CANINE CAR T CELL THERAPY FOR SOLID TUMORS

by

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Dedicated to the people who helped me throughout my education

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ABSTRACT

Adoptive cell transfer of chimeric antigen receptors (CAR) T cells has successfully targeted hematological malignancies in human patients. However, unpredicted side effects experienced after injection of the CAR T cells suggests the need for an optimal predictive preclinical animal model. Dogs have intact immune systems and develop solid tumors spontaneously with similar morphology and genetics to humans. I hypothesize that generating CAR T cells for dogs will closely mimic human patients' outcomes, thus providing new understandings of the safety of this immunotherapy. In addition to the dog as a preclinical model, we propose using a universal CAR T cell to overcome various tumor-related immunosuppressive challenges and control the killing of the target cells. To achieve this, we established methods for activating and expanding canine T cells to a clinically relevant scale. Then, we expressed a second-generation anti-FITC-8-41BB-ζ CAR T cell via lentiviral transduction. In the presence of the correct low-molecular-weight bispecific adapter, we showed *in-vitro* CAR-mediated function. Our results proved that it is feasible to generate functional canine anti-FITC-8-BB-ζ CAR T cells for therapy.

CHAPTER 1. ADVANCES AND CHALLENGES OF CAR T CELL THERAPY AND SUITABILITY OF ANIMAL MODELS

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Abstract

Chimeric antigen receptors (CARs) recently gained momentum in cancer treatment due to their ability to promote T-cell mediated responses to a specific tumor-associated antigen. CARs are part of the adoptive cell transfer (ACT) strategies that utilize patients' T lymphocytes, genetically engineered to kill cancer cells. However, despite the therapy's success against blood-related malignancies, treating solid tumors has not reached its fullest potential yet. The reasons include the complex suppressive tumor microenvironment, mutations on cancer cells' target receptors, lethal side-effects, restricted trafficking into the tumor, suboptimal persistence in vivo and the lack of animal models that faithfully resemble human tumor's immunological responses. Currently, rodent models are used to investigate the safety and efficacy of CAR therapies. However, these models are limited in representing the human disease faithfully, fail to predict the adverse treatment events and overestimate the efficacy of the therapy. On the other hand, spontaneously developed tumors in dogs are more suited in CAR research and their efficacy has been demonstrated in a number of diseases, including lymphoma, osteosarcoma and mammary tumors. The present review discusses the design and evolution of CARs, challenges of CAR in solid tumors, human and canine clinical trials, and advantages of the canine model.

1.1 Introduction

Treatment of cancer by standard methods, surgery, radiation, and chemotherapy, is less effective in advanced-stage disease and causes numerous side effects. Consequently, researchers are in the quest to explore the possibility of developing more effective, less toxic therapy. Recently immunotherapy has emerged as a sound approach that includes immune checkpoint inhibitors, T-cell transfer therapy, monoclonal antibodies, vaccines, and immune system modulators. The most studied type of immuno- therapy is T-cell transfer therapy or adoptive cell transfer (ACT).

ACT is the collection and the use of patients' immune cells to treat their cancer. Currently, there are a few types of ACT-based therapies, tumor-infiltrating lymphocytes (TIL), engineered T cell receptor (TCR), natural killer (NK) cells, iNKT cells, Chimeric antigen receptors (CAR) T-cell [1], and γδT cells [2]. TIL uses T cells around or in a patient's tumor tissues. These T cells are collected, and the best that recognizes and kills the tumor ex-vivo is selected, expanded, and adoptively transferred back to the patient to eliminate tumor cells. TCR or transduced T-cell is the genetic engineering of T-cells to express new specific TCR to recognize tumors ex vivo. NK cells therapy depends on the immune system's activation against abnormal cells. Unlike TLs, NK cell receptors interact with target cells independent of antigen processing and presentation. Γδ T cells are T cells that express a unique TCR composed of one γ-chain and one δ-chain [3,4]. In CAR T cell therapy, the T lymphocytes undergo modification with a receptor based on a recognition sequence of an antibody, called CAR, a non-MHC restricted receptor, to attach to specific proteins (antigens) on cancer cells' surface ex-vivo. The T cells in CAR therapy have an improved ability to attack and kill the cancer cells compared to T cells in TIL therapy [5]. In these therapies, the lymphocyte undergoes modification via plasmids or viral vectors, such as adenovirus, retrovirus, or lentivirus [6].

CAR T therapy showed promising success in treating malignant blood diseases such as acute lymphoblastic leukemia (ALL) and diffused-large B cell lymphoma (DLBCL) in children and young adults. Therefore, the FDA authorized cluster of differentiation 19 (CD19) specific CAR T cell therapies for these diseases. Tisagenlecleucel (Kymriah™) against ALL and DLBCL for children/young adults, Axicabtagene ciloleucel (Yescarta™) against adult non-Hodgkin lymphoma (NHL) and DLBCL, Brexucabtagene autoleucel (Tecartus™) for relapsed or refractory (R/R) mantle cell lymphoma (MCL) treatments [7–10], and most recently, Breyanzi (lisocabtagene maraleucel) for the treatment of relapsed or refractory (R/R) large B cell lymphomas (LBCL) in adult patients [11].

However, these CAR T therapies have limited success in solid tumors. CAR T cells treatment directed against antigens such as vascular endothelial growth factor receptor 2 (VEGF-R2), CD171, folate receptor alpha, disialoganglioside GD2, human epidermal growth factor receptor 2, mesothelin, EGFRvIII, or carbonic anhydrase IX, in patients with solid tumors failed to produce similar beneficial outcomes as seen in blood-related malignancies [12].

Translating successful CAR T-cell therapies to solid tumors requires overcoming several barriers, including identifying an ideal tumor-associated antigen to target and overcome antigen expression heterogeneity, addressing the tumor-suppressive microenvironment, and employing a preclinical model that faithfully represents the disease. The review collected data using PubMed, Google Scholar and other publicly available databases and discusses the design and evolution of CARs and the challenges facing CAR therapies in solid tumors. Also, it discusses the advantages and disadvantages of preclinical animal models emphasizes the advantages of using the canine model (Fig. 1)

1.2 General Design of CARs

The discovery of the CARs started around the 1980s. Several factors are essential for CAR T cell therapy to be effective, such as recruitment, activation, expansion, and persistence of bioengineered T cells at the tumor site. Even though ~41 years have passed since the first CAR T cell's creation, some essential components of its structure remained the same [13]. However, these components have undergone numerous modifications to enhance CAR T therapeutic capabilities over the years. The structure consists of four components: the ectodomain (the domain of a membrane protein outside the cytoplasm) a hinge, the transmembrane domain, and the intracellular signaling endodomain. Each domain has a specific function and optimal molecular design. The extracellular domain, the target-binding domain, is usually a single-chain variable fragment (scFv) of the antigen-binding region of a monoclonal antibody's light and heavy chain. It recognizes any antigen and binds targets with high affinity. The hinge connects the extracellular antigen-binding domain to the intracellular signaling domains and regulates the extracellular domain flexibility, facilitating the migration and binding capacity to tumor cell receptors. The length and composition of the hinge can affect antigen binding and signal through the CAR. Generally, the hinge domain consists of amino acid sequences from CD8, CD28, IgG1, or IgG4. The transmembrane domains anchor the CAR in the T cell membrane. It consists of a hydrophobic alpha helix that spans the membrane, such as CD3ζ, CD28, CD4, or CD8α. The primary function of the transmembrane domain is to stabilize the CAR. The endodomain domain (intracellular signaling domain) comprises of the activation domain, a TCR-derived CD3ζ-derived immunoreceptor tyrosine-based activation motifs, and intracellular costimulatory domains derived from CD28 or 4-1BB (CD137) [14,15]. The first CAR generations with CD3-ζ transmembrane domains suffered detachment from

the surface of T cells. Consequently, CAR T structure is subjected to modification with a well-balanced transmembrane domain composed of the CD4, CD8, or CD28 molecules [16]. Antigen-specific T cell activation, in nature, requires three signals to gain full functionality that enables proliferation, differentiation, and survival. Co-stimulation plays a vital role in the CAR T-cell functionality as it triggers the T-cell immune response against foreign antigens. The absence of co-stimulation can enter T cells in a state of anergy, leading to its unresponsiveness to antigen binding [17]. Unfortunately, cancer cells promote co-stimulatory-ligand deficient environments generating unfavorable antitumor responses. Therefore, CAR T is designed with various costimulatory molecules to overcome the tumor cell suppressing environment. The conserved region of a CD3-ζ domain, the immunoreceptor tyrosine-based activation motifs (ITAMs), carries out signaling transduction pathways on CAR T cells to build sufficient T cell activation [18].

Also, CARs function without relying on the major histocompatibility complex (MHC), allowing it to target various antigens without antigen presentation for activation since activated with the single-chain Fv domain interaction with the targeted TAA [19]. The MHC independence is an essential feature of CAR design since the tumor microenvironment consistently down-regulates the MHC complexes.

1.3 Generation of CAR T cells

Although CAR T therapy can lead to long-lasting remissions for some patients with very advanced malignant disease, it can cause severe and fatal side effects such as cytokines storm and neurological problems, including termer, delirium, and seizures. Therefore, scientists modified CAR T cells to create safe and more effective therapy by building on the CAR T cell's original components and information gained from clinical trials. These include:

CAR 1st generation. It consists of a single-chain variable fragment (scFv) ectodomain and a TCR-derived signaling CD3-ζ constant region representing the endodomain fragment. These 1st generation CAR cannot maintain the CAR stable on the T cell membrane and T cell activation for a considerable amount of time [20].

CAR 2nd and 3rd generations. The second and the third generation compared to the 1st generation were modified to enhance the receptor cohesion toward the lymphocyte surface, thus allowing optimal functionality. As a result, these CARs generations have one (2nd generation) or

two (3rd generation) costimulatory signals that augment T cell proliferation, differentiation, and survival despite the effect of tumor-suppressing environments [17](17).

CAR 4th generation. The fourth generation compared to 2nd and 3rd generation CAR, create a robust immune attack to eliminate the tumor before they re-generate or mutate. The 4th generation CAR T cells redirected for universal cytokine killing (TRUCK), has the same structure and physiology as the 2nd and the 3rd CAR generations with a slight genotypic difference [20]. These TRUCKs contain a nuclear factor of the activated T cells (NFAT), codifying a transgenic cytokine. NFATs are found in T cells and play a crucial role in cytokine expression. TRUCKs deliver a considerable amount of IL-12 on the tumor site stimulating T cells and recruiting other immunological cells to target tumor cells not recognized by the (svFc) fragment of a CAR [21].

CAR 5th generation. The 5th generation have the same structure as the second generation of CARs, but they contain a truncated cytoplasmic IL-2 receptor β-chain domain with a binding site for the transcription factor STAT3. The antigen-specific activation of this receptor simultaneously triggers TCR (through the CD3ζ domains), costimulatory (CD28 domain), and cytokine (JAK-STAT3/5) signaling required physiologically to drive full T cell activation and proliferation.

1.4 CAR T-cell therapies common side effects

CAR-based therapy's common side effects are the body's immunological defense impulses triggered by the T cell artificial receptor. These autoimmune consequences can affect the patient's prognosis and disease outcomes. The most common side effects include.

Cytokine release syndrome (On-target on-tumor toxicity). One of the most frequent setbacks in using CAR T therapies is releasing proinflammatory cytokines into the body or cytokine release syndrome (CRS) due to excessive antigen-CAR T cell engagement. These cytokines are small proteins that act as cell messengers to help direct the body's immune response. Increased cytokine levels lead to chronic inflammation throughout the body, which can be harmful and interfere with several body functions. CRS is characterized by increased serum levels of cytokines, fever, diarrheas, hypotension, hypoxemia, low blood pressure, and organ dysfunctions. Most patients have a mild CRS form, but it may be severe or life-threatening in some individuals due to organ failure. The severity of CRS depends upon the disease burden. Generally, splitting the initial dose and strictly monitoring the vital parameters can mitigate the risk. Also, treating

specific symptoms to lower the immune response, such as tocilizumab and siltuximab, interferes with IL-6 or corticosteroids to help reduce inflammatory and immune response [22].

Immune effector cell-associated neurotoxicity syndrome (ICANS). Although CAR T neurotoxicity is the most common side effect, its pathophysiology is not entirely understood. Recent studies suggested that blood-brain barrier disfunction (BBB) causes CAR T cells' infiltration into the cerebrospinal fluid [23]. Symptoms include confusion, myoclonus, seizures, delirium, aphasia, memory loss, and coma [8,9,22]. Neurotoxic issues are reported in patients within the first two months of CAR T treatment lasting between 6-17 days, depending on the type of blood cancer treated and the specific drug-infused [24]. Trials studying GD2 in treating neuroblastoma with high-affinity GD2 specific CAR T and ERBB2 with ERBB specific CAR T for metastatic colorectal cancer found It to cause severe neurotoxicity and multi-organ failure, respectively [25,26].

On-target toxicities (On-target off-tumor toxicity). On-target off-tumor effect arises in patients with target antigens expressed on both tumors and healthy tissues. The condition was first noticed in patients who experienced uncommon reductions of healthy B-lymphocytes, B-cell aplasia, in trials utilizing a CD-19 specific CAR T cell due to the binding of the engineered T cells to both CD-19 malignant and healthy B cell [27,28]. Similarly, low-level ERBB2, CAIX, and CEACAM5 expression on healthy lung, liver, and gastro- intestinal epithelia resulted in deadly toxicities in these organs [25,29]. Thus, it is crucial to know the background expression of the target antigen in healthy tissues to determine whether its levels are over the threshold that may cause toxicity and the potential severity.

Off-target toxicity. Off-target toxicity occur when CAR T cells attack an antigen other than those for which the CAR T was meant to bind or activate themselves independently from their specificity. The risk of off-target toxicity occurs due to the inherited CAR T makeup [23]. For example, patients treated with CAR T-anti-HER2/neu. CAR T-anti-HER2/neu carries IgG1-derived CH2CH3 domain as an extracellular spacer which can interact with the Fc receptor expressed on innate immune cells and, as a result, lead to antigen-independent activation [29].

1.5 Future generations of CAR T therapy

Even though treatment with CAR-T cells has produced remarkable clinical responses with specific subsets of B cell leukemia or lymphoma, a number of challenges (mentioned above) limit

the therapeutic efficacy of CAR-T cells in solid tumors and hematological malignancies. However, researchers are working restlessly to overcome these limitations by pursuing various new CAR concepts and models to generate the next generation of CAR therapies. These concepts include:

The bispecific adaptor platform. Among numerous platforms to improve CAR T therapy, the adaptor CAR platforms have received much attention and immense research. The platform separates the tumor-targeting and signaling moieties of conventional CARs resulting in a system consisting of an adaptor CAR or universal CAR and soluble, tumor-specific adaptor molecules. The universal CAR construct contains cytoplasmic activation domains in conventional CAR and an extracellular single-chain variable fragment (scFv) that recognizes fluorescein (anti-FITC CAR T cell). The bispecific adapter molecule comprises fluorescein linked to a tumor-specific ligand. Such an adaptor brings the CAR T cell to the tumor cell triggering CAR T-cell activation and the subsequent destruction of the cancer cell-the omission of the bispecific adapter prevents CAR T cell engagement with the cancer cell and the tumor cell killing. A cocktail of orthogonal fluorescein-linked bispecific adapters in which each fluorescein-linked adapter is attached to a unique tumor-specific ligand capable of binding one of the cancer cell's antigens could be prepared [30,31]. Developing this platform improves conventional CAR T cells' flexibility, tumor specificity, and controllability [32].

Dual CAR T-cells. Despite the great successes with Tisagenlecleucel and Axicabtagene, Anti-CD19 chimeric CAR T cell, therapy in leukemia, up to 60% of patients relapse due to CD19 antigen loss. A new approach to overcoming antigen loss targets more than one antigen on cancer cells, such as autologous CD19/CD22 CAR T cell therapy, which demonstrated to be safe and had anti-leukemic activity in patients with relapsed/refractory B-ALL [33].

Dominant-negative receptor CAR T cells. In addition to the target antigen scFv, dominant-negative receptor CAR T cells are transduced with an additional co-inhibitory receptor that controls inhibitory signals sent by the tumor milieu to the T cell. Those receptors include PD-1 and TGF-βRII [34,35]. Other upregulated receptors when the T cell is exhausted, and potential candidates for this type of method are CTLA-4, TIM-3, and TIGIT.

Off-the-shelf CAR T cells. These Off-the-shelf CAR T cells are a third-party, healthy donor-derived alternative. Because the preparation of autologous CAR T cells takes time, the patient needs to be stable to withdraw their T cells by leukapheresis; pre-made CAR T cells offer a ready-to-use therapy for advance stage cancer patients.

1.6 CAR T therapies in hematological malignancies

The FDA gave authorization for five CAR T therapies up to date. The first four therapies utilize slightly different methods of genetic engineering to transform the patient's T cells into CAR T cells. However, all therapies produced CAR T cells that bind to the cluster differentiation 19 (CD19) protein on the B-cell surface.

The first approved CAR T therapy is tisagenlecleucel (Kymriah; Novartis), approved in August 2017. In this therapy, the T cells are induced by a vector that encodes a second-generation CAR with scFv, derived from the CD19-specific monoclonal antibody FMC63 and the costimulatory domain from 4-1BB and CD3ζ. The therapy is indicated to treat acute lymphoblastic leukemia, the most common cause of cancer-related deaths among children in the USA age 25 or younger [36].

The second FDA-approved CAR T therapy is Axicabtagene ciloleucel (Yescarta™), developed by Kite, a Gilead Science, Inc company, in October 2017. In this therapy, patient-derived T cells are transduced using a gamma-retroviral vector expressing a second-generation CAR that targets CD19. Yescarta is created from CD3+ enriched autologous T cells, while Kymriah is generated from autologous CD4/CD8 T-cell. The therapy works similarly to Kymriah but is indicated for treating adults with certain non-Hodgkin lymphomas, including diffuse large B-cell lymphoma [37].

The third FDA-approved CAR T therapy is brexucabta- gene autoleucel (Tecartus), on July 24, 2020, developed by Kite Pharma to treat relapsed or refractory (R/R) mantle cell lymphoma (MCL), which is a form of non-Hodgkin lymphoma occurring in cells from the 'mantle' zone of the lymph node. It is aggressive cancer that primarily affects men 60 years and over. Tecartus is similar to Yescarta in generation and CAR structure. It is the first and only CAR-T cell therapy for adult patients suffering from R/R mantle cell lymphoma [38].

In February 2021, the FDA approved the fourth CAR T therapy, Lisocabtagene maraleucel (Breyanzi®; Bristol Myers Squibb). Breyanzi® is indicated for adult patients with relapsed or refractory large B-cell lymphoma, including diffuse large B-cell lymphoma (DLBCL) not otherwise specified (including DLBCL arising from indolent lymphoma), high-grade B-cell lymphoma, primary mediastinal large B-cell lymphoma, and follicular lymphoma grade 3B after two or more lines of systemic therapy.

However, these treatments caused two potentially fatal side effects: neurologic toxicity and cytokine release syndrome (CRS). CRS occurred in 94% of patients; 13% experienced symptoms that required aggressive treatment or were considered life-threatening in the phase II ZUMA-1 trial [11,39].

Recently, in March 2021, FDA approved the first B-cell maturation agent (BCMA)-directed CAR T cell therapy, idecabtagene vicleucel (Abecma®) developed by Bristol Myers Squibb. It is indicated for relapsed or refractory multiple myeloma treatment after four or more prior lines of therapy [40]. BCMA is a member of the tumor necrosis factor superfamily and only expressed by some B cells, normal plasma cells, and malignant plasma cells and not expressed by hematopoietic stem cells and normal essential non-hematopoietic tissues [41].

Ongoing hematological malignancies clinical trials. Currently, numerous trials used CAR T cells against different hematological malignancies: A Phase I clinical trial (NCT03778346) against Refractory/Recurrent Multiple Myeloma using BCMA-7x19 CAR T cells by Wenzhou Medical University. The CAR T cell targets BCMA antigens and expresses IL-7 and CCL19. This design provides superior T cells differentiation, migration, expansion, and tumor killing. Both patients enrolled achieved complete remission (CR) and very good partial response (VGPR) with a response of over 12 months. Side effects included Grade 1 cytokine release syndrome one month after the first infusion. A Phase II clinical trial evaluated the efficacy and safety of anti-CD19 CAR T cells alone or in combination with anti-B cell maturation antigen CAR T cells therapy against relapsed/refractory multiple myeloma. The disease targeted immunoglobulin D (IgD) multiple myeloma, a rare subtype with a worse prognosis. A total of 7 patients enrolled in the trial. Six achieved stringent complete remissions (CR), and one with extracellular disease achieved minimal response (MR) 60 days after the first infusion.

Clinical trials conducted by Kite Pharma, Inc., the developers of Yescarta™, are currently underway to demonstrate safety and clinical benefits to patients with R/R Indolent Non-Hodgkin Lymphoma (iNHL). ZUMA-5 is a Phase II multicenter trial in which participants receive an infusion of axi-cel CAR T cells ($2x10^6$ cells/kg). The participants included 124 patients with follicular lymphoma (FL) and 22 with marginal zone lymphoma (MZL). Out of the evaluated 104 patients, the ORR was 92%, with a CR of 76% after a 17.5-month follow-up. FL patients (n=84) responded with an ORR of 94% and CR of 80% compared to the MZL patients (n=20) with 85% ORR and a 60% CR.

Three different clinical trials ELIANA (NCT02435849), ENSIGN (NCT02228096), and B2101J (NCT01626495), tested Kymriah™ (Novartis Pharmaceuticals Corp.) in CD19-positive R/R B cell acute lymphoblastic leukemia. The patients of all three trials experienced a minimum of 69-95% overall remission rates (ORR) with durable remission. A Phase I clinical trial using m971 anti-CD22 CAR T cells targeting R/R B-cell ALL patients previously received an infusion of CD19 CAR T cells. Even though CD19 CAR T has impressive results treating ALL patients, some patients relapse. The trial consisted of two cohorts of patients with R/R Large B cell lymphoma (n=9) and patients with R/R B-cell ALL (n=6) that undergo allogeneic hematopoietic stem cell trans- plant. Patients that experienced R/R Large B cell lymphoma received an infusion of 1x106 (n=3) and 3x106 cells/kg (n=6), while all R/R B-cell ALL received $1x10^6$ cells/kg. Large B cell lymphoma patients experienced ORR of 78% and CR of 56%. Five of the R/R B-cell ALL patients were minimal disease negative in the 28 days, while all subjects except one experienced relapse. Flow cytometry analysis showed that ALL patients downregulate CD22, promoting relapse.

1.7 CAR T cells in solid tumors

T cell therapy's potential to induce successful immunological responses in patients with solid tumors has been demonstrated in immune checkpoint therapy [42] and TIL and TCR therapies in melanoma, sarcoma, cholangiocarcinoma, and breast cancer in a few patients [43], suggesting T cells can eliminate solid tumors under adequate condition. However, few CAR T cell therapy attempts have been reported in glioblastoma and neuroblastoma [44,45]. The Key challenges posed to CAR T cell therapy success in solid tumors can be described in three steps: finding, entering, and surviving in the tumor. These challenges include the lack of tumor-specific target antigens and tumor cell heterogeneity, CAR T cell trafficking/infiltration towards tumor sites, T cell inhibitory signals in solid tumors, physical barriers in the solid tumor microenvironment, and the immunosuppressive microenvironment [26,46,47].

Antigen selection and heterogeneity in solid tumors. Target selection in solid tumors is a major hurdle in implementing CAR T-cell therapy against solid tumors. Also, in contrast with hematological malignancies, where the surface antigen expression is uniform and intense, solid tumor cells rarely express uniformly one specific antigen, and even when present, the levels may be quite variable [47]. The antigen is also more common to be enriched on tumors and at low levels

on healthy tissues, increasing the potential risk of significant on-target off-tumor toxicity. Almost all currently targeted TAAs for solid tumors display this heterogeneity, including CEA, ERBB2, EGFR, GD2, mesothelin, MUC1, and PSMA. The lack of antigen specificity and the acceptance of low levels of the target antigen on normal tissues have led to a number of catastrophic events. A patient with metastatic colon cancer died after receiving an infusion of CAR T cells targeted to the HER2 (ERBB2) antigen [48]. Another patient died from encephalitis when infused with a high-affinity anti-GD2 CAR for neuroblastoma [49]. CAR targets used for the treatment of solid malignancies include:

Prostate-specific membrane antigen (PSMA). PSMA is a Glutamate carboxypeptidase 2, a type II membrane protein highly expressed on most prostate-cancer cells and tumor-associated neovasculature of numerous solid tumors [50].

Mesothelin (MSLN). MSLN is a protein present in malignant pleural mesothelioma, ovarian, pancreatic, and lung cancers. Also, mesothelin is expressed on non-transformed peritoneal, pleural and pericardial mesothelial cells [51].

Fibroblast activation protein-α (FAP). FAP is a type-II transmembrane serine protease expressed almost exclusively in pathological conditions including fibrosis, arthritis, and cancer, where explicitly expressed on cancer-associated stromal cells present in epithelial cancers [52].

Epidermal growth factor receptor (EGFR). EGFR is a transmembrane protein that serves as receptors for numerous epidermal growth factor families of extracellular protein ligands. Different human tumors, including non-small cell lung cancer (NSCLC), breast, head, neck, gastric, colorectal, esophageal, prostate, bladder, renal, pancreatic, and ovarian cancers, express EGFR. EGFR signaling causes increased proliferation, decreased apoptosis, and enhanced tumor cell motility and neo-angiogenesis.

Carcinoembryonic antigen (CEA). CLA are glycosylphosphatidylinositol (GPI) cell-surface-anchored glycoproteins, characterized as members of the CD66 cluster of differentiation. These proteins serve as functional colon carcinoma L-selectin and E-selectin ligands [53]. Currently, CEA-targeted CAR T cell is used to treat patients with liver metastases that are positive for CEA expression.

The human epidermal (HER2). HER2 is a receptor tyrosine-protein kinase member of the human epidermal growth factor receptor (HER/EGFR/ERBB) family. HER2 is expressed on

epithelial cells in the gastrointestinal, respiratory, reproductive, and urinary tract, and it is amplification or over-expression on breast cancer denote aggressive types of breast cancer [54].

CAR T trafficking in solid tumors. In hematological malignancies, infused CAR T Cells and tumor cells co-circulate in the blood and have a higher propensity to migrate to similar areas such as bone marrow and lymph nodes. On the other hand, CAR T cells in solid tumors encounter a number of hurdles, including difficulty migrating to and adequately penetrating the tumor, binding to receptors, and completing their cyto- toxic function. Chemokines, such as CXCL12 and CXCL5, secreted by the tumor inhibit T-cell migration into the tumor. In some instances, the chemokine receptors on T cells do not adequately match the tumors' chemokine signature, resulting in little migration to the tumor site. For example, it has been shown that T cells genetically modified to express CXCR2 migrate towards tumor cells expressing CXCL1. Chemokines secreted by the tumor's stroma, the chemokine repertoire in the tumor location, and the local 'normal' cytokine milieu also affect the CAR T cell mov'ment and migration. Furthermore, solid tumor stroma sends chemokines signals that mismatch the chemokine-receptors on T cells' surface, resulting in dysregulation and cancer progression [55].

T cell inhibitory signals in solid tumors. Endogenous suppressive signal and their upregulation reduce CAR T cells' therapeutic ability. Intrinsic inhibitory T cells and upregulation inhibitory receptors CTLA-4/PD-1 may cause T cell exhaustion and prevent T cell persistence by interacting with ligands overexpressed on tumor cells.

Physical barriers in the solid tumor microenvironment. Physical barriers generated by excessive tumor-stromal density favors tumor progression and aggressiveness. The physical barriers that affect CAR T cell function in solid tumors include:

Hypoxia. Abnormal vascularization and rapidly growing tumor cells limit the amount of oxygen (hypoxia) in the tumor. Hypoxia impacts CAR T cells' attributes by decreasing CAR T cells' expansion ability, blocking their differentiation into effector memory cells, and enriching the cultures with T cells with a central memory cell phenotype [56]. Also, abnormal hypoxia-derived tumor vessels affect T cell adhesion and extravasation towards the solid tumor. Additionally, abnormalities of blood vessels, known as high endothelial venules (HEV), compromise immune cell trafficking to the tumor [47,57].

Extracellular matrix. Peritumoral extracellular matrix (ECM) collagen fibers limit T cell access to tumors, and it is known that tumors with high collagen density present lower levels of infiltrating T cells.

Tumor vasculature. The tumor's core exhibits immature vessel formation, leading to low permeability [46].

Fibroblasts. Other non-immune cells that enhance tumorigenesis are stromal cells, such as cancer-associated fibroblast (CAF) [47]. The cells are involved in the secretion of pro-tumorigenic molecules contributing to tumor vasculature and anti-inflammatory reaction to immune cells [47,57]. In addition, fibroblast differentiation can express activation makers that support matrix degradation and remodeling [46].

Tumor microenvironment. The immunosuppressive nature of the tumor microenvironment plays an essential role in tumor survival, metastatic progression, and influences immunotherapies' outcomes [57]. Numerous suppressive immune cells and molecular factors in the tumor microenvironment can block CAR T cell's antitumor immune function. These immune cells include immune suppressor cells, such as Tregs, myeloid-derived suppressor cells, and tumor-associated macrophages. In contrast, molecular factors include cytokines and soluble factors associated with immunosuppression, such as TGF-β and IL-10, promoting T cell anergy by indirect contact. Another factor known to condition the antitumor effect of T cells in solid tumors is soluble factors such as trans- forming growth factor B (TGF-β) and vascular endothelial growth factors (VEGF) secreted mainly by stromal and tumor cells [47]. TGF-β can also be secreted by regulatory T cells (Tregs), platelets, macrophages, and fibroblasts to suppress T cell proliferation and effect function [25]. Evidence suggests that it promotes Treg maturation and modulate CD8+ effector cell function [26,58].

CAR T solid tumors trials. The accomplishments surrounding CAR T-cell-based therapies hinge on their success in hematological diseases; however, for the reasons mentioned above, much work is needed to sure their success in solid tumors [59].

The CAR T cells' persistence in the stromal micro-environment was the main setback in two clinical trials targeting neuroblastoma and ovarian tumors. Neuroblastoma CARs were generated with the use of EBV-specific cytotoxic T lymphocytes (EBV-CTLs) and activated T cells (ATCs) targeting GD2 (45). Although both engineered T cells were found to circulate the

system at higher concentrations demonstrating improved functionality for CAR-T cell therapy purposes, only three out of eleven patients with active disease completed remission [45].

Furthermore, few clinical trials used CAR T-EGFR to treat biliary tract cancers (BTC), cholangiocarcinomas, and gall- bladder carcinomas that express EGFR. The results reported that out of 19 patients, one achieved complete remission and ten stable diseases, concluding that CAR T-EGFR treatment was a safe and promising strategy for EGFR-positive advanced biliary tract cancers [60]. Also, trials targeted carcinoembryonic antigens (CEA), utilizing CAR T-CEA. CEA is overexpressed In lung, gastrointestinal, and breast cancers and is used as a tumor marker for cancer patients' diagnosis and prognosis [61]. In this Phase I trial, a total of 8 patients with CEA-positive liver metastases were included, of which 4 have more than ten metastatic foci in the liver. Patients received treatment with anti-CEA CAR T cells via hepatic arterial infusions. In addition to CAR T cell infusion, half of the patients received IL-2 cytokine. The trial results indicated that patients experienced no fatal side effects or adverse unpredictable outcomes and that patients tolerated very well the anti-CEA CAR T therapy with or without IL2 administration [62].

1.8 Animal models

Preclinical animal testing requires using a relevant animal model that truly represents the human disease and can elicit a biological response similar to what would happen in humans. However, the preclinical model used in testing the safety and efficacy of CAR T cell therapy fell short to adhere to the standard due to variability in cross-species reactivity to non-human target antigens and, therefore, difficult to identify potential adverse events in humans and often offer a false sense of safety.

Rodent models. Before testing new therapeutic approaches in human patients for clinical trial purposes, safety and efficacy are usually assessed pre-clinically in animal models such as mice, zebrafish, among others. Rodent models have been critical for understanding pathways, identifying tumor-target antigens, and understanding the tumor physiology and the microenvironment [63]. However, despite rodent models' role in preclinical trials, which led to numerous breakthroughs in modern medicine, it has a number of limitations. For example, among drugs that showed strong efficacy and inhibited tumor growth in mice, only 11% are approved for human use by FDA. Furthermore, side effects seen in humans were not observed in mice [64].

Also, rodent models do not appropriately portray the complex microenvironment relationship between the immune cells and tumor cells [65]. These animals do not develop spontaneous tumors. Their living condition, which is pathogen-free, impacts their immune system flora [64]. Thus, rodents do not produce 'normal' immune cell lines found in humans or animals exposed to natural environments, resulting in the same immune milieu between them and identical gene sequence composition. Therefore, studies using animals with none functioning immune systems have limited translational impact. In the case of toxicities involving immune system signaling, brain swelling after CAR T cells therapy is not detectable in studies using immunodeficient mice. All these mentioned factors make rodent models less trustworthy and raise questions regarding whether their contribution is sufficient to use them as preclinical models.

Non-human primate model. Of all the animal models mentioned, the one that more accurately resembles the human genetic composition are the non-human primates. Although similar, these models are not adequate for comparative studies since they experience low spontaneous cancer rates (64), high maintenance, and ethical regulation surrounding these models. Taraseviciute et al studied how neurotoxicities can affect the non-primate model, rhesus macaque, after transferring autologous CD20-specific CAR T cells. The group demonstrated that CD20 CAR and non-CAR T cells infiltrate and accumulate in the cerebrospinal fluid (CSF) and brain parenchyma, causing high levels of proinflammatory cytokines in the CSF and pan-encephalitis [66].

Canine model. Unlike the rodent models, dogs develop spontaneous tumors that resemble human disease in morphology, molecular aspects, and genetic behavior [67]. Also, dogs have intact immune systems with considerable similarities to humans' immune milieu because dogs and humans cohabitate in the same household, therefore, sharing the same environ- mental risk factors [64]. Furthermore, the genetic diversity displayed by different dog breeds provides an ideal tool that enriches the preclinical studies by providing similar challenges seen in humans' studies from different ethnic groups. Also, cancer is the number one cause of death in dogs [63]. All hematological malignancies and solid tumors in dogs are similar to human diseases. These included mammary tumors (breast), osteosarcoma, prostate, bladder cancer, and leukemia.

Canine mammary tumors. Studies revealed that spontaneous invasive mammary carcinomas are closely similar in pathology, epidemiology, and immunohistochemical characterization with human breast cancers [68]. Commonly overexpressed estrogen and progesterone hormone receptors, the conglomeration of similar tumor-infiltrating lymphocyte

ratios, and homologous cancer risk factors such as obesity and age are similar between humans and canines' tumors [64,69].Clinical outcomes after tumor progression are closely related to these two species. Furthermore, molecular markers such as the nuclear protein Ki-67, the p53 tumor suppressor gene, and the BCRA genes provide valuable information regarding both species' prognosis status [70]. Clinical trials using canine CAR T therapy in canine mammary tumors are not initiated yet. However, CAR T cell therapies' benefits in humans breast cancer have been explored over the last years. The following trials are ongoing and centered on improving the safe dose and uncovering the different effects (good and bad). Phase I trials are ongoing targeting HER2+ breast cancer (NCT04650451 and NCT03740256) in patients with advanced-stage III (NCT04650451) or metastatic (stage IV) (NCT04650451 and NCT03740256) cancer with no other treatment option available using BPX-603 and HER2 specific CAR T cells, respectively. City of Hope Medical Center conducted a trial using HER2 specific CAR T cells targeting HER2+ breast cancer cells (NCT03696030) in patients with brain or leptomeningeal metastases. Two trials (NCT02414269 and NCT02792114) at the Memorial Sloan Kettering Cancer Center are ongoing targeting Mesothelin in patients with metastatic (stage IV) breast cancer that spread to the pleura (iCasp9M28z CAR T-cells-Phase I/II) and HER2-cells (Mesothelin CAR T cells-Phase I), respectively. Tmunity Therapeutics using CAR T-TnMUC1 (NCT04025216) in patients with triple-negative and ER-low, HER2-breast cancer with TnMUC1 positive cells. Minerva Biotechnology Corporation conducts a trial targeting MUC1* (NCT04020575) utilizing huMNC2-CAR44 CAR T cells in patients with metastatic (stage IV) breast cancer. Fred Hutchinson Cancer Research Center conducts a phase I trial on triple-negative and ER-low breast cancer (NCT02706392), targeting ROR1 positive cells. Lastly, patients that received a minimum of two therapies for advanced cancer expressing GD2 antigen are carried on by Baylor's College of Medicine (NCT03635632) using a C7R-GD2 CAR T cell.

Canine osteosarcoma. Canine develops osteosarcoma (OSA) at a much higher rate than humans [71], serving as a remarkable model for developing treatments and overcoming the numerous challenges in solid tumor therapies. There are a number of similarities between the canine and humans concerning this disease. The tumor location, the pattern of metastasis, genetic drivers of the disease, and response to therapy are similar in both species. Canine OSA is a spontaneous, naturally occur- ring disease as in humans. Canine OSA has aggressive biology and an increased rate of metastasis, and the animal often dies within six months, and almost 96% of dogs with OSA

perish from the disease. Canine trials or in-vitro experiments related to osteosarcoma are scarce in the literature. Mata et al [65] developed a CAR T cell targeting HER2 overexpressing tumor cells in-vitro. Canine and human-derived transmembrane and signaling domains were tested on tumor cells, demonstrating little to no difference in tumor suppression [65] On the other hand, Baylor College of Medicine is conducting a Phase I clinical trial (NCT03635632) in human patients with relapsed or refractory osteosarcoma with increased expression of GD2 antigen utilizing C7R-GD2 CAR T cells. The National Cancer Institute (NCI) has completed a Phase I clinical targeting GD2 positive solid tumors with anti-GD2 CAR T cells in children and young adults that suffer osteosarcoma (NCT02107963), no final data has been posted yet.

Canine prostate cancer. Canines are a few animal models that develop spontaneous prostate cancer as humans [72,73]. Both dogs and humans share similar risk factors, including advanced age, low mortality rates, clinical outcomes, and prostate gland functionality, suggesting that these animals may be ideal models for future clinical trials [72–74]. Unfortunately, a lack of prostate cancer screening in canine augments the malignancy's mortality rate and aggressiveness, thus not allowing proper treatment strategies [74,75]. On the other hand, human screening methods have strengthened over the last few years, enabling rapid diagnosis [76]. ACT therapy for prostate cancer has been developed mainly in humans. CAR T cells against TCRγ chain alternative reading frame protein (TARP), prostate stem cell antigen (PSCA), and prostate-specific membrane antigen (PSMA) were developed and used to suppress tumor growth in vitro [77–80]. Phase I clinical trials are currently conducted in patients with castrate-resistance prostate cancer targeting PSMA with doses of CART-PSMA-TGFβRDN, LIGHT-PSMA CAR T P-PSMA-101 CART cells (NCT03089203, NCT04053062, and NCT04249947). The City of Hope Medical Center conducted another trial against metastatic castration-resistance prostate cancer, targeting the PSCA antigen's overexpression with anti-PSCA-4-1BB/TCRζ-CD19 CART cells (NCT03873805). Phase I/II clinical trial (NCT02744287), sponsored by Bellicium Pharmaceuticals, PSCA-CART (BPX-601), is currently used to treat patients with previously treated advanced tumors, including metastatic prostate and metastatic castrate-resistance prostate cancer. Finally, the First Affiliated Hospital of Chengdu Medical College targeted EpCAM positive prostate cancer with an EpCAM-specific CAR T cell (NCT03013712), a second-generation CAR (CD28/CD3ζ) targeting PSMA.

Canine bladder cancer. Invasive Urinary bladder cancer (InvUC), Invasive transitional cell carcinoma (TCC), and invasive urothelial carcinoma (UC) are three different subtypes of bladder cancer spontaneously developed in canines that resemble 'humans' malignancies [79,81,82]. Similarities in clinical outcomes, histological features, and progression sites make canines straightforward compared to humans (79). Canine trials or CAR T generations are not seen in literature, but human clinical trials are currently under investigation. A Phase I/II clinical trial, conducted by Shenzen Geno-Immune Medical Institute (NCT03185468), is currently evaluating the safety and efficacy of a 4SCART-PSMA CART cell against PSMA-expressing bladder cancer.

Canine leukemias. As mentioned above, preclinical trials driven with canine models could represent an enormous step in adoptive T cell therapy development. Unfortunately, preclinical trials using canine models are scarce in the scientific literature. The few clinical trials available are primarily performed in B cell lymphomas. Panjwani conducted a trial in patients with B cell lymphomas, targeting the CD20 antigen. The study concluded the need for stable CAR T cell expression and that further studies must be performed [83]. Nonetheless, the second trial showed stable CAR T transduction using lentiviral vectors [84]. Their CD20-BB-ζ CAR T cell, alongside cyto- kines IL7 and IL5, proved to be durable and antigen-specific against DLBCL. Non-Hodgkin's Lymphoma (NHL) is the most common cancer in dogs, and the most common sub-type is Diffuse Large B-Cell Lymphoma (DLBCL). While combi- nations of chemotherapy agents lead to clinical remission in ~75% of dogs, most dogs relapse within six to nine months of standard treatment, a statistic that has remained unchanged for the past 30 years. An urgent need exists for new therapies for canine lymphoma. Furthermore, evaluating these new therapies in pet dogs with naturally occurring cancer may also provide vital information to advance novel therapies for individuals.

1.9 Conclusions

The remarkable progress that adoptive immunotherapy has experienced these past years, especially in blood-related cancers, provides optimism for CARs therapy. Trials of CAR T in leukemia and lymphomas had shown positive outcomes, with some cases experiencing mild side effects. Notwithstanding, trials conducted in solid tumors represent a daunting task to achieve. Tumor microenvironment, CARs tracking and duration, and the various toxicities experienced by a number of patients represent significant setbacks that need addressing. The animal model that

faithfully resembles humans is another milestone in this endeavor. Up to date, all preclinical studies of CAR T safety and efficacy are conducted in mice, including syngeneic, transgenic, and xenograft, and humanized mouse models to represent humans' immune responses and diseases to test the safety and efficacy of CART therapy. However, these models fell short in representing the disease and its adverse effect. The dog's importance is recently recognized as a preclinical model for cancer CAR T therapy because of its human physiology, immune responses, and disease similarities. The development of reagents and the use of the dogs in clinical trials will help advance the CAR T therapy field for both species.

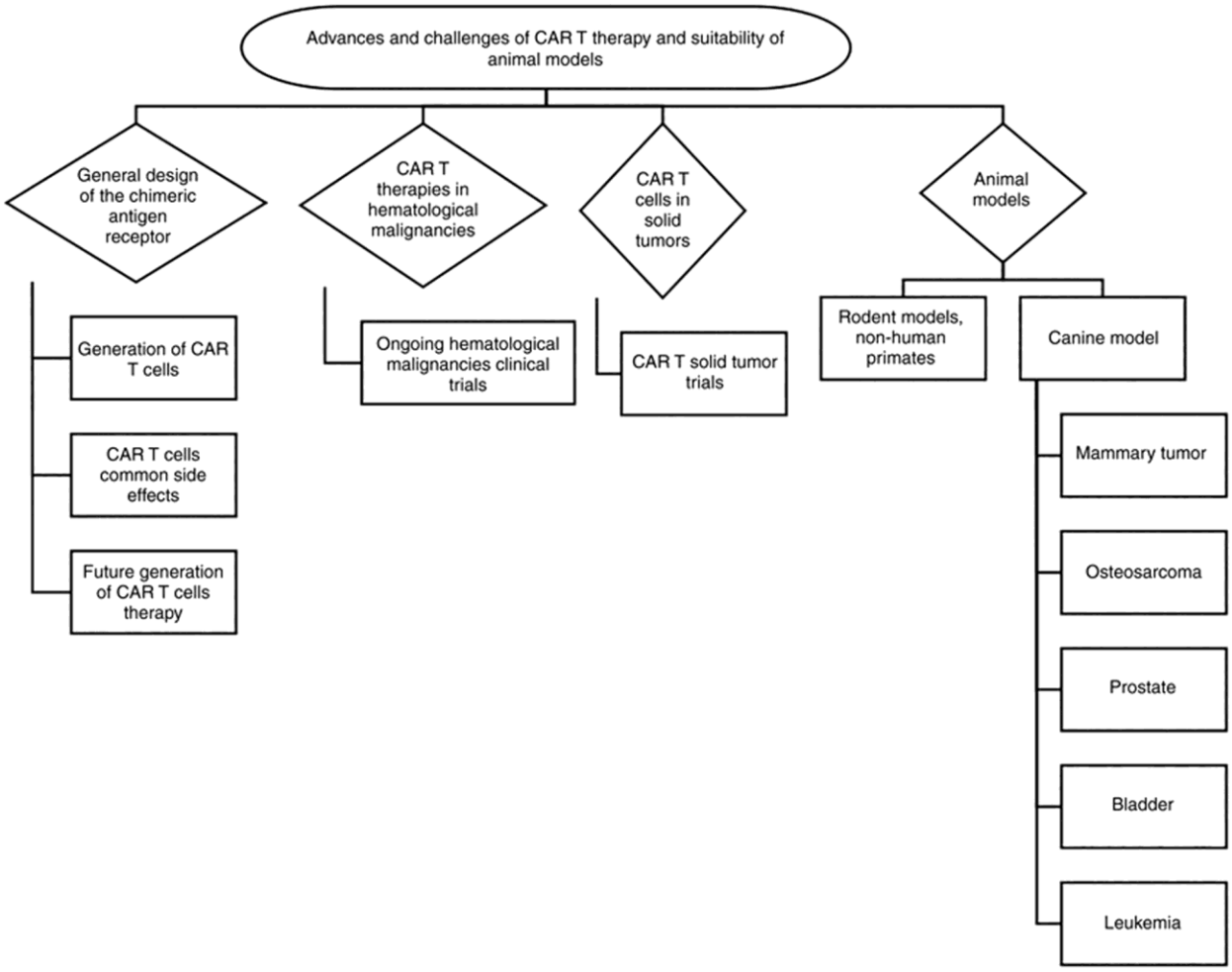

Figure 1.1. Schematic diagram of the advances and challenges of CAR T cell therapy in animal models. General design of the chimeric antigen receptor, trials and differences between hematological malignancies and solid tumors are discussed in the present review paper. In addition, comparison between different animals used as pre-clinical models are discussed presenting their potential translational impact in CAR T cell development. CAR, chimeric antigen receptor.

CHAPTER 2. GENERATION OF ANTI-FITC CHIMERIC ANTIGEN RECEPTOR ON CANINE T CELLS

2.1 Introduction

Chimeric antigen receptor (CAR) T cell therapy is a type of adoptive cell therapy that utilizes the patient's own T lymphocytes to treat cancer. The lymphocytes are separated from the blood, genetically engineered, and infused back into the patient. The CAR T cells expresses a receptor specific to its target antigen, improving the ability to attack and kill cancer cells. Genetic modifications of T lymphocytes are commonly performed via viral transduction with lentivirus or retrovirus. CAR T cell therapy has shown significant progress in treating relapsed/refractory B cell malignancies such as acute lymphoblastic leukemia (ALL) and diffused-large B cell lymphoma (DLBCL) in children and young adults. Currently, there are five FDA Cluster of Differentiation 19 (CD19) specific CAR T cell therapies for these diseases.

Despite this success, CAR T cell therapy has failed to produce similar beneficial outcomes when treating tumor malignancies. Rodent models are used as the preclinical animal model for this immunotherapy, and although they have been critical for understanding pathways and tumor physiology, they do not appropriately portray human clinical outcomes. When treating solid tumors, there are several barriers to overcome that includes: identifying an ideal tumor-associated antigen to target in a heterogeneous tumor, addressing the tumor-suppressive microenvironment, and the incapability to terminate or gradually inactivate CAR T cell killing. This preclinical animal model restricts translational research between animal and human patients due to their limited immunological and genetic resemblance.

As a solution to these limitations and challenges, we propose to use canines as the preclinical animal model. Dogs develop spontaneous tumors that resemble human diseases in morphology, molecular aspects, genetic behavior, and treatment regimens/clinical outcomes. Additionally, dogs have intact immune systems with considerable similarities to humans' immune milieu. The genetic diversity displayed by different dog breeds provides an ideal tool that enriches preclinical studies by providing similar challenges seen in human studies from different ethnic groups. In addition to the dog as a preclinical model for this therapy, we propose using a universal CAR T cell to overcome the antigenically heterogeneous tumor suppressive microenvironment and potentially control the immediate killing of the CAR T cells. The universal CAR T cell is a

second-generation CAR construct that consists of a leader sequence, hinge, and transmembrane sequence of canine CD8α and the stimulatory domains from canine 4-1BB and CD3ζ. The antibody-based binding domain exclusively recognizes fluorescein (FITC), thus being an anti-fluorescein CAR T cell. Our idea is to use this anti-FITC CAR T cell with a cocktail of low-molecular-weight bispecific adapters, each made of fluorescein linked to a different specific tumor-associated antigen of interest, that serves as a bridge between the CAR T cells and the cancer cell. This bispecific adapter cocktail permits targeting multiple antigens with the use of one universal CAR T cell, providing the opportunity to reach several antigens in a heterogeneous tumor. Tumor cell killing can only be achieved in the presence of the bispecific molecule designed to last 20 minutes on the patient's body, controlling potential side effects related to the gradual inactivation of the cells.

In this study, we established a suitable CAR T cell generation system for preclinical testing in canine models. In order to do so, activation of T lymphocytes is essential for the expansion and proliferation of the CAR T cells. To determine the optimal activation method, activation between artificial antigen-presenting cells (APC), artificial antigen-presenting cells knock-down (APC-KO), anti-canine CD3/CD28 magnetic beads, and plate-bound anti-CD3 will be compared. Canine T cells will be transduced using a VSV-G lentiviral pseudotype and CAR-mediated functionality will be evaluated *in-vitro*.

2.2 Material and Methods

2.2.1 Cell lines and culture conditions:

Canine breast cancer cell lines composed of mammary carcinoma P114 and CF41 were purchased from (ATCC, Manassas, Virginia). Canine prostate carcinoma (TR5P) was kindly provided by Dr. Knapp from Purdue University, and the B cell lines CLBL-1,17-71 and GL-1 were kindly supplied by Dr. Stuter from North Carolina State University. Human oral squamous carcinoma, KB, and the human embryonic kidney, HEK 293 T, cell lines were kindly provided by Phillip Low from Purdue University. Human bladder cell lines HT1376 and UMUC3 were purchased from ATCC. We have stably transduced the gene-modified K562 (purchased from ATCC, Manassas, Virginia) expressing human CD80, CD86, CD137L, and CD64 with a self-inactivating lentiviral vector in our laboratory. The artificial antigen-presenting cell expressing

human CD3 and CD28 with disrupted endogenous expression of the low-density lipoprotein receptor (aAPC-ΔLDLR) was kindly provided by Dr. Maus. FRα-expressing cells KB and P114 were cultured in folic acid-free Rosewell Park Memorial Institute (RMPI) 1640 (Thermo Fisher Scientific Gibco, Waltham Massachusetts) containing 10% heat-inactivated FBS (Cytiva, Marlborough, Massachusetts) and 1% penicillin-streptomycin (Thermo Fisher Scientific Gibco, Waltham Massachusetts) and cultured at 37°C in 5% CO2. Human bladder cancer cell lines were maintained in Eagle's Minimum Essential Medium (EMEM) (ATCC, Mannasas, Virginia) containing 10% heat-inactivated FBS (Cytiva, Marlborough, Massachusetts) and 1% penicillin-streptomycin (Thermo Fisher Scientific Gibco, Waltham Massachusetts) and cultured at 37°C in 5% CO2. CF41 and HEK 293T were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Thermo Fisher Scientific Gibco, Waltham Massachusetts) containing 10% heat-inactivated FBS (Cytiva, Marlborough, Massachusetts) and 1% penicillin-streptomycin (Thermo Fisher Scientific Gibco, Waltham Massachusetts) and cultured at 37C in 5% CO2. Canine prostate TR5P, canine leukemias and K562-APC cell lines were maintained in Rosewell Park Memorial Institute (RMPI) 1640 (Thermo Fisher Scientific Gibco, Waltham Massachusetts) containing 10% heat-inactivated FBS (Cytiva, Marlborough, Massachusetts) and 1% penicillin-streptomycin (Thermo Fisher Scientific Gibco, Waltham Massachusetts) and cultured at 37C in 5% CO2.

2.2.2 Generation of artificial antigen-presenting cell (APC):

Human cDNA molecules were amplified: CD86 (GenBank accession no. NM_175862.4), CD80 (GenBank accession no. NM_005191.3), CD137L (GenBank accession no. NM_003811.3) and CD64 (GenBank accession no. NM_000566.3) were cloned individually into a self-inactivated vector, pLV. Gene expression of all the vectors controlled by the cytomegalovirus (CMV) promoter. Lentivirus particles were generated by transient transfection of 293 T cells with the different expression vectors and pPACK packaging plasmid mix (System Biosciences, Palo Alto, California), providing gag/pol and VSV-G. CD86-pLV, CD80-pLV, CD137L-pLV, and CD64-pLV lentiviral vectors were used simultaneously for transduction of the parental K562 cell. Transduction efficiency was examined by flow cytometry.

2.2.3 Generation of anti-canine CD3/CD28 magnetic beads:

Agonistic mouse anti-canine CD3 (BioRad, Hercules, California) clone CA17.2A12 and mouse anti-canine CD28 (Thermo Fisher Scientific Invitrogen, Waltham, Massachusetts) clone 1C6 were coupled to magnetic tosylactivated Dynabeads (Thermo Fisher Scientific Invitrogen, Waltham Massachusetts) following the manufacturer protocol, briefly described elsewhere 84. 10^8 beads were incubated with 50µg of antibodies (25µg CD3 and 25µg CD28) for 24hrs at room temperature in 0.1 M sodium phosphate buffer, pH 7.4 (Buffer 2) in the presence of 0.01% bovine serum albumin to avoid non-specific binding. Then, the beads were incubated for 4hrs at 37C in 0.2 M Tris w/0.1% BSA, pH 8.5 (Buffer 3) to deactivate free tosyl groups. Conjugated anti-canine CD3/CD28 beads were stored in Buffer 2 at a density of $4x10^8$ beads/ml at 4C.

2.2.4 Anti-canine CD3 plate-bound coated well:

Agonistic mouse anti-canine CD3 (BioRad, Hercules, California) clone CA17.2A12 was coupled to a 48-well plate tissue culture plate (Cell treat, Pepperell, Massachussets) by incubating for 2hrs 5ug CD3 with 250ul of PBS. Wash 3 times with PBS after the incubation period.

2.2.5 PBMC isolation and T cell culture, expansion, and activation:

Canine whole blood was obtained from healthy client-owned canines treated at Purdue University College of Veterinary Medicine on an institutionally approved protocol. Peripheral blood mononuclear cells (PBMCs) were isolated over Lymphoprep discontinuous density gradient centrifugation (StemCell Technology, Vancouver, Canada) containing TexMACS medium (Militenyi) 10% canine beagle serum (Cytiva, Marlborough, Massachusetts) and 100 U/ml penicillin and 100ug/ml streptomycin (Thermo Fisher Scientific Gibco, Waltham Massachusetts), filtered by through a 0.22um filter Stericup (Thermo Fisher Scientific Gibco, Waltham Massachusetts).

PBMCs were enumerated by hemocytometer using trypan blue exclusion (Corning, New York) and plated on 48-well plates (Cell treat, Pepperell, Massachusetts) at $1x10^6$ cells/mL and incubated with its specific activation method at 37°C, 5% CO2 and 95% humidity. Before using the APC-K562 and APC-K562-KO for lymphocyte activation and further expansion, those cells

were subject to γ-irradiation at 50Gy, washed once with TCM, and used for co-incubation in a 1:2 ratio of K562-APC: PBMCs to achieve a final concentration of $5x10^5$ APC and $1x10^6$ PBMCs per mL with 0.5ug/ml mouse anti-canine CD3 (BioRad, Hercules, California). When the antibody-conjugated CD3/CD28 beads were used for stimulation, the beads were washed twice with Dubelcco's phosphate-buffered saline (DPBS) and once with TCM before co-incubation with PBMCs in 3:1 bead: PBMCs ratio. For activation with plate-bound CD3 antibody, cells were cultured in $1x10^6$ per ml in 48-well plates. Cytokines were used on the day of stimulation and every other day- 100 IU/ml of recombinant human IL-2 (R&D Biosystem, Minneapolis, Minnesota),10ng/ml of recombinant human IL-21 (R&D Biosystem, Minneapolis, Minnesota).

2.2.6 Purification of $CD3^+$ T cells by MACS separation:

Purification of $CD3^+$ T lymphocytes was achieved by MACS separation with anti-FITC microbeads (Miltenyi Biotec Inc., USA) following manufacturer protocol and Cordeiro Guinchetti's lab [85]. I prepared a cell suspension of 3 $x10^7$ PBMCs in a 1mL tube with isolation buffer (PBS 1×, pH 7.2, 0.5% BSA, 2 mM EDTA). Monoclonal mouse anti-canine CD3 (BioRad, Hercules, California) clone CA17.2A12 was added to 2µg/ml of total PBMCs, and incubated at room temperature for 15 min. Then 10µl/ml of the anti-FITC magnetic beads were added and incubated for 15 min at 4C. Finally, the cells were loaded to the MACS®column (Mil-tenyi Biotec Inc., USA), passed through the magnetic field of the MACS®separator and the unlabeled cells ran thorough while the $CD3^+$ cells were retained. After removing the separation column, the cells were eluted by washing with 5mL of isolation buffer. Purity was evaluated by flow cytometry.

2.2.7 RealTime-Glo™ MT Cell Viability Assay:

Gene-modified APC-K562 and APC-K562-KO cells were subject to 10, 50, and 100 Gray radiation. Irradiated K562-APC cells were seeded at a 750 cells/well density in 80ul of complete culture media containing 1 x RealTime-Glo™ reagents (Promega, Madison, Wisconsin) at a 96-well, black-walled, transparent flat bottom tissue culture plate (Costar, Washington D.C) The plate was read at 0hr, 24hr, 48hr and 72hr on a (SpectraMAx i3x, Molecular Devices, San Jose, California) (37°C/5% CO2). All the conditions had triplicates, and the manufacturer's continuous read method: and reagent addition at cell plating recommendations were followed.

2.2.8 Generation and use of lentiviral vector encoding anti-FITC CAR:

The second-generation CAR construct consists of canine CD8α leader, fluorescein (FITC) scFv, canine CD8α hinge and transmembrane region, canine 4-1BB intracellular domain, and CD3ζ intracellular domain. After codon optimization for dogs, the nucleotide sequence was synthesized and cloned to the pCDH-EF1-MCS-(PGK-GFP) lentiviral expression vector (System Bioscience). The sequence was confirmed by DNA sequencing at Purdue Genomic Core Facility. Canine-purified $CD3^+$ T cells were activated for 24 to 48 hours and then infected with the mentioned lentivirus in the presence of 8µg/mL of Polybrene (Santa Cruz Biotechnology, Dallas, Texas) or 1:100 ratio of LentiBoost (Sirion Biotech, Gräfelfing, Germany) as transduction enhancers. After three days post-transduction, T cells were recovered and analyzed by flow cytometry to determine transduction efficiency.

2.2.9 In Vitro Analysis of cytotoxicity of anti-FITC CAR T cells:

LDH assay (Pierce, ThermoFisher Scientific, Waltham, Massachusetts) was used to determine the cytotoxicity of anti-FITC CAR T cells *in vitro* against FAα-expressing cells KB and P114. Briefly, cells were seeded at a density of 5000 cells/100µl and grown overnight in 96-well tissue culture plates (Cell treat, Pepperell, Massachusetts). CAR T cells were added at a 50,000 and 100,000 density in the absence or presence of the bispecific adaptor for 18 to 24 hours. The plates were centrifuged at 350g for 10 minutes, and the supernatants were analyzed.

2.2.10 Flow cytometry:

BD C6 Acurri and Attune Acoustic flow cytometers were used to obtain immunofluorescence data. K562-APC receptor expression was assessed by staining with anti-human CD64-APC clone: 10.1 (Cat. 305013) (BioLegend, San Diego, California), anti-human 4-1BB- PE clone: 5F4 (Cat. 311503) (BioLegend, San Diego, California), anti-human CD80 PE/Cy5 clone: 2D10(Cat. 305209) (BioLegend, San Diego, California) and anti-human CD86- Alexa Fluor 488 clone: IT2.2 (Cat. 305413) (BioLegend, San Diego, California). Canine T and B cell markers surface expression were evaluated by the three-color reagent cocktail: anti-dog pan T cell marker-APC, anti-Dog T cell activation marker- FITC, and anti-B cell marker-PE (BD Bioscience). Positive selection (just in case we mention it) FITC labeled mouse anti-canine CD3: FITC (BioRad,

Hercules, California). CAR efficiency was assessed using a FITC-AlexaFluor488 small molecule kindly provided by Dr. Low [30]. CAIX and PSMA expression on the cell surface was measured by anti-CAIX-FITC and anti-PSMA-FITC provided by Dr. Low. Cell viability was measured by 7-AAD cell viability dye (BioLegend, San Diego, California). All cells collected for antibody staining were washed with PBS, counted to achieve a cell density of $1x10^5$ prior to staining, and stained for 30min in ice. Then, they were washed with cell sorting buffer (PBS containing 2% FBS) twice before analysis.

2.3 Results

2.3.1 Purification of $CD3^+$ T cells

From canine peripheral blood mononuclear cells (PBMC), we obtained high purity levels of the $CD3^+$ subpopulation after positive selection. Approximately 80% (Figure 2.1) of the population was $CD3^+$ expressing T cells following Cordeiro Guinchetti et al. guidelines [85] and the manufacturer protocol. On average, canine PBMC comprises 12.3% granulocytes, 10.2% monocytes, and 59.5% lymphocytes [85]. Purifying these cells from the rest of the PBMCs is essential in assuring optimal activation and transduction efficiency.

2.3.2 Generation of Artificial Antigen Presenting Cell (APC)

In nature, three signals are required for antigen-specific T-cell activation. The first signal comprises antigen presentation by the MHC class molecules recognized by the TCR of the T cell, followed by costimulatory signals mainly triggered by the CD28 receptor interactions with CD80/CD86. The third signal is triggered by cytokines presented to the T cell to promote differentiation [86]. These signals help the T cells expand, proliferate, and differentiate to function in the immune system. For growing cells *in-vitro*, the goal is to mimic these signals to achieve optimal cell activation, thus, proliferation. Standards methodologies to activate canine T cells include using plate-bound canine CD3 antibodies or the mitogenic lectins phytohemagglutinin and Concanavalin (ConA) [83]. These methods can activate and expand cells, but they are not vigorous enough to grow cells on a clinically relevant scale. Therefore, we will use methods commonly used in human T-cell immunotherapies. One popular activation and proliferation method is artificial antigen-presenting cells (APC).

Parental cell line K562 was transduced with four lentiviral pseudotypes that code for the human CD80, CD86, CD137L, and CD64 receptors (Figure 2.2A). CD80 and CD86 are known costimulatory signals that interact with the CD28 receptor on T cells. CD137L engages to CD137 of a T cell and is known to increase T cell survival, proliferation, and cytokine production [87]. Lastly, the CD64 receptor exhibits a high affinity towards Fc receptors, which will recognize the canine anti-CD3 antibody that will trigger activation through the TCR. Single-cell clones were obtained by cell sorting with the BD FACS Aria III Cell Sorter at Purdue University Bindley Bioscience Center (Figure 2.2B).

2.3.3 Irradiation of Artificial Antigen Presenting Cell (APC)

In preparation for co-incubation of the APCs with the canine T cells, irradiation is required. Radiation-induced cell death is necessary since the APCs viability is not our interest. The objective of the irradiated APCs is to interact with the T cells receptors, thus proliferate and differentiate. It is known by others that APC remains post-initial culture for 4-5 days [83,88]. Typically, Gray radiation of cells ranges between 10-100Gy; therefore, with Purdue University's X-RAD320 Precision X-Ray, we irradiated at 10, 50, and 100Gy to investigate the minimal dose needed to achieve mitotic death. RealTime-Glo™ MT Cell Viability Assay was performed to measure the viability of the irradiated cells. The non-irradiated APC exhibited significant luminescence compared to all the irradiated cells independent of the dose (Figure 2.2C).

Additionally, we counted the cells with the Countess 3 Automated Cell Counter using trypan blue. The non-irradiated cells grew from 2.0×10^6 to 7.3×10^7 after 10 days of incubation, while any irradiated APCs showed no significant growth over the same period of incubation (Figure 2.3D). As seen, even the lowest irradiation dose can lethally affect the DNA of the APCs.

2.3.4 Activation and expansion of canine T cells

As mentioned, we will use standard human T cell activation methods to reach clinically relevant numbers of canine T cells. In addition to the cell-based APC-K562 generated in our laboratory, we used the artificial antigen-presenting cell generated by Dr. Maus (APC-K562-KO). The K562 parental cell was genetically engineered to express the T cell stimulation and costimulatory receptors CD3 and CD28 [89]. The third activation method involves conjugating

agonistic anti-canine CD3 and anti-canine CD28 antibodies to magnetic tosylactivated Dynabeads. The fourth activator is the plate-bound agonistic anti-canine CD3 antibody to tissue culture plates. Freshly isolated canine T cells from healthy donors were stimulated with APC-K562, APC-K562-KO, CD3/CD28 beads, plate-bound CD3 with the addition of a non-stimulated group as control.

After 24hr co-incubation of the T cells and the stimulation methods, CD25 activation marker upregulation was measured on the cell surface. $CD3^+$ T cells activated with APC-K562 (75%) and beads (60%) showed significant upregulation in comparison to the non-stimulated control (22%) (Figure 2.3A). After nine days of co-culture, beads (21-fold) activation resulted in more significant T cell division compared to APC-K562 (9.8-fold), APC-KO (4.3-fold), and coated plates (4.7-fold) (Figure 2.3B-C).

2.3.5 Fresh versus frozen T cell activation

Typically, fresh blood is drawn from dogs to isolate T cells. Frozen T cells are an alternative way to save cells for future experiments. Frozen beagle PBMCs are commercially available, which is an excellent option for researchers that do not have the facilities or protocols to withdraw canine blood. Fresh and frozen T cells CD25 frequency was measured after 24hr stimulation with APC-K562 and CD3/CD28 beads (Figure 2.4A). There was no significant difference between fresh and frozen lymphocyte activation after coculture.

2.3.6 Breed variability on T cell activation

To predict variability, we tested how activation can vary when using the two best stimulation methods against canine T cells in different breeds. The breeds compared are golden retriever, beagle, pitbull, and Boston terrier, all from healthy donor dogs. After staining for the frequency of the CD25 activation marker, almost all breeds were responsive to both activation methods (Figure 2.4B). Boston terriers showed no significance when APC-K562 was used as stimuli due to the high CD25 frequency exhibited even with no previous stimulation. This data suggest that these breeds can be activated by CD3/CD28 beads successfully.

2.3.7 Culture plate comparison

Another condition that can affect expansion is the culture plates used for co-incubation. U-shaped plates versus flat-shaped plates using APC-K562 and CD3/CD28 beads as activation methods were compared. Both activation methods showed greater expansion in U-shaped plates than flat plates, with beads once again more significant than APC-K562 (Figure 2.5A). The U-shaped plates augment the interaction between cells and the stimulant.

2.3.8 Recombinant human IL-2 and beagle serum supplementation

With CD3/CD28 beads stimulation, recombinant human IL-2 in combination with different percentages of beagle serum was compared. IL-2 supplementation is essential in the T cells' expansion compared to IL-2 absence (Figure 2.5B) after five days of culture. Higher percentages of serum significantly expand the lymphocytes compared to no serum in the culture media. Lower percentages of serum are common when activating human T cells, but dog T cells need higher percentages, as shown in figure 2.5B.

2.3.9 Design and function of anti-FITC-8-41BB-ζ lentivirus pseudotype

To evaluate canine CAR T cells' immunotherapeutic capacity, a second-generation anti-FITC CAR was designed as described by Yong et al.[30]. The scFv is flanked by CD8α hinge and transmembrane region, 4-1BB, and CD3ζ intracellular domains (Figure 2.6A). This gene was cloned in pCDH-EF1-MCS-(PGK-GFP) vector with VSV-G as the envelope gene [84] (Figure 2.6 B and C). Before utilizing the VSV-G pseudotype on canine T cells, the virus will be titrated on the K562 cell line following Mason et al. protocol and equation [90]. Dilution six was used to measure titration of the viral batch (titer: 3.16 x 105 TU/mL) since transduction efficiencies that range from 1-20% are associated with accurate estimates [91] (Figure 2.7).

2.3.10 Transduction enhancers comparison and donor variability

Polybrene and LentiBoost are two common transduction facilitators for T-cell gene modification. We examined both transduction agents on T cells from three dogs of the same species (beagle) with VSV-G anti-FITC lentivirus pseudotypes with a similar multiplicity of infection (MOI). Polybrene was added at a concentration of 8µg/mL, while LentiBoost was at a 1:100 ratio

(culture total volume: LentiBoost volume) to the T cells-virus suspension incubation. As measured by flow cytometry 72 hours after transduction, Polybrene showed higher transduction rates. Dog 1= 11.45%, Dog 2= 6.89%, and Dog 3= 36.71%, while LentiBoost efficiency was Dog 1= 4.69%, Dog 2= 5.03%, and Dog 3= 15.53% (Figure 2.8). Variability between the different dogs plays a role in the transfection efficiency.

2.3.11 Evaluation of anti-FITC-8-41BB-ζ CAR-T cells functionality *in-vitro*

To determine the ability of CAR-mediated effector functions, we cocultured our anti-FITC CAR T cells with a target cell that expresses the tumor-associated antigen of interest in the presence of the bispecific adaptor that binds to the antigen. This test will also assess the capacity of the bispecific adaptor to engage immunological synapses between the CAR T cell and the target cell [30]. First, we determine the surface expression of folate receptor (FR), prostate-specific membrane antigen (PSMA), and carbonic anhydrase IX (CAIX) in our target cells. The human cell line KB expressed 99.5% of folate receptor, while HT1376 and UMUC3 expressed 11.66% and 35.15% of CAIX, respectively (Table 2.1). Only one canine malignant cell line, P114, overexpressed one of the antigens of interest at a percentage of 40-98%. Next, we cocultured the target (T) cells with the effector (E) cells (CAR T cells) in a 10:1 and 20:1 E: T ratio with different adaptor concentrations. As seen in Figure 2.9A, cancer-killing is enabled by adding the bispecific adaptor compared to the non-conjugated control with a 10:1 E:T ratio. No significant killing was observed with the 20:1 ratio. In Figure 2.9B, both ratios, 10:1 and 20:1, function lysing the cells significantly compared to the non-conjugated control. Both target cell lines were influenced by E:T and bispecific adaptor concentrations. Higher effector: target ratio does not correlate with a higher killing percentage. Higher bispecific adapter concentration can block intracellular bridges on both cell lines due to monovalent saturation, as explained by [30]. The addition of the correct bispecific adaptor, compared to the no-conjugation and wrong bispecific adaptor molecule co-incubation, showed more significant killing (Figure 2.9C). Co-incubation with the wrong bispecific molecule showed effective lysis when compared to no-conjugation. When CAR T cells recognize the FITC portion of the adaptor, modest CAR-mediated killing can occur.

2.4 Discussion

Canine preclinical animal models develop spontaneous tumors resembling morphology, molecular aspects, genetics, and treatment outcomes as human patients [67]. This animal model can potentially facilitate the clinical translation of new immunotherapeutic approaches relevant to the success of CAR T cells in hematological malignancies. We conducted a first-in-species study of universal CAR T cell therapy using low-molecular-weight adapters with canine T cells. We demonstrated successful activation, expansion, transduction, and CAR T cell antigen-specific functionality.

CAR T cell generation depends on *in-vitro* activation and expansion after isolation from the PBMCs. We compared four activation and expansion methods widely used in human and canine T cell cultures. APC-K562 provided the highest activation marker (CD25) percentage after 24 hours of co-incubation (Figure 2.3A). However, the agonistic canine anti-CD3/CD28 beads achieved an average of 21.5-fold expansion after nine days post-stimulation (Figure 2.3C), while APC-K562 had only a 9.8-fold expansion. APC-K562 has three costimulatory molecules (human CD80, CD86, and CD137L) in addition to the fc binding CD64 receptor that can provide various effects through additional uncharacterized surface ligand receptors and secreted factors [83]. After 24 hours of co-incubation with the T cells, these signals elicit a higher activation marker percentage, while the beads, since they are a reductionist system (CD3 and CD28), do not trigger expression of CD25 has the artificial presenting cells. On the other hand, the artificial presenting cells are an irradiated xenogenic coculture; by day six, they die in culture, potentially sending other non-identified signals that can affect the proper expansion of the primary T cells. Contrary to others [65,83], beads showed better T cell expansion, with the additional benefit that they do not serve as a viral sink like the APC-K562 and are easier to remove from culture.

One benefit of using dogs as preclinical models is the genetic diversity that different breeds represent, providing similar challenges seen in human studies from other ethnic groups [92]. In this study, we activated with APC-K562 and beads canine T cells from the golden retriever, beagle, pitbull, and Boston terrier breeds to see if there is variation when activating the cells (Figure 2.4B). Almost all the breeds showed significant expression of the T cell activation marker compared to the non-stimulated control after activation with the two best stimulants, except Boston terriers with APC-K562. The non-stimulating control had higher CD25 expression on the cell surface than the other breeds. Expansion and transduction susceptibility of the T cells from different breeds should

be addressed in future experiments. We also compared how cryopreserved T cells react to APC-K562 and beads stimulus compared to freshly isolated T cells (Figure 2.4A). Both conditions showed no significant difference, as seen in Figure 2.4A. The use of cryopreserved T cells is advantageous to researchers that do not have the facilities or the protocols to withdraw blood from canines, as beagle PBMCs are commercially available.

With the beads being the optimal T cell stimulant compared to others, we study how recombinant human IL-2 supplementation enhances canine T cell expansion with different concentrations of beagle serum in media instead of fetal bovine serum as others (Figure 2.5B). rhIL-2 combined with 10% beagle serum (4-fold expansion in five days of culture) resulted in the optimal condition to culture canine T cells.

We demonstrated that canine T cells can be genetically engineered using the VSV-G pseudotyped lentiviral particles encoding anti-FITC-8-41BB-ζ. Transduction efficiency ranged from 1-20% consistently, although they were cases that CAR expression reached 36.7% with polybrene as the transduction enhancer (Figure 2.8). Polybrene and LentiBoost AB transduction enhancers are suitable options for canine CAR T cell experiments, although polybrene showed higher efficiency (Figure 2.8). Transduction efficiency could be improved by combining both transduction enhancers since both can act synergistically on different pathways [93]. Polybrene is a cationic polymer that can neutralize charge repulsion between virions and the cell surface, while LentiBoost interacts with poloxamers of the lipid bilayer. Following canine CAR T cell production, we evaluated CAR-mediated functionality by coincubation with the folate receptor-expressing cell lines KB and P114. The low-molecular-weight adaptor served as an intracellular bridge between the CAR T cell and the target cell, as suggested in Figure 2.9. The absence of the bispecific adaptor molecule showed no significant killing, while its presence showed, in most cases, killing functionality.

In conclusion, we developed a feasible activation, expansion, and transduction protocol to generate canine anti-FITC expressing CAR T cells. This is a first-in-species canine universal CAR T cell system for solid tumor treatments. These techniques can allow the evaluation of this immunotherapy in large animal models.

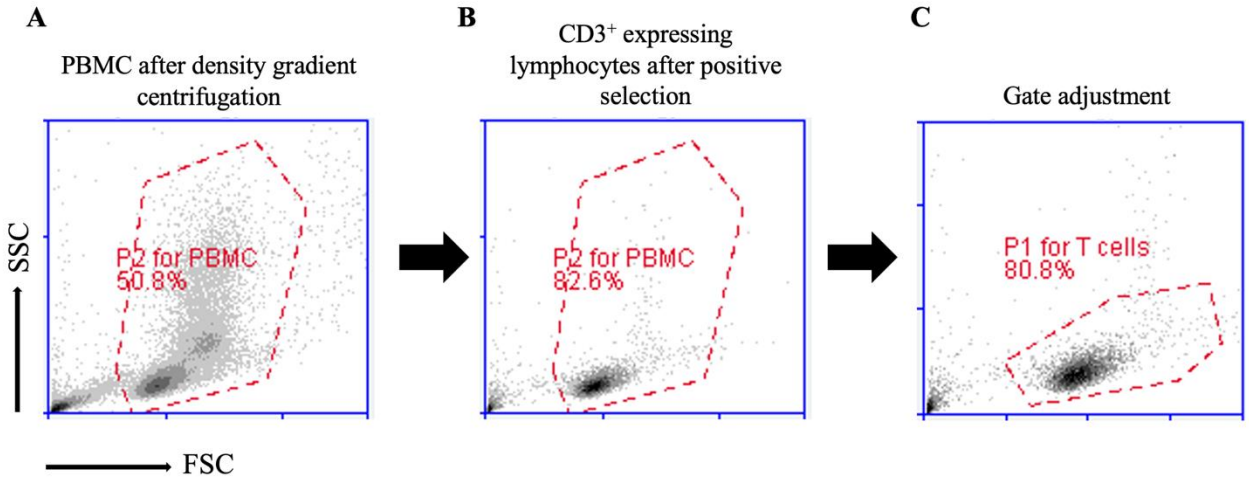

Figure 2.1: $CD3^+$ T cell purification by MACS separation

(A-C) Dot plot illustration of PBMCs and $CD3^+$ T cells before and after MACS positive selection. (A) Immediately after Lymphoprep density gradient centrifugation, the PBMC population is divided mainly of granulocytes, monocytes, and lymphocytes. (B and C) After selection, the majority the cells were $CD3^+$ T cells.

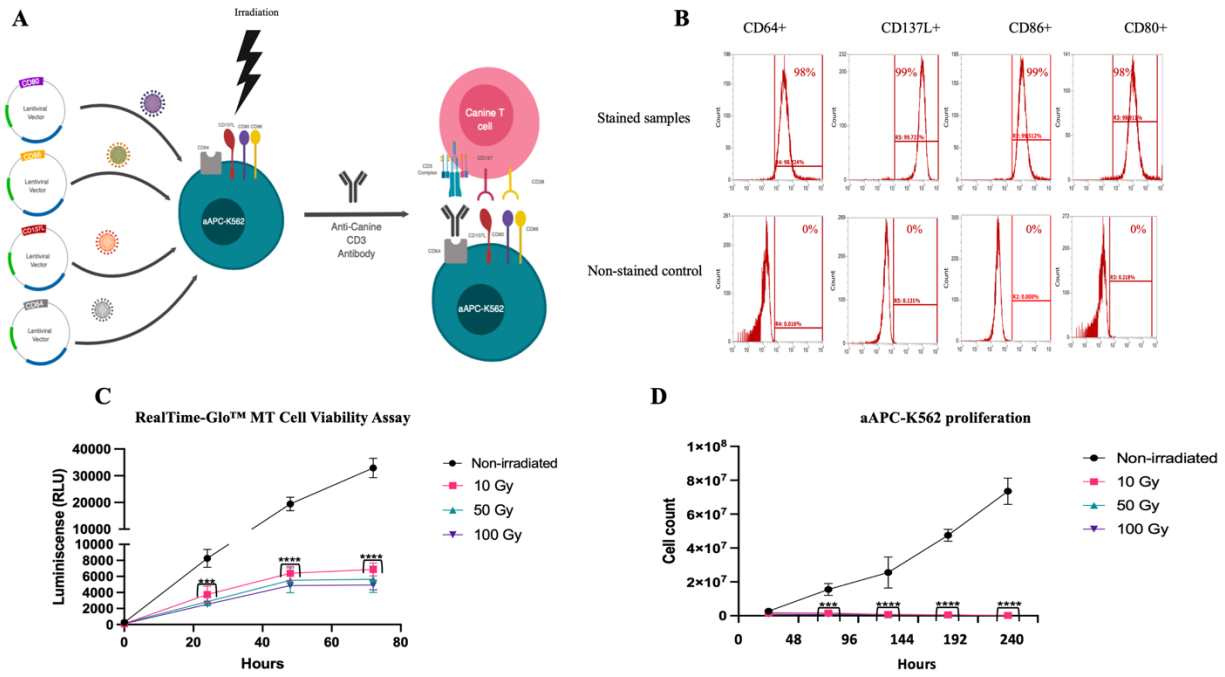

Figure 2.2: Generation of the Artificial Antigen Presenting Cell (APC)

(A)Diagram summarizing the key steps for generation and use of the APCs. Lentiviral-mediated expression of the receptors in the parental K562 cell line, followed by irradiation, anti-CD3 addition and co-incubation with T cells. (B) Histogram of the expression of CD64, CD137L, CD86 and CD80 after cell sorting. (C and D) RealTime-Glo™ MT Cell Assay and counting to measure viability of the irradiated cells in comparison with the non-irradiated control (D) APCs were enumerated by trypan blue exclusion and compared to the initial number added in culture. In all cases, ***p< 0.001 and ****p<0.0001 as measured by two-way ANOVA.

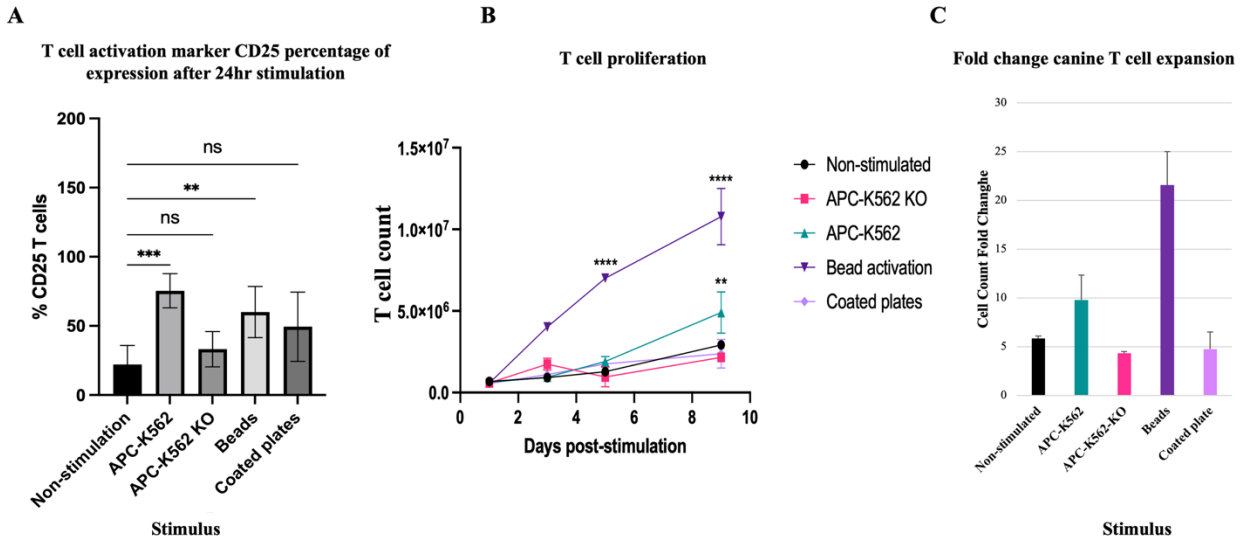

Figure 3.3: Canine T cell expansion in response of the different activation methods.

Purified $CD3^+$ T cells from healthy dog donors were stimulated with APC-K562, APC-K562-KO, CD3/CD28 beads and CD3 coated plate. (A) Surface expression frequency of CD25 was measured in flow cytometry (n=5). (B) Enumeration of canine T cells after stimulation at the time point indicated (n= 2). (C) Fold-change of total $CD3^+$ T cells after nine days of stimulation (n=2). In all cases, **p< 0.01, ***p< 0.001 and ****p<0.000 as measured by two-way ANOVA.

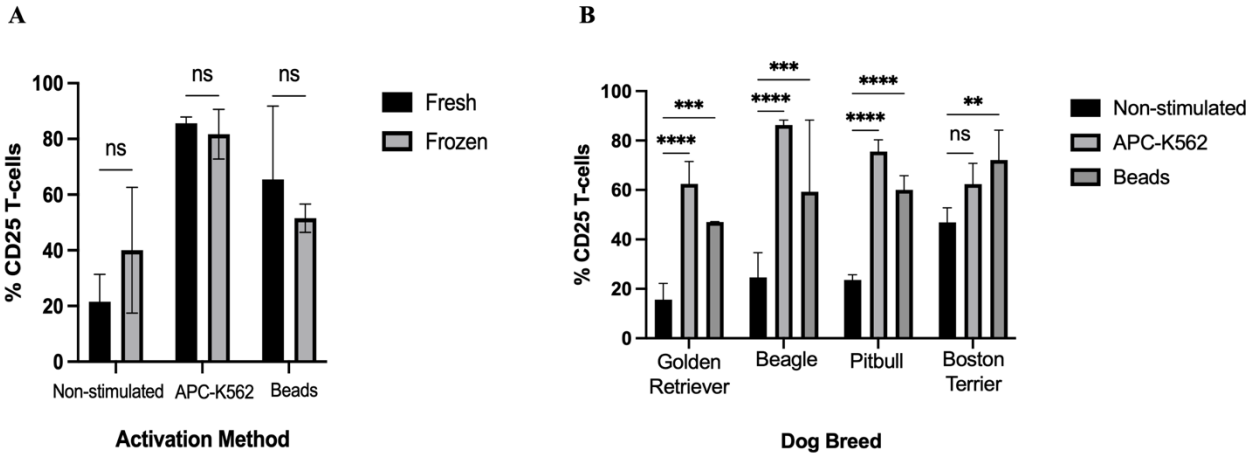

Figure 4.4: Breed variability and cryopreservation effects in canine T cell activation

Surface expression of CD25 frequency was measured by flow cytometry. (A) Effect of APC-K562 and CD3/CD28 beads in different canine breeds (n=2). (B) Comparison between fresh and frozen canine T cells when activated by APC-K562 and CD3/CD28 beads (n=3). In all cases, **p< 0.01, ***p< 0.001 and ****p<0.0001 as measured by two-way ANOVA.

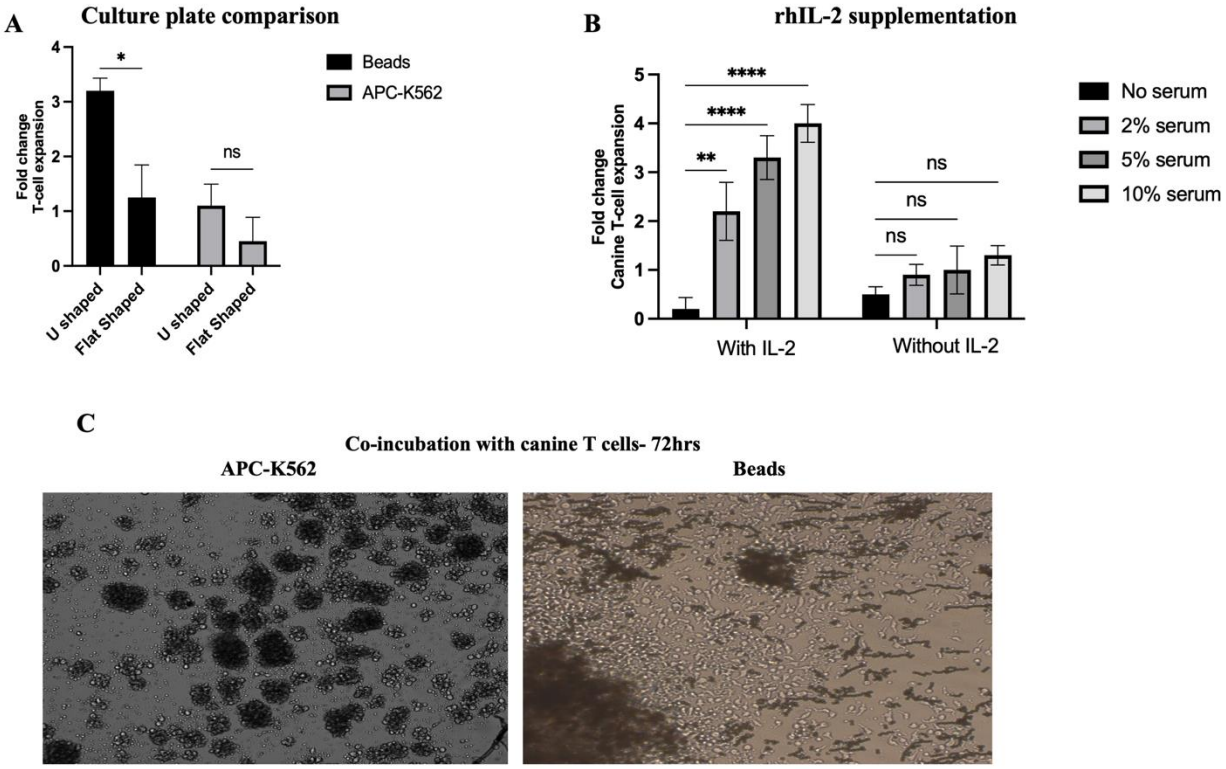

Figure 5.5: Culture plate comparison, rhIL-2 supplementation, and serum concentration.

(A)Comparison between U-shaped and Flat-shaped wells after activation with APC-K562 and CD3/CD28 beads (n=3). (B) Effect of rhIL-2 supplementation with different concentrations of beagle serum (n=2). (C) Cluster formation of APC-K562 and CD3/CD28 bead co-incubation with T cells. Bead activated cells appeared to be enlarged with a spindle-shaped morphology, indicating proper engagement. In all cases, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$ as measured by two-way ANOVA.

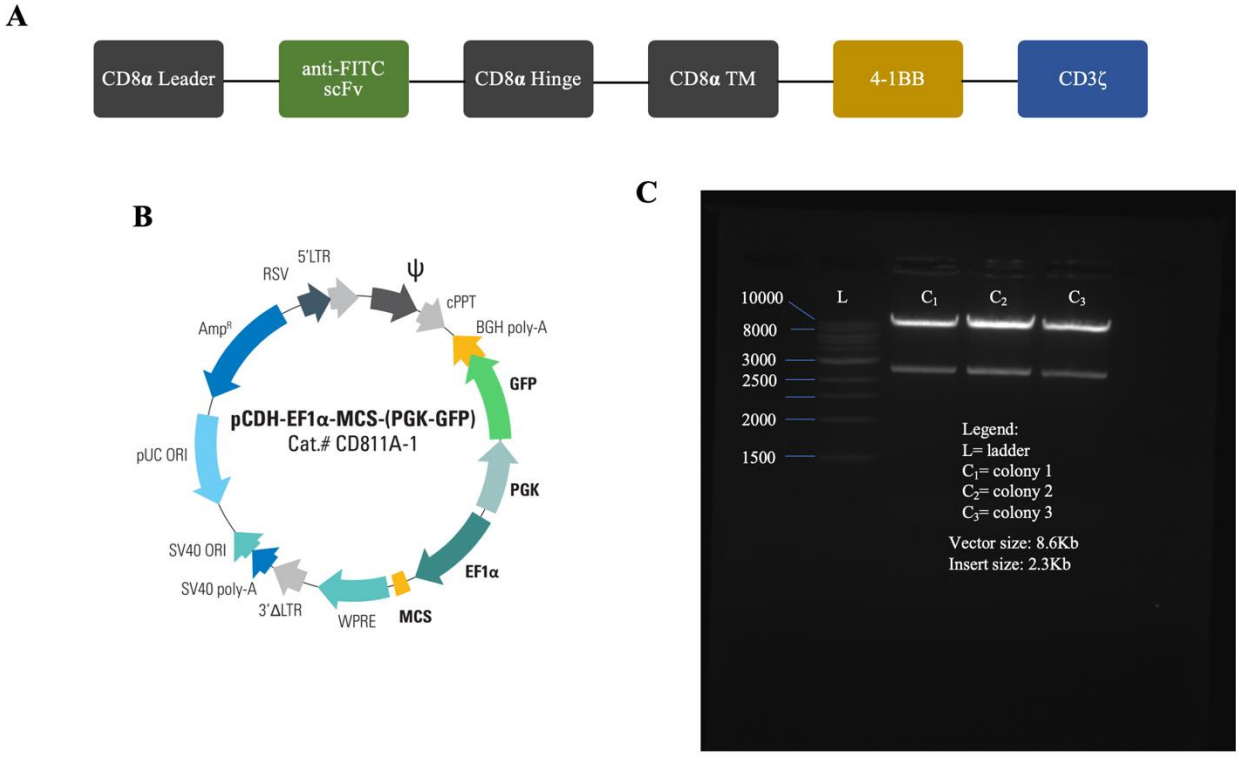

Figure 6.6: anti-FITC-8-41BB-ζ construct design and restriction enzyme digestion after cloning.

(A)Diagram of canine anti-FITC-8-41BB-ζ construct. (B and C) pCDH-EF1-MCS-(PGK-GFP) lentiviral vector was cloned to the anti-FITC CAR gene. One percent agarose gel run with 1 Kb ladder. L represents ladder, C represent colonies.

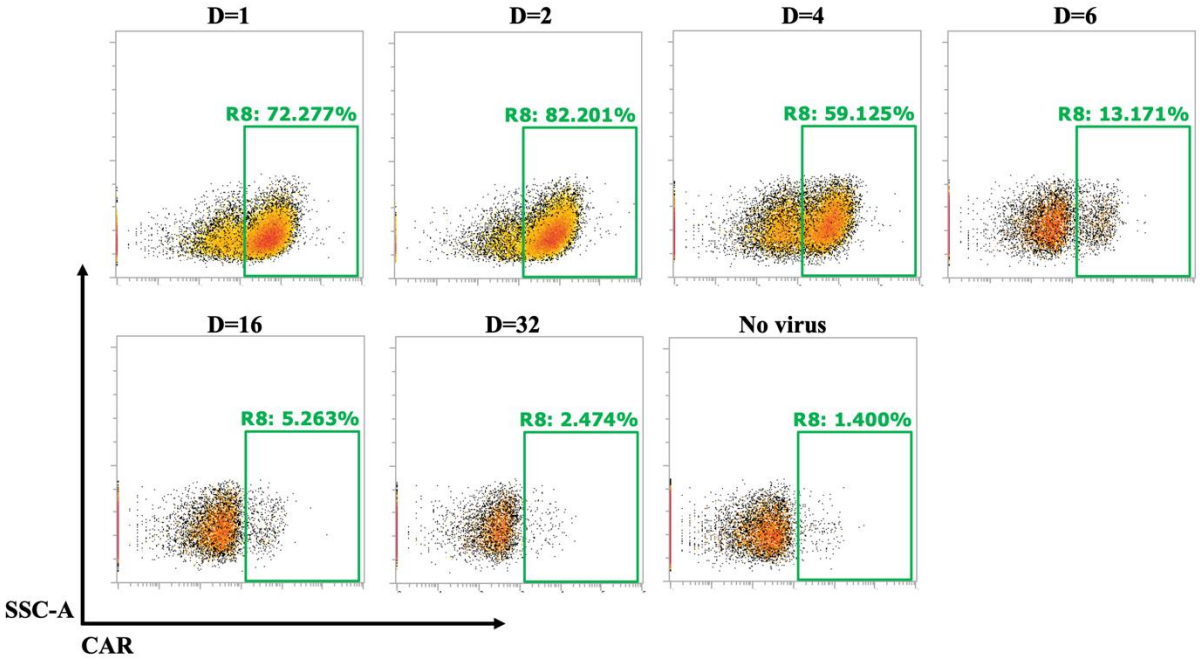

Figure 7.7: Titration of anti-FITC-8-41BB-ζ VSV-G pseudotype on K562 cells.

K562 transduced cells were stained with FITC-Alexa-647. Titration was calculated by the following equation: TU/mL=FxNxDx1000/V.

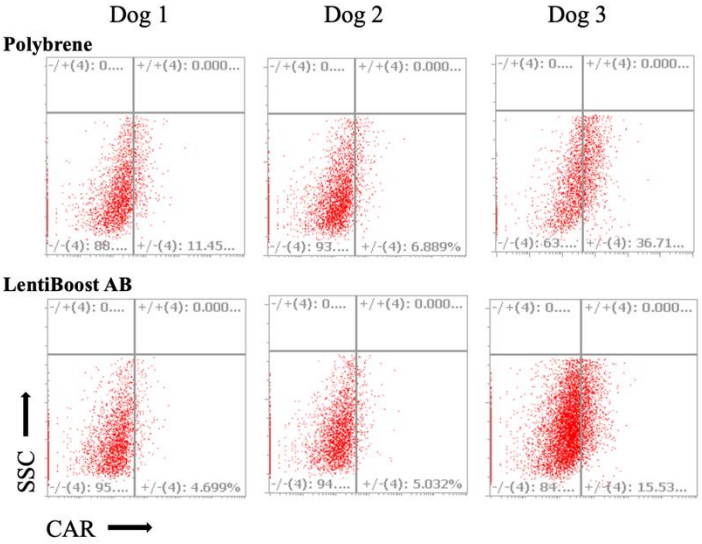

Figure 8.8: CAR surface expression measured by flow cytometry after transduction with two different transduction enhancers.

Table 2.1.: Tumor associated antigen identification

Cell Line	Classification	Species	Folate Expression	CAIX Expression	PSMA Expression
KB	Oral Squamous Carcinoma	Human	99.5%	0%	-
HT1376	Bladder Cancer	Human	5%	11.66%	-
UMUC3	Bladder Cancer	Canine	0%	35.16%	-
P114	Breast Cancer	Canine	40-98%	4.1%	-
CF41	Breast Cancer	Canine	0%	1.9%	-
TR5P	Prostate Cancer	Canine	0%	2.5%	3.3%
CLBL-1	Leukemia	Canine	0%	0%	-
17-71	Leukemia	Canine	0%	0%	-
GL-1	Leukemia	Canine	4%	0%	-

Tumor associated target identification in human and canine cell lines. Expression of each receptor on the desired cancer cell line was confirmed by flow cytometry after staining with the appropriate FITC-labeled CAR T cell Adaptor Molecule (CAM).

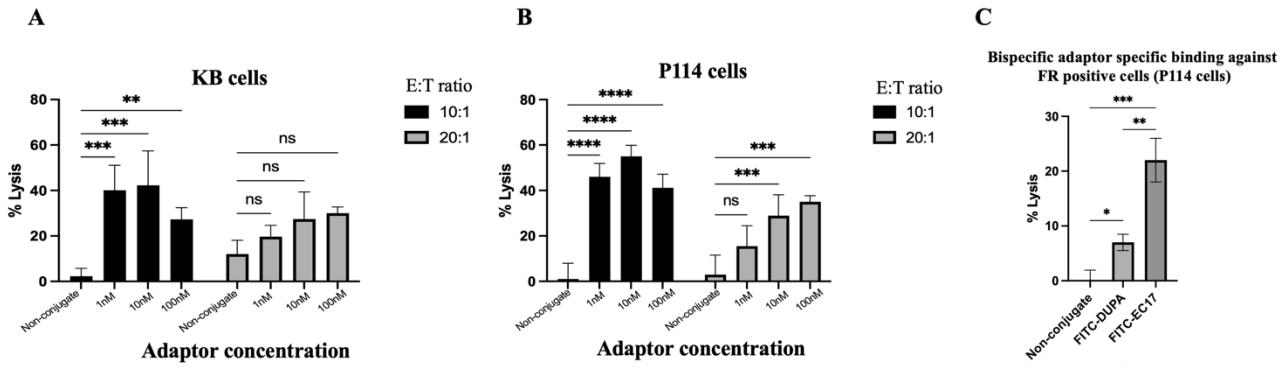

Figure 9.9: *In-vitro* analysis of cytotoxicity of canine anti-FITC CAR T cells using LDH assay.

Naturally occuring folate receptor expressing (A) KB cells and (B and C) P114 cells incubated at different Effector:Target cell ratios with different concentrations of FITC-folate bispecific adapter prior to analysis of tumor cell lysis with Pierce LDH Assay. (n=3). In all cases, *p< 0.05, **p< 0.01, ***p< 0.001 and ****p<0.0001 as measured by two-way ANOVA.

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