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Commentary

Theme: Precision Medicine: Implications for the Pharmaceutical Sciences
Guest Editor: Marilyn N. Martinez and Adel Karara

CAR T Cell Immunotherapy in Human and Veterinary Oncology: Changing the Odds Against Hematological Malignancies

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Abstract. The advent of the genome editing era brings forth the promise of adoptive cell transfer using engineered chimeric antigen receptor (CAR) T cells for targeted cancer therapy. CAR T cell immunotherapy is probably one of the most encouraging developments for the treatment of hematological malignancies. In 2017, two CAR T cell therapies were approved by the US Food and Drug Administration: one for the treatment of pediatric acute lymphoblastic leukemia (ALL) and the other for adult patients with advanced lymphomas. However, despite significant progress in the area, CAR T cell therapy is still in its early days and faces significant challenges, including the complexity and costs associated with the technology. B cell lymphoma is the most common hematopoietic cancer in dogs, with an incidence approaching 0.1% and a total of 20–100 cases per 100,000 individuals. It is a widely accepted naturally occurring model for human non-Hodgkin's lymphoma. Current treatment is with combination chemotherapy protocols, which prolong life for less than a year in canines and are associated with severe dose-limiting side effects, such as gastrointestinal and bone marrow toxicity. To date, one canine study generated CAR T cells by transfection of mRNA for CAR domain expression. While this was shown to provide a transient anti-tumor activity, results were modest, indicating that stable, genomic integration of CAR modules is required in order to achieve lasting therapeutic benefit. This commentary summarizes the current state of knowledge on CAR T cell immunotherapy in human medicine and its potential applications in animal health, while discussing the potential of the canine model as a translational system for immuno-oncology research.

KEY WORDS: ommuno-oncology; CAR T cell; lymphoma; One Health.

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INTRODUCTION

Research in cancer immunotherapy has two major current and complementary approaches: (1) immune checkpoint inhibitors such as those that recently garnered a Nobel Prize in Medicine (1) and (2) chimeric antigen receptor (CAR) T cell programming. The former focuses on activation of intrinsic properties of T cells. The latter involves the exogenous "education" of T cells to seek out and target T cells expressing a particular antigen found on specific cancer cell types (2). These methods are considered complementary, and progress on combining these approaches is being reported (3). Cancer immunotherapy is an extremely promising new approach in oncology that has the profound potential for curative endpoints. CAR T cell therapies are particularly promising for hematologic malignancies, garnering two FDA approvals in 2017 using autologous cells (4,5) representing the first for both these classes of immunotherapies in addition to serving as the inaugural class of gene

therapy–based strategies for personalized medicine. Over 700 potential Investigative New Drug applications are in the queue for cellular and/or gene therapy applications (6) demonstrating the sustained future for these classes of drugs in the therapeutic pipeline. B cell neoplasms are the most common hematopoietic cancer in both humans and dogs (7). In canines, genetic background can impact disease onset and progression as some breeds show a substantially higher risk of this blood disease, including 11 small-breed dogs, with English bulldogs presenting years earlier than the overall cohort (8).

The present commentary provides a review of the current knowledge on the biology of CAR T cell therapy and its applications in human oncology. With the success at treating B cell lymphoma using CAR T cell therapies in people, and the conserved nature of the blood systems between dogs and humans, this review also provides a perspective for developing these cell therapies for conquering canine cancer.

DEFINITION AND PROCESS OF MANUFACTURING CAR T CELLS FOR CANCER THERAPY

What Are CAR T Cells?

The evolution of CAR T cell therapies at an unprecedented pace in the world of immuno-oncology marks an exciting time for the development of new strategies for cancer treatment. There are currently three main generations of CAR T cells. The original CAR structure (*first generation*) was described in 1989 and included an *antigen-binding domain* (usually derived from a single-chain variable fragment (scFv) or a protein receptor), a *hinge* that connects the scFv to a *transmembrane domain* and a *signaling domain* composed of CD3ζ (Fig. 1). The hinge region of the CAR is important for optimal tumor antigen binding, while the activation domain directs CAR T cell response. In most cases, the scFv binding domain of these CARs was of murine origin, leading to anti-CAR cytotoxic T cell responses (10,11). Therefore, the first-generation CAR T cell therapy resulted in weak proliferation, brief survival, and limited anti-tumor effect in patients (12–14).

Later, it was found that T cells require a second signal for full activation, and therefore, *second-generation* CAR T cells were developed, with two recently FDA-approved products in the USA and Europe. The second-generation CAR T typically includes an antigen-binding domain, a *hinge*, *one co-stimulatory domain*, and a CD3ζ *signaling domain*. The addition of a co-stimulatory molecule (e.g., CD28 or 4-1BB) leads to improved expansion and persistence of CAR T cells and has been shown to increase their anti-tumor effect in human cancer patients (15,16). CD28 and 4-1BB (CD137) are the two most commonly used co-stimulatory molecules thus far. CD28 is a member of the immunoglobulin family of co-stimulatory receptor, which also includes cytotoxic T lymphocyte-associated antigen-4 and programmed death receptor (PD-1). CD28 signaling increases the effect of T cell and receptor antigen engagement and results in proliferation of T cells at otherwise submitogenic antigen concentrations (17). Consequently, cytokine production, most importantly IL-2, is significantly increased. Therefore, CD28 co-stimulation increased T cell survival by inducing expression of anti-apoptotic proteins such as Bcl-X_L (18). 4-1BB, on the other hand, is a member of the tumor necrosis factor (TNF) receptor family and is expressed primarily on activated lymphocytes. It results in proliferation and differentiation of CD8+ T cells, while inhibiting programmed cell death (19). While CD28 co-stimulation expands naïve T cells, 4-1BB co-stimulation expands memory T cells, resulting in enrichment of antigen-reactive T cells upon recognition of previously primed antigens. Co-stimulation with 4-1BB domain has shown enhanced *in vivo* persistence, higher expansion, and enhanced cytolytic ability compared with CD28 co-stimulation (19,20).

Finally, some authors have recently suggested that combining two co-stimulatory domains would result in a more efficient and persistent anti-tumor activity, through a combination of early tumor-killing with late persistence and engraftment (21). This has led to the concept of *third-generation* CAR that now includes *two co-stimulatory domains* along with the activation domain, resulting in ≥ 3 signaling domains in the CAR T structure. Thus far, the insertion of additional stimulatory domains has not resulted in improved CAR T cell response in preclinical or early clinical trials. However, further research should elucidate if this is a promising approach.

T Cell Isolation, Expansion, and Generation of CAR T Cells

The following steps are required to generate clinical-grade autologous, patient-specific CAR T cells (Fig. 2):

1. T cells are collected from patients by leukapheresis.
2. T cells are then cultured in a good manufacturing process-compliant facility.
3. T cells are stimulated using stimulating beads, antibodies or artificial antigen-presenting cells.
4. T cells are transduced with the CAR of interest. At this stage, the non-tumor-specific T cells acquire the ability to recognize tumor antigens. To insert the CAR gene into T cells, viral vectors (lentivirus or retrovirus) or non-viral approaches are used (transposon, CRISPR, TALEN, RNA). While the use of viruses raises concerns for insertional mutagenesis, third-generation lentiviruses have been shown to be safe after decades of patient follow-up.
5. T cells are cultured for a period of 7–14 days. During that time, they expand by several folds, express the CAR T construct of choice, and are tested to pass prespecified release criteria (i.e., sterility, safety, efficacy). They will then be cryopreserved for future infusion in patients.
6. CAR T cells are finally administered as intravenous infusion in patients following a low-dose lymphodepleting chemotherapy.

After infusion, CAR T cells are stimulated through the CAR receptor after they recognize their target antigen on tumor cells. This is followed by a massive expansion of T cells *in vivo*, associated with cytokine production, and the release of cytotoxic granules (Fig. 3). During this time, T cells exhibit their anti-tumor effect and patients are at risk of developing cytokine release syndrome. Following T cell expansion, CAR

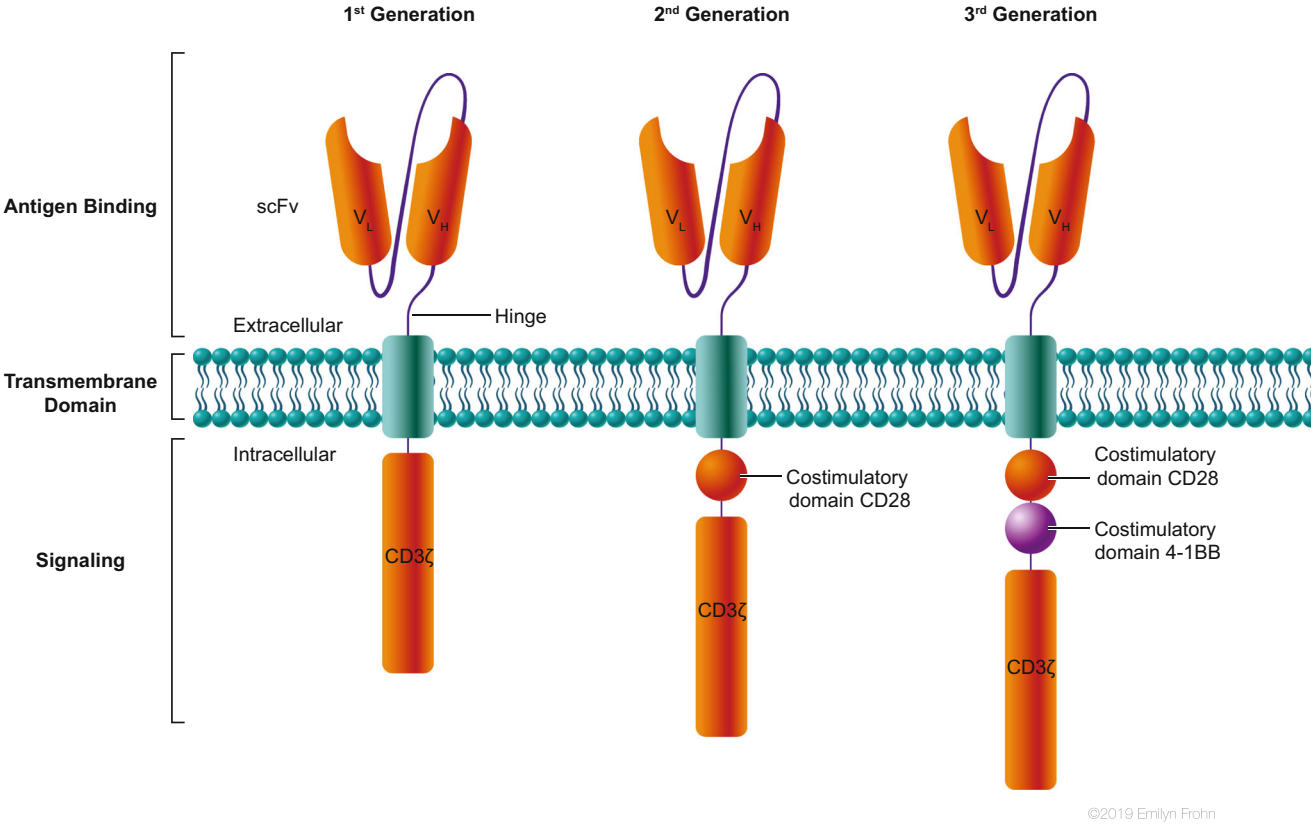

Fig. 1. Evolution of the chimeric antigen receptor (CAR). The first CAR generation consists of an antigen-binding domain, usually derived from a single-chain variable fragment (scFv), a hinge that connects the scFv to a transmembrane domain, and a signaling domain composed of CD3ζ. The second generation includes an antigen-binding domain, a hinge, a co-stimulatory domain (typically CD28), and a CD3ζ signaling domain. The third-generation CAR includes two co-stimulatory domains (e.g., CD28 and 4-1BB) along with the activation domain, resulting in ≥ 3 signaling domains in the CAR structure. Adapted from (9). Additional details on CAR T cell structure and response can be found in "Definition and Process of Manufacturing CAR T Cells for Cancer Therapy" section, under *What Are CAR T Cells?*

T cells have the ability to differentiate into a stable population of memory T cells to prevent potential cancer relapses (16).

CURRENT APPLICATIONS IN HUMAN ONCOLOGY

In the pre-CAR T therapy era, prognosis of relapsed/refractory B cell acute lymphoblastic leukemia (ALL) was dismal with median overall survival reported in few weeks–months and survival at 5 years around 7–8% (23–25). *Tisagenlecleucel* (previously CTL019) was the first FDA-approved gene therapy for the treatment of relapsed/refractory B cell ALL in patients up to 25 years of age. The initial report included two children from the University of Pennsylvania, one of whom had an ongoing response at 11-month follow-up (and we know is ongoing to date), while the other relapsed with CD19-negative blast cells after an ephemeral response lasting for 2 months (26). In the subsequent report of 30 patients with relapsed/refractory ALL, 27 (90%) patients achieved a complete response and 22 (73%) patients had no detection of disease using sensitive multiparametric flow cytometry at 1 month after infusion (27). In a follow-up multicenter clinical study (ELIANA trial) including 75 ALL patients under tisagenlecleucel therapy, remission was noted in 83% of patients with an overall survival rate of 90% at 6 months and 76% at 12 months (5).

Following the remarkable activity in ALL, trials with CAR T cells targeting CD19 (CART19) were initiated in B cell lymphomas. Diffuse large B cell lymphoma (DLBCL) is a heterogeneous group within non-Hodgkin's lymphomas (NHL) with varying molecular profiles, gene sequencing patterns, and clinical responses, some of which are associated with poorer outcomes representing an area of therapeutic unmet need (28).

The now FDA-approved *axicabtagene-ciloleucel* (KTE-019) therapy was initially developed at the National Cancer Institute (NCI) (29). Subsequent clinical studies showed an objective positive response in 75–80% of DLBCL patients treated with axicabtagene-ciloleucel, including long-lasting responses (30). This construct was further pursued by Kite Pharma, as KTE-019, in the pivotal ZUMA-1 trial which paved the way for FDA approval of this modality for DLBCL. The phase 1 of the ZUMA trial enrolled seven patients with one patient experiencing a dose-limiting toxicity, while grade ≥ 3 cytokine release syndrome (CRS) and neurotoxicity were reported in 14% and 57% of patients, respectively. In this report, five out of the seven (71%) patients showed an objective positive response, with four (57%) being complete responses. In the phase 2 ZUMA-1 study of 111 DLBCL patients, overall positive response was reported in 82% of patients, with a complete response in 54% of the cases (4). Of the 108 patients who had at least 1-year

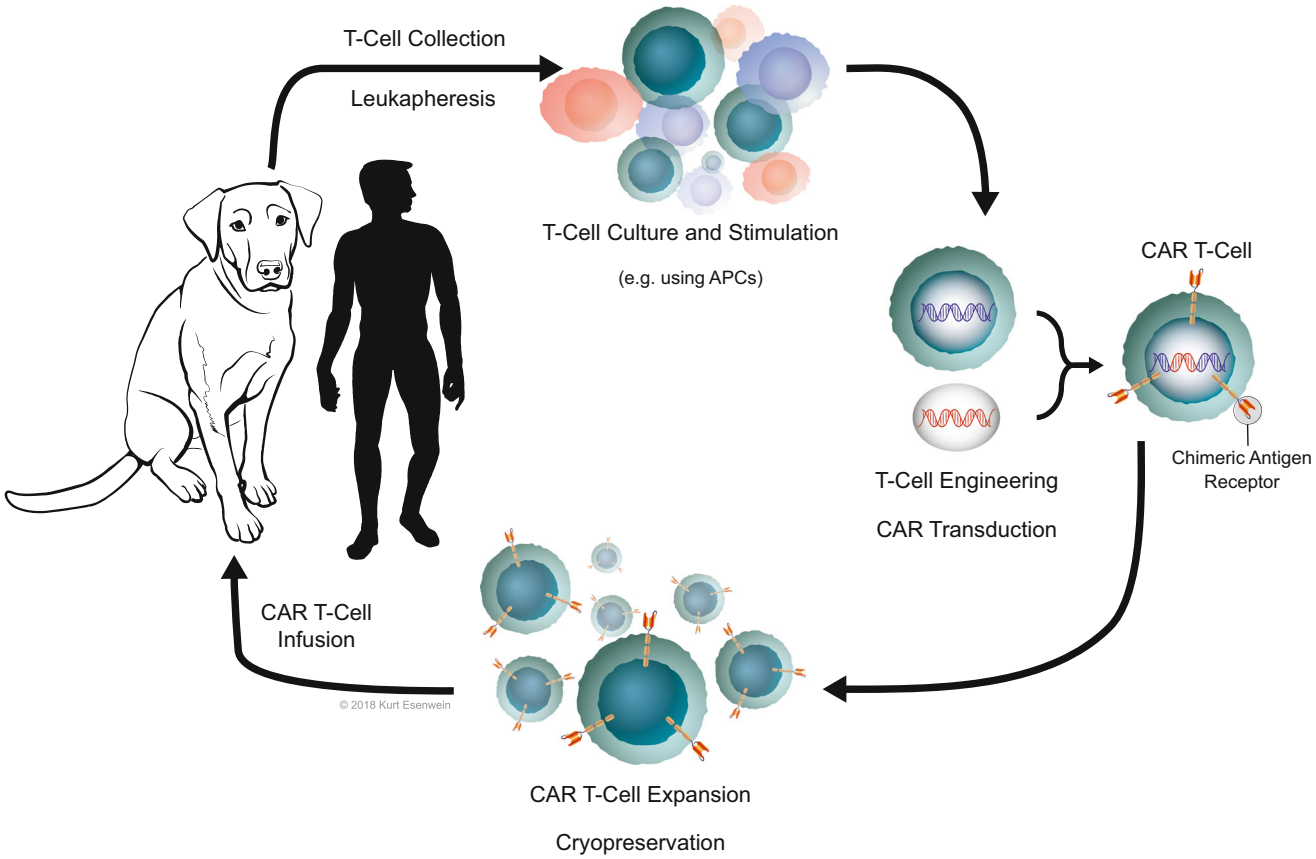

Fig. 2. An overview of the basic steps of CAR T cell therapy production: (1) a patient (human, dog) or donor is undergoing leukapheresis to isolate T cells; (2) T cells are then cultured *ex vivo* and stimulated using antigen-presenting cells (APCs); (3) T cells are genetically engineered to express CAR by gene transduction; (4) CAR-expressing T cells are expanded to a significant population size *in vitro*. They will be cryopreserved prior to future use in patients; (5) CAR T cells are finally infused in patients following a low-dose lymphodepleting chemotherapy

follow-up in phase 1 and phase 2 of the ZUMA-1 trial, an overall response was seen in 82% of patients, with a complete response in 58% of the cases. The progression-free survival rate was estimated at 49% in patients at 6 months, 44% at 12 months, and 41% at 15 months, while the overall survival rate was 78%, 59%, and 52% at 6, 12, and 15 months, respectively. Response to treatment was associated with a higher expansion of CAR T cells. One-year follow-up data presented at the Annual Meeting of the American Society of Hematology and the Bone Marrow Transplantation Tandem Meetings in 2018 (31) suggested loss of CD19 expression and gain of PD-L1 expression as possible mechanisms for resistance following CAR T cell therapy.

Next to axicabtagene-ciloleucel, *tisagenlecleucel* has also been approved by the FDA for use in patients with relapsed/refractory DLBCL (not including primary mediastinal large cell lymphoma). Approval was based on a phase 2 study (JULIET) that enrolled 160 patients with primary analysis available on 81 patients with at least 3-month follow-up or earlier discontinuation (32). Best overall response rate was estimated at 53.1% in these evaluable patients (39.5% complete response and 13.6% partial response). At 6 months, the probability of being relapse-free was estimated at 73.5% with an overall survival of 64.5%. Ninety-five percent of patients in complete response at 3 months also maintained a positive response at 6 months. Another case series for the same product enrolled 38 patients with DLBCL or follicular lymphoma, of which 28 were able to receive cell infusion (33). At 3 months, 18 of the 28 patients had a positive response (64%). At 6 months, 16 out of 28 (57%) patients had a complete response, and these remained in remission at a median time of 29.3 months (range 7.7–37.9 months).

Overall, multiple CD19 targeting CAR T cell therapy constructs are currently in development and expected to receive FDA approvals for different B cell malignancies in the next 2–3 years. One example is the B cell maturation antigen (BCMA) directed CAR T cell therapy which is showing promising activity in multiple myeloma (34).

UNIQUE TOXICITIES OF CAR T CELL THERAPY

Due to their specific mode of action, CAR T cells are less likely to produce off-target toxicity as compared with standard chemotherapeutics. As such, the off-target recognition of cross-reactive antigens by the scFv portion of the CAR has not been reported in clinical trials to date. Nevertheless, CAR T cell therapy is associated with various adverse reactions, including the development of cytokine release syndrome, neurotoxicity, and B cell aplasia resulting in hypogammaglobulinemia.

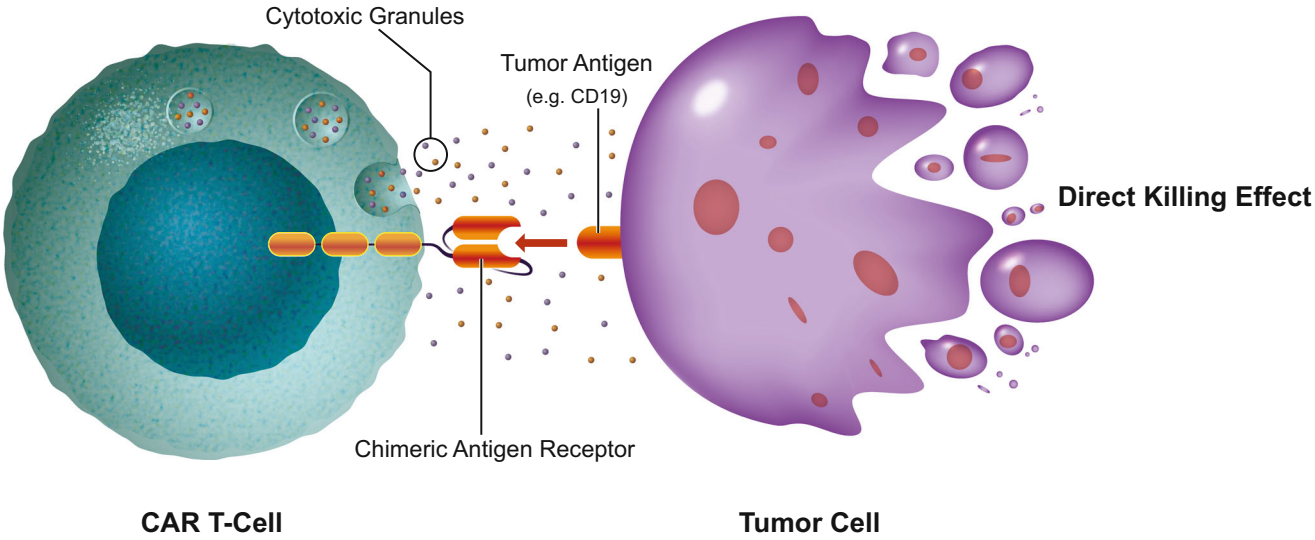

Fig. 3. In chimeric antigen receptor (CAR) therapy, a patient's T cells are reprogrammed to seek out and target cells expressing a particular antigen found on specific cancer cell types (2). Activation of CAR T cells leads to direct killing of tumor cells through the release of cytotoxic granules, such as granzyme and perforin. Tumor cell killing can also be mediated by activation of other components of the immune system such as macrophages and natural killer cells (22). Consult Fig. 2 for additional technical details on CAR T cell production

Cytokine Release Syndrome

Cytokine release syndrome (CRS) is one of the most feared toxicities related to CAR T cell therapy. As its name suggests, CRS is a systemic inflammatory state resulting from the excessive production of cytokines associated with CAR T cell activation. Time to development of CRS is widely variable and depends on the CAR construct, the disease type, and the tumor burden. Rates of CRS have ranged from 45 to 100% in various reports with serious or ≥ 3 grade in up to 50% of patients (35). Clinical manifestations can range from mild fever to life-threatening vasodilatory shock causing hypoxia, hypotension, and organ toxicity mandating management in the intensive care unit. Death related to CRS has been reported in multiple studies (4,15,36). It has also been suggested that a higher burden of tumor antigens is associated with higher rates and severity of CRS (37).

Various biomarkers have been studied to elucidate the mechanisms of CRS, of which interleukin (IL)-6/IL-6 receptor interaction has been most consistently shown to correlate with the occurrence of CRS. Consequently, blockade of the IL-6 pathway typically results in alleviation of symptoms related to CRS (11). C-reactive protein and ferritin are clinically available laboratory tests that are known to be elevated in patients who develop CRS and are monitored closely at some institutions, including the Mayo Clinic Cancer Center (38,39). Other cytokines associated with inflammation such as interferon-gamma, soluble IL-2 receptor, and IL-10 have been implicated as well.

Teachey *et al.* (40) at the University of Pennsylvania identified a set of 24 cytokines, including interferon-gamma, IL-6, and soluble glycoprotein-130 that are associated with severe CRS in ALL patients receiving 4-1BB/ CD3ζ CAR T cell therapy. More recently, studies in murine models of CRS have demonstrated that the severity of CRS does not only depend on CAR T cell–derived cytokines but also on IL-1, IL-6, and nitric oxide release by host macrophages (41). This finding can potentially open additional avenues for preventative or therapeutic measures.

Currently, the mainstay of treatment for CRS remains tocilizumab (a humanized monoclonal antibody against the IL-6 receptor) since its use in the first patient treated with CART19 for ALL (25). Subsequent data showed that the use of tocilizumab for CRS does not adversely affect the expansion of CD28/CD3ζ CAR T cells, unlike that of high-dose steroids (39). Another agent of potential utility for this indication is siltuximab, a chimeric monoclonal antibody, which, in contrast to tocilizumab, directly inhibits IL-6.

Neurotoxicity

The risk of neurotoxicity with CAR T cell therapy became apparent when 5 patients died of cerebral edema in one of the early phase ROCKET trial being conducted by Juno Pharmaceuticals using JCAR015 in adult patients with B cell ALL. Additional deaths have been reported in both B cell NHL and ALL trials (10,39). Non-fatal but clinically significant neurotoxicity has additionally been reported in around 40–50% of patients across various clinical trials with the different CAR constructs in various malignancies (42). Clinical presentation can vary from headache, confusion, tremor, to delirium, expressive aphasia, obtundation, myoclonus, or seizure. Whether there are pre-existing risk factors in the form of CNS disease is currently unknown, as patients with active CNS disease were typically excluded from clinical trials. Various hypotheses have been put forth to explain the development of neurotoxicity, but the exact mechanism remains elusive. One hypothesis is that CAR T cell activation results in elevated cytokine levels triggering macrophage activation and subsequent neurotoxicity. More recently, with the use of the CD28-CD3ζ therapy in lymphoma, IL-10 as well as IL-15 were noted to achieve higher peak levels in patients with grade 3 or 4 neurotoxicity compared with those with grade < 3 neurotoxicity (43). Endothelial activation and

multifocal vascular lesions, resulting in disruption of the blood-brain barrier, were reported in patients experiencing neurotoxicity within 28 days of infusion with CD19 CAR T cells in B cell ALL and NHL (44). Humanized mice model studies have shown a role for IL-1 and IL-6 derived from host monocytes in neurotoxicity, thereby providing a rationale for the use of anakinara (IL-1 receptor antagonist) in this indication (41). Additionally, the direct inhibition of IL-6 by siltuximab justifies its use over tocilizumab for the treatment of CRS, as it reduces the likelihood of IL-6 passive diffusion into the CNS and its related neurotoxicity (45). As of today, the mainstay of therapy to resolve CAR T-associated neurotoxicity remains corticosteroids.

Hypogammaglobulinemia

B cell aplasia is an example of "on-target/off-tumor" activity of CAR T cell therapy because CD19 is expressed not only on the malignant B cells but also on normal B-lymphocytes. Since B cells are assigned with the task of producing immunoglobulins, B cell aplasia following CAR T cell therapy results in prolonged hypogammaglobulinemia. Thus, it is not surprising that all patients from the University of Pennsylvania ALL cohort who had a positive clinical response to CAR T cell therapy also developed B cell aplasia (5). Hypogammaglobulinemia leads to an increased risk of infections and the need for regular intravenous immunoglobulin replacement for the duration of B cell aplasia.

Introduction of the inducible caspase-9 (iCasp9) suicide gene has been described as one of the therapeutic approaches to limit the toxicity of CAR T cells *in vivo* (45). Upon activation with a bio-inert small molecule AP1903, iCasp9 operates as a safety switch resulting in T cell apoptosis. By significantly improving the safety profile of CAR T cells, the iCasp9/AP1903 suicide gene approach is likely to facilitate the widespread use of cell-based therapy in clinical practice.

APPLICATIONS IN VETERINARY ONCOLOGY

A Critical Need for New and Innovative Therapies in Canine B Cell Lymphoma

It is estimated that more than 4.2 million dogs (5300/100,000 per population rate) in the USA are diagnosed with cancer each year (47). The epidemiology of canine cancer is, however, not well defined in the literature. Most of the available incidence data comes from a limited number of tumor registries and the European Union where there is a higher percentage of insured dogs. Very little to no published data is available to indicate what percentage of dogs diagnosed with cancer are then treated or how they are treated in the USA. This makes any assessment of the actual market potential for veterinary oncology therapeutics extremely challenging. Clinical experience would indicate that the most common canine malignant cancers diagnosed and treated include lymphoma, mast cell tumor, osteosarcoma, soft tissue sarcoma, hemangiosarcoma, and melanoma.

This clinical impression is supported by a Swiss Canine Cancer Registry study that outlined the most common neoplasms diagnosed in over 120,000 dogs during a 53-year period as follows: adenoma/adenocarcinoma (18.1%), mast cell tumor (6.5%), lymphoma (4.3%), melanoma (3.6%), fibroma/fibrosarcoma (3.4%), hemangioma/hemangiosarcoma (2.8%), squamous cell carcinoma (1.9%), and osteoma/osteosarcoma (1.2%) (48). The high occurrence of carcinoma (mammary) is related to the less frequent implementation of ovariohysterectomy at a young age which is more common in the USA.

Lymphoma, with an estimated incidence rate of 20–100 per 100,000 dogs (49), is one of the most widely treated canine cancers given its frequent occurrence and typically robust response to chemotherapeutics. Based on the current approximation of 75 million dogs in the USA, estimates are that 16,000–80,000 new cases of canine lymphoma are diagnosed each year (50). Other estimates place the number of diagnosed canine lymphoma cases at over 250,000 annually in the USA, accounting for 12–18% of annual death-related malignant cancers in dogs (47). This makes the canine lymphoma market a very appealing potential opportunity for therapeutic development.

There is abundant recent literature highlighting the pathologic, biologic, immunophenotypic, genetic, and treatment response similarities between human and canine lymphoma (50–53). Specifically, DLBCL is the most common subtype of lymphoma in both species (54), and it is the subtype most studied with genomic profiling in veterinary medicine (47). Utilizing immunohistochemistry and gene expression profiling, similar profiles were noted between human and canine DLBCL, and certain markers were able to separate the canine DLBCL cases into two groups with significantly different clinical outcomes (7). Provided this robust and expanding body of data supporting the parallels between the most common types of human and canine lymphoma, the opportunities for therapeutic development in one species to inform and progress that in the other species will only continue to grow.

The majority of canine cancer treatments rely on the use of human generic chemotherapeutics. Yet, the clinical responses to these therapeutics for the most common canine cancers (lymphoma, osteosarcoma, hemangiosarcoma) have remained static for the past 10–20 years.

Focusing on canine B cell lymphoma in particular, the standard of care for dogs with high-grade lymphoma over the last 35 years has ranged from single-agent protocols (using prednisone or doxorubicin) to combination chemotherapy regimens of variable duration. Most veterinary oncologists agree that a doxorubicin-based (e.g., CHOP) combination chemotherapy protocol provides the longest period of disease control and overall survival (54). However, the response to chemotherapy is often suboptimal with recurrent or refractory disease representing a significant clinical challenge. The combination of chemotherapy with half- and total-body irradiation has also been evaluated in some dogs with lymphoma. The reported median survival rate in these instances is no longer than that achieved with chemotherapy alone, thereby questioning the utility of this adjunctive therapy (54). Transplantation of autologous bone marrow has recently facilitated the safe dose escalation of cyclophosphamide that resulted in long-term remission and prolonged patient survival in dogs (55). However, autologous bone marrow transplantation is logistically challenging to perform in a veterinary hospital setting which limits its widespread application.

With only a handful of FDA-approved or USDA-licensed veterinary oncology therapeutics currently available to veterinarians, there is a dire need for canine-specific treatment options (Table I). To date, there is only one therapeutic with conditional FDA approval, rabacfosadine (Tanovea®-CA1, VetDC), for the treatment of canine B cell lymphoma. Rabacfosadine is an intravenously administered prodrug of the active nucleotide analog 9-(2-phosphonylmethoxyethyl) guanine (PMEG), a cytotoxic therapeutic agent. Rabacfosadine effectively loads lymphoid cells with active PMEG while reducing circulating levels of PMEG in plasma and target organs of toxicity. Tanovea-CA1 received conditional approval from FDA in January 2017 for the treatment of lymphoma in dogs and became available to veterinarians in the spring of 2017.

Immuno-oncology innovations are starting to make their way to veterinary oncology but remain limited with extremely sparse supporting data. Rituximab has been evaluated in dogs *ex vivo* and found not to bind or deplete canine B cell lymphocytes (56,57). Although an anti-CD20 (BLONTRESS®, Aratana) and an anti-CD52 (TACTRESS®, Aratana) monoclonal antibody are both fully licensed by the USDA, the company has stated that neither antibody is as specific to their respective targets as expected. No peer-reviewed data is available on either of these therapeutics to date, and they are not commercially available. Another immunotherapeutic, the canine lymphoma vaccine, DNA (Boehringer Ingelheim) is currently available. This is a xenogeneic murine CD20 DNA therapeutic vaccine for use in dogs with B cell lymphoma that was conditionally licensed by the USDA in 2015. No peer-reviewed data is available on this therapeutic either. Therefore, with current median survival times for dogs with lymphoma stagnant at less than 1 year, the opportunity for new, advanced, specific therapeutics remains clear.

Preliminary Data in Dogs

In a first-ever canine study, Panjwani and colleagues (58) have reported successful mRNA electroporation of primary canine T cells to generate CAR T cells. In brief, a novel expansion methodology was developed that yields large numbers of canine T cells from normal or lymphoma-diseased dogs. In this study, the authors had modified previous methods to activate and expand canine T cells *ex vivo* by using artificial antigen-presenting cells genetically modified to express human CD32 and canine CD86. These artificial antigen-presenting cells were loaded with a canine CD3 monoclonal antibody and used in combination with human IL-2 and IL-21 to preferentially expand $CD8^+$ T cells. The mRNA electroporation procedure was utilized to express a first-generation, canine CD20-specific CAR in expanded T cells as primary therapy. Treatment in one dog with relapsed B cell lymphoma was well tolerated and led to a modest, but transient, anti-tumor activity, suggesting that stable CAR expression is required for sustained clinical remission. Other possible factors that could have contributed to the partial anti-tumor activity include limited CAR T cell expansion and the development of canine anti-mouse antibodies directed against the murine scFv construct. Future studies are currently underway to investigate the clinical efficacy of a stably transduced canine CAR T cell line expressing fully canine, second-generation CAR constructs. Lymphodepleting chemotherapy should also reduce the risk of inducing canine anti-mouse antibodies.

The high cost of current human treatments, $475,000 for tisagenlecleucel and $373,000 for axicabtagene ciloleucel (59) not including hospitalization and other costs, raises an important potential challenge for the accessibility of this technology for use in dogs. In humans, non-viral genome engineering tools are in development with the potential to reduce the cost of goods through obviating the need for the generation of an infective engineered virus. For example, the *Sleeping Beauty* (60) and *piggyBac* (61) transposons are in ongoing CAR T cell clinical trials. In addition, gene editing approaches for targeted knock-in using electroporation and ssDNA as donor (62) and new approaches using enhanced dsDNA as donors for efficient targeted gene knock-in (63) hold the potential for additional and more accessible, non-viral methods for CAR T cell generation.

COMPARATIVE ONCOLOGY: AN OPPORTUNITY TO ACCELERATE PARALLEL DRUG DEVELOPMENT

As opposed to small molecule drugs, CAR T cells are considered biological products and are therefore regulated by the FDA Center for Biologics Evaluation and Research for humans and the USDA/APHIS Center for Veterinary Biologics for canine applications. According to a recent report from the National Academy of Medicine (64), only one out of ten oncology candidates that appear promising in preclinical mouse models are in fact effective and safe in human clinical trials. This overtly high attrition rate highlights the need for alternative models at the early stage of the Drug Research and Development lifecycle (65), as shown in other therapeutic areas (66–71).

Although murine models have been extremely useful for studying the biology of cancer initiation, promotion, and progression, mice typically do not faithfully represent many of the features constitutive of human cancer, including genomic instability, tumor heterogeneity, and long periods of latency (72). Additionally, study mice are often immunocompromised and bred in sterile laboratories, unlike domesticated dogs that share the same habitat and are exposed to same environmental carcinogens (e.g., UV light, pollution, and food contaminants) as humans.

As opposed to mice, cancers develop spontaneously in dogs (i.e., without genetic manipulation) and in the context of an intact immunity with a syngeneic host and tumor microenvironment. Canine tumors typically have similar features to human malignancies, such as histological appearance, cytogenic abnormalities, therapeutic response, acquired resistance, and background genetics (72). Indeed, as the dog genome became available, multiple comparative genomic studies have shown significant homologies between canine and human cancer-associated genes, including MET, mTOR, KIT, and TRAF3 (73). Given the large number of breeds and their shared ancestry (74), inheritable germline mutations associated with cancer are easier to identify in purebred dogs than in human populations (75). The outbred nature of dogs (relative to most murine models) contributes to their biological relevance for studying new cancer therapies. At

Table I. Approved or Licensed Veterinary Oncology Therapeutics (USA)

Trade name	Compound name	Company	Indication	Regulatory status, USA (year)	Species	Commercial availability
Blontress®	Canine lymphoma MAb, B cell	Aratana	B cell lymphoma	USDA Licensed (2015)	Canine	No
NA	Canine lymphoma vaccine, DNA	Merial/BI	B cell lymphoma	USDA Conditional License (2015)	Canine	Yes
NA	Canine osteosarcoma vaccine, live listeria vector	Aratana	Osteosarcoma	USDA Conditional License (2017)	Canine	Yes
NA	Feline interleukin-2 immunomodulator	Merial/BI	Primary stage I fibrosarcoma	USDA Conditional License (2015)	Feline	Yes
Immunocidin ®	Mycobacterium cell wall fraction	NovaVive	Mammary tumors	USDA Licensed (2009)	Canine	Yes
Oncept®	Canine melanoma vaccine, DNA	Merial/BI	Melanoma	USDA Licensed (2010)	Canine	Yes
Palladia®	Toceranib phosphate	Zoetis	Grade II/III mast cell tumor	FDA Approved (2009)	Canine	Yes
Tactress®	Canine lymphoma MAb, T cell	Aratana	T cell lymphoma	USDA Licensed (2016)	Canine	No
Tanovea®-CA1	Rabacfosadine for injection	VetDC	Lymphoma	FDA Conditional Approval (2017)	Canine	Yes

the same time, the rapid progression of cancer associated with the shorter lifespan of dogs provides an opportunity to study the efficacy and safety of candidate therapeutic drugs in a much faster timeframe than clinical trials in human patients (76).

Biological similarities between canine and human cancer provide an impetus for the study of novel therapeutics in dog clinical trials (Fig. 4). In fact, the evaluation of oncology drugs in dogs with naturally occurring cancers is not new, with a few descriptions already available in the early 1970s (77–79). Over the last decade, multiple reports have demonstrated the relevance of the dog model to bridge the knowledge gap between murine experiments and human clinical trials and exemplify the value of a comparative oncology approach to drug development (80,81). For instance, both canine and human DLBCL patients share similar constitutive NF-κB activity that drives overexpression of anti-apoptotic NF-κB target genes which promote lymphocyte proliferation (82,83). Studies indicate that administration of a targeted inhibitor of constitutive NF-κB activity, NEMO binding domain (NBD), induces apoptosis of canine malignant B cells *in vitro*. Moreover, pilot trials have demonstrated that intranodal administration of NBD peptide to dogs with relapsed B cell lymphoma inhibits the expression of NF-κB target genes leading to reduced tumor burden (84). In a separate phase 1 clinical trial, these same investigators showed that NBD peptide administered intravenously is safe and effective at inhibiting constitutive NF-κB activity in a subset of dogs with lymphoma (85).

Additionally, the use of established canine tumor cell lines has proven beneficial in studying tumor biology and preclinical therapeutics. A CD40 ligand-dependent culture system for canine malignant B cells has been recently designed to test compounds for treatment in primary tumor samples from dogs and humans (86). The tumor cells retain their original phenotype, clonality, and known karyotypic abnormalities after expansion and culture. This canine cell culture system is reported to be potentially robust to perform *in vitro* preclinical cytotoxic assays with primary B cell malignancies.

The opportunity to synergize quantitative information available from humans and animals sharing clinical analogs to develop improved therapies for both species is known as "reverse translation" (65). A significant component of the success of comparative oncology in drug development is the creation of consortia that link drug development stakeholders to veterinary clinicians with access to tumor-bearing pet animals. This supports the implementation of clinical trials carried out in pets and the collection of high-quality clinical data and biologic specimens that are critical to defining pharmacokinetic/pharmacodynamic (PK/PD), tolerability, and efficacy of novel therapeutic approaches destined for human use.

To this end, the Comparative Oncology Program of the NCI has established a multicenter collaborative network of 24 veterinary academic partners known as the Comparative Oncology Trials Consortium (COTC) (72,87). The mission of the COTC is to answer biological questions geared to inform the development path of chemotherapeutics for future use in human cancer patients. The COTC operates as a platform for collaborative work between the NCI and extramural academic comparative oncology centers to design and execute clinical studies in dogs with cancer. Support for

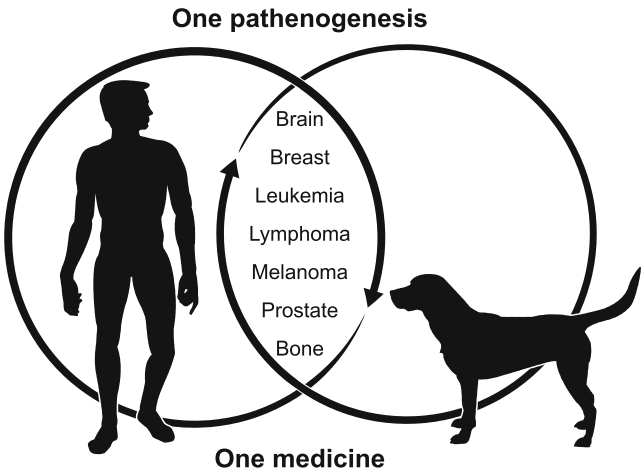

Fig. 4. Common cancers that have clinical analogs in humans and dogs. Approximately 4.2 million dogs (*vs.* 1.7 million human patients) are diagnosed with cancer each year, representing about 5300 new canine cases for a standard 100,000 population size (47)

the oversight and management of the COTC comes from the NCI. Trial sponsors, most often pharmaceutical companies, support the costs associated with clinical studies in dogs in established COTC academic centers.

Several published examples of COTC trials exemplify the functionality and impact of such studies (87–89). COTC trials do not focus exclusively on small molecules or biologic agents; instead, they can be designed and implemented to answer a range of drug development questions that are key to the forward progress of an agent or group of candidate molecules, medical devices, or molecular profiling platforms. One such example illustrating the value of the dog model pertains to the development of the inflammatory cytokine IL-12 for the treatment of human malignant melanoma. The use of cytokines to enhance anti-tumor immunity has been recognized as an important immunomodulatory approach in cancer management. Yet, historically, the high risk for systemic toxicity presented by IL-12 dosing had prevented development of this cytokine into a therapeutic drug. A strong genetic similarity exists between canine and human IL-12 (i.e., 84% homology for the ligand and 68% homology for the receptor), which motivated studies on the characterization of IL-12 PK/PD, efficacy, and toxicity in dogs with naturally occurring malignant melanoma (81). Results showed that a fully human necrosis-targeted immunocytokine NHS-IL-12 could be safely administered subcutaneously to canine patients with malignant melanoma, while maintaining both systemic immunological and clinical activity. This was demonstrated by measuring serum IL-12 and other representative biomarkers (e.g., IL-10 and IFN-gamma) over time and establishing PK/PD models of IL-12. These findings in dogs were key to guide the sponsor's decision to move forward with a phase I clinical trial of this agent in humans. In turn, preliminary studies focusing on IL-12 gene electrotransfer in dog patients with melanoma have shown promising results for the treatment of spontaneous canine tumors (90,91).

With respect to CAR T cell therapy research and development, the COTC infrastructure stands ready to support the implementation of cell-based trials for pivotal go/no-go decision-making prior to clinical testing in humans. Through strategic partnerships with study sponsors whom can provide the necessary cell manufacturing, quality control/assurance, and distribution support for such trials, the COTC can provide the requisite scientific input and execution for such trials to be carried out in the veterinary academic setting. Similarly, the COTC Pharmacodynamic Core Laboratory can provide access to providers of canine-specific assay support for critical immunological assays such as flow cytometric assessment of immune cell subsets, gene expression profiling, histopathology, immunohistochemistry, proteomics, multiplex cytokine analysis, and related diagnostic assays (92).

Besides applications in oncology, efforts are on the way to harness the immunosuppressive property of CAR T cell for the treatment of autoimmune diseases, such as inflammatory bowel disease (IBD) (93), thereby opening new avenues for comparative medicine and parallel drug development as the dog is a spontaneous animal disease model for IBD as well (94).

CONCLUSIONS

CAR T cells are one of the most promising therapies for the treatment of hematological malignancies. Specifically, CART19 cells have demonstrated unprecedented clinical results in human B cell malignancies with two constructs already approved by the FDA in 2017.

Yet, the technology is still in its early phase, and significant challenges need to be resolved before these novel therapies can be used for large-scale clinical trials. Obvious limitations include the complexity and costs (direct: related to the manufacturing and indirect: related to hospital costs and patient care) of CAR T cell therapy. The requirement for GMP materials and the individualized nature of the therapy are the main causes that drive up the cost. The possibility to generate allogeneic off-the-shelf universal CAR T cells (95) would lead to easier and more cost-effective manufacturing, reduced time to CAR T cell infusion, and faster translation of novel combination strategies with CAR T cells in early phase clinical trials. Also, allogeneic CAR T cells will be generated from healthy donors with a functional immune system, providing advanced stage cancer patients the option to undergo CAR T cell therapy when their own T cells lack the ability to expand and be reprogrammed *ex vivo* (95). Importantly, the management of toxicities after CAR T cell therapy requires specialized expertise and care level, making it available only in specialized tertiary centers. Strategies to modulate cytokine production after CAR T cell therapy are being developed and could represent a new paradigm in the management of CAR-T cell-related adverse reactions.

There is currently a lack of robust preclinical models to recapitulate the microenvironment and toxicities following CAR T cell therapy. Canines have long been used for the preclinical testing of human cell therapies and represent an attractive spontaneous disease model to study innovative CAR T cell strategies and to develop novel off-the-shelf approaches. In return, information on CAR T cell efficacy and safety from human clinical trials can guide the development of future cell-based therapies in veterinary oncology, under the so-called One Health initiative (65). Preliminary data in dogs using a canine CD 20-specific CAR in expanded T cells showed promising, but transient results. However, these preliminary findings lay the foundation for future studies in dogs where both tumor biology and the microenvironment more reliably model the human disease.

Finally, multiple studies in humans are currently evaluating the effect of CAR T cell therapy for the treatment of solid tumors, with modest results thus far (96). Potential strategies to increase the efficacy of CAR T in this context include combinations with immune stimulants, secondary modifications of CAR T cells, re-engineering of the T cell, and specific targeting of the tumor microenvironment.

AUTHOR CONTRIBUTIONS

All authors (JPM, SE, CJ, AJ, KA, ABM, MK, SB WW, AKL, SSK) have contributed to the writing of the manuscript. JPM was responsible for the final production of the commentary. All authors have read and approved the final manuscript.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest JPM, SE, CJ, AJ, KA, WW, and SSK are founders of LifEngine Animal Health Laboratories, Inc. SSK is inventor on patents in the CAR T cell therapy field that are licensed to Novartis. This work was partially supported (AKL) by the Intramural Program of the National Cancer Institute, NIH (Z01-BC006161).

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