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TITLE:
Safety and immunogenicity of a potential checkpoint blockade vaccine for canine melanoma

ABSTRACT:
Human immunotherapy with checkpoint blockades has achieved significant breakthroughs in recent years. In this study, a checkpoint blockade vaccine for canine melanoma was tested for safety and immunogenicity. Five healthy adult dogs received a mixture of three replication-defective chimpanzee-derived adenoviral vectors, one expressing mouse fibroblast-associated protein (mFAP) and the others expressing canine melanoma-associated antigens Trp-1 or Trp-2 fused into Herpes Simplex-1 glycoprotein D, a checkpoint inhibitor of herpes virus entry mediator (HVEM) pathways. The vaccine mixture was shown to be well tolerated and increased frequencies of canineTrp-1-specific activated CD8(+) and CD4(+) T cells secreting interferon-(IFN)-γ, tumor necrosis factor (TNF)-α, or interleukin (IL)-2 alone or in combinations in four and five out of five dogs, respectively. To avoid excessive bleeds, responses to cTrp-2 were not analyzed. All dogs responded with increased frequencies of mFAP-specific activated CD8(+) and CD4(+) T cells. The results of this safety/immunogenicity trial invite further testing of this checkpoint blockade vaccine combination in dogs with melanoma. ELECTRONIC SUPPLEMENTARY MATERIAL: The online version of this article (10.1007/s00262-018-2201-5) contains supplementary material, which is available to authorized users.

Introduction:
The incidence of cancer is high in dogs with 100 male and 272 female cases per 100,000 dog-years, respectively [1]. Malignant melanoma is one of the most common canine cancers with an estimated annual 20.3 cases of oral melanoma per 100,000 dogs. The incidence increases with age and in certain breeds, Cocker Spaniels, Golden Retrievers, and Labrador Retrievers, are at a higher risk than others such as Dachshunds or Beagles [2]. Treatment options include radical surgery [3], immunotherapy [4], radiation therapy [5], chemotherapy, [6] or hyperthermia [7]. The two latter treatment options are generally ineffective. Prognosis varies according to tumor stage and treatment [8, 9]. Even after radical surgery, dogs with stage II or III melanoma die on average within less than a year [5].
In humans, immunotherapies using checkpoint blockades [10, 11], transfer of in vitro expanded tumor-infiltrating T lymphocytes (TILs) [12], T cells with a chimeric antigen receptor (CAR) [13], or cancer vaccines [14] have achieved significant breakthroughs in recent years. The overall efficacy of cancer vaccines, the most cost-effective option for cancer immunotherapy, warrants further improvement. Major barriers are exhaustion of tumor antigen (TA)-specific CD8+ TILs within the tumor microenvironment (TME) [15, 16], the recruitment of immunoinhibitory cells [17], and a metabolically hostile milieu [18]. T-cell exhaustion is characterized by increased expression of immunoinhibitory molecules such as PD-1, LAG-3, BTLA, CTLA-4, and others, leading to loss of functions and eventual cell death. Immunosuppression can in part be reversed by antibodies that block the interactions between the immunoinhibitors on T cells and their ligands [19, 20]. Human checkpoint blockade therapy has mainly focused on treatments with blocking antibodies to PD-1, its ligand PD-L1, or CTLA-4, that have resulted in tumor regression in certain cancer patients [21]. We have used a different, two-pronged approach to enhance vaccine-induced, TA-specific CD8+ T-cell responses and to reduce CD8+ T cell exhaustion within the TME. Fusion of TA into Herpes simplex virus (HSV-1) glycoprotein (g)D blocks the binding of the immunoinhibitory B- and T-cell lymphocyte attenuator (BTLA)/CD160 to the Herpes virus entry mediator (HVEM) to enhance CD8+ T-cell stimulation [22]. The immunoinihbitory milieu of the TME can further be reduced by depleting cells of the tumor stroma, such as fibroblasts, with a vaccine targeting cancer-associated fibroblasts (CAF) with fibroblast-associated protein (FAP) to elicit a vaccine-induced anti-CAF T-cell response [23], which in turn reduces T-cell exhaustion and delays tumor progression.
We previously tested melanoma vaccines based on chimpanzee-origin replication-defective adenovirus vectors of serotype C68 (AdC68) expressing epitopes from different MAAs administered together with a second AdC68 vector expressing FAP and demonstrated significant protection against transplanted melanoma cells or in transgenic melanoma-prone Tyr::CreER, BrafCA/+Ptenlox+/lox+mice [23]. To further the development of a combination MAA–FAP vaccine for the treatment of dogs with melanoma, we conducted a safety and immunogenicity study in healthy Beagles using AdC68 vectors expressing canine (c) tyrosinase-related protein (Trp-1)

within gD, tyrosinase-related protein 2 (Trp-2) within gD, or mFAP. The vaccine mixture was well tolerated and induced T-cell responses to Trp-1 and mFAP. To avoid taking excessive blood volumes from the dogs, immune responses to Trp-2 were not analyzed.

Construction and purification of the vaccines ::: Materials and methods:
The AdC68-mFAP vector has been described previously [23]. The cTrp-1 and the cTrp-2 sequences were synthesized (Integrated DNA Technologies) and cloned into the Age I (bp 1996) and BamHI (bp 2008) sites of the gD gene carried by the pShuttle vector. The expression cassette was excised with the rare cutters I-Ceu I and PI-Sce I and cloned into the molecular AdC68 clone cut with the same enzymes. Recombinant molecular clones were identified in transformed bacteria through diagnostic restriction enzyme digests. For each construct, a molecular clone with the correct insert was digested with PacI. Unique sites for this restriction enzyme are located directly adjacent to the 5′ and 3′ ITR of the viral genome of the AdC68 molecular clone. The DNA was transfected into semi-confluent monolayers of HEK 293 cells from the American Tissue Culture Collection (ATCC® CRL-1573™) for viral rescue. When viral plaques became visible, cells were harvested and the virus was released by freeze–thawing. The virus was then expanded over several passages on HEK 293 cells. When a large-scale stock of 40 T175 flasks was produced, the virus was purified by buoyant density ultracentrifugation on CsCl gradients followed by desalting with a Bio-Gel P-6DG column.

Titration of vaccines ::: Materials and methods:
The virus particle (vp) content was determined by spectrophotometry at 260 and 280 nm, with the latter determining purity of the preparation. The titer in vp was determined using the formula: OD260 × dilution × 1.1 × 1012. The content of infectious units (IU) was determined by nested reverse transcription PCR with hexon-specific primers on cells infected with serial dilutions of the vectors.

Quality control of vaccines ::: Materials and methods:
The genetic integrity and identity of the vaccines were assessed upon isolation of viral DNA. The recombinant DNA was digested with a set of restriction enzymes (in parallel to the molecular clone) and analyzed by gel electrophoresis. The vaccines' genome showed the expected banding patterns. The same assay was used to analyze the DNA after 15 serial passages of the viral vectors on HEK 293 cells. The vector genomes' DNA restriction enzyme banding patterns were conserved, indicating that the vaccines were genetically stable.
Batches were tested for endotoxin by the limulus amebocyte lysate (LAL) gel-clot method using a commercial kit. Vaccines contained no detectable endotoxin. Vectors were tested for sterility by their culture in LB medium for 24 h at 37 °C. No bacterial or fungal growth was observed.

Treatment of dogs ::: Materials and methods:
Dogs were housed in individual ~ 4 ft × 4 ft × 42′′ elevated pens constructed of expanded galvanized metal. The floor of each cage was equipped with a solid, polypropylene resting panel (~ 24″ × 24″) and individual food and water containers. Pens were located in a climate-controlled room equipped with environmental control systems. Dogs were maintained in conditions ranging from 71.4 to 81.3 °F and 41–90% relative humidity over the course of the trial. Artificial lighting was provided by covered, overhead fluorescent fixtures set with a timer to provide a cycle of alternating periods of 12 h darkness/12 h light. Pens were cleaned daily and sanitized at least once weekly. Water pails were emptied and rinsed daily, and cleaned as necessary. Dogs were confined individually, and were removed from their pens for individual and group enrichment prior to day 0. Thereafter, dogs were removed from their housing for daily cleaning and measurement of rectal temperature, body weights/physical exams, treatment, or blood collection. Dogs were offered dry, commercial dog food (Lab Diet® Canine 5006) at ~ 2 cups per day, divided into similar a.m. and p.m. portions. While in confinement, fresh potable water was supplied by a local, commercial utility. Water was available ad libitum in 1-L stainless steel pails, and was replenished at least twice daily.
Prior to initiation of the study, the protocol was reviewed and approved by the East Tennessee Clinical Research (ETCR, Rockwood, TN) Institutional Animal Care and Use Committee. Dogs were acclimatized for 2 weeks. Dogs were immunized intramuscularly. The three vaccines were given at different sites paralumbar and prescapular in 0.5 ml of sterile saline containing 2 × 1011 vp of AdC68-mFAP, 1 × 1011 vp of AdC68-gDcTrp-1, or 1 × 1011 vp of AdC68-gDcTrp-2. The health status of the dogs was assessed prior to vaccination and at different time points after

vaccination. Dogs were bled before vaccination, and at 24 and 48 h after vaccination for blood chemistry, which included testing for glucose, total bilirubin, total protein, urea nitrogen, phosphorus, sodium, chloride, magnesium, and alanine aminotransferase (ALT). In addition, samples underwent routine complete blood count (CBC); the hematological studies showed no abnormalities (data not shown). Dogs were bled before and at 7, 14, and 42 days after vaccination. Blood was immediately transferred into EDTA-containing tubes and then shipped to The Wistar Institute for testing of T-cell responses.

Preparation of PBMCs ::: Materials and methods:
PBMCs were isolated by Ficoll® Paque Plus (GE Healthcare) gradient centrifugation for 30 min at room temperature at 2200 rpm. Red blood cells were lysed by 1× RBC lysis buffer (eBioscience). Cells were washed, counted, and cryopreserved in 90% fetal bovine serum and 10% DMSO at – 80 °C until testing.

Intracellular cytokine staining ::: Materials and methods:
Antibodies, as shown in Table 1, were either directed against canine sequences or the manufacturer confirmed their cross-reactivity with canine proteins. To confirm appropriate reactivity, the antibodies against surface markers were initially tested against PBMCs from a normal dog that was not part of the study. These data are shown in Suppl. Table 1 as % positive cells within the live lymphoid cell gate for most markers; for CD95 we show % cells that are double-positive for CD8 and CD95, and for CD28 we show % cells that are triple-positive for CD8, CD95, and CD28. For the intracellular stains, we initially tested pre-bleeds from two of the enrolled (younger) dogs upon polyclonal activation. Percent of cytokine positive cells within the CD3 gate is shown in Suppl. Table 2.

Frequencies of T cells to cTrp-1 and mFAP were assessed by intracellular cytokine staining (ICS) after stimulation with cTrp-1 or mFAP peptide pools. Frozen PBMCs were thawed and immediately washed with RPMI medium containing 10% FBS. Cells were resuspended in RPMI media, and then co-stimulated with CD28/CD49d for 6 h at 37 °C 5%CO2 with peptides for cTrp-1, mFAP, or cultured for 6 h with PMA (20 ng/ml) and ionomycin (1 μg/ml) both from Sigma-Aldrich (St. Louis, MO). During the 6-h culture, the medium contained GolgiStop (containing Monensin from BD Biosciences, San Jose, CA) to block protein secretion. Peptides were 15 amino acids in length and overlapped by five amino acids with each adjacent peptide. All peptides were used at a final concentration of 2 μg of each peptide per ml. Control cells were cultured with the same volume of acetonitrile:water (no peptide). Following incubation, cells were stained with live/dead aqua-fluorescent reactive dye, anti-CD14-AF-700 as a dump gate, anti-CD3-FITC, anti-CD8-Pacific blue, anti-CD4-PE-Cy7, anti-CD95-BV650, and anti-CD28-APC-A for 30 min at 4 °C (Table 1). Following surface staining, the cells were fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences, San Jose, CA) for 30 min at 4 °C, cells were stained with anti-IFNγ-PE-A antibody, anti-IL-2-Biotin counterstained with BV605-Streptavidin and anti-TNF-alpha-DyLight 755 for 1 h at room temperature (Table 1). Cells were washed once, fixed with BD stabilizing fixative, and analyzed by FACS using BD FACSCelesta (BD Biosciences, San Jose, CA) and DiVa software. Flow cytometric acquisition and analysis of samples were performed on at least 1,000,000 events. Post-acquisition analyses were performed with FlowJo (TreeStar, Ashland, OR). Data shown on graphs represent values for cTrp-1 or mFAP peptide-stimulated cells from which background values were subtracted. BD CompBeads (BD Biosciences, San Jose, CA) were used as single-color controls for compensation.

Statistical analyses ::: Materials and methods:
Significance of post- and pre-vaccination data were determined by Fisher's least significant difference (LSD) test with two-way analysis of variance (ANOVA).

Quality control of vaccines ::: Results:
Vaccine antigens were expressed by replication-defective recombinant chimpanzee adenoviruses of serotype SAdV-25, also called AdC68 [26]. One vaccine for mouse (m)FAP has been described previously [23]. The others were constructed to carry cTrp-1 or cTrp-2 fused into HSV-1 gD. Genetic integrity of the purified AdC68-cTrp-1 and AdC68-cTrp-2 vectors was confirmed by restriction enzyme digestion of the viral DNA followed by gel electrophoresis. Genetic stability was

confirmed by propagation of the vectors over 15 passages followed by purification of viral DNA and restriction enzyme digestion, which revealed the same banding patterns as the digests conducted with viruses from an early passage. Protein expression was tested by Western blots on HEK 293 cells infected with 1000 infectious units (IU) of vector and probed with an antibody to gD. Viruses were sterile and free of endotoxin (detection limit of the assay: 0.125 EU/ml). The AdC68mFAP and AdC68gDTrp-1 vectors had virus particle (vp) to infectious units ratios of 55:1 and 303:1, respectively.

Vaccine toxicity ::: Results:
Five male healthy Beagles of 9.5–10 months of age were enrolled at ETCR. Dogs had received standard vaccination against canine viruses (adenovirus type 2, parainfluenza, distemper, parvo virus, corona virus, rabies virus, papilloma virus). They had received the oral Bordetella vaccine and they had been checked and if needed treated for intestinal parasites before purchase. Dogs were acclimatized for 2 weeks. The dogs' weight, temperature, and overall health were checked. Blood was collected before vaccination for clinical chemistry and hematology. Animals were vaccinated intramuscularly into different sites with 1 × 1011 vp of the AdC68-gDcTrp-1 vector, 1 × 1011 vp of the AdC68-gDcTrp-2 vector and 2 × 1011 vp of the AdC68mFAP vector. Safety of the vaccine was assessed by once daily evaluation of dogs. General health observations, including rectal temperature measurements, clinical health observations were made at 1, 3, 6, 12, and 24 h after vaccination, physical examinations on days 2 and 7, and clinical pathology analyses (hematology and plasma chemistry) at 24, 48, and 168 h post-vaccination. Four of the five dogs transiently lost weight during the first 2 days after vaccination (Fig. 1a). The treating veterinarian concluded that the initial weight loss was caused by stress due to handling and frequent bleeds. Temperatures stayed within normal range during the study period (Fig. 1b). Blood chemistry included tests for ALT (Fig. 1c) and total bilirubin (0–0.1 for the latter for all dogs at all time points, data not shown) for liver functions, glucose for pancreas functions (Fig. 1d), urea nitrogen for kidney functions (Fig. 1e) as well as electrolytes and total protein (not shown). All blood chemistry values remained within normal range throughout the study. No hematological abnormalities were observed (data not shown). From vaccination until trial termination on day 42 after vaccination, six minor and temporary alimentary abnormalities in form of vomition and mucus in feces were observed (Table 2). Only one dog vomited within the initial period after vaccination when adverse events due to inflammatory reactions would be expected to peak.

No further health abnormalities were identified following vaccination, or in physical examinations conducted on days 2 and 7 post-vaccination. None of the animals required veterinary treatment following vaccination. At the end of the study, dogs were released for adoption.

CD8+ T-cell responses to cTrp-1 and mFAP ::: Results:
Dogs were bled before vaccination and at 7, 14, and 42 days after vaccination. Peripheral blood mononuclear cells (PBMCs) were prepared and tested by intracellular cytokine staining (ICS) after a 6-h stimulation period in the presence of a secretion inhibitor with 15-mer peptides overlapping at each end by five amino acids with the adjacent peptide and spanning the sequences of cTrp-1 and mFAP. Positive control cells were stimulated with phorbol 12-myristate 13-acetate (PMA), negative control cells were cultured without peptide. To avoid excessive bleed volumes, immune responses to Trp-2 were not analyzed. After stimulation, cells were stained with panels of antibodies. CD8+ and CD4+ T cells were identified as cells that were positive for CD3 and CD8 or CD4 and negative for CD14. T cells were further subdivided into activated T cells by stains for CD95, which increases upon stimulation, and CD28, which is higher on resting than activated T cells. Cells were tested for intracellular interferon (IFN)-γ, interleukin (IL)-2, and tumor necrosis factor (TNF)-α. After staining, they were analyzed by flow cytometry and gated by FlowJo as shown in Suppl. Fig. 1 for a positive control sample.
Dogs showed increased frequencies of cytokine-producing CD8+ T cells to both cTrp-1 and mFAP. Upon gating of the different cytokine responses, Boolean gating was used to calculate the overall response. The sum of cytokine-producing CD8+ T cells to cTrp-1 was highest on day 7, reflecting mainly increases in TNF-α production (Fig. 2a). Responses to mFAP peaked later by day

14 and were dominated by CD8+ T cells producing IL-2 (Fig. 2b). Restricting the gate to activated CD95+CD8+ T cells increased frequencies of cells responding to the cTrp-1 and mFAP peptide pools (Fig. 2c, d) and broadened the cytokine profile with higher frequencies of cells producing IFN-γ with or without IL-2. CD95+CD8+ cells also had increased frequencies of IFN-γ-producing mFAP-specific cells. Further narrowing the gate by excluding CD28+ cells showed a shift in kinetics with peak frequencies to both peptide pools by day 42, at which time cells secreted mainly IL-2 and IFN-γ alone or in combination (Fig. 2e, f).

Individual dogs developed peak responses at different times as shown in Fig. 3a, b. Rates of responders were defined as dogs in which the sum of T-cell responses or responses for individual cytokines were ≥ 0.1% of the tested CD8+ T cell population and at least double the frequency of cytokine+ CD8+ T cells at baseline. Individual dogs showed differences in responses to the two antigens and between the different CD8+ T cell populations as well as between the different cytokines (Fig. 3). Analyzing responsiveness for the sum of all cytokine-secreting CD8+ T cells showed that only two of the five dogs met the criteria for positive responses to cTrp-1. Both showed increases in IL-2 and IFN-γ, only one was positive for TNF-α. Upon analysis of CD95+CD8+ T cells, response rates to cTrp-1 increased to three of five dogs (Fig. 3c, d) and further restricting the gate to CD95+CD28−CD8+ T cells, which markedly reduced baseline responses showed cTrp-1-specific responses in four of five dogs (Fig. 3c, d). Increases in responses were mainly seen for IFN-γ and IL-2. Response rates were higher for mFAP. Upon gating on all CD8+ T cells, four of five dogs qualified as responders; the gates for CD95+CD8+ and CD95+CD28−CD8+ cells showed responses in all animals. Responses again mainly reflected increases in IFN-γ- and IL-2-producing CD8+ T cells.

CD4+ T-cell responses to cTrp-1 and mFAP ::: Results:
CD4+ T cells, unlike CD8+ T cells, did not show a distinctive population of CD28− cells within the CD95+ gate. We therefore only analyzed all CD4+ cells and CD95+CD4+ cells for cytokine responses. CD4+ T-cell responses to both antigens peaked on day 14 after vaccination with increases seen mainly in cells producing TNF-α. Responses contracted sharply by day 42 after vaccination (Fig. 4a, b). Responses of cTrp-1-specific CD95+CD4+ cells peaked earlier by day 7 and again mainly reflected TNF-α+ cells, while responses to mFAP were highest on day 14 and showed increases in both TNF-α- and IFN-γ-producing cells (Fig. 4c, d). Overall the kinetics of CD4+ T-cell peak responses were fairly uniform, in most dogs responses peaked on day 14, cTrp-1-specific CD95+CD4+ T cells came up earlier and peaked on day 7 in two dogs, while one dog showed a delayed peak response by day 42 (Fig. 5a, b).

All dogs showed positive CD4+ T-cell responses to cTrp-1; one did not score as responsive upon restricting the analysis to CD95+CD4+ cells. Most dogs were responsive for IL-2 and TNF-α; only one dog showed a positive IFN-γ response (Fig. 5c). The CD4+ T-cell response was less pronounced for mFAP; only three of five dogs scored positive upon analysis of all CD4+ T cells. Response rates increased to 100% when the gate was narrowed to CD95+CD4+ T cells (Fig. 5d).

Discussion:
Cancer vaccines are being developed to combat malignancies in companion animals for which other approaches of active immunotherapy that are being explored for use in humans are prohibitively expensive, such as checkpoint blockade or adoptive T-cell transfer. One canine cancer vaccine, Oncept®, a xenogeneic DNA vaccine encoding human tyrosinase protein, has been developed and is licensed in the US for treatment of dogs with malignant melanoma [27]. This vaccine, based on a DNA vector, was generally well tolerated in a study of 111 dogs. No systemic reactions occurred other than local hematomas and pain at the injection site [4]. Initial

studies indicated that Oncept® improved the dogs' prognosis [27]. In another retrospective study, medical records from 45 dogs were reviewed, including 30 dogs with stage II and III disease treated with the Oncept® vaccine after appropriate achievement of locoregional cancer control. The review found that dogs that received the vaccine did not achieve a greater progression-free survival, disease-free interval, or median survival time than dogs that did not receive the vaccine [28]. Similar results were obtained in a study conducted in the UK [29]. The vaccine was shown to induce a B-cell response, which is unlikely to limit melanoma progression. Another canine melanoma vaccine, termed K9-ACV, based on autologous tumor lysate; also induced antibody responses and was reported to improve survival in some dogs [30]. Additional vaccines targeting other types of common canine cancers have produced promising results [31–34], underscoring the use of vaccines as a viable option for treatment of dogs with cancer.

Here, we describe the results of a preclinical dog trial that assessed the safety and immunogenicity of a checkpoint blockade cancer vaccine. Our efforts focused on melanoma using a combination melanoma vaccine based on AdC68 vectors expressing MAAs fused into the checkpoint inhibitor gD combined with a second vector expressing mFAP for induction of T cells against CAF. A similar vaccine demonstrated efficacy in melanoma-bearing mice [23]. AdC68 vectors were chosen as vaccine delivery vehicles as previous studies showed that they are exceptionally immunogenic and induce very potent and sustained transgene product-specific CD8+ T-cell responses [35]. It should be pointed out, however, that an AdC68-based vaccine, unlike a DNA vaccine, should only be given once because neutralizing antibodies induced against the vaccine carrier will reduce its efficacy upon repeated administration. This can be overcome using a serologically distinct Ad vector for a booster immunization [36]. Dogs are commonly immunized against canine adenovirus serotype 2 (CAV-2) or acquire neutralizing antibodies to CAV-2 upon natural infections [37]. Neutralizing antibodies to adenoviruses are serotyope- and species-specific, and such antibodies should thus not interfere with the immunogenicity of a chimpanzee-derived Ad vector [38]. FAP is highly conserved between different species. It is expressed on fibroblasts within human or canine cancers [39] and was, therefore, selected to target the tumor stroma. To increase responses to FAP, the vaccine expressed the mouse sequence, which shows 87% homology with the corresponding canine protein. FAP is selectively expressed on CAFs and at sites of wound healing or chronic inflammation [40, 41]. Previous studies in mice showed that FAP+ fibroblasts are essential drivers of tumor progression [42]. Additional studies are needed to confirm that FAP expression and the role of FAP in targeting CAF are also crucial to inhibiting the growth of canine melanomas. Eliminating FAP+ cells by CAR-T cells resulted in significant toxicity in mice [43]. Our approach of inducing T cells by a mFAP-expressing vaccine was well tolerated in mice and, as shown here, in dogs. mFAP was immunogenic in dogs and all the treated dogs developed CD8+ and CD4+ T-cell responses to this protein.

cTrp-1, which was shown to be overexpressed on canine melanoma cells [44], was used as the vaccine antigen. In mice, this MAA induces a strong CD8+ T-cell response [22, 23]. To further increase the response and to preserve CD8+ T cell functions in dogs with melanoma, cTrp-1 was expressed as a fusion protein within HSV-1 gD. HVEM was first identified as a receptor for HSV-1 [45]. HSV-1 gD is a bimodal switch expressed on many cells, including antigen-presenting cells, that can interact with immunoregulatory molecules on lymphocytes [46]. Binding of HVEM to LIGHT or lymphotoxin provides stimulatory signals; binding to BTLA or CD160 activates inhibitory pathways [46]. Coactivators and coinhibitors bind to different domains of HVEM and can form a trimolar complex, in which signaling through coinhibitors dominates. The N terminus of HSV-1 gD binds to a site on HVEM that is close to the BTLA/CD160 binding site and thereby blocks immunoinhibitory but not costimulatory HVEM signaling [47]. As we have shown previously, vaccines that express antigens fused into the C-terminus of gD elicit enhanced T-cell responses that are linked to the blockade of the immunoinhibitory HVEM pathways [48]. Adjuvanting a vaccine antigen with gD is especially effective to augment CD8+ T-cell responses in aging mice [49] and in mice with advanced cancers [48]. The effect of gD is local at the site of immunization rather than systemic as are antibody-based checkpoint inhibitors, thus minimizing side effects. As expressed within gD, cTrp-1 was immunogenic and elicited both CD4+ and CD8+ T-cell responses in healthy dogs. We did not include a control AdC68 vector, expressing wild-type cTrp-1, and we can thus not assess if gD augmented the T-cell response to cTrp-1.

Surface markers for canine T cells are less well defined than non-human primate or human T-cell markers. In the latter species, activated CD8+ T cells can be identified by increased expression of CD95 while levels of CD28 expression distinguishe CD28low effector cells from naïve or memory CD8+ T cells that are CD28high [50, 51]. Using these two additional stains revealed for both CD8+

and CD4+ T cells distinct populations of CD95low and CD95high cells with a trend toward increased frequencies of Trp-1- and FAP-specific CD8+ T cells in the CD95high gate. The CD28 stain only showed two distinct populations within the CD95high gate of the CD8+ T-cell population and here, frequencies of Trp-1 and FAP-specific CD28– T cells selectively increased at the day 42 time point, which may suggest that loss of CD28 expression on CD95+CD8+ T cells might not necessarily reflect canine effector T cells, which would be expected to show maximal frequencies early after immunization.

In summary, the pre-clinical trial showed that the canine melanoma vaccine combination was safe, with no local or systemic side effects related to vaccination. The vaccines also induced transgene product-specific CD8+ and CD4+ T-cell responses, opening the path toward their testing in companion dogs with melanoma.