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

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**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   **1   Introduction**

2    Research in cancer immunotherapy has two major current and complementary approaches: (1)  
3    immune checkpoint inhibitors such as those that recently garnered a Nobel Prize in Medicine [1],  
4    and (2) chimeric antigen receptor (CAR) T-cell programming. The former focuses on activation of  
5    intrinsic properties of T-cells. The latter involves the exogenous ‘education’ of T cells to seek-out  
6    and target cells expressing a particular antigen found on specific cancer cell types [2]. These  
7    methods are considered complementary, and progress on combining these approaches is being  
8    reported [3]. Cancer immunotherapy is an extremely promising new approach in oncology that has  
9    the profound potential for curative endpoints. CAR T-cell therapies are particularly promising for  
10   hematologic malignancies, garnering two FDA approvals in 2017 [4,5] representing the first for both  
11   these classes of immunotherapies in addition to serving as the inaugural class of gene therapy-  
12   based strategies. Over 700 potential Investigative New Drug applications are in the queue for cellular  
13   and/or gene therapy applications [6] demonstrating the sustained future for these classes of drugs  
14   in the therapeutic pipeline. B-cell neoplasms are the most common hematopoietic cancer in both  
15   humans and dogs [7]. In canine, genetic background can impact disease onset and progression as  
16   some breeds show a substantially higher risk of this blood disease, including 11 small-breed dogs,  
17   with English Bulldogs presenting years earlier than the overall cohort [8].  
18   The present Commentary provides a review of the current knowledge on the biology of CAR T-cell  
19   therapy and its current applications in human oncology. With the success at treating B-cell  
20   lymphoma using CAR T-cell therapies in people, and the conserved nature of the blood systems  
21   between dogs and humans, this review also provides a perspective for developing these and related  
22   living therapies for conquering canine cancer.

## 23   **2   Definition and Process of Manufacturing CAR T-cells for Cancer Therapy**

### 24    ***What are CAR T-cells?***

25    The original CAR structure was described in 1989 and included a receptor fused to a signaling  
26    domain composed of CD3 $\zeta$  (Fig. 1). This first-generation CAR T-cell therapy resulted in weak  
27    proliferation, short survival and limited anti-tumor effect in patients [9-11]. Subsequently, it was found  
28    that T-cells require a second signal for full activation and, therefore, second-generation CAR T-cells  
29    were developed, with two recently FDA approved products in the U.S and Europe. The structure of  
30    this new CAR includes a co-stimulatory molecule (e.g. CD28 or 4-1BB) that leads not only to  
31    improved expansion and persistence but also to superior anti-tumor effect [12,13]. The basic  
32    second-generation CAR T includes an antigen-binding domain, usually derived from a single chain  
33    variable fragment (scFv) or a protein receptor, a hinge that connects the scFv to a transmembrane

34 domain, a co-stimulatory domain, and a CD3 $\zeta$  signaling domain. This allows for antigen presentation  
35 bypassing the major histocompatibility complex and results in direct activation of T cells upon  
36 exposure to tumor surface antigens. In most cases, the scFv has been murine derived and been  
37 implicated in anti-CAR cytotoxic T-cell responses upon subsequent CAR T infusion, rendering them  
38 ineffective [14,15]. It is hypothesized that such responses against autologous T-cells expressing  
39 CAR transgene may be less pronounced with the human derivatives.

40 The transmembrane hinge region allows for optimal structure of antigen binding while the activation  
41 domains direct CAR T-cell phenotype and function into specific ways. CD28 and 4-1BB (CD137)  
42 are the two most commonly used co-stimulatory molecules thus far. CD28 is a member of the  
43 immunoglobulin family of co-stimulatory receptor, which also includes cytotoxic T-lymphocyte  
44 associated antigen-4 and programmed death receptor (PD-1). The extracellular domain of CD28  
45 binds to B7 proteins and initiates the co-stimulatory signal transduction [16]. CD28 signaling  
46 increases the effect of T-cell and receptor antigen engagement and results in proliferation of T cells  
47 at otherwise sub-mitogenic antigen concentrations [17]. Consequently, cytokine production, most  
48 importantly IL-2, is significantly increased. Therefore, CD28 co-stimulation increases cell survival by  
49 inducing expression of anti-apoptotic proteins such as Bcl-X<sub>L</sub> [18]. 4-1BB, on the other hand, is a  
50 member of the TNF receptor family and is expressed primarily on activated lymphocytes. It results  
51 in proliferation and differentiation of CD8<sup>+</sup> T cells, while inhibiting programmed cell death [19]. While  
52 CD28:B7 co-stimulation expands naïve T-cells, 4-1BB co-stimulation expands memory T-cells,  
53 resulting in enrichment of antigen-reactive T-cells upon recognition of previously primed antigens.  
54 Co-stimulation with 4-1BB domain has shown enhanced *in vivo* persistence, higher expansion and  
55 enhanced cytolytic ability compared to CD28 co-stimulation [19,20]. It has also been suggested that  
56 combining these 2 co-stimulatory domains result in a more efficient and persistent anti-tumor activity,  
57 by combining their strengths of early tumor-killing with late persistence and engraftment. This has  
58 led to the concept of third-generation CAR that now include 2 co-stimulatory domains along with the  
59 activation domain, resulting in  $\geq 3$  signaling domains in the CAR T structure [21]. To date, the  
60 incorporation of more stimulatory domains did not enhance CAR T-cell function in preclinical or early  
61 clinical trials. This evolution at an unprecedented pace in the world of immuno-oncology has  
62 generated a tremendous enthusiasm and has led to an exciting time for developing new strategies  
63 for cancer treatment.

64

### 65 ***T Cell isolation, expansion and generation of CAR T-cells***

66 The following steps are required to generate clinical grade CAR T-cells (Fig. 2):

- 67 1) T-cells are collected from patients by leukapheresis;
- 68 2) T-cells are then cultured in a good manufacturing process-compliant facility;

- 69 3) T-cells are stimulated using stimulating beads, antibodies or artificial antigen presenting cells;  
70 4) T-cells are transduced with the CAR of interest. At this stage, the non-tumor specific T cells  
71 acquire the ability to recognize tumor antigens;  
72 5) To insert the CAR gene into T-cells, viral vectors (lentivirus or retrovirus), or non-viral  
73 approaches are used (transposon, CRISPR, TALEN, RNA). While the use of viruses raises  
74 concerns for insertional mutagenesis, third generation lentiviruses have been shown to be safe  
75 after decades of follow-up;  
76 5) T-cells are cultured for a period of 7-14 days. During that time, they expand by several folds  
77 and express the CAR T construct of choice;  
78 6) The final product needs to pass pre-specified release criteria (i.e. sterility, safety, efficacy)  
79 and is then cryopreserved for future infusion into patients;  
80 7) Patients receive low-dose lymphodepleting chemotherapy, followed by infusion of the CAR T  
81 cells.

82 After infusion, CAR T-cells are stimulated through the CAR receptor after they recognize their target  
83 antigen on tumor cells. This is followed by a massive *in vivo* T-cell expansion, associated with  
84 cytokine release, and the release of toxic granules (Fig. 3). During this time, T-cells exhibit their  
85 antitumor effect and patients are at risk of developing clinical cytokine release syndrome. Following  
86 expansion, T-cells contract and, in some instances, differentiate into a memory phenotype.

### 87 3 Applications in Human Oncology

88 CD19 targeting CAR T-cell has been the most successful therapy to date in relapsed/refractory acute  
89 lymphoblastic leukemia (ALL). In the pre-CAR T therapy era, prognosis of relapsed/refractory B-cell  
90 ALL has been dismal with median overall survival reported in few weeks-months and survival at 5  
91 years around 7-8% [22-24]. B-cell ALL was the first indication for which any CAR T therapy was  
92 approved by the U.S FDA. Tisagenlecleucel (previously CTL019) was the first gene FDA-approved  
93 therapy for the treatment of relapsed/refractory B cell ALL in patients up to 25 years of age. The initial  
94 report included 2 children from the University of Pennsylvania, one of whom had an ongoing response  
95 at 11 months follow-up (and we know is ongoing to date), while the other relapsed with CD19 negative  
96 blast cells after an ephemeral response lasting for 2 months [25]. In the subsequent report of 30  
97 patients with relapsed/refractory ALL, 27 (90%) patients achieved a complete response and 22 (73%)  
98 patients had no detection of disease using sensitive multiparametric flow cytometry at 1 month after  
99 infusion [26]. Interestingly, one patient had relapsed T-cell ALL post-transplantation with aberrant  
100 CD19 expression and achieved a morphological response with tisagenlecleucel but with only minimal  
101 residual disease. Data from clinical trials were expanded from single center experience to multi-  
102 center studies with the ELIANA trial that included 92 patients; 75 (82%) of which received infusion of

103 tisagenelecleucel [5]. Remission was noted in 83% patients with overall survival rate of 90% at 6  
104 months and 76% at 12 months. From the intention-to-treat analysis of 92 enrolled patients, complete  
105 response (with or without complete hematological recovery) was observed in 66% patients.

106 Following the remarkable activity in ALL, trials with CART19 cell therapy were initiated in B-cell  
107 lymphomas. Diffuse large B-cell lymphoma (DLBCL) is a heterogeneous group within Non-Hodgkin's  
108 lymphomas (NHL) with varying molecular profiles, gene sequencing patterns and clinical responses;  
109 some of which are associated with poorer outcomes and represent an area of therapeutic unmet  
110 need. Clinically, patients who achieved stable or progressive disease as best response during the  
111 entire course of therapy, or those who relapsed within 12 months of autologous stem cell  
112 transplantation, have been shown to have a rather low overall survival rate of around 6.3 months  
113 [27]. The now FDA-approved axicabtagene-ciloleucel (KTE-019) therapy was initially developed at  
114 the National Cancer Institute (NCI). Preclinical work done at the NCI consisted of developing CAR-  
115 transduced T-cells that could specifically recognize murine CD19 and resulted in eradication of  
116 intraperitoneally injected lymphoma cells and subcutaneous lymphoma masses in a murine model  
117 [28].

118 Subsequent clinical studies showed an objective positive response in 75-80% patients treated with  
119 axicabtagene-ciloleucel, including some longer lasting responses [29]. This construct was further  
120 pursued by Kite Pharma, as KTE-019, in the famous ZUMA-1 trial which paved the way for FDA  
121 approval of this modality for DLBCL. The Phase 1 part of the ZUMA trial enrolled 7 patients with 1  
122 patient experiencing a dose limiting toxicity, while grade  $\geq 3$  cytokine release syndrome (CRS) and  
123 neurotoxicity were reported in 14% and 57% patients, respectively. In this report, 5 out of the 7 (71%)  
124 patients showed an objective positive response, with 4 (57%) being complete responses. The Phase  
125 2 ZUMA-1 study enrolled 111 patients, of whom 101 were able to receive the CAR T-cell infusion [4].  
126 Overall positive response was reported in 82% patients with a complete response in 54% of the  
127 cases. Complete response was maintained in 40% patients at a median follow-up of 15.4 months. Of  
128 the 108 patients who had at least 1 year follow-up in Phase 1 and Phase 2 of the ZUMA-1 trials, an  
129 overall response was seen in 82% patients, with a complete response in 58% of the cases. Of the  
130 60 patients who had a partial response or a stable disease at the first assessment 1 month post CAR  
131 T-cell therapy, 23 had a subsequent complete response. The progression free survival rate was  
132 estimated at 49% in patients at 6 months, 44% at 12 months and 41% at 15 months, while the overall  
133 survival rate was 78%, 59% and 52% at 6, 12 and 15 months, respectively. Response to treatment  
134 was not affected by CD19 expression intensity, CD4-to-CD8 cell ratio, or the use of tocilizumab; but  
135 was associated with a higher expansion of CAR T-cells instead. However, CAR T-cell expansion  
136 within the first 28 days was noted to be higher in patients who had a positive response compared to  
137 those who did not. One-year follow-up data presented at the Annual Meeting of the American Society



138 of Hematology and the Bone Marrow Transplantation Tandem Meetings in 2018 [30] suggested loss  
139 of CD19 expression and gain of PD-L1 expression as possible mechanisms for resistance following  
140 CAR T-cell therapy. Another product, tisagenlecleucel (CTL019), is now FDA-approved for use in  
141 patients with relapsed/refractory DLBCL (not including primary mediastinal large cell lymphoma).  
142 Approval was based on a Phase 2 study (JULIET) that enrolled 160 patients with primary analysis  
143 available on 81 patients with at least 3 month follow-up or earlier discontinuation [31]. Best overall  
144 response rate was 53.1% in these evaluable patients (39.5% complete response and 13.6% partial  
145 response). At 6 months, probability of being relapse-free was estimated at 73.5% with an overall  
146 survival of 64.5%. 95% patients in complete response at 3 months also maintained positive response  
147 at 6 months. Another case-series for the same product enrolled 38 patients with DLBCL or follicular  
148 lymphoma, of which 28 were able to receive cell infusion [32]. At 3 months, 18 of the 28 patients had  
149 a positive response (64%). Three patients with follicular lymphoma and 1 patient with DLBCL who  
150 had partial response at 3 months had a complete response by 6 months. At 6 months, 16 out of 28  
151 (57%) patients had a complete response and these remained in remission at a median time of 29.3  
152 months (range: 7.7 – 37.9 months). In this study, peak expansion of CAR T-cells was not different  
153 between patients who responded compared to those who did not.

154 Overall, multiple CD19 targeting CAR T-cell therapy constructs are currently in development and  
155 expected to receive FDA approvals for different B cell malignancies in the next 2-3 years. One  
156 example is the B-Cell Maturation Antigen (BCMA) directed CAR T-cell therapy which is showing  
157 promising activity in multiple myeloma [33].

#### 158 **4 Unique Toxicities of CAR T-Cell Therapy**

159 Due to its specific mode of action, CAR T-cell therapy is associated with various adverse effects,  
160 including the development of cytokine release syndrome, neurotoxicity and B-cell aplasia resulting in  
161 hypogammaglobulinemia.

162

##### 163 ***Cytokine Release Syndrome***

164 Cytokine release syndrome (CRS) is one of the most feared toxicities related to CAR T-cell  
165 therapy. As its name suggests, CRS is a systemic inflammatory state resulting from the excessive  
166 production of cytokine associated with CAR T-cell activation. Time-to-development of CRS is widely  
167 variable and depends on the CAR construct, the disease type and the tumor burden. Rates of CRS  
168 have ranged from 45 to 100% in various reports with serious or  $\geq 3$  grade in up to 50% of patients  
169 [34]. Clinical manifestations can range from mild fever to life-threatening vasodilatory shock causing  
170 hypoxia, hypotension and organ toxicity mandating management in the intensive care unit. Death  
171 related to CRS has been reported [4,14,35]. It has also been suggested that a higher burden of

172 tumor antigens may be associated with higher rates and severity of CRS [36]. Various biomarkers  
173 have been studied to elucidate the mechanism, of which interleukin(IL)-6/ IL-6 receptor interaction  
174 has been most consistently shown to correlate with CRS. Consistently, blockade of the IL-6 pathway  
175 has resulted in alleviation of symptoms related to CRS [37]. C-reactive protein and ferritin are  
176 clinically available laboratory tests that have been shown to be elevated in patients who develop  
177 CRS and are monitored closely at some institutions, including the Mayo Clinic Cancer Center  
178 [38,39]. Other cytokines associated with inflammation such as interferon-gamma, soluble IL-2  
179 receptor and IL-10 have been implicated. Teachey et al. [40] at the University of Pennsylvania  
180 identified a set of 24 cytokines, including interferon-gamma, IL-6, and soluble glycoprotein-130 that  
181 are associated with severe CRS in ALL patients receiving 4-1BB/ CD3 $\zeta$  CAR T-cell therapy. More  
182 recently, studies in murine models of CRS have demonstrated that the severity of CRS does not  
183 only depend on CAR T-cell derived cytokines but also on IL-1, IL-6 and nitric oxide release by host  
184 macrophages [41]. This finding can potentially open additional avenues for preventative or  
185 therapeutic measures. Currently, the mainstay of treatment for CRS remains tocilizumab since its  
186 use in the first patient treated with CART19 for ALL [25]. Subsequent data showed that the use of  
187 tocilizumab for CRS does not adversely affect the expansion of CD28/CD3 $\zeta$  CAR T-cells, unlike that  
188 of high-dose steroids [38]. Another agent of potential utility for this indication is siltuximab which, in  
189 contrast to tocilizumab, directly inhibits IL-6. This direct inhibition may result in less reliance on  
190 competitive binding to IL-6 receptor and eliminate the risk of passive diffusion of unbound IL-6 into  
191 the central nervous system (CNS) resulting in neurotoxicity [42].

192

### 193 ***Neurotoxicity***

194 The risk of neurotoxicity with CAR T-cell therapy became apparent when 5 patients died of  
195 cerebral edema in one of the early phase ROCKET trial being conducted by Juno Pharmaceuticals  
196 using JCAR015 in adult patients with B-cell ALL. Additional deaths have been reported in both B-  
197 cell ALL and NHL trials [14,39]. Non-fatal but clinically significant neurotoxicity has additionally been  
198 reported in around 40-50% patients across various clinical trials with the different CAR constructs in  
199 various malignancies [43]. Clinical presentation can vary from headache, confusion, tremor, to  
200 delirium, expressive aphasia, obtundation, myoclonus or seizure. Whether there are pre-existing  
201 risk factors in the form of CNS disease is currently unknown, as patients with active CNS disease  
202 were typically excluded from clinical trials. Various hypotheses have been put forth to explain the  
203 development of neurotoxicity, but the exact mechanism remains elusive. One hypothesis is that  
204 CAR T-cell activation results in elevated cytokine levels triggering macrophage activation and  
205 subsequent neurotoxicity. More recently, with the use of the CD28-CD3 $\zeta$  therapy in lymphoma, IL-  
206 10 as well as IL-15 were noted to achieve higher peak levels in patients with grade 3 or 4

207 neurotoxicity compared to those with < grade 3 neurotoxicity [44]. Endothelial activation and  
208 multifocal vascular lesions, resulting in disruption of the blood-brain-barrier were reported in patients  
209 experiencing neurotoxicity within 28 days of infusion with CD19 CAR T-cells in B cell ALL, NHL and  
210 CLL [45]. Humanized mice model studies have shown a role for IL-1 and IL-6 derived from host  
211 monocytes in neurotoxicity which would provide a rationale for the use of anakinara (IL-1 receptor  
212 antagonist) in this indication [41]. However, the mainstay of therapy to resolve CAR T-associated  
213 neurotoxicity remains corticosteroids.

214

### 215 ***Hypogammaglobulinemia***

216 B-cell aplasia is an example of 'on-target/off-tumor' activity of CAR T-cell therapy since CD19 is  
217 expressed not only on the malignant B-cells but also on normal B-lymphocytes. B-cells are assigned  
218 with the task of producing immunoglobulins and hence, B-cell aplasia following CAR T-cell therapy  
219 results in prolonged hypogammaglobulinemia. Hence, it is not surprising that all patients from the  
220 University of Pennsylvania ALL cohort who had a positive clinical response to CAR T-cell therapy  
221 also developed B-cell aplasia [5]. Hypogammaglobulinemia leads to an increased risk of infections  
222 and the need for regular intravenous immunoglobulin replacement for the duration of B-cell aplasia.

## 223 **5 Applications in Veterinary Oncology**

### 224 ***A critical need for new and innovative therapies in canine B-cell lymphoma***

225 It is estimated that more than 4.2 million dogs (5300/100,000 per population rate) in the U.S are  
226 diagnosed with cancer each year [46]. The epidemiology of canine cancer is, however, not well  
227 defined in the literature. Most of the available incidence data comes from a limited number of tumor  
228 registries and the European Union where there is a higher percentage of insured dogs. Very little to  
229 no published data is available to indicate what percentage of dogs diagnosed with cancer are then  
230 treated or how they are treated in the U.S. This makes any assessment of the actual market potential  
231 for veterinary oncology therapeutics extremely challenging. Clinical experience would indicate that  
232 the most common canine malignant cancers diagnosed and treated include lymphoma, mast cell  
233 tumor, osteosarcoma, soft tissue sarcoma, hemangiosarcoma and melanoma.

234 This clinical impression is supported by a Swiss Canine Cancer Registry study that outlined the most  
235 common neoplasms diagnosed in over 120,000 dogs during a 53-year period as follows:  
236 adenoma/adenocarcinoma (18.09%), mast cell tumor (6.5%), lymphoma (4.35%), melanoma  
237 (3.63%), fibroma/fibrosarcoma (3.40%), hemangioma/hemangiosarcoma (2.80%), squamous cell  
238 carcinoma (1.95%) and osteoma/osteosarcoma (1.24%) [47]. The high occurrence of carcinoma  
239 (mammary) is related to the less frequent implementation of ovariohysterectomy at a young age  
240 which is more common in the U.S.

241 Lymphoma, with an estimated incidence rate of 20-100 per 100,000 dogs [48], is one of the most  
242 widely treated canine cancers given its frequent occurrence and typically robust response to  
243 chemotherapeutics. Based on the current approximation of 75 million dogs in the U.S, estimates are  
244 that 16,000-80,000 new cases of canine lymphoma are diagnosed each year [49]. Other estimates  
245 place the number of diagnosed canine lymphoma cases at over 250,000 annually in the U.S,  
246 accounting for 12-18% of annual death-related malignant cancers in dogs [46]. This makes the  
247 canine lymphoma market a very appealing potential opportunity for therapeutic development.

248 There is abundant recent literature highlighting the pathologic, biologic, immunophenotypic, genetic  
249 and treatment response similarities between human and canine lymphoma [49-52]. Specifically,  
250 DLBCL is the most common subtype of lymphoma in both species [52], and it is the subtype most  
251 studied with genomic profiling in veterinary medicine [46]. Utilizing immunohistochemistry and gene  
252 expression profiling, similar profiles were noted between human and canine DLBCL, and certain  
253 markers were able to separate the canine DLBCL cases into two groups with significantly different  
254 clinical outcomes [53]. Provided this robust and expanding body of data supporting the parallels  
255 between the most common types of human and canine lymphoma, the opportunities for therapeutic  
256 development in one species to inform and progress that in the other species will only continue to  
257 grow.

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

261 Focusing on canine B-cell lymphoma in particular, the standard of care for dogs with high grade  
262 lymphoma over the last 35 years has ranged from single agent protocols (using prednisone or  
263 doxorubicin) to combination chemotherapy regimens of variable duration. Most veterinary  
264 oncologists agree that a doxorubicin-based (e.g. CHOP) combination chemotherapy protocol  
265 provides the longest period of disease control and overall survival [54]. However, the response to  
266 chemotherapy is often sub-optimal with recurrent or refractory disease representing a significant  
267 clinical challenge. The combination of chemotherapy with half- and total-body irradiation has also  
268 been evaluated in some dogs with lymphoma. The reported median survival rate in these instances  
269 is no longer than that achieved with chemotherapy alone, thereby questioning the utility of this  
270 adjunctive therapy [54]. Transplantation of autologous bone marrow has recently facilitated the safe  
271 dose escalation of cyclophosphamide that resulted in long-term remission and prolonged patient  
272 survival in dogs [55]. However, autologous bone marrow transplantation is technically and  
273 logistically challenging to perform in a veterinary hospital setting which limits widespread application.  
274 With only a handful of FDA-approved or USDA-licensed veterinary oncology therapeutics currently  
275 available to veterinarians, there is a dire need for canine-specific treatment options (Table 1). To

276 date, there is only one therapeutic with conditional FDA approval, rabacfosadine (Tanovea®-CA1,  
277 VetDC), for the treatment of canine B-cell lymphoma. Rabacfosadine is an intravenously  
278 administered cytotoxic therapeutic agent which is a prodrug of the nucleotide analogue 9-(2-  
279 phosphonylmethoxyethyl) guanine (PMEG). It effectively loads lymphoid cells while reducing levels  
280 of PMEG in plasma and target organs of toxicity. Tanovea-CA1 received conditional approval from  
281 FDA in January 2017 for the treatment of lymphoma in dogs and became available to veterinarians  
282 in the spring of 2017.

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

295

### 296 ***Preliminary data in dogs***

297 In a first ever canine study, Mason et al. [58], has reported successful mRNA electroporation of  
298 primary canine cells to generate CAR T-cells. In brief, a novel expansion methodology was  
299 developed that yields large numbers of canine T-cells from normal or lymphoma-diseased dogs. In  
300 this study, the authors had modified previous methods to activate and expand canine T cells *ex vivo*  
301 by using artificial antigen-presenting cells genetically modified to express human CD32 and canine  
302 CD86. These artificial antigen-presenting cells were loaded with a canine CD3 monoclonal antibody  
303 and used in combination with human IL2 and IL21 to preferentially expand CD8<sup>+</sup> T-cells. The mRNA  
304 electroporation procedure was utilized to express a first-generation, canine CD20-specific CAR in  
305 expanded T-cells as primary therapy. Treatment in 1 dog with relapsed B-cell lymphoma was well  
306 tolerated and led to a modest, but transient, anti-tumor activity, suggesting that stable CAR  
307 expression is required for sustained clinical remission. Other possible factors that could have  
308 contributed to the partial antitumor activity include limited CAR T-cell expansion and the  
309 development of canine antimouse antibodies directed against the murine scFv construct. Future  
310 studies are currently underway to investigate the clinical efficacy of a stably-transduced canine CAR

311 T-cell line expressing fully canine, second-generation CAR constructs. Lymphodepleting  
312 chemotherapy should also reduce the risk of inducing canine antimouse antibodies.  
313 The high-cost of current human treatments, \$475,000 for tisagenlecleucel and \$373,000 for  
314 axicabtagene ciloleucel [59] not including hospitalization and other costs, raises an important  
315 potential challenge for the accessibility of this technology for use in dogs. New, non-viral genome  
316 engineering tools are in development with the potential to reduce the cost of goods through obviating  
317 the need for the generation of an infective engineered virus. For example, the *Sleeping Beauty* [60]  
318 and *piggyBac* [61] transposons are in ongoing CAR T-cell clinical trials. In addition, gene editing  
319 approaches for targeted knock-in using electroporation and ssDNA as donor [62] and new  
320 approaches using enhanced dsDNA as donors for efficient targeted gene knock-in at diverse loci  
321 [63] hold the potential for additional and more accessible, non-viral methods for CAR T-cell  
322 generation.

## 323 **6 Comparative Oncology: An Opportunity to Accelerate Parallel Drug Development**

324 According to a recent report from the National Academy of Medicine [64], only 1 out of 10 oncology  
325 candidates that appear promising in preclinical mouse models are in fact effective and safe in human  
326 clinical trials. This overtly high attrition rate highlights the need for alternative models at the early  
327 stage of the Drug Research and Development lifecycle [65], as shown in other therapeutic areas [66-  
328 71]. Although murine models have been extremely useful for studying the biology of cancer initiation,  
329 promotion and progression, mice typically do not faithfully represent many of the features constitutive  
330 of human cancer, including genomic instability, tumor heterogeneity and long periods of latency [72].  
331 Additionally, study mice are often immunocompromised and bred in sterile laboratories, unlike  
332 domesticated dogs that share the same habitat and are exposed to same environmental carcinogens  
333 (e.g. UV light, pollution and food contaminants) as humans.

334 Importantly, cancers develop spontaneously in dogs (i.e. without genetic manipulation) and in the  
335 context of an intact immunity with a syngeneic host and tumor microenvironment. Canine tumors  
336 typically have similar features to human malignancies, such as histological appearance, cytogenic  
337 abnormalities, therapeutic response, acquired resistance and background genetics [72]. Indeed, as  
338 the dog genome became available, multiple comparative genomics studies have shown significant  
339 homologies between canine and human cancer-associated genes, including MET, mTOR, KIT and  
340 TRAF3 [73]. Given the large number of breeds and their shared ancestry [74], inheritable germline  
341 mutations associated with cancer are easier to identify in purebred dogs than in human populations  
342 [75]. The outbred nature of dogs (relative to most murine models) contributes to their biological  
343 relevance for studying new cancer therapies. At the same time, the rapid progression of cancer

344 associated with the shorter lifespan of dogs provides an opportunity to study the efficacy and safety  
345 of candidate therapeutic drugs in a much faster timeframe than clinical trials in human patients [76].  
346 Biological similarities between canine and human cancer provide an impetus for the study of novel  
347 therapeutics in dog clinical trials (Fig. 4). In fact, the evaluation of oncology drugs in dogs with  
348 naturally occurring cancers is not new, with a few descriptions already available in the early 1970s  
349 [77-79]. Over the last decade, multiple reports have demonstrated the relevance of the dog model to  
350 bridge the knowledge gap between murine experiments and human clinical trials, and exemplify the  
351 value of a comparative oncology approach to drug development [80-81].

352 For instance, both canine and human DLBCL patients share similar constitutive NF- $\kappa$ B activity that  
353 drives overexpression of anti-apoptotic NF- $\kappa$ B target genes which promote lymphocyte proliferation  
354 [82-83]. Studies indicate that administration of a targeted inhibitor of constitutive NF- $\kappa$ B activity,  
355 NEMO Binding Domain (NBD), induces apoptosis of canine malignant B cells in vitro. Moreover, pilot  
356 trials have demonstrated intranodal administration of NBD peptide to dogs with relapsed B-cell  
357 lymphoma inhibits the expression of NF- $\kappa$ B target genes leading to reduced tumor burden [84]. In a  
358 separate Phase 1 clinical trial, these same investigators showed that NBD peptide administered  
359 intravenously is safe and effective at inhibiting constitutive NF- $\kappa$ B activity in a subset of dogs with  
360 lymphoma [85]. Additionally, the use of established canine tumor cell lines has proven beneficial in  
361 studying tumor biology and pre-clinical therapeutics. A CD40 ligand-dependent culture system for  
362 canine malignant B-cells has been recently designed to test compounds for treatment in primary  
363 tumor samples from dogs and humans [86]. The tumor cells retain their original phenotype, clonality,  
364 and known karyotypic abnormalities after expansion and culture. This canine cell culture system is  
365 reported to be potentially robust to perform in vitro preclinical cytotoxic assays with primary B-cell  
366 malignancies.

367 The opportunity to synergize quantitative information available from humans and animals sharing  
368 clinical analogs to develop improved therapies for both species is known as 'Reverse Translation'  
369 [65]. A significant component of the success of comparative oncology in drug development is the  
370 creation of consortia that link drug development stakeholders to veterinary clinicians with access to  
371 tumor-bearing pet animals. This supports the implementation of clinical trials carried out in pets and  
372 the collection of high-quality clinical data and biologic specimens that are critical to defining PK/PD,  
373 tolerability and efficacy of novel therapeutic approaches destined for human use. To this end, the  
374 Comparative Oncology Program of the NCI has established a multi-center collaborative network of  
375 24 veterinary academic partners known as the Comparative Oncology Trials Consortium [72,87]. The  
376 mission of the COTC is to answer biological questions geared to inform the development path of  
377 chemotherapeutics for future use in human cancer patients. The COTC operates as a platform for

378 collaborative work between the NCI and extramural academic comparative oncology centers to  
379 design and execute clinical studies in dogs with cancer. Support for the oversight and management  
380 of the COTC comes from the NCI. Trial sponsors, most often pharmaceutical companies, support the  
381 costs associated with clinical studies in dogs in established COTC academic centers.

382 Several published examples of COTC trials exemplify the functionality and impact of such studies  
383 [87-89]. COTC trials do not focus exclusively on small molecules or biologic agents; instead they can  
384 be designed and implemented to answer a range of drug development questions that are key to the  
385 forward progress of an agent or group of candidate molecules, medical devices, or molecular profiling  
386 platforms. One such example illustrating the value of the dog model pertains to the development of  
387 the inflammatory cytokine IL-12 for the treatment of human malignant melanoma. The use of  
388 cytokines to enhance antitumor immunity has been recognized as an important immunomodulatory  
389 approach in cancer management. Yet, historically, the high risk for systemic toxicity presented by IL-  
390 12 dosing had prevented development of this cytokine into a therapeutic drug. A strong genetic  
391 similarity exists between canine and human IL-12 (i.e. 84% homology for the ligand and 68%  
392 homology for the receptor), which motivated studies on the characterization of IL-12 PK/PD, efficacy,  
393 and toxicity in dogs with naturally occurring malignant melanoma [90]. Results showed that a fully  
394 human necrosis-targeted immunocytokine NHS-IL-12 could be safely administered subcutaneously  
395 to patients with malignant melanoma, while maintaining both systemic immunological and clinical  
396 activity. This was demonstrated by measuring serum IL-12 and other representative biomarkers (e.g.  
397 IL-10 and IFN-gamma) over time, and establishing PK/PD models of IL-12. These findings in dogs  
398 were key to guide the sponsor's decision to move forward with a Phase I clinical trial of this agent in  
399 humans. In turn, preliminary studies focusing on IL-12 gene electrotransfer in dog patients with  
400 melanoma have shown promising results for the treatment of spontaneous canine tumors [91-92].

401 With respect to CAR T-cell therapy research and development, the COTC infrastructure stands ready  
402 to support the implementation of cell-based trials for pivotal go/no-go decision-making prior to clinical  
403 testing in humans. Through strategic partnerships with study sponsors whom can provide the  
404 necessary cell manufacturing, quality control/assurance, and distribution support for such trials, the  
405 COTC can provide the requisite scientific input and execution for such trials to be carried out in the  
406 veterinary academic setting. Similarly, the COTC Pharmacodynamic Core laboratory can provide  
407 access to providers of canine-specific assay support for critical immunological assays such as flow  
408 cytometric assessment of immune cell subsets, gene expression profiling, histopathology,  
409 immunohistochemistry, proteomics, multiplex cytokine analysis, and the like [93].

410



## 411 **7 Conclusions**

412 CAR T-cells are one of the most promising development for the treatment of hematological  
413 malignancies. Specifically, CART19 cells have demonstrated unprecedented clinical results in  
414 human B-cell malignancies with two constructs being approved by the U.S FDA in 2017.

415 Yet, the technology is still in its early phase and significant challenges need to be resolved before it  
416 can be used for large scale clinical trials. Obvious limitations include the complexity and costs (direct:  
417 related to the manufacturing, and indirect: related to hospital costs and patient care) of CAR T-cell  
418 therapy. The requirement for GMP materials and the individualized nature of the therapy are the main  
419 causes that drive-up the cost. The possibility to generate allogeneic off-the-shelf universal CAR T-  
420 cells [94] would lead to easier and more cost-effective manufacturing, reduced time to CAR T-cell  
421 infusion, improved CAR T health and faster translation of novel combination strategies with CAR T-  
422 cells in early phase clinical trials. In addition, the management of toxicities after CAR T-cell therapy  
423 requires specialized expertise and care level, making it available only in specialized tertiary centers.  
424 Strategies to modulate cytokine production after CAR T-cell therapy are being developed and could  
425 represent a new paradigm in the management of CAR-T cell-related side effects.

426 Importantly, there is currently a lack of robust preclinical models to recapitulate the microenvironment  
427 and toxicities following CAR T-cell therapy. Canine models have long been used in development of  
428 human cell therapies and allogeneic transplantation procedures and represent an attractive model to  
429 further investigate novel CAR T-cell strategies in liquid and solid tumors, as well as to develop novel  
430 off-the-shelf approaches. Preliminary data in dogs using a canine CD 20-specific CAR in expanded  
431 T-cells showed promising, but transient results. However, these preliminary findings lay the  
432 foundation for future studies in dogs where both tumor biology and the microenvironment more  
433 faithfully recapitulate that of humans.

434 Multiple studies are currently evaluating the effect of CAR T-cell therapy for the treatment of solid  
435 tumors, with modest results thus far [95]. Potential strategies to increase the efficacy of CAR T in this  
436 context include combinations with immune stimulants, secondary modifications of CAR T-cells, re-  
437 engineering of the T cell, and specific targeting of the tumor microenvironment. Lastly, efforts are on  
438 the way to harness the immunosuppressive property of CAR T-cell for the treatment of autoimmune  
439 diseases, such as Inflammatory Bowel Disease (IBD) [96], thereby opening new avenues for  
440 comparative medicine and parallel drug development as the dog is a spontaneous animal disease  
441 model for IBD as well [97].

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443 None.

444 **9 Conflict of Interest**

445 JPM, SE, CJ, AJ, KA, WW and SSK are founders of LifEngine Animal Health Laboratories, Inc. SSK  
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451 **10 Author Contributions**

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

455

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## Tables

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

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

## Figure Captions

**Figure 1.** Evolution of the Chimeric Antigen Receptor (CAR). The 1<sup>st</sup> CAR generation consists of a receptor fused to a signaling domain composed of CD3 $\zeta$ . The 2<sup>nd</sup> generation includes 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, a co-stimulatory domain (typically CD28) and a CD3 $\zeta$  signaling domain. The 3<sup>rd</sup> generation CAR includes 2 co-stimulatory domains along with the activation domain, resulting in  $\geq 3$  signaling domains in the CAR structure. Adapted from Zhao et al. [98].

**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 genetically engineered to express CAR by gene transfection; (3) CAR-expressing T cells are expanded to a significant population size *in vitro*; (4) CAR T-cells are then introduced back into the patient.

**Figure 3.** In Chimeric Antigen Receptor (CAR) therapy, a patient's T cells are reprogrammed to specifically to seek-out and target cells expressing a particular antigen found on specific cancer cell types (Kenderian, 2014). Activation of T cells leads to direct killing of tumor cells through the release of cytolytic proteins, such as granzyme and perforin. 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) get diagnosed with cancer each year, representing ca. 5,300 new canine cases for a standard 100,000 population size [46].

Figure 1

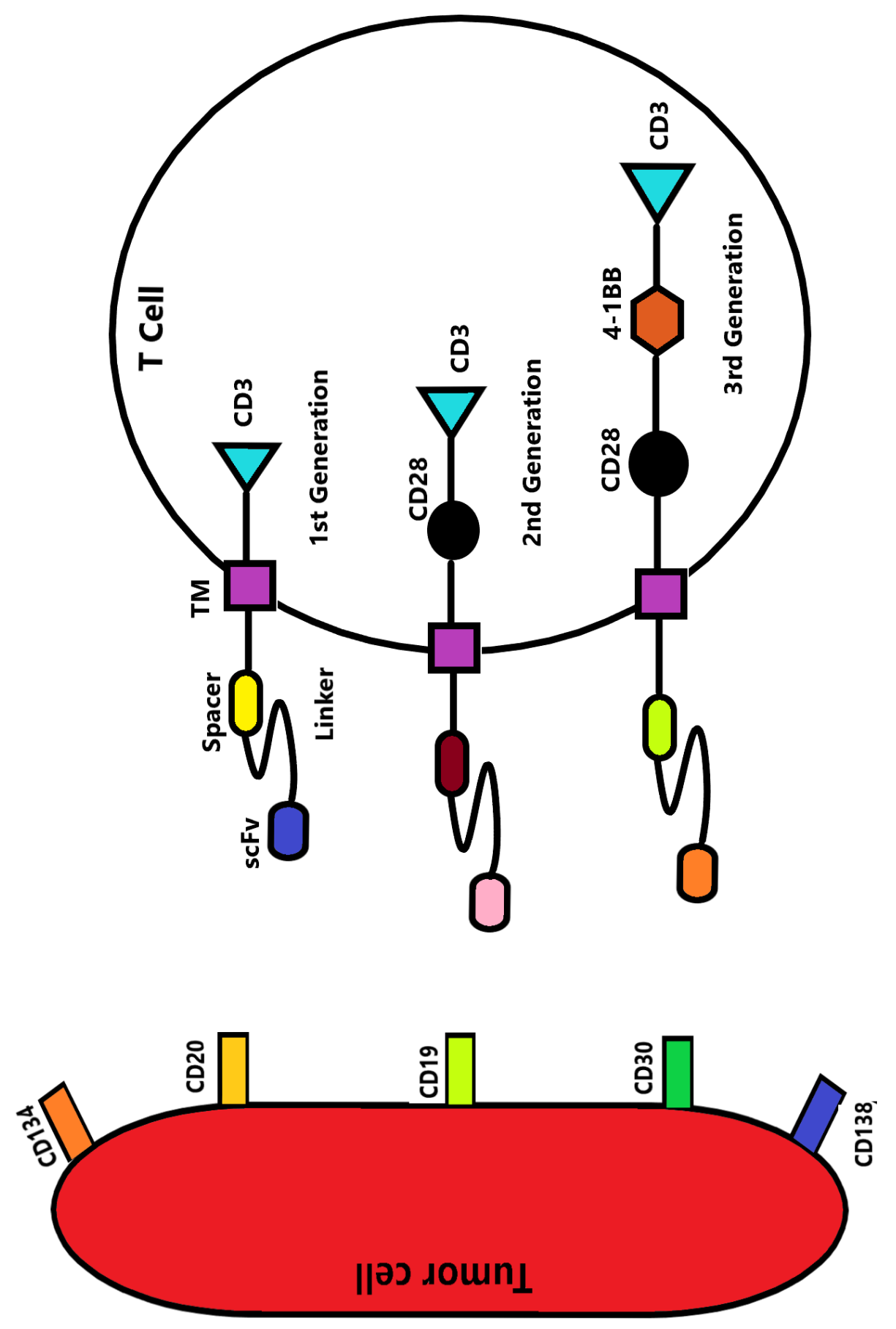


Figure 2

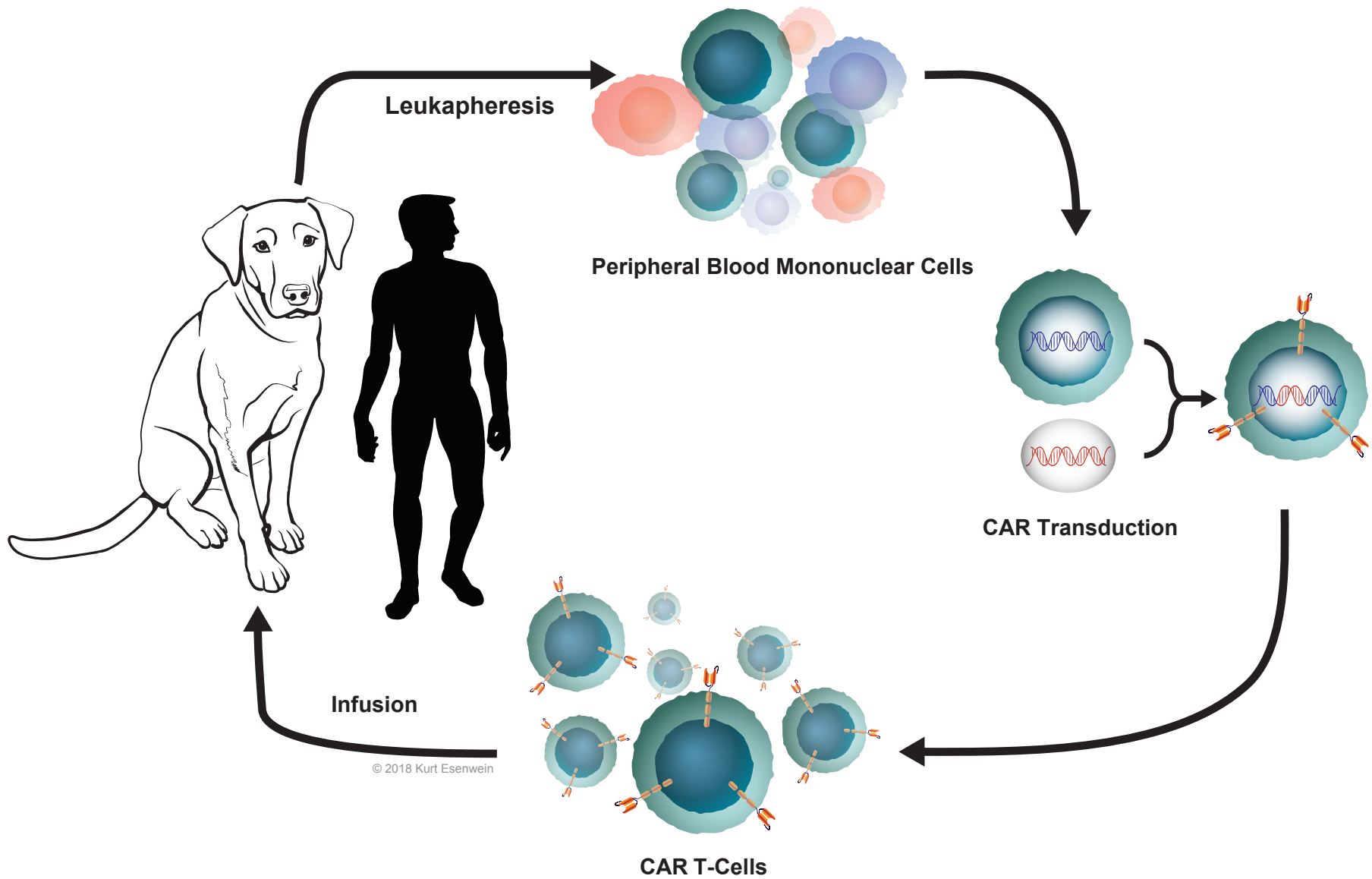




Figure 3

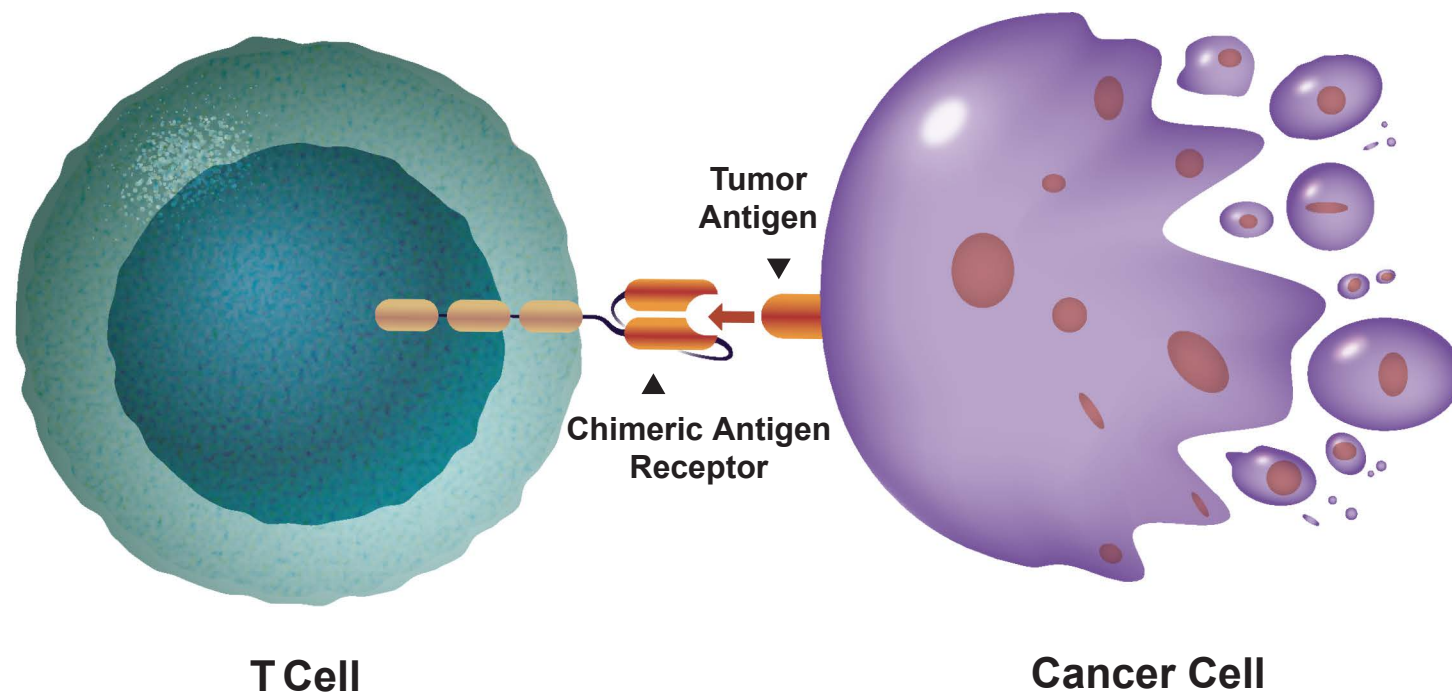
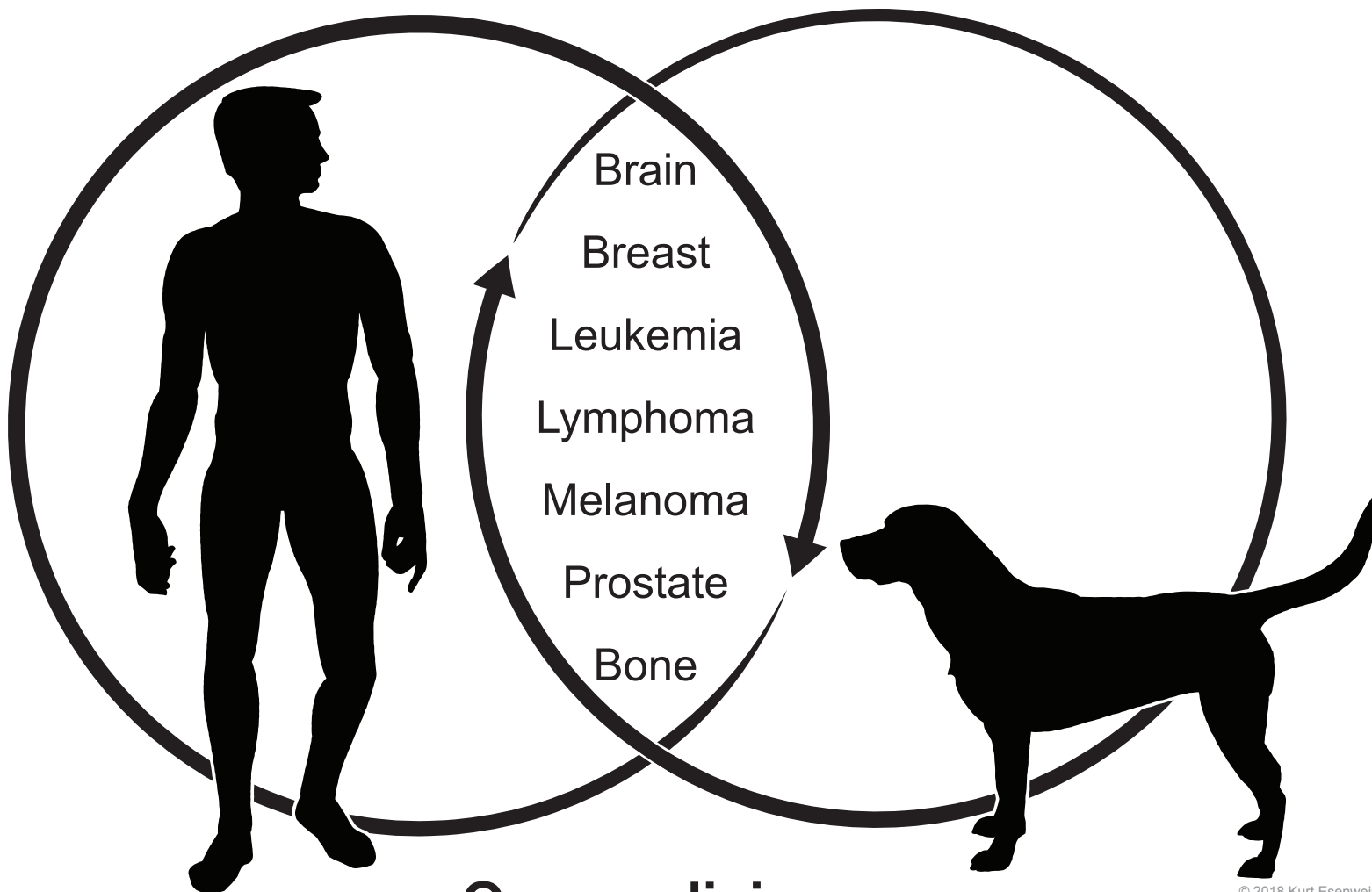


Figure 4

# One pathenogenesis



# One medicine