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Racing CARs to veterinary immuno-oncology

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Chimeric antigen receptors (CARs) have demonstrated remarkable promise in human oncology over the past two decades, yet similar strategies in veterinary medicine are still in development. CARs are synthetically engineered proteins comprised of a specific antigen-binding single chain variable fragment (ScFv) fused to the signaling domain of a T cell receptor and co-receptors. Patient T cells engineered to express a CAR are directed to recognize and kill target cells, most commonly hematological malignancies. The U.S Food and Drug Administration (FDA) has approved multiple human CAR T therapies, but translation of these therapies into veterinary medicine faces many challenges. In this review, we discuss considerations for veterinary use including CAR design and cell carrier choice, and discuss the future promise of translating CAR therapy into veterinary oncology.

KEYWORDS

applied immunology, cancer, cell therapy, immunotherapy, translational medicine

1. Introduction

Cell-based immunotherapy has progressed exponentially over the past few decades as a cutting-edge treatment option for multiple cancers. Adoptive cell therapy (ACT) involves harvesting immune cells from the patient, expanding them under good manufacturing practice (GMP) conditions, and reinfusing a clinically relevant dose. One of the first human ACTs used isolated tumor infiltrating lymphocytes (TILs) and selected for cells with a T cell receptor (TCR) specific toward a tumor neoantigen presented on MHC I of the tumor (1–3). Although promising (4–6), a significant advance in ACT that takes advantage of the specificity and affinity of antibodies against a tumor surface antigen, rather than relying on endogenous T cell receptors (TCRs), is chimeric antigen receptors (CARs). The FDA has approved multiple CAR T therapies against human B cell maturation antigen expressed on antibody-secreting plasma cells (7, 8), and CD19, which is expressed on the surface of almost all B cells (9–12). Similar to humans, lymphomas are common in companion animals. Retrospective analysis of 171 canine and feline non-Hodgkin's lymphoma samples revealed 79.9% of canine cases were B cell lymphomas that were predominantly multicentric, while 64.6% of feline cases were T cell lymphomas that were predominantly alimentary (13). While chemotherapy remains the standard of care in veterinary medicine (14), CARs are an attractive alternative or add on therapy for refractory veterinary lymphomas. Clinical trials have only recently been initiated in dogs. In this review, we outline the design of CARs and the future outlook of the therapy for veterinary use.

2. CAR construct design

Development of a CAR therapy requires multiple steps, each of which presents unique challenges for translation to veterinary medicine (Figure 1). In this section, we summarize basic CAR design and methods of expressing the CAR in primary cells.

CARs are created by stitching together an ScFv, a hinge, a transmembrane domain, and one or more cytoplasmic signaling domain(s) derived from the TCR signaling complex (15, 16). ScFvs are developed from the variable light and heavy chains of a specific monoclonal antibody targeting a tumor-associated antigen. Some CAR approaches use endogenous ligands or receptors, rather than ScFvs, to target tumors and may be a good alternative when cross-reactive or veterinary-specific antibodies are not available (17). Newer high-throughput fluorescence-activated cell sorting (FACS) screens can also be used to identify potential antibodies or ScFvs (18), but it is unclear if this strategy would be practical for clinical manufacturing in veterinary medicine.

The cytoplasmic signaling domains are critical to drive T cell activation and can lead to different effector functions in the patient. Use of one signaling domain, CD3ζ, resulted in low-level signaling, and poor persistence or anergy in patients (19, 20). CAR T therapies approved for human use have additional costimulatory receptor signaling domains like 4-1BB (Kymriah®, Breyanzi®, Abecma®, and Carvykti™) or CD28 (Yescarta®, Tecartus®). Human primary T cells transduced with a CAR containing the CD28 signling domain preferentially generated effector memory T cells *in vitro* ($CCR7^{-}CD45RO^{+}$) while the 4-1BB signlaing domain drove a central memory phenotype ($CCR7^{+}CD45RO^{+}$) (21). Using NSG mice with a xenografted osteosarcoma, infused human CAR T cells with 4-1BB had lower expression of exhaustion markers than those with CD28 (22). Some CARs use two costimulatory domains and have increased efficacy in preclinical animal models (23, 24). Comparison of efficacy of different CAR components in veterinary oncology remains limited and will likely require additional empirical testing (25).

CARs are frequenty delivered to patient primary T cells using a replication-incompetent lentivirus or γ-retrovirus (26, 27). Pre-activation is required because the viruses can only (γ-retrovirus), or preferentially (lentivirus), integrate into dividing cells (26, 27).

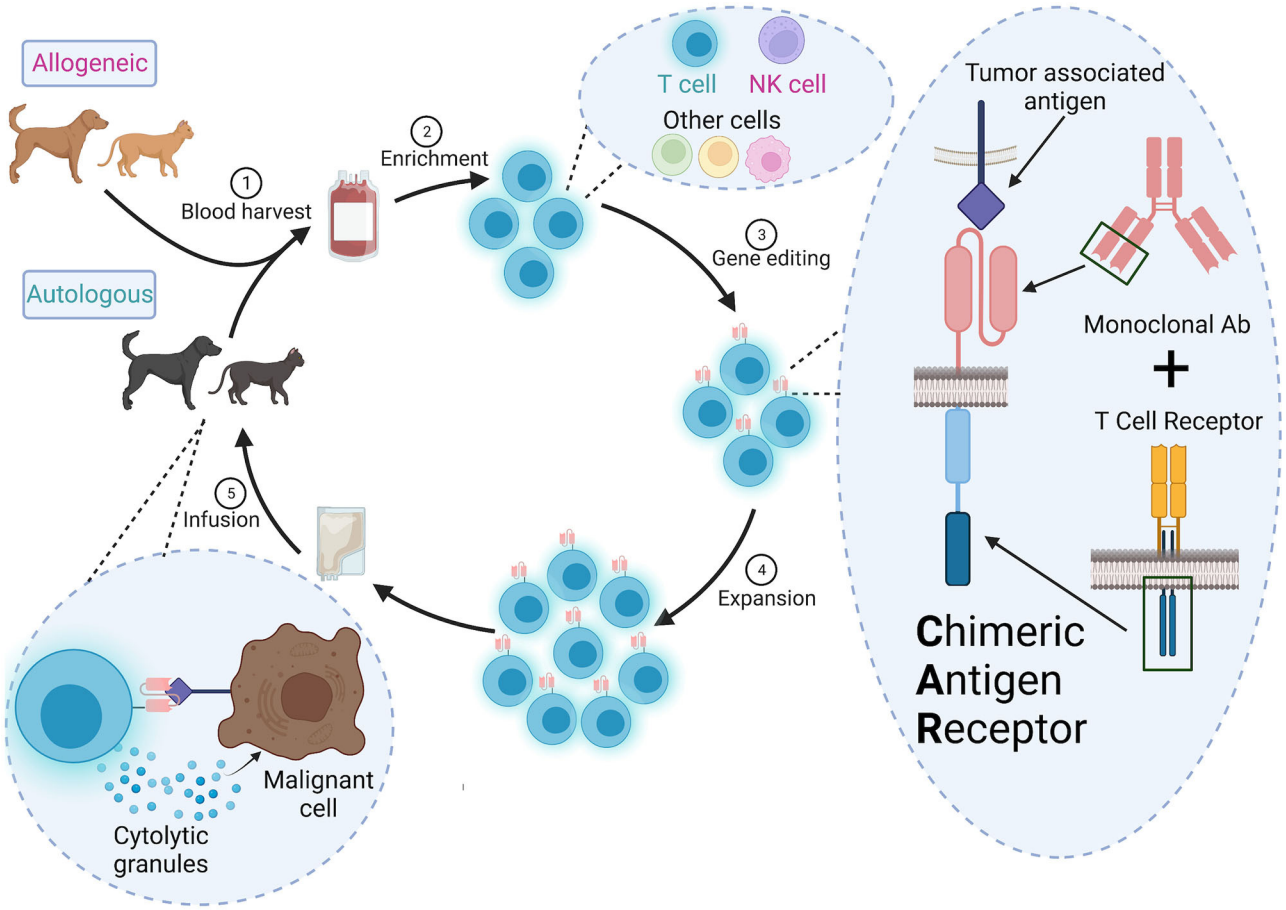

FIGURE 1
Overall scheme for CAR therapy in veterinary medicine. (1) Autologous (from the patient) or allogeneic (from a donor) cells are harvested from peripheral blood or apharesis, (2) enriched and (3) engineered to a express a CAR *ex vivo*. The CAR contains the variable heavy and light chains of a monoclonal antibody specific for a tumor-associated antigen and signaling domains from the TCR signaling complex. (4) The CAR^{+} cells are expanded to a clinically relevant dose and (5) infused into the patient. These CAR cells will detect and destroy cells expressing the target antigen.

However, other approaches have used transposons to integrate the CAR-encoding DNA (28, 29). To avoid delivery of viruses to patients, anti-canine CD20 CAR mRNA was directly electroporated into canine T cells (30, 31). However, CAR expression by mRNA delivery was transient and waned after 14 days (30). Lipid nanoparticles may enhance delivery of CAR mRNA and can be used *in vivo* (32). Transient CAR expression could be an advantage for veterinary therapy since it will limit immune reaction against the xenogeneic antibody components of the ScFv. Regardless of which CAR is developed, the sequences should be species-matched as much as possible to reduce host anti-CAR immune responses.

Gene editing tools like transcription activator-like effector nucleases (TALEN®) and clustered regularly interspaced palindromic repeats (CRISPR)/Cas9 allow for simultaneous delivery of the CAR and reduced graft-vs-host and host-vs-graft responses (33–36). For example, CAR insertion into the TCR locus allows for expression of the CAR under the endogenous transcriptional regulation of the TCR promoter, which limits exhaustion, and elimination of TCR expression, which reduces graft vs. host disease (37). Conversely, deletion of β2-microglobulin, part of MHC I, reduces CAR T cell rejection by the host. However, loss of MHC I increases detection and destruction by natural killer (NK) cells, which can be mitigated in part by knock-in of human leukocyte antigen E into the *B2M* locus (38). Inhibitory receptors such as PD-1, which limit CAR T cytotoxicity, can also be deleted using these gene editing tools (39). However, CRISPR can induce unwanted mutations (40, 41), or multiple donor DNA insertions (42). Unpredicted translocations have also occured when TALEN® was used to delete the TCR alpha chain and CD52 to make "universal" CAR T cells (35). Fortunately, these off-target events are relatively rare (43).

3. Cell manufacturing

While GMP guidelines must be followed for clinical-stage ACT in both human and veterinary medicine, there are some specific considerations for veterinary application. In this section we will outline methods that are under investigation for veterinary CAR T cell expansion, as well as systems employed in human CAR T cell production that could be adapted for veterinary use.

Production of cells for clinical use requires validation of standard operating procedures and GMP-grade reagents and materials in all manufacturing steps with individual certificates of analysis. Growth of cells for human medicine requires serum-free, xeno-free, GMP-grade, commercial media formulations. There may not be commercially available GMP-grade species-specific sera for veterinary applications. Anti-canine CD20 CAR T cells failed to grow in OpTmizer™ serum-free media, and while there was some growth in LymphoONE™ serum-free media, CAR expression levels were low, suggesting empirical identification of optimal growth conditions for each veterinary CAR application may be necessary (44). Moreover, veterinary species cytokine supplements are limited (45) and thus validated cross-reactive reagents may be required (46). Feeder cells or special additives can enhance *ex vivo* expansion. For example, K562 cells can be engineered to express human CD32 and canine CD86, thereby acting as artificial antigen presenting cells (aAPCs). Co-culture of canine T cells with these aAPCs resulted in nearly six-fold expansion, and was even able to stimulate proliferation in T cells that were unresponsive to agonistic anti-canine CD3/CD28 beads (30). High $CD8^+$ subset expansion and reduced PD-1/PD-L1 expression on canine CAR T cells occurred when the cells were grown with thyroid adenocarcinoma aAPCs expressing CD80, CD83, CD86, and 4-1BBL in the presence of phytohemagglutinin (47). Phytohemagglutinin also increased retroviral transduction efficiency (44). Additional advancements in cell culture using closed-system bioreactors can further enhance *ex vivo* expansion yield, reduce contamination risks, and minimize technician handling (26, 48–52). These devices will likely be employed more frequently in future veterinary clinical trials.

4. Choosing the right "CAR driver"

Currently all FDA approved human CAR therapies are T cell-based, and T cells are also the "driver" for canine CAR therapy. However, many different immune cells could potentially be used to carry a CAR (Table 1). In this section, we describe the major advantages and limitations of each cell type, as well as the development and therapeutic potential in veterinary medicine. The focus is on canine CARs since they have advanced the furtherest in veterinary medicine, but we will discuss potential use in felines and highlight findings in human medicine that have potential for veterinary applicability.

4.1. T cells

The most advanced CAR therapeutics in veterinary medicine are T cells. The first clinical trial of CAR T cells in canine patients delivered a CD20 CAR mRNA by electroporation. The CAR contained a murine anti-canine CD20 ScFv with human CD8α leader, hinge, transmembrane, and a CD3ζ signaling domain (30). One canine patient with relapsed spontaneous B cell lymphoma was infused in three separate doses and had reduced $CD20^+$ cell numbers with no adverse events. A follow up study treated diffuse large B cell lymphoma with anti-CD20 CAR containing the same ScFv, but canine signaling domains (78). No adverse events were documented following infusion in three dogs, but this therapy had lower efficacy and *in vivo* persistence of the cells was poor. Eventually, an escape-variant of CD20 was detected on peripheral blood B cells post-infusion. Additionally, two of the dogs developed anti-mouse ScFv CAR serum antibodies, which peaked at day 50 post-infusion. These types of anti-CAR immune responses can be reduced by generating a "caninized" ScFv where all but the complementarity determining regions of the ScFv are canine.

Preclinical and clinical investigation of canine CAR T cells has also begun to target solid tumors, which have notoriously been resistant in human CAR therapy. A HER2 CAR T cell therapy (79) with canine CD28 and CD3ζ signaling domains secreted IFNγ and was cytotoxic against $HER2^+$ osteosarcoma and breast cancer target cell lines *in vitro* (80). IL13Rα canine CAR T cells secreted IFNγ when incubated with $IL13R\alpha^+$ targets (81). A canine glioma cell line implanted into mouse brains was effectively eliminated using canine CAR T cells against IL13Rα with either a human or a canine 4-1BB signaling domain. B7-H3 CAR T

TABLE 1 Summary of species-specific surface markers that define immune cells and can be used to enrich desired populations through FACS or magnetic bead enrichment.

Immune cell	Human phenotypic markers	Murine phenotypic markers	Canine phenotypic markers	Feline phenotypic markers	References
T cell	$CD3^+CD56^-$ $\alpha\beta TCR^+$	$CD3^+$ $\alpha\beta TCR^+NK1.1^-$	$CD3^+CD5^{bright}NKp46^-$ $\alpha\beta TCR^+$	$CD3^+CD56^-$ $\alpha\beta TCR^+$	(53–58)
NK cell	$CD3^-CD56^+CD7^+$	$CD3^-$ $NK1.1^+$ $\alpha\beta TCR^-$	$CD3^{-/+}$ $CD5^{dim}CD8^+TCR\alpha\beta^-TCR\gamma\delta^-$ $CD21^-CD4^-$ $CD94^+$ $NKp46^+$	$CD3^-CD56^+$	(53, 54, 59–64)
NKT cell	$CD3^+CD56^{+/-}iTCR^+$	$CD3^+NK1.1^+iTCR^+$	$CD3^+CD5^{intermediate}$ $NKp46^+CD94^+$ $iTCR^+$	$CD3^+CD56^+$	(53, 54, 59–61, 65–68)
γδ T cell	$CD3^+\gamma\delta TCR^+$	$CD3^+\gamma\delta TCR^+$	$CD3^+\gamma\delta TCR^+$	$CD3^+\gamma\delta TCR^+$	(69–71)
Macrophage	$CD68^+$: $CD80^+CD206^{dim}$ (M1) or $CD80^-CD206^{bright}$ (M2)	$F4/80^+$: $CD38^+$ (M1) or $CD38^-$ (M2)	$Iba1^+$: $CD204^-$ (M1) or $CD204^+$ (M2)	$Iba1^+$: $CD204^-$(M1) or $CD204^+$(M2)	(72–77)

cells (82) were more cytotoxic than HER2 CAR T cells toward canine osteosarcoma spheroids, but cytotoxicity was similar for the constructs incorporating CD28 or 4-1BB signaling domains (83). Two healthy canine subjects were then infused with either frozen or fresh autologous B7-H3 CAR T cells. The fresh infusion did induce a grade 2 toxicity but no other adverse events were observed, while the recipient of frozen cells had an allergic reaction 67 days later that was likely unrelated to the infusion. Together, these results show that canine CAR T cells are safe and well-tolerated, even for some solid tumors.

The most notable drawback of human CAR T therapy is cytokine release syndrome, which presents with pyrexia, delirium, hypotension, and increased serum IL6, and often requires administration of the IL6 receptor antagonist tocilizumab and steroids (84). To enhance safety and rapidly deplete infused cells in the event of an adverse reaction, drug-sensitive "kill switches" can be incorporated into the CAR (85–89). Since some adverse reactions have been observed in canine CAR T trials, including a case report of increased serum cytokines consistent with cytokine release syndrome (90), incorporating kill switches in the CAR construct may be needed in veterinary medicine as well.

4.2. Natural killer cells

NK cells have reduced risk of inducing a graft vs. host reponse and have shown promise in human preclinical studies. Moreover, human NK cells can be sourced allogeneically (91, 92), and infused at higher doses (93, 94). Allogeneic sourcing may allow mass production of an "off the shelf" product, reducing manufacturing costs, which is a significant concern in veterinary medicine.

Major challenges to using NK cells for veterinary CAR therapy include the lack of consensus on surface markers, limited antibody reagents, and lack of robust purification and expansion protocols. Feline NK cells are $CD56^+CD3^-$ (53, 59) and feline CD3 and CD56 antibodies exist (clones NZM1 and SZK1, respectively) (95, 96). However, there is not a consensus on canine NK markers. NKp46 is a common NK marker across species and $CD3^-NKp46^+$ cells enriched by FACS from canine peripheral blood mononuclear cells (PBMCs) exhibited cytotoxicity toward canine osteosarcoma and canine thyroid adenocarcinoma targets (60). Coculture of canine PBMCs with K562 cells expressing membrane bound IL15 and 4-1BBL, and added human IL2 and IL15, expanded large granular lymphocytes with cytotoxic activity (61). These presumptive NK cells were $CD5^{dim}CD3^+CD8^+TCR\alpha\beta^-TCR\gamma\delta^-CD21^-CD4^-$ and although they did not have mRNA for CD56, they did have mRNAs for other NK receptors like NKG2D, NKp30, and NKp46. CD5 depleted canine PBMCs cultured with IL2 alone or IL2 and IL15 for 14 days also had NK-like cytotoxicity yet were $CD56^-$ (97). $CD94^+$ cells enriched from canine PBMCs were $CD5^{dim}NKp46^+CD3^-$ (54). A first-in-canine clinical trial infused expanded cells with a similar phenotype into ten sarcoma patients in combination with intratumoral rhIL2 following focal radiotherapy (98). Five of the patients remained metastasis free at the 6-month primary endpoint (98). Despite NK cells being safe (99), their clinical efficacy does not yet match CAR T. Moreover, NK cells have a shorter *in vivo* lifespan than T cells. Addition of the *IL15* gene may provide sufficient signaling to overcome these limitations (100, 101).

4.3. Other cells

Immune cells such as natural killer T (NKT) cells, γδ T cells, and macrophages have been explored preclinically and clinically as human CAR drivers. Human NKT cells are rare $CD3^+$ lymphocytes expressing an invariant αβ TCR, and may coexpress CD56 (65, 102, 103). Feline NKT cells are $CD56^+CD3^+$ (53, 59); however, canine NKT markers are more controversial. Originally defined as $CD3^+$ lymphocytes that bound to complexes of α-galactosylceramide and murine CD1d (68), one group identified a $CD5^{intermediate}NKp46^+CD94^+CD3^+$ subset of large granular lymphocytes that may be NKT cells (54). Clinical isolation protocols for NKT cells may require dual CD56/CD3 enrichment for felines or NKp46/CD3 for canines, and there are currently no expansion protocols to obtain clinically useful numbers of these feline or canine cells. Regardless, human CD19 CAR NKT cells against lymphoma (104), and GD2 CAR NKT cells against neuroblastoma (105), have demonstrated preclinical efficacy, with

CD19 CAR NKT cells exerting anti-lymphoma activity through both the CAR and the invariant TCR interaction with CD1d. However, not all tumors express CD1d and much of the activity will be *via* the CAR (106, 107). Human GD2 CAR NKT cells, co-expressing IL15, infused in pediatric neuroblastoma patients, were well-tolerated and reduced metastasis in one patient. This study provided safety data for human CAR NKT cells co-expressing self-supporting growth factors (108). NKT cells may soon be explored for CAR therapy in veterinary medicine.

In veterinary medicine, γδ T cells play an important role in mucosal immunity (109), and can comprise nearly half of the PBMC compartment in young ruminants (110). γδ T cells express TCRs with broad specificity and are MHC independent, yet they have *in vitro* cytotoxic activity similar to NK and T cells. Human GD2 CAR γδ T cells demonstrated *in vitro* cytotoxicity to the LAN1 neuroblastoma cell line (111). Both canine and feline *TCRG* loci have been identified and subsets can be classified through PCR, but robust isolation and expansion protocols are lacking (69, 70, 112). Moreover, many γδ T cells are located in peripheral tissues and may be difficult to enrich from peripheral blood in sufficient numbers to expand for clinical use (113). Enrichment of human Vδ1 cells from peripheral blood and expansion in cell culture bags using IFNγ, anti-CD3, and IL4, for 2 weeks followed by IL15 for 1 week, did generate a clinically relevant product yield and upregulation of effector markers (NKG2D, DNAM-1, NKp30, NKp44, and 2B4) (114). However, further research is needed to determine if γδ T cells will be useful in veterinary CAR therapy.

Macrophages are abundant in tumors of many different species, can exhibit anti-tumor activity, and have therapeutic potential as CAR drivers (115, 116). Macrophages can polarize to many different functional states from the extremes of proinflammatory M1 to anti-inflammatory/immunosuppressive M2 cells. Tumor-associated macrophages also adapt to the tumor microenvironment in ways that promote rather than eliminate tumors (117). In dogs, high numbers of macrophages in tumors is correlated with increased aggressiveness and worse prognosis for mammary cancer (72). Human THP-1 monocytic cells engineered to express CD19, HER2, or mesothelin CARs, phagocytosed target cells *in vitro* (118). Primary human HER2 CAR macrophages extended survival in a mouse ovarian xenograft model, suggesting that they still demonstrated antitumor activity despite the immunosuppressive tumor microenvironment (118). Macrophage immunotherapy in veterinary oncology has largely focused on *in vivo* activation of macrophages rather than *ex vivo* manipulation and reinfusion, but there is potential to develop them as CAR drivers (119–122), A limitation is that macrophages, and their precursor monocytes, are notoriously difficult to genetically modify regardless of species. Some approaches to overcome this limitation include using a replication-incompetent adenovirus (118, 123). Despite their limitations, macrophages and other CAR drivers warrant a basic science investigation to understand their true potential for use in veterinary medicine.

5. Discussion

Cell-based immunotherapy has gained traction as a promising therapeutic modality for multiple cancers in both human and veterinary patients. Although clinical veterinary studies are still in the beginning phases, the potential for breakthrough therapies, like has happened for human hematologic oncology, is high. Veterinary clinical trials involving infusions of T cells and NK cells have demonstrated the feasibility and safety of harvesting and manufacturing cells for clinical use (30, 78, 83, 98). However, to fully break into the cellular immunotherapy sector the way human medicine has, veterinary schools or other hospitals will need appropriate infrastructure for cellular manufacturing and genetic modification, or identify industry partners. Current manufacturing systems are designed for clinical production of human cellular therapeutics, but as interest in veterinary cell therapy grows, so will the market for xeno-free GMP-grade media, reagents, and supplements to be used for species-specific cell isolation and clinical expansion. The potential cost of the therapy also presents a major hurdle, and possibly the biggest challenge toward translation to clinical veterinary use. Insurance coverages that can defray the six-figure prices of human CAR T cell therapies would not be an option in veterinary medicine. Thus, a significant focus of future veterinary CAR research must be to develop more generally tolerable therapies with low levels of side effects to create a product that could be administered at a general veterinary practice. These will likely include a product where endogenous TCRs are deleted and other modifications are made to reduce cytokine release syndrome. Overall, companion animal patients may greatly benefit from immunotherapies that have seen success thus far in human patients due to their shared spontaneous disease development. As the field progresses in veterinary medicine, future treatment modalities designed for companion animals may one day translate back to human medicine.

Author contributions

JC and CL conceived of the review, wrote, and edited the manuscript. JC performed searches in PubMed and Google Scholar databases including, but not limited to: CAR T therapy, chimeric antigen receptor, canine, feline, CAR NKT, macrophage markers, T cell phenotype, gamma delta T cell veterinary, and murine T cells. Both authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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