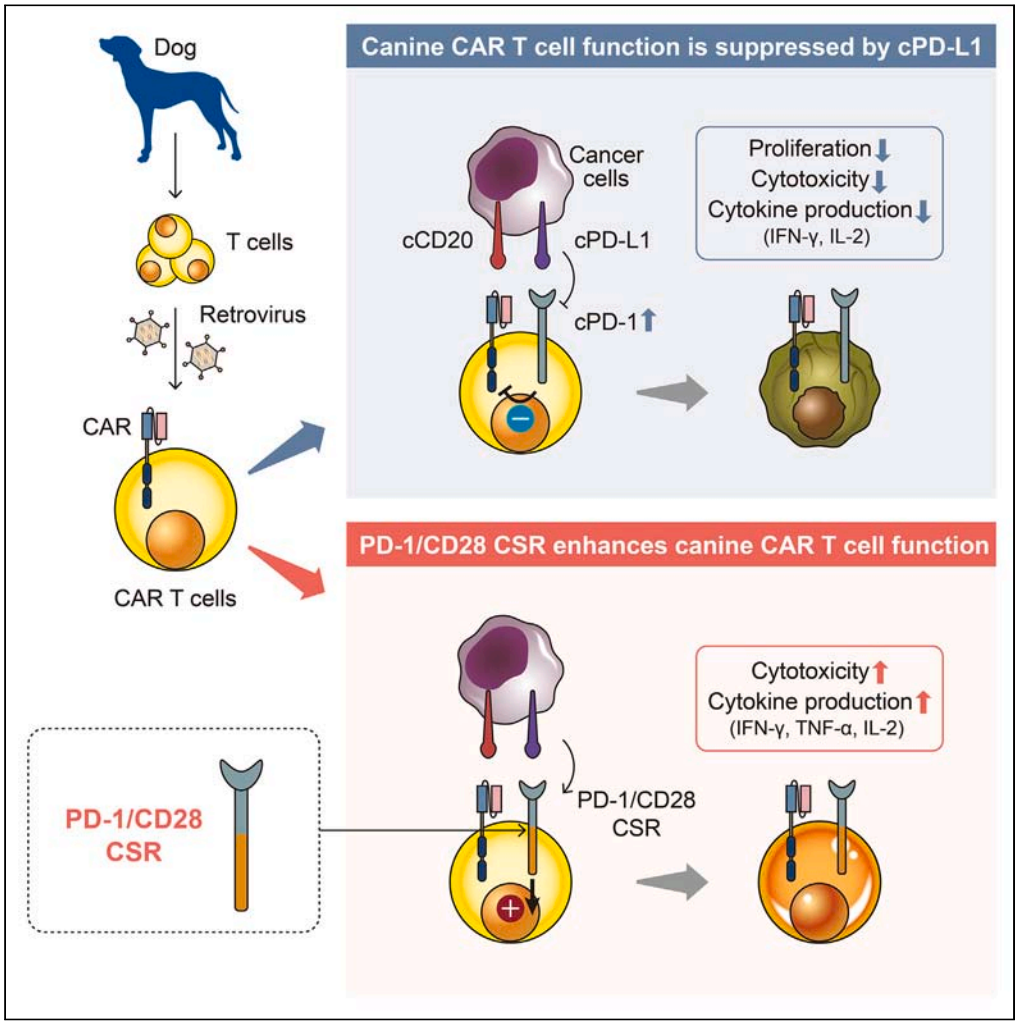
iScience

CellPress
OPEN ACCESS

Article

Validation of a PD-1/CD28 chimeric switch receptor to augment CAR-T function in dogs with spontaneous B cell lymphoma



Sho Yoshimoto, Ayano Kudo, Antonia Rotolo, Kay Foos, Lauren Olenick, Satoshi Takagi, Nicola J. Mason

nmason@vet.upenn.edu

Highlights

Second generation canine CAR-T cells are inhibited by PD-L1 on target cells

PD1/CD28 switch receptors enhance canine CAR-T function against PD-L1^{+} targets

PD1/CD28 switch receptors maintain canine CAR-T central memory phenotype

Switch receptor effects on canine CAR-T require an active CD28 signaling domain

Yoshimoto et al., iScience *27*, 110863
September 20, 2024
https://doi.org/10.1016/j.isci.2024.110863

iScience

CellPress
OPEN ACCESS

Article

Validation of a PD-1/CD28 chimeric switch receptor to augment CAR-T function in dogs with spontaneous B cell lymphoma

Sho Yoshimoto,[1] Ayano Kudo,[1] Antonia Rotolo,[2,4] Kay Foos,[3,4] Lauren Olenick,[3] Satoshi Takagi,[1] and Nicola J. Mason[2,4,5,*]

SUMMARY

Chimeric antigen receptor (CAR) T cell therapy has achieved unprecedented clinical outcomes in patients with relapsed/refractory B cell leukemias; however, response rates in patients with large B cell lymphoma (LBCL) are less impressive. Expression of PD-1 on activated T cells and PD-L1 on malignant, stromal, and immune cells within the tumor microenvironment (TME) contribute to CAR-T exhaustion, hypofunction, and treatment failures. Here, a comparative approach is taken to develop a chimeric switch receptor (CSR) with potential to augment CAR-T persistence, function, and clinical efficacy in immune competent, pet dogs with spontaneous B cell lymphoma (BCL). We show that similar to human CAR-T cells, expression of a PD-1/CD28 CSR in canine CAR-T cells results in enhanced function against PD-L1$^+$ targets and preserves central memory phenotype. We also demonstrate that these effects depend upon active CSR signaling. This work paves the way for *in vivo* studies in canine BCL patients to inform human trial design.

INTRODUCTION

Chimeric antigen receptor (CAR) T cells have shown remarkable therapeutic activity in patients with relapsed, refractory hematological malignancies including B cell leukemias and multiple myeloma.[1–4] However, outcomes for patients with large B cell lymphomas (LBCLs) have been less impressive with over 50% of patients either failing to respond or experiencing disease relapse.[3,5] Factors that contribute to this failure include characteristics of the CAR-T product itself and the immune suppressive tumor microenvironment (TME) which promotes T cell dysfunction and drives exhaustion.[6–8] In particular, expression of the inhibitory checkpoint programmed cell death protein-1 (PD-1) on CAR-T cells correlates with poor response in patients with hematological and solid tumors,[9,10] and amplification of its ligand PD-L1 has been associated with resistance to CART-19 therapy in large B cell lymphomas.[11] PD-1 is up-regulated on the surface of activated T cells, B cells, and natural killer (NK) cells,[12] and its interaction with PD-L1 suppresses T cell activation and promotes hypofunction and exhaustion principally through recruitment of SHP-1/2 phosphatases that de-phosphorylate ZAP70, PI3K, and CD28 and inhibit T cell receptor (TCR) and CD28 signaling.[13,14] Engagement of PD-1 on CAR-T cells in the TME contributes to CAR-T hypofunction,[10] and strategies aimed at disrupting the PD-1:PD-L1 axis to improve the clinical efficacy of CAR-T cells are being explored across tumor histologies. Concurrent administration of anti-PD-1 antibodies with CAR-T cells improved CAR-T function in pre-clinical mouse models and showed early promise in a phase I clinical trial in patients with malignant pleural disease.[15–17] Furthermore, anti-PD-1 antibodies are being evaluated in patients with diffuse large B cell lymphoma (DLBCL) that is refractory to CD19 CAR-T therapy, with early reports suggesting PD-1 blockade might revitalize previously administered CART-19 cells.[18,19] Other strategies to address PD-1 mediated T cell hypofunction include advanced engineering of CAR-T cells to constitutively secrete an anti-PD-1 minibody,[20,21] editing of CAR-T cells to disrupt PD-1 expression (abstract: Rupp LJ., CRISPR-Cas9-mediated PD-1 disruption enhances anti-tumor efficacy of human chimeric antigen receptor T cells. Sci Rep. 2017; 7:737. https://doi.org/10.1038/s41598-017-00462-8),[22] and expression of chimeric switch receptors (CSRs) consisting of the extracellular PD-1 domain linked to a transmembrane and intracellular, co-stimulatory CD28 domain.[23,24] Unlike other strategies which block and inhibit PD-1 mediated suppression, the CSR approach aims to augment effector function following PD-L1 and PD-L2 engagement by delivering an activating co-stimulatory signal to the CAR-T cell. Human CAR-T cells expressing a PD-1/CD28 CSR showed superior tumor infiltration and tumor regression, decreased functional impairment, and reduced expression of other inhibitory receptors compared to CAR-T cells without a CSR in murine xenograft models.[23,25] Furthermore, administration of CD19 CAR-T cells expressing a PD-1/CD28 CSR in a pilot study of relapsed/refractory DLBCL patients with

[1]Laboratory of Small Animal Surgery, Department of Veterinary Medicine, School of Veterinary Medicine, Azabu University, Sagamihara, Kanagawa, Japan
[2]Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA, USA
[3]Department of Cancer Biology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA
[4]Center for Cellular Immunotherapies, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA
[5]Lead contact
*Correspondence: nmason@vet.upenn.edu
https://doi.org/10.1016/j.isci.2024.110863

disease progression after CD19 CAR-T therapy led to complete tumor regression in 3/6 patients.[24] Together, these data suggest that CAR-T cells expressing a PD-1/CD28 CSR will provide superior clinical responses to CAR-T cells alone in human cancer patients. However, it remains unknown whether PD-1/CD28 CSR CAR-T cells will be safe, show enhanced persistence and function, and lead to improved clinical outcomes in patients poorly responsive to conventional CAR-T cell therapy. Additionally, the value of PD-L1 expression on tumor cells and tumor-infiltrating immune cells in patient stratification and prediction of clinical response to PD-1/CD28 CSR CAR-T requires further exploration. Investigations in an immune competent, large animal model of spontaneous B cell lymphoma (BCL) will enable these critical questions to be addressed over a compressed time frame and aims to provide valuable insights for the effective implementation of PD-1/CD28 CSR-based strategies in the human clinic.

Immune competent pet dogs develop spontaneous tumors that share similar features to human tumors including chromosome aberrations, molecular subtypes, tumor heterogeneity, immune signatures, immune suppressive microenvironments, metastatic behavior, and chemotherapeutic response. These similarities have identified pet dogs as a relevant parallel patient population to advance next generation adoptive cell therapies (ACTs) that may rapidly address current barriers to clinical response and durable remissions in human patients.[26–29] We have previously developed robust protocols to generate canine CAR-T cells targeting CD20[30] and have demonstrated similar barriers to CAR-T efficacy following CAR-T administration to pet dogs with BCL as in human LBCL patients, including primary tumor resistance, target antigen down-regulation and loss associated with epitope splicing, and lack of CAR-T cell persistence.[31,32] We have also observed mild CAR-T associated toxicity similar to cytokine release syndrome in human patients with comparable clinical signs and cytokine profiles.[33]

Here, we demonstrate that canine CAR-T cells up-regulate canine (c)PD-1 after engagement of the CAR with its target antigen and are inhibited by cPD-L1$^+$ target cells. We show that canine CAR-T cells engineered to express a PD-1/CD28 CSR exhibit superior effector functions against cPD-L1$^+$ targets compared to CAR-T without CSR and that this functional enhancement requires active CD28 signaling. These findings parallel those of human CAR-T cells expressing a PD-1/CD28 CSR and now enable pilot studies in canine cancer patients to address the hypothesis that CAR-T cells expressing a functional CSR can improve outcomes in patients with LBCL. Due to the compressed time course of LBCL progression in dogs with spontaneous disease, this hypothesis can now be addressed and outcomes determined in canines in an expedited manner.

RESULTS

Canine CAR-T cells express PD-1 following activation

Negatively selected canine T cells were transduced with a second-generation canine CAR comprised of a murine anti-canine CD20 scFv and canine CD8α hinge, transmembrane, 4-1BB and CD3ζ domains (Figure 1A). Cell surface expression was confirmed on healthy canine CD4$^+$ and CD8$^+$ T cells by flow cytometry 4 days post-transduction (Figure 1B). Target K562 cells engineered to express canine (c)CD20 either alone (K562-cCD20) or together with canine cPD-L1 (K562-cCD20-cPD-L1) were confirmed to express comparable levels of cCD20 either with or without cPD-L1 (Figure 1C). To assess whether cPD-1 was up-regulated following cCD20-specific CAR-T cell engagement and activation, CAR-T cells were co-cultured at different E:T ratios with K562, K562-cCD20, and K562-cCD20-cPD-L1 target cells and cPD-1 expression was determined using a validated anti-canine PD-1 antibody 72 h later (Figure 1D).[34] Following co-culture with CD20 expressing target cells, cPD-1 was up-regulated on CD8$^+$ T cells in a dose-dependent manner with a greater percentage of cPD-1$^+$ CAR-T cells occurring at lower E:T ratios, conceivably due to higher number of CAR-T cells effectively engaged and activated by target cells. Similarly, cPD-1 was up-regulated in a dose dependent manner on CD8$^-$ T cells after activation. CAR-T cells co-cultured with K562-WT cells did not up-regulate cPD-1, suggesting that CAR engagement is required to induce cPD-1 expression on canine CAR-T cells (Figure 1D).

Canine CAR-T cell function is suppressed by cPD-L1

Engagement of PD-1 with PD-L1 in human and mouse T cells inhibits TCR and CD28 signaling and suppresses T cell proliferation, cytokine production, and cytotoxicity.[13] To determine whether cPD-1:cPD-L1 also inhibits canine CAR-T cell effector function, CAR-T cells from 4 healthy outbred donor dogs were labeled with CellTraceViolet (CTV) dye and co-cultured with K562-cCD20 or K562-cCD20-cPD-L1 target cells at an E:T of 1:1. Co-culture of CAR-T cells with cPD-L1 expressing targets resulted in significant inhibition of CD4$^+$ and CD8$^+$ CAR-T proliferation (Figure 2A). To determine whether cPD-L1 also inhibited cytotoxic function, CAR-T cells were co-cultured with luciferase expressing K562-WT, K562-cCD20, K562-cPD-L1, or K562-cCD20-cPD-L1 cells at increasing E:T ratios and cytotoxicity was determined 24 h later (Figure 2B). CAR-T cells from all 4 dogs showed dose-dependent cytotoxicity against target cells expressing cCD20. Untransduced canine T cells not expressing a CAR showed no significant cytotoxicity against any of the target cells, demonstrating the antigen-specific cytotoxicity of canine CAR-T cells. While CD20-targeted CAR-T cells were capable of killing CD20$^+$ target cells, expression of cPD-L1 by the target cells resulted in significant inhibition of cytotoxicity at each E:T ratio tested, confirming the inhibitory effect of cPD-1:cPD-L1 interaction on CAR-T cytotoxicity. Expression of cPD-L1 on target cells also significantly inhibited the production of interferon-γ (IFN-γ) and interleukin-2 (IL-2) by CAR-T cells after 24 h of culture, although tumor necrosis factor α (TNF-α) production was not significantly affected, possibly due to donor variability (Figure 2C). Finally, the effect of cPD-1 engagement on the memory phenotype of CAR-T cells was evaluated. Previous studies have shown that CAR-T cells exhibiting central memory phenotypic markers are associated with improved clinical outcomes for patients with hematological malignancies[36] and expression of PD-1 inhibits the development of central memory T cells in response to acute viral infections.[37] To test the hypothesis that CAR-T cells co-cultured with cPD-L1 expressing targets would show a loss of cells with central memory-like features, the memory profile of CAR-T cells undergoing repeated stimulation with CD20$^+$ target cells expressing cPD-L1 was determined. After three stimulations, there was a reduction in the percentage of CD8$^+$ CAR-T cells with central memory (CD62L$^+$CD45RA$^-$) and naive

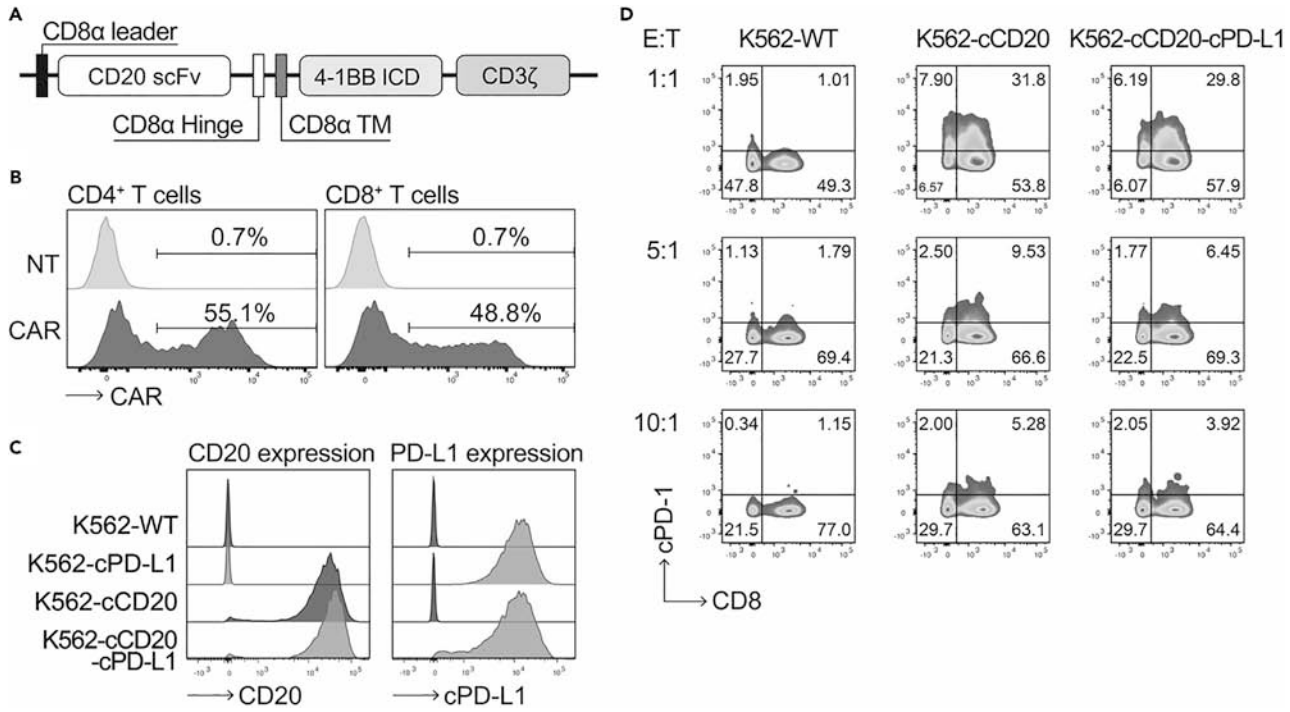


Figure 1. Canine CD20-BB-ζ CAR-T cells express cPD-1 after CAR engagement

(A) Schematic representation of cCD20-BB-ζ CAR construct. ICD, intracellular domain; TM, transmembrane domain.

(B) Surface expression of cCD20-BB-ζ CAR on $CD4^+$ and $CD8^+$ T cells evaluated by flow cytometry 4 days post transduction. Plots are gated on lymphocytes>single>live>$CD5^+$>$CD4^+$ or $CD8^+$ cells.

(C) Surface expression of cCD20 and cPD-L1[35] on engineered K562 cells. Plots are gated on live single cells.

(D) cPD-1 expression[34] on CAR-T cells after co-culture with indicated target cells at different E:T ratios. CAR-T cells were generated as described and 6 days after transduction (8 days post activation) cells were co-cultured with either K562-WT or K562-cCD20 cells with or without cPD-L1, and cPD-1 expression was determined by flow cytometry after 72 h. Plots are gated on lymphocyte>single>live>$CD5^+$>CAR^+ cells.

($CD62L^+CD45RA^+$) phenotype, while the percentage of $CD8^+$ effector memory CAR-T cells ($CD62L^-CD45RA^-$) increased compared to CAR-T cells stimulated with cPD-$L1^-$ target cells (Figures 2D and 2E). Taken together, these data indicate that interaction with cPD-L1 drives canine CAR-T cells away from the more favorable central memory-like phenotype that in human patients is associated with deeper, durable remissions. Therefore, cPD-L1 expression on antigen-positive target cells may reduce the long term effector function of canine CAR-T cells, mirroring findings in human studies.[38] These comparable findings between human and canine CAR-T cells provide the rationale for evaluating novel CSR-based strategies in the canine system to inform optimal design of human trials.[39]

A canine PD-1/CD28 CSR enhances CAR-T cell function against engineered cPD-$L1^+$ target cells

A fully canine CSR containing the extracellular domain of cPD-1 linked to the transmembrane and intracellular domains of canine CD28 (CSR_{WT}) was constructed and linked to the cCD20-BB-ζ CAR via FMD2A and cloned into the MSGV1 retroviral vector (CAR+ CSR_{WT}) (Figure 3A). To determine the mechanism of CSR effect on canine CAR-T function, a mutant CSR (CSR_{MUT}) was constructed. In this control receptor, the extracellular cPD-1 moiety was intact and still capable of competing with endogenous cPD-1 for cPD-L1 interaction. However, the functionality of the intracellular CD28 costimulatory moiety was abrogated by mutating the YMNM, PRRP, and PYAP motifs, known binding sites for Grb2 and Lck, to FFFF, ARRA, and AYAA respectively as previously described in a human switch receptor (Figures 3A and 3B).[10] As such, CSR_{WT} was expected to enhance CAR-T cells to a greater extent than CSR_{MUT} due to additional CD28 signaling. Canine T cells from four healthy donor dogs were transduced with either the CAR alone, CAR+CSR_{WT}, or CAR+CSR_{MUT}. Expression of the CAR and the CSR_{WT} or CSR_{MUT} was determined by flow cytometry. Greater than 74% of canine $CD4^+$ T cells and 42% of $CD8^+$ T cells expressed the CAR plus CSR and expression levels were comparable between CSR_{WT} and CSR_{MUT} (Figures 3C and 3D). CAR expression in CAR-T cells alone was significantly lower than in CAR-T cells expressing either CSR, a finding that was previously reported for human CAR-T cells engineered with CSR constructs.[25]

To determine whether the cPD-1/CD28 CSR could enhance CAR-T function against cPD-L1 expressing target cells, CAR, CAR+ CSR_{WT}, or CAR+CSR_{MUT} generated from four healthy donor dogs were co-cultured with K562-cCD20-cPD-L1 target cells at a 1:1 E:T ratio and IFN-γ, TNF-α, and IL-2 were measured in culture supernatants after 24 h (Figure 4A). CAR+ CSR_{WT} produced more IFN-γ, IL-2, and TNF-α than CAR-T cells without a CSR or CAR+CSR_{MUT}, when co-cultured with K562-cCD20-cPD-L1 target cells, demonstrating that active CD28 signaling is the central mechanism responsible for the increase in cytokine production. Furthermore, this effect was mediated by cPD-L1

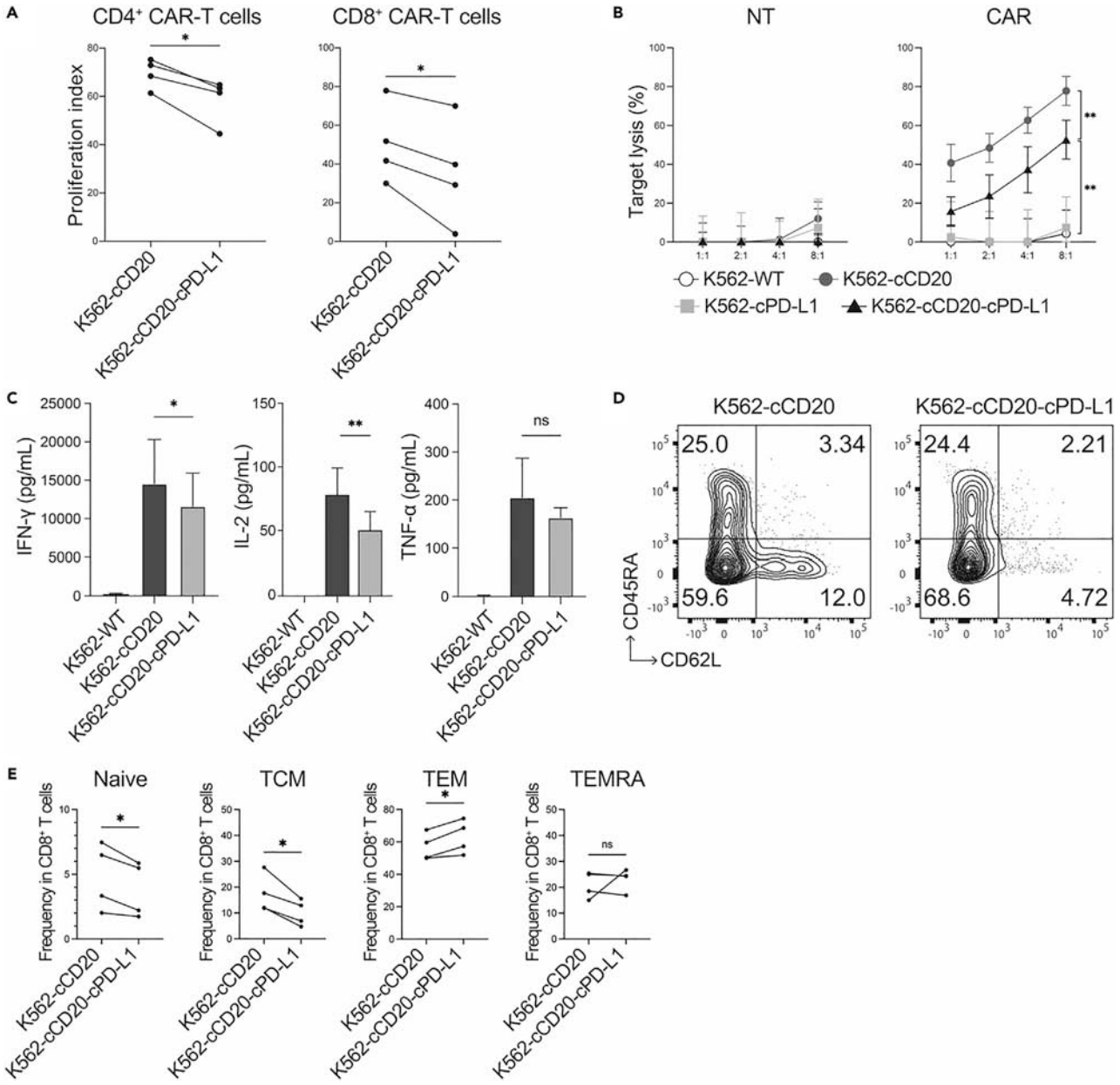


Figure 2. cPD-L1 expressing targets suppress canine CAR-T cell effector functions

(A) Proliferation index of canine $CD4^+$ and $CD8^+$ CD20-BB-ζ CAR T cells from 4 healthy donors, 72 h after stimulation with the indicated K562 target cells at an E:T ratio of 1:1.

(B) cCD20-BB-ζ CAR-T cells were co-cultured with the indicated K562 target cells at different E:T ratios and cytotoxicity was measured after 24 h. Data are mean ± SD (n = 4 biological replicates).

(C) IFN-γ, IL-2, and TNF-α production by cCD20-BB-ζ CAR T cells cultured with K562 (wild-type) or K562-cCD20 cells with or without PD-L1 at an E:T ratio of 1:1. Data are mean ± SD (n = 4 biological replicates).

(D and E) Effects of target cPD-L1 expression on CAR-T cell phenotype. cCD20-BB-ζ CAR T cells were stimulated three times with K562-cCD20 or K562-cCD20-cPD-L1 cells and immunophenotyped 6 days after the third stimulation. (D) Representative two-dimensional dot plot of CD45RA and CD62L expression on $CD8^+$ CAR T cells. (E) Data for each of the four dogs are shown. *$p < 0.05$; **$p \leq 0.01$; ns: not significant. Naive-like ($CD45RA^+CD62L^+$), TCM-like ($CD45RA^-CD62L^+$), TEM-like ($CD45RA^-CD62L^-$), TEMRA-like ($CD45RA^+CD62L^-$).

as CAR+ CSR_{WT} did not augment cytokine production against K562-cCD20 targets. There was no significant difference between cytokine production by CAR-T cells and CAR+CSR_{MUT}, against K562-cCD20-cPD-L1 target cells suggesting that competition between the CSR and endogenous cPD-1 for cPD-L1 does not contribute to augmented cytokine production seen with CAR+CSR_{WT} cells.

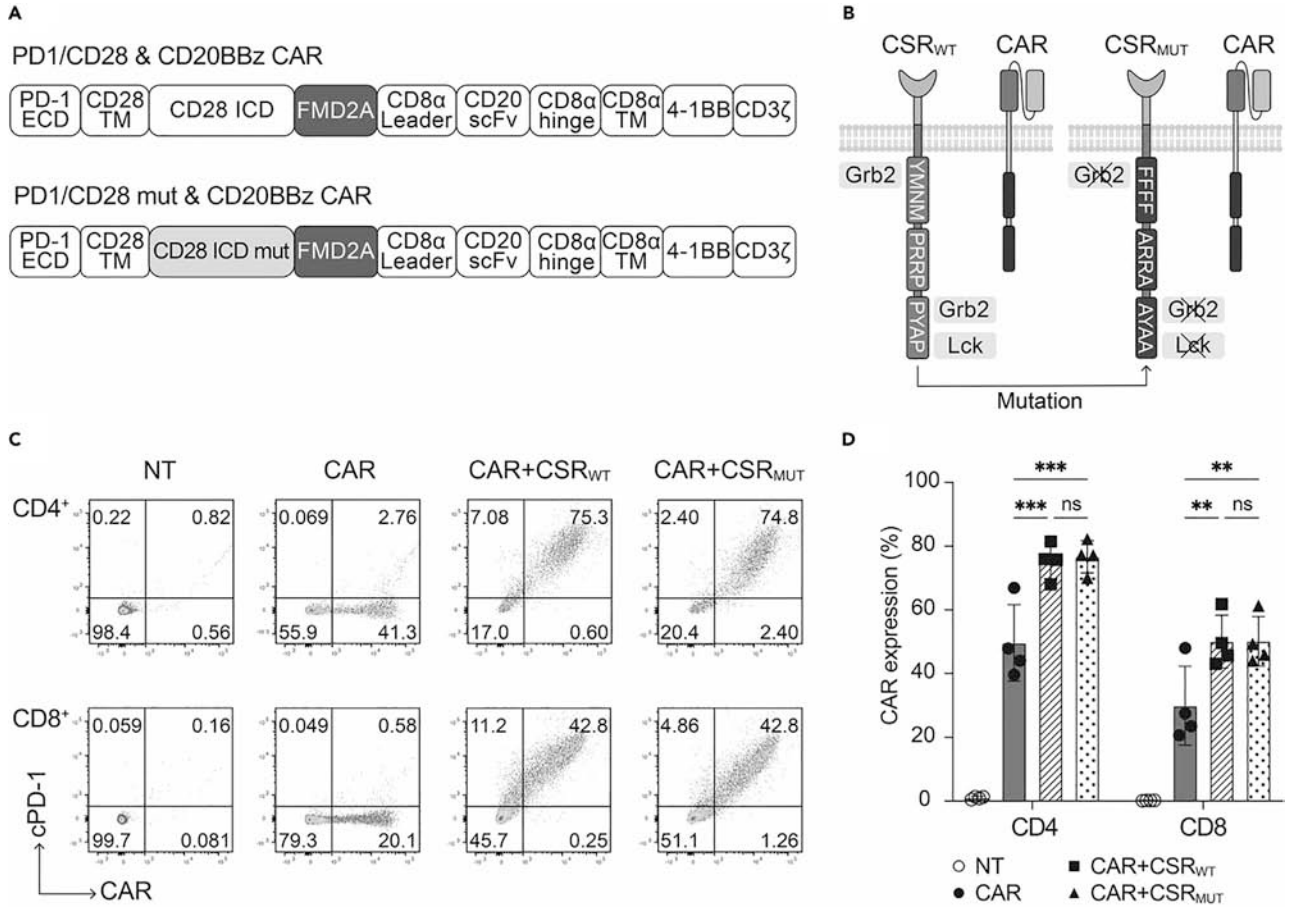


Figure 3. An active PD-1/CD28 CSR augments cCD20-BB-ζ CAR T cell function against cPD-L1 expressing target cells

(A) Design of CSR_{WT} and CSR_{MUT} CAR constructs. ECD, extracellular domain; TM, transmembrane; ICD, intracellular domain.

(B) Cartoon showing the CD28 mutations that prevent Grb2 and Lck binding in the CSR_{MUT} construct.

(C) Canine $CD4^+$ and $CD8^+$ T cells successfully co-express the cCD20-BB-ζ CAR and the CSR_{WT} and CSR_{MUT} constructs. Representative data from one of four CAR-T cell products generated from 4 healthy donor dogs 6 days post-transduction. Plots are gated on lymphocyte>single>live>$CD5^+$>$CD4^+$ or $CD8^+$ cells.

(D) CAR expression on $CD4^+$ and $CD8^+$ T cells from 4 healthy donor dogs transduced with CAR alone, CAR+CSR_{WT} or CAR+CSR_{MUT}. Data are mean ± SD ($n = 4$ biological replicates). $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; ns: not significant.

To determine whether the CSR_{WT} also enhances cytotoxicity against cPD-$L1^+$ target cells, CAR, CAR+CSR_{WT}, or CAR+CSR_{MUT} were co-cultured with K562-cCD20 or K562-cCD20-cPD-L1 target cells and cytotoxicity was assessed after 24 h (Figure 4B). As previously seen, cytotoxicity of CAR-T cells was reduced by cPD-L1 expression on target cells. However, expression of the CSR_{WT} but not the CSR_{MUT} restored cytotoxicity against cPD-$L1^+$ targets compared to CAR alone. As seen with cytokine production, increased cytotoxicity observed with the CAR+CSR_{WT} only occurred against cPD-L1-expressing target cells, indicating the effect was mediated by cPD-L1 interaction with the CSR_{WT}. Since CAR-T cell interaction with cPD-$L1^+$ target cells reduced the central memory phenotype and increased effector memory phenotype of $CD8^+$ CAR-T cells (Figure 2D), the hypothesis that expression of a CSR will prevent cPD-L1-mediated phenotypic changes was explored. CAR-T cells with or without a CSR were stimulated with K562-cCD20 or K562-cCD20-cPD-L1 target cells at 1:1 ratio three times and the effects of the CSR_{WT} and CSR_{MUT} on CAR-T phenotype were determined after the third stimulation (Figure 4C). CAR-T cells and CAR+CSR_{MUT} showed a significant reduction in the frequency of $CD8^+$ central memory-like T cells after stimulation with cPD-$L1^+$ targets. By contrast, expression of the CSR_{WT} prevented the reduction in $CD8^+$ central memory-like T cells. Taken together, these data demonstrate that expression of an active CSR enhances CAR-T effector function against cPD-$L1^+$ target cells and maintains $CD8^+$ central memory CAR-T phenotype following repetitive stimulation through the CAR.

CSR_{WT} expression augments canine CAR-T cell function against B cell lymphoma cells

While CAR-T cells expressing the CSR_{WT} enhance CAR-T effector function against target cells engineered to express high levels of cPD-L1, it is unknown whether similar effects would be observed against target cells that express endogenous levels of cPD-L1. For this set of experiments, the $CD20^+$ canine B cell lymphoma cell line (CLBL-1) was used as the target. The CLBL-1 cell line was derived from a canine patient with

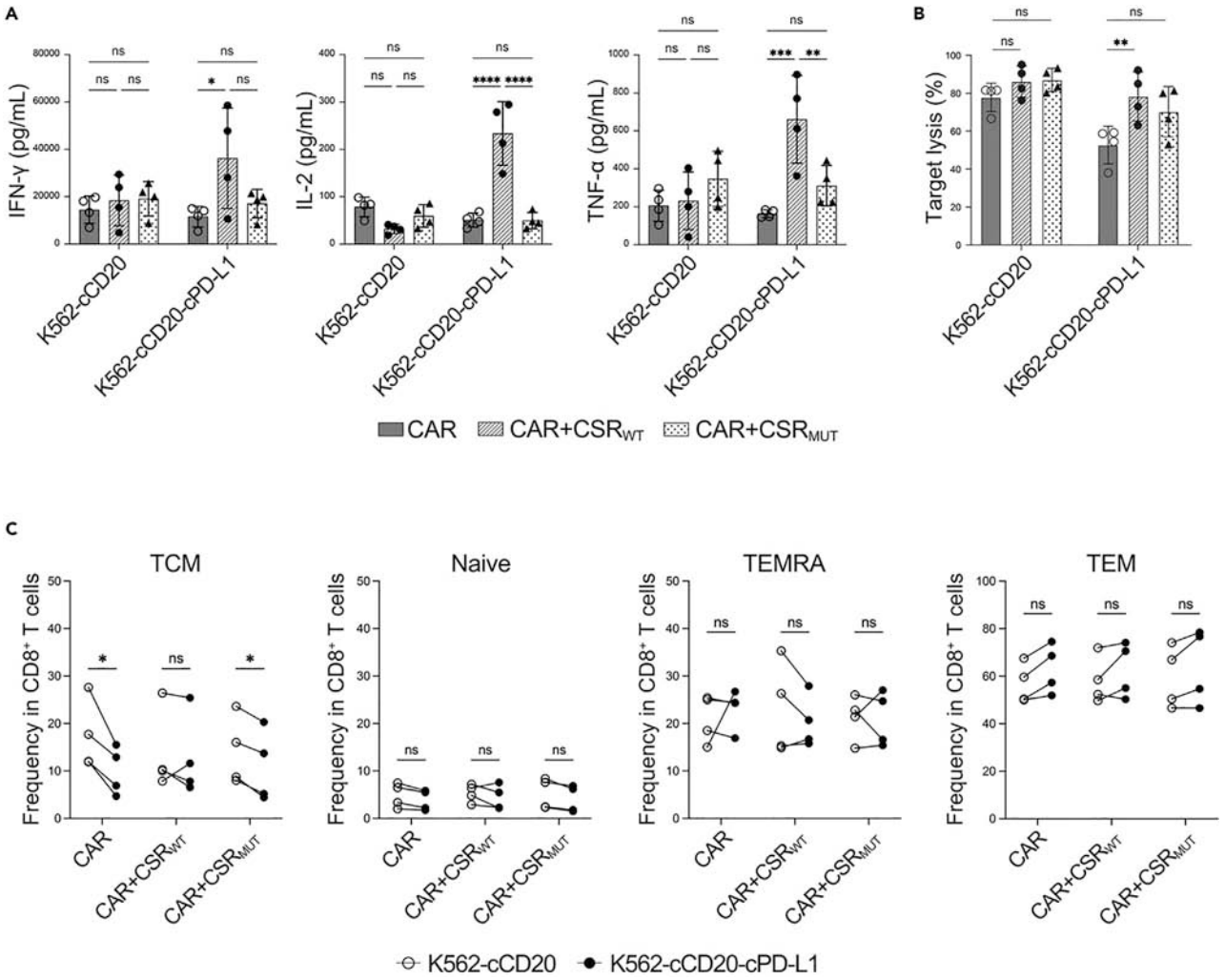


Figure 4. An active PD-1/CD28 CSR augments CAR-T function against PD-L1 engineered target cells

(A) IFN-γ, TNF-α, and IL-2 production by CAR+CSR$_{WT}$ or CAR+CSR$_{MUT}$ after 24 h co-culture with K562-cCD20 or K562-cCD20-cPD-L1 target cells at an E:T ratio of 1:1. Data are mean ± SD (n = 4 biological replicates).

(B) Cytotoxicity of CAR, CAR+CSR$_{WT}$, or CAR+CSR$_{MUT}$ against K562-cCD20 and K562-cCD20-cPD-L1 target cells at E:T ratio of 8:1. Data are mean ± SD (n = 4 biological replicates).

(C) Memory phenotype of CAR, CAR+CSR$_{WT}$, or CAR+CSR$_{MUT}$ T cells determined 6 days after the third stimulation with K562-cCD20 and K562-cCD20-cPD-L1 target cells at an E:T ratio of 1:1. Data for each of the four dogs are shown. *$p < 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$, ns: not significant.

spontaneous B cell lymphoma and naturally expresses both cCD20 and cPD-L1.[40] The experimental design is outlined in Figure 5A. CLBL-1 cells were first edited to disrupt cPD-L1 (CLBL-1ΔcPD-L1) and lack of cPD-L1 expression was confirmed by flow cytometry (Figure 5B). CAR-T cells and CAR+CSR$_{WT}$ were generated from three healthy donor dogs and CAR$^+$ cells were enriched by positive selection. Analysis by flow cytometry confirmed that there was no significant difference in the percentage of CAR-expressing CD4$^+$ or CD8$^+$ T cells between the CAR only and CAR+CSR$_{WT}$ populations (Figure 5C), nor was there any difference in the CD4:CD8 CAR-T ratio between constructs (data not shown). To determine whether endogenous levels of cPD-L1 on CLBL-1 cells inhibited CAR-T function, CAR, and CAR+CSR$_{WT}$ T cells were repetitively stimulated with CLBL-1 cells or CLBL-1ΔcPD-L1 cells. Supernatants were collected 24 h after the 5th stimulation and analyzed for IFN-γ and TNF-α (Figure 5D). CAR-T cells from two out of three dogs cultured with CLBL-1ΔcPD-L1 cells produced significantly more IFN-γ and TNF-α then those cultured with unedited CLBL-1 cells. These findings indicate that intrinsic expression of cPD-L1 by CLBL-1 cells suppresses CAR-T cytokine production. Furthermore, in all three dogs evaluated, CAR+CSR$_{WT}$ produced significantly more IFN-γ and (in two out of the three dogs) more TNF-α when co-cultured with CLBL-1 cells compared to CAR-T cells without the CSR$_{WT}$ (Figure 5E). To determine whether incorporation of the CSR$_{WT}$ enhanced cytotoxicity against CLBL-1 cells, CAR-T, and CAR-T+CSR$_{WT}$ cells were first stimulated with K562-CD20-cPD-L1 target cells 3 times at 3-day intervals before co-culture with CLBL-1 cells and assessment of cytotoxicity. In CAR-T cells derived from 2 healthy dogs, the inclusion of the CSR$_{WT}$ showed a modest increase in cytotoxic activity against CLBL-1 cells at lower E:T ratios (Figure 5F).

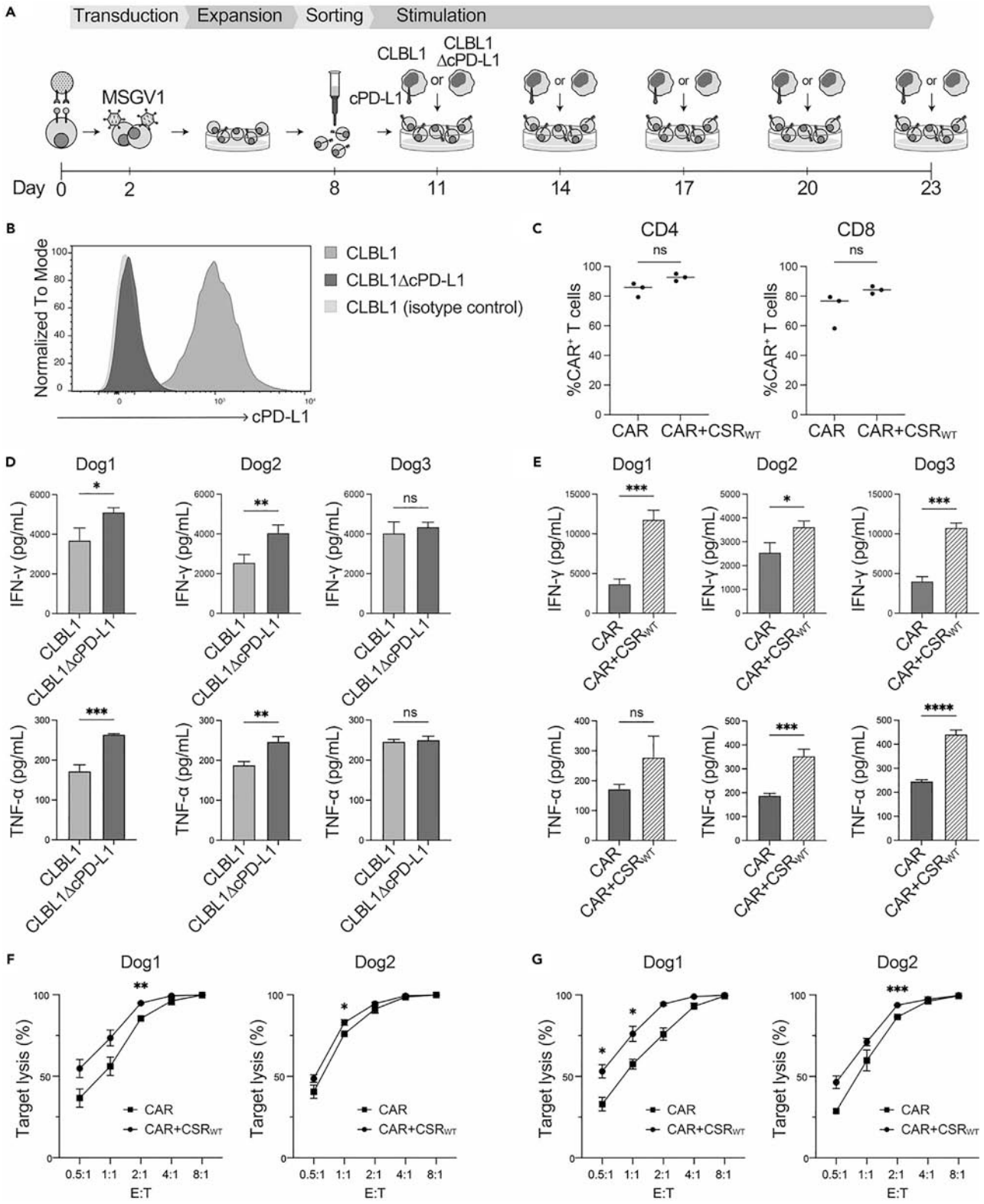


Figure 5. An active PD-1/CD28 CSR augments CAR-T function against canine B cell lymphoma

(A) Schematic of experimental timeline of repeat stimulation of CAR-T cells with WT or CLBL-1ΔcPD-L1 target cells.

(B) Confirmation of lack of cPD-L1 expression by CLBL-1ΔcPD-L1 cells by flow cytometry. Plots are gated on 7AAD^{-} cells.

Figure 5. *Continued*

(C) Cell surface expression of CAR on $CD4^+$ and $CD8^+$ CAR-T and CAR+CSR_{WT} T cells from healthy donor dogs following cell sorting as determined by flow cytometry. Data from three individual dogs are shown.

(D) IFN-γ and TNF-α production by CAR-T cells stimulated with CLBL-1 cells or CLBL-1ΔcPD-L1 cells after 5 rounds of stimulation at an E:T of 1:1. IFN-γ and TNF-αproduction was assessed in supernatants 24 h after the fifth stimulation. Data are mean ± SD of 3 technical replicates for each dog.

(E) CAR-T and CSR_{WT}CAR-T cells from healthy dogs were co-cultured with CLBL-1 target cells at an E:T ratio of 1:1. Supernatants were harvested at 24 h after the 5^{th} stimulation and assayed for IFN-γ and TNF-α production.

(F and G) CAR-T and CSR_{WT} CAR-T cells from 2 healthy dogs were stimulated 3 times every 3 days with K562-cCD20-cPD-L1 target cells and then co-cultured with (F) CLBL-1 cells or (G) CLBL-1 cells pre-treated with IFN-γ for 48 h, at increasing E:T ratios. Cytotoxicity was determined by luciferase assay at 24 h. Data are mean ± SD of 3 technical replicates for each dog are shown. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns: not significant.

Similarly, when CLBL-1 cells were pre-treated with IFN-γ to increase surface expression of cPD-L1 (Figure S1), CAR-T+CSR_{WT} showed greater cytotoxicity against the pre-treated CLBL-1 cells compared to CAR-T cells alone (Figure 5G). The enhancement of CAR-T cell cytotoxicity against CLBL-1 cells by inclusion of the CSR_{WT} was only modest in these *in vitro* studies and may be of questionable significance in improving anti-tumor activity of CAR-T cell *in vivo*. However, collectively, these results provide proof of concept that endogenous levels of cPD-L1 on canine B cell lymphoma cells are sufficient to suppress CAR-T function and to augment the function of CAR-T cells expressing a functional CSR, particularly when cPD-L1 is up-regulated by IFN-γ.

DISCUSSION

The clinical responses of patients with relapsed/refractory hematological malignancies and solid tumor histologies to CAR-T cells and checkpoint inhibitors respectively has accelerated investigations to further improve therapeutic efficacy. Tumor heterogeneity, the immune suppressive tumor microenvironment, and a low central memory, highly exhausted T cell phenotype have emerged as key factors responsible for clinical failures of different immunotherapeutic approaches. Despite the increasing sophistication and complexity of genetically engineered mouse models, the barriers to effective immunotherapies are challenging to faithfully recapitulate, emphasizing the need for spontaneous, immune competent, cancer "models" where the complex, evolving interplay between tumor cells and the immune system can more closely parallel that of human patients. To this end, attention has turned to immune competent pet dogs that spontaneously develop cancers which share similar clinical, biological, and molecular features to human cancers. Superior to other preclinical models, pet dogs also share the same environment and have comparable gut microbiota as their owners, which may influence the clinical response to immune therapies including checkpoint inhibitors.[41–43] Furthermore, their shorter life span and compressed disease time course expedites clinical trial performance and outcomes. Therefore, pet dogs with cancer can serve as a parallel patient population in which to advance next generation immunotherapeutic approaches designed to overcome current barriers and improve therapeutic response. These efforts are supported by the National Cancer Institute and a network of comparative immune-oncology translational researchers (www.precinctnetwork.org) performing preclinical trials in canine cancer patients with the ultimate goal to identify correlative biomarkers that may inform human trial design and accelerate human translation.

B cell lymphoma in dogs is often highly aggressive and mimics human activated B cell (ABC)-DLBCL with constitutive canonical nuclear factor κB (NF-κB) activity and Myc:IgH translocations.[44,45] These features are associated with poor prognosis in humans, and suggest that dogs with spontaneous DLBCL are a valuable patient population to evaluate next generation CAR-T approaches to improve therapeutic response. *In vitro*, canine $CD20^-$CD28-ζ and CD20-BB-ζ CAR-T cells exhibited antigen-specific proliferation, cytokine production, and cytotoxicity.[31,46] *In vivo* use of autologous cCD20-CD28-ζ and cCD20-BB-ζ CAR-T cells in dogs with BCL resulted in cCD20 down-regulation, target antigen loss, and cytokine release, although durable clinical responses have yet to be realized.[31,33] These findings underscore the comparable barriers to effective CAR-T therapy in canine and human patients and suggest that shared mechanisms of primary resistance might contribute in part to the lack of clinical effect of CAR-T cells in dogs with BCL.

High expression of PD-L1 on malignant B cells of patients with ABC-DLBCL correlates with shorter overall survival and less favorable clinical response to CAR-T therapy.[47–49] Therefore, various approaches are being explored to overcome PD-1:PD-L1 mediated suppression of CAR-T cells in patients with BCL and other solid tumors, including the use of anti-PD-1 and anti-PD-L1 antibodies, constitutive expression of an anti-PD-1 scFv by CAR-T cells, and knockout/knockdown of PD-1 in CAR-T cells.[10,15,21,38] In a recent small study using RNA scope, PD-L1 expression in canine DLBCL also correlated with shorter time to progression and lymphoma specific survival.[50] Therefore, we hypothesized that PD-L1 expression on malignant B cells may contribute to CAR-T hypofunction and lack of clinical response to CD20 targeted CAR-T therapy in the dog. Our *in vitro* data supports this hypothesis showing for the first time that target cell expression of cPD-L1 inhibits the function of canine CAR-T cells and reduces their central memory T cell phenotype, which correlates with clinical responses to CART-19 in human CLL patients.[36] These findings parallel those of human CAR-T cells and support the use of canine DLBCL patients to overcome PD-L1 mediated CAR-T cell hypofunction.

Unlike concurrent administration of PD-1 or PD-L1 blocking antibodies, editing CAR-T cells to secrete anti-PD1 minibodies or to disrupt PD-1, the use of a PD-1/CD28 CSR augments CAR-T function following PD-L1 engagement both *in vitro* and *in vivo* in murine models using human and murine CAR-T cells.[25,51] Furthermore, pilot studies of CAR-T cells expressing a PD-1/CD28 CSR have shown promise in human patients with relapsed/refractory B cell lymphoma, including those who had disease progression after CD19 CAR-T treatment.[24,52] However, the safety, *in vivo* efficacy, and the identification of correlative biomarkers of response to CSR CAR-T cells need further investigation

in a faithful preclinical model. In particular, questions of safety emerge from concerns of more dramatic cytokine release together with off-tumor targeting of PD-L1$^+$ antigen-presenting cells and/or endogenous T cells.

We therefore generated canine PD-1/CD28 CSR constructs that parallel those used in human *in vitro* CAR-T studies and determined whether they would augment canine CAR-T function against PD-L1$^+$ targets. We found that transduction efficiency of the CAR-CSR constructs was higher than the CAR construct alone, a finding also reported in human T cells.[25] This finding could be associated with cPD-L1 expression on T cells and enhanced proliferation of CAR-CSR$_{WT}$ cells through CD28 signaling of CSR. However, the same phenomenon is observed for CAR-CSR$_{MUT}$ T cells suggesting competition with endogenous cPD-1 may also contribute to the higher percentage transduction efficacy. Our results suggest that cPD-1 signaling (via cPD-L1 expression on target cells) inhibits TCM-like differentiation of canine CAR-T cells containing the 4-1BB intracellular signaling domain. These results are consistent with the literature that shows that PD-1 engagement limits TCM differentiation of 4-1BB containing CAR-T cells.[53] Mechanistically, it is proposed that PD-1 signaling abrogates oxidative phosphorylation driven by the 4-1BB co-stimulatory domain of the CAR in human CAR-T cells and that this down-regulation of OXPHOS suppresses TCM differentiation. Further, PD-1 blockade via the inclusion of anti-PD1 antibody in these published studies enhanced central memory differentiation of CAR-T cells.[53]

Our finding that the active CD28 domain of the CSR$_{WT}$ was necessary to maintain the central memory-like phenotype following CAR-T stimulation with PD-L1$^+$ target cells suggests that this effect of the CSR$_{WT}$ is not a result of reduced PD-1 signaling but is more likely associated with the known role of CD28 signaling in providing pro-survival signals to CD8$^+$ memory T cells.[54] It is also consistent with the findings of third-generation CAR-T cells containing both 4-1BB and CD28 co-stimulatory domains that show enhanced expansion, persistence, and higher frequency of central memory T cells compared to second-generation constructs.[55] Additional studies in canine CAR-T cells are required to determine whether 4-1BB containing CARs drive OXPHOS and promote TCM differentiation and the effects of PD-1 signaling and the PD-1/CD28 CSR signaling on canine T cell metabolism.

In our experiments, we used CD45RA and CD62L to define canine T cell memory-like subsets as previously described, where assignment of subset was based on changes in subset proportions and their differential production of TNF-α in response to mitogen stimulation.[56] We recognize the limitations of this approach and additional transcriptomic and functional studies are needed to better validate this phenotypic subset distinction in the dog. A recent single-cell RNA sequencing (scRNA-seq) analysis of canine peripheral blood leukocytes was published that characterizes leukocyte subsets and reveals gene signatures for Tnaive, Teff, and TCM for CD4$^+$ and CD8$^+$ T cell clusters.[57] However, transcriptomic data of T cell subsets defined by the combination of CD45RA and CD62L phenotypic markers has not been reported.

Enhancement of T cell function by CSR has been shown to be dependent upon PD-L1 expression levels in tumor cells.[58] Although expression of cPD-L1 on the canine B cell lymphoma cell line (CLBL-1) was lower than our engineered K562 target cells, it was sufficient to inhibit CAR-T function. Furthermore, incubation of CLBL-1 cells with cIFN-γ increased cPD-L1 expression, mimicking the likely *in vivo* scenario in which IFN-γ from activated CAR-T cells augments PD-L1 expression on tumor cells and further inhibits CAR-T function. Expression of the CSR$_{WT}$ overcame PD-L1-mediated inhibition, promoting cytokine production, and cytotoxicity, although CSR$_{WT}$ effects on cytotoxicity were modest. While these results provide proof of concept for evaluating this CSR strategy to augment CAR-T efficacy against PD-L1$^+$ tumor targets *in vivo* studies are required to determine whether the observed *in vitro* effects of the CSR$_{WT}$ will translate into improved clinical outcome in canine cancer patients.

The implications of our findings that canine CAR-T cells expressing a PD-1/CD28 CSR overcome cPD-L1 mediated suppression in a similar manner to human CAR-T cells expressing a PD-1/CD28 CSR, provide compelling support for initiating clinical trials of CAR-T plus PD-1/CD28 CSR in dogs with PD-L1$^+$ BCL to provide information regarding safety, persistence, efficacy, and potential correlative biomarkers of response that may direct human patient stratification to future switch receptor clinical trials. Canine trials also offer the opportunity to address important aspects related to CAR-T cell exhaustion in clinical settings, including whether a PD-1/CD28 switch receptor can rescue CAR-T cells from inhibition caused by other checkpoint molecules such as LAG-3, TIM-3, and TIGIT.

Limitations of the study

Augmenting CAR-T function against PD-L1 expressing tumor cells aims to overcome a significant barrier to clinical efficacy however, it is unknown whether CAR-T cells expressing CSR as described, are safe and effective *in vivo*. Concerns exist over increased risk of cytokine release or activation induced cell death of the CSR CAR-T, reducing *in vivo* clinical effect. Our study provides proof of concept that CSR expressing canine CAR-T cells display similar enhanced functional characteristics against PD-L1$^+$ target cells as CSR-expressing human CAR-T cells and set the stage for *in vivo* evaluation of this approach to enhance CAR-T activity in dogs with naturally occurring BCL. However, it is recognized that the target cells used in this study were either an engineered human K562 cell line or a canine B cell lymphoma cell line. As such, it is possible that xenogenic or allogeneic activation of canine T cells contributed to their functionality against CD20$^+$ target cells. Such allogeneic activation would be absent using an autologous approach in the canine clinic and as such, *in vivo* cytotoxicity and cytokine secretion may not be as robust as suggested by our *in vitro* studies. In addition, our studies used CAR-T cells generated from healthy donor dogs, and it is unknown whether similar antigen-specific effector functions would be seen in CAR-T cells derived from canine BCL patients where the disease process itself and/or prior rounds of cytotoxic chemotherapy may adversely affect the function of autologous CAR-T cells and the effects of the CSR. We have previously demonstrated that CAR-T cells can be generated from canine patients with relapsed BCL and show evidence of activation and CD20$^+$ B cell targeting *in vivo*.[31,33] Further, our studies have focused on cPD-1:cPD-L1 and it remains unknown whether other checkpoints such as TIM3, LAG3, or TIGIT will have a dominant inhibitory effect on human and canine CAR-T cells despite the expression of the PD1/CD28 CSR. In total, our work serves as a proof of concept to inspire future studies dissecting mechanisms and effects of other pathways/inhibitory cytokines on CAR-T cell outcome through pre-clinical trials in dogs with relapsed/refractory BCL or solid tumors that are comparable with their human counterparts.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Nicola J Mason (nmason@vet.upenn.edu).

Materials availability

Unique reagents generated in this study include anti-canine PD-1 antibody, cCD20-BB-ζ CAR T cells, cCD20-BB-ζ CAR T cells with PD-1/CD28 CSR_{WT} and cCD20-BB-ζ CAR T cells with PD-1/CD28 CSR_{MUT}. Requests for reagents should be addressed to N.J.M.

Data and code availability

- All data presented in this study will be shared upon reasonable request by the lead contact, Nicola J Mason (nmason@vet.upenn.edu).
- This paper does not report any original code.
- Additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

ACKNOWLEDGMENTS

This research was funded by the Mason Cancer Research Fund (N.J.M). N.J.M. is supported by U01-CA-272270-01 and U24-CA-224122-02. S.Y. is supported by the Japan Society for the Promotion of Science KAKENHI program grant numbers JP21J01155 and JP22K15018. A.K. is supported by the Japan Society for the Promotion of Science, KAKENHI program grant number JP24KJ2120. A.R. is supported by U01-CA-272270-01 and U24-CA-224122-02. We would like to thank Dr. Barbara Rütgen for her generous gift of the CLBL-1 cells, Ms. Amiko Saito Yoshimoto for her assistance preparing the manuscript figures, and Dr. Aimee Payne for her helpful review and comments.

AUTHOR CONTRIBUTIONS

Conceptualization and methodology, S.Y., A.R., and N.J.M.; validation, S.Y., A.K.,and L.O.; formal analysis, S.Y., A.K., A.R., and L.O.; investigation, S.Y., A.K., L.O., and K.F.; resources, S.T. and N.J.M.; data curation, S.Y., L.O., and N.J.M.; writing—original draft preparation, S.Y., K.F., and N.J.M.; writing, review, and editing, S.Y., A.R., K.F., S.T., and N.J.M; supervision, S.T. and N.J.M.; funding acquisition, S.Y., S.T., and N.J.M. All authors have read and agreed to the published version of the manuscript.

DECLARATION OF INTERESTS

N.J.M is a co-founder of Vetigenics.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS
  - Cells, cell lines and cell culture
  - Generation of cPD-L1 edited CLBL-1 cells
- METHOD DETAILS
  - Vectors and constructs
  - Generation of canine CAR-T cells
  - Enrichment of canine CAR-T cells
  - Flow cytometry
  - Cytotoxicity assay
  - Cytokine Quantification
  - Proliferation assay
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.110863.

Received: January 10, 2024
Revised: July 7, 2024
Accepted: August 29, 2024
Published: August 31, 2024

REFERENCES

1. Maude, S.L., Laetsch, T.W., Buechner, J., Rives, S., Boyer, M., Bittencourt, H., Bader, P., Verneris, M.R., Stefanski, H.E., Myers, G.D., et al. (2018). Tisagenlecleucel in Children and Young Adults with B-Cell Lymphoblastic Leukemia. N. Engl. J. Med. *378*, 439–448. https://doi.org/10.1056/NEJMoa1709866.

2. Munshi, N.C., Anderson, L.D., Jr., Shah, N., Madduri, D., Berdeja, J., Lonial, S., Raje, N., Lin, Y., Siegel, D., Oriol, A., et al. (2021). Idecabtagene Vicleucel in Relapsed and Refractory Multiple Myeloma. N. Engl. J. Med. *384*, 705–716. https://doi.org/10.1056/NEJMoa2024850.

3. Neelapu, S.S., Locke, F.L., Bartlett, N.L., Lekakis, L.J., Miklos, D.B., Jacobson, C.A., Braunschweig, I., Oluwole, O.O., Siddiqi, T., Lin, Y., et al. (2017). Axicabtagene Ciloleucel CAR T-Cell Therapy in Refractory Large B-Cell Lymphoma. N. Engl. J. Med. *377*, 2531–2544. https://doi.org/10.1056/NEJMoa1707447.

4. Shah, B.D., Ghobadi, A., Oluwole, O.O., Logan, A.C., Boissel, N., Cassaday, R.D., Leguay, T., Bishop, M.R., Topp, M.S., Tzachanis, D., et al. (2021). KTE-X19 for relapsed or refractory adult B-cell acute lymphoblastic leukaemia: phase 2 results of the single-arm, open-label, multicentre ZUMA-3 study. Lancet *398*, 491–502. https://doi.org/10.1016/S0140-6736(21)01222-8.
5. Newsam, A.D., Coughlin, C.A., Trabolsi, A., and Schatz, J.H. (2023). Functional drivers of resistance to anti-CD19 CAR-T cell therapy in diffuse large B cell lymphoma. Leuk. Lymphoma *64*, 2217–2224. https://doi.org/10.1080/10428194.2023.2258244.
6. Joyce, J.A., and Fearon, D.T. (2015). T cell exclusion, immune privilege, and the tumor microenvironment. Science *348*, 74–80. https://doi.org/10.1126/science.aaa6204.
7. Newick, K., O'Brien, S., Moon, E., and Albelda, S.M. (2017). CAR T Cell Therapy for Solid Tumors. Annu. Rev. Med. *68*, 139–152. https://doi.org/10.1146/annurev-med-062315-120245.
8. Deng, Q., Han, G., Puebla-Osorio, N., Ma, M.C.J., Strati, P., Chasen, B., Dai, E., Dang, M., Jain, N., Yang, H., et al. (2020). Characteristics of anti-CD19 CAR T cell infusion products associated with efficacy and toxicity in patients with large B cell lymphomas. Nat. Med. *26*, 1878–1887. https://doi.org/10.1038/s41591-020-1061-7.
9. Jackson, Z., Hong, C., Schauner, R., Dropulic, B., Caimi, P.F., de Lima, M., Giraudo, M.F., Gupta, K., Reese, J.S., Hwang, T.H., and Wald, D.N. (2022). Sequential Single-Cell Transcriptional and Protein Marker Profiling Reveals TIGIT as a Marker of CD19 CAR-T Cell Dysfunction in Patients with Non-Hodgkin Lymphoma. Cancer Discov. *12*, 1886–1903. https://doi.org/10.1158/2159-8290.CD-21-1586.
10. Moon, E.K., Wang, L.C., Dolfi, D.V., Wilson, C.B., Ranganathan, R., Sun, J., Kapoor, V., Scholler, J., Puré, E., Milone, M.C., et al. (2014). Multifactorial T-cell hypofunction that is reversible can limit the efficacy of chimeric antigen receptor-transduced human T cells in solid tumors. Clin. Cancer Res. *20*, 4262–4273. https://doi.org/10.1158/1078-0432.CCR-13-2627.
11. Sworder, B.J., Kurtz, D.M., Alig, S.K., Frank, M.J., Shukla, N., Garofalo, A., Macaulay, C.W., Shahrokh Esfahani, M., Olsen, M.N., Hamilton, J., et al. (2023). Determinants of resistance to engineered T cell therapies targeting CD19 in large B cell lymphomas. Cancer Cell *41*, 210–225.e5. https://doi.org/10.1016/j.ccell.2022.12.005.
12. Sharpe, A.H., and Pauken, K.E. (2018). The diverse functions of the PD1 inhibitory pathway. Nat. Rev. Immunol. *18*, 153–167. https://doi.org/10.1038/nri.2017.108.
13. Freeman, G.J., Long, A.J., Iwai, Y., Bourque, K., Chernova, T., Nishimura, H., Fitz, L.J., Malenkovich, N., Okazaki, T., Byrne, M.C., et al. (2000). Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. J. Exp. Med. *192*, 1027–1034. https://doi.org/10.1084/jem.192.7.1027.
14. Hui, E., Cheung, J., Zhu, J., Su, X., Taylor, M.J., Wallweber, H.A., Sasmal, D.K., Huang, J., Kim, J.M., Mellman, I., and Vale, R.D. (2017). T cell costimulatory receptor CD28 is a primary target for PD-1-mediated inhibition. Science *355*, 1428–1433. https://doi.org/10.1126/science.aaf1292.
15. John, L.B., Devaud, C., Duong, C.P.M., Yong, C.S., Beavis, P.A., Haynes, N.M., Chow, M.T., Smyth, M.J., Kershaw, M.H., and Darcy, P.K. (2013). Anti-PD-1 antibody therapy potently enhances the eradication of established tumors by gene-modified T cells. Clin. Cancer Res. *19*, 5636–5646. https://doi.org/10.1158/1078-0432.CCR-13-0458.
16. Serganova, I., Moroz, E., Cohen, I., Moroz, M., Mane, M., Zurita, J., Shenker, L., Ponomarev, V., and Blasberg, R. (2017). Enhancement of PSMA-Directed CAR Adoptive Immunotherapy by PD-1/PD-L1 Blockade. Mol. Ther. Oncolytics *4*, 41–54. https://doi.org/10.1016/j.omto.2016.11.005.
17. Adusumilli, P.S., Zauderer, M.G., Rivière, I., Solomon, S.B., Rusch, V.W., O'Cearbhaill, R.E., Zhu, A., Cheema, W., Chintala, N.K., Halton, E., et al. (2021). A Phase I Trial of Regional Mesothelin-Targeted CAR T-cell Therapy in Patients with Malignant Pleural Disease, in Combination with the Anti-PD-1 Agent Pembrolizumab. Cancer Discov. *11*, 2748–2763. https://doi.org/10.1158/2159-8290.CD-21-0407.
18. Chong, E.A., Alanio, C., Svoboda, J., Nasta, S.D., Landsburg, D.J., Lacey, S.F., Ruella, M., Bhattacharyya, S., Wherry, E.J., and Schuster, S.J. (2022). Pembrolizumab for B-cell lymphomas relapsing after or refractory to CD19-directed CAR T-cell therapy. Blood *139*, 1026–1038. https://doi.org/10.1182/blood.2021012634.
19. Chong, E.A., Melenhorst, J.J., Lacey, S.F., Ambrose, D.E., Gonzalez, V., Levine, B.L., June, C.H., and Schuster, S.J. (2017). PD-1 blockade modulates chimeric antigen receptor (CAR)-modified T cells: refueling the CAR. Blood *129*, 1039–1041. https://doi.org/10.1182/blood-2016-09-738245.
20. Li, S., Siriwon, N., Zhang, X., Yang, S., Jin, T., He, F., Kim, Y.J., Mac, J., Lu, Z., Wang, S., et al. (2017). Enhanced Cancer Immunotherapy by Chimeric Antigen Receptor-Modified T Cells Engineered to Secrete Checkpoint Inhibitors. Clin. Cancer Res. *23*, 6982–6992. https://doi.org/10.1158/1078-0432.CCR-17-0867.
21. Rafiq, S., Yeku, O.O., Jackson, H.J., Purdon, T.J., van Leeuwen, D.G., Drakes, D.J., Song, M., Miele, M.M., Li, Z., Wang, P., et al. (2018). Targeted delivery of a PD-1-blocking scFv by CAR-T cells enhances anti-tumor efficacy *in vivo*. Nat. Biotechnol. *36*, 847–856. https://doi.org/10.1038/nbt.4195.
22. Choi, B.D., Yu, X., Castano, A.P., Darr, H., Henderson, D.B., Bouffard, A.A., Larson, R.C., Scarfò, I., Bailey, S.R., Gerhard, G.M., et al. (2019). CRISPR-Cas9 disruption of PD-1 enhances activity of universal EGFRvIII CAR T cells in a preclinical model of human glioblastoma. J. Immunother. Cancer *7*, 304. https://doi.org/10.1186/s40425-019-0806-7.
23. Chen, C., Gu, Y.M., Zhang, F., Zhang, Z.C., Zhang, Y.T., He, Y.D., Wang, L., Zhou, N., Tang, F.T., Liu, H.J., and Li, Y.M. (2021). Construction of PD1/CD28 chimeric-switch receptor enhances anti-tumor ability of c-Met CAR-T in gastric cancer. OncoImmunology *10*, 1901434. https://doi.org/10.1080/2162402X.2021.1901434.
24. Liang, Y., Liu, H., Lu, Z., Lei, W., Zhang, C., Li, P., Liang, A., Young, K.H., and Qian, W. (2021). CD19 CAR-T expressing PD-1/CD28 chimeric switch receptor as a salvage therapy for DLBCL patients treated with different CD19-directed CAR T-cell therapies. J. Hematol. Oncol. *14*, 26. https://doi.org/10.1186/s13045-021-01044-y.
25. Liu, X., Ranganathan, R., Jiang, S., Fang, C., Sun, J., Kim, S., Newick, K., Lo, A., June, C.H., Zhao, Y., and Moon, E.K. (2016). A Chimeric Switch-Receptor Targeting PD1 Augments the Efficacy of Second-Generation CAR T Cells in Advanced Solid Tumors. Cancer Res. *76*, 1578–1590. https://doi.org/10.1158/0008-5472.CAN-15-2524.
26. Mason, N.J., Chester, N., Xiong, A., Rotolo, A., Wu, Y., Yoshimoto, S., Glassman, P., Gulendran, G., and Siegel, D.L. (2021). Development of a fully canine anti-canine CTLA4 monoclonal antibody for comparative translational research in dogs with spontaneous tumors. mAbs *13*, 2004638. https://doi.org/10.1080/19420862.2021.2004638.
27. Mata, M., Vera, J.F., Gerken, C., Rooney, C.M., Miller, T., Pfent, C., Wang, L.L., Wilson-Robles, H.M., and Gottschalk, S. (2014). Toward immunotherapy with redirected T cells in a large animal model: ex vivo activation, expansion, and genetic modification of canine T cells. J. Immunother. *37*, 407–415. https://doi.org/10.1097/CJI.0000000000000052.
28. Sakai, O., Igase, M., and Mizuno, T. (2020). Optimization of canine CD20 chimeric antigen receptor T cell manufacturing and *in vitro* cytotoxic activity against B-cell lymphoma. Vet. Comp. Oncol. *18*, 739–752. https://doi.org/10.1111/vco.12602.
29. Zhang, S., Black, R.G., Kohli, K., Hayes, B.J., Miller, C., Koehne, A., Schroeder, B.A., Abrams, K., Schulte, B.C., Alexiev, B.A., et al. (2022). B7-H3 Specific CAR T Cells for the Naturally Occurring, Spontaneous Canine Sarcoma Model. Mol. Cancer Therapeut. *21*, 999–1009. https://doi.org/10.1158/1535-7163.MCT-21-0726.
30. Rotolo, A., Atherton, M.J., Kasper, B.T., Haran, K.P., and Mason, N.J. (2021). Genetic re-direction of canine primary T cells for clinical trial use in pet dogs with spontaneous cancer. STAR Protoc. *2*, 100905. https://doi.org/10.1016/j.xpro.2021.100905.
31. Panjwani, M.K., Atherton, M.J., MaloneyHuss, M.A., Haran, K.P., Xiong, A., Gupta, M., Kulikovsaya, I., Lacey, S.F., and Mason, N.J. (2020). Establishing a model system for evaluating CAR T cell therapy using dogs with spontaneous diffuse large B cell lymphoma. OncoImmunology *9*, 1676615. https://doi.org/10.1080/2162402X.2019.1676615.
32. Shah, N.N., and Fry, T.J. (2019). Mechanisms of resistance to CAR T cell therapy. Nat. Rev. Clin. Oncol. *16*, 372–385. https://doi.org/10.1038/s41571-019-0184-6.
33. Atherton, M.J., Rotolo, A., Haran, K.P., and Mason, N.J. (2022). Case Report: Clinical and Serological Hallmarks of Cytokine Release Syndrome in a Canine B Cell Lymphoma Patient Treated With Autologous CAR-T Cells. Front. Vet. Sci. *9*, 824982. https://doi.org/10.3389/fvets.2022.824982.
34. Yoshimoto, S., Chester, N., Xiong, A., Radaelli, E., Wang, H., Brillantes, M., Gulendran, G., Glassman, P., Siegel, D.L., and Mason, N.J. (2023). Development and pharmacokinetic assessment of a fully canine anti-PD-1 monoclonal antibody for comparative translational research in dogs with spontaneous tumors. mAbs *15*, 2287250. https://doi.org/10.1080/19420862.2023.2287250.
35. Choi, J.W., Withers, S.S., Chang, H., Spanier, J.A., De La Trinidad, V.L., Panesar, H., Fife, B.T., Sciammas, R., Sparger, E.E., Moore, P.F., et al. (2020). Development of canine PD-1/PD-L1 specific monoclonal antibodies and

amplification of canine T cell function. PLoS One *15*, e0235518. https://doi.org/10.1371/journal.pone.0235518.

36. Fraietta, J.A., Lacey, S.F., Orlando, E.J., Pruteanu-Malinici, I., Gohil, M., Lundh, S., Boesteanu, A.C., Wang, Y., O'Connor, R.S., Hwang, W.T., et al. (2018). Determinants of response and resistance to CD19 chimeric antigen receptor (CAR) T cell therapy of chronic lymphocytic leukemia. Nat. Med. *24*, 563–571. https://doi.org/10.1038/s41591-018-0010-1.
37. Allie, S.R., Zhang, W., Fuse, S., and Usherwood, E.J. (2011). Programmed death 1 regulates development of central memory CD8 T cells after acute viral infection. J. Immunol. *186*, 6280–6286. https://doi.org/10.4049/jimmunol.1003870.
38. Cherkassky, L., Morello, A., Villena-Vargas, J., Feng, Y., Dimitrov, D.S., Jones, D.R., Sadelain, M., and Adusumilli, P.S. (2016). Human CAR T cells with cell-intrinsic PD-1 checkpoint blockade resist tumor-mediated inhibition. J. Clin. Invest. *126*, 3130–3144. https://doi.org/10.1172/JCI83092.
39. Shen, L., Li, H., Bin, S., Li, P., Chen, J., Gu, H., and Yuan, W. (2019). The efficacy of third generation anti-HER2 chimeric antigen receptor T cells in combination with PD1 blockade against malignant glioblastoma cells. Oncol. Rep. *42*, 1549–1557. https://doi.org/10.3892/or.2019.7263.
40. Rütgen, B.C., Hammer, S.E., Gerner, W., Christian, M., de Arespacochaga, A.G., Willmann, M., Kleiter, M., Schwendenwein, I., and Saalmuller, A. (2010). Establishment and characterization of a novel canine B-cell line derived from a spontaneously occurring diffuse large cell lymphoma. Leuk. Res. *34*, 932–938. https://doi.org/10.1016/j.leukres.2010.01.021.
41. Matson, V., Fessler, J., Bao, R., Chongsuwat, T., Zha, Y., Alegre, M.L., Luke, J.J., and Gajewski, T.F. (2018). The commensal microbiome is associated with anti-PD-1 efficacy in metastatic melanoma patients. Science *359*, 104–108. https://doi.org/10.1126/science.aao3290.
42. McCulloch, J.A., Davar, D., Rodrigues, R.R., Badger, J.H., Fang, J.R., Cole, A.M., Balaji, A.K., Vetizou, M., Prescott, S.M., Fernandes, M.R., et al. (2022). Intestinal microbiota signatures of clinical response and immune-related adverse events in melanoma patients treated with anti-PD-1. Nat. Med. *28*, 545–556. https://doi.org/10.1038/s41591-022-01698-2.
43. Routy, B., Le Chatelier, E., Derosa, L., Duong, C.P.M., Alou, M.T., Daillère, R., Fluckiger, A., Messaoudene, M., Rauber, C., Roberti, M.P., et al. (2018). Gut microbiome influences efficacy of PD-1-based immunotherapy against epithelial tumors. Science *359*, 91–97. https://doi.org/10.1126/science.aan3706.
44. Breen, M., and Modiano, J.F. (2008). Evolutionarily conserved cytogenetic changes in hematological malignancies of dogs and humans–man and his best friend share more than companionship. Chromosome Res. *16*, 145–154. https://doi.org/10.1007/s10577-007-1212-4.
45. Richards, K.L., Motsinger-Reif, A.A., Chen, H.W., Fedoriw, Y., Fan, C., Nielsen, D.M., Small, G.W., Thomas, R., Smith, C., Dave, S.S., et al. (2013). Gene profiling of canine B-cell lymphoma reveals germinal center and postgerminal center subtypes with different survival times, modeling human DLBCL. Cancer Res. *73*, 5029–5039. https://doi.org/10.1158/0008-5472.CAN-12-3546.
46. Panjwani, M.K., Smith, J.B., Schutsky, K., Gnanandarajah, J., O'Connor, C.M., Powell, D.J., Jr., and Mason, N.J. (2016). Feasibility and Safety of RNA-transfected CD20-specific Chimeric Antigen Receptor T Cells in Dogs with Spontaneous B Cell Lymphoma. Mol. Ther. *24*, 1602–1614. https://doi.org/10.1038/mt.2016.146.
47. Kiyasu, J., Miyoshi, H., Hirata, A., Arakawa, F., Ichikawa, A., Niino, D., Sugita, Y., Yufu, Y., Choi, I., Abe, Y., et al. (2015). Expression of programmed cell death ligand 1 is associated with poor overall survival in patients with diffuse large B-cell lymphoma. Blood *126*, 2193–2201. https://doi.org/10.1182/blood-2015-02-629600.
48. Schuster, S.J., Bishop, M.R., Tam, C.S., Waller, E.K., Borchmann, P., McGuirk, J.P., Jäger, U., Jaglowski, S., Andreadis, C., Westin, J.R., et al. (2019). Tisagenlecleucel in Adult Relapsed or Refractory Diffuse Large B-Cell Lymphoma. N. Engl. J. Med. *380*, 45–56. https://doi.org/10.1056/NEJMoa1804980.
49. Schuster, S.J., Svoboda, J., Chong, E.A., Nasta, S.D., Mato, A.R., Anak, Ö., Brogdon, J.L., Pruteanu-Malinici, I., Bhoj, V., Landsburg, D., et al. (2017). Chimeric Antigen Receptor T Cells in Refractory B-Cell Lymphomas. N. Engl. J. Med. *377*, 2545–2554. https://doi.org/10.1056/NEJMoa1708566.
50. Aresu, L., Marconato, L., Martini, V., Fanelli, A., Licenziato, L., Foiani, G., Melchiotti, E., Nicoletti, A., and Vascellari, M. (2021). Prognostic Value of PD-L1, PD-1 and CD8A in Canine Diffuse Large B-Cell Lymphoma Detected by RNAscope. Vet. Sci. *8*, 120. https://doi.org/10.3390/vetsci8070120.
51. Kobold, S., Grassmann, S., Chaloupka, M., Lampert, C., Wenk, S., Kraus, F., Rapp, M., Düwell, P., Zeng, Y., Schmollinger, J.C., et al. (2015). Impact of a New Fusion Receptor on PD-1-Mediated Immunosuppression in Adoptive T Cell Therapy. J. Natl. Cancer Inst. *107*, djv146. https://doi.org/10.1093/jnci/djv146.
52. Liu, H., Lei, W., Zhang, C., Yang, C., Wei, J., Guo, Q., Guo, X., Chen, Z., Lu, Y., Young, K.H., et al. (2021). CD19-specific CAR T Cells that Express a PD-1/CD28 Chimeric Switch-Receptor are Effective in Patients with PD-L1-positive B-Cell Lymphoma. Clin. Cancer Res. *27*, 473–484. https://doi.org/10.1158/1078-0432.CCR-20-1457.
53. Li, F., Zhang, Z., Xuan, Y., Zhang, D., Liu, J., Li, A., Wang, S., Li, T., Shi, X., and Zhang, Y. (2020). PD-1 abrogates the prolonged persistence of CD8(+) CAR-T cells with 4-1BB co-stimulation. Signal Transduct. Targeted Ther. *5*, 164. https://doi.org/10.1038/s41392-020-00277-6.
54. Borowski, A.B., Boesteanu, A.C., Mueller, Y.M., Carafides, C., Topham, D.J., Altman, J.D., Jennings, S.R., and Katsikis, P.D. (2007). Memory CD8+ T cells require CD28 costimulation. J. Immunol. *179*, 6494–6503. https://doi.org/10.4049/jimmunol.179.10.6494.
55. Ramos, C.A., Rouce, R., Robertson, C.S., Reyna, A., Narala, N., Vyas, G., Mehta, B., Zhang, H., Dakhova, O., Carrum, G., et al. (2018). In Vivo Fate and Activity of Second- versus Third-Generation CD19-Specific CAR-T Cells in B Cell Non-Hodgkin's Lymphomas. Mol. Ther. *26*, 2727–2737. https://doi.org/10.1016/j.ymthe.2018.09.009.
56. Withers, S.S., Moore, P.F., Chang, H., Choi, J.W., McSorley, S.J., Kent, M.S., Monjazeb, A.M., Canter, R.J., Murphy, W.J., Sparger, E.E., and Rebhun, R.B. (2018). Multi-color flow cytometry for evaluating age-related changes in memory lymphocyte subsets in dogs. Dev. Comp. Immunol. *87*, 64–74. https://doi.org/10.1016/j.dci.2018.05.022.
57. Ammons, D.T., Harris, R.A., Hopkins, L.S., Kurihara, J., Weishaar, K., and Dow, S. (2023). A single-cell RNA sequencing atlas of circulating leukocytes from healthy and osteosarcoma affected dogs. Front. Immunol. *14*, 1162700. https://doi.org/10.3389/fimmu.2023.1162700.
58. Schlenker, R., Olguín-Contreras, L.F., Leisegang, M., Schnappinger, J., Disovic, A., Rühland, S., Nelson, P.J., Leonhardt, H., Harz, H., Wilde, S., et al. (2017). Chimeric PD-1:28 Receptor Upgrades Low-Avidity T cells and Restores Effector Function of Tumor-Infiltrating Lymphocytes for Adoptive Cell Therapy. Cancer Res. *77*, 3577–3590. https://doi.org/10.1158/0008-5472.CAN-16-1922.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse anti-canine CD28, (clone 1C6)	eBioscience	Cat#16-0283-82; RRID:AB_2573076
Mouse anti-canine CD3, (clone CA17.2A12)	Bio-Rad	Cat# MCA1774GA; RRID:AB_2291174
Mouse anti-canine CD11b, (clone CA16.3E10), Purified	Bio-Rad	Cat# MCA1777S; RRID:AB_322922
Mouse anti-canine CD11c, (clone CA11.6A1), Purified	Bio-Rad	Cat# MCA1778S; RRID:AB_322942
Mouse anti-human CD14, (clone TuK4), Purified	Bio-Rad	Cat# MCA1568GA; RRID:AB_566514
Mouse anti-canine CD21, Clone CA2.1D6, Purified	Bio-Rad	Cat# MCA1781R; RRID:AB_323665
Dog Gamma Globulin	Jackson ImmunoResearch	Cat# 004-000-002; RRID:AB_2336980
Biotin-SP (long spacer) AffiniPure Rabbit Anti-Mouse IgG (H+L)	Jackson ImmunoResearch	Cat# 315-065-003; RRID:AB_2340089
Rat anti-canine CD5, Clone YKIX322.3, PE	eBioscience	Cat# 12-5050-42; RRID:AB_10718841
Rat anti-canine CD5, Clone YKIX322.3, APC-eFluor780	eBioscience	Cat# 47-5050-42; RRID:AB_2784709
Rat anti-canine CD4, Clone YKIX302.9, PE-Cy7	eBioscience	Cat# 25-5040-42; RRID:AB_2573458
Rat anti-canine CD4, Clone YKIX302.9, PB	Bio-Rad	Cat# MCA1038PB; RRID:AB_2077612
Rat anti-canine CD8, Clone YCATE55.9, FITC	Bio-Rad	Cat# MCA1039F; RRID:AB_324550
Rat anti-canine CD8, Clone YCATE55.9, APC	eBioscience	Cat# 17-5080-42; RRID:AB_10597006
Mouse anti-human CD62L, Clone FMC46, PE	Bio-Rad	Cat#MCA1076PE; RRID:AB_321523
Mouse anti-canine CD45RA, Clone CA4.1D3	Bio-Rad	Cat# MCA2036S; RRID:AB_323368
Mouse anti-canine CD20, Clone 6C12	InvivoGen	Cat# dcd20-mab11
Mouse anti-canine PD-L1, Clone JC071	QED Bioscience	Cat# 34091
Canine anti-canine PD-1, (clone P3C6mut3.1), from canine scFv phage display library	Vetigenics	N/A
HA-tagged canine anti-canine PD-1, (clone P3C6mut3.1), from canine scFv phage display library	Vetigenics	N/A
Mouse anti-HA.11 Epitope Tag Antibody, Clone 16B12, APC	BioLegend	Cat# 901524; RRID:AB_2734658
Goat anti-mouse IgG, Clone Poly4053, DyLight 649	BioLegend	Cat# 405312; RRID:AB_1575128
Biological samples		
Canine Peripheral Blood Mononuclear Cells	Healthy canine donors, University of Pennsylvania	N/A
Chemicals, peptides, and recombinant proteins		
Dynabeads M-450 Tosylactivated	Invitrogen	Cat# 14013
Streptavidin MicroBeads	Miltenyi Biotec	Cat# 130-048-101
Goat anti-Mouse IgG Microbeads	Miltenyi Biotec	Cat# 130-048-401
Lipofectamine 2000	Invitrogen	Cat# 11668019

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
RetroNectin Recombinant Human Fibronectin Fragment	Takara	Cat# T100A
Recombinant human IL-2	PeproTech Inc.	Cat# 200-02
Recombinant human IFN-gamma	PeproTech Inc.	Cat# 300-02
7-AAD viability dye	BioLegend	Cat# 420404
Streptavidin, APC-Cy7	BD Biosciences	Cat# 554063; RRID:AB_10054651
APC Conjugation Kit - Lightning-Link	Abcam	Cat# ab201807
Iscove's Modified Dulbecco's Medium (IMDM), 25 mM Hepes	Gibco	Cat# 12440053
Roswell Park Memorial Institute Medium (RPMI) 1640	Corning	Cat# 10-040-CV
Opti-MEM (minimal essential medium)	Gibco	Cat# 31985070
Heat Inactivated Fetal Bovine Serum (FBS)	Biotechne	Cat# S11150
Penicillin/Streptomycin solution	Invitrogen	Cat# 15140122
Sodium pyruvate 100mM	Invitrogen	Cat# 11360070
Minimum Essential Media Non-Essential Amino Acids (MEM NEAA), 100×	Invitrogen	Cat# 11140050
Bovine serum albumin	Sigma-Aldrich	Cat# A3294
EDTA (0.5M)	Invitrogen	Cat# AM9260G
Human TruStain FcX™	BioLegend	Cat# 422302
TrueCut™ Cas9 Protein v2	Invitrogen	Cat# A36498
Critical commercial assays		
Canine IFN-γ quantikine ELISA kit	R&D Systems	Cat# CAIF00
Canine IFN-γ DuoSet ELISA	R&D Systems	Cat# DY781B
Canine TNF-α quantikine ELISA kit	R&D Systems	Cat# DY1507
Canine IL-2 DuoSet ELISA	R&D Systems	Cat# DY1815
DuoSet ELISA Ancillary Reagent Kit 2	R&D Systems	Cat# DY008
CellTrace™ Violet Cell Proliferation Kit	Invitrogen	Cat# C34557
ONE-Glo™ Luciferase Assay System	Promega	Cat# 6120
Experimental models: Cell lines		
HEK 293T	ATCC	CRL-3216; RRID:CVCL_0063
K562	ATCC	CCL-243; RRID:CVCL_0004
Jurkat	A kind gift of Dr Jim Riley, University of Pennsylvania	N/A
CLBL1	A kind gift of Dr. Barbara Rutgen, University of Vienna	N/A
Oligonucleotides		
sgRNA (canine PD-L1): GAC CUG UAU GUG GUA GAG UA	Synthego, Menlo Park, CA	N/A
Recombinant DNA		
pCMV Gag-Pol	Dr. Andrei Thomas-Tikhonenko	N/A
phCMV RD114 TR	Dr. Daniel Powell	N/A
phCMV VSV-g	Michael Atchison Lab	N/A
pMx-puromycin-cPD-L1	This paper	N/A
pCMV-GFP-Luciferase	This paper	N/A
pMSGV1-cCD20-4-1BB-ζ	This paper	N/A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
pMSGV1-cPD-1-cCD28-cCD20-4-1BB-ζ	This paper	N/A
pMSGV1-cPD-1-cCD28$_{MUT}$-cCD20-4-1BB-ζ	This paper	N/A
Software and algorithms		
FlowJo v10.8.1	FlowJo, LLC	https://www.flowjo.com; RRID:SCR_008520
Graph Pad Prism v10.0.1	GraphPad	https://www.graphpad.com; RRID:SCR_002798

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Cells, cell lines and cell culture

Canine peripheral blood mononuclear cells (PBMCs) were obtained from healthy, intact, 1-1.5 year old, male donor hound-mix dogs (30-35kg), obtained from Marshall BioResources. PBMCs were harvested either by leukapheresis using the Terumo Optia Spectra and continuous mononuclear cell collection or from peripheral blood samples following density gradient centrifugation. PBMC collection was performed by a board-certified veterinary internist (N.J.M) and carried out in accordance with the Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals and were fully approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania (IACUC protocol# 807025). All dogs were re-homed after use and no dogs were euthanized in the performance of these studies. PBMCs were washed twice in complete (c)RPMI media containing RPMI 1640 with 2mM L-Glutamine (Mediatech, Manassas, VA), 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Flowery Branch, Georgia), 10mM HEPES (Gibco, Grand Island, NY), and 100U/ml penicillin and 100μg/ml streptomycin (Gibco) prior to cryopreservation or immediate use. For cryopreservation, cells were resuspended in cold RPMI and mixed with an equal volume of cold 2x freezing medium (20% DMSO/FBS, final concentration 10% DMSO). For thaw, cryovials were warmed in a bead bath at 37°C until almost completely thawed. Cells were washed twice in warm cRPMI. After the second wash, cell viability was determined using trypan blue exclusion. The human erythroleukemic cell line K562, and human embryonic renal cell line HEK 293T cells were grown in (c) RPMI supplemented with 1mM sodium pyruvate (Mediatech) and 30μg/mL gentamicin. CLBL-1 cells were grown in cRPMI and passaged every 3 days.

For canine (c)PD-L1 expressing target cells, K562 expressing canine CD20 (K562-cCD20) were generated as previously described.[46] Briefly, cCD20 was amplified from PBMC cDNA and cloned into the pCLPS lentiviral vector where the cCD20 transgene is under the control of a cytomegalovirus (CMV) promoter. Lentivirus was generated via transfection of 3T3 cells and concentrated supernatant was used to infect K562 to generate cCD20-expressing K562 cells. K562-cCD20 cells were transduced with a RD114/VSVg pseudotyped pMx-puromycin retroviral vector containing cPD-L1 to generate K562-cCD20-cPD-L1 cells. K562 cells were incubated with retroviral particles at 37°C for 3 days and then assessed for the expression of cPD-L1 using a mouse anti-canine PD-L1 antibody (QED Bioscience). K562-cCD20-PD-L1 cells were sorted using flow cytometry and expanded in supplemented cRPMI as described above. Target K562 cell lines and CLBL-1 cells were irradiated with 10,000 rads prior to use in proliferation and cytokine production experiments.

Generation of cPD-L1 edited CLBL-1 cells

sgRNA (Synthego, Menlo Park, CA) oligos were re-hydrated in nuclease free 1xTE buffer to 100μM and stored at -80°C. CLBL-1 cells were washed in warm OptiMEM media twice, counted, and re-suspended in warm OptiMEM at 5 x 10^7 cells/ml. Cas9/gRNA ribonucleoproteins (RNP) were prepared immediately before electroporation by mixing 1nmol of sgRNA against cPD-L1 (GAC CUG UAU GUG GUA GAG UA) and 0.24nmol of Cas9 protein (Thermo Fisher Scientific) and incubating at RT for 15mins to develop the RNP complex. 100μl of CLBL-1 cells was added to the Cas9/gRNA RNP complex and gently mixed to avoid any bubble formation. Cells plus RNP were placed in a 0.2cm cuvette and electroporated with the BTX ECM 830 Square wave electroporation system (BTX Technologies, Holliston, MA, USA) set at 500V for 700ms. Cells were quickly removed from the cuvette following electroporation and transferred into a 6 well plate containing 3mls of warm cRPMI media. Seventy-two hours after electroporation, edited CLBL-1 cells were labeled with mouse anti-canine PD-L1 antibody (QED Bioscience), and editing efficiency was determined by flow cytometry. Five days post transduction, edited cells were bulk sorted by FACS Aria II Cell Sorter (BD Biosciences). Bulk sorted cells were cryopreserved as described above stored in liquid nitrogen prior to use.

METHOD DETAILS

Vectors and constructs

A canine CAR construct consisting of a canine CD8α leader sequence, a mouse anti-canine CD20 scFv (a kind gift from Laurence Cooper), canine CD8α hinge and transmembrane domain, canine 4-1BB co-stimulatory domain and canine CD3ζ intracellular signaling domain has been previously described.[31] The canine CAR construct was amplified by PCR and cloned into the MSGV1 retroviral vector, and its sequence was confirmed by Sanger sequencing. For the PD-1/CD28 CSRs, a truncated extracellular domain of canine PD-1 joined to the transmembrane and intracellular co-stimulatory domains of canine CD28 was synthesized upstream of a FMD2A ribosomal skip site and the canine CD20-c4-1BB-ζ CAR (Genewiz, South Plainfield, NJ). For the mutant PD-1/CD28 CSR, the YMNM signaling domain was mutated to FFFF

and the proline residues in the two proline-rich motifs PRRP and PYAP were changed to alanine residues, becoming ARRA and AYAA respectively as previously described.[25] Both constructs were cloned into the MSGV1 vector by Genewiz, and sequences were confirmed by Sanger sequencing.

Retrovirus was produced through transient transfection of HEK 293T cells using Lipofectamine 200 (Invitrogen) as previously described.[30] Briefly, 15μl of Lipofectamine 2000 was gently mixed with 1.4mls of transfection media (97% DMEM high glucose, 1% 100mM sodium pyruvate, 1% Glutamax, and 1% 1M Hepes) per plate of HEK 293T cells and incubated for <14 mins at 20°C -25°C. A master mix of Gag/Pol (GP) packaging plasmid, the RD114 (R) envelope plasmid and the MSGV1 plasmid containing the CAR, CAR+CSR_{WT} or CAR+CSR_{MUT} construct was prepared using a molar ratio of GP:R:M=1:1.5:3-5 for a total of 21-23μg plasmid DNA/dish in up to 100μl of transfection media/dish. The plasmid mixture was added to the lipofectamine/transfection media, mixed and incubated for <25 mins at 20°C -25°C. 1.5mls of lipofectamine/plasmid mix was added dropwise to each 10cm dish of HEK293 cells at 80-90% confluency. 3.5mls of warm HEK 293T media was then added to each dish and plates are incubated for 6 hours at 37°C. After incubation, the transfection media was completely replaced with 6mls of warm HEK 293T media and plates were incubated for 48 hours prior to harvest. At harvest, supernatants were centrifuged at 500 × *g* for 5 min at 20°C–25°C to remove cellular debris. Supernatants were then filtered using a 0.45 μM PES filter to remove contaminating cells and large cell debris and then snap-frozen and cryopreserved at -80°C prior to use. Viral titers were determined using Jurkat cells as previously described.[30] Briefly, 2-fold serial dilutions of virus were prepared and added in triplicate to 4 × 10^4 Jurkat cells in a 96 well plate. Plates were incubated at 37°C for 72 hours, prior to flow cytometry to evaluate % transduction. Viral titer was calculated as follows: TU/mL: number of transducing particles (viral vectors) per mL. F: fraction of CAR positive Jurkat cells (% of positive cells/100). N: number of Jurkat cells at the time of infection (4 × 10^4). D: dilution factor. 1000: correction factor to provide the number of TU per mL. V: volume of diluted virus added to each well (100 μL).

Generation of canine CAR-T cells

Canine CAR-T cells were generated as previously described.[30] Briefly, T cells were negatively selected from PBMCs labeled with anti-CD11b, anti-CD11c, anti-CD14 and anti-CD21, followed by goat anti-mouse IgG microbeads using Miltenyi LS columns. Negatively selected T cells were washed twice in cIMDM media (containing IMDM with 1mM Sodium Pyruvate, 1% MEM NEAA, 10% heat-inactivated fetal bovine serum, 100U/mL penicillin, and 100μg/mL streptomycin), counted and activated using tosylactivated dynabeads coated with anti-CD3/CD28 antibodies at a bead:T cell ratio of 3:1, in the presence of 100IU/mL of recombinant human (rh) IL-2. Forty-eight hours after activation, cells were harvested, counted, and added to non-treated culture plates coated with RetroNectin and pre-loaded with RD114/VSVg pseudotyped MSGV1 retrovirus (MOI=5) expressing the canine CD20-4-1BB-ζ CAR. Cells were incubated at 38.8°C in cIMDM. Mock transductions were carried out in the absence of virus. Cells were expanded in rhIL-2 at 100U/ml. Four days post transduction, and at the indicated timepoints, cells were analyzed for CAR expression using flow cytometry.

Enrichment of canine CAR-T cells

Transduced T cells were washed twice in MACS buffer (0.5%BSA, 2mM EDTA in 1xPBS), counted and blocked with canine IgG for 10mins on ice. Cells were then labeled with recombinant biotinylated rabbit anti-mouse IgG (15μg of antibody per 10 million cells) for 30 mins on ice. After washing twice with MACS buffer, cells were incubated with 10μL of Streptavidin MACS MicroBeads (Miltenyi Biotec) per 10 million cells for 15mins on ice. Cell suspensions were then washed twice and CAR^+ cells were enriched by MACS sorting as per manufacturer's instructions.

Flow cytometry

Canine T cells, canine cell lines and human cell lines were washed twice in FACS buffer. Cells were blocked with either 5μl of Human TruStain FcX (Fc receptor blocking solution; BioLegend, San Diego, CA, USA) per 1x10^6 cells in 100μl FACS buffer, or 10μl of canine IgG per 1x10^6 cells in 100μl FACS buffer, for human- or canine-derived cells respectively for 10mins at RT prior to cell surface labelling with indicated antibodies (please see key resources table for antibodies used in flow cytometry). Surface expression of CAR was detected using recombinant biotinylated rabbit anti-mouse IgG (Jackson ImmunoResearch) and APC-Cy7-conjugated streptavidin (BD Biosciences). Surface expression of the two chimeric switch receptors and endogenous PD-1 were detected using an anti-canine PD-1 antibody generated from a canine scFv phage display library and validated as previously described,[34] conjugated to APC using Lightning-Link APC-conjugation kit (Lightning-Link®; Abcam). All other antibodies used were commercially available and their clone, host species, commercial source and catalogue number are reported in the key resources table. 7-AAD (BioLegend) was used to exclude dead cells. Cells were acquired on a FACS Canto II flow cytometer (BD Biosciences) or FACS LSRFortessa (BD Biosciences), and data was analyzed using FlowJo software version 10 (Treestar, Ashland, OR).

To determine whether CAR engagement with target antigen led to increased expression of cPD-1, CAR-T cells were harvested 6 days post transduction, washed in cRPMI and co-cultured at 38.8°C with irradiated K562 cells, K562-cCD20 cells or K562-cCD20-cPD-L1 at 1:1, 5:1 and 10:1 E:T ratios. Seventy- two hours post co-culture, cells were harvested, washed twice in FACS buffer, and labeled for CD5, CD8, and cPD-1. Dead cells were identified and excluded from analysis using 7AAD exclusion.

Cytotoxicity assay

Luciferase expressing target cells were generated using the retroviral vector pCMV containing GFP and Luciferase. RD114 pseudotyped retroviral particles were generated as previously described[30] and used with RetroNectin coated plates to transduce CLBL-1 cells and K562 target cells. Luciferase-transduced cell lines were purified using FACS sorting by gating on GFP^+ cells. Where indicated, CAR-T cells were stimulated with target cells every 3 days for 3 to 5 stimulations prior to their evaluation in cytotoxicity assays. For cytotoxicity assays, CAR T cells were co-cultured at 38.8°C with the indicated target cells at different effector (total T cells): target ratios for 24 hours. In some experiments, CLBL-1 target cells were pre-treated with 100ng/ml of IFN-γ for 48 hours to up-regulate cPD-L1, washed and then used in cytotoxicity assays. Promega ONE-Glo™ luciferase assay reagent (Promega, Madison, WI, USA) was then added and cells were incubated at RT for 5mins. 100% lysis of target cells was achieved using 0.5% triton. Luminescence was measured using a Glomax® microplate reader (Promega) at 1.0 second integration per well. Cytotoxicity was calculated using the following formula:

$$\% \text{ cytotoxicity} = 100 \times (\text{spontaneous death RLU- test RLU})/(\text{spontaneous death RLU- maximal killing RLU}).$$

Cytokine Quantification

Supernatants from *in vitro* assays were collected at the time points indicated and analyzed by ELISA using the canine IFN-γ quantikine ELISA kit (R&D Systems), canine IFN-γ Duoset ELISA (R&D Systems), canine TNF-α quantikine ELISA kit (R&D Systems) and canine IL-2 DuoSet ELISA (R&D Systems). All assays were performed as per manufacturer's instructions. However, for the canine IL-2 assay, the capture antibody was used at 4 times the recommended concentration in the manufacturer's instructions (Dr. Naoya Maekawa, personal communication). The remainder of the protocol was followed as per manufacturer's instructions.

Proliferation assay

Canine CAR T cells were labelled with 5mM of CellTrace™Violet (CTV) solution (Invitrogen) in PBS according to manufacturer's instructions. Cells were washed twice in PBS, resuspended in cIMDM and co-cultured at 38.8°C with irradiated (10,000rads) K562 target cell lines (with or without cCD20 and cPD-L1) at an E:T ratio of 1:1. After 72hrs incubation, cells were harvested, washed twice in FACS buffer, and labelled with biotinylated Rabbit anti-mouse IgG antibody (Jackson ImmunoResearch) at RT for 30 mins for CAR detection. Cells were washed twice in FACS buffer and labelled with rat anti-canine CD5 antibody (Clone: YKIX 322.3 eBioscience), rat anti-canine CD4 antibody (Clone: YKIX302.9, eBioscience), rat anti-canine CD8 antibody (Clone:YCATE55.9 BioRad) and APC-Cy7-conjugated streptavidin (BD Biosciences) for 30 mins at RT. Cells were washed twice in FACS buffer prior to acquisition on a FACS Canto II flow cytometer (BD Biosciences), and data was analyzed using FlowJo software version 10 (Treestar, Ashland, OR). The proliferation index of $CD4^+$ and $CD8^+$ CAR-T cells was calculated according to the following: proliferation index (%) = 1-(MFI (target cells)/MFI (control target))x100 where K562-WT were the control target cells.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data were expressed as mean +/- sd as indicated and analyzed using GraphPad Prism 10 (GraphPad Software, Boston, MA). One or two-tailed paired or un-paired t-tests were used when comparing two groups. For comparisons of more than two groups, a one- or two-way ANOVA with appropriate post hoc testing was used. Statistical significance was defined as $P < 0.05$ (* $P < 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$, ns: not significant).