expansion approaches. Regarding immune cells, ex vivo T-cell expansion/activation procedures result in potential modifications in the T-cell compartment when compared with the baseline status. Furthermore, myeloid cells for such in vitro experiments are often obtained from peripheral blood due to tumor-infiltrating myeloid leukocytes' limited availability and viability. In fact, the inclusion of myeloid cells in this type of culture is limited in duration for time-sensitive cells such as neutrophils and macrophages.[23] Experiments involving ex vivo-modified monocyte-derived or B cell-derived APCs are also cumbersome and blighted with potential biases.[17–19 21 22 24]

3D cultures

Over the last decades, 3D in vitro culture techniques have emerged as intermediate steps between conventional 2D cultures and more resource-consuming in vivo methods. In the following sections, we summarize the main methodologies available to increase the architectural complexity of in vitro cultures and the cellular composition of the distinct non-tumoral cells present in the tumor microenvironment (TME) in attempts to resemble the stroma of solid tumors.

Organoids

The *organoid* concept derives from self-assembled self-reproducing 3D multicellular structures that were initially described to recapitulate multicellular structures and functions of some tissues in the tissue culture dish. This technology has been rapidly adapted to culture patient-derived tumor cells for a variety of applications.[25] Conventional tumor organoids are built by culturing variably processed primary tumors in a medium containing extracellular matrix components and tissue-specific growth factors (reviewed by Zhao *et al*[25]), but these typically lose stromal, vascular and immune cells as a result of serial passages in culture. In fact, it has not been until recently that specific efforts have been described to incorporate immune cells to organoids.

One of the pioneering and most relevant tumor-organoid and T-cell co-culture methods was published by E. Voest's group.[26] In this work, mismatch repair deficient (MMRd) colorectal cancer (CRC) and non-small cell lung cancer (NSCLC)-derived conventional organoids were cocultured with autologous peripheral blood mononuclear cells (PBMCs). Authors remarkably showed T-cell reactivity to autologous tumor organoids, which was able to spare autologous healthy tissue-derived organoids. This piece of work proved that individualized tumor-reactive T-cell expansion from peripheral blood was feasible in a proportion of patients with immunogenic tumors. Clinical correlative data from a small cohort of patients with CRC showed that $CD8^+$ T-cell reactivity was enriched in cocultures derived from clinical responders to neoadjuvant nivolumab plus ipilimumab.[27] Recently, other groups have confirmed clonal expansion of tumor-specific tumor-infiltrating lymphocytes (TILs with similar ex vivo culture methods.[28 29] An additional recent article on patient-derived organoids has described the 3D killing kinetics by gamma-delta TCR-transduced allogeneic T cells.[30]

A relevant potential application of organoids in anticancer drug development is testing therapeutic agents against healthy tissue-derived organoids. This is of particular interest for adoptive T-cell therapy (ACT) and T-cell engagers, where on-target off-tumor toxicity is a source of major concern. The exposure of normal epithelium-derived organoids to effector T cells or T-cell-engaging bispecific antibodies (BsAbs) could preclinically aid in the identification of toxicities.[20 31]

Regarding limitations, some groups have attempted to overcome the admitted lack of the lymph node component in these ex vivo models by co-culturing tumor cells and tumor-draining lymph node (TDLN)-derived cells.[32] Additionally, organoids might face tumor purity issues due to the outgrowth of normal epithelial cells, which may require periodical verification.[33–35] Organoid development was fuelled as a tool to test tumor cell-targeted drugs with clinically concordant results, prior to the immunotherapy era.[36 37] However, correlative evidence for organoids incorporating human immune cells remains limited.[27]

Microphysiological systems

Microphysiological or so-called organ-on-a-chip systems are devices intended to recapitulate the compartmentalized

and dynamic configuration of organs/tumors in tissue-culture conditions. Experimentation on some tumor-immune cell interactions requires microfluidic devices in order to be reproduced in vitro. For instance, a microfluidic device was required to reliably induce some cytokines in human tumor spheroids.[38] These devices were also used to confirm the role of functional LKB1 for NSCLC spheroid infiltration by T lymphocytes.[39] Microfluidic devices have also been used to study interactions between human cancer cells exposed to a variety of treatments (including ICIs) and allogeneic dendritic cells (DCs).[40 41] For instance, a report described that these devices can be used to monitor peripheral blood lymphocyte migration into patient-derived tumor cells on exposure to indoleamine 2,3-dioxygenase 1 inhibitors or programmed death-ligand 1 (PD-L1) blockers.[42] Overall, the distinct compartments of these devices are particularly well suited to study circulating immune cell migration into tumors. In a recent report, a microfluidic device has been used to describe the interactions between CRC organoids and multiple autologous cell types such as TILs, cancer-associated fibroblasts and monocyte-derived DCs.[43] Of note, enhanced antitumor activity of TILs by atezolizumab was observed only in samples derived from an immunogenic microsatellite instability-high (MSI) tumor.[43]

Modeling the tumor microvasculature poses a serious obstacle. Advances in microfluidic device designs and tubular structure formation protocols[43–45] might significantly contribute to addressing specific questions regarding tumor infiltration by circulating lymphocytes and their drainage by lymphatic vessels. In this regard, in vitro generation of lymphoid follicles can significantly contribute to a better recapitulation of adaptive immune responses in these devices.[46] Nevertheless, the recapitulation of the finely regulated multifactorial process comprising endothelial barriers and the entire cancer immunity cycle involving the TDLN and lymphatic vessels seems currently beyond the reach of this sort of organ-on-a-chip devices.[47] Another approach in biomedical research involves the use of multi-organ or patient-on-a-chip models. These models consist of multiple organ tissue cultures that are exposed to culture medium containing experimental drugs. These models have been described to uncover relevant pharmacokinetics/pharmacodynamics (PD) aspects of multiple non-immunotherapeutic agents.[48–51] While these methods are emerging as valuable tools for exploring critical interactions between tumors and TDLN, they have important limitations, particularly in their inability to replicate physiological cues related to leukocyte migration, such as the presence of lymphatic vessels. Thus, further research is required to validate this approach for cancer immunotherapy applications.

Early passage organoids and tumor-fragment cultures

A piece of seminal work in this field was developed by Jenkins *et al*, who reported on small partially digested tumor fragment cultures in microfluidic devices that reportedly retained the immune cell composition of original tumors.[38] In mouse-derived tumor fragments, they showed that ex vivo killing induced by antiprogrammed death-1 (anti-PD-1) treatment was associated with the in vivo response to each transplantable tumor cell line. This group also showed that the TME secretome could be evaluated in patient-derived tumor samples. However, there was no apparent association between the ex vivo secretome and clinical benefit from PD-1 blockade in a series of matched patient-derived samples.[38] Regarding tumor-cell killing, evidence of an association between ex vivo and clinical responses remains limited to short series of cultures derived from anti-PD-1-resistant melanomas.[52] The method described by this group is somehow a hybrid between conventional organoids and tumor-fragment cultures as later described by D. Thommen's group,[53] because the tumor fragments used were directly derived from original tumors but retained proliferative capacity ex vivo.[38] In a somewhat similar air-liquid culture platform, enzymatic digestion-free processed tumor fragments were cultured for several days and showed sustained organoid growth but progressive and substantial decline of stromal and immune components in culture over time.[54] Importantly, a proportion of tumor organoids derived from anti-PD-1-responsive tumor subtypes (NSCLC, melanoma, and renal cell carcinoma) showed $CD8^{+}$ T cell expansion and activation on PD-1 blockade ex vivo.[54] Other approaches have described that responsiveness to anti-PD-1 agents can be tested in similar short-term cultures.[55]

So far, the most relevant contribution to patient-derived tumor fragment (PDTF) cultures was published by D. Thommen's group.[53] They developed a straightforward method to culture so-called PDTFs obtained from patients with a variety of tumor subtypes, with and without an anti-PD-1 monoclonal antibody (mAb). Following an unsupervised hierarchical clustering of samples regarding dynamics of the cytokine secretome and TIL activation markers by flow cytometry, tumors were classified as 'PDTF-responders' and 'non-responders'. A weighted score was developed with the most discriminative parameters. Of utmost importance, they showed that the PDTF response score was statistically associated with the radiological response of patients to anti-PD-1 therapy.[53]

Nevertheless, tumor fragment cultures have a number of limitations that future studies and alternative methods should contribute to overcoming. Cytokine and TIL activation readouts in such an approach are limited to just a few days after culture initiation,[56] although more prolonged tissue preservation has been described using perfusion bioreactors.[57] Furthermore, tumor fragment cultures show a remarkable interfragment heterogeneity, which demands testing several fragments per experimental condition.[53] In this regard, it remains to be addressed whether preserving the original tissue architecture in tumor fragments is crucial for a proper PD assessment of ICIs. Lastly, the short viability of tumor fragment cultures limits direct anti-tumor activity readouts beyond 2–3 days. In this regard, the majority of published reports have

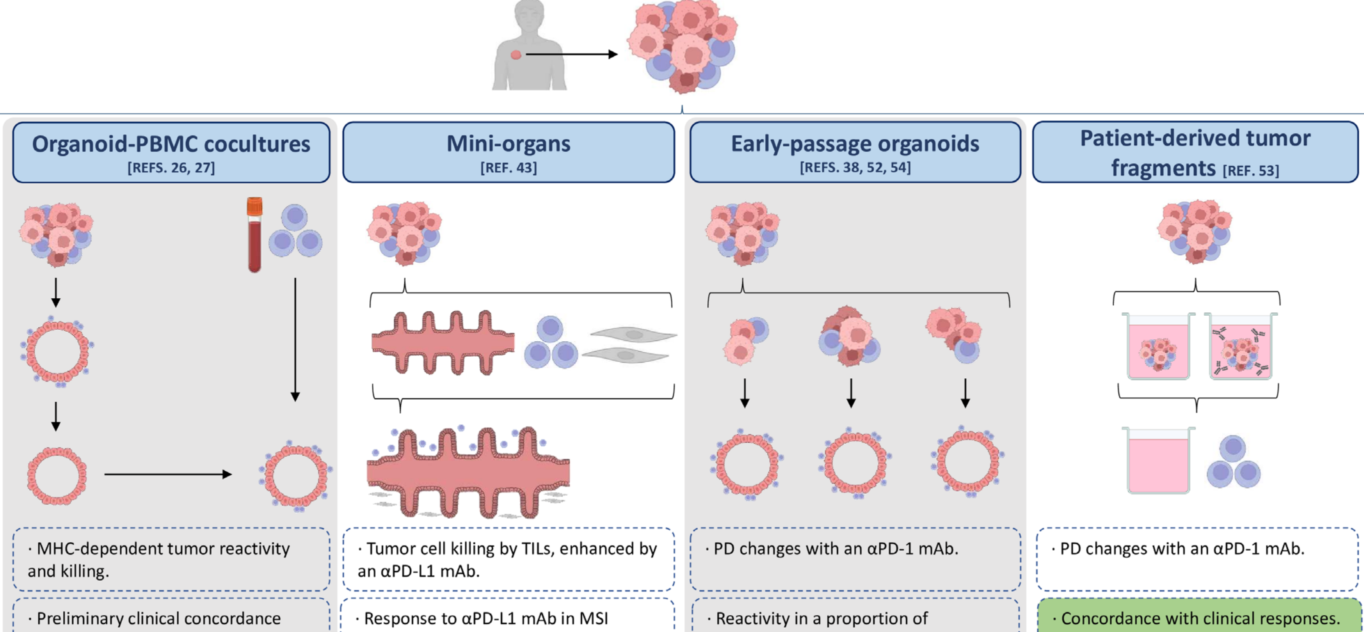

Figure 1 Summary of the most relevant in vitro methodologies for cancer immunotherapy applications with patient-derived samples and corresponding seminal works. Dashed line boxes in the first row represent the key findings of corresponding publications. Dashed line boxes in the second row represent correlative clinical data of corresponding publications. *Patient-derived tumors can be processed either manually (Voabil *et al*,[53] Roelofsen *et al*[56]) or with a vibrating microtome (see Sivakumar *et al*,[233] Corrales *et al*,[234] Hewitt *et al*[235]). αPD-L1, anti-PD-L1; mAb, monoclonal antibody; MHC, major histocompatibility complex; MSI, microsatellite instability-high; PBMCs, peripheral blood mononuclear cells; PD, pharmacodynamic; TIL, tumor-infiltrating lymphocyte. Images created in Biorender.com.

used fluorescence-based approaches to evaluate tumor cell death in those cultures.[26 30 38 54] Overall, antitumor efficacy readouts and long-term outcomes remain less reliable in vitro than in vivo. A summary of features of the most relevant in-culture methodologies for cancer immunotherapy applications with patient-derived samples is provided in figure 1.

In vivo models

Human tumor xenografts are typically included among the preclinical steps required to support the translation of promising chemotherapies and tumor cell-targeted drugs into early clinical trials. With the advent of antiangiogenics and immunotherapy, the stromal TME components are increasingly recognized for playing a relevant role in tumor regression both in drugs that directly target these compartments and in the immune system's contribution to the response to conventional treatments.[58 59] This notion is challenging our understanding of the utility of the distinct fully murine and humanized models to an unprecedented level of sophistication.

A critical appraisal of fully murine models

In situ tumor development mouse models: spontaneous, carcinogen-induced, and genetically engineered mouse models

Spontaneous[60 61] and carcinogen-induced cancer models[62] were the first in vivo models to be developed. In fact, many popular cell lines have been derived by isolating these tumors and serially passaging the cells in vitro. Later, key discoveries on the role of oncogenes and tumor suppressor genes in cancer led to the development of genetically engineered mouse models (GEMMs).

Early seminal observations led to the description of protection against tumor rechallenge with methylcholanthrene (MCA)-induced sarcomas in mice that had been immunized by surgical ligation of previously inoculated tumors.[63] Of note, the development of immune protection did not occur when experiments were performed with spontaneous mammary tumors. These observations led to pioneering speculations that carcinogen-induced tumors could be more immunogenic than spontaneous mammary counterparts as a result of high numbers of mutations triggered by the carcinogen giving rise to neoantigens.[63] More recently, several reports by R. Schreiber's group have provided evidence in support of the cancer immunoediting hypothesis and the existence of a transient immune-mediated equilibrium phase with MCA-induced fibrosarcomas.[64–66] In addition, the seminal works that showed the in vivo effectiveness of cytotoxic T-lymphocyte antigen-4 and PD-1 blocking strategies readily used transplantable models that were originally carcinogen-induced, among others.[1 2] Overall, in situ carcinogen-induced models have enabled key insights on the role of the immune system in cancer immune surveillance, but high costs related to a variable penetrance and the stochastic genomics of developed tumors have reduced

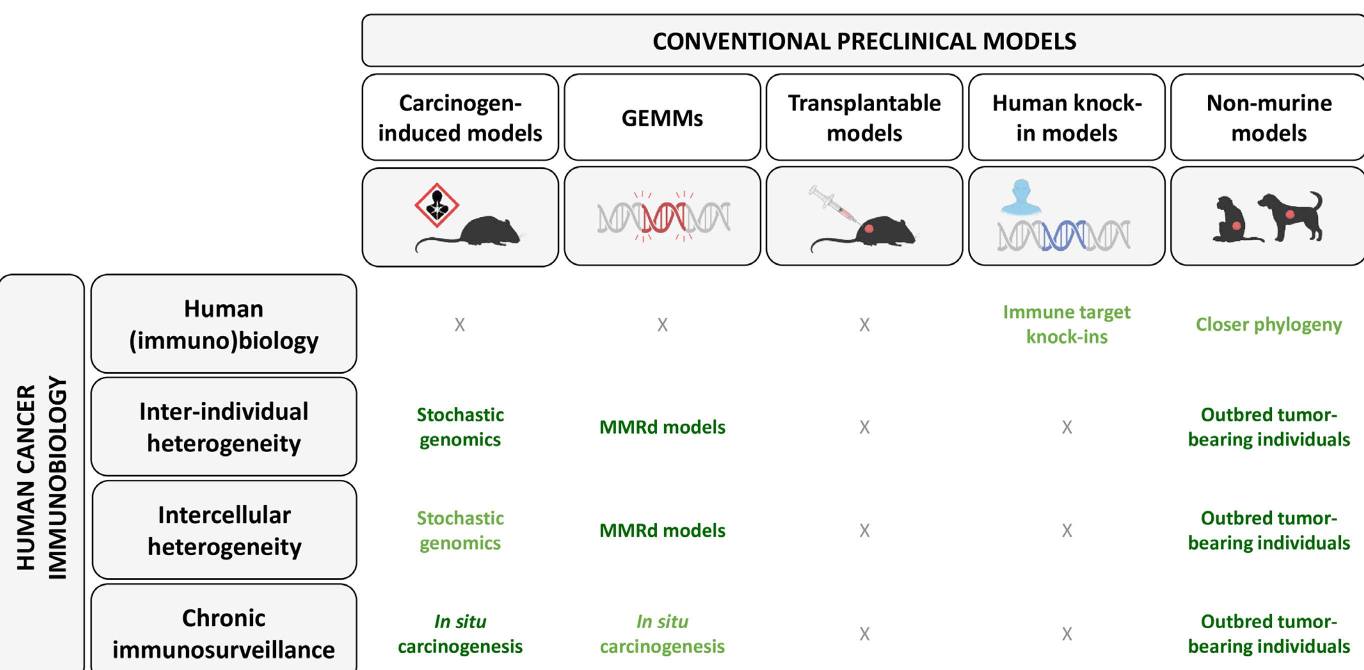

Figure 2 Degree of recapitulation of key human cancer immunobiology characteristics by conventional non-humanized in vivo models. Key human cancer immuno-biology aspects are depicted in the rows: human immuno-biology includes all the aspects related to immune system's structure and function, highlighting its ability to distinguish self from non-self, mount specific and adaptive responses to pathogens, and maintain homeostasis through complex cellular and molecular interactions. Interindividual heterogeneity includes both germline differences between individuals (eg, human leukocyte antigen gene polymorphisms) and intertumor heterogeneity between patients. Intercellular heterogeneity refers to the variations in gene expression, function, and behavior among individual cells within the same tissue or population, contributing to diverse responses and adaptability in biological systems. Chronic immunosurveillance refers to the sustained monitoring and response of the immune system to detect and control persistent threats such as tumors, latent infections, or chronic inflammation, often involving ongoing immune activation and adaptation. In the columns, all the conventional preclinical models are depicted, along with their ability to recapitulate the key aspects of human cancer immunobiology. 'X' indicates insufficient recapitulation; light green letters represent low recapitulation; darker green letters represent high recapitulation. GEMMs, genetically engineered mouse models; MMRd, mismatch repair deficiency. Images created in Biorender.com.

their use in recent years in favor of more reproducible transplantable versions and GEMMs.

GEMMs replicate to some extent the in situ development of the carcinogenic process in constant interaction with the stroma and immune cells (figure 2). Initial germline tissue-agnostic GEMMs have evolved to modifications under the control of tissue-specific promoters[67] or the local delivery of Cre-recombinase by viral vectors to elicit the expression of transgenic oncogenes.[68] The timing of gene silencing or induction can also be controlled to better mimic the progressive accumulation of genomic alterations in human carcinogenesis through post-transcriptional control of the activity of an estrogen receptor-fused Cre-recombinase by tamoxifen administration[69] or tetracycline-regulated Cre-recombinase expression.[70] In addition, dual-recombinase systems have been developed to metachronously induce these events.[71] Furthermore, recent advances have reduced thymic 'leaky' expression of exogenous antigens and ensuing secondary immune tolerance.[72]

Regarding applications, GEMMs have been used for a variety of experimental objectives in cancer immunotherapy, mainly involving TME immune phenotypes driven by cancer cell genomics.[73] Interestingly, GEMM has also been exploited to trace antigen-specific T-cell exhaustion dynamics during carcinogenesis.[74] Recently, exquisite knowledge of the regulation of bone marrow myelopoiesis has allowed a refined exploration of the role of myeloid cell progenitors in the progression of the Kras/Trp53-mutant (KP) lung adenocarcinoma GEMM.[75] An interesting variation of GEMMs is the generation of multifocal hepatocellular carcinomas by means of hydrodynamic gene transfer of hepatocytes on high-pressure intravenous injection of expression cassettes encoding oncogenes, sequences for CRISPR/Cas9 deletion of tumor suppressor genes and model antigens.[76 77] Overall, these models have allowed for the establishment of immune surveillance concepts and the testing of combination immunotherapy strategies.[78 79]

Despite the highly valuable contribution of GEMMs to mechanistic discoveries, their capacity to replicate the intricate mutational landscape of carcinogen-induced highly mutated human tumors remains to be established, as illustrated by a very low mutational burden of a model of KRAS-driven lung adenocarcinoma.[80] GEMMs of MSI tumors have also been developed recently, providing

relevant insights on immunosurveillance of intratumor clonal heterogeneity[81] (figure 2). However, important caveats remain regarding the recapitulation of key characteristics of human MMRd tumors such as sensitivity to ICIs.[81] Nevertheless, recent evidence suggests that GEMMs are a valuable platform for studying immunosurveillance of early carcinogenesis in models encompassing a very low mutational burden.[82]

Transplantable syngeneic tumor models

The still most commonly used mouse tumor models are built by transplantation of syngeneic in vitro-expanded tumor cells into immunocompetent mice, with obvious practical advantages. In fact, transplantable mouse models were used to prove that ICIs could successfully restore an effective immune response against cancer.[1–3] It is similarly apparent, however, that transplantable mouse models fail to reproduce the chronic interaction between tumor and immune cells through the carcinogenic process[83 84] and most of the intercellular heterogeneity of cancer cells (figure 2).

Some widely used variants of syngeneic transplantable models (eg, B16-OVA) bear endogenous or surrogate antigens for which epitope-specific TCRs are expanded after immunization. These have provided essential insights on antigen specificity and spreading in terms of mechanistic clarifications. However, many of the most widely used antigens/epitopes are exogenous or allogeneic, limiting the translatability of the findings.[85–87] Nevertheless, endogenous immunodominant antigens have also been identified for some of the most widely used transplantable syngeneic models, in some instances encompassing derepressed retroviral sequences or cancer testis family antigens.[88 89] Furthermore, transgenic mouse strains for antigen-specific TCRs have also been developed, with a wide range of applications in ACT and ex vivo experiments.[90–92] Human antigen-specific TCRs have also been identified and sequenced successfully,[93] but due to human leukocyte antigen (HLA) allele restriction combined with the high degree of individual privacy of human neoantigens, these are typically targeted to tumor-associated antigens.[94] Personalized mutation-specific TCR identification still depends on costly and hardly scalable methodologies,[18 19] but shared hotspot mutation antigens are emerging.[95 96]

Non-murine animal models

The mouse is the dominant species selected for preclinical experiments due to an acceptable equilibrium between the phylogenetic distance, ethical considerations, and breeding costs. However, other species with a closer phylogenetic relationship with humankind can also be used to address defined scientific needs.

From the perspective of the recapitulation of spontaneous tumors, pet dogs diagnosed with cancer are an interesting setting where preclinical trials of immunotherapy strategies have been conducted.[97 98] In this regard, an interesting opportunity comes from veterinary clinical trials in which pets diagnosed with spontaneous tumors are treated with agents that are active across species, such as TLR agonists or viral vectors encoding immune mediators (reviewed by Bergman[99] and Finocchiaro and Glikin[100]). Nevertheless, these genuinely preclinical trials are expensive and require an often-non-available deep understanding of the underlying biology. These issues may limit their application in favor of FIH clinical trials.

Regarding the latter, the use of healthy non-human primates (NHPs) is a common previous step aimed at ruling out unexpected adverse events and having an estimate of the first dose level to be tested in human beings in phase I clinical trials. Here, the phylogenetic closeness between NHPs and humans translates into a cross-reactivity of many drugs designed to target human proteins expressed by the immune system.[101 102] Nevertheless, preclinical NHP and clinical toxicity discordance examples[101 103 104] illustrate that interspecies differences extend beyond receptor sequence similarity and underscore the need for cautious FIH clinical trial designs. Of note, human proteins including immunoglobulins are immunogenic in macaques, and antidrug antibodies may constitute a confounding factor.

Human knock-ins

With current genome editing techniques, specific regions of the mouse genome can be substituted by human genomic sequences or human genes can be introduced as transgenes. The most commonly used human knockin mouse models for cancer immunotherapy have been designed to replace mouse orthologue immune checkpoint receptors to allow the interaction with the anti-human mAbs under development. Frequently, chimeric constructs are introduced in the homologous locus that encodes the extracellular domain, while the intracellular murine segments are kept for optimal signal transduction.[105–107] Other models incorporate transgenes encoding for human Fc receptors onto mouse strains that lack murine homologs.[108] Overall, these models are attractive because they might uncover relevant interactions between clinical-grade drugs and cognate target membrane proteins. In addition, they might be useful to comparatively test a panel of antibodies for their in vivo PD properties.[105 106 109 110] In this regard, mouse strains with a replacement of the immunoglobulin loci by human orthologs have played a relevant role in the development of fully human therapeutic mAbs.[111] Nevertheless, knockin models pose a number of limitations regarding the prediction of their effects in human patients, derived from an often-inaccurate recapitulation of gene expression, post-transcriptional/translational modifications, and interactions with its upstream/downstream proteins under natural conditions.[107 112] Cellular humanization with viable human immune cells, discussed in the following section, should provide a complementary recapitulation of the PD changes induced by targeting proteins expressed in human immune cells, which might be relevant to *de-risk* drug development. A summary of

the recapitulation of key human tumor biology characteristics by non-humanized in vivo models is provided in figure 1.

Humanized mouse models

Humanized models are a highly intertwined combination of their three main components: the tumor, the murine host, and the human immune system. Every component should be carefully selected, because their characteristics will critically influence both the degree to which the myriad of components of the human immune system are recapitulated in the mouse model, as well as the tolerance of the mouse host tissues to various forms of xenoreactivity produced by the interaction of competent human immune cells with host murine tissues (figure 3).

The tumor

There are two main origins of human tumor cells: cell lines and patient-derived tumor xenografts (PDX). Human tumor cell lines are widely available and reproducible, with very similar experimental advantages and disadvantages compared with transplantable syngeneic murine cell lines. By contrast, PDX will better recapitulate a good number of the essential features of human cancer such as tumor heterogeneity, tissue architecture, and even the presence of native stromal and immune components, particularly in early graft passages. In addition, PDX models can potentially be humanized with autologous immune cells obtained from the same patient from whom the tumor was excised.

A relatively simple but interesting approach, called iPDX,[113] is based on exploiting the native immune infiltrate that is present in the original tumor sample and remains viable during the first passage of the PDX.[114] A variety of immunomodulatory strategies have been tested on first-passage PDX, confirming that the highly valuable native tumor-infiltrating immune cells can be modulated in vivo.[115 116] More recently, this methodology has been used to explore phenotypic changes in TILs on exposure to PD-L1 blockade.[117] Nevertheless, this strategy is limited by the initial tumor sample size, progressive loss of the immune infiltrate on serial passage, and tumor engraftment failure. In fact, conventional tumor growth and shrinkage dynamics often cannot be properly assessed and surrogate end points of pathological response (eg, tumor necrosis/viability) are to be considered, as well as the serial quantitation of circulating tumor DNA in the serum of xenografted mice.

Another aspect of tumor engraftment, not specific to humanized models, is the site of tumor engraftment. For practical reasons, the majority of groups working with PDX uses the subcutaneous compartment. However, it is admitted that, bridging potential interspecies gaps, the microenvironment of the tumor-hosting tissue might significantly influence tumor engraftment and evolution.[118] Nonetheless, to our knowledge, the influence of the murine host tissue microenvironment on shaping the human immune phenotype of the tumor graft has yet to be established.

The host

Currently, the most widely used mouse strains combine a variety of immune defects to allow human cell engraftment. These mice typically have a *scid* phenotype, that is, absence of mature T and B lymphocytes due to an inability to rearrange the immunoglobulin and the TCR loci.[119] This can be conferred by mutations in (i) the DNA protein kinase complex[120] or (ii) Rag-1 or Rag-2 recombinases involved in T/B cell receptor recombination processes.[121 122] In addition, xenograft-oriented mouse strains frequently have a non-obese diabetic (NOD) polygenic background, with a number of immune dysfunctions such as reduced peripheral blood T lymphocyte counts,[123] a lack of a functional C5 component of complement,[124] defective macrophage maturation and antigen presentation,[125 126] reduced phagocytic capacity,[127] reduced NK cell activity,[128 129] and a signal regulatory protein alpha (SIRPα) polymorphism with a greater affinity for human CD47 to avoid phagocytosis of engrafted human cells.[130] Finally, these mice typically include a targeted mutation in interleukin (IL)-2Rγ (common component of the receptors for IL-2, 4, 7, 9, 15, and 21), which additionally confers a lack of functional NK cells and identifiable peripheral lymph nodes (with the exception of mesenteric rudiments).[131 132] Other genetic modifications favoring other aspects can be found in different immunodeficient murine strains. A detailed review has recently been published by Chuprin *et al*.[133]

The grafted human immune system cells

Two primary sources of human immune cells are used to humanize immunocompromised mice: mature cells (comprising PBMCs and TILs) and hematopoietic stem and progenitor cells (HSPCs).

Peripheral blood mononuclear cell

PBMCs are the most readily available source of human immune cells and can be obtained from healthy donors or cancer patients. Circulating mature immune cells typically lead to a predominantly lymphocytic expansion in humanized mice.[134] The expansion process of mature lymphocytes in a lymphopenic host has received the classic name of *homeostatic proliferation*. This process is better characterized in mouse-to-mouse lymphocyte transfer experiments and leads to the expansion of donor cells to fill the 'void' left by absent recipient counterparts due to an extra availability of non-consumed IL-7 and IL-15.[135] In fact, this phenomenon underlies part of the basis for the application of conditioning lymphodepletion prior to ACT in the clinic.

Of note, murine T cells undergoing homeostatic proliferation have been reported to retain antitumor potential and cytokine production capacity on exposure to antigens ex vivo.[135 136] However, lymphocytes undergoing homeostatic proliferation acquire a memory phenotype

		HUMANIZATION EXPERIMENTAL CHALLENGES				OVERALL CAPABILITIES		
		HLA education	Myeloid cells	Autologous	xGVHD	Strength	Weakness	Feasibility
HUMAN TUMOR	iPDX	Optimal	Poorly described, but probably short-lived	Yes	-	Fully human TME	Short-term readouts Limited by the original sample size	+
	PDX	N/A	N/A	Feasible	N/A	Tumor infiltration & TIL phenotype description	Low engraftment rate	++
	CDX	N/A	N/A	No	N/A	Tumor-driven TME shaping analysis	Cell line drawbacks (similar to murine)	+++
MURINE HOST	Immuno-deficient host	N/A	N/A	N/A	Influenced by the degree of immuno-deficiency	-	-	+++
	HLA knock-ins	Partially matched	N/A	N/A	N/A	-	Lack of MiHA education	+
	Human cytokine knock-ins	N/A	Facilitated (for HSPCs)	N/A	Late myeloid cell activation syndromes	Myeloid cell co-option by the tumor	-	+
	mMHC knock-out	N/A	N/A	N/A	-	Improved antitumor activity readouts	-	++
HUMAN IMMUNE SYSTEM	PBMCs	Optimal	Short lived, function not well described	Feasible	+++	Short time for ready-to-use engraftment	T cells: shift toward CM phenotype, tumor-specific clone scarcity	+++
	Adult HSPCs	Depends on HLA expression	Partially preserved, depend on human cytokines	Feasible	-/+	-	Risk of pseudo-allo-reactivity	++ (+ if autologous)
	B(L)T	Matched if autologous	Partially preserved, depend on human cytokines	No*	+	Non-tumoral tissue available Controlled oncogenesis*	Risk of pseudo-allo-reactivity (MiHA)	+
	TILs	Optimal	Absent after expansion ex vivo	By definition	-/+	Enrichment of tumor-reactive clones	Ex vivo expansion required	++

Figure 3 Different humanized models, experimental challenges, and overall capabilities. Different humanized models are classified based on the three main components of these models: human tumor source, murine host features, and human immune system source. The main challenges to recapitulate the human antitumor immune response are depicted, along with the strengths, weaknesses, and feasibility of each model. 'Pseudo-alloreaction' refers to a potentially spurious recognition of autologous tumor cells in HSPC-humanized models due to an incomplete negative selection of autoreactive clones because of a lack of physiological expression of HLA genes and/or MiHAs. *See Moquin-Beaudry *et al*.[200] B(L)T, bone marrow, (liver), thymus model; CDX, cell line-derived xenograft; CM, central memory; HLA, human leukocyte antigen; HSPCs, hematopoietic stem and progenitor cells; iPDX, immune patient-derived xenograft (early-passage); MiHA, minor histocompatibility antigen; mMHC, murine major histocompatibility complex; N/A, not applicable; PBMCs, peripheral blood mononuclear cells; PDX, patient-derived xenograft; TILs, tumor-infiltrating lymphocytes; TME, tumor microenvironment; xGVHD, xenograft-versus-host disease; Images created in Biorender.com.

even in the absence of an antigen.[137] Humanized mice, on transfer of PBMCs, share many of the features described for homeostatic proliferation in fully murine models, such as extensive proliferation and acquisition of a predominantly memory phenotype,[138 139] even in the absence of murine major histocompatibility complex (MHC).[134]

Regarding other immune cells, B cells can be detected in PBMC-humanized mice, although at low levels.[139] Functionally, antigen-specific IgM and IgG have been described in HBV-vaccinated mice.[140] Mature myeloid cells, whose proliferative capacity and lifespan are limited, will only survive for a few days in PBMC-humanized mice,[139 141] posing a significant limitation for their use in experimental settings where a substantial influence by these immune cell populations can be expected.

Mouse humanization with mature human immune cells is associated with the development of xenograft-versus-host disease (xGVHD), which typically limits the survival of conventional immunocompromised mice to a few weeks following human PBMC transfer.[134 138] In fact, the MHC mismatch between the two species leads to the promiscuous activation of a relatively high proportion of involved T-cell clones.[142]

MHC class I and II double knockout mice have consistently been shown to minimize xGVHD on PBMC transfer.[134 141] Comparative studies on lymphocyte engraftment in MHC-deficient mice show a shift toward a predominantly effector memory phenotype (≈80%) in peripheral blood after intravenous administration.[134] However, several groups, including ours, have shown that, within the $CD8^+$ compartment, MHC-double knockout mice preserve a small proportion of circulating naïve cells.[134 143] Functionally, antigen-specific IgG-producing B cells and antigen-specific $CD8^+$ T cells have been described in MHC-double knockout mice on infectious stimulus.[141] Regulatory T (Treg) cells are also detectable in the recipient mice,[134 143] but the suitability of the models to test Treg-targeted immunotherapy approaches, although promising, remains to be seen.

Tumor-infiltrating lymphocytes

TILs are an additional interesting source to humanize mice for cancer immunotherapy applications. As an advantage, tumor-specific T lymphocytes are enriched in the TME compared with peripheral blood.[21 144] By definition, TILs are autologous to tumor cells and show reduced xGVHD incidence due to tumor-specificity enrichment and a lower proportion of naïve cells.[116 145–147] However, obtaining sufficient numbers for in vivo experiments requires in vivo/ex vivo TIL expansion procedures.[145–147] Since TIL-humanized mouse models are a subtype of humanized mouse models that is specific for cancer research applications, reports using this approach will be discussed in further detail in the corresponding section below.

Hematopoietic stem and progenitor cells

There are three main sources of HSPCs that have been used to construct humanized mouse models. Early in the development of these models, it was demonstrated that umbilical cord blood $CD34^+$ cells were a more successful source compared with adult bone marrow[148] or granulocyte colony-stimulating factor ()-mobilized peripheral blood precursors.[149] However, the limited availability and the origin of these cells in tumor-free individuals have hindered the application of the former in the cancer immunotherapy experimentation field. Regarding adult HSPCs, remarkable efforts have been made to collect these cells from patients with cancer in order to construct autologous models, but these remain limited due to the ethical and procedural complexities associated with sample collection.[150 151]

Initial humanization descriptions in NOD scid gamma (NSG) mice confirmed multilineage differentiation in mice transplanted with umbilical cord or mobilized peripheral blood cells. $CD19^+CD10^+$ immature B cells,[152 153] myeloid precursors,[128] T cells, and NK cells are typically detectable.[154] In contrast with PBMC-humanized mice, naïve T cells are dominant across organs in HSPC-humanized models with no antigenic stimulus.[154 155] In addition, no clinically overt xGVHD is typically described.[154 155] Both observations suggest that negative selection of murine-MHC-responsive TCR clones takes place in the murine thymus, although the contribution of de novo generated naïve cells cannot be ruled out.

Regarding the B-cell compartment, mature cells are detectable in the spleen and low levels of IgM and IgG are detectable in plasma.[152–154] In terms of adaptive immunity capabilities, some reports have shown that antigen-specific T-cell expansion on influenza vaccination was significantly better in HSPC-engrafted versus PBMC-engrafted mice.[156] Regarding the engraftment of myeloid progenitor lineages and mature cells, proportions are typically low, particularly in peripheral blood.[157] Overall, it is considered that HSPC-humanized models in conventional immunocompromised mice only partially recapitulate the various functions of the human innate immune compartment. This has been attributed to a lack of functional myeloid lineage-promoting cytokines in these mice. In the following section, a variety of approaches developed to overcome this limitation will be reviewed.

Multigene knockin mice to favor engraftment and development of human immune cells

Even though all the successful and a majority of potentially effective immunotherapies under development are targeted to lymphocytes, the influence of myeloid cells on T-cell targeted immunotherapy outcomes is increasingly reported.[158] Furthermore, a variety of strategies are being developed to specifically target myeloid cells in order to improve cancer patient outcomes.[159] Therefore, it is critical to develop strategies to better recapitulate myeloid cell biology in humanized mouse cancer models.

In this regard, the most recent advances in genomic mouse humanization came from combining various human genes in a single mouse strain. As such, the MISTRG (M-CSF, IL-3, GM-CSF, SIRPα, TPHO—Rag2$^{-/-}$IL-2Rγ$^{-/-}$ strain) and the NSG-SGM-3 (SCF, GM-CSF, IL-3—NSG strain) are two of the most widely used strains for cancer immunology applications. The MISTRG mouse combines the replacement of mouse M-CSF, IL-3, GM-CSF, and TPHO by their human orthologues, in combination with a transgene encoding for human SIRPα (to prevent phagocytosis of human CD47^{+} cells) generated on a Rag2$^{-/-}$IL-2Rγ$^{-/-}$ background.[160] Compared with control NOD/SCID/IL-2Rγ^{null} mice, MISTRGs showed a higher proportion of CD33^{+} cells in peripheral blood.[160] Of note, MISTRG mice also showed a better representation of monocyte subsets based on CD14 and CD16 expression. Interestingly, there was also a higher presence of NK cells in MISTRG mice (markedly under-represented in control NSG mice), which was attributed to the transpresentation of human IL-15 by the myeloid compartment.[160] MISTRG mice have been further modified with the incorporation of the human *IL6* gene into its mouse orthologous locus. In a recent work by R. Flavell's group, HSPC engraftment was compared between MISTRG-6, MISTRG, and NSG mice.[150] Regardless of the source of HSCPs, human cell engraftment was increased in MISTRG-6 mice compared with MISTRG mice. The results described in this work regarding tumor immune infiltration and therapeutic interventions will be reviewed in the corresponding section below.

An additional multiple knockin mouse strain that has been frequently described is the NSG-SGM3, which includes a transgene for human KIT-L (also known as stem cell factor (SCF)), IL-3, and GM-CSF. Compared with conventional NSGs, NSG-SGM3 mice had a better engraftment of CD33^{+} cells. NSG-SGM3 mice also showed higher proportions of mature B cells and higher plasma levels of total and antigen-specific IgG on infection.[161 162] Advanced multiple knockin MISTRG and NSG-SGM3 strains have been directly compared.[163] In peripheral blood, there was a better representation of CD33^{+}SSC^{high} granulocytes in the NSG-SGM3 strain, but the proportion of morphologically mature (segmented) neutrophils was lower than in MISTRG mice.[163] Within the CD33^{+}SSC^{low} compartment, MISTRG mice recapitulated significantly better the physiological human monocyte subsets based on CD14 and CD16 expression.[163] In addition, MISTRG mice showed CD68^{+} tissue macrophages in lungs and livers that better recapitulated their presence in human organs.[163]

The latter two mouse strains face constraints regarding long-term survival. The MISTRG model is characterized by an anemic syndrome that has been associated with phagocytosis of murine red blood cells by human macrophages, whose SIRPα does not interact with mouse CD47.[160] NSG-SGM3 mice, due to the transgenic supraphysiological expression of human cytokines, often develop a severe inflammatory condition that resembles clinical macrophage-activation syndromes.[164 165]

Additional mouse strains have been developed for NK-cell targeted approaches. For instance, hIL-15 knockin and transgenic mice have been described to have significantly more abundant circulating NK cells and have been used for cancer immunotherapy applications involving this killer subpopulation of lymphocytes.[166 167] Regarding the engraftment of neutrophils, a human G-CSF knockin has been generated in the MISTRG background.[168] In addition, given the important functions of DCs in adaptive immunology, models supplemented with FLT3-ligand have been reported to enhance immune responses on viral vaccination[169] and human tumor cell exposure.[170] A detailed review on additional human cytokine knockin mouse strains is provided by Chuprin *et al*.[133]

Recapitulating key features of cancer adaptive immune responses

For a valid recapitulation of the neoantigen-specific adaptive cancer immunology in HSPC-humanized mouse models, it is essential to reproduce the MHC-education of lymphocytes that takes place under physiological conditions. This is a significant challenge that has been addressed by two different approaches in HSPC-humanized mouse models: (i) engraftment of human fetal thymus fragments and (ii) knockins of HLA alleles.

Human thymus engraftment

The first robust evidence of the fact that human thymus grafts could provide physiological T cell maturation came from experiments where mice were engrafted with fetal liver (as a source of HSPCs) and thymus fragments.[171] The model was later refined by the addition of ex vivo purified CD34^{+} HSPCs to thymus and liver engraftments placed underneath the kidney capsule,[172 173] to build the so-called bone marrow-liver-thymus (BLT) model. The BLT model has been shown to be capable of negative selection of TCR-engineered autoreactive lymphocytes when the thymus graft is HLA-matched with the transgenic TCR.[174–176] Another article showed that building an allogeneic HLA-matched adult bone marrow-fetal thymus (BT) model was feasible and yielded T lymphocytes that were self-tolerant but alloreactive against a third donor.[177] The latter approach might facilitate the translatability of the BT model to study adult cancer, but reports of this kind have not been developed to our knowledge.

Functionally, adaptive immune responses have been described in BLT models on infectious stimulation.[173 178] Nevertheless, reports on the inability of BLT models to reject HLA-mismatched human allografts suggest that immune reconstitution is incomplete.[179] An additional drawback of BLT models is the development of a late xGVHD-like syndrome,[180 181] which has been attributed to the carry-over of mature lymphocytes within the solid organ grafts,[177] the maturation of mouse MHC-reactive T cell clones,[182] and macrophage activation.[164] Availability

of human thymus samples is obviously another major limitation, particularly in adults.

HLA transgenic mice

The alternative approach to BLT models for thymocyte education is HLA transgenes of alleles of the latter. Evidence of positive selection of lymphocyte precursors has been described for single-allele knockins for either type I or II HLA.[183–186] For instance, Giannoni *et al* reported that *HLA-A2*02:01* knockin mice increased the engraftment of HLA*02:01-restricted MART-1_{26-35}-specific TCR-transduced HSPCs.[185] *HLA-A2*$^{+}$ NSG mice have also been successfully engrafted with bone marrow HSPCs from non-metastatic cancer patients, demonstrating the development of self-tolerant alloreactive T lymphocytes.[187] In addition, mice carrying transgenes for both HLA type I and II have been generated, which have shown superior adaptive immunity capabilities compared with HLA-negative mouse strains on infectious stimuli.[188–190] Overall, the restriction of T-cell precursors by the HLA transgene(s) has been consistently described in the literature; however, evidence of the application of these models for assessing tumor-specific T-cell development remains very limited, as discussed in the corresponding section below.

Defective lymph nodes

TDLNs are increasingly recognized as relevant structures when it comes to mounting adaptive immune responses to cancer.[47] In fact, the in situ presence of TDLNs is considered one of the main factors behind the recent success of neoadjuvant plus adjuvant PD-1 blockade in comparison with the same treatment given fully adjuvant in patients with melanoma.[191]

As previously described, IL-2Rγ knockout mice are defective in lymph node development. Throughout the mouse body, macroscopic lymph nodes are typically observed as rudiments restricted to the mesenteric region.[131 132] These are usually engrafted with human CD3^{+} and CD20^{+} cells on HSPC transfer,[154 192] but lack follicular structures.[154]

Recent approaches have attempted the challenging task of rescuing lymph node development in immunodeficient mice while keeping the immune system of the host severely suppressed. One of such strategies involves the overexpression of thymic stromal lymphopoietin (TSLP), which signals through heterodimeric receptors composed of IL-7Rα (CD127) and TSLP-R.[193] The overexpression of TSLP (T) restores lymph node development in Rag$2^{-/-}$IL-2R$\gamma^{-/-}$ and Balb/c Rag$2^{-/-}$IL-2Rγ–/–SIRPα^{NOD} (BRGST) mice,[194] suggesting a limited role of colonization by mature lymphocytes on lymph node organogenesis. Of note, B-cell compartmentalization was observed in lymph nodes in humanized BRGST mice, with a less organized T-cell distribution.[195] BRGST mice also developed enhanced cellular and humoral antigen-specific responses on immunization with an exogenous protein.[195]

An independent group has created an additional IL-2Rγ^{null} mouse strain with enhanced lymph node development. Takahashi *et al* created a NOD-scid IL-2Rγ^{null} mouse with a transgene encoding murine IL-2Rγ regulated by endogenous control elements of the RORγt gene, which restricts the expression of mouse IL-2Rγ to lymphoid tissue inducer cells.[196] This approach significantly enhances the development of lymph nodes beyond the mesenteric region, facilitates T-cell engraftment in peripheral blood, and increases plasma IgG levels.[196] In this regard, the biology of lymphotoxin-β also offers opportunities for the induction of lymphoid tissue.

It should be noted, nevertheless, that genetic modifications designed to improve lymph node development have introduced genes encoding murine proteins. These have, in fact, improved the development of murine lymph nodes, but the replication of the complex interactions taking place in secondary lymphoid organs remains to be characterized in this context.

Cancer immunotherapy studies in humanized mice

When it comes to cancer immunotherapy testing, the key question is whether humanized mouse models are able to recapitulate the very essential pillars of cancer immunology in order to be predictive of effects in patients. As such, it is important to have a look at the conditions that are present in each humanized mouse model prior to the tumor challenge in order to correctly interpret the results.

Mature T lymphocytes contained in PBMCs have the key advantage of having undergone the exquisite negative and positive selection processes in the thymus of the donor. Importantly, PBMCs contain a representation of tumor-specific T-cell clones in the range of 0.002%–0.4% in patients with advanced melanoma.[16 17] Provided each mouse is typically engrafted with over 5×10^{6} T lymphocytes, the infusion product should contain a representation of tumor-specific clones, at least in patients with immunogenic tumors.

Regarding specific contributions, spontaneous antitumor activity of autologous PBMCs has been demonstrated by several reports.[197 198] Responses of autologous PBMCs have also been described by Moquin-Beaudry *et al* in tumor models derived from genetically engineered adult skin fibroblasts, in an approach that resembles GEMMs and might not be devoid of immunogenic artifacts due to lentiviral transformation.[199 200] Regarding antitumor activity of ICIs, responses to PD-1 blockade have also been described, which confirms that the immune regulatory function of the pathway is at least partially preserved in the model.[197 200–202] Regarding additional functional capabilities of PBMC-humanized models, intratumoral clonal expansion of lymphocytes has been described in an allogeneic model.[197]

The development of xGVHD in PBMC-humanized models influences the survival of conventional immunodeficient mice. Furthermore, the overt T-cell activation induced by xenoreactivity impacts antitumor activity

readouts.[143] Provided that the stromal and vascular components are of murine origin in cell line-derived xenografts and late-passage PDX, xGVHD might lead to spurious antitumor effects. In fact, PDX in autologous PBMC-humanized conventional NSG mice has been reported to be more infiltrated by T-cells than original tumors.[198] In this regard, a recent report has described a modest antitumor response to the combination of nivolumab and ipilimumab in an autologous melanoma PDX model in MHC double knockout mice.[203]

In addition, PBMC-humanized models are particularly well suited for T cell-targeted therapeutic strategies such as T cell-engaging bispecific antibodies and CAR-T cells.[204–206] In fact, many bispecific T-cell engagers under clinical development (such as those targeting HER2, PSMA, DLL3, and CD19, among others) have been tested in humanized mouse models.[207–212]

TILs are a cancer-specific source for mouse humanization with mature lymphocytes. In parallel to the recent successful development of this treatment modality for patients with advanced melanoma,[213] some groups have reported on TIL-humanized mouse models.[146] Spontaneous antitumor response[141] and enhancement by PD-L1 blockade has been described in such models.[145] Of note, Jespersen *et al* reported responses in melanoma PDX models humanized with TILs that were concordant with clinical responses of patients to TIL therapy in a clinical trial (n=6).[147] This work is, to our knowledge, the one with the most relevant correlative data between humanized mice and matched patients. Data are also remarkable because they prove that a T-cell enriched product induces effective antitumor responses regardless of the lack of human professional APCs and functional lymph nodes. Nevertheless, the need for ex vivo expansion procedures prior to the infusion differentiates TIL models from mouse humanization models with minimal ex vivo processing such as humanization with PBMCs. In fact, it remains to be established which of the two models better reproduces the natural immunity reinvigoration on exposure to systemic ICIs in patients responding to treatment. Moreover, the paucity of TILs in less immunogenic tumors may limit the broader application of this experimental approach.

Models engrafted with HSPCs rely on MHC-driven thymic education in vivo. In conventional models, this renders them tolerant to mouse MHC, but potentially reactive to HLA, even if the latter enter the system as a tumor that is autologous to the HSPC donor.[150 151] In fact, experiments that have explored the influence of HLA-mismatches between immune cells and tumors in HSPC-humanized mouse models have not detected an association with tumor responses.[214 215] By contrast, a previously mentioned innovative work by Moquin-Beaudry *et al*, in which fetal HSPCs and autologous thymus are co-engrafted, reported that the antitumor activity was more pronounced when the genetically engineered fibroblast-derived tumors were allogeneic.[200] Although questions remain regarding minor histocompatibility antigen (MiHA) education in the B(L)T model prior to tumor cell inoculation, Moquin-Beaudry *et al* open new avenues for matched HLA education along with autologous fetal tissue-derived engineered tumor cells. In this regard, in situ transformations of HSPCs/ induced pluripotent stem cell grafts[216] or the engraftment of untransformed tissues along with (L)T grafts to improve such education prior to tumor inoculation might significantly refine these models. HLA knockin mouse strains could provide a similar T-cell education from the beginning of the engraftment of human immune cells, but descriptions of their potential application for cancer immunotherapy remain limited.[203] In fact, the most advanced HLA-knockin strains harbor no more than one transgene for each HLA class, which overlooks the influence of the rest of the alleles of the HLA haplotypes of the patient and thymic human MiHA presentation. This likely leads to alloreactivity on tumor challenge.

Regarding other experimental capabilities, interesting reports have shown that the antitumor capacity of HSPCs is shaped by characteristics of the tumor such as the MSI/MSS status.[217] In addition, experiments of T-cell transplantations from HSPC-humanized tumor-bearing mice to secondary recipients have suggested that tumor-specific T-cell expansion can occur in HSPC-humanized models.[218] Other reports have supported this hypothesis with ex vivo experiments.[214] Nevertheless, these observations have so far been made in allogeneic experiments.

In addition, the multilineage differentiation in HSPC models allows for the modulation of cytokine release syndromes on exposure to T-cell engaging BsAbs, with clinically meaningful insights on the role of myeloid cells on syndrome-driving IL-6 and IL-1 production[219] or on the influence of anti-inflammatory treatments on antitumor efficacy.[220]

For antitumor immune responses to begin, tumor cell antigens are presumably released by cancer cell death and captured by professional APCs that can either present them in situ or travel through afferent lymphatic vessels to the nearest TDLN to do so.[221] In fact, the requirement of certain professional specialized APC subsets such as $CD11c^{+}Clec9a/DNGR1^{+}XCR1^{+}$ DCs, capable of cross-presenting exogenous endosomal antigens onto MHC-I molecules in murine models,[47] suggests that similar processes might be conserved in human cancer immunobiology. For the differentiation of such specialized DCs, it is likely that FLT3L has to be made available in the model.[133]

Myeloid cells are known for their plasticity and have also been recognized as relevant tumor-promoting cells in the TME. In this regard, as previously discussed, a variety of immunocompromised strains have been developed to increase the engraftment of myeloid leukocytes in mice transferred with HSPCs and used for cancer immunotherapy applications. For instance, in the seminal publication of the MISTRG mouse strain by Rongvaux *et al*,[160] tumors were described to be more infiltrated by human $CD11b^{+}$ and $CD163^{+}$ immune cells than

in conventional NSG mice. Strikingly, human tumors grew significantly faster in MISTRG mice.[160] These observations have recently been confirmed by Chiorazzi *et al* with the MISTRG-6 mouse strain,[150] in one of the most ambitious mouse humanization pieces of work published to date in the cancer immunotherapy field. MISTRG-6 mice were engrafted with patient-derived CD34$^+$ HSPCs derived from bone marrow aspirates followed by autologous PDX (melanoma, NSCLC, pancreatic ductal adenocarcinoma, and head and neck squamous cell carcinoma (HNSCC)). Consistently, PDX grew significantly larger in humanized versus non-humanized MISTRG mice.[150] This article set a proof-of-concept of the feasibility of autologous HSPC-humanized cancer models and elegantly shows that the immune system can be co-opted by cancer cells to promote tumor progression, which is consistent with other works.[222–224] Regarding the adaptive immune response in the model, T cells were under-represented as compared with CD19$^+$ and CD33$^+$ cells,[150] and authors did not report data on the outcomes on treatment with ICIs. The work by Chiorazzi *et al* is to be commended for building such an ambitious model, but illustrates that further research is needed to evaluate its adequacy for cancer immunotherapy testing.

The recapitulation of the migration of APCs to the draining lymph node for lymphocyte priming is challenging in these mouse models. Even though recently developed mouse strains have shown improved lymph node development,[195 196] these remain to be tested for cancer immunotherapy applications. Therefore, this surely important step in the immune recognition of cancer cells awaits specific characterizations in humanized models.

It must be acknowledged that there is no humanized in vivo model developed so far that can reliably recapitulate the various key points involved in cancer immunobiology, and each modality comes with its limitations (figure 3). In addition, robust concordance data between humanized mice and matched patients are largely missing in reliable series of cases.

FUTURE DIRECTIONS

The increasing number of potential immunotherapy combinations makes it impractical to test them all in the clinic. The report by D. Thommen's group on a positive association between PD changes induced by anti-PD-1 treatment on human tumor fragments ex vivo and patient-matched tumor responses in the clinic has propelled this technology as a valuable tool to explore other immunotherapy strategies and combinations[225–227] and to confirm preclinical hypotheses.[228]

Although it is tempting to consider that patient-derived 'collect and deploy' in vitro methods could be used to prospectively select the best treatment modality for a given patient, this would require extensive prospective validation and a level of complexity that challenges cost/benefit ratios. With current methods, it is the benefit of future patients that seems to be the most reasonable objective of these methodologies.[53] For instance, a drug that is being considered for entering clinical development could be reasonably prioritized if supporting preclinical data are accompanied by positive results in a series of human tumor fragment cultures and effects in humanized mouse models. In addition, tumor fragment cultures could potentially be useful to prioritize some tumor histologies or subtypes over others for early clinical trials seeking the initial signs of efficacy. Nevertheless, the scientific community should encourage the validation of ex vivo methodologies whenever a significant modification to either the method, the tumor type, or the treatment modality is introduced.

For humanized mouse model development, investigators should consider the strengths and limitations of each selected component in order to address specific scientific questions. Complementarily, ex vivo cultures of lymphocytes gathered from humanized mice with autologous tumor-cell suspensions, with Enzyme-Linked Immuno Spot Assay (ELISPOT), flow cytometry, and histopathology as readouts, could provide relevant mechanistic insights. Techniques with spatial resolution including multiplex tissue immunofluorescence and spatial transcriptomics on the human xenografted tumors may offer important information on the remodeling of the TME with immunotherapy approaches. In addition, other techniques such as TCR sequencing could provide valuable information regarding tumor-specific clonal expansion of lymphocytes in humanized mice.

Finally, the advent of artificial intelligence (AI) is expected to boost preclinical drug development in fully integrated in silico simulations[229] or by assisting the analysis of high-throughput data generated by genomic, transcriptomic, or multiplexed imaging methodologies. In particular, in silico simulations of 3D configurations of proteins and the prediction of protein-to-protein interactions are expected to boost drug development, while reducing the inherent costs of wet lab approaches. The recent success of personalized mRNA vaccines in patients with melanoma, which relies critically on epitope binding prediction algorithms,[230] nicely illustrates the potential of these technologies. Readers are directed to specific reviews for further information.[231 232] Conversely, results from the models can be fed back into the AI for further training.

CONCLUSIONS

In vitro and in vivo methodologies to work with human samples in the laboratory are appealing strategies to refine translational cancer immunotherapy research. Overall, these methodologies pursue the following objectives: (i) confirming hypotheses generated from fully animal models, (ii) predicting clinical

outcomes and aiding clinical trial designs, (iii) being useful as platforms to improve our understanding of drugs that we currently use but lack strong predictive biomarkers of response and (iv) generating new hypotheses directly from human samples.

Given the formidable task of reproducing in the laboratory the intricate complexities of human cancer immunobiology, it is reasonable to find guidance on G. Box's famous quote that states that "no model is perfect, but some are useful". These experimental models and their future refinements are meant to be useful to increase the rate of success in clinical immunotherapy drug development as very much needed by the scientific community involved in cancer immunotherapy research.

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