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Review

Applying a clinical lens to animal models of CAR-T cell therapies

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Chimeric antigen receptor (CAR)-T cells have emerged as a promising treatment modality for various hematologic and solid malignancies over the past decade. Animal models remain the cornerstone of pre-clinical evaluation of human CAR-T cell products and are generally required by regulatory agencies prior to clinical translation. However, pharmacokinetics and pharmacodynamics of adoptively transferred T cells are dependent on various recipient factors, posing challenges for accurately predicting human engineered T cell behavior in non-human animal models. For example, murine xenograft models did not forecast now well-established cytokine-driven systemic toxicities of CAR-T cells seen in humans, highlighting the limitations of animal models that do not perfectly recapitulate complex human immune systems. Understanding the concordance as well as discrepancies between existing pre-clinical animal data and human clinical experiences, along with established advantages and limitations of each model, will facilitate investigators' ability to appropriately select and design animal models for optimal evaluation of future CAR-T cell products. We summarize the current state of animal models in this field, and the advantages and disadvantages of each approach depending on the pre-clinical questions being asked.

INTRODUCTION

The field of chimeric antigen receptor (CAR)-T cell therapy has grown exponentially since the first CAR product, tisagenlecleucel (tisa-cel) was approved by the FDA in 2017,[1,2] paving the way for the approval of five additional CAR-T cell products for lymphoid malignancies as of June 2022, targeting CD19 for lymphomas or acute lymphoblastic leukemia or B cell maturation antigen (BCMA) for multiple myeloma. Clinical testing has begun for multiple new CAR-T cell approaches targeting additional antigens, including CD22 for lymphoid malignancies, CD33 and CD123 for myeloid malignancies, and GD2 and mesothelin for solid tumors, giving hope that CAR-T cell therapies can be extended to a broader range of diseases.

As with any developmental therapy, *in vivo* animal studies establishing biological plausibility and pre-clinical safety are generally required before moving CAR-T cell therapies to the clinic.[3,4] Very few products identified as promising in the laboratory ultimately make it to clinical trials, let alone gain FDA approval. Successful clinical translation of new cell therapy products is even more challenging than for new drug therapies, partly due to difficulty generating clinically relevant pharmacology, toxicology, and safety data from non-human animal models.[5] In contrast to regular pharmacological agents, CAR-T cells are "living drugs" that proliferate, migrate, and persist in patients to varying degrees depending on *in vivo* environmental signals that they receive, making design of animal models particularly challenging. As clinical data accumulates for pioneering CAR-T cell therapies, it has become increasingly apparent that individual pre-clinical animal models do not fully predict clinical behaviors of CAR-T cells. Indeed, a comparative study that assessed the divergence of marketing authorization applications from the expected regulatory data requirements revealed that the applications for cell therapies reflected difficulties of using animal models to address toxicology and mechanisms of action more so than those for other biological agents.[6] In this review, we summarize available information generated in pre-clinical CAR-T cell animal models and assess their utility and limitations, in light of results in humans. We then discuss animal studies on select timely topics, namely CAR-T cell trafficking and CAR-T cell differentiation, and we assess their implications for future engineered immune cell therapies.

GENERAL CLASSIFICATIONS OF ANIMAL MODELS USED IN CAR-T CELL RESEARCH

Xenograft mouse models

Human tumor cell lines and some types of primary human cells can be transplanted as xenografts into immunodeficient mice that lack fully functional immune systems, permitting sustained engraftment of human cells. Less immunodeficient strains, such as athymic nude mice that lack T cells and CB17-scid mice that lack both T and B cells, continue to produce natural killer (NK) cells, which can potentially mediate anti-tumor activity and confound data. Mouse strains created on the non-obese diabetic (NOD)-severe combined immunodeficiency (SCID) background are severely immunodeficient, with complete absence of an adaptive immune system and varying degrees of impairments in innate

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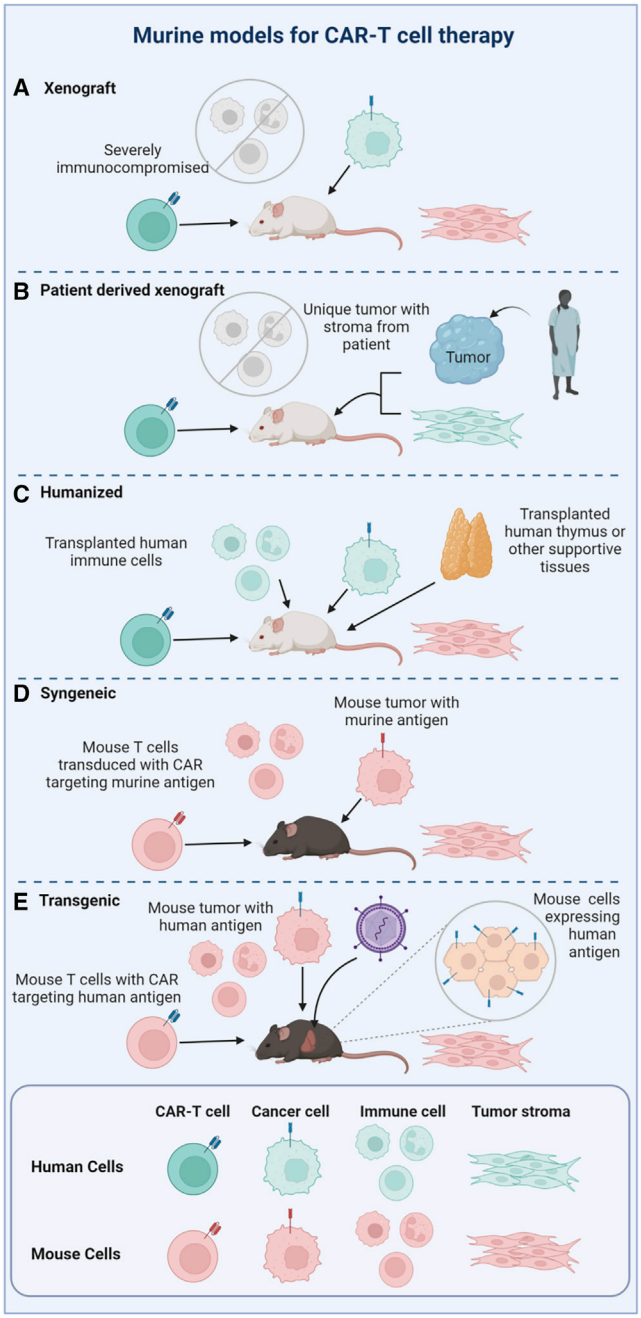

Figure 1. Murine models for CAR-T therapies
Human cells are depicted in aqua and mouse cells in pink throughout the figure.

immunity. NOD/SCID/IL2Rγc-KO (NSG) mice lack B, T, and NK cells and have become one of the most commonly utilized mouse strains for studying human CAR-T cell therapies. A broad range of human tumor cells robustly engraft in these mice, providing a model for development of adoptive immune cell therapies directed at a wide variety of human cancers.[7]

Patient-derived xenograft (PDX) models, in which primary patient-derived tumors are implanted into immunodeficient mice, enable evaluation of CAR-T cell activity against cells with more physiologic characteristics, including unique clonal dynamics that arise from patient-inherent tumor heterogeneity and thus variable susceptibility to CAR-T cells.[8] In general, primary tumors able to engraft in PDX animals have a tendency to closely mimic characteristics of metastatic rather than primary disease.[9,10]

Human xenografts can also be established in mice that are reconstituted with a human immune and hematopoietic system. These "humanized" models may better capture interactions of adoptively transferred CAR-T cells in the context of tissue microenvironments and other human immune cells, potentially recapitulating human physiology more closely compared with standard xenografted mice lacking these components of the human immune system. Additional genetic modifications to improve the engraftment and support of human cells include strains such as the NSG-SGM3 mouse, engineered to express the human cytokines stem cell factor, GM-CSF, and IL3 that facilitate engraftment of human hematopoietic cells.[11] A comparison of commonly utilized mouse models is depicted in Figure 1.

While xenograft mouse models have many advantages for studying human T cell therapies, notable limitations include xenogeneic graft-versus host disease (GVHD) and still imperfect human immunity due to incomplete cross-species cellular and soluble factor interactions. Lack of human stromal cells and other supportive elements may also interfere with maintenance and trafficking of human CAR-T as well as preclude the study of therapies specifically targeting the stromal elements of solid tumors since tumor-associated stroma supporting injected human tumor cells develop from murine fibroblasts.

Syngeneic mouse models

Syngeneic murine models allow analyses of tumor and CAR-T cell interactions in the context of an intact host immune system, including species-specific cytokines, stromal and other microenvironmental elements, inhibitory cells, and other potentially influential factors. These models are particularly beneficial for testing hypotheses based on complex interactions between adoptively transferred T cells and host elements. Pitfalls include the rapid growth of tumor cell lines in these models, rapid enough to preclude generation of an immunosuppressive tumor microenvironment playing a major role in tampering down immune responses to human solid tumors. Alternatively, mouse models genetically engineered to develop spontaneous tumors may permit more physiologic tumor stromal development and better model characteristics of primary tumor cells, as opposed to immortalized tumor cell lines.[12,13] Historically, transgenic mouse strain generation is expensive and time consuming, with transgene integration variability sometimes creating undesirable phenotypic differences. In recent years, CRISPR-Cas9 targeted genome editing has greatly reduced the time and cost required to create these models. Knocking in

human target antigens into murine tissues also allows evaluation of human antigen-directed T cells in otherwise syngeneic systems.[14] Foreign proteins encountered in tumor cell lines, such as luciferase and GFP, are known to be highly immunogenic when given to immunocompetent mice. Transgenic mouse models have been developed allowing tolerance to these immunogenic reporter molecules, enabling the use of imaging to track tumor burden in syngeneic mouse models over time.[15–17]

Non-murine species

Though mice are the most frequently used model species for adoptive cell therapies, based on availability, cost, and familiarity, non-murine species have also been used for *in vivo* studies of CAR-T cells. Approximately four million dogs in the United States per year spontaneously develop cancer and therefore may serve as natural models for the spontaneous process of oncogenesis. This stands in contrast with the artificial introduction of cancer cell lines or germline genetic manipulation of mice to induce "spontaneous" tumor development. Across breeds, dogs harbor a much greater degree of genetic diversity than mice, which are inbred for genetic homogeneity. Breed predilections for certain tumor types exist, with large-breed dogs at greater risk for osteosarcoma, golden retrievers at risk for mast cell tumors, and smaller terrier breeds at greater risk for transitional cell carcinomas, for example.[18] Humans also have diverse genetic backgrounds, making canine heterogeneity an advantage of this model system. Environmental factors leading to cancer in humans often similarly impact their canine companions, and these vary widely across different parts of the world.[19] Due to dogs' domestic evolution alongside humans, they have been exposed to many of the same pathogens and have developed immune systems that function similarly to those of their human counterparts. Dogs are immunologically competent at birth, but like humans, postnatal maturation of the immune system develops with pathogenic exposures and antigen priming.[20] Pet owners and veterinarians can be highly motivated to contribute to the clinical development of new therapies, both to extend the life and health of their companions, as well as contribute to medical advances relevant to humans.

Non-human primates (NHPs) most closely resemble humans, regarding lifespan, size, telomere length, immune system characteristics, and genetic heterogeneity, conferring high predictive value for the potency and safety of cell therapies. Taraseviciute et al. established an NHP model of anti-CD20 CAR-T cell therapy, recapitulating human CAR-T cell phenotype, expansion, persistence, and toxicities.[21] This achievement underscores the importance and unique strengths of NHP models. Drawbacks include limited animal availability and high costs of both animal purchase and husbandry, limiting experiments to small cohorts and thus limited statistical power, as well as lack of sufficient animals with spontaneous tumors. Induction of tumors is not feasible to date in NHP models. For these reasons, NHPs have limited utility in anti-tumor efficacy studies, but they remain the gold standard for evaluating toxicity. Each model has distinct advantages and disadvantages, which have been outlined in Table 1.

STUDYING EFFICACY OF CAR-T CELLS IN ANIMAL MODELS

Murine models

Estimation of a cell product's ability to induce complete remission

Xenograft murine models are often the most accessible and commonly utilized to test proof-of-concept activity of a new CAR-T cell product. Immunodeficient mice are first engrafted with human cancer cell lines or PDX tumors, followed by treatment with human T cells transduced with CARs, which allows direct observation of the effects of the therapeutic human T cell product *in vivo*. All CAR-T cell products currently in clinic were tested in xenograft models and most have been described in publications reporting *in vivo* anti-tumor activity, including CARs targeting hematologic tumor antigens,[22,23] dual antigen targeting (e.g., CD20 and CD19,[24] CD19 and CD22[25,26]) and solid tumor antigens (e.g., EGFRvIII,[27] mesothelin[28]).

Estimation of clinically effective and safe cell dose

Predicting biologically active dose levels and identifying a safe starting dose are considered central objectives of pre-clinical studies by regulatory bodies such as the FDA.[3] Unfortunately, xenograft mouse models have been of limited utility in defining appropriate doses of CAR-T cells for first-in-human trials. Cell doses in xenograft studies are often chosen arbitrarily or start very high in order to quickly assess an anti-tumor response. For example, anti-CD19 and CD22 human CAR-T cell doses employed in many early pre-clinical studies were often as high as 1×10^6 to 1×10^7 cells per mouse,[29–31] which extrapolates to 5×10^7 to 5×10^8 cells per kilogram, based on an average mouse weighing 20 grams. In contrast, clinically safe and effective cell doses of anti-CD19, CD22, and BCMA CAR-T cells in humans are far lower, only approximately 2×10^5 to 5×10^6 cells/kg.[32–40] These stark differences in cell doses reflect the fact that mice tend to be much less sensitive to CAR-T cell-mediated toxicities than humans (discussed further later in the context of toxicity models). Lower CD19 CAR-T cell doses in NSG xenograft models (5×10^4 to 2×10^5 cells/mouse) do have some *in vivo* anti-leukemia activity,[41] more in alignment with clinically feasible doses. In humans, duration of clinical response and long-term survival are the critical relevant outcomes. However, the lifespan of xenografted mice is limited by both rapid progression of engrafted tumor cells and xeno-GVHD-related morbidity and mortality. Thus, dose-finding in these models is not feasible, and investigators are more often forced to rely on dosing data from previous human clinical trials to set starting parameters for new therapies, sometimes with unanticipated intolerable toxicities at doses required for efficacy.

Evaluation of lymphodepletion and long-term therapeutic effects

The syngeneic murine system models physiologic species-specific cytokine and immune cell interactions without the complication of xenogeneic GVHD, overcoming some of the major limitations of xenograft models. While direct evaluation of actual human CAR-T cells is not possible in a syngeneic murine system, studies of equivalent murine CAR-T cells can provide translationally valuable

Table 1. A comparison of animal model systems for pre-clinical CAR-T cell testing

Species		Advantages	Disadvantages	Efficacy	Toxicity
Zebrafish		• Rapid *in vivo* system (24–48 h) • Visualization of CAR-T-tumor interactions • Inexpensive • Large study size • Low cell numbers	• Limited ability for longitudinal studies • Genetic distance frm humans • No spontaneous tumor development	*In vivo* efficacy reflects similar results as *in vitro* cytotoxicity assays	Unclear utility for toxicity modeling
Mouse	Xenograft	• Rapid *in vivo* system (3–8 weeks) • Avoid interspecies immune rejection • Direct evaluation of human T cell product • Broad range of human tumor cell lines available	• Longitudinal studies limited by xenogeneic GVHD • Lacks spontaneous tumors • Immunodeficient	Most CAR-T undergo proof-of-concept efficacy studies in xenografts. • Different CAR generations • Single, tandem, bicistronic, loop CARs • Hematologic, solid tumor targets • Gated CARs	Limited reports of toxicity, but some exist
	Patient-derived xenograft (PDX)	• Direct study of clinically relevant heterogeneous tumors • Tumor includes human stroma, microenvironment	• Work and time intensive • Lacks spontaneous tumors • Immunodeficient	Advantageous for testing efficacy of dual-target and gated CAR-T cells	Limited toxicity data
	Genetically engineered	• Spontaneous tumor development • More accurate tumor microenvironment	• Historically expensive, time intensive • Limited control over timing of tumor development	No CAR-T studies published to date	
	Humanized	• Direct evaluation of human T cell product • Modeling of human CAR-T in the presence of human immune and hematopoietic cells	• Lacks human stroma • The human immune systems established in mice are incomplete	Important for modeling efficacy in the face of other human immune cells and tissues Improved B and T cell maturation in NSG-SGM3 BLT mice	Allows study of the role other cell types play in toxicity
	Syngeneic	• Immune competent • No xenogeneic GVHD • Ideal for longitudinal studies	• Unable to directly evaluate human T cell product • Murine and human biology are different	Critical for understanding role of lymphodepletion on CAR-T efficacy	On-target off-tumor toxicity may not directly apply to humans HLH, MAS modeled in perforin-deficient CD19 CAR-T cells
	Transgenic	• Direct evaluation of CAR transduced in murine T cells directed against human antigen expressed on murine tumor and healthy cells • Immune competent	• Transgenic mouse strains are available for a limited number of target antigens	Evaluation of human CAR construct in otherwise all murine system	Ideal for modeling on-target off-tumor toxicity Evaluation of host immune system involvement
Dog		• Intraspecies genetic heterogeneity • Spontaneous tumors • Shortened lifespan, rapid translation	• Less established than other model systems • Limited lab/specialist availability	Preliminary efficacy with similar escape mechanisms in humans	No published CAR-T studies of toxicity
Non-human primate		• Genetically similar to humans • Immune competent • Ability for longitudinal CAR-T studies, target dependent	• Expensive • Small study numbers • Lack of relevant tumor models	Efficacy in infectious disease models and anti-B cell antigen CAR-T (B cell aplasia) Limited utility in anti-tumor efficacy	Ideal for modeling cytokine-mediated cytotoxicity (CRS, ICANS) Useful for on-target off-tumor toxicity modeling in antigens with cross-species reactivity

information. One of the earliest syngeneic mouse CAR-T cell studies was conducted by Cheadle et al. using a retroviral vector containing a first-generation CAR-T cell targeting murine CD19. This work helped establish that efficacy of CAR-T cells is enhanced by lymphodepletion with irradiation or cyclophosphamide prior to CAR-T infusion and also uncovered the limited persistence of first-generation CAR-T cells.[42,43] Pre-clinical syngeneic studies of a second-generation CAR-T construct targeting CD19 and containing co-stimulatory domains demonstrated the ability of these CAR-T cells to persist long term and induce B cell aplasia for up to 3–12 months following treatment, enabling the evaluation of long-term therapeutic outcomes.[43–48] Work by Davila et al. used syngeneic tumor-prone Eμ-myc C57BL/6 transgenic mice to demonstrate anti-leukemia CD19 second-generation CAR-T cell efficacy, as well as showing that intensive preconditioning with cyclophosphamide was more central to efficacy than CAR-T cell dose escalation.[49,50] Clinical studies

have confirmed the importance of achieving the appropriate degree of lymphodepletion, with higher overall response rates and progression-free survival in patients receiving higher doses of lymphodepletion treatment prior to CAR-T cell infusion.[51] The similarity of the results from syngeneic mouse models and human trials highlights that the presence of an intact immune system and ability to follow animals long term are valuable for pre-clinical optimization of cell therapies.

One of the most important outcomes that can be modeled most easily in a syngeneic model is post-CAR-T cell relapse. Despite the remarkable ability of CAR-T cells to induce complete remissions, sustained disease control is achieved in no more than half of patients.[52,53] Disease relapse occurs in part due to the emergence of cell-surface antigen-low or negative tumor subclones that evade CAR-T cell targeting. Interestingly, lineage switch of B cell malignancies to non-B cell lineages under CAR-T cell pressure has been shown to contribute to post CAR-T cell therapy relapses as well.[44,54,55] Jacoby et al. demonstrated leukemia lineage reprogramming in a syngeneic model as an escape mechanism from CD19 CAR-T cell pressure, characterized by epigenetic suppression of B cell lineage transcription factors such as *Pax5* and *Ebf1*, with concurrent upregulation of myeloid-lineage transcription factors such as *Cebpa*.[44] In clinic, KMT2A-rearranged leukemias, most often seen in infants, are particularly vulnerable to CD19 negative relapses after CD19-directed therapies.[54,56] Extensive efforts are underway to model KMT2A-rearranged leukemia (as reviewed in Liao et al.[57]), which will further elucidate the mechanisms of leukemia lineage switch provoked by CAR-T cell pressure.

Comparing the potency of CARs with different structural designs

There are substantial ongoing efforts to improve CAR-T cell efficacy by fine-tuning CAR designs, including co-stimulatory domains, linker lengths, and signaling strength. Animal models, especially xenograft mouse models, have been essential to evaluate the function of various parts of human antigen-directed CARs. As summarized by Cappell and Kochenderfer in a recent review,[58] various murine studies demonstrated heterogeneous results regarding the efficacy between varying co-stimulatory domains. Mouse xenograft models and immunocompetent syngeneic models have been used to evaluate the effects of modulation of CAR signaling by partially inactivating CD3z immunoreceptor tyrosine-based activation motifs (ITAMs)[59] or by incorporating mutations in subdomains of a co-stimulatory molecule.[60] In addition to different combinations of hinge and transmembrane domains,[61,62] the length of linker connecting the variable heavy (V_H) and light chain (V_L) of the scFv were also found to influence anti-tumor efficacy of CAR-T cells in murine xenografts,[63] informing the design of subsequent clinical trials (NCT02650414 and NCT03620058).

Evaluating CAR-T cell efficacy in non-murine species

Zebrafish

One group has created a xenografted CAR-T cell model in embryonic zebrafish, arguing that this system is fast, inexpensive, and offers the ability to perform live imaging for the study of CAR-T cell migration and killing.[64] They used human anti-CD19 CAR-T cells labeled with the DiI membrane dye in fish engrafted with human GFP-expressing human leukemia cells, and they measured *in vivo* cytotoxicity over 24 hours via luminescence imaging. Mouse studies, on the other hand, typically require 2–6 weeks at a minimum to evaluate *in vivo* anti-tumor activity. The related downside of this short-term zebrafish model is that it does not allow analysis of CAR-T cell persistence, exhaustion, or tumor escape, all requiring much longer periods of *in vivo* observation. Furthermore, application to solid tumor models, in which orthotopic tumor inoculation is desired, may be difficult due to the size of zebrafish embryos. Lastly, the zebrafish embryonic immune system varies significantly from humans in that it consists of only innate immune cells with unclear cross-species cytokine reactivity. The lack of adaptive endogenous immunity prevents immediate rejection of human CAR-T cells, but it does not accurately recapitulate the immune milieu of humans.

Canine models

Dogs with spontaneous hematologic malignancies have been treated with canine CAR-T cells targeting CD20.[65–67] Dogs bearing sarcomas have also been treated with B7-H3 CAR-T cells, which are also in human clinical trials (NCT046670068), demonstrating feasibility and safety.[68] Many of the observations made in human CAR-T cells trials have been recapitulated in dogs, including the development of cytokine release syndrome, the emergence of antigen-negative tumor relapse, and the development of anti-mouse antibodies generated against the murine portion of the scFv used in many human and dog CAR constructs (Figure 2).[69] While thus far use of the canine CAR-T cell models and clinical utilization by veterinarians is lagging behind, canine models can serve as a promising translational bridge, and activity in this area is increasing.

Non-human primate models

The primary limitation for efficacy testing in NHP models is the lack of high incidence spontaneous malignancies in these animals, in part because cancer is often a disease of aging, and housing enormous (and thus expensive) colonies of aging NHPs for many decades would be required to obtain any animals with relevant primary tumors. Use of tumor cell lines is not feasible in non-inbred and immunocompetent NHPs, and any tumor cell artificially introduced from another animal would be immediately rejected. However, B cell aplasia can be used as a surrogate marker for modeling persistence of CAR-T cell therapies directed at B lymphoid target antigens.[21]

NHPs, specifically various old world macaques, have been central platforms for the pre-clinical development of immune therapies, including vaccines, cell therapies, and monoclonal antibodies against infectious pathogens, given the many macaque viruses that are highly homologous to human pathogens such as HIV, CMV, EBV, Ebola, Zika, and SARS-CoV-2.[70–73] The first CAR-T cells given to humans were actually targeting HIV-infected cells via CARs with a CD4/CD3zeta first-generation chimeric receptor binding HIV envelope expressed on the surface of HIV-infected T cells. Pre-clinical testing of

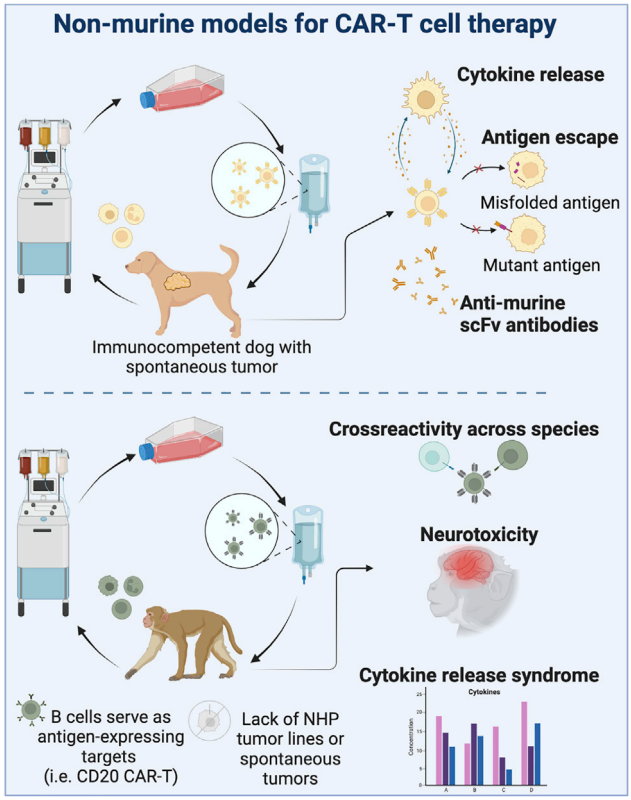

Figure 2. Non-murine models for CAR-T cell therapies

CAR-transduced human hematopoietic stem and progenitor cells (HSPCs) resulted in efficacy against an HIV-infected leukemia cell line in a xenograft model.[74] However, pioneering clinical trials using first-generation CAR-T cells in HIV-infected patients were ineffective, with limited *in vivo* expansion in the absence of lymphodepletion.[75,76] More recently, Zhen et al. developed a lentiviral vector expressing a CD4/CD3z CAR along with a C46 fusion inhibitor to prevent viral infection of CAR-expressing T cells.[77] These investigators also engineered HSPCs rather than peripheral T cells to express CARs, with successful differentiation into CAR-expressing T cells *in vivo* following autologous transplantation. CAR-T cells then persisted for over 2 years and provided sustained anti-viral immunity. The NHP SIV model also enabled testing of the combination therapy of CD4 CAR-T cells plus immune checkpoint blockade or antigen boosting.[78]

MODELING CAR-T CELL TOXICITIES

Cytokine-driven toxicities

CAR-T cell therapies are associated with unique toxicities associated with systemic inflammation, linked to various cytokines secreted by both CAR-T cells and the recipients' endogenous immune cells. The spectrum of toxic manifestations has been categorized into cytokine release syndrome (CRS), immune effector cell-associated neurotoxicity syndrome (ICANS), hemophagocytic lymphohistiocytosis (HLH), and macrophage activation syndrome (MAS).[79,80] Prior to the advent and clinical translation of CAR-T cells, the potential for cytokine-driven toxicities resulting from adoptive cell transfer was not anticipated, in large part due to lack of similar events in standard murine models. The unexpected nature of the toxicities may be reflected in the study design of the first-in-human clinical trial of CD19 CAR-T cells administered together with high-dose IL-2.[81] Exogenous IL-2 continues to be frequently co-administered with polyclonal or TCR-based T cell products, but it is not now administered with CAR-T cells, given the well-recognized risks of cytokine-related toxicities that occur even without exogenous cytokine administration. Over the past decade, significant progress has been made in establishing animal models for CAR-T-associated toxicities. This section will highlight representative animal models recapitulating the spectra of inflammatory toxicities seen in humans (Figure 3), with a particular focus on B cell antigen-directed CAR-T cells.

Difficulties predicting toxicities using animal models

Novel human CAR-T cell products intended for clinical translation are often tested in murine immunodeficient xenograft models to demonstrate pre-clinical anti-tumor activity, as discussed above. However, NSG mice receiving CAR-T cells targeting CD19, CD22, or other human B cell antigens generally do not manifest obvious CRS or other CAR-T toxicities, likely due to incomplete cross-species reactivity of cytokines, lack of human immune effector cells other than CAR-T cells, and lack of additional cell targets such as normal B cells. Xenogeneic GVHD can further complicate interpretation of possible CAR-T toxicities in immunodeficient mice. Syngeneic,[46,82] transgenic,[83] and humanized murine models[84] along with non-human primate models[21] have been more valuable tools for understanding the pathophysiology of CAR-T cell-mediated toxicities. Despite this, animal models are somewhat limited in pre-clinical ability to predict all possible untoward effects of a given human CAR-T cell product, since no model can fully represent the complex immune system and diversity of cell types and antigen expression within a patient with cancer. These models have been more robust in advancing our understanding of the pathophysiology of these side effects and initial exploration of the efficacy of potential interventions.

Xenograft models for studying pathophysiology of cytokine-driven toxicities

Multiple models suggest the important role that recipient-derived myeloid cells, such as monocytes and macrophages, play in cytokine-mediated toxicities. In a xenograft mouse model using SCID-beige mice, which lack B and T cells but have a relatively normal myeloid compartment, severe CRS developed in the context of high intraperitoneal disease burden in mice treated with human CD19-CAR-T cells.[85] Taking advantage of the xenograft model, the cellular source of cytokines could be distinguished by specifically measuring levels of human versus murine cytokines generated from either adoptively transferred human CAR-T cells or murine immune cells, respectively. Mice with severe CRS had elevated mouse-derived IL-6, CCL2, and CXCL9, and murine myeloid cells with high levels of pro-inflammatory gene expression were recruited to the sites of

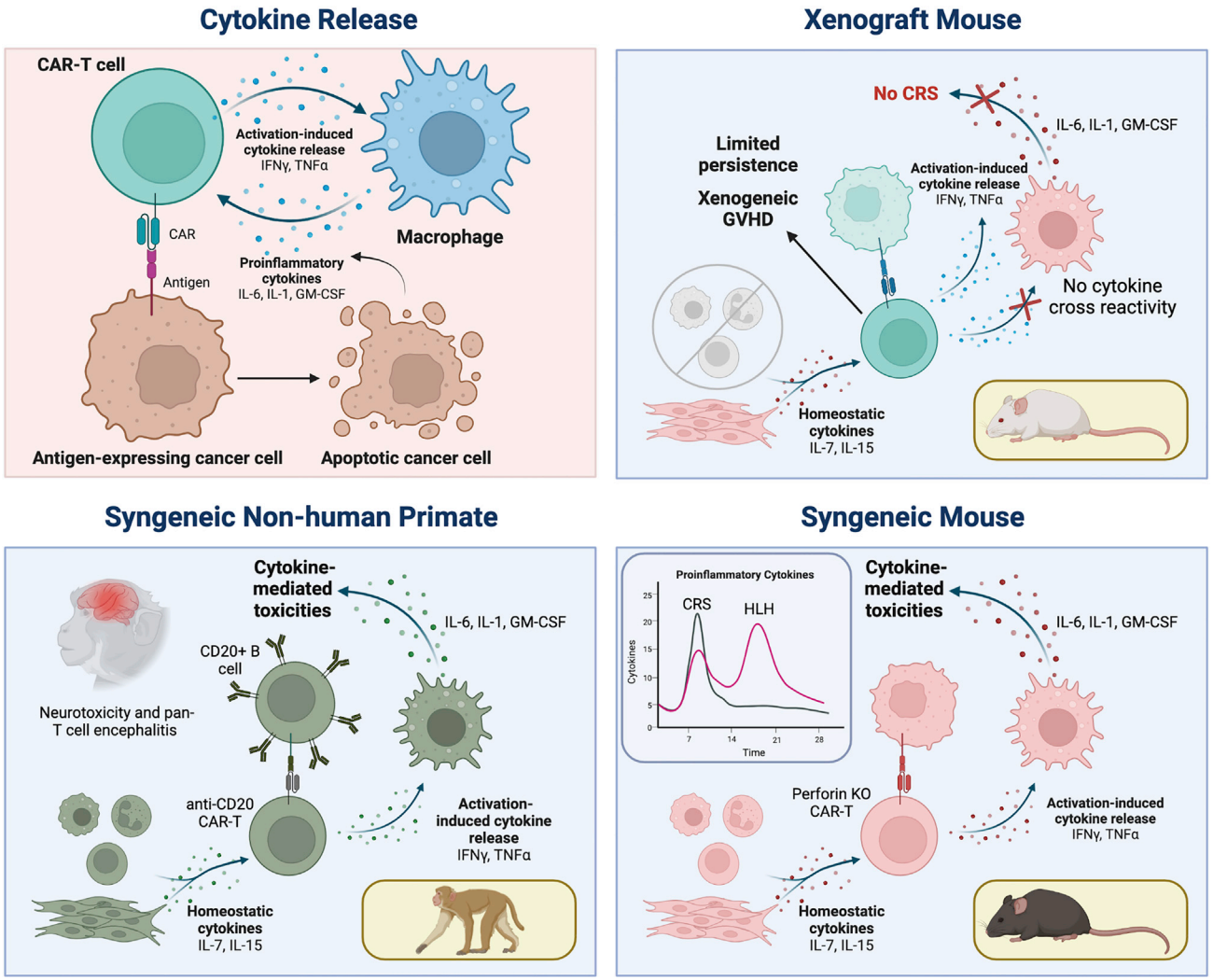

Figure 3. Modeling cytokine-mediated CAR-T cell toxicities

The mechanism of CAR-T-mediated cytokine-related toxicities involves a surge of T cell activation, resulting in release of cytokines, which in turn results in myeloid cell release of other pro-inflammatory cytokines. CAR-T-mediated lysis and induction of tumor cell apoptosis further feeds the cycle of pro-inflammatory cytokine production. Limitations of the xenograft mice for modeling toxicity stem from lack of interspecies cytokine cross-reactivity and inadequate host immune functions, although CRS and neurotoxicity have been successfully modeled in humanized mice. Both CRS and HLH have been successfully modeled in syngeneic or transgenic immunocompetent mouse models. Similarly, neurotoxicity and CRS modeling in NHPs has mimicked human toxicities and provided valuable insight into toxicity mechanisms.

tumor in the peritoneum. Notably, human CAR-T cells engineered to co-express mouse CD40L resulted in more severe CRS, indicating that physical CD40/CD40L-mediated interactions between CAR-T and recipients' myeloid cells expressing mouse CD40 receptor contributed to toxicity. IL-6 and genes involved in IL-1 signaling were upregulated in recipient mice myeloid cells, consistent with observations in humans,[32,86,87] and blockade of IL-6, IL-1, and inducible nitric oxide synthase improved CRS without negatively impacting anti-leukemia efficacy of CAR-T cells.

The critical roles played by recipients' myeloid cells and IL-6/IL-1 cytokine axes were further emphasized in a humanized NSG-SGM3 murine model expressing human SCF, GM-CSF, and IL-3. Newborn NSG-SGM3 mice engrafted with human cord blood HSPCs develop human T cells xenotolerant to murine antigens (termed nHuSGM3 T cells). Use of nHuSGM3 T cells for CAR-T generation mitigated the confounding issue of xeno-GVHD.[84] Compared with SGM3 recipient mice without human hematopoietic reconstitution, humanized SGM3 recipient mice with human myeloid cells experienced more severe and sometimes fatal CRS within a week of CAR-T cell infusion. Depletion of monocytes, determined to be the major source of IL-6 and IL-1, mitigated CRS and further underscored the central role of recipient myeloid cells. Neurotoxicity was also observed in humanized SGM3 recipients, characterized by multifocal meningeal

thickening and human macrophage infiltration into the subarachnoid space. Importantly, blockade of IL-1 with anakinra effectively treated both CRS and neurotoxicity, but IL-6 blockade with tocilizumab only addressed CRS, mirroring the efficacies of each drug in human CRS versus ICANS.[88,89]

In addition to IL-6 and IL-1, other myeloid cell-derived cytokines such as GM-CSF have also been implicated in CRS and neurotoxicity in murine xenograft models.[90] Mice that suffered neurotoxicity had CD11b+ myeloid cell infiltration into the brain accompanied by inflammation. Pharmacologic GM-CSF blockade or genetic deletion of GM-CSF from CAR-T cells improved toxicities while enhancing the anti-tumor activity of CAR-T cells. These data provided the basis for an ongoing clinical trial prospectively evaluating the use of lenzilumab, a human GM-CSF-neutralizing antibody, with axicabtagene ciloleucel (NCT04314843).

The role of catecholamines as an upstream myeloid factor influencing cytokine release has also been studied in the context of xenograft NSG-SGM3 models.[91] The myeloid-specific deletion of tyrosine hydroxylase, which blocks conversion of tyrosine to L-DOPA, led to the reduction of catecholamine production following bacterial infection or other inflammatory stimuli. CD19 CAR-T cell-driven CRS in NSG-SGM3 mice was improved by pharmacologic blockade of tyrosine hydroxylase without impacting anti-leukemia efficacy, demonstrating an alternative avenue to address cytokine-mediated toxicities.

Syngeneic immunocompetent murine models for toxicities

Syngeneic and transgenic murine models allow for the study of CAR-T cell biology in the presence of a fully competent host immune system. It is important to note, however, that mice maintained in a standard animal facility have an antigen-inexperienced immune system due to facility cleanliness standards, not accurately resembling humans exposed to a multitude of exogenous antigens.[92,93] This may partially explain why symptomatic CRS or neurotoxicity typically does not occur in standard syngeneic murine models with CD19 CAR-T cells on a C57BL/6 background.[45,46] One of the most faithful recapitulations of clinical CRS and neurotoxicity was achieved in a transgenic mouse system in which mice with B cell-restricted human CD19 expression were engrafted with a mouse lymphoma cell line (TBL12) engineered to co-express human CD19 and given syngeneic murine T cells expressing a human CD19-directed CAR.[83] CAR-T cell dose-dependent acute and lethal cytokine-mediated toxicities consistent with CRS and neurotoxicity occurred, accompanied by depletion of brain microglial cells. Blockade of IL-6 and IFNγ ameliorated toxicities. Another syngeneic murine model utilized perforin-deficient T cells to capture late-onset cytokine-mediated toxicities resembling the poorly understood clinical CAR-T toxicities of HLH and MAS.[46] Perforin-deficient T cells expressing anti-CD19 CAR had initial *in vivo* expansion concurrent with leukemia clearance, and then re-expanded in the absence of detectable antigens several weeks after CAR-T cell infusion. This secondary expansion was accompanied by concomitant expansion and activation of recipient-derived lymphocytes and myeloid cells, splenomegaly, and pro-inflammatory cytokine upregulation, resembling HLH. The model mirrored clinical observations of patients treated with anti-CD22 CAR-T cells who experienced biphasic inflammation that was chronologically and phenotypically distinguishable, manifesting as CRS in the first phase and HLH/MAS in the second.[32,94] IL-1 family cytokines were among many pro-inflammatory cytokines significantly elevated in the murine model as well as in patients who experienced HLH/MAS compared with patients with CRS alone. Anakinra, an IL-1 receptor antagonist antibody, is increasingly utilized in clinic as an adjunctive treatment to manage CAR-T-related toxicities.[95] Murine models recapitulating different aspects of human clinical manifestations may aid in elucidating the pathophysiology of each toxicity, which may be distinct from one another.

NHP model of CRS and neurotoxicity

Lastly, CRS and neurotoxicity have been modeled in NHPs.[21] Feasible study sizes for NHP studies are small but offer the unparalleled advantage of studying clinical toxicities in animals with immune systems closely related to humans and body size permitting longitudinal sampling of CSF and other relevant tissues. CD20-specific CAR-T cells were administered to four rhesus macaques preconditioned with cyclophosphamide.[21] All experienced CRS followed by overt neurotoxicity. Various cytokines, including IL-6, GM-CSF, IL-1β, and MCP-1, were significantly elevated in CSF compared with concurrent blood samples from each animal. Scheduled autopsies at the onset of neurotoxicity, corresponding to maximal *in vivo* CAR-T expansion, demonstrated diffuse infiltration of brain parenchyma with CAR-positive T cells as well as CAR-negative T cells. These findings suggested that CAR-T-associated neurotoxicity is not target antigen dependent.

Antigen-specific toxicities

CAR-T cells also have the potential for inducing antigen-specific toxicity via on-target/off-tumor and off-target reactivities. In contrast to CRS, ICANS, and HLH/MAS, in which clinical manifestations are driven by systemic inflammatory responses, antigen-specific toxicity is a direct result of CAR-T cell-mediated damage to otherwise healthy tissues. Off-target toxicity occurs when the CAR cross-reacts with an unintended target expressed on healthy tissues. Off-target toxicities and clinical trial fatalities have occurred with TCR-engineered T cell infusions,[96–98] but none have been reported with CAR-T cells to date. Off-target toxicities of human engineered T cells cannot be studied in animal models because they do not express the complete repertoire of human antigens required to study these side effects. In the absence of suitable animal models, off-target screening of CAR-T cells is generally achieved via *in vitro* evaluations using cell lines and tissue arrays, in addition to extrapolating experiences from antibody therapies, from which the antigen-recognition domains of CARs (i.e., scFv) are often derived.

On-target off-tumor toxicities occur when a known target antigen is shared by both tumor and normal tissues. B cell aplasia after B cell lineage antigen-directed CAR-T cell therapy is the classic example

of on-target off-tumor toxicity. B cells are uniquely "dispensable" normal cells, since B cell aplasia and resultant hypogammaglobulinemia can be managed with intravenous immunoglobulin (IVIG) supplementation, but the vast majority of other potential target antigens are found on cells and organs indispensable for survival. On-target toxicities can be avoided if antigen expression is restricted to cancerous cells alone (i.e., tumor-specific antigens); however, many, if not all, cell-surface proteins on tumors are shared by normal cells. Target antigen vetting and *in vitro* methods of antigen expression evaluation have been previously reviewed.[99] The following section will discuss examples of animal models employed to define and avoid adverse effects of on-target off-tumor toxicities.

HER2 and ERBB family antigens

HER2 (ERBB2) is an example of tumor-associated antigen with known variable protein-level expression by various normal tissues.[100] The first clinical trial of trastuzumab-based CAR-T cells targeting HER2 led to acute respiratory compromise and a fatality,[101] hypothesized to be due to on-target off-tumor targeting of lung epithelial cells encountered upon first pass circulation. However, subsequent clinical trials of different anti-HER2 constructs based on the alternative FRP5 antibody given at lower cell doses demonstrated improved safety profiles without on-target off-tumor toxicities.[102–104] These examples imply that antigen expression on normal tissue is not the only determinant of on-target off-tumor toxicity, underscoring the importance of pre-clinical models to predict the therapeutic window of CAR-T cells directed against tumor-associated antigens. NSG mice gene-transferred to express human HER2 in liver cells experienced on-target off-tumor hepatic toxicities when given human HER2 CAR-T cells.[105] Interestingly, when low- and high-affinity CAR-T cells were compared in these mice, the low-affinity CAR-T cells mediated superior anti-tumor activity compared with high-affinity CAR-T cells due to on-target off-tumor sequestration of the high-affinity CAR-T cells in the liver. The study demonstrated the importance of evaluating on-target off-tumor effects not only to achieve a better understanding of toxicity but also to identify potential mechanisms for improving CAR-T cell efficacy. CAR-T cells that target several ErbB dimers, named T1E28z CAR-T cells,[106] have been evaluated in SCID-beige mice[107] and cross-react with both human and mouse ErbB, making this murine model suitable for on-target off-tumor toxicity evaluation. Mice treated with intravenous or intratumoral injection of T1E28z CAR-T cells did not experience toxicities, but intraperitoneal delivery led to toxicities reminiscent of CRS. Toxicities were considered on-target off-tumor effects since mice developed toxicity regardless of the presence of tumor in the peritoneum. The model highlights the relevance of T cell administration route.

GD2

GD2 is a tumor target antigen also known to be expressed by normal brain cells. A recent pioneering clinical study suggested that GD2 CAR-T cells, given either intravenously or intracerebroventricularly, are surprisingly safe and potentially effective, without apparent on-target off-tumor toxicities.[108] There is strong homology of the GD2 antigen between human and rodents, and certain scFvs may bind GD2 expressed by both species,[109] enabling the modeling of on-target off-tumor toxicities in a murine system. Pre-clinical murine models have shown different degrees of toxicity depending on the CAR construct, ranging from complete absence to lethality.[109–111] Murine GD2 CAR-T cell models have been valuable for testing the effectiveness of various toxicity mitigation strategies, such as a ligand-inducible system to degrade CAR on the cell surface[110] and SynNotch CAR designs.[112] In the case of SynNotch-gated GD2-B7H3 CAR-T cells interrogated in murine xenograft models of neuroblastoma, GD2 and anti-GD2 interactions were required to induce B7H3 CAR expression, ensuring that CAR-T cells are reactive only with cells that co-express both GD2 and B7H3, reducing the likelihood of tumor non-specific targeting by CAR.[112]

CD33 and CD123

Animal models have been particularly important for pre-clinical evaluation of predicted serious on-target off-tumor toxicities of CAR-T cells directed against myeloid leukemia target antigens such as CD33 and CD123, also expressed by normal HSPCs and mature myeloid cells. When NSG mice engrafted with human HSPCs were treated with anti-CD123 CAR-T cells, human hematopoiesis was depleted,[113] contrasting with *in vitro* hematopoietic colony assays suggesting CD123 CAR-T could mediate specific cytotoxicity against leukemia cells while sparing normal HSPCs,[114,115] emphasizing the importance of *in vivo* models. CD33 CAR-T cell on-target off-tumor toxicity to human hematopoiesis was mitigated by engrafting mice with CD33-knockout (KO) human HSPCs generated utilizing CRISPR-Cas9 technology.[116] Importantly, the authors also demonstrated that CD33-KO HSPCs were fully functional and capable of establishing long-term multilineage hematopoiesis in rhesus macaques transplanted with autologous CD33-KO HSPCs, setting the stage for the approach to genetically engineer the recipients' cells to mitigate on-target, off-tumor toxicities.

Other on-target toxicities studied in NHP models

NHP models have been particularly useful in assessing the impact of potential on-target off-tumor toxicities for antigens that are homologous between humans and macaques. For example, ROR1 is a tumor-associated antigen expressed by B cell malignancies and some epithelial cancers. Berger and colleagues treated macaques with autologous ROR1 CAR-T cells after demonstrating that ROR1 is highly homologous between the two species, with similar tissue expression patterns.[117] ROR1 CAR-T cells did not induce toxicities against normal tissues expressing ROR1 at low levels. Another example is a study of EPHB4-CAR-T cells[118] that exploited the fact that the human EPHB4 ligand Ephrin B2-based CAR binds to both human and NHP EPHB4. Macaques given lymphodepleting conditioning followed by human T cells expressing the EPHB4-CAR experienced no acute on-target off-tumor toxicity. However, given expected xenogeneic T cell rejection, this model could not be used to study later-onset toxicities. Nonetheless, the study demonstrated that lymphodepleted NHPs may serve as a model to study acute on-target toxicities of human CAR-T cells.

CAR-T CELL BIOLOGY AND ANIMAL MODELS

Trafficking

An obvious prerequisite for effective CAR-T cell therapy involves the cells' ability to reach the tumor site, which is a concept that cannot be adequately studied in *in vitro* and necessitates use of animal models. T cell migration relies on a variety of factors, including cytokines, chemokines, adhesion molecules, and their respective receptors. Though many of these factors are species specific, some adhesion molecules and chemokine receptors can engage with murine receptors/ligands.[119] Evaluation of the impact of different routes of CAR-T cells administration also requires animal studies. Murine xenograft studies by Parente-Pereira et al. demonstrated that intravenous administration mimicked patterns seen with either human or murine T cells in recipients of the same species.[120] Locoregional administration of CAR-T cells via either intraperitoneal or subcutaneous routes showed no systemic trafficking of CAR-T cells, which may reduce systemic exposure and on-target off-tumor toxicity.[121] This work led to a clinical trial (NCT01818323) of intratumoral administration of CAR-T cells for head and neck cancers. Trials in murine xenografts have also shown improved results when CAR-T cells for brain tumors were administered directly into the CNS compared with systemic administration.[122,123] Safety and feasibility of implanted CNS reservoirs for delivery of CAR-T cells were demonstrated in NHPs,[124] paving the way for clinical trials of locoregional administration of CAR-T cells for glioblastoma.[125,126]

Non-invasive tracking systems using reporter molecules and radiotracers have enabled researchers to visualize CAR-T cells in animal models using PET/SPECT imaging. Although immunodeficient mice lack chemokines and cytokines that might otherwise influence cellular trafficking patterns, murine xenografts have allowed proof-of-concept studies to demonstrate that CAR-T cells can be effectively labeled and imaged, which is necessary prior to conducting large mammal studies to ensure tracking methods are tolerated. The sodium iodide symporter (NIS)[127,128] and somatostatin receptor 2 (SSTR2) [129] are reporter molecules successfully employed for non-invasive imaging of CAR-T cells in murine xenografts.

CAR-T cell stemness, differentiation, and exhaustion

T cell terminal differentiation is an undesirable state. Less differentiated T cells mediate superior anti-tumor responses in *in vivo* models characterized by enhanced persistence and sustained anti-tumor activities (excellent reviews on the concept are available[130,131]). The hypothesis that CD8+ effector memory (T_{EM}) and central memory (T_{CM}) T cells may variably survive and persist following adoptive transfer has been investigated in both non-human primates[132,133] and murine xenografts,[134] influencing later phase I clinical trial development evaluating the safety and feasibility of CD19 CAR T_{CM} therapy post-autologous hematopoietic stem cell transplantation (HSCT) in relapsed/refractory diffuse large B cell lymphoma (DLBCL).[135] T memory stem cells (T_{SCM}) are even less differentiated and retain stem cell-like properties such as high self-renewal capacity and multipotency,[136,137] and CAR-T_{SCM} cells demonstrate superior anti-tumor responses in murine xenografts compared with conventional CAR-T cells.[138] Similarly, CAR-T cells generated from pre-selected naive and stem cell memory T cells demonstrated superior and sustained anti-leukemia activity in a humanized mouse model compared with bulk CAR-T cells.[139] CAR-T_{SCM} cells can be reliably generated at clinical scale and were tested in a clinical trial (NCT01087294).

The concept of combining human CAR-T cells with various agents that prevent T cell terminal differentiation, either during the CAR-T manufacturing process or as combination systemic therapy, has been tested in murine xenograft models.[140–142] Some CAR constructs are prone to tonic signaling that induces CAR-T cell exhaustion, leading to suboptimal anti-tumor efficacy in murine xenograft models.[111] A drug-regulatable platform allowing control of CAR surface expression with dasatinib, which is known to suppress CAR-T cell activation, mitigated exhaustion of anti-GD2 CAR-T cells in murine xenografts,[143] which provided a basis for an ongoing clinical trial (NCT04539366).

CONCLUSION

It is important to understand the benefits and limitations of available animal models for pre-clinical evaluation of CAR-T cells. Retrospective interpretation of animal model-derived data under the light of amassing human clinical observations helps us better delineate the appropriate application of each model. Establishment of safety may be the most important objective of pre-clinical animal studies, but animal models remain an imperfect system for evaluating the toxicology of human CAR-T cell products. Nonetheless, animal models have been indispensable to advancing the cell therapy field, and increasingly refined models will continue to aid us in better understanding the biology of CAR-T cells and provide critical information for the development of novel next-generation cell therapy products.

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AUTHOR CONTRIBUTIONS

B.B.D., K.I., and C.E.D. wrote the manuscript.

DECLARATION OF INTERESTS

No conflict of interest.

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