Defining the optimal murine models to investigate immune checkpoint blockers and their combination with other immunotherapies

M. F. Sanmamed1*, C. Chester2, I. Melero3 & H. Kohrt2

1Department of Immunobiology, Yale University School of Medicine, New Haven; 2Department of Medicine, Division of Oncology, Stanford University School of Medicine, Stanford, USA; 3Division of Immunology and Immunotherapy, Center for Applied Medical Research (CIMA), University of Navarra, Pamplona, Spain

Received 16 December 2015; revised 21 January 2016; accepted 22 January 2016

The recent success of checkpoint blockers to treat cancer has demonstrated that the immune system is a critical player in the war against cancer. Historically, anticancer therapeutics have been tested in syngeneic mouse models (with a fully murine immune system) or in immunodeficient mice that allow the engraftment of human xenografts. Animal models with functioning human immune systems are critically needed to more accurately recapitulate the complexity of the human tumor microenvironment. Such models are integral to better predict tumor responses to both immunomodulatory agents and directly antineoplastic therapies. In this regard, the development of humanized models is a promising, novel strategy that offers the possibility of testing checkpoint blockers’ capacity and their combination with other antitumor drugs. In this review, we discuss the strengths and weaknesses of the available animal models regarding their capacity to evaluate checkpoint blockers and checkpoint blocker-based combination immunotherapy.

Key words: checkpoint blocker, immunotherapy, murine model, humanized mice, tumor microenvironment

introduction

Anticancer drug discovery and development is an inexact and inefficient process, as reflected by the high attrition rate of potential anticancer drugs that enter preclinical testing: 85%–89% fail to gain FDA approval [1, 2]. This high rate of failure is costly on both economic and social levels. Most drugs fail in late stages of development after investments of more than $500 million [3]. The high rate of ineffective compounds entering clinical testing indicates a need for more accurate models to predict efficacy before in-human clinical trials are launched.

The underlying causes of the low predictive value of current models are not fully understood. Evidence suggests that the process of generating cancerous murine or human cell lines, the foundation of historical animal models, results in major and irreversible alterations in biologic properties, including gain and loss of genetic information, alteration in growth and invasive properties, and loss of specific cell populations [4]. Another contributing factor may be the absence of appropriate interactions between the human tumor and human stroma-associated cells, in the case of human xenografts [5].

The advent of immunotherapeutic agents like checkpoint inhibitors adds additional complexity and further jeopardizes the validity of animal models to predict clinical response. While most of the current drugs in oncology have been explored in human tumor xenografts growing in immunodeficient mice, this approach is inadequate to test immunotherapy, because the mice lack a human immune system. Therefore, 'humanization' of the mouse with human functional hematopoietic cells or human knock-in (KI) genes is needed to test drugs that target human immune cells.

In this review, we will describe the different animal models available, and under development, to study checkpoint blockade drugs and their combination with other immunotherapies (Figure 1).

advantage and disadvantage of classical animal models to investigate immune checkpoint blockers

syngeneic murine models

Early efforts in cancer drug screening relied on murine tumor models, particularly leukemia systems, such as L1210 or P388, grafted into immunocompetent syngeneic mice [6]. The US National Cancer Institute (NCI) promoted such an effort from the mid-1950s, adding mouse solid tumor models, as they became available (Colon 38, B16 melanoma, Lewis lung, and M5076 reticulosarcoma) [7].
These models are easily established and have been successfully used to identify the antitumor activity of currently approved checkpoint blockers, including anti-CTLA-4 [8], anti-programmed death (PD)-1 and anti-PD-L1 [9]. In this scenario, the main advantage of syngeneic models is the interaction between tumor cells and a fully competent immune system. However, the dawn of the era of target therapies moved the scientific community toward therapies directed against human tumor targets. Syngeneic models were then partially replaced by human xenograft models and genetically engineered mouse models (GEMMs).

In addition to the absence of human targets, syngeneic models have other important caveats. The rapid growth of murine tumors in syngeneic mouse models does not facilitate the development of the chronic inflammatory environment characteristic of human tumors. In human tumorigenesis, immunologic inhibitory pathways associated with inflammation, such as the PD-1/PD-L1 axis, contribute strongly to tumor microenvironment (TME) remodeling [10]. Finally, murine tumors do not normally reflect the genetic complexity of human tumors because they have lower mutational loads [11] (Table 1).

Despite these limitations, the accessibility of syngeneic models and the importance of working with an intact immune system made them the first experimental approach in immuno-oncology experiments.

**Figure 1.** Description of different available mouse models to study checkpoint blockers. Tumor cell, stroma cell, and immune cell compartments in the different mouse models have been depicted. Green has been used to indicate murine cells, whereas blue indicates human cells. In some models, both human and murine cells coexist in the same compartment; in these cases, green/blue cells appear in the same box. In ‘human xenograft’ and ‘patient-derived xenograft’ models, an ‘X’ highlights the absence of an adaptive immune system. Scars in the flank of the mouse indicate that surgery is needed to engraft the tumor cells. PDX, patient-derived xenograft; HSPC, hematopoietic stem and progenitor cells; PBMCs, peripheral blood mononuclear cells; TIL, tumor infiltrated lymphocytes.

**Genetically engineered mouse models**

Increased knowledge of oncogenesis and the adoption of genetic engineering techniques have made it possible to create spontaneous tumor models in mice. These models provide a more physiologically relevant TME recapitulating some of the oncogenesis steps and localizing tumor growth to a specific and appropriate (orthotopic) site to recapitulate the tissue microenvironment [12].

An extensive array of technologies is employed to engineer the mouse germline with great precision. It is possible to direct expression of a gene of interest throughout a tissue (for example, targeted germline mutations) or the entire organism (for example, targeted germline mutations) [13]. Also, tet-regulated [14] and CRE-inducible alleles [15, 16] allow us to control the timing, duration, and tissue compartment of gene expression or inactivation.

Several groups have precisely modified oncogenes and tumor suppressor genes directly in somatic cells of adult mice, significantly improving the feasibility and flexibility of this genetic engineering approach [17, 18]. These models also better mimic human cancer relative to standard germline GEMMs since tumors typically arise from fewer cells in the context of normal stroma.

One important disadvantage of these models is the low mutation burden since the tumors develop from 1 to 2-mutated
oncogene transgenes even though surrogate tumor antigens can be engineered into them [19]. Transgene expression is commonly driven to supra-physiological levels, such that protein expression often exceeds that ever encountered in patients [20]. Also, random integration of a transgene can result in unexpected phenotypes and increased variability in the presentation and quantity of tumor. Furthermore, GEMMs are time-consuming and expensive since extensive experience and infrastructure are required to ensure the use of accurate models and to achieve sufficiently populated preclinical studies. Notably, with the advent of CRISPR/Cas9 technology [21], and with it the ability to perform complex gene editing with relative ease and speed, it is expected to improve most of these drawbacks and dramatically enhanced the value of GEMMs.

Table 1. Advantage and disadvantage of murine models to investigate immune checkpoint blockers

<table>
<thead>
<tr>
<th>Model</th>
<th>Advantage</th>
<th>Disadvantage</th>
<th>Complexity</th>
<th>Cost</th>
<th>Human Relevance</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syngeneic murine</td>
<td>- Easiness and economic</td>
<td>- No human targets</td>
<td>Low</td>
<td>$</td>
<td>Low</td>
<td>Proof of concept of checkpoint blockers antitumor effect and mechanism of action</td>
</tr>
<tr>
<td></td>
<td>- Intact immune system</td>
<td>- No chronic inflamed tumor environment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genetically engineered mice (GEM)</td>
<td>- Orthotopic tumors</td>
<td>- Time-consuming and expensive</td>
<td>Medium</td>
<td>$$</td>
<td>Medium</td>
<td>Proof of concept of checkpoint blockers antitumor effect and mechanism of action</td>
</tr>
<tr>
<td></td>
<td>- More physiological oncogenesis</td>
<td>- Unexpected phenotypes and increased variability in the presentation and quantity of tumor secondary to random integration of transgenes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Intact immune system</td>
<td>- Supraphysiological levels of transgene expression</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Nonhuman immune system</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human knock-in mice</td>
<td>- Similar to GEM</td>
<td>- Low tumor mutation burden</td>
<td>Medium</td>
<td>$</td>
<td>Medium</td>
<td>Selection of antihuman checkpoint blockers</td>
</tr>
<tr>
<td></td>
<td>- Human targets</td>
<td>- Nonhuman immune system</td>
<td></td>
<td></td>
<td></td>
<td>Study toxicity of checkpoint blockers.</td>
</tr>
<tr>
<td>Human xenografts</td>
<td>- Reproduce human genetic tumor complexity</td>
<td>- Not intact immune system</td>
<td>Low</td>
<td>$</td>
<td>Low</td>
<td>Not applicable for study checkpoint blockers lymphocytes dependent</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Not reproduce human tumor microenvironment (TME) complexity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Murine stroma, non-species-specific interactions</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient-derived xenograft (PDX)</td>
<td>- Reproduce human TME architecture and genetic complexity</td>
<td>- Not intact immune system</td>
<td>High</td>
<td>$$$</td>
<td>High</td>
<td>Not applicable for study checkpoint blockers lymphocytes dependent</td>
</tr>
<tr>
<td></td>
<td>- Easiness</td>
<td>- Murine stroma, non-specie-specific interactions</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Human tumor-immune cells interface</td>
<td>- Xenograft versus host disease (xGVHD)</td>
<td>Medium</td>
<td>$$</td>
<td>Medium</td>
<td>Screening of checkpoint blockers antitumor effect and mechanism of action</td>
</tr>
<tr>
<td>Immunoavatar</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemato-lymphoid humanized mice</td>
<td>- Development of a complete human immune system</td>
<td>- Not fully and physiologic maturation of human immune cells</td>
<td>High</td>
<td>$$$</td>
<td>High</td>
<td>Study of checkpoint blockers antitumor effect and mechanism of action</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Sophisticated and expensive</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immune-PDX</td>
<td>- Human tumor complexity with human tumor infiltrated lymphocytes (TILs)</td>
<td>- Limited tissue for performing experiments</td>
<td>High</td>
<td>$$$</td>
<td>High</td>
<td>Study mechanism of action checkpoint blockers (modification of human TME)</td>
</tr>
<tr>
<td></td>
<td>- Human tumor complexity with human TILs</td>
<td>- Not immune cell recruitment to the tumor site</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Organoids/explants</td>
<td>- No murine stroma</td>
<td>- No in vivo model (different temperature, and hormones, cytokines)</td>
<td>Medium</td>
<td>$</td>
<td>Medium</td>
<td>Study mechanism of action checkpoint blockers (modification of human TME)</td>
</tr>
<tr>
<td></td>
<td>- Cheap</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
All in all, GEMMs provide the only opportunity to evaluate checkpoint blockers delivery, therapeutic response, and biomarker expression in the presence of a competent immune system.

human KI mice

One attractive advantage of GEMMs is the opportunity to introduce more than one transgene or KI gene for human checkpoint molecules in a murine system. KI models thus allow researchers to study drugs that only recognize human version of the checkpoint molecule. When the human checkpoint molecule does not cross-react with its corresponding murine counterpart, both the human version of the ligand and the receptor have to be KI genes. In this regard, KI mice offer the possibility to study clinical grade checkpoint blockers targeting human checkpoints in the context of a fully functional immune system.

KI models also provide the opportunity to explore the capacity of checkpoint blockers to elicit cell-mediated cytotoxic effector functions such as antibody-dependent cellular cytotoxicity and phagocytosis. The recent emphasis on the importance of the IgG isotype of anticancer antibodies and their engagement of activating and inhibitory Fc gamma receptors (FcγR) necessitates drug screening in human systems [22, 23]. Checkpoint blocker mAbs with different human Fc may be tested in human FcR KI mice to select the IgG isotype with the greatest antitumor effects and least toxicity [24].

Perhaps, the most important advantage of the KI model is our ability to evaluate autoimmune or pro-inflammatory side effects associated with candidate human therapeutic antibodies. One illustrative example of this is the human CTLA-4 gene KI mouse developed by Lute et al. [25]. In this model, after treating CTLA-4 KI mice with anti-hCTLA-4 mAbs, the mice developed the autoimmune effects observed in patients treated with anti-CTLA-4 mAbs [25].

The caveats of these KI models are common to syngeneic and GEMMs. In all systems, conclusions are limited by working with a murine immune system. With the adoption of CRISPR/Cas9 technology, the combination of human KI mice bearing human FcRs and human checkpoint molecules may be developed, increasing the relevance and interest of these models.

human xenografts

Starting in the mid-1980s, the availability of athymic mice (nu/nu) and subsequent severe combined immunodeficiency (SCID) mice allowed the possibility of screening anticancer therapeutics against human tumor xenotransplants in mice [26].

The introduction of immunodeficient models promised to accelerate the identification of drugs more likely to have activity in human solid tumors. However, the results obtained with these surrogate xenograft model systems have inadequately predicted human clinical outcomes, particularly in the case of targeted therapeutics [4, 27]. One of the given reasons is the need to grow tumors in immunodeficient mice, which are not reflective of the dynamic process of tumor–immune surveillance and immune-mediated editing [5] and where immunomodulatory effects of the antineoplastic drugs cannot be studied [28].

Another common concern comes from the limitations of artificially maintained tumor cell lines that do not stably recapitulate the phenotypes or genotypes of human tumors. Finally, the interactions between human tumor cells and mouse stroma cells can impair the interpretation and translation of the results.

Despite these pitfalls, human xenografts have been extensively used to directly test cytotoxic drugs. Chemotherapy and more advanced antitumor drugs that target mutated or overexpressed tumor proteins are suitable agents for testing in human xenografts. The ease by which cells can be genetically manipulated ex vivo before xenotransplantation makes human xenografts a compelling preclinical model to screen cytotoxic drugs. However, the need to work with immunodeficient mice limits investigation with checkpoint blockers.

patient-derived xenograft

To better preserve the genomic integrity and tumor heterogeneity observed in patients, some groups xenograft freshly resected pieces of primary or metastatic solid tumors into immunodeficient mice. This procedure, known as patient-derived xenograft (PDX), allows serial passage and expansion of tumors through successive generations of murine hosts without intervening cell culture (reviewed elsewhere [29–31]).

Tumors are implanted as pieces or single-cell suspensions either alone, embedded in Matrigel, or mixed with human fibroblasts or mesenchymal stem cells. The most common site of implantation is subcutaneous implantation on the dorsal region of mice although orthotopic implantation is sometimes used. Orthotopic PDX models may better replicate the TME than subcutaneous models, and thus may be more physiopathologically relevant [32]. Regardless of the transplantation site, the cellular complexity and architecture of PDX tumor models remains remarkably faithful to the tumor in its natural state [33].

The main advantage of PDX models is that tumor growth occurs in a physiologically relevant milieu of oxygen, nutrients, and hormones (in cases where the murine hormones are recognized by the human tumors). In contrast to in vitro propagated cultures, cytogenetic analyses of PDX models reveal strong preservation of the chromosomal architecture observed in patient tumors [29]. Many PDX models have proved to exhibit levels of chemoresponsiveness that mirror the cytotoxicity observed in the chemo-treated patient who donated material for the PDX [30]. Recently, comprehensive screening of anticancer drugs in PDX models has validated this model as a very promising platform [34]. Cumulatively, these results emphasize the fidelity of these models to the natural disease state.

However, although the incorporation of PDX models in cancer research brings substantial improvements, this model suffers the same primary limitation as human xenografts when attempting to study immune checkpoint blockers: PDX mice lack a fully functional immune system. A novel variant of PDX models, called immune-PDX (iPDX) model, provides the advantages of PDX in the context of human tumor-infiltrating lymphocytes (see ‘iPDX model’ section below).

humanized mice: new animal models to study checkpoint blockers

Traditional in vivo studies of cancer therapeutics have prioritized tumoral targets and de-emphasized the characteristics of the host. In stark contrast, the study of checkpoint blockers requires a focus on host–tumor interactions, specifically the immune–tumor interface. As mentioned above, fully murine
models can be used to study checkpoint-mediated immunity because the mouse immune system remains intact. However, inherent differences among human and murine immune systems and the lack of human targets in the mouse limit investigations of the antitumor effects of immunotherapies [35]. Therefore, efforts have been made to ‘humanize’ the murine immune system.

For many years, the biggest limitation to humanizing the murine immune system was the absence of mouse models that allow engraftment of non-tumoral tissues. In 1983, Bosma et al. [36] reported a spontaneous, autosomal-recessive mutation in the protein kinase, DNA-activated, catalytic polypeptide (prkd) gene, which severely impairs lymphopoiesis, mimicking the clinical phenotype of human severe combined immunodeficiency (SCID) and subsequently named as SCID mice. The lack of both T and B cells in prkd mutants allowed for the successful transplantation of human-derived hematopoietic stem cells (HSCs) [37], peripheral blood mononuclear cells (PBMCs) [38], and fetal tissues [39]. In 1980, Makino et al. described non-obese diabetic (NOD) mice, which are characterized by an impaired innate immunity [40]. Crossing SCID and NOD strains resulted in mice with defects in both innate and adaptive immunity [41]. The NOD/SCID mouse has served as the model of choice for the engraftment of functional human cells for the last two decades. Later, other strains have been developed using genetic engineering to induce mutations at the recombination-activating gene 1 (Rag1) and Rag2 loci that prevent mature T- and B-cell development [42, 43]. Arguably, the most relevant improvement in immunodeficient mice in the genetic engineer era has been the development of homozygous mice for a targeted mutation in the interleukin-2 receptor γ chain (IL-2γ) gene. The absence of the IL-2γ completely prevents natural killer (NK)-cell development; NK cells are the primary innate cell population mediating tissue engraftment and rejection. Crossing IL-2γnull mice with mice carrying the Rag 1 or 2null (Rag 1 or 2null IL-2γnull; i.e. BRG mice) [44] or prkd mutation (NOD/SCID IL-2γnull; i.e. NSG mice) [45] results in even more profound immunological defects, improving the success of engraftments of tumoral and non-tumoral tissues.

PBMC, HSC, or human tumor explants are engrafted in last-generation immunodeficient mice to create humanized animal models where the interrelation between a human tumor and human immune cells can be studied.

immunoavatar models

One simple and economic method to generate humanized mice is to engraft human PBMCs in severe immunodeficient mice. PBMC engraftment allows human tumor xenografts from either cell lines or tumor explants to be studied in an autologous or heterologous immunologic context [46–48]. The use of PBMCs obfuscates the need for sophisticated cell isolation techniques and leads to a stable engraftment of activated T-cell populations.

Early work with immunoavatars demonstrated the feasibility and applicability of this approach. Immunoavatars were used to screen and identify antihuman CTLA-4 mAb clones with the capacity to activate human PBMCs (hPBMCs) in xenografted SCID mice [49]. Also, Fisher et al. [46] tested the antitumor efficacy of PF-05082566 (a fully human anti-4-1BB IgG2 mAb) in an NOD/SCID IL-2γnull mouse bearing human prostate carcinoma (PC3) cell line xenograft and adoptively transferred with human PBMCs intraperitoneally. PF-05082566 lacks rodent 4-1BB cross-reactivity, thereby precluding testing in murine syngeneic models. In the immunoavatar model, it was possible to demonstrate a significant control in tumor growth in mice treated with the anti-4-1BB mAb [46]. Our group has also reported tumor control in immunoavatar models using BALB/c Rag2–/–IL2rg–/– mice: human colon xenograft growth was significantly slower in mice engrafted with human PBMCs and treated with urelumab (a fully human anti-4-1BB IgG4 mAb) or nivolumab (a fully human anti-PD-1 IgG4 mAb). Moreover, we replicated the same results using a human gastric explant and autologous PBMCs [47].

The primary drawback of the immune-avatar model is the robust human xenograft versus host disease (xGvHD) that develops a few weeks after hPBMC engraftment. The xGvHD is thought to be due to major histocompatibility complex (MHC) mismatch between human T cells and mouse cells [50, 51]. To avoid this xenoreaction, some groups have proposed using knockout mice devoid of the genes encoding MHC class I or II molecules. As an alternative, we have reported that depleting CD4-positive cells from PBMCs before engraftment dramatically reduces xGvHD, and mice remain asymptomatic after up to 3 months of follow-up [47]. This finding suggests that xGvHD in immunoavatar models is human CD4+ T-cell-dependent. Also, in these models, cultures of T cells derived from tumor-infiltrating lymphocytes can be used to enhance antitumor reactivity and potentially mitigate xenoreactivity.

The importance of testing immune-modulating agents in a model with a human immune–tumor interface makes immunoavatar an attractive first screening model for checkpoint blockers and other immunomodulatory drugs.

hemato-lymphoid humanized mice

A more sophisticated method to xenograft human immune cells in an immunodeficient mouse is the transplantation of CD34+ human hematopoietic stem and progenitor cells (HSPCs). In these models, current protocols vary widely. Human CD34+ cells can be isolated from cord blood, bone marrow, peripheral blood, and fetal liver [35]. Isolation is usually carried out by Ficoll separation and subsequent incubation with human CD34+ magnetic selection beads. Currently, human cord blood is the most commonly used HSPC reservoir, because it is readily available and demonstrates a higher rate of successful engraftment compared with cells isolated from adult bone marrow [32].

Interspecies differences in the specificity of growth factors and cytokines represent another serious hurdle when constructing a human immune system in immunodeficient mice. Without the proper milieu of cytokines, hormones, and growth factors, the human hematopoietic and immune system cannot develop and progress. Human-specific cytokines are necessary for the generation of functional human immune cells and their proper hierarchical organization. This is of special importance for the proper development of the innate immune system. To provide human cytokines, researchers administer exogenous cytokines or cytokine-encoding plasmids [53, 54]. However, high systemic concentrations of cytokines can result in artificial
effects such as the mobilization and exhaustion of HSCs or supraphysiological cell frequencies [55]. In contrast, knocking in human cytokine genes to replace their mouse counterparts has the advantage of ensuring appropriate tissue-, cell-, and context-specific expression of the human cytokine [56].

Recently, Rongvaux et al. have generated immunodeficient mice named MITRG in which the genes encoding the human version of macrophage colony-stimulating factor M-CSF, IL-3, granulocyte-macrophage colony-stimulating factor GM-CSF, and thrombopoietin were knocked in to their respective mouse loci in a BALB/c Rag2-/-IL2rg-/- background. All these cytokines favor the development and survival of monocytes and macrophages. The same group generated a variant of MITRG named MISTRG, in which the mice carry a bacterial artificial chromosome transgene encoding human signal regulatory protein α (SIRPα) in addition to other human KI genes. Human SIRPα binds to human CD47, a transmembrane protein constitutively expressed on human cells. In the MISTRG model, SIRPα is expressed in murine myeloid cells and ligation of SIRPα by CD47 transmits a ‘don’t eat me’ signal, enabling mouse phagocytes to ‘tolerate’ and not engulf engrafted human cells. MISTRG mice develop functional macrophages with an adequate response to Listeria and Influenza infection, and also can produce functional NK cells, which has historically been a challenge in earlier humanized models. In the initial proof-of-concept experiment, MITRG and MISTRG mice were used to explore the immune infiltration of a human melanoma cell line (Me290); interestingly, within the mice, human immune cells developed from CD34+ HSPCs and infiltrated melanoma cells. The infiltrating cells displayed a M2 polarized phenotype [56]. These results signify the initial successes of a new generation of humanized mouse models that faithfully recapitulate of human–immune interactions.

A crucial next step in the development of hemato-lymphoid humanized mice for cancer research will be to study the immune infiltration of a patient-derived tumor xenograft using autologous human CD34+ HSPCs. Autologous models are more relevant, but also more challenging. The collection of tumor cells and HPSC from the same individual can be difficult to coordinate and co-engraftment of HPSC and patient tumor presents new unexplored challenges, as the engraftment of HPSC and patient tumor may occur at different rates.

Despite the progress in hemato-lymphoid humanized mouse models, many challenges remain before their widespread adoption in translational research. Crucially, any established adaptive immune system is naive and an appropriate antigen-specific T-cell response is limited as human T cells cannot be adequately educated through human leukocyte antigen restriction within the mouse thymus; the mouse thymus lacks the expression of human MHC molecules [57]. Furthermore, human CD47 is absent on mouse red blood cells (RBCs). Without human CD47, RBCs are phagocytosed by human macrophages and mice develop anemia, limiting the duration of experiments. Finally, even after rigorous depletion of T cells, there is a risk of xGvHD caused by de novo T-cell generation although the onset of the wasting disease can be expected at a later time point than in immunoavatar models.

Hemato-lymphoid humanized mice represent one of the most attractive animal models to study checkpoint blockers and other treatments targeting immune cells. The potential to recapitulate human tumor–immune interactions in a complete, human-developed system differentiates them from other models.

**iPDX models**

PDX models represent the best available models for faithfully reproducing the human TME architecture and complexity. However, these models do not recapitulate the dynamics of infiltrating immune populations because human tumor infiltrated lymphocytes (TILs) do not survive passage between different mouse hosts. One variation in the traditional PDX model is the iPDX, which differs primarily in that the experiments are conducted in the first passages, before the human stroma is replaced by murine stroma. In this model, human TILs are available in the TME and are suitable to be targeted with mAbs administered systemically to the mice.

The first reference to iPDX models was published in 1996, when Williams et al. [58] successfully xenografted human lung cancer biopsy tissue into the subcutis of SCID mice. In 143 of 148 xenografted mice, architecture of the tumor and tumor/TIL interface was maintained up to 22 weeks [58]. Later, the same group demonstrated the use of exogenous IL-12 as an effective antitumor treatment in the same model [59–61]. Recently, Yokota et al. have successfully engrafted omental metastasis from ovarian cancer patients in NSG mice peritoneum. This model allowed the authors to study a viable human TME in an orthotopic site. TILs remain immunosuppressed in this context and the mechanism of human T-cell immunosuppression was able to be characterized [62].

The primary advantage of the iPDX model is the human species-specific interaction among tumor and immune cells. iPDX model offer a very attractive approach for studying human tumors in the same context as they grow in patients. Additionally, the repertoire of human T cells is enriched for clones recognizing tumor antigens, minimizing the xGvHD, and increasing the number of specific antitumor T cells.

The greatest limitation to successful iPDX models is the paucity of tissue available to perform experiments. Because passing the tumor to expand it is not desired in iPDX settings, researchers are confined to the material in the biopsy sample. In our experience, the average piece of tumor material allows experiments with 6–12 mice. Experiments are also constrained by their duration: after 3–4 weeks postengraftment, murine innate cells partially replace their human counterparts. Finally, and critically, iPDX models do not have the capacity to study immune cell recruitment to the tumor site. When considering the factors involved in long-term tumor surveillance, the inability to assess tumor trafficking might be a serious limitation.

Despite these drawbacks, the iPDX model is the best candidate for evaluating agents that target checkpoint blockade pathways in the human TME. In iPDX models, the TIL populations and their phenotypes are preserved and the TME mirrors that of the cancer patient donor.

**animal models to study synergy between checkpoint blockers and other immunotherapies**

The enormous success of checkpoint blockers, namely anti-PD-1 and anti-PD-L1 mAbs, has accelerated interest in combination
studies of these therapies to improve their efficacy and widen their applicability [63]. Recently, the success of the combination of anti-PD-1 and anti-CTLA-4 has encouraged multiple clinical trials combining anti-PD-1 mAbs with other agents [64].

Beyond the anti-CTLA-4 and anti-PD-1 mAb combination, checkpoint blockers combined with other immunomodulatory agents have demonstrated synergistic effects in a variety of preclinical animal studies (reviewed elsewhere [65]). Most of these preclinical studies have been developed in syngeneic murine models that appeal because of their ease-of-use and intact and functional immune system. Clinical trials testing many of these combinations are currently ongoing and efforts are underway to appraise the accuracy of the traditional preclinical models for predicting combination clinical success.

The spontaneous carcinomas that occur in GEMM are highly resistant to immunotherapy approaches and likely to represent a more predictive model for translational research [66]. In such a way, Morales-Kastresana et al. [67] have tested combinations of three immunostimulatory monoclonal antibodies (anti-OX40, -PD-L1, and -CD137) and adoptive therapy with specific tumor antigen-activated T cells in a transgenic mouse model that spontaneously develops hepatocellular carcinoma tumors. A clear synergy was observed between the triple combination of three mAbs and adoptive transfer of antitumor-specific cells. A similar result was achieved by Uno et al. [68] with the combination of three agonistic mAbs targeting members of the TNFR family (anti-DR-5, -CD40, and -CD137) in an autochthonous, methylcholanthrene-induced primary fibrosarcoma. The onco-genes of GEMMs favors the development of tolerogenic TMEs with dysfunctional T cells displaying expression of multiple checkpoint molecules. The upregulation of checkpoint molecules and the presence of dysfunctional T cells make these models more relevant for testing synergies between checkpoint blockers and other immunostimulatory agents.

Immunomodulatory agents that do not cross-react with mouse molecules necessitate the use of human KI or humanized models to better predict the clinical outcome of combinatorial regimens. To date, there is limited experience with testing checkpoint inhibitor combinations in humanized mouse models. To our knowledge, only Sanmamed et al. have reported results in immunoavatar mice, combining a checkpoint inhibitor (nivolumab, anti-hPD-1) with an immunostimulatory mAb (urelumab, anti-hCD137). In this model, the authors were able to detect the expression of PD-1 (TILs) and PD-L1 (tumor cells and antigen-presenting cells) in the human TME and also CD137 (TILs) expression. Even when differences in tumor growth were not significantly different between monotherapy and combination therapy, the model was suitable to develop mechanistic studies that support this combination in the clinic [47]. This experience represents an initial proof of concept that combination strategies can be modeled in humanized mouse models.

alternatives to animal models to study checkpoint blockers

Humanized animal models are the only model that allows us to study, in vivo, the interactions between a human immune system and tumors. However, the murine–human interaction could limit the relevance and translational value of these models. In this context, innovative patient-unique ex vivo systems provide another compelling alternative. Foremost in this class of models are 3D organoids and 3D human tumor explant cultures. These systems offer ‘cleaner’ experimental conditions free of murine cells and enjoy a comparable architecture and complexity to the human TME.

Organoids are three-dimensional organized clusters of epithelial and mesenchymal cells growing in culture from an embryonic or pluripotent stem cell. These systems combine the accurate multilineage differentiation and physiology of in vivo systems with facile in vitro manipulation of transformed cell lines [69]. On the other hand, human explants can be obtained directly from surgery or biopsy of cancer patients and grown in vitro for short periods of time. In short-term conditions, slices of tumor tissue can maintain many in vivo properties, including three-dimensional growth, maintenance of tissue organization/structure, and tumor–immune cells interactions [70].

In combination with accurate and sensitive read-outs, these systems could facilitate our understanding of the mechanism of action and biology of checkpoint blockers. New technology platforms that provide single-cell analyses, such as drop-seq or CyTOF [71], could be useful companion tools to explore the effects of checkpoint blockade on immune subsets, and tumor, endothelial, or other stroma cells.

conclusions

All existing animal models have certain caveats that constrain their utility. Knowledge of these limitations is essential for obtaining preclinical results that are meaningful for clinical translation.

Fully murine models are the best approach for initial screening. It is more desirable to use GEMMs, as they mimic oncogenesis better and facilitate the development of characteristics of chronic inflammation, where most of the checkpoint molecules are expressed. The flexibility of human KI mice offers a good possibility to study, in an intact immune system, checkpoint blockers that exclusively react with human targets.

Next-generation models could be immunoavatar mice that, even with limitations such as xGvHD, offer a powerful approach for studying the effect of checkpoint blockers on the antitumor activity of human immune cells against human xenografts.

In our opinion, hemato-lymphoid humanized mouse models are the most promising animal models to test the antitumor effects of checkpoint blockers and other immunotherapy strategies. Hemato-lymphoid models allow the development of a complete human immune system in a human tumor-bearing mouse. However, we need to overcome important obstacles related to the physiological maturation of human immune cells in these models.

iPDX models provide an accessible model for studying how checkpoint blockers modify the human TME, but their broader utility is limited. Xenografts require among 1–2 months to start to grow and very few animals can be xenografted per sample.

Finally, we come from an era dominated by a strong focus on parenchyma tumor cells, where xenografts growing in immunodeficient mice were considered sufficient for drug screening. The arrival of therapies targeting the immune system against the
malignant tissue highlights the importance of the host. This new perspective in understanding oncogenesis has improved the treatment and outcome of cancer patients across a range of histologies. Hopefully, the development of animal models based on these principles will improve our capacity to screen and develop more efficacious anticancer therapies, substantially improving the outcomes of patients.

acknowledgements

A special thanks to our dear friend, Holbrook, who dedicated his life to cancer research, patients, and working towards a cure. We, the rest of the authors, will keep pushing as inspired by him to make a difference in this world, where time is always too short.

funding

This work was partially supported by Sociedad Española de Oncología Médica to MFS (no grant numbers apply). Financial support was from MICINN (SAF2011-22831 and SAF2014-52361-R) to IM.

disclosure

The authors have declared no conflicts of interest.

references

49. Matsunuma T, Kametani Y, Ando K et al. Functional CD5+ B cells develop predominantly in the spleen of NOD/SCID/gamma(null) (NOD) mice transplanted either with human umbilical cord blood, bone marrow, or mobilized peripheral blood CD34+ cells. Exp Hematol 2003; 31(9): 789–797.