MABS
2021, VOL. 13, NO. 1, e2004638 (11 pages)
https://doi.org/10.1080/19420862.2021.2004638

Taylor & Francis
Taylor & Francis Group

REPORT

OPEN ACCESS Check for updates

# Development of a fully canine anti-canine CTLA4 monoclonal antibody for comparative translational research in dogs with spontaneous tumors

Nicola J. Mason[a,b,c,*], Nicholas Chester[d,*], Ailian Xiong[a], Antonia Rotolo[a], Ying Wu[e], Sho Yoshimoto[a,f], Patrick Glassman[g], Gayathri Gulendran[h], and Don L. Siegel[c,h]

[a]Department of Clinical Sciences and Advanced Medicine, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA, USA; [b]Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA, USA; [c]Center for Cellular Immunotherapy, Department of Pathology and Laboratory Medicine, School of Medicine, University of Pennsylvania, Philadelphia, PA, USA; [d]Vetigenics LLC, Philadelphia, PA, USA; [e]Department of Immunobiology, Yale University, New Haven, CT, USA; [f]Laboratory of Small Animal Surgery, Department of Veterinary Medicine, School of Veterinary Medicine, Azabu University, Kanagawa, Japan; [g]Department of Systems Pharmacology and Translational Therapeutics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA; [h]Department of Pathology and Laboratory Medicine, School of Medicine, University of Pennsylvania, Philadelphia, PA, USA

**ABSTRACT**

The immune checkpoint inhibitor (ICI) ipilimumab has revolutionized the treatment of patients with different cancer histologies, including melanoma, renal cell carcinoma, and non-small cell lung carcinoma. However, only a subset of patients shows dramatic clinical responses to treatment. Despite intense biomarker discovery efforts linked to clinical trials using CTLA4 checkpoint blockade, no single prognostic correlate has emerged as a valid predictor of outcome. Client-owned, immune competent, pet dogs develop spontaneous tumors that exhibit similar features to human cancers, including shared chromosome aberrations, molecular subtypes, immune signatures, tumor heterogeneity, metastatic behavior, and response to chemotherapy. As such, they represent a valuable parallel patient population in which to investigate novel predictive biomarkers and rational therapeutic ICI combinations. However, the lack of validated, non-immunogenic, canine ICIs for preclinical use hinders this comparative approach. To address this, fully canine single-chain variable fragments (scFvs) that bind canine CTLA4 were isolated from a comprehensive canine scFv phage display library. A lead candidate for clinical development was selected based on its subnanomolar binding affinity to canine CTLA4 and its ability to prevent CTLA4 binding to CD80/CD86 and promote T cell proliferation and effector function. In vivo mouse studies revealed pharmacokinetics similar to isotype control IgG with no evidence of short-term adverse effects. This work paves the way for in vivo analysis of the first fully canine, anti-canine CTLA4 antibody to promote anti-tumor immunity in dogs with immune-responsive cancers and provide an important comparative tool to investigate correlative biomarkers of response and mechanisms of resistance to CTLA4 checkpoint inhibition.

**ARTICLE HISTORY**
Received 31 May 2021
Revised 20 October 2021
Accepted 1 November 2021

**KEYWORDS**
Checkpoint inhibitor; canine; CTLA4; large animal model; monoclonal antibody; single chain variable fragment; immunotherapy

## Introduction

Cytotoxic T lymphocyte associated protein 4 (CTLA4) or CD152 is a member of the immunoglobulin superfamily of transmembrane receptors that is highly homologous with the T cell co-stimulatory molecule CD28 and shares the same receptor ligands, CD80 and CD86. CTLA4 expression is up-regulated following activation of $CD4^+$ and $CD8^+$ T cells and, due to its high affinity binding to CD80 and CD86, it out-competes CD28 for interaction with these ligands. Engagement of CTLA4 with CD80 and CD86 delivers an inhibitory signal to the effector T cell, negatively regulating the T cell response.[1,2] Lymphocyte inhibition is achieved following engagement via recruitment of SHP2 and PP2A, which dephosphorylate the T cell receptor ζ chain and target downstream effectors of phosphoinositide 3-kinase, respectively.[3,4] The high binding affinity of CTLA4 for CD80/86 on antigen-presenting cells (APCs) can also lead to the removal of these co-stimulatory ligands via transendocytosis, promoting a tolerogenic phenotype which further impairs effector T cell responses.[5] In contrast to effector T cells, regulatory T cells (Tregs) constitutively express high levels of CTLA4 on their surface, which is essential for their suppressor activity, particularly their ability to inhibit dendritic cell maturation.[6,7] The importance of CTLA4 as a negative regulator of T cell responses is underscored by the rapid development of fatal lymphoproliferative disease and autoimmunity in CTLA4-deficient mice and in mice with CTLA4-deficient regulatory T cells.[6,8] Monoclonal antibodies (mAbs) that bind CTLA4 and inhibit binding to CD80/86 enhance endogenous T cell responses and inhibit Treg function, thereby promoting anti-tumor immunity.[9,10] These findings led to the use of CTLA4-specific mAbs to block checkpoint signaling and enhance anti-tumor immunity in syngeneic tumor-bearing mouse models.[11] Systemic administration of anti-CTLA4 mAb led to tumor rejection and long-lasting protection against

**CONTACT** Nicola J. Mason nmason@vet.upenn.edu Room 315, Hill Pavilion, 380 South University Avenue, Philadelphia, PA 19104, USA
*These authors contributed equally to this work.
Supplemental data for this article can be accessed on the publisher's website.

re-challenge in multiple different murine xenograft models.-[10,12–14] Furthermore, these responses correlated with the immunogenicity of the tumor type.[15] Ensuing clinical trials of anti-CTLA4 mAb in patients with tumors that carry a high mutational burden (melanoma, renal cell carcinoma, non-small cell lung carcinoma (NSCLC) and prostate adenocarcinoma) showed that a subset of patients experienced dramatic durable clinical responses to treatment.[16–20] Additional data in mice suggest that clinical anti-tumor responses associated with anti-CTLA4 mAbs may be associated in part with their ability to deplete intratumoral Tregs, which constitutively express high levels of CTLA4.[21–23] However, despite intense efforts, robust correlative biomarkers that predict responders to such checkpoint inhibition remain elusive.

An unfortunate consequence of disrupting immune homeostasis and immunological self-tolerance through checkpoint inhibition is the induction of auto-immunity.[24] Such immune-related adverse events frequently affect the gastrointestinal tract, endocrine glands, skin and liver, although any organ system can be affected.[25] These side effects are more common and more severe with CTLA4 inhibition than with inhibition of the PD-1 axis[26] affecting up to 60% of recipients.[16] To reduce the adverse systemic effects of anti-CTLA4 therapy, intra- and peri-tumoral administration of anti-CTLA4 mAb has been investigated with promising results in mouse models.[27–29]

Dogs are closely related phylogenetically to humans and spontaneously develop cancer that shares similar biological, behavioral and genetic features with human counterparts.[30–32] As such, immune competent, canine cancer patients can serve as a relevant parallel patient population to assist in unraveling the mechanisms of action of anti-CTLA4 mAbs, identify correlative biomarkers of response and understand mechanisms of resistance. Further, they may also play an important role in informing human clinical trial design regarding the safety and efficacy of combination therapies. Currently, little is known about the expression of CTLA4 in canine T cells or the role that CTLA4 blockade and targeted depletion of CTLA4$^+$ Tregs might play in promoting anti-tumor immunity. Canine CTLA4 shares 88% identity with human CTLA4 and was shown to induce tolerance to sheep red blood cells in dogs, suggesting a conserved mechanism of action for CTLA4 between human and dog.[33] In addition, canine patients with histiocytic sarcoma[34] and B cell lymphoma-[35] have a higher percentage of CTLA4$^+$ CD4$^+$ and CD8$^+$ T cells in their peripheral blood when compared to control dogs, suggesting the presence of an exhausted phenotype that adversely affects anti-tumor immunity.[36] Finally, recent reports using RNAseq have confirmed that canine CD4$^+$CD25$^{hi}$ T cells express a regulatory phenotype that includes expression of high levels of CTLA4 transcripts.[37] Together, these findings provide the rationale for development of a therapeutic fully canine, anti-canine CTLA4 antibody that could promote anti-tumor immunity and provide a valuable reagent for comparative canine studies to investigate correlative biomarkers of clinical response and optimal combination therapies.

## Results

### *Isolation of canine anti-canine CTLA4 scFv*

A canine IgM/IgG/λ/κ single-chain variable fragment (scFv) phage display library containing an estimated 40 billion individual *E. coli* transformants was constructed from canine B cell mRNA and the pComb3X phagemid vector as described[38] using oligonucleotide primers based on published canine immunoglobulin heavy and light chain germline genes[39] (data not shown). An aliquot of the library underwent four rounds of solid phase selection ("panning") against biotinylated avitag-labeled canine CTLA4 (cCTLA4, supplementary methods). Substantial enrichment of phage for cCTLA4 specific binders began in the second round of panning (P2) and increased through the fourth round of panning (P4) (Supplemental Table 1).

To verify that selected phage contained cCTLA4-specific scFv phage particles, polyclonal scFv phage from each round of panning was evaluated by scFv phage ELISA using cCTLA4 as the target antigen (Figure 1a). scFv-phage captured through positive selection on cCTLA4 reacted only with cCTLA4-loaded wells, confirming the presence of cCTLA4 specific scFvs in P2, P3 and P4.

In an initial screening to demonstrate the presence of unique anti-cCTLA4 antibodies, 12 clones from the third panning round and 12 clones from the fourth panning round were randomly selected and tested for their ability to bind to

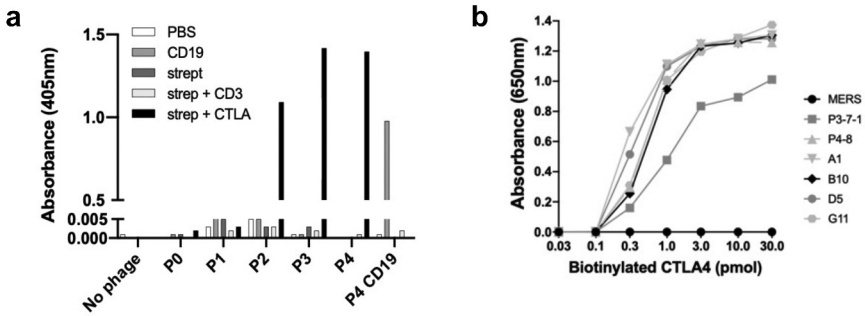


**Figure 1.** Enrichment and isolation of canine scFv that bind canine CTLA4 antigen. (a). Initial library (P0) and polyclonal libraries obtained after each round of panning (P1 through P4) were analyzed for their ability to bind to cCTLA4 by phage ELISA. Bound phage was detected using HRP-conjugated anti-M13 mAb and ABTS. Plates coated with no antigen (PBS), canine CD19, human avitag-CD3ε or SA alone were used as negative controls. Polyclonal phage from the 4$^{th}$ round of panning (P4) of library against canine CD19 was used as a positive control. (b). Unique, soluble, HA-tagged and purified scFvs were generated from clones randomly selected from P3 and P4 and tested for their ability to bind to increasing concentrations of cCTLA4 by ELISA. 0.25 ug soluble scFv were added to each well containing the indicated amount of streptavidin-captured cCTLA4 and bound scFv was detected with an AP-conjugated anti-HA antibody. An irrelevant MERS specific soluble scFv was used as a negative control.

cCTLA4 by phage ELISA. All 24 clones bound to cCTLA4 and nucleotide sequencing of these 24 scFvs revealed 20 unique single chains, of which 17 had λ light chains and 3 had κ light chains (data not shown).

A further 88 clones were randomly selected from both P3 and P4 (total of 176 clones) and expression extracts containing soluble scFvs were generated, purified and analyzed for their ability to bind to cCTLA4 by scFv ELISA (data not shown). Ten clones showing comparable or better binding to cCTLA4 than any of the first 20 unique clones were selected and sequenced. From these, three more unique clones were identified from P3 (A1, B10 and C5) and two more new clones (D5 and G11) were identified from P4. The binding of soluble monoclonal scFvs (A1, B10, D5 and G11) to cCTLA4 was confirmed by ELISA along with two clones from the initial screening (Figure 1b). Clone C5 was not evaluated as a purified, soluble scFv. Thus, a total of 24 unique, fully canine, soluble scFvs that bound to the extracellular domain of cCTLA4 were available for further analysis.

Next, we sought to determine whether any of the unique cCTLA4-specific scFvs were able to block the interaction between cCTLA4 and CD80/CD86, a property that might bestow therapeutic potential by enhancing T cell responses in tumor-bearing canine patients. First, an ELISA-based interaction assay was developed that centered on the ability of biotinylated cCTLA4 to bind to commercially available human (hu) CD86-Fc and huCD80-Fc chimeric proteins in a dose-dependent manner (data not shown). Next, the ability of 18 scFvs from the original group of 20 unique clones, along with the additional 4 (A1, B10, D5 and G11) identified from expression extracts, to inhibit cCTLA4: huCD80 and cCTLA4:huCD86 interactions was determined (Figure 2a and b and data not shown). Seventeen of 18 and 4/4 clones from the original and additional screening groups, respectively, showed varying degrees of inhibition of cCTLA4 binding to huCD86. Thirteen of the original clones that inhibited binding of cCTLA4 to huCD86 also inhibited the binding of cCTLA4 to huCD80, with clones P4-8 and P3-7 showing the greatest inhibition. Clones from the additional screening round showed comparable or greater inhibition of cCTLA4 binding to huCD80/86 when compared to P4-8 and P3-7. In all cases, use of a higher molar ratio of scFv:cCTLA4 resulted in greater inhibition (6:1 compared to 2:1).

### Production of full-length canine anti-cCTLA4 IgG

Based on their ability to inhibit cCTLA4 binding to CD80 and CD86, clones A1, D5, B10, G11 and P4-8 were chosen to be reformatted into fully canine IgG for further functional analysis. B10 and D5 were successfully reformatted as canine $IgG_A$ (producing 78 μg/10 ml culture media and 35 μg/10 ml culture media, respectively), but only low levels of clone A1 could be expressed (6 μg/10 ml culture media). Clones G11 and P4-8 could not be expressed as full-length canine $IgG_A$. Given the superior capability of clone A1 to block cCTLA4 binding, efforts to improve its production through complementarity-determining region (CDR) grafting were made. Using heavy and light chain swapping experiments, low productivity was localized to the heavy chain variable region (VH) of A1 (data not shown). Since the full-length $IgG_A$ of clone B10 produced well and its framework regions showed high sequence homology with those of clone A1, the CDR regions of A1 were grafted into the VH backbone of clone B10. The resulting chimeric A1 heavy chain combined with the original A1 light chain resulted in high production (70–80 μg/10 ml culture media) of the fully canine $IgG_A$ now designated A1mut2. The ability of full-length A1mut2, B10 and D5 IgG to bind cCTLA4 in ELISA was confirmed (Supplemental Figure S1A). Further, the ability of A1mut2, B10 and D5 to inhibit the interaction of cCTLA4 with canine CD80 and canine CD86 was evaluated in an inhibition ELISA, which confirmed the superior ability of A1mut2 to block cCTLA4 binding to its canine ligands (Supplemental Figure S1B). The amino acid sequences of A1, B10 and A1mut2 are shown in Supplemental Figure S2.

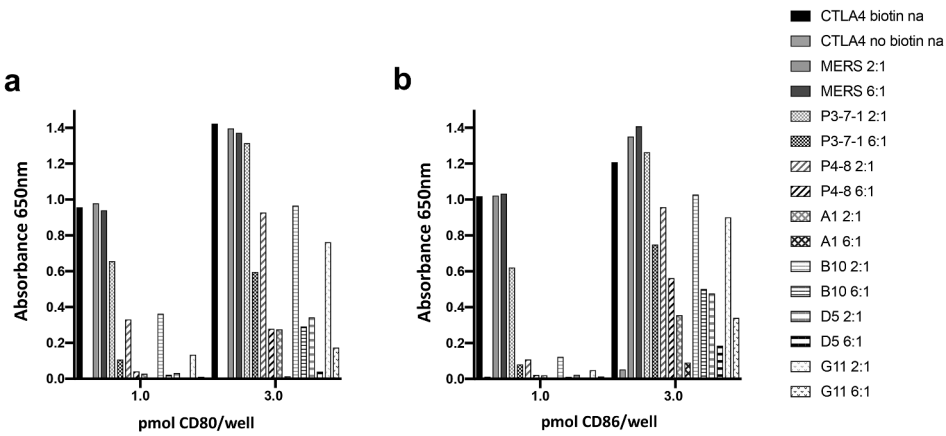


**Figure 2.** CTLA4 specific soluble scFv inhibit the binding of cCTLA4 to CD80 and CD86. 1.0 and 3.0 pmol of recombinant human CD80-Fc (a) or human CD86-Fc (b) were bound to ELISA plates overnight. Biotinylated avitag-cCTLA4 was pre-incubated with each soluble scFv at the indicated molar ratios for 1 hr before being added to the plate. Bound cCTLA4 was detected using a streptavidin-AP conjugate and AP colorimetric substrate. Biotinylated and unbiotinylated cCTLA4 in the absence of any antibody were used as positive and negative controls. na = no antibody present.

### *CTLA4-specific IgG bind cell surface expressed canine and feline CTLA4*

To determine whether A1mut2, B10 and D5 $IgG_A$s bound to membrane-expressed cCTLA4, KTδ32 cells were genetically modified to express cCTLA4 (KTδ32.cCTLA4) and used as target cells in flow cytometry experiments (Figure 3). All clones bound to KTδ32.cCTLA4, but not to the parent KTδ32 cells. Furthermore, pre-incubation of each IgG with soluble cCTLA4 to block antigen binding sites abolished antibody binding, indicating that membrane binding was antigen specific. Next, the ability of the three selected $IgG_A$s to bind to CTLA4 on the surface of activated canine T cells was assessed (Figure 4). Negligible amounts of CTLA4 were detected on the surface of $CD5^+CD4^+$ and $CD5^+CD4^-$ cells prior to activation; however, both T cell subsets demonstrated a significant increase in CTLA4 expression at 48 and 72 hours post activation. At each timepoint a greater percentage of CD4 T cells expressed CTLA4 compared with CD8 T cells (50.7% vs 29.5% at 48 hrs and 56.6% vs. 40.5% at 72 hrs, respectively). Next, the surface and intracellular expression of CTLA4 on canine $CD45^+CD5^+CD4^+FoxP3^+$ Tregs was determined using A1mut2 $IgG_A$. A1mut2 bound to both surface and intracellular CTLA4 on Tregs isolated from the peripheral blood of a dog with T cell lymphoma, confirming that CTLA4 is constitutively expressed by canine peripheral blood Tregs (Supplemental Figure S3). Finally, given that canine and feline CTLA4 share 99% identity, feline leucocytes were incubated with A1mut2 to assess antibody cross-reactivity. Feline $CD5^+$ cells showed minimal surface staining with A1mut2, but the antibody bound strongly to intracellular CTLA4 (Supplemental Figure S4).

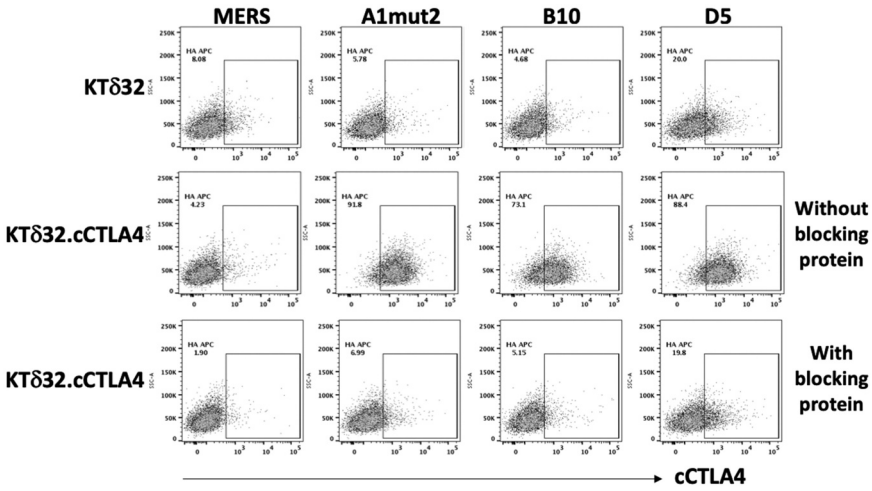


**Figure 3.** Evaluation of anti-CTLA4 IgG binding to membrane expressed canine CTLA4. K562 cells lacking the FcγRII (CD32) were genetically engineered to express canine CTLA4 (KT32δ.cCTLA4). KT32δ cells (top row) and KT32δ.cCTLA4 cells (middle row) were incubated with three anti-CTLA4 antibodies reformatted as $IgG_B$, and surface labeling was detected using an anti-HA antibody. To confirm antigen-specific binding, anti-CTLA4 clones were first incubated with soluble cCTLA4 protein to block antigen binding sites and then used for cell surface labeling (bottom row).

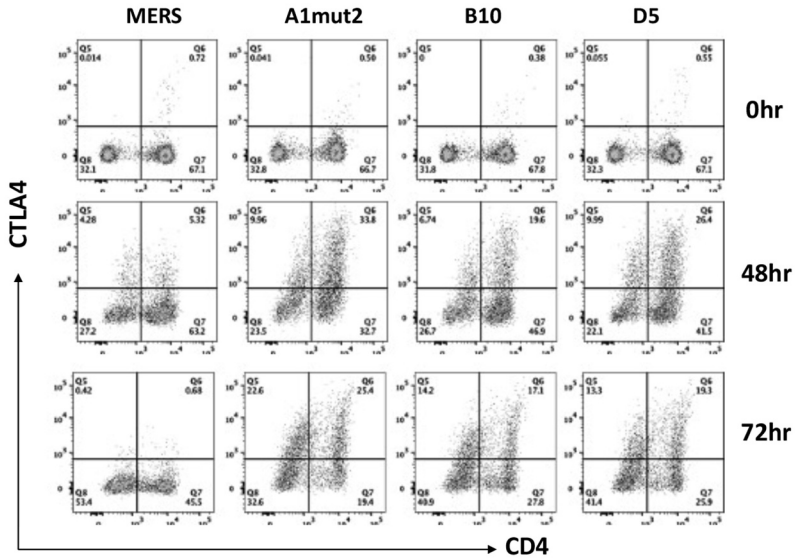


**Figure 4.** Evaluation of binding of anti-CTLA4 mAb clones to activated canine T cells. Canine PBMCs were activated with 2.5 ug/ml Concanavalin A, harvested at 48 hr and 72 hr post activation and labeled with each HA-tagged anti-CTLA4 IgG as indicated. Bound anti-CTLA4 antibody was detected using an anti-HA antibody. Plots are gated on $CD5^+$, $7AAD^-$ cells.

### Canine anti-CTLA4 IgG demonstrate high binding affinity for soluble cCTLA4

The affinities and binding kinetics of A1mut2, D5 and B10 for cCTLA4 were measured to determine whether they may be suitable for evaluation as therapeutics. Using surface plasmon resonance (SPR), the on and off rates of these three $IgG_A$ against cCTLA4 were determined (Table 1). The dissociation constant ($K_D$) of A1mut2 was in the subnanomolar range and demonstrated the highest binding affinity of the three clones. The other two clones displayed single digit nanomolar affinities. For comparison, the $K_D$ of ipilimumab to human CTLA4is 18.2 nM.[40] Thus, all three IgGs displayed sufficient binding affinity to cCTLA4 to serve as potential therapeutic checkpoint inhibitors.

### Canine anti-CTLA4 IgG subclasses ($IgG_B$ and $IgG_C$) fix complement

The ability of CTLA4-targeted antibodies to deplete intratumoral Tregs is being increasingly recognized as contributing to anti-tumor immunity in mice. Given this, we assessed the ability of A1mut2 IgG subclasses to fix complement in vitro. Canine $IgG_B$ and $IgG_C$ effectively fixed complement whereas $IgG_A$ and $IgG_D$ did not, a finding that is consistent with the literature[41] (Supplemental Figure 5A). A control ELISA confirmed binding of the four canine IgG subclasses to plate-bound cCTLA4 (Supplemental Figure 5B). This result indicates that the $IgG_B$ and $IgG_C$ subclasses of A1mut2 have the potential to initiate complement-mediated cytotoxicity of $CTLA4^+$ cells, which may preferentially deplete intratumoral Tregs that express high levels of CTLA4.

Table 1. Binding kinetics and affinities of canine anti-cCTLA4 IgG to canine and murine CTLA4.

| | A1mut2 (canine CTLA4) | A1mut2 (murine CTLA4) | B10 (canine CTLA4) | D5 (canine CTLA4) |
|---|---|---|---|---|
| $k_{on}$ | 2.1 x $10^5$ $M^{-1}s^{-1}$ | 2.9 x $10^5$ $M^{-1}s^{-1}$ | 1.6 x $10^5$ $M^{-1}s^{-1}$ | 1.2 x $10^5$ $M^{-1}s^{-1}$ |
| $k_{off}$ | 1.8 x $10^{-4}$ $s^{-1}$ | 1.8 x $10^{-5}$ $s^{-1}$ | 2.5 x $10^{-4}$ $s^{-1}$ | 1.8 x $10^{-4}$ $s^{-1}$ |
| $K_D$ | 0.85 nM | 0.061 nM | 1.5 nM | 1.5 nM |
| $R_{max}$ | 122 RU | 112 RU | 84 RU | 118 RU |

### A1mut2 increases T cell proliferation and IFN-γ production

To determine whether A1mut2 can increase T cell proliferation, canine peripheral blood mononuclear cells (PBMCs) were labeled with Cell Trace Violet and stimulated with ConA in the presence of either A1mut2 ($IgG_A$) or the irrelevant Middle East respiratory syndrome (MERS)-specific negative control $IgG_A$. Cells were harvested at 72 or 96 hr, and the responder frequency and proliferative capacity of T cells activated in the presence of A1mut2 versus MERS IgG were determined by flow cytometry (Figure 5a). A1mut2 ($IgG_A$) increased the percentage of T cells responding to the mitogen (responder frequency) and the average number of daughter cells produced per responding cell (proliferative capacity) in 8/9 dogs at 96 hours post stimulation when compared to the negative control MERS $IgG_A$ (Figure 5b). Interferon-γ (IFN-γ) production was evaluated in the supernatants of T cells stimulated with ConA in the presence of A1mut2 $IgG_A$. T cell cultures from 4/4 dogs showed an increase in IFN-γ production at 96 hrs when A1mut2 was added (Figure 5c). Given that a recent report has shown that ipilimumab, (anti-CTLA4 $IgG_1$), but not ipilimumab reformatted as an $IgG_4$, inhibits antigen-specific $CD4^+$ T cell proliferation in a $CD16^+$ cell-dependent manner in vitro,[42] a repeat T cell proliferation experiment was performed using PBMCs from 2 healthy donor dogs to determine whether use of A1mut2 $IgG_B$ (the functional equivalent of human $IgG_1$ subclass) would inhibit T cell responses. A1mut2 $IgG_B$ did not inhibit $CD5^+$ T cell proliferation and no difference was identified between the effects of A1mut2 $IgG_A$ and $IgG_B$ on $CD5^+$ T cell proliferation (data not shown). Together, these results suggest that A1mut2-mediated checkpoint inhibition promotes canine T cell proliferation and IFN-γ production and further support its clinical evaluation as an IgG to enhance tumor-specific T cell priming and effector responses within the tumor microenvironment.

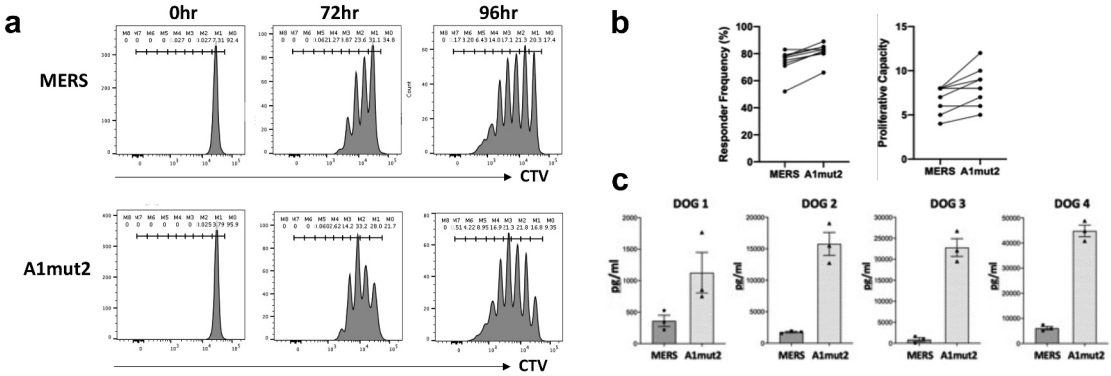


Figure 5. A1mut2 increases the effector function of canine T cells. Canine PBMCs were labeled with CTV and stimulated with ConA at 2.5ug/ml in the presence of 10ug/ml of either A1mut2 or the irrelevant MERS antibody. Cells were harvested at 72 or 96 h and labeled with an anti-CD5 mAb and the viability dye 7-AAD. Cells were acquired on a FACS Canto II and analyzed by FlowJo software. Responder frequency (number of cells undergoing at least one division) and proliferative capacity (average number of daughter cells produced per cell) were determined. (a). Representative histogram from one dog. Plots are gated on $7AAD^-$>single cell>$CD5^+$ cells. (b). Responder frequency and proliferative capacity of 9 healthy dogs calculated at 96 hours post stimulation. (c) IFN-γ present in culture supernatants as determined by ELISA at 96 hrs. Data from experimental triplicates of three healthy donor dogs and one dog with B cell lymphoma (dog 3) are shown. Mean with SEM is shown.

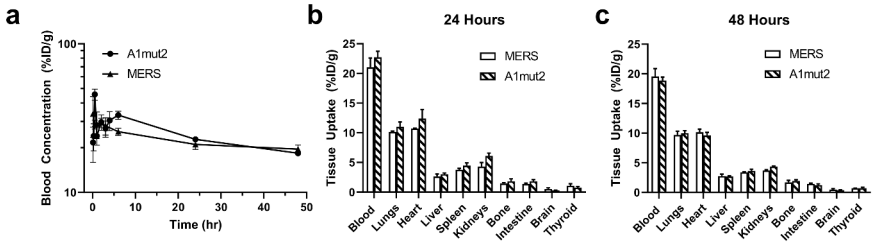


**Figure 6.** Blood and tissue pharmacokinetics of A1mut2 and control MERS IgG in C57BL/6 mice following an intravenous bolus dose. (a). Blood concentration versus time profile; (b). Tissue biodistribution 24 h post-injection; (c). Tissue biodistribution 48 h post-injection.

### *A1mut2 $IgG_B$ displays linear kinetics in vivo*

Following confirmation that A1mut2 binds murine CTLA4 by ELISA (data not shown) and displays subnanomolar binding affinity and similar binding kinetics to the extra cellular domain of murine CTLA4 as for cCTLA4, albeit with a protracted off-rate (Table 1), the pharmacokinetics (PK) of A1mut2 (subclass $IgG_B$) in mice were determined. After intravenous injection into naïve mice, A1mut2 displayed blood (Figure 6a) and tissue (Figure 6b and c) PK similar to that of the untargeted control $IgG_B$ (MERS). The blood area under the concentration vs. time ($AUC_{0\text{-}48h}$) was similar between A1mut2 and MERS (1174 ± 51%ID/g*h vs. 1073 ± 56%ID/g*h, respectively, $p > .05$ by Student's *t*-test), suggesting that overall exposure was the same between the two IgG. A1mut2 appeared to circulate well in mice, with 18.4 ± 0.4%ID/g remaining in blood 48 hours post-injection (vs. 19.6 ± 1.3 for MERS), which represents ~30% of the total injected dose in the circulation. These results suggest that at a potentially therapeutically relevant dose, binding between A1mut2 and mouse CTLA4 does not affect disposition in naïve mice. These results are consistent with observations in humans where anti-CTLA4 displays dose-proportional PK in humans at doses of 3 and 10 mg/kg. Further, there were no differences in either body weight or in complete blood count parameters between mice treated with A1mut2 and MERS (Supplemental Figure S6), demonstrating that the injection of A1mut2 was well-tolerated and did not result in untoward hematological effects.

## Discussion

CTLA4 blockade has proven to be a powerful strategy to promote anti-tumor immunity by inducing the expansion of Th1-like $CD4^+$ effector T cells and exhausted $CD8^+$ T cells.[43] These effects have led to clinically relevant anti-tumor immunity, particularly in human patients with malignant melanoma, NSCLC and renal carcinoma. Interestingly, in mouse models of cancer, anti-CTLA4 antibodies eliminate intra-tumoral Tregs, but in human cancer patients treated with either ipilimumab or tremelimumab, intra-tumoral depletion of Tregs was not observed.[27,44] A greater understanding of mechanisms of acquired and/or innate resistance of tumors to anti-CTLA4 therapy, identification of biomarkers of response, optimized protocols and combination approaches to improve outcome, and understanding of the mechanisms related to checkpoint toxicity in clinical patients is needed to increase overall response rate and reduce toxicity.[45] Here, we used scFv phage display to identify multiple unique canine scFvs that specifically bind with nanomolar and sub-nanomolar affinities to canine CTLA4. From these scFvs, we generated and further selected full-length canine lead candidate IgG based on developability and in vitro functional capacity to evaluate in clinical trials in dogs with spontaneous cancers to further investigate mechanisms of resistance and correlative biomarkers of response. The use of fully canine anti-CTLA4 IgG in immune competent canine cancer patients with spontaneous tumors that share similar features to their human counterparts should yield informative results for human clinical trial design. To this end, in 2017 the Beau Biden Cancer Moonshot Initiative launched a canine cancer immunotherapy clinical trials network aimed at evaluating novel immunotherapies and combination immunotherapies in pet dogs with spontaneous cancer with the ultimate aim to accelerate human clinical translation.-[46] The development of a fully canine anti-CTLA4 provides an important comparative tool for translational research and enables such combination therapies being evaluated to include checkpoint inhibition.

In the absence of a fully validated, anti-canine CTLA4 antibody, little is known about CTLA4 biology in the dog. Similar to results from human donors, we observed minimal cell surface expression of CTLA4 on resting peripheral T cells from healthy canine donors.[47] However, as for human T cells, CTLA4 was up regulated on the surface of $CD4^+$ and $CD8^+$ T cells following activation. We observed higher CTLA4 expression in the $CD4^+$ T cell subset compared to $CD8^+$ T cells after activation, consistent with findings in human $CD4^+$ and $CD8^+$ T cells[48] and peak expression of CTLA4 on canine T cells at or after 72 hours. Similarly, studies in human T cells have shown peak expression of CTLA4 on mitogen-activated total T cells at 72 hrs post stimulation.[49] Together, these results reveal comparable CTLA4 cell surface expression kinetics between canine and human T cells. Furthermore, we confirmed at the protein level the transcriptomic findings of Garden et al. that showed CTLA4 is constitutively expressed in canine Tregs and as such may serve as a target for A1mut2-dependent complement mediated cytotoxicity.[37] In human patients, the higher level of CTLA4 expression on intra-tumoral Tregs might enable their selective depletion following anti-CTLA4 treatment compared to Tregs in the peripheral blood, and this may contribute to an increase in intra-tumoral CD8:Treg following treatment. This mechanism of action in human patients, however, remains controversial.[50,51] Future studies to directly compare expression levels of CTLA4 on canine peripheral versus intra-tumoral Tregs will determine whether similar differences exist

in canine cancer patients and will allow clinical investigations into the effect of local or systemic delivery of anti-CTLA4 antibodies on selective intra-tumoral Treg depletion in pet dogs with spontaneous cancers.

In human PBMCs, monotherapy with ipilimumab showed minimal effects on IFN-γ production and T cell proliferation in allogeneic T:DC mixed lymphocyte response assays and only modest increases in interleukin-2 production following mitogen activation of human T cells.[47] Furthermore, response varied amongst individual samples tested. Although in vitro assay response in human T cells has been modest, clinical responses in a subset of human patients have been dramatic and underscore the different effects that CTLA4 blockade exerts in vivo, as well as the influence that patient-specific factors may have on clinical response. We also observed marked inter-donor variability in the magnitude of T cell proliferation and IFNγ production that occurred in response to CTLA4 blockade amongst mitogen activated canine T cells. Interestingly, a recent study demonstrated that ipilimumab, an IgG1 monoclonal antibody and therapeutic checkpoint inhibitor, inhibits antigen-specific $CD4^+$ T cell responses in vitro and this effect is dependent upon the presence of $CD16^+$ cells in the culture.[42] As such, use of the same antibody reformatted as an $IgG_4$, which lacks FcR binding capability, did not inhibit $CD4^+$ T cell responses. Our results using A1mut2 $IgG_A$ in effector T cell assays are similar to those observed with ipilimumab $IgG_4$. However, both A1mut2 $IgG_A$ (weakly binds to FcγRI and FcγRIIb, no binding to FcγRIII) and $IgG_B$ (weakly binds to FcγRIIb and FcγRIII, strongly binds to FcγRI)[41] promoted effector T cell proliferation in our studies. Given the significant variability amongst human donors of $CD4^+$ T cell responses to anti-CTLA4 IgG1 in vitro, it is possible that larger numbers of canine donors would be required to see a statistically significant inhibitory effect of A1mut2 $IgG_B$ on $CD4^+$ T cell responses.

Given the restricted genetic heterogeneity that occurs within canine breeds, this parallel patient population may now enable us to more readily determine genetic factors that might influence a patient's ability to respond to checkpoint inhibition.[52] Likewise, within canine breeds, studies to evaluate the influence of the individual's genetic background and the gut microbiome on the severity of immune-mediated side effects of checkpoint inhibition now become possible and might shed light on these important questions that remain unanswered in human immune-oncology.

We have now engineered A1mut2 as an $IgG_B$, which is the functional analog of the human IgG1 subtype that efficiently fixes complement and induces ADCC.[41] Evaluation of this antibody in mice revealed linear PK and no evidence of toxicity over 48 hours. In these studies, A1mut2 was not found to accumulate preferentially in lymphoid-rich tissue of naïve mice (housed in a sterile environment), possibly due to the lack of CTLA4 expressed on the surface of conventional resting T cells. However, additional PK studies will need to be performed in dogs before A1mut2 can be tested in clinical trials. Our SPR analysis showed that A1mut2 has a higher binding affinity than both ipilimumab and tremelimumab (anti-human CTLA4 $IgG_2$), which is associated with the comparatively slow dissociation rate of A1mut2 from CTLA4 ($1.8 \times 10^{-4} M^{-1}s^{-1}$ compared to ipilimumab at $6.96 \times 10^{-3} M^{-1}s^{-1}$).[40] As such, we anticipate that the enhanced binding of A1mut2 will influence its PK properties, which might be reflected as a lower plasma AUC but improved duration of receptor occupancy, and thereby prolonged pharmacodynamic effects. This is a hypothesis that needs to be tested via direct comparison of variants with different binding affinities. It remains to be determined whether this would be associated with greater autoimmune adverse events, but it might enable clinical effects to be seen at a lower administered dose and increased inter-dosing interval.

The field is now poised to investigate outstanding questions in the field of CTLA4 checkpoint inhibition in a clinically relevant, immune competent, large animal model, including understanding mechanisms that might mediate resistance to anti-CTLA4 therapy. The role of the gut microbiome in determining susceptibility to autoimmunity and predicting clinical response to CTLA4 blockade[53,54] is of particular interest for this comparative approach, given comparable features of gut microbiota between humans and canines.[55] Furthermore, studies in canine cancer patients using anti-CTLA4 IgG to augment immunogenic effects and clinical responses to vaccine strategies, adoptive cell therapies, radiation therapy and chemotherapy can aid in accelerating the most promising combination approaches into human clinical studies. Finally, if clinical responses to CTLA4 inhibition in canine malignancies mirror those of human patients with melanoma, renal cell carcinoma and urothelial carcinoma, incorporation of this therapy into the veterinary arsenal will improve the outcome of pet dogs with similar malignancies.

## Materials and methods

### Cells and cell lines

PBMCs were isolated from blood of either healthy donor dogs or blood from a dog with T cell lymphoma (for Treg staining) or a dog with relapsed B cell lymphoma by discontinuous density centrifugation over Ficoll-Paque PLUS (GE Healthcare, Chicago, IL). For feline cells, residual heparinized blood from clinical hematological assessments of two cats with hematological malignancies was first subject to red blood cell lysis (ACK lysing buffer, ThermoFisher Scientific, Waltham, MA). Cells were washed twice in complete (c)RPMI media containing RPMI 1640 with 2 mM L-Glutamine (Mediatech, Manassas, VA), 10% heat-inactivated fetal bovine serum (FBS; Atlanta Biologicals, Flowery Branch, Georgia), 10 mM HEPES (Gibco, Grand Island, NY), and 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco) prior to use. Where indicated negatively selected canine T cells were used. PBMCs were washed in (c)RPMI, labeled with mouse anti-dog CD11b (Clone CA16.3E10), CD11c (Clone CA11.6A1), mouse anti-human CD14 (Clone TuK4), and mouse anti-Dog CD21 (Clone CA2.1D6) all from ABD Serotec/Biorad, followed by goat anti-mouse IgG Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). T cells were negatively selected using MACS LS columns (Miltenyi). T cell purity was determined by CD5 labeling and flow cytometric analysis. Human cell lines (K562 and 293 T cells) were grown in RPMI-1640

supplemented with HEPES, 1 mM sodium pyruvate (Mediatech), glutamine and penicillin and streptomycin (Thermo Fisher Scientific) supplemented with 10% FBS (cRPMI).

### Generation of a canine CTLA4 expressing target cell line

Full-length cCTLA4 was amplified from cPBMC cDNA by RT-PCR as described above using the following primers (cCTLA4 Forward: 5′-acgctGAATTCatggctggctttggattccggaggcat – 3′, cCTLA4 Reverse: 5′- acagtGTCGACtcaattgatgggaataaaataa-3′). The resulting 1856bp amplicon was cloned into the pMX-puromycin retroviral expression vector (Cell Biolabs, Inc. San Diego CA). Retrovirus was generated and used to stably transduce the human erythroleukemic K562 cell line, previously edited using CRISPR/Cas9 to remove FcγRII (KTδ32) and reduce nonspecific mAb binding. Transduced cells were selected in 2.5 μg/ml of puromycin dihydrochloride (Sigma, St. Louis, MO) to yield KTδ32.cCTLA4. Expression of the cCTLA4 transgene was confirmed by RT-PCR.

### scFv phage ELISA

For ELISAs to detect binding of phage-displayed scFvs, microplate wells coated with streptavidin were used to capture biotinylated avitag cCTLA-4 and control antigens as described for phage display library panning (see Supplementary Methods). To cCTLA-4-coated plates, samples were added of polyclonal phage from the PEG-concentrated initial phage library (P0) and antigen enriched libraries obtained after each round of panning (P1 through P4) diluted 1:1000 in 2% nonfat dry milk in phosphate-buffered saline (MPBS) or monoclonal phage prepared from randomly picked phage clones from output plates of the third round (P3) and fourth round (P4) of panning (non-PEG-concentrated phage diluted 1:100 in MPBS). After a 1-hour incubation at 37°C, plates were washed with 0.1% Tween 20 in PBS (PBST) and horseradish peroxidase (HRP)-conjugated anti-M13 mAb (GE Healthcare., Chicago, IL) diluted 1:5000 in MPBS was added. Plates were washed again and bound HRP-conjugated secondary antibody was detected with 2,2′-azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS). Optical density was read at 405 nm after 30 min using a Molecular Devices SpectraMax 340 spectrophotometer. Plates coated with no antigen, canine CD19, streptavidin alone, and streptavidin with irrelevant human avitag-CD3ε (Acro Biosystems, Newark, DE) were used as negative controls.

### Flow cytometry

Canine PBMCs or isolated T cells, feline leucocytes, KTδ32. cCTLA4, and KTδ32 (WT) cell lines were washed twice in fluorescence-activated cell sorting (FACS) buffer (1% heat-inactivated FBS in 1X PBS with calcium and magnesium). Cells were blocked with 10 μg of canine IgG (Jackson ImmunoResearch Labs Inc) for 10 minutes at room temperature (RT) prior to cell surface labeling with 5 μg of canine scFv, or 500 ng canine $IgG_A$. Specificity of IgG binding was evaluated in blocking experiments where 250 ng $IgG_A$ were pre-incubated with 1.25 μg cCTLA4 extracellular domain (ECD) protein for one hr at RT prior to incubation with target cells. Cells were washed in FACS buffer and incubated with either $IgG_A$ alone or pre-incubated $IgG_A$. After washing, an APC-labeled anti-HA.11 epitope tag (BioLegend, San Diego, CA) and viability dye 7-AAD (BioLegend) were added, and cells were incubated for 30 min at RT. For experiments using PBMCs, where indicated, cells were also labeled with rat anti-canine CD45 (Clone YKIX716.13, BioRad, Hercules, CA), rat anti-canine CD5 mAb (Clone: YKIX 322.3 ThermoFisher Scientific) or mouse anti-feline CD5 mAb (Clone: FE1.1B11, BioRad) and/or rat anti-canine CD4 (Clone YKIX302.9 BioRad). Following cell surface labeling, cells were washed twice in FACS buffer and fixed in 1% paraformaldehyde (Thermo Fisher Scientific, Waltham, MA). Cells were acquired on a FACS Canto II flow cytometer (BD Biosciences) and data was analyzed using FlowJo software version X (Treestar, Ashland, OR). For intracellular staining, cells were surface stained where applicable, washed twice in FACS buffer and resuspended in fixation/permeabilization FOXP3/Transcription Factor Staining Buffer Set (ThermoFisher Scientific, Waltham, MA). Cells were incubated on ice for 30 min in the dark and then washed once with permeabilization buffer. Cells were then incubated with APC-conjugated anti-mouse FoxP3 (clone: FJK-16s, ThermoFisher Scientific) or Rat IgG2a kappa isotype control APC (17–4321-81, ThermoFisher Scientific), anti-CTLA4 IgGs (0.5ug) or an anti-MERS antibody (0.5 ug, negative control) plus anti-HA.11 epitope tag PE (BioLegend) on ice for 30 min. Cells were washed once with permeabilization buffer and twice with 1x FACS buffer prior to acquisition.

### Soluble scFv ELISA

Thirty μg/ml streptavidin was added to ELISA plates and incubated overnight at 4°C. Plates were subsequently blocked with 5% milk/PBS-Tween. Titrations of biotinylated CTLA4 were added and after washing, 0.25 μg/ml of soluble scFvs were added and incubated for 2 hr at RT. For expression extract ELISA, 10–100 μl of extracts were added to ELISA plates and incubated for 2 hr at RT. Bound scFvs were detected using anti-HA mouse IgG AP conjugate or an anti-rabbit AP conjugate for the positive polyclonal rabbit anti-canine CTLA4 control (Sino Biological, Wayne, PA) incubated for 1 hr at RT, washed and detected by an AP colorimetric substrate. Plates were read at 650 nm.

### ELISA-based cCTLA-4 receptor blocking assay

0.3, 1.0 and 3.0 pmol of recombinant human CD86-Fc chimera (carrier free) or human CD80-Fc chimera (carrier free) (BioLegend) or recombinant canine CD86-Fc or canine CD80-Fc were bound to ELISA plates overnight. Wells were blocked with 5% milk/PBS 0.05% Tween. Three pmol of biotinylated or unbiotinylated cCTLA4 protein in PBS/Tween were added to the wells and incubated for 2 hr at RT. For experiments aimed at evaluating blocking activity, cCTLA4 was pre-incubated with scFv or full-length $IgG_A$ at the indicated molar ratios for 1 hr at RT prior to being added to the plate with human or canine CD80 and CD86 and incubated on a shaker for 2 hr at RT. After washing, a streptavidin-AP conjugate

(Jackson ImmunoResearch, West Grove, PA) was added for 1 hr at RT. Three TBS-Tween washes were performed and cCTLA4 bound to human or canine CD80 or CD86 was detected using an AP colorimetric substrate (InvivoGen, Carlsbad, CA).

### *In vitro PBMC stimulation assays*

Canine PBMCs were washed once with PBS and red blood cells were removed by ACK lysing buffer (ThermoFisher Scientific). Cells were washed once in complete IMDM media supplemented with 10% FBS, labeled with Cell Trace Violet (ThermoFisher Scientific) at 0.5 μM and incubated for 20 min at RT. Cells were washed in complete IMDM and resuspended to $1 \times 10^6$ cells/ml and cultured in triplicate in 96-well, round-bottom plates with Concanavalin A (Sigma-Aldrich) at 2.5 μg/ml, in the presence or absence of 10 μg/ml of anti-canine CTLA4 $IgG_A$ or $IgG_B$ or the negative anti-MERS $IgG_A$ or $IgG_B$ control. Proliferation was determined by flow cytometry at 72 and 96 h. At 72 h, supernatants were harvested and the amount of IFN-γ present in the supernatants was measured by canine IFN-γ ELISA (R&D Systems, Minneapolis, MN). For experiments to detect cell surface expression of CTLA4 after activation, canine PBMCs were resuspended to $1 \times 10^6$ cells/ml and cultured in 96-well, round-bottom plates with Concanavalin A at 2.5 μg/ml for 48 and 72 hours prior to analysis.

### *Determination of binding kinetics and affinity*

A Biacore T200 SPR instrument with version 2.0 and HC30M (Xantec Bioanalytics, Duesseldorf, Germany) sensor chips were used to determine the binding parameters of anti-canine CTLA4 antibodies to soluble canine and murine CTLA4 ECD. Anti-CTLA4 antibodies ($IgG_A$) were immobilized on the surface of a protein A/G coated linear carboxylate SPR sensor chip. Briefly, each anti-CTLA4 IgG was diluted to 2.5 ug/ml in 20 mM acetate at a pH of 5.0 and injected into the experimental flow cell over 60 seconds. For B10, 440–475 RU were captured, for D5, 400–500 RU were captured and for A1mut2, 450–535 RU were captured. Any remaining activated sites were blocked with 1 M ethanolamine, pH 8.5 for 10 mins. 10 x 1:2 serial dilutions of canine or murine CTLA4 ECD (Sino Biological) ranging from 200 nM to 0 nM (for canine CTLA4) or 100 nM to 0 nM (for murine CTLA4) were prepared in duplicate in running buffer (10 mM HEPES, pH 7.4, 150 mM NaCl and 0.05% Tween20). The flow rate across the chip was 30 uL/min and the contact time of the cCTLA4 ECD sample with the chip surface was 240 s. Dissociation was monitored for 720–960s. Report points were recorded before and after each injection and the amount of antigen binding for each analysis cycle was reported in relative response units (RU). Curves of response versus antigen concentration were generated using the BIAcore Wizard program. After each injection of CTLA4, the chip surface was regenerated with 20 mM glycine at pH 2.0 for 60 s after the RU for each concentration of analyte was recorded. All experiments were carried out at 25°C. Assay data was processed using Biacore Evaluation Software, version 2.0 to obtain kinetic and affinity values and reported as $k_{on}$, $k_{off}$ and $K_D$.

### *Radiolabeling*

For in vivo murine studies, proteins were radiolabeled with $^{125}I$ (PerkinElmer, Waltham, MA) using the Iodogen method. Labeled proteins were purified using Zeba 7 kDa spin columns and radiochemical purity was confirmed to be >95% via thin layer chromatography.

### *In vivo studies*

All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania and carried out in 6–8-week-old, male C57BL/6 mice (body weight ~ 20–25 g). Radiolabeled A1mut2 ($IgG_B$) (3 mg/kg) or control anti-MERS $IgG_B$, (0.8 mg/kg) were injected intravenously via the retro-orbital plexus. Blood was collected in staggered sampling approach at designated times post-injection (5, 15, 30 min, 1, 2, 3, 4, 6, 24, 48 h) and tissues were collected following animal sacrifice at 24 and 48 h. All samples were analyzed via gamma counting (PerkinElmer Wizard 2) and blood and tissue concentrations were represented as percent of injected dose per gram of tissue (%ID/g). In addition to PK samples, animals were weighed throughout the study and complete blood counts (Abaxis VetScan HM5 Hematology Analyzer) were measured at 48 h post-injection.

## Abbreviations

APC, Antigen-presenting cells; ConA, Concanavalin A; CTLA4, Cytotoxic T lymphocyte associated protein 4; ELISA, Enzyme-linked immunoassay; mAb, Monoclonal antibody; MERS, Middle East respiratory syndrome; NSCLC, Non-small cell lung carcinoma; scFv, Single-chain variable fragments; SPR, Surface plasmon resonance; Tregs, Regulatory T cells.

## Acknowledgments

This work was supported by the V Foundation and the NCI/SBIR under Contract 75N91018C000042. PG is supported by NCI/NHLBI HL157189. We would like to thank Joel Cassel for his assistance and expertise in the performance of SPR.

## Disclosure statement

Authors NJM, DLS and NC have equity in Vetigenics LLC.

## Funding

This work was supported by the National Cancer Institute; National Heart, Lung, and Blood Institute; Japanese Society for the Promotion of Science; V Foundation for Cancer Research.

## References

1. Linsley PS, Greene JL, Brady W, Bajorath J, Ledbetter JA, Peach R. Human B7-1 (CD80) and B7-2 (CD86) bind with similar avidities but distinct kinetics to CD28 and CTLA-4 receptors. Immunity. 1994;1:793–11. doi:10.1016/S1074-7613(94)80021-9
2. Walunas TL, Lenschow DJ, Bakker CY, Linsley PS, Freeman GJ, Green JM, Thompson CB, Bluestone JA. CTLA-4 can function as a negative regulator of T cell activation. Immunity. 1994;1:405–13. doi:10.1016/1074-7613(94)90071-X

3. Chuang E, Fisher TS, Morgan RW, Robbins MD, Duerr JM, Vander Heiden MG, Gardner JP, Hambor JE, Neveu MJ, Thompson CB, *et al*. The CD28 and CTLA-4 receptors associate with the serine/threonine phosphatase PP2A. Immunity. 2000;13:313–22. doi:10.1016/S1074-7613(00)00031-5.
4. Marengere LE, Waterhouse P, Duncan GS, Mittrücker H-W, Feng G-S, Mak TW. Regulation of T cell receptor signaling by tyrosine phosphatase SYP association with CTLA-4. Science. 1996;272:1170–73. doi:10.1126/science.272.5265.1170
5. Walker LS, Sansom DM. Confusing signals: recent progress in CTLA-4 biology. Trends Immunol. 2015;36:63–70. doi:10.1016/j.it.2014.12.001
6. Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, Nomura T, Sakaguchi S. CTLA-4 control over Foxp3 + regulatory T cell function. Science. 2008;322:271–75. doi:10.1126/science.1160062
7. Onishi Y, Fehervari Z, Yamaguchi T, Sakaguchi S. Foxp3+ natural regulatory T cells preferentially form aggregates on dendritic cells in vitro and actively inhibit their maturation. Proc Natl Acad Sci U S A. 2008;105:10113–18. doi:10.1073/pnas.0711106105
8. Waterhouse P, Penninger JM, Timms E, Wakeham A, Shahinian A, Lee KP, Thompson CB, Griesser H, Mak TW. Lymphoproliferative disorders with early lethality in mice deficient in CTLA-4. Science. 1995;270:985–88. doi:10.1126/science.270.5238.985
9. Egen JG, Kuhns MS, Allison JP. CTLA-4: new insights into its biological function and use in tumor immunotherapy. Nat Immunol. 2002;3:611–18. doi:10.1038/ni0702-611
10. Leach DR, Krummel MF, Allison JP. Enhancement of antitumor immunity by CTLA-4 blockade. Science. 1996;271:1734–36. doi:10.1126/science.271.5256.1734
11. Grosso JF, Jure-Kunkel MN. CTLA-4 blockade in tumor models: an overview of preclinical and translational research. Cancer Immun. 2013;13:5.
12. Shrikant P, Khoruts A, Mescher MF. CTLA-4 blockade reverses CD8+ T cell tolerance to tumor by a CD4+ T cell- and IL-2-dependent mechanism. Immunity. 1999;11:483–93. doi:10.1016/S1074-7613(00)80123-5
13. Sotomayor EM, Borrello I, Tubb E, Allison JP, Levitsky HI. In vivo blockade of CTLA-4 enhances the priming of responsive T cells but fails to prevent the induction of tumor antigen-specific tolerance. Proc Natl Acad Sci U S A. 1999;96:11476–81. doi:10.1073/pnas.96.20.11476
14. Yang YF, Zou JP, Mu J, Wijesuriya R, Ono S, Walunas T, Bluestone J, Fujiwara H, Hamaoka T. Enhanced induction of antitumor T-cell responses by cytotoxic T lymphocyte-associated molecule-4 blockade: the effect is manifested only at the restricted tumor-bearing stages. Cancer Res. 1997;57:4036–41.
15. van Elsas A, Hurwitz AA, Allison JP. Combination immunotherapy of B16 melanoma using anti-cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and granulocyte/macrophage colony-stimulating factor (GM-CSF)-producing vaccines induces rejection of subcutaneous and metastatic tumors accompanied by autoimmune depigmentation. J Exp Med. 1999;190:355–66. doi:10.1084/jem.190.3.355
16. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, Gonzalez R, Robert C, Schadendorf D, Hassel JC, *et al*. Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med. 2010;363:711–23. doi:10.1056/NEJMoa1003466.
17. Kwon ED, Hurwitz AA, Foster BA, Madias C, Feldhaus AL, Greenberg NM, Burg MB, Allison JP. Manipulation of T cell costimulatory and inhibitory signals for immunotherapy of prostate cancer. Proc Natl Acad Sci U S A. 1997;94:8099–103. doi:10.1073/pnas.94.15.8099
18. Yang JC, Beck KE, Blansfield JA, Tran KQ, Lowy I, Rosenberg SA. Tumor regression in patients with metastatic renal cancer treated with a monoclonal antibody to CTLA4 (MDX-010). J Clin Oncol. 2005;23:2501. doi:10.1200/jco.2005.23.16_suppl.2501
19. Lynch TJ, Bondarenko I, Luft A, Serwatowski P, Barlesi F, Chacko R, Sebastian M, Neal J, Lu H, Cuillerot J-M, *et al*. Ipilimumab in combination with paclitaxel and carboplatin as first-line treatment in stage IIIB/IV non-small-cell lung cancer: results from a randomized, double-blind, multicenter phase II study. J Clin Oncol. 2012;30:2046–54. doi:10.1200/JCO.2011.38.4032.
20. Weintraub K. Drug development: releasing the brakes. Nature. 2013;504:S6–8. doi:10.1038/504S6a
21. Arce Vargas F, Furness AJS, Litchfield K, Joshi K, Rosenthal R, Ghorani E, Solomon I, Lesko MH, Ruef N, Roddie C, *et al*. Fc effector function contributes to the activity of human anti-CTLA-4 antibodies. Cancer Cell. 2018;33:649–663 e644. doi:10.1016/j.ccell.2018.02.010.
22. Selby MJ, Engelhardt JJ, Quigley M, Henning KA, Chen T, Srinivasan M, Korman AJ. Anti-CTLA-4 antibodies of IgG2a isotype enhance antitumor activity through reduction of intratumoral regulatory T cells. Cancer Immunol Res. 2013;1:32–42. doi:10.1158/2326-6066.CIR-13-0013
23. Simpson TR, Li F, Montalvo-Ortiz W, Sepulveda MA, Bergerhoff K, Arce F, Roddie C, Henry JY, Yagita H, Wolchok JD, *et al*. Fc-dependent depletion of tumor-infiltrating regulatory T cells co-defines the efficacy of anti-CTLA-4 therapy against melanoma. J Exp Med. 2013;210:1695–710. doi:10.1084/jem.20130579.
24. Bertrand A, Kostine M, Barnetche T, Truchetet ME, Schaeverbeke T. Immune related adverse events associated with anti-CTLA-4 antibodies: systematic review and meta-analysis. BMC Med. 2015;13:211. doi:10.1186/s12916-015-0455-8
25. Myers G. Immune-related adverse events of immune checkpoint inhibitors: a brief review. Curr Oncol. 2018;25:342–47. doi:10.3747/co.25.4235
26. Khoja L, Day D, Wei-Wu Chen T, Siu LL, Hansen AR. Tumour- and class-specific patterns of immune-related adverse events of immune checkpoint inhibitors: a systematic review. Ann Oncol. 2017;28:2377–85. doi:10.1093/annonc/mdx286
27. Fransen MF, van der Sluis TC, Ossendorp F, Arens R, Melief CJ. Controlled local delivery of CTLA-4 blocking antibody induces CD8+ T-cell-dependent tumor eradication and decreases risk of toxic side effects. Clin Cancer Res. 2013;19:5381–89. doi:10.1158/1078-0432.CCR-12-0781
28. Marabelle A, Kohrt H, Sagiv-Barfi I, Ajami B, Axtell RC, Zhou G, Rajapaksa R, Green MR, Torchia J, Brody J, *et al*. Depleting tumor-specific Tregs at a single site eradicates disseminated tumors. J Clin Invest. 2013;123:2447–63. doi:10.1172/JCI64859.
29. Wang Z, Wu VH, Allevato MM, Gilardi M, He Y, Luis Callejas-Valera J, Vitale-Cross L, Martin D, Amornphi moltham P, Mcdermott J, *et al*. Syngeneic animal models of tobacco-associated oral cancer reveal the activity of in situ anti-CTLA-4. Nat Commun. 2019;10:5546. doi:10.1038/s41467-019-13471-0.
30. Dow S. A role for dogs in advancing cancer immunotherapy research. Front Immunol. 2019;10:2935. doi:10.3389/fimmu.2019.02935
31. Gardner HL, Fenger JM, London CA. Dogs as a model for cancer. Annu Rev Anim Biosci. 2016;4:199–222. doi:10.1146/annurev-animal-022114-110911
32. Schiffman JD, Breen M. Comparative oncology: what dogs and other species can teach us about humans with cancer. Philos Trans R Soc Lond B Biol Sci. 2015;370:20140231. doi:10.1098/rstb.2014.0231
33. Graves SS, Stone D, Loretz C, Peterson L, McCune JS, Mielcarek M, Storb R. Establishment of long-term tolerance to SRBC in dogs by recombinant canine CTLA4-Ig. Transplantation. 2009;88:317–22. doi:10.1097/TP.0b013e3181ae3285
34. Tagawa M, Maekawa N, Konnai S, Takagi S, Bayry J. Evaluation of costimulatory molecules in peripheral blood lymphocytes of canine patients with histiocytic sarcoma. PLoS One. 2016;11:e0150030. doi:10.1371/journal.pone.0150030
35. Tagawa M, Kurashima C, Takagi S, Maekawa N, Konnai S, Shimbo G, Matsumoto K, Inokuma H, Kawamoto K, Miyahara K, *et al*. Evaluation of costimulatory molecules in dogs with B cell high grade lymphoma. PLoS One. 2018;13:e0201222. doi:10.1371/journal.pone.0201222.

36. Wherry EJ, Ha S-J, Kaech SM, Haining WN, Sarkar S, Kalia V, Subramaniam S, Blattman JN, Barber DL, Ahmed R, *et al*. Molecular signature of CD8+ T cell exhaustion during chronic viral infection. Immunity. 2007;27:670–84. doi:10.1016/j.immuni.2007.09.006.
37. Wu Y, Chang Y-M, Stell AJ, Priestnall SL, Sharma E, Goulart MR, Gribben J, Xia D, Garden OA. Phenotypic characterisation of regulatory T cells in dogs reveals signature transcripts conserved in humans and mice. Sci Rep. 2019;9:13478. doi:10.1038/s41598-019-50065-8
38. Andris-Widhopf J, Steinberger P, Fuller R, Rader C, and Barbas CF. Generation of antibody libraries: PCR amplification and assembly of light- and heavy-chain coding sequences. In: Barbas CF, Burton DR, Scott JK, and Silverman GJ, editors. Phage display: a laboratory manual. Chapter. 9, 9.1-9.113: Cold Spring Harbor Laboratory Press, NY; Cold Spring Harbor Laboratory Press.
39. Lefranc MP, Giudicelli V, Ginestoux C, Jabado-Michaloud J, Folch G, Bellahcene F, Wu Y, Gemrot E, Brochet X, Lane J, *et al*. IMGT, the international ImMunoGeneTics information system. Nucleic Acids Res. 2009;37:D1006–1012. doi:10.1093/nar/gkn838.
40. He M, Chai Y, Qi J, Zhang CWH, Tong Z, Shi Y, Yan J, Tan S, Gao GF. Remarkably similar CTLA-4 binding properties of therapeutic ipilimumab and tremelimumab antibodies. Oncotarget. 2017;8:67129–39. doi:10.18632/oncotarget.18004
41. Bergeron LM, McCandless EE, Dunham S, Dunkle B, Zhu Y, Shelly J, Lightle S, Gonzales A, Bainbridge G. Comparative functional characterization of canine IgG subclasses. Vet Immunol Immunopathol. 2014;157:31–41. doi:10.1016/j.vetimm.2013.10.018
42. Rosskopf S, Leitner J, Zlabinger GJ, Steinberger P. CTLA-4 antibody ipilimumab negatively affects CD4(+) T-cell responses in vitro. Cancer Immunol Immunother. 2019;68:1359–68. doi:10.1007/s00262-019-02369-x
43. Wei SC, Levine JH, Cogdill AP, Zhao Y, Anang NAAS, Andrews MC, Sharma P, Wang J, Wargo JA, Pe'er D, *et al*. Distinct cellular mechanisms underlie anti-CTLA-4 and anti-PD-1 checkpoint blockade. Cell. 2017;170:1120–1133 e1117. doi:10.1016/j.cell.2017.07.024.
44. Sharma A, Subudhi SK, Blando J, Scutti J, Vence L, Wargo J, Allison JP, Ribas A, Sharma P. Anti-CTLA-4 immunotherapy does not deplete FOXP3 + regulatory T cells (Tregs) in human cancers. Clin Cancer Res. 2019;25:1233–38. doi:10.1158/1078-0432.CCR-18-0762
45. Weiss SA, Wolchok JD, Sznol M. Immunotherapy of melanoma: facts and hopes. Clin Cancer Res. 2019;25:5191–201. doi:10.1158/1078-0432.CCR-18-1550
46. Diagnosis, N.C.I.D.o.C.T.a. NCI-Funded Canine Immunotherapy Trials Network Treats Pet Dogs to Study Cancers Common to Humans. 2019. https://dctd.cancer.gov/NewsEvents/20190327_canine_immunotherapy.htm
47. Selby MJ, Engelhardt JJ, Johnston RJ, Lu L-S, Han M, Thudium K, Yao D, Quigley M, Valle J, Wang C, *et al*. Preclinical development of ipilimumab and nivolumab combination immunotherapy: mouse tumor models, in vitro functional studies, and cynomolgus macaque toxicology. PLoS One. 2016;11:e0161779. doi:10.1371/journal.pone.0161779.
48. Chan DV, Gibson HM, Aufiero BM, Wilson AJ, Hafner MS, Mi Q-S, Wong HK. Differential CTLA-4 expression in human CD4+ versus CD8+ T cells is associated with increased NFAT1 and inhibition of CD4+ proliferation. Genes Immun. 2014;15:25–32. doi:10.1038/gene.2013.57
49. Liu MF, Liu HS, Wang CR, Lei HY. Expression of CTLA-4 molecule in peripheral blood T lymphocytes from patients with systemic lupus erythematosus. J Clin Immunol. 1998;18:392–98. doi:10.1023/A:1023226621966
50. Du X, Tang F, Liu M, Su J, Zhang Y, Wu W, Devenport M, Lazarski CA, Zhang P, Wang X, *et al*. A reappraisal of CTLA-4 checkpoint blockade in cancer immunotherapy. Cell Res. 2018;28:416–32. doi:10.1038/s41422-018-0011-0.
51. Jie HB, Gildener-Leapman N, Li J, Srivastava RM, Gibson SP, Whiteside TL, Ferris RL. Intratumoral regulatory T cells upregulate immunosuppressive molecules in head and neck cancer patients. Br J Cancer. 2013;109:2629–35. doi:10.1038/bjc.2013.645
52. Postow MA, Sidlow R, Hellmann MD, Longo DL. Immune-related adverse events associated with immune checkpoint blockade. N Engl J Med. 2018;378:158–68. doi:10.1056/NEJMra1703481
53. Dubin K, Callahan MK, Ren B, Khanin R, Viale A, Ling L, No D, Gobourne A, Littmann E, Huttenhower C, *et al*. Intestinal microbiome analyses identify melanoma patients at risk for checkpoint-blockade-induced colitis. Nat Commun. 2016;7:10391. doi:10.1038/ncomms10391.
54. Lo B, Abdel-Motal UM. Lessons from CTLA-4 deficiency and checkpoint inhibition. Curr Opin Immunol. 2017;49:14–19. doi:10.1016/j.coi.2017.07.014
55. Coelho LP, Kultima JR, Costea PI, Fournier C, Pan Y, Czarnecki-Maulden G, Hayward MR, Forslund SK, Schmidt TSB, Descombes P, *et al*. Similarity of the dog and human gut microbiomes in gene content and response to diet. Microbiome. 2018;6:72. doi:10.1186/s40168-018-0450-3.