Slov Vet Res 2022; 59 (4): 173–84
DOI 10.26873/SVR-1513-2022
UDC 606:616-056.7:616-006.4:602.68:606:61:615.37:57.084

Review Article

PRECLINICAL MOUSE MODELS IN ADOPTIVE CELL THERAPIES OF CANCER

Uroš Rajčević[1]*, Anže Smole[2]*

[1]Lek, d.d., Technical Research and Development, Kolodvorska cesta 27, 1234 Mengeš, Slovenia, [2]National Institute of Biology, Department of Genetic Toxicology and Cancer Biology, Immunology and Cellular Immunotherapy (ICI) Group, 1000 Ljubljana, Slovenia

*Corresponding author, E-mail: uros.rajcevic@novartis.com, anze.smole@nib.si

Abstract: Engineered T cell-based therapies are an advanced approach for cancer immunotherapy using genetically modified T cells. To date, CD19 and BCMA targeting Chimeric Antigen Receptor (CAR) T cells have been approved for the treatment of certain hematologic malignancies. The success of CAR-T cells is offset by limited efficacy, particularly in solid tumors, and safety risks. Preclinical *in vivo* research, which is highly dependent on reliable mouse models, has been a cornerstone of the success story of adoptive cell therapies and continues to provide invaluable information for the development of the next generation of cellular immunotherapies. In this review we describe four of the most common preclinical mouse models: xenograft models, syngeneic models, immunocompetent transgenic models and humanized mouse models. All of these have advantages and disadvantages and no mouse model can fully recapitulate the human situation because of inherent differences and treatment complexity. Reports suggest that using a combination of mouse models in preclinical *in vivo* research prior to translating the treatment to humans in clinical trials can help incrementally improve the quality, safety, and efficacy of the treatment and provide more comprehensive information than a single model.

Key words: mouse model; xenograft; syngeneic; transgenic; humanized; CAR-T; adoptive cell therapy

Introduction

Adoptive cell therapy (ACT) is a next-generation approach to treating cancer based on immune cells engineered to specifically recognize and effectively eliminate cancer cells. T-cell therapy using chimeric antigen receptors (CARs) has emerged as a leading approach in ACT (1). CARs are designed receptor molecules that merge specificity of monoclonal antibodies with the signalling capacity and effector functions of the T cell receptor (TCR) in T cells (2). The initial CAR designs, referred to as first-generation CARs, include an extracellular antigen-binding domain, usually in the form of a single-chain variable fragment (scFv) derived from an antibody, linked to intracellular signalling domains, most often derived from the components of the TCR complex, such as the CD3 zeta chain (3, 4). This molecule is capable of recognizing antigens independently of HLA (human leukocyte antigens) presentation but does not support long-term T cell persistence and effector responses due to its limited signalling capacity (5). The second-generation CAR design incorporates additional co-stimulatory domains such as CD28 and 4-1BB (TNFRSF9) that enhance expansion, effector functions and persistence of CAR-T cells (2). The second-generation CAR-T design was the basis for successful clinical trials in relapsed or refractory paediatric and adult blood malignancies (6-10) that have led the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) to approve CD19-targeting CAR-T cells in 2017

Received: 29 June 2022
Accepted for publication: 16 September 2022

and 2018, respectively and BCMA-targeting CAR-T cells in 2021 (11). However, adoptive cancer immunotherapy is associated with safety risks such as cytokine release syndrome (CRS) and neurologic toxicities (12), which have led to life-threatening complications (13). In addition, the efficacy of cellular immunotherapy in solid tumor as well as hematologic malignancies is limited, due in part to the immunosuppressive tumor microenvironments, intrinsic T-cell dysfunction (14) and lack of unique surface antigens (15). Various approaches have been pursued to increase the efficacy of adoptive immunotherapy in cancer, and preclinical mouse models are currently irreplaceable to validate their efficacy and safety. Excellent reviews present the use of preclinical models for adoptive cell therapies (16-18). Here, we focus on how preclinical mouse models have supported recent advances in the development of next-generation cellular immunotherapies.

Human xenograft models

A xenograft is a cell, tissue, or organ transplant from a donor that is of a different species then the recipient. In the use of mouse models for the study of human diseases, the most common type of xenograft is the transfer of human tissue, including cells and biopsies, to a mouse recipient. The recipient must be immunocompromised to avoid rejecting foreign human cells. Cells are applied either non-original place (ectopic model) or in the organ from which the tissue is derived (orthotopic model) (reviewed in (19)). In this way, researchers can translate *in vitro* findings into preclinical *in vivo* stage to evaluate the efficacy and safety of cellular immunotherapy in a given disease.

Patient-derived cancer xenografts (PDX) are a simulation of a specific patient's tumor in an animal model. The various types of immunocompromised mice used in tumor xenograft models lack part or most of the immune system so they cannot reflect the immune system of humans. Their immune response to tumors is simplified and does not capture the full complexity of the response. In the past, different types of immunocompromised mice were developed to mimic the human immune system to varying degrees.

The development of immunocompromised mice dates back to athymic mice reported as early as 1966 (20), non-obese diabetic severe combined immune-deficient mice (NOD SCID) and improved NOD SCID mice with Interleukin 2 Receptor Subunit Gamma (IL2Rg) deficiency (16, 21, 22). Since then, the use of NSG (NOD SCID IL2Rg-) (reviewed in (23)) in CAR-T has been reported by several authors including (24). The NSG mouse was developed by backcrossing the *Il2rg−/−*mouse resulting from a complete null mutation onto NOD/ShiLtSz-Prkdc SCID mouse (25). The NOG mouse was developed by backcrossing the *Il2rg−/−* mouse resultin from a truncated intracellular signaling domain onto NOD/ShiLtSz-Prkdc SCID mouse (26). In NOG mice, the *Il2rg* mutant gene is expressed and produces a protein that binds cytokines but does not signal. Conversely, *Il2rg* gene expression does not occur in NSG mice (27, 28). The use of the xenograft models gives us opportunity to assess the effect of the human CAR-T cells on human tumors but there are no interactions with other immune cells or healthy tissues. Nevertheless, xenograft models are often used as the initial preclinical mouse model in proof-of-concept studies in the development of next-generation cellular immunotherapies. They also allow functional validation of engineered human T cells including tumor targeting, anti-tumor activity, secretion of cytokines, tonic signaling, and intrinsic dysfunction such as T cell exhaustion. Here are selected recent examples that represent only a snapshot of numerous studies in this highly active and explored field of research.

As described in the introduction, the development of second-generation CAR-T cells that contain costimulatory domains in addition to CD3 zeta has been critical for clinical efficacy. Currently, so-called third-generation CAR-T cells, which integrate multiple costimulatory domains into the same CAR molecule, are being tested for efficacy and safety (29). Xenograft models have been useful in the development of second and third generation CAR-T cells (30). Route of administration is important in solid tumors (31). Xenograft models revealed the main differences between the two domains, CD28 and 4-1BB (TNFRSF9). Exhaustion was ameliorated by 4-1BB (TNFRSF9) and exacerbated by CD28 (32). CD28 promoted faster tumor regression, while 4-1BB (TNFRSF9) promoted multiple cytokine secretion (33).

CAR-T cells, which can be remotely controlled by the addition of small-molecule were tested

in immunodeficient NSG mice. The authors demonstrated that the use of a split receptor, in which antigen recognition and intracellular signaling domains assemble into a functional unit only after the addition of a heterodimerizing small molecule, allows remote control of the activity of the engineered CAR-T cells. Such regulation provides additional control of the T cell activity, with the rationale of improving safety (34).

Another landmark study at the interface between cellular immunotherapy and synthetic biology is the development of designer T cells equipped with tailored therapeutic response programs through the use of synthetic Notch receptors (synNotch). Using NSG mice with subcutaneously implanted CD19 negative or CD19 positive target cells, authors demonstrate that their system functions as designed *in vivo*. Specifically, they demonstrated *in vivo* expression of cytokines and bi-specific tumor-targeting antibodies by the SynNotch T Cells (35).

Distinct approach that allows additional control over the injected CAR-T cells to increase safety, but also to allow multiple antigen targeting to mitigate potential antigen escape in CAR-T cell therapy, is the prototype universal immune receptor called SpyCatcher. This universal immune receptor enables covalent binding of targeting ligands to the T cell surface using SpyCatcher-SpyTag chemistry. The SpyCatcher immune receptor redirects primary human T cells by addition of SpyTag-labeled targeting ligands *in vivo* in a solid tumor xenograft model. (36).

As mentioned in the introduction, intrinsic dysfunction of T cells is one of the important limiting factors in cellular immunotherapies. One such example is T cell exhaustion, which leads to defects in T cell functionalities. In an attempt to counteract exhaustion, CAR-T cells were engineered to overexpress the transcription factor c-Jun. In this study, human xenograft models in immunocompromised NOD-SCID-*Il2rg*−/− (NSG) mice were used as models to demonstrate enhanced expansion, improved function, limited terminal differentiation and enhanced anti-tumor activity (37).

Current clinically used CAR-T cells use lentiviral or retroviral vectors to introduce CARs into primary T cells. While this is effective, it poses certain problems related to random integration. With the advent of genome editing approaches most notably CRISPR/Cas systems, the CARs (38) or TCRs (39, 40) were introduced into the endogenous TCR genomic locus. These protocols utilize CRISPR/Cas9 and the homology-directed repair (HDR) pathway with either viral (38) or non-viral (39, 40. 41) donor template delivery. Both landmark approaches were validated in NOD/SCID/ IL2gr-null (NSG) xenograft models. In these studies, xenograft models were sufficient to provide evidence of the concept of these novel platforms, specific targeting and tumor control, as well as improved functionalities of human T cells engineered with designed immune receptors.

Tasian reports that CAR-T's orchestration of off-target toxicities may only be found in early clinical trials (41). Mouse xenografts are useful in screening for basic CAR-T efficacy and for answering specific human biology questions. Additional studies in immune competent hosts are required to evaluate CAR safety. The hostile tumor microenvironment (TME) includes Tregs and MDSCs, but it's largely ignored in preclinical immunocompromised models (42). Tregs may partially explain worse CAR-T clinical trial results in solid tumors, and their inclusion in xenograft models may provide more accurate results.

In xenograft models it is difficult to distinguish between xenogeneic rejection, graft versus host disease (GVHD), allogenic response of human CAR-T cells to the tumor and actual CAR-T therapeutic efficacy. Therefore, appropriate and rigorous controls in experimental design are very important to obtain reliable results and draw correct conclusions. One such control that aids in differentiating the above-mentioned effects from the on-target responses of designed cellular immunotherapies are T cells engineered with a non-targeting immune receptor, such as CAR directed against target that is not expressed on tumor cells. In the absence of the host immune system, it is not possible to test the TME, tumor metastatic potential, or host response to CAR-T.

Taken together, xenograft models provide key insights into the function of human CAR-T cells against human tumors *in vivo*, which has been a basis for clinical success. This allowed the study of basic properties of human CAR-T cells such as anti-tumor activity, secretion of cytokines, expansion and persistence *in vivo*. However, in the absence of an interacting immune system, these models do not allow for comprehensive evaluation of immune-mediated mechanisms as well as on-target off-tumor toxicities (Figure 1).

To gain deeper insight into mechanism of action, interactions with the endogenous immune system and the role of endogenous immune response, xenograft models need to be complemented with syngeneic mouse models.

Syngeneic mouse models

Syngeneic models refer to genetically identical or sufficiently identical and immunologically compatible individuals to allow transplantation. The main feature of syngeneic mouse models is their immunocompetence, including full mouse immunity and comprehensive stroma. Important factor in their comprehensive use is their relative simplicity compared with other immunocompetent models.

The field of engineered cellular immunotherapies is moving towards increasing the efficacy and improving safety. Often, designed T cells rely upon recruiting endogenous immune response or counteracting immunosuppressive TME. Elucidation of the effects and mechanisms cannot be performed in a comprehensive manner in immunocompromised mice because the interacting immune system is lacking. Here, we list selected reports of upgraded CAR-T cells whose functions have been studied in various syngeneic mouse models.

In preclinical studies of adoptive cell therapy in a syngeneic model, the CAR-T, tumors, and target antigens are all mouse derived. Thus, the model allows observation of the CAR-T cells in the context of a functional immune system (16). This model can reveal-on-target off-tumor toxicity (43). Its major drawback is that mouse biology does not fully recapitulate human biology. Mouse syngeneic models have largely been unable to mimic CRS. Mouse CAR-T have shorter persistence and are more susceptible to activation–induced cell death compared to human. Syngeneic models do not provide much insight into the mechanisms of human CAR-T cells (44).

Initial syngeneic models of CAR-T demonstrated the superior efficiency of CAR-T over monoclonal antibodies. Preconditioning of the patient by irradiation or chemoablation was crucial for the efficacy of CAR-T therapy (45, 46). In this way, B-cell aplasia was shown as toxicity – mice were clear of lymphoma, but B cells were also absent. Cheadle (47) showed that first generation CAR-T were efficient in removing lymphoma, but not persistent. Second generation CAR-T cells induced B-cell aplasia and chronic toxicity accompanied by CD11b+Gr1+ myeloid derived suppressor cells; elevated TNFα and IFNγ point toward CRS–BALB/c (48), but not in C3H/HeJ or C57BL/6J – side effects vary between strains.

Better and more accurate preclinical models are needed for CAR-T in solid tumors. There the combination with checkpoint blockade regimens was shown to be successful (49). Syngeneic model also demonstrated potential toxicities and strain-specific effects (50). Chinnasamy (51) emphasized that mouse strains must be carefully selected or tested on multiple strains to ensure that toxicity is accurately modeled.

Widely varying results in studies using the same tumor associated antigen (TAA) but different mouse strains as seen in anti-CD19, anti-NKG2D and anti-VEGF studies caution against using a single mouse strain to determine safety before moving to clinical trials (16).

One approach to generate CAR-T cells with improved functionalities is to overexpress an additional accessory molecule along with the immune receptor. Constitutive expression of IL-12 in CAR-T cells was shown to augment CAR-T cell functions in a syngeneic model. Additional modification of infused hCD19-targeted CAR-T cells to secrete IL-12 allowed for efficient eradication of systemic EL4 (hCD19) tumors, as well as induction of B-cell aplasias, in the absence of prior cyclophosphamide conditioning. This outcome was dependent on both CD4 and CD8 T-cell subsets and required continued *in vivo* autocrine stimulation of IL-12 as well as modified T cell–IFNγ secretion, which in turn resulted in resistance to Treg-mediated suppression (52).

In addition to IL-12, IL-18 emerged as a promising candidate (53-56). In one of these studies (54) syngeneic model of pancreatic and lung tumors revealed that release of IL-18 modulated the immune cell landscape in the tumor. Increased numbers of CD206- M1 macrophages and NKG2D+ NK cells were observed, while Tregs, suppressive $CD103^{+}$ DCs, and M2 macrophages decreased. These observations were possible because an intact interaction immune system was present.

CAR-T cells were also engineered to secrete a combination of IL-7+CCL19 with the rationale that these factors contribute to the maintenance of T-cell zones in lymphoid organs. Upgraded CAR-T cells eradicated established solid tumors

and prolonged survival compared to conventional CAR-T cells. The syngeneic model showed increased infiltration of dendritic cells and T cells into tumor tissues. Depletion of recipient T cells reduced the therapeutic effects of upgraded CAR-T cell treatment, demonstrating that endogenous immune responses were induced (57).

In another study, CAR-T cells were engineered to secrete single-chain variable fragments (scFv) that block PD-1 (PDCD1). Clinically relevant syngeneic model with PD-L1 (CD274) positive tumor targets made it possible to demonstrate that secreted scFv acted on both CAR-T cells and bystander tumor-specific T cells to improve anti-tumor activity (58).

CAR-T cells constitutively expressing the immune-stimulatory molecule CD40 ligand (CD40L) demonstrated improved anti-tumor activity. In relevant syngeneic models, the authors investigated the underlying mechanisms and found that CD40L+ CAR-T cells were able to counteract tumor antigen escape variants via CD40/CD40L-mediated cytotoxicity and induction of an endogenous immune response. After adoptive cell transfer, upgraded CAR-T cells licensed antigen-presenting cells and recruited endogenous tumor-recognizing T cells (59).

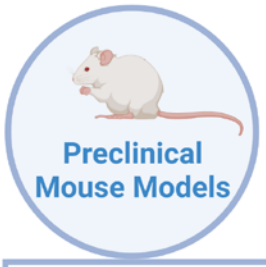
Another study demonstrating the importance of syngeneic mouse models explored how depletion of immunosuppressive M2 tumor-associated macrophages (TAMs) may improve the efficacy of CAR-T cells. The authors found that a folate receptor β (FRβ) positive subset of TAMs exhibited an immunosuppressive M2-like profile. When CAR-T cells were engineered to eliminate these FRβ+ TAMs, an enrichment of proinflammatory monocytes, a recruitment of endogenous tumor-specific CD8+ T cells, delayed tumor growth, and prolonged survival were observed (60).

Syngeneic models are useful for evaluating immunotherapies, *e.g.* in combination studies, particularly using checkpoint inhibitors. Syngeneic model panels can be extensively characterized (e.g. RNA sequencing of cell lines and tumors, immunophenotyping, biomarker identification), and these data can be combined with *in vivo* efficacy benchmarking profiling results from common checkpoint inhibitors (anti-PD-1, PD-L1, CTLA-4).

Syngeneic models are therefore an indispensable for evaluating the safety and efficacy of cellular immunotherapy, as they allow the study of adoptively transferred cells in the context of an interacting immune system. This allows for a rigorous assessment of immune-mediated mechanisms involved in successful therapy as well as toxicities. Potential drawbacks of syngeneic models include difficulties in generating cellular products and limited persistence and efficacy after infusion (Figure 1).

Immunocompetent transgenic mice

Transgenic animals have been around for several decades (61). Immunocompetent transgenic mice tolerant to human tumor associated antigens (TAAs) have been described in hematologic and solid tumors and for evaluating the safety and efficacy of antitumor immunotherapies (62-67). Although most anti CD19 CAR-Ts are studied in syngeneic or xenograft models, immunocompetent transgenic mouse models can also be used to better determine CAR-T safety. In transgenic mice, human TAA (murine TAA knockout and human TAA knock in) are expressed in mice to highlight the on-target off-tumor effect in healthy tissues. The mice are bred to have TAA expression similar to humans (68). Mice have their own T cells and an intact immune system (like in syngeneic models), but allow the use of human TAA-specific CAR-Ts (like xenografts) (16). A study in C57BL/6J with mouse CD19 knockout and human CD19 knockng restricted to B cells showed no toxicities other than B-cell aplasia (52). CARs targeting different antigens (CD19, CEA, HER2) have been tested in the clinic. The positive effect of preconditioning on adoptively transferred cells was confirmed in transgenic and not xenograft models (16, 69, 70). Engrafted tumors do not recapitulate many of the properties of naturally occurring tumors. A transgenic model in which tumors develop spontaneously can better mimic clinical progression and predict off-tumor toxicities (16). Transgenic CEA mice were developed by Zimmerman and colleagues (71) and have been frequently used to test CEA-targeting- CAR-T-cell therapy. In this model with high CEA expression, the toxicity associated with CAR-T cells appears to be limited to high-affinity CAR-T cells (65, 67). One of transgenic mouse models with CEA levels equivalent to those in humans (72) has also shown severe side effects associated with CAR-T-cell therapy (73, 74).



	Cancer cells	CAR-T cells	Interacting Immune/Host Environment	Pros	Cons
Human Xenograft	Human	Human	Immunodeficient Mouse	•Human CAR-T vs human cancer cells •Evaluation of fundamental CAR-T cells properties	•Interacting immune system is lacking •No ON-target OFF-tumor toxicity evaluation •GVHD
Syngeneic	Mouse	Mouse	Immunocompetent Mouse	•Interacting immune system is present •Evaluation of immune mediated mechanisms	•Tumor engraftment can be challanging •Preconditioning may be required •Limited persistance of transfered CAR-T cells
Transgenic	Mouse	Mouse	Immunocompetent Mouse	•Interacting immune system and expression of target antigens on host tissues enables the studies of ON-target OFF-tumor effects	•Expression of target antigens may not correlate between human and mouse •Limited recapitulation of spontaneous tumors
Humanized	Human	Human	Human (HSPCs)	•Human CAR-T cells vs human cancer cells in the presence of the engrafted human immune cells •CRS and neurotoxicity evaluation	•Difficult and complex model •Requirement of HSPCs engraftment

Figure 1: Schematic representation of preclinical mouse models for adoptive cell therapies

Figure created with BioRender.com

Humanized models

Currently, NOD/SCID/*Il2rg*−/− (NSG) or BALB/c/RAG2−/−/*Il2rg*−/− (BRG) mice are the standard recipients in the generation of humanized mice because they are deficient in mouse T cells, B cells, and NK cells (75-77). Transfer of human CD34+ hematopoietic stem and progenitor cells (HSPCs) into newborn NSG or BRG mice results in long-term engraftment of CD34+ cells and reconstitution of multilineage human immune cells (77-79). The comprehensive transfer protocol involves transfer of human CD34+ cells into NSG recipient mice and implantation of human fetal thymus and liver tissue under the kidney capsule of these mice (77, 80, 81). The resulting humanized mice are called bone marrow-liver-thymus (BLT) mice. They support the long-term engraftment and systemic reconstitution of a nearly complete human immune system, including multilineage human adaptive and innate immune cells consisting of T cells, B cells, NK cells, dendritic cells, and macrophages (77, 80, 81). Importantly, human immune cells developed in BLT mice, particularly T cells, are functional and have shown productive responses to skin xenografts and various viral/bacterial infections (77, 80-82). The next generation of humanized mouse models including NSG-SGM3 (or NSGS), NSGW41, NOG-EXL and MISTRG, support human myelopoiesis at varying degrees and through different strategies (28).

Humanized mouse models may bridge the gap between the syngeneic and xenograft models, because they are tolerant to human cells and exhibit aspects of a functional human immune system (16).

Until recently, CRS, which typically develops within the first few days after infusion of CD19 CAR-T cells, and neurotoxicity, the two major toxicities associated with clinically used CD19 CAR-T cells in humans, could not be reproduced in preclinical mouse models. This changed with

the development of a novel xenograft model in humanized mice that faithfully recapitulated both major toxicities (83). This model was developed by engrafting human cord blood hematopoietic stem and progenitor cells (HSPCs) into sub lethally irradiated newborn triple transgenic NSG (SGM3) mice. These mice express human stem cell factor, granulocyte-macrophage colony-stimulating factor (CSF2), and IL-3 to support the engraftment. Successful reconstitution of hematopoiesis was demonstrated, which included human B cells, monocytes, and T cells as well as cells from other lineages. Interestingly, the timing of HSC injection shortly after birth was important for successful human T lymphopoiesis. Circulating T cells exhibited a physiological CD4/CD8 ratio and differentiated into all major T cell differentiation subsets. T cells from these mice were then used to generate second-generation CAR-T cells targeting either CD19 or CD44v6. These CAR-T cells were injected into adult SGM3 mice that had previously been engrafted with ALL-CM leukemia cells. CAR-T cells cleared leukemia, which was associated with CRS characterized by weight loss, fever, and elevated systemic levels of human inflammatory cytokines including IL-6, resembling CRS in humans receiving CD19-targeting CAR-T cells. The authors used this model to show that monocytes are the major sources of IL-1 and IL-6 during CRS and that the syndrome could be prevented by depleting monocytes or by blocking the IL-6 receptor with tocilizumab (IL-6 receptor-blocking antibody). This model also recapitulated the lethal neurotoxicity, characterized by inflammation of the meninges. Interestingly, authors demonstrated that anakinra (IL-1 receptor antagonist) but not tocilizumab ameliorated neurotoxicity.

In the follow-up study by the same group, this model was further developed to investigate the efficacy and safety of CAR-T cells derived from preselected T cell subsets. When preselected naive/stem memory T cells were used to generate the CAR-T cellular product, superior anti-tumor activity, expansion and functional phenotype were observed compared to unselected bulk T cells. Surprisingly, this was accompanied by limited incidence of severe CRS and neurotoxicity. Overall, this model reveled improved efficacy and safety when preselected T cells are used to generate CAR-T cell products (84).

Therefore, in contrast to all other *in vivo* models using mice, humanized mouse models were able to reveal and investigate critical toxicities associated with CAR-T cell therapy (Figure 1).

In addition to humanized mouse models for T cell-based therapies, models have also been developed that allow adoptive transfer of engineered B cells. In this example, B cells were first isolated from the spleens of humanized donor mice, then genetically engineered and infused into "autologous" humanized recipient mice. Because the recipient mice were humanized with the same source of CD34+ cells as the humanized donor mice, such approach rendered enigneered human B cells tolerant to the host (85).

Conclusions

Cellular immunotherapy with CAR-T cells is a paradigm shifting approach for the treatment of certain blood cancers. However, limited efficacy and safety risks are the major barriers to advance the field and plethora of academic research groups, biotech and pharmaceutical companies are developing innovative next-generation cellular immunotherapies. After a novel approach has been validated *in vitro*, the next steps are experiments in preclinical mouse models. Typically, the initial experiments are conducted in human xenograft models, which are well suited for evaluating of novel genetic constructs and designs, immune receptor signaling, anti-tumor activity and intrinsic properties of human CAR-T cells, such as expansion and persistence, as well as certain dysfunctions, including T cell exhaustion. When more in-depth information about the immune mechanisms is required, which is the case when improved cellular immunotherapies aim to recruit endogenous immune responses and/or counteract an immunosuppressive tumor microenvironment, xenograft models are inadequate because they lack an interacting immune response. To answer such questions, syngeneic models are needed and have already provided valuable information for clinical translation. Since syngeneic models are associated with certain challenges including difficulties in the manufacturing of mouse cellular products, *in vivo* expansion, persistence, and efficacy, studies that systematically develop optimized protocols and procedures are very important (86).

In human clinical trials using CAR-T cells, certain toxicities including CRS and neurotoxicity

were observed that were not predicted by any of the preclinical models available at the time. Recently, a humanized mouse model was successfully developed to specifically address this challenge and replicate the pathologies observed in humans in preclinical mouse models as well (83). This example highlights the importance of continued development of preclinical mouse models as the field of cellular immunotherapy rapidly grows.

Preclinical mouse models continue to be a cornerstone in the development of the next generation of cellular immunotherapies. In this review recent advances and use of the four most common preclinical mouse models are presented. In addition, we presented the selected recent studies in which these models have been used to demonstrate innovative CAR-T cell approaches. The use of multiple models may provide a better understanding of a particular CAR-T therapy than a single model and we anticipate that increasingly sophisticated models will be developed, aided in part by recent advances in genome editing technologies, to comprehensively address the complexities of immunology and cellular immunotherapy. However, we must be aware of the limitations of preclinical mouse models, including the inherent differences between human and mouse biology and immunology. Careful design of properly controlled experiments is essential to generate reliable, high-quality data and draw the right conclusions required for clinical translation of cellular immunotherapies.

Acknowledgements

We thank K. Butina Ogorelec for reviewing and providing valuable feedback on the manuscript. A.S. received funding from the Slovenian Research Agency (ARRS) for Project J3-3084 and Program P1-0245, and from Research fund of the National institute of biology for Project 10ICIGEN (ICI). Figures created with BioRender.com

References

1. June CH, Sadelain M. Chimeric antigen receptor therapy. N Engl J Med 2018; 379: 64–73.

2. Sadelain M, Brentjens R, Rivière I. The promise and potential pitfalls of chimeric antigen receptors. Curr Opin Immunol 2009; 21: 215–23.

3. Kuwana Y, Asakura Y, Utsunomiya N, et al. Expression of chimeric receptor composed of immunoglobulin-derived V regions and T-cell receptor-derived C regions. Biochem Biophys Res Commun 1987; 149: 960–8.

4. Eshhar Z, Waks T, Gross G, Schindler DG. Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody-binding domains and the gamma or zeta subunits of the immunoglobulin and T-cell receptors. Proc Natl Acad Sci U S A 1993; 90: 720–4.

5. Brocker T, Karjalainen K. Signals through T cell receptor-zeta chain alone are insufficient to prime resting T lymphocytes. J Exp Med 1995; 181: 1653–9.

6. Grupp SA, Kalos M, Barrett D, Aplenc R et al. Chimeric antigen receptor-modified T cells for acute lymphoid leukemia. N Engl J Med 2013; 368: 1509–18.

7. Maude SL, Laetsch TW, Buechner J, et al. Tisagenlecleucel in children and young adults with B-cell lymphoblastic leukemia. N Engl J Med 2018; 378: 439–48.

8. Lee DW, Kochenderfer JN, Stetler-Stevenson M, et al. T cells expressing CD19 chimeric antigen receptors for acute lymphoblastic leukaemia in children and young adults: a phase 1 dose-escalation trial. Lancet 2015; 385: 517–28.

9. Schuster SJ, Svoboda J, Chong EA, et al. Chimeric antigen receptor T cells in refractory B-cell lymphomas. N Engl J Med 2017; 377: 2545–54.

10. Sommermeyer D, Hudecek M, Kosasih PL, et al. Chimeric antigen receptor-modified T cells derived from defined CD8+ and CD4+ subsets confer superior antitumor reactivity i*n vivo*. Leukemia 2016; 30: 492–500.

11. Mullard A. FDA approves first BCMA-targeted CAR-T cell therapy. Nat Rev Drug Discov. 2021; 332: 20–5.

12. Brudno JN, Kochenderfer JN. Recent advances in CAR T-cell toxicity: mechanisms, manifestations and management. Blood Rev 2019; 34: 45–55.

13. Neelapu SS, Tummala S, Kebriaei P, et al. Chimeric antigen receptor T-cell therapy - assessment and management of toxicities. Nat Rev Clin Oncol 2018; 15: 47–62.

14. Fraietta JA, Lacey SF, Orlando EJ, et al. Determinants of response and resistance to CD19 chimeric antigen receptor (CAR) T cell

therapy of chronic lymphocytic leukemia. Nat Med 2018; 24: 563–71.

15. Johnson LA, June CH. Driving gene-engineered T cell immunotherapy of cancer. Cell Res 2017; 27: 38–58.

16. Siegler EL, Wang P. Preclinical models in chimeric antigen receptor–engineered T-cell therapy. Human Gene Ther 2017; 29: 534–46.

17. Kaushik G, Venkatesha S, Verma B, et al. Preclinical in vitro and *in vivo* models for adoptive cell therapy of cancer. Cancer J 2022; 28: 257–62.

18. Magee MS, Snook AE. Challenges to chimeric antigen receptor (CAR)-T cell therapy for cancer. Discov Med 2014; 18: 265–71.

19. Rajcevic U. A rodent brain orthotopic model to study human malignant glioma. Slov Vet Res 2011; 48: 5–14.

20. Flanagan SP. 'Nude', a new hairless gene with pleiotropic effects in the mouse. Genet Res 1966; 8: 295–309.

21. Goldman JP, Blundell MP, Lopes L, et al. Enhanced human cell engraftment in mice deficient in RAG2 and the common cytokine receptor gamma chain. Br J Haematol 1998; 103: 335–42.

22. Tyagi RK, Jacobse J, Li J, Allaman MM, et al. HLA-restriction of human treg cells is not required for therapeutic efficacy of low-dose IL-2 in humanized Mice. Front Immunol 2021; 24: e630204. doi: 10.3389/fimmu.2021.630204

23. Rodriguez-Garcia A, Watanabe K, Guedan S. Analysis of antitumor effects of CAR-T cells in mice with solid tumors. Methods Mol Biol 2020; 2086: 251–71.

24. Walsh NC, Kenney LL, Jangalwe S, et al. Humanized mouse models of clinical disease. Annu Rev Pathol 2017; 24: 187–215.

25. Shultz LD, Lyons BL, Burzenski LM, Shultz LD. Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2R gamma null mice engrafted with mobilized human hemopoietic stem cells. J Immunol 2005; 174: 6477–89.

26. Ito M, Hiramatsu H, Kobayashi K, et al. NOD/SCID/gamma(c)(null) mouse: an excellent recipient mouse model for engraftment of human cells. Blood 2002 100: 3175–82.

27. Covassin L, Jangalwe S, Jouvet N, et al. Human immune system development and survival of non-obese diabetic (NOD)-scid IL2rγnull (NSG) mice engrafted with human thymus and autologous haematopoietic stem cells. Clin Exp Immunol 2013; 174: 372–88.

28. Arranz L. The hematology of tomorrow is here-preclinical models are not: cell therapy for hematological malignancies. Cancers (Basel) 2022; 14(3): e580. doi: 10.3390/cancers14030580.

29. Ramos CA, Rouce R, Robertson CS, et al. *In vivo* fate and activity of second- versus third-generation CD19-specific CAR-T cells in B cell non-Hodgkin's lymphomas. Mol Ther 2018; 26: 2727–37.

30. Maher J, Brentjens RJ, Gunset G, et al. Human T-lymphocyte cytotoxicity and proliferation directed by a single chimeric TCRzeta / CD28 receptor. Nat Biotechnol 2002; 20: 70–5.

31. Kakarla S, Chow KKH, Mata M., et al. Antitumor effects of chimeric receptor engineered human T cells directed to tumor stroma. Mol Ther 2013; 21: 1611–20.

32. Long AH, Haso WM, Shern JF, et al. 4-1BB costimulation ameliorates T cell exhaustion induced by tonic signaling of chimeric antigen receptors. Nat Med 2015; 21: 581–90.

33. Carpenito C, Milone MC, Hassan R, et al. Control of large, established tumor xenografts with genetically retargeted human T cells containing CD28 and CD137 domains. Proc Natl Acad Sci U S A 2009; 106: 3360–5.

34. Wu CY, Roybal KT, Puchner EM, et al. Remote control of therapeutic T cells through a small molecule-gated chimeric receptor. Science 2015; 350(6258): aab4077. doi: 10.1126/science.aab4077

35. Roybal KT, Rupp LJ, Morsut L, et al. Engineering T cells with customized therapeutic article engineering T cells with customized therapeutic esponse programs using synthetic notch receptors. Cell 2016; 167: 419–32.

36. Minutolo NG, Sharma P, Poussin M, et al. Quantitative control of gene-engineered T-cell activity through the covalent attachment of targeting ligands to a universal immune receptor. J Am Chem Soc 2020; 142: 6554–68.

37. Lynn RC, Weber EW, Sotillo E, et al. c-Jun overexpression in CAR T cells induces exhaustion resistance. Nature 2019; 576: 293–300.

38. Eyquem J, Mansilla-Soto J, Giavridis T, et al. Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. Nature 2017; 543: 113–7.

39. Roth TL, Li PJ, Blaeschke F, et al. Pooled knockin targeting for genome engineering of cellular immunotherapies. Cell 2020; 181: 728–44.

40. Roth TL, Puig-Saus C, Yu R, et al. Reprogramming human T cell function and specificity with non-viral genome targeting. Nature 2018; 559: 405–9.

41. Tasian SK, Teachey DT, Li Y, et al. Potent efficacy of combined PI3K/mTOR and JAK or ABL inhibition in murine xenograft models of Ph-like acute lymphoblastic leukemia. Blood 2017; 129: 177–87.

42. Lee JC, Hayman E, Pegram HJ, et al. *In vivo* inhibition of human CD19-targeted effector T cells by natural T regulatory cells in a xenotransplant murine model of B cell malignancy. Cancer Res 2011; 71: 2871–81.

43. Sanmamed MF, Chester C, Melero I, Kohrt H. Defining the optimal murine models to investigate immune checkpoint blockers and their combination with other immunotherapies. Ann Oncol 2016; 27: 1190–8.

44. Holzapfel BM, Wagner F, Thibaudeau L, et al. Concise review: humanized models of tumor immunology in the 21st century: convergence of cancer research and tissue engineering. Stem Cells 2015; 33: 1696–704.

45. Kochenderfer JN, Yu Z, Frasheri D, et al. Adoptive transfer of syngeneic T cells transduced with a chimeric antigen receptor that recognizes murine CD19 can eradicate lymphoma and normal B cells. Blood 2010; 116: 3875–86.

46. Davila ML, Brentjens R. Chimeric antigen receptor therapy for chronic lymphocytic leukemia: what are the challenges? Hematol Oncol Clin North Am 2013; 27: 341–53.

47. Cheadle EJ, Hawkins RE, Batha H, et al. Natural expression of the CD19 antigen impacts the long-term engraftment but not antitumor activity of CD19-specific engineered T cells. J Immunol 2010; 184: 1885–96.

48. Cheadle EJ, Sheard V, Rothwell DG, et al. Differential role of Th1 and Th2 cytokines in autotoxicity driven by CD19-specific second-generation chimeric antigen receptor T cells in a mouse model. J Immunol 2014; 192: 3654–65.

49. Burga RA, Thorn M, Point GR, et al. Liver myeloid-derived suppressor cells expand in response to liver metastases in mice and inhibit the anti-tumor efficacy of anti-CEA CAR-T. Cancer Immunol Immunother 2015; 64: 817–29.

50. VanSeggelen H, Hammill JA, Dvorkin-Gheva A, et al. T cells engineered with chimeric antigen receptors targeting NKG2D ligands display lethal toxicity in mice. Mol Ther 2015; 23: 1600–10.

51. Chinnasamy D, Tran E, Yu Z, et al. Simultaneous targeting of tumor antigens and the tumor vasculature using T lymphocyte transfer synergize to induce regression of established tumors in mice. Cancer Res 2013; 73: 3371–80.

52. Pegram HJ, Lee JC, Hayman EG, et al. Tumor-targeted T cells modified to secrete IL-12 eradicate systemic tumors without need for prior conditioning. Blood 2012; 119: 4133–41.

53. Kunert A, Chmielewski M, Wijers R, et al. Intra-tumoral production of IL18, but not IL12, by TCR-engineered T cells is non-toxic and counteracts immune evasion of solid tumors. Oncoimmunology 2017; 7(1): e1378842. doi: 10.1080/2162402X.2017.1378842.

54. Chmielewski M, Abken H. CAR T cells releasing IL-18 convert to T-Bet high FoxO1 low effectors that exhibit augmented activity against advanced solid tumors. Cell Rep 2017; 21: 3205–19.

55. Hu B, Ren J, Luo Y, et al. Augmentation of antitumor immunity by human and mouse CAR T cells secreting IL-18. Cell Rep 2017; 20: 3025–33.

56. Zimmermann K, Kuehle J, Dragon AC, et al. Design and characterization of an all-in-one lentiviral vector system combining constitutive anti-G D2 CAR expression and inducible cytokines. Cancers (Basel) 2020; 12(2): e375. doi: 10.3390/cancers12020375

57. Adachi K, Kano Y, Nagai T, et al. IL-7 and CCL19 expression in CAR-T cells improves immune cell infiltration and CAR-T cell survival in the tumor. Nat Biotechnol 2018; 36: 346–51.

58. Rafiq S, Yeku OO, Jackson HJ, et al. Targeted delivery of a PD-1-blocking scFv by CAR-T cells enhances anti-tumor efficacy *in vivo*. Nat Biotechnol 2018; 36: 847–56.

59. Kuhn NF, Purdon TJ, van Leeuwen DG, et al. CD40 ligand-modified chimeric antigen receptor T cells enhance antitumor function by eliciting an endogenous antitumor response. Cancer Cell 2019; 35: 473–88.

60. Rodriguez-Garcia A, Lynn RC, Poussin M, et al. CAR-T cell-mediated depletion of immunosuppressive tumor-associated macrophages promotes endogenous antitumor immunity and augments adoptive immunotherapy. Nat Commun 2021; 12(1): e877. doi: 10.1038/s41467-021-20893-2

61. Hogan B. Manipulating mouse embryos. Cold Spring Harbor : Cold Spring Harbor Laboratory Press, 1994.

62. Eades-Perner AM, van der Putten H, Hirth A, et al. Mice transgenic for the human

carcinoembryonic antigen gene maintain its spatiotemporal expression pattern. Cancer Res 1994; 54: 4169–76.

63. Peat N, Gendler SJ, Lalani N, et al. Tissue-specific expression of a human polymorphic epithelial mucin (MUC1) in transgenic mice. Cancer Res 1992; 52: 1954–60.

64. Beavis PA, Henderson MA, Giuffrida L, et al. Targeting the adenosine 2A receptor enhances chimeric antigen receptor T cell efficacy. J Clin Invest 2017; 127: 929–41.

65. Chmielewski M, Hahn O, Rappl G, et al. T cells that target carcinoembryonic antigen eradicate orthotopic pancreatic carcinomas without inducing autoimmune colitis in mice. Gastroenterology 2012; 143: 1095–107.

66. Wang LX, Westwood JA, Moeller M, et al. Tumor ablation by gene-modified T cells in the absence of autoimmunity. Cancer Res 2010; 70: 9591–8.

67. Wang LX, Kang G, Kumar P, et al. Humanized-BLT mouse model of Kaposi's sarcoma-associated herpesvirus infection. Proc Natl Acad Sci U S A 2014; 111: 3146–51.

68. Bhattacharya-Chatterjee M, Saha A, Foon KA, Chatterjee SK. Carcinoembryonic antigen transgenic mouse models for immunotherapy and development of cancer vaccines. Curr Protoc Immunol 2008; 80: 20.8.1–12.

69. Brentjens RJ, Rivière I, Park JH, et al. Safety and persistence of adoptively transferred autologous CD19-targeted T cells in patients with relapsed or chemotherapy refractory B-cell leukemias. Blood 2011; 118: 4817–28.

70. Parkhurst MR, Yang JC, Langan RC, et al. T cells targeting carcinoembryonic antigen can mediate regression of metastatic colorectal cancer but induce severe transient colitis. Mol Ther 2011; 19: 620–6.

71. Eades-Perner AM, Zimmermann W. Carcinoembryonic antigen-transgenic mice: a model for tumor immunotherapy. Tumour Biol 1995; 16: 56–61.

72. Chan CHF, Stanners CP. Novel mouse model for carcinoembryonic antigen-based therapy. Mol Ther 2004; 9: 775–85.

73. Blat D, Zigmond E, Alteber Z, et al. Suppression of murine colitis and its associated cancer by carcinoembryonic antigen-specific regulatory T cells. Mol Ther 2014; 5: 1018–28.

74. Aranda F, Barajas M, Huarte E. Transgenic tumor models for evaluating CAR T-cell immunotherapies. Curr Protoc Pharmacol 2019; 86(1): e66. doi: 10.1002/cpph.66.

75. Brehm MA, Shultz LD, Greiner DL. Humanized mouse models to study human diseases. Curr Opin Endocrinol Diabetes Obes 2010; 17: 120–5.

76. Takenaka K, Prasolava TK, Wang JC, et al. Polymorphism in Sirpa modulates engraftment of human hematopoietic stem cells. Nat Immunol 2007; 8: 1313–23.

77. Smith DJ, Lin LJ, Moon H, et al. Propagating humanized BLT mice for the study of human immunology and immunotherapy. Stem Cells Dev 2016; 25: 1863–73.

78. Traggiai E, Chicha L, Mazzucchelli L, et al. Development of a human adaptive immune system in cord blood cell-transplanted mice. Science 2004; 304: 104–7.

79. Chicha L, Tussiwand R, Traggiai E, et al. Human adaptive immune system Rag2-/-gamma(c)-/- mice. Ann N Y Acad Sci 2005; 1044: 236–43.

80. Melkus MW, Estes JD, Padgett-Thomas A, et al. Humanized mice mount specific adaptive and innate immune responses to EBV and TSST-1. Nat Med 2006; 12: 1316–322.

81. Lan P, Tonomura N, Shimizu A, et al. Reconstitution of a functional human immune system in immunodeficient mice through combined human fetal thymus/liver and CD34+ cell transplantation. Blood 2006; 108: 487–92.

82. Wege AK, Melkus MW, Denton PW, et al. Functional and phenotypic characterization of the humanized BLT mouse model. Curr Top Microbiol Immunol 2008; 324: 149–65.

83. Norelli M, Camisa B, Barbiera G, et al. Monocyte-derived IL-1 and IL-6 are differentially required for cytokine-release syndrome and neurotoxicity due to CAR T cells. Nat Med 2018; 24: 739–48.

84. Arcangeli S, Bove C, Mezzanotte C, et al. CAR T cell manufacturing from naive/stem memory T lymphocytes enhances antitumor responses while curtailing cytokine release syndrome. J Clin Invest 2022; 132(12): e15080. doi: 10.1172/JCI150807

85. Page A, Laurent E, Nègre D, et al. Efficient adoptive transfer of autologous modified B cells: a new humanized platform mouse model for testing B cells reprogramming therapies. Cancer Immunol Immunother 2022; 71: 1771–5.

86. Lanitis E, Rota G, Kosti P, et al. Optimized gene engineering of murine CAR-T cells reveals the beneficial effects of IL-15 coexpression. J Exp Med 2021; 218(2): e20192203. doi: 10.1084/jem.20192203

PREDKLINIČNI MIŠJI MODELI PRI ADOPTIVNIH CELIČNIH TERAPIJAH RAKA

U. Rajčević, A. Smole

Izvleček: Napredne terapije na osnovi biotehnološko spremenjenih limfocitov T predstavljajo moderen pristop k imunoterapiji raka z uporabo genetsko spremenjenih limfocitov T. Do danes sta bili za zdravljenje hematoloških malignosti odobreni terapiji s himernimi antigenskimi receptorji usmerjenimi proti antigenoma CD19 in BCMA. Uspeh zdravljenja s celicami CAR-T pa ovirajo omejena učinkovitost, še posebej pri solidnih tumorjih in varnostna tveganja. Predklinične raziskave *in vivo*, ki so močno odvisne od zanesljivih mišjih modelov, so bile kritični dejavnik zgodbe o uspehu adoptivnih celičnih terapij in še vedno zagotavljajo neprecenljive podatke za razvoj naslednje generacije celičnih imunoterapij. V preglednem članku povzemamo štiri najpogostejše predklinične mišje modele: ksenografte, singenetske modele, imunokompetentne transgenske modele in humanizirane mišje modele. Vsi opisani modeli imajo svoje prednosti in slabosti in noben mišji model ne more do popolnosti preslikati situacije v človeškem pacientu zaradi medvrstnih razlik ter izjemne zapletenosti zdravljenja. Podatki iz literature kažejo na to, da lahko uporaba kombinacije mišjih modelov v predkliničnih in vivo raziskavah pred translacijo zdravljenja na ljudi v kliničnih poskusih pripomore k postopnemu izboljšanju kakovosti, varnosti in učinkovitosti zdravljenja in zagotovi bolj celostni nabor podatkov kot en sam model.

Ključne besede: mišji model; ksenograft; singenetski; transgenski; humanizirani; CAR-T; adoptivna celična terapija