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

---Manuscript Draft---

<table>
<thead>
<tr>
<th>Manuscript Number:</th>
<th>AAPSJ-D-18-00401R1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full Title:</td>
<td>CAR T-Cell Immunotherapy in Human and Veterinary Oncology: Changing the Odds Against Hematological Malignancies</td>
</tr>
<tr>
<td>Article Type:</td>
<td>Commentary</td>
</tr>
<tr>
<td>Section/Category:</td>
<td>INVITED ONLY: Precision Med: Implications for the Pharmaceutical Sciences (GE. Martinez, Karara)</td>
</tr>
<tr>
<td>Keywords:</td>
<td>Immuno-Oncology; CAR T-cell; Lymphoma; One Health</td>
</tr>
<tr>
<td>Corresponding Author:</td>
<td>Jonathan Paul Mochel, DVM, MS, PhD, DECVPT Iowa State University College of Veterinary Medicine Ames, Iowa UNITED STATES</td>
</tr>
<tr>
<td>Corresponding Author's Institution:</td>
<td>Iowa State University College of Veterinary Medicine</td>
</tr>
<tr>
<td>First Author:</td>
<td>Jonathan Paul Mochel, DVM, MS, PhD, DECVPT</td>
</tr>
<tr>
<td>Order of Authors:</td>
<td>Jonathan Paul Mochel, DVM, MS, PhD, DECVPT Stephan C Ekker, Ph.D Chad M Johannes, DVM, DACVIM Albert E Jergens, DVM, Ph.D, DACVIM Karin Allenspach, DVM, Ph.D, DECVIM Agnes Bourgois-Mochel, DVM Michael Knouse, BS Sebastien Benzekry, Ph.D Wesley Wierson, Ph.D Amy K LeBlanc, CVM, Ph.D Saad S Kenderian, MD</td>
</tr>
<tr>
<td>Manuscript Region of Origin:</td>
<td>UNITED STATES</td>
</tr>
</tbody>
</table>
CAR T-Cell Immunotherapy in Human and Veterinary Oncology:  
Changing the Odds Against Hematological Malignancies  

Subject: Revision of Manuscript ID # AAPSJ-D-18-00401

Dear Pr. Fung,

On behalf of the co-authors I would like to thank the Reviewers for their comprehensive review of our Commentary.

The Manuscript has been edited accordingly, and all comments/concerns from the Referees have been addressed in our revised files using the ‘Track Changes’ option of Microsoft Word.

You will find herein our responses to the Reviewers’ comments supporting our revised manuscript submitted online on February 26, 2019.

With best regards,

Jonathan P. Mochel
CAR T-Cell Immunotherapy in Human and Veterinary Oncology: Changing the Odds Against Hematological Malignancies

Jonathan P Mochel¹, Stephen C Ekker², Chad M Johannes³, Albert E Jergens³, Karin Allenspach³, Agnes Bourgois-Mochel³, Michael Knouse¹, Sebastien Benzekry⁴, Wesley Wierson⁵, Amy K LeBlanc⁶, Saad S Kenderian⁷,⁸

¹Iowa State University, Department of Biomedical Sciences, Ames, IA 50011, USA.
²Mayo Clinic Cancer Center Department of Biochemistry and Molecular Biology, Rochester, MN 55905, USA.
³Iowa State University, Department of Veterinary Clinical Sciences, Ames, IA 50011, USA.
⁴Institut National de Recherche en Informatique et en Automatique, Team MONC, Bordeaux, France.
⁵Iowa State University, Department of Genetics, Development, and Cell Biology, Ames, IA 50011, USA.
⁶Comparative Oncology Program, Center for Cancer Research National Cancer Institute, Bethesda, MD 20892, USA.
⁷Mayo Clinic Division of Hematology, Department of Medicine, Rochester, MN 55905, USA.
⁸Department of Immunology, Mayo Clinic, Rochester, MN 55905.

Correspondence:
Jonathan P. Mochel, DVM, MS, Ph.D, DECVPT
Associate Professor of Pharmacology
Iowa State University College of Vet. Medicine
2448 Lloyd, 1809 S Riverside Dr.
Ames, IA 50011-1250
Phone 515-294-7424
Email: jmochel@iastate.edu

Running head: CAR T-Cell Immunotherapy in Comparative Oncology.
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 U. S Food and Drug Administration; one for the treatment of pediatric Acute Lymphoblastic Leukemia (ALL), 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.
**Keywords:** Immuno-Oncology; CAR T-cell; Lymphoma; One Health.
1 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.

2 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 immunoncology marks an exciting time for the development of new strategies for cancer treatment. There are currently 3 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 [9,10]. Therefore, the first-generation CAR T-cell therapy resulted in weak proliferation, brief survival and limited anti-tumor effect in patients [11-13].

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 U.S 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 [14,15]. 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 sub- mitogenic antigen concentrations [16]. 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-XL [17]. 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 [18]. 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 to CD28 co-stimulation [18,19].

Finally, some authors have recently suggested that combining 2 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 [20]. This has led to the concept of third-generation CAR that now include 2 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 pre-specified 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 antitumor effect and patients are at risk of developing cytokine release syndrome. Following T-cell expansion, CAR T-cells have the ability to differentiate into a stable population of memory T-cells to prevent potential cancer relapses [15].

3 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% [21-23]. 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 2 children from the University of Pennsylvania, one of whom had an ongoing response at 11 months 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 [24]. 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 [25]. In a follow-up multi-center 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 [26].

The now FDA-approved *axicabtagene-ciloleucel* (KTE-019) therapy was initially developed at the National Cancer Institute (NCI) [27]. Subsequent clinical studies showed an objective positive response in 75-80% of DLBCL patients treated with axicabtagene-ciloleucel, including long-lasting responses [28]. 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 7 patients with 1 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, 5 out of the 7 (71%) patients showed an objective positive response, with 4 (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 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 [29] 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 [30]. 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%. 95% 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 [31]. 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 [32].

4 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.

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 [33]. 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 [4,14,34]. It has also been suggested that a higher burden of tumor antigens is associated with higher rates and severity of CRS [35].

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 [36]. 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 [37,38]. Other cytokines associated with inflammation such as interferon-gamma, soluble IL-2 receptor and IL-10 have been implicated as well.

Teachey et al. [39] 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 [40]. 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 [24]. 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 [38]. 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 [9,38]. 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 [41]. 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 to those with < grade 3 neurotoxicity [42]. 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 [43]. 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 [40]. 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 [44]. 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.

5 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 U.S are diagnosed with cancer each year [46]. 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 U.S. 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%) [47]. 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 U.S.

Lymphoma, with an estimated incidence rate of 20-100 per 100,000 dogs [48], 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 U.S, estimates are that 16,000-80,000 new cases of canine lymphoma are diagnosed each year [49]. Other estimates place the number of diagnosed canine lymphoma cases at over 250,000 annually in the U.S, accounting for 12-18% of annual death-related malignant cancers in dogs [46]. 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 [49-52]. Specifically, DLBCL is the most common subtype of lymphoma in both species [52], and it is the subtype most studied with genomic profiling in veterinary medicine [46]. 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 [53]. 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 sub-optimal 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 1). 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 analogue 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 one 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 1 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 antitumor activity include limited CAR T-cell expansion and the development of canine antimouse 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 antimouse 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.

6   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 1 out of 10 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 genomics 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 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 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 pre-clinical 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 multi-center 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 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 antitumor 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 [90]. 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 [91-92].

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 [93].
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) [94], thereby opening new avenues for comparative medicine and parallel drug development as the dog is a spontaneous animal disease model for IBD as well [95].

7 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 [96] 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 [96]. 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-cells 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 [97]. 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.

8 Funding

None.

9 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). The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

10 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.
References


We thank the Reviewers and Editor for their insightful and helpful critiques which have helped to improve the quality of our manuscript. We have responded to Reviewers comments and have revised the manuscript text and presentation of figures in response to their critiques in order to improve the clarity of our Commentary. Changes to the manuscript text have been made using the ‘Track Changes’ option of Microsoft Word.

Referee 1
Overall the article is well written and comprehensive. The following comments and edits should be addressed before publication:

Response: We would like to thank the Referee for her/his positive evaluation of our work.

1. Line 15: canine should be plural.
Response: Thank you. The manuscript has been revised accordingly (L15).

2. Line 22: define "living therapies".
Response: We agree with the Referee that the term ‘living therapies’ is confusing and we have replaced it with ‘cell therapies’ in our revision of the manuscript (L22).

3. Figure 1: The figure should be replaced with a more accurate presentation of the 3 generations of CAR T-cell therapy. All components should have a legend or be defined such as TM, and the co-stimulatory molecules. the hinge should be identified.
Response: Thank you. Figure 1 has been significantly edited to address the Referee’s comments. Details on CAR structure and co-stimulatory domains are available in the Figure legend.

4. Line 39 should be modified to read "... CAR transgene may be less pronounced with human derived scFv."
Response: Thank you. This section has been significantly edited and this sentence is no longer present in our revision of the manuscript.
4. Line 44: the sentence states "The extracellular domain of CD28..." Figure 1 shows CD28 as intracellular. This should be reconciled.
Response: The paragraph describing the 3 generations of CAR T-cells has been revised and simplified to improve understanding of this sub-section (also following recommendations from the second Referee). Details on B7 proteins binding were deemed superfluous and this sentence was removed from the revised version of our manuscript. Figure 1 has been significantly edited in agreement with the Reviewer’s suggestions (details above).

5. Line 48: modify the sentence to read "Therefore, CD28 co-stimulation increased T cell survival by..."
Response: Thank you. The manuscript has been revised accordingly (L47).

6. Line 50: spell out TNF.
Response: The manuscript has been revised accordingly (L49).

7. Line 59 - 61: The sentence "To date..." needs another sentence following it such as "However they show promise".
Response: Thank you. The manuscript has been revised accordingly and this paragraph now reads as follows (L60-62):

‘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.

8. Lines 61 - 63: The sentence is awkward. Recommend revising to "The evolution of CAR T-cell therapy is at an unprecedented pace in the world of immuno-oncology and has generated a tremendous enthusiasm which has led to ..."
Response: We agree with the Reviewer. This sentence has been edited in our revision of the manuscript (L26-27):

‘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’.
9. Figure 2 needs more legends and descriptions, such as adding "culture" at the top, "Isolated T-cells and stimulate", "Expansion" at the bottom. Also need to use transduction and transfection in a consistent manner. Figure 2 uses "transduction", The Figure Captions uses "transfection". The text for the article uses "transduced".
Response: Thank you. Figure 2 has been significantly edited and now include all necessary details on CAR T-cell production to address the Referee’s comments. Additionally, the term 'transduction/transduced' is now being used consistently in the body of the manuscript, Figure 2 and the figure legend.

10. Line 75: Add "patient" to "...after decades of patient follow-up."
Response: This change is now included in our revision of the manuscript (L73).

11. Figure 3 needs more information within the drawing on the right, possibly add "lysis" or "direct killing". Figure 3 captions: the reference within the description should be replaced with the number (2?). Also recommend the description is revised to "In Chimeric Antigen Receptor (car) therapy, a patient’s T cells are reprogrammed to seek out and target cells..."
Response: Figure 3 has been edited to address the Reviewer’s comments. The figure caption now includes a numbered citation in reference to the work from Kenderian et al. (2014). In addition, the wording of the legend has been amended based on the Referee’s suggestion.

12. Lines 85 and 86: The last sentence starting with "Following expansion,..." is a hanging thought and does not flow well with the paragraph. Is there any references or evidence to support the statement that T-cells contract? Is this in size? Any references to support that the T-cells differentiate into a memory phenotype?
Response: The Reviewer makes a good point. The paragraph was clarified and the term ‘contraction’ was removed to simplify the description of CAR T-cell differentiation into a memory phenotype. A reference to the work by Kalos et al. (2011) has been included to support our statement. The revised sentence now reads as follows (L82-84):
'Following T-cell expansion, CAR T-cells have the ability to differentiate into a stable population of memory T-cells to prevent potential cancer relapses [15].

13. Throughout the section on Applications in Human Oncology: insert "of" when describing % of patients such as on line 103. Also provide a reference for the grading system used for adverse reactions.
Response: Our apologies for this oversight. This issue has been resolved in our revision of the manuscript.

14. Lines 92 and 93: Revise sentence to: "...first FDA-approved gene therapy..."
Response: Thank you. The manuscript has been revised accordingly (L88-89).

Response: CART19 is now defined as ‘CAR T cells targeting CD19’ in our revision of the manuscript (L98).

16. Line 120: delete "famous" famous to whom?
Response: The wording has been changed to 'pivotal ZUMA-1 trial' (L106-107).

17. Line 140: delete "Another product..." because the product was previously discussed.
Response: Thank you. The manuscript has been revised accordingly.

18. Line 159: change "adverse effects" to "adverse reactions".
Response: Thank you. The manuscript has been revised accordingly (L142).

19. Line 166: cytokine should be plural.
Response: Thank you. The manuscript has been revised accordingly (L147).

20. Line 185: describe "tocilizumab".
Response: A definition for tocilizumab has been added in our revision of the manuscript (L167-168).
21. Line 188: describe "siltuximab".
Response: A definition for siltuximab has been added in our revision of the manuscript (L171).

22. Lines 188 - 191: The last 2 sentences describe neurotoxicity. These sentences seem to belong in the next section under Neurotoxicity.
Response: Thank you for noticing this. The manuscript has been edited and these 2 sentences are now included in the Neurotoxicity section (L190-195) which reads as follows:

‘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 [40]. 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 [44].’

The numbering of the citations has been revised accordingly.

23. Line 197: switch ALL and NHL because reference 14 is for NHL and reference 39 is for ALL.
Response: Thank you for noticing this. These changes have been made in our revision of the manuscript (L177).

24. Line 216: change "since" to "because" for proper grammar.
Response: This change has been made in our revision of the manuscript (L197).

25. Lines 217 and 218 both uses "hence" in the sentences. Please revise for clarity.
Response: Thank you. This section has been revised and now reads as follows (L198-202):

‘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].’
26. Lines 236 - 238: either include or drop the trailing zero to consistently use 1 or 2 digits after the decimal point.
Response: Thank you. The percentages are now presented with a single digit after the decimal point.

27. Line 279: what is "it" in the sentence "It effectively loads lymphoid cells..." also provide more of an explanation about what is loaded, PMEG?
Response: Our apologies for the lack of clarity of our original statement. This sentence has been revised to address the Referee’s concern and now reads as follows (L263-266):

Rabacfosadine is an intravenously administered prodrug of the active nucleotide analogue 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’.

28. Line 297: The reference "Mason et al" should be "Panjwani et al".
Response: The reference has been edited to ‘Panjwani et al’.

29. Line 298: "...primary canine cells..." add: "...primary canine T cells..."
Response: This sentence has been revised as suggested by the Referee (L283).

30. Line 316: clarify this is for human use.
Response: This paragraph has been edited and now reads as follows (L301-303):

‘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’.

31. Section 6: Comparative Oncology: An Opportunity to Accelerate Parallel Drug Development: CAR T-Cells are considered biological products and are regulated by FDA/CBER for humans and USDA/APHIS/Center for Veterinary Biologics. Please clarify and provide an explanation that these products are not considered drugs such as small molecules.
Response: Thank you for this valuable addition. An introductory sentence has been included at the beginning of Section 6 to address the Reviewer’s comment (L309-311):
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.

32. Line 372: spell out PK/PD.
Response: The manuscript has been revised accordingly (L361).

33. Line 375: Provide the acronym COTC following Comparative Oncology Trials Consortium.
Response: The manuscript has been revised accordingly (L365).

34. Line 395: clarify if patients are humans or dogs.
Response: Our apologies for this oversight. The sentence was revised to clarify that the results were observed in canine patients (L385).

35. Line 409: delete "and the like". This is slang and not appropriate for the article.
Response: Agreed, and our apologies for this poor choice of words. This sentence was edited and now reads as follows (L398-400):

‘(...) such as flow cytometric assessment of immune cell subsets, gene expression profiling, histopathology, immunohistochemistry, proteomics, multiplex cytokine analysis, and related diagnostic assays [93].’

36. Conclusion: The last paragraph introduces a new topic (treatment of IBD). New topics should not be introduced in a conclusion. This would fit better under Section 6.
Response: We agree with the Referee. This change is now included in our revision of the manuscript. The numbering of the citations has been modified accordingly (L401-404).

37. Line 412: replace "development" with "therapies".
Response: The manuscript has been revised accordingly (L406).

38. Line 414: delete "being" and "U.S."
Response: The manuscript has been revised accordingly.
39. Line 416: at the end of the line, what is "it"? Recommend replace "it" with "these novel therapies".
Response: Thank you. We have replaced ‘it’ by ‘these novel therapies’ in our revision of the manuscript (L410).

40. Line 421: states "...improved CAR T health..." What does this mean? this was not discussed in the body of the paper. Is there evidence to support this statement?
Response: Our apologies for the lack of clarity of our original statement. This sentence was revised to clarify our messaging and now reads as follows (L416-419):

‘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 [96].’

This issue was discussed in previous work by Ruella and Kenderian (last author in our paper). A reference to their work was included in our revised manuscript to support this statement.

41. Line 425: replace "side effects" with "adverse reactions" for consistent use of terms.
Response: The manuscript has been revised accordingly (L422).

42. Line 434: revise the sentence to clarify "Multiple studies in humans..."
Response: The manuscript has been revised accordingly (L432).

43. The conclusion section needs a conclusion about translational medicine from dogs to humans and vice versa.
Response: Thank you for this excellent suggestion. The Conclusion section has been amended to address the Referee’s comment, and now reads as follows (L424-428):

‘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-cells 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].’
44. Reference 98 is not cited in the paper.
Response: The reference can be found in the legend of Figure 1.

45. Figure 4 is not mentioned in the body of the article. The figure captions for Figure 4 should be revised: "...million dogs (vs. 1.7 million human patients) are diagnosed..." What is "ca." at the end of the same line?
Response: Figure 4 is mentioned on L347 of the original submission (now on L336). The Figure caption has been revised to address the Reviewer's comments. The abbreviation 'ca' (from the Latin circa) was replaced by 'about' in the legend to avoid any confusion.
Referee 2

I submit this review as one who is a novice to this area. As such, you will see questions that others may be asking as they read through this manuscript. This was a very good article with many citations. That is very helpful.

Response: We would like to thank the Referee for her/his positive evaluation of our work.

The following as comments for your consideration.

1. Since this will be going into an article on personalized medicine, please provide within the introduction how CAR T therapy represents personalized medicine and how T cells are host specific.

Response: The Reviewer raises an excellent point. The FDA-approved CAR T-cell therapies are all autologous (i.e. host-specific) and thus represent a personalized medicine approach. Revisions to the manuscript can be found are on L9-12 of the Introduction.

‘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’.

2. Also, go into the reduced risk of off-target toxicity compared to traditional chemotherapeutic interventions. If this is host-specific how are these generated for the individual patient in the hospital?

Response: Thank you for this valuable comment. A description of the reduced risk for off-target toxicity is now presented in our revision of the manuscript (L139-141):

‘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’.
In addition, a detailed description of the steps required to generate and administer CAR T-cells is provided in a dedicated paragraph titled ‘T-cell isolation, expansion and generation of CAR T-cells’ (L63-78). We edited the introductory sentence of this section to make it clearer that these steps are for the generation of autologous, patient-specific CAR T-cells. Briefly, to generate autologous CAR T-cells, patients undergo apheresis. T-cells are isolated and then shipped to a GMP facility for culture. At the GMP facility, T-cells are isolated, stimulated and expanded over a period of 7-10 days. During this period, the CAR transgene is introduced and the T-cells are cryopreserved and sent back to the hospital to be administered to this individual patient after a low-dose lymphodepleting chemotherapy.

3. How can there be 'off the shelf' CAR T cells (as you mention in the conclusion)?
Response: Our apologies for the lack of clarity of our original statement. With allogenic, off-the-shelf CAR T-cell therapy, immune cells are collected from a healthy donor rather than the patient. The approach is now being clearly described in our revision of the manuscript (L416-419):

> ‘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 lose the ability to expand and be reprogrammed in vitro [96].’

4. Finally, is there a need to deactivate these cells so that they don’t continue functioning? Is there a way to terminate their activity if needed be?
Response: The Referee raises an excellent point. CAR T-cell therapy must carefully balance effective T-cell activation for antitumor activity with the potential for uncontrolled activation that may produce some of the adverse reactions described in our Commentary. To limit on-target, off-tumor toxicities of CAR T-cells, the inducible caspase-9 suicide gene has been described as one of the approaches to control the infused cells in vivo through its activation with a nontherapeutic chemical inducer of dimerizer (CID). This important addition is now included in our revision of the manuscript (L204-208). The Reference section has been adjusted accordingly.
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.

5. Line 27: Change short survival to brief survival.
Response: The manuscript has been revised accordingly (L35).

6. Line 28: Is this two-signal requirement true for both human and dog CAR-T cells? What does this imply about normal function of T cells; or is this solely a function of the chimeric derivative of host T cells? Does this imply a change in host tissue over time (e.g., changes in CD3ζ) or is it that the T cells themselves that normally require multiple signals (e.g., original signal and a confirmatory signal) to function (even for bacterial infections)?
Response: The Referee raises an excellent point. T-cell co-stimulation with ‘a second signal’ is an essential component for normal T-cell activation and effector function. This is also true for CAR T-cell activation and functions in humans. Whether a co-stimulatory signal is needed for canine CAR T-cells to exert their full effector function is currently unknown and remains an area for further investigation. The manuscript has been edited to specify that the superior anti-tumor effect of 2nd generation CAR T-cells has been demonstrated in human patients only (L39-41):

‘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 [14,15].’

7. Paragraph 40 - 63 is difficult to understand for those of us with limited knowledge in this area. It would have been helpful if you started the paragraph with the statement that we are now dealing with three generations of CAR T cells. Then, there would be sub-bullets that would describe each generation and the rationale, pros and challenges associated with each.
Response: We agree with the Referee’s comment that this technical section describing the various CAR generations was difficult to read. To address the Reviewer’s concerns, we have therefore reformatted this sub-section, while making some significant changes to the narrative (L25-62). Specifically, and as suggested by the Reviewer, we have organized the section in multiple paragraphs, with each paragraph describing a unique CAR generation. The nomenclature of the CAR structure has also been revised to fully match the molecular features presented in Figure 1. The numbering of the citations has been adjusted accordingly.

8. Line 41: You state "domains direct CAR T-cell phenotype and function into specific ways" Do you mean domains direct CAR T-cell phenotype, functionality, and specificity?
Response: Thank you for this valuable comment. As described above, this paragraph was significantly edited as per the Referee’s recommendation. This sentence now reads as follows (L31-33):

‘The hinge region of the CAR is important for optimal tumor antigen binding, while the activation domain directs CAR T-cell response’.

9. Just wordsmithing: to maintain consistency in format (starting Line 78):
(6) Ensure that the final product passes pre-specified release criteria (i.e. sterility, safety, efficacy) and is then cryopreserved for future infusion into patients;

(7) Administer low-dose lympho-depleting chemotherapy to patients. This is followed by infusion of the CAR T-cells.

Response: Our apologies for this oversight. These 2 bullet points were edited to address the Reviewer’s comments (L74-78):

‘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 pre-specified 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’.
10. Line 106: this is the first time you mention CAR T19 cells. Please clarify what this implies. You have only referred to things as CAR T cells.
Response: CART19 is now defined as ‘CAR T cells targeting CD19’ in our revision of the manuscript (L98).

11. I found the in-depth description of each of the clinical trials to be very wordy and difficult to read. Would you consider summarizing key points in a table, with some summary statements about how these all lead to our current state of knowledge and where we are now in terms of therapeutic options?
Response: We agree with the Referee’s comment that Section 3 (Current Applications in Human Oncology) was overtly too long and difficult to read. To address this issue, we have removed unnecessary details while focusing on take-home messages on CAR T-cells clinical development instead. As a result of these changes, Section 3 is now considerably shorter than in our original submission. In addition, our previous description of human clinical trials was significantly edited to improve the overall readability of this section. We would like to keep the bulk of this information in the body of the manuscript, as we consider these data to be critical for the structure/organization and the logical flow of our paper.

12. Divide into multiple paragraphs. For example, a new paragraph can begin on Lines 172 (Various biomarkers), 179 (Teachey), and 185 (Currently, the mainstay of treatment for CRS).
Response: Thank you for this valuable suggestion. These changes have been made in our revision of the manuscript.

13. Line 208, add a comma after barrier.
Response: The manuscript has been revised accordingly (L188).

14. Line 248 - new paragraph should start.
Response: The manuscript has been revised accordingly (L234).
15. Line 275: To date should start new paragraph.
Response: Thank you. We have now created a new paragraph starting with a statement of the need to develop new canine-specific therapeutic options in veterinary oncology (L260).

Response: Details presented on L390 belong to the previous paragraph describing the use of the canine model for translational research on IL-12 in the treatment of malignant melanoma. As such, we think it is important this information in a single block. However, to improve the structure of this section, we have included a line space for a new paragraph introducing the future support of the COTC for research on CAR T-cell therapy (L392).

17. Line 395: to patients - human or canine? I know you say dog a few sentences later but this clarification up front would be of value.
Response: Our apologies for this oversight. The sentence was revised to clarify that the results were observed in canine patients (L385).

18. In the conclusions, you mention allogeneic off-the-shelf universal CAR T cells [94]. How can this be given the personalized method you describe in the earlier part of the text?
Response: Allogeneic CAR T-cells are now described in detail on Lines 413-419 of the revised manuscript. Of course, the development of off-the-shelf CARs would supersede any autologous (i.e. personalized) cell approaches such as those currently approved by the FDA as human therapies (as noted on L9-12 of our revision).

19. Figures are relevant and very useful.
Response: Thank you for this positive feedback, this is greatly appreciated.
# Tables

## Table 1. Approved or Licensed Veterinary Oncology Therapeutics (U.S.)

<table>
<thead>
<tr>
<th>Trade Name</th>
<th>Compound Name</th>
<th>Company</th>
<th>Indication</th>
<th>Regulatory Status, U.S. (Year)</th>
<th>Species</th>
<th>Commercial Availability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blontress®</td>
<td>Canine lymphoma MAb, B-cell</td>
<td>Aratana</td>
<td>B-cell lymphoma</td>
<td>USDA Licensed (2015)</td>
<td>Canine</td>
<td>No</td>
</tr>
<tr>
<td>NA</td>
<td>Canine lymphoma vaccine, DNA</td>
<td>Merial/Bi</td>
<td>B-cell lymphoma</td>
<td>USDA Conditional License (2015)</td>
<td>Canine</td>
<td>Yes</td>
</tr>
<tr>
<td>NA</td>
<td>Canine osteosarcoma vaccine, live listeria vector</td>
<td>Aratana</td>
<td>Osteosarcoma</td>
<td>USDA Conditional License (2017)</td>
<td>Canine</td>
<td>Yes</td>
</tr>
<tr>
<td>NA</td>
<td>Feline interleukin-2 immunomodulator</td>
<td>Merial/Bi</td>
<td>Primary stage I fibrosarcoma</td>
<td>USDA Conditional License (2015)</td>
<td>Feline</td>
<td>Yes</td>
</tr>
<tr>
<td>Immunocidin®</td>
<td>Mycobacterium cell wall fraction</td>
<td>NovaVive</td>
<td>Mammary tumors</td>
<td>USDA Licensed (2009)</td>
<td>Canine</td>
<td>Yes</td>
</tr>
<tr>
<td>Onccept®</td>
<td>Canine melanoma vaccine, DNA</td>
<td>Merial/Bi</td>
<td>Melanoma</td>
<td>USDA Licensed (2010)</td>
<td>Canine</td>
<td>Yes</td>
</tr>
<tr>
<td>Palladia®</td>
<td>Toceranib phosphate</td>
<td>Zoetis</td>
<td>Grade II/III mast cell tumor</td>
<td>FDA Approved (2009)</td>
<td>Canine</td>
<td>Yes</td>
</tr>
<tr>
<td>Tactress®</td>
<td>Canine lymphoma MAb, T-cell</td>
<td>Aratana</td>
<td>T-cell lymphoma</td>
<td>USDA Licensed (2016)</td>
<td>Canine</td>
<td>No</td>
</tr>
<tr>
<td>Tanovea®- CA1</td>
<td>Rabacfosadine for injection</td>
<td>VetDC</td>
<td>Lymphoma</td>
<td>FDA Conditional Approval (2017)</td>
<td>Canine</td>
<td>Yes</td>
</tr>
</tbody>
</table>
Figure Captions

**Figure 1.** Evolution of the Chimeric Antigen Receptor (CAR). The 1\textsuperscript{st} 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 2\textsuperscript{nd} generation includes an antigen-binding domain, a hinge, a co-stimulatory domain (typically CD28) and a CD3ζ signaling domain. The 3\textsuperscript{rd} generation CAR includes 2 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 [98]. Additional details on CAR T-cell structure and response can be found in Section 2, under What are CAR T-cells?

**Figure 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 \textit{ex vivo} and stimulated using antigen presenting cells (APCs); (3) T-cells are genetically engineered to express CAR by gene \textit{transduction}; (4) CAR-expressing T-cells are expanded to a significant population size \textit{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.

**Figure 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 [99]. Consult Figure 2 for additional technical details on CAR T-cell production.

**Figure 4.** Common cancers that have clinical analogues in humans and dogs. Approximately 4.2 million dogs (vs. 1.7 million human patients) are diagnosed with cancer each year, representing about 5,300 new canine cases for a standard 100,000 population size [46].
Extracellular Antigen Binding

1st Generation

2nd Generation

3rd Generation

Transmembrane Domain

Intracellular

Signaling

CD3ζ

Costimulatory domain CD28

Costimulatory domain 4-1BB

scFv

V_L

V_H

Hinge

©2019 Emilyn Frohn

Figure 1
CAR T-Cell Tumor Cell Direct Killing Effect

Tumor Antigen (e.g. CD19)

Cytotoxic Granules

Chimeric Antigen Receptor

CAR T-Cell

Tumor Cell

Direct Killing Effect
One pathogenesis

Brain
Breast
Leukemia
Lymphoma
Melanoma
Prostate
Bone

One medicine

© 2018 Kurt Esenwein
American Association of Pharmaceutical Scientists
Transfer of Copyright Agreement

Journal:  □ The AAPS Journal or □ AAPS PharmSciTech
Title: [Enter Title Here]

Author(s) name(s): [Enter Author Names]

Corresponding Author’s name, address, affiliation and email: [Provide Corresponding Author Information]

The transfer of copyright gives AAPS the right to develop, promote, distribute, sell, and archive a body of scientific works in the United States and throughout the world (for government employees: to the extent transferable). The Author hereby grants and assigns to AAPS all rights in and to Author’s work in and contributions to the Work. In connection with this assignment, the Author acknowledges that AAPS will have the right to print, publish, create derivative works, and sell the Work throughout the world, all rights in and to all revisions or versions or subsequent editions of the Work in all languages and media throughout the world, and shall be the sole owner of the copyright in the Work throughout the world. AAPS shall register the Work with the Copyright Office of the United States in its own name within four months after first publication.

If the Author is an employee of the U.S. Government and performed this work as part of their employment, the contribution is not subject to U.S. copyright protection. If the work was performed under Government contract, but the Author is not a Government employee, AAPS grants the U.S. Government royalty-free permission to reproduce all or part of the contribution and to authorize others to do so for U.S. Government purposes. If any of the above Authors on this agreement is an officer or employee of the U.S. Government reference will be made to this status in the signature.

An author may self-archive his/her accepted manuscript on his/her personal website provided that acknowledgement is given to the AAPS publication and a link to the published article on the journal website is inserted. An author may also deposit the accepted manuscript in a repository 12 months after publication in the journal, provided that acknowledgement is given to the AAPS publication and a link to the published article on the journal website is inserted. The Author must ensure that the publication by AAPS is properly credited and that the relevant copyright notice is repeated verbatim.

The Author reserves the following rights: (a) All proprietary rights other than copyrights, such as patent rights, (b) The right to use all or part of this article, including tables and figures in future works of their own, provided that the proper acknowledgment is made to the Publisher as copyright holder, and (c) The right to make copies of this article for his/her own use, but not for sale.

I warrant and represent that the Work does not violate any proprietary or personal rights of others (including, without limitation, any copyrights or privacy rights); that the Work is factually accurate and contains no matter libelous or otherwise unlawful; that I have substantiately participated in the creation of the Work and that it represents my original work sufficient for me to claim authorship. I further warrant and represent that I have no financial interest in the subject matter of the Work or any affiliation with an organization or entity with a financial interest in the subject matter of the Work, other than as previously disclosed to the Association.

I have the consent of each author to transfer and assign any and all right, title, and interest; including copyright of the article referenced above. I hereby assign and transfer to the American Association of Pharmaceutical Scientists copyright and all rights under it. I further confirm that this article has not been published elsewhere, nor is it under consideration by any other publisher.
For applicable government employees only:

☐ Jonathan P Mochel (Author Name), an Author on this paper, is an employee of _________________________________________ and, by law, is not allowed to assign copyright. As corresponding author, I therefore consent below to have this article published without transfer of copyright.

Signature: Jonathan Mochel Date: 11.19.2018

After completion of this form, please either mail the original signed form to the AAPS Editorial Office at the address below; fax the signed form to AAPS at +1.703.243.9532; or include the signed form in your manuscript submission.