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# Developing a translational murine-to-canine pathway for an IL-2/agonist anti-CD40 antibody cancer immunotherapy

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## Abstract

Human and canine sarcomas are difficult to treat soft tissue malignancies with an urgent need for new improved therapeutic options. Local recurrence rates for humans are between 10%–30%, and 30%–40% develop metastases. Outcomes for dogs with sarcoma vary with grade but can be similar. Pet dogs share the human environment and represent human cancer with genetic variation in hosts and tumours. We asked if our murine studies using genetically identical mice and cloned tumour cells were translatable to larger, genetically diverse domestic dogs with naturally occurring tumours, to (i) develop a canine cancer therapeutic, and (ii) to use as a translational pathway to humans. Our murine studies showed that intra-tumoral delivery of interleukin-2 (IL-2) plus an agonist anti-CD40 antibody (Ab) induces long-term curative responses ranging from 30% to 100%, depending on tumour type. We developed an agonist anti-canine-CD40 Ab and conducted a phase I dose finding/toxicology 3 + 3 clinical trial in dogs ($n = 27$) with soft tissue sarcomas on account of suitability for intratumoral injection and straightforward monitoring. Dogs were treated with IL-2 plus anti-CD40 antibody for 2 weeks. Three dose levels induced tumour regression with minimal side effects, measured by monitoring, haematological and biochemical assays. Importantly, our mouse and canine studies provide encouraging fundamental proof-of-concept data upon which we can develop veterinary and human immunotherapeutic strategies.

**KEYWORDS**
anti-CD40 antibody, dogs, immunotherapy, interleukin-2, soft tissue sarcoma

## 1 | INTRODUCTION

Our pre-clinical mouse studies using intra-tumoral delivery of interleukin-2 (IL-2)[1] and an agonist anti-CD40 antibody (Ab),[2] or their combination,[3] in mesothelioma and lung cancer induced 100% and 30% curative responses, respectively. The intra-tumoral route was more effective and less toxic than the intravenous route.[3] The IL-2 or anti-CD40 Ab monotherapies were only effective with small tumour burdens, ≤25 $mm^{21}$ whilst the IL-2/CD40 combination induced curative regression of larger tumours that were resistant to either monotherapy.[3] This approach induced a systemic immune response that cured secondary untreated tumours on the contralateral side of the mouse.[3] Moreover,

long-term protective memory immune responses were generated that persisted to old age,[3] and elderly mice could be given the same treatment, albeit with a reduced but still significant cure rate of 30%–40%.[4]

CD40, a member of the tumour necrosis factor superfamily, is expressed on multiple immune cell types and ligating CD40 sends powerful activation signals.[5–7] IL-2 is a potent growth factor that induces T cell expansion[8] and downstream dendritic cells (DC) activation.[9] Our investigations of IL-2/CD40-driven mechanisms underlying tumour regression showed: induction of tumour-antibody producing B cells[2]; DC and effector/memory T cell activation[3]; conversion of tumour-associated macrophages into M1-like anti-tumour macrophages[10]; and neutrophil and T cell recruitment into tumours.[3] $CD8^+$ T cells and neutrophils were key effector cells that collaborated to mediate a curative response.[3] We found that $CD40^+$ tumour endothelia became permissive to T cell infiltration,[11] and that regulatory T cells were not expanded.[12]

Whilst onco-immunology mouse models provide important proof-of-principle data, they consist of genetically identical mice implanted with clonally selected tumour cells. We hypothesized that our mouse studies were translatable to larger mammals with natural tumours. Dogs with soft tissue sarcomas were chosen on account of being easily able to inject intratumorally, monitor tumour growth, and recruit sufficient numbers for the study. Pet dogs with spontaneous sarcoma live in the same environment as humans and better represent human cancer with genetic variation in hosts and tumours.[13–16] We developed agonist anti-canine-CD40 Abs and the lead candidate was combined with canine IL-2. A dose finding/toxicology standard 3 + 3 phase I clinical trial using the same therapeutic regimen given to mice, that is, three doses a week for 2 weeks, was conducted.

## 2 | MATERIALS AND METHODS

### 2.1 | Generating antibodies targeting canine CD40

Sera from mice immunized with recombinant canine CD40 protein were tested for CD40 reactivity by ELISA. Splenic cells from the mouse with the highest titre were fused to SP2/0-Ag14 myeloma cells by standard polyethylene glycol techniques. Hybridoma clones secreting canine-specific CD40-IgG were identified using MATF's proprietary microarray and ELISA. Positive clones were screened by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide assay[17] measuring $2 \times 10^5$ healthy dog peripheral blood mononuclear cell (PBMC) proliferative responses to hybridoma supernatant at 37°C, 5% $CO_2$ (8 days); 2 mg/ml thiazolyl-blue-tetrazolium-bromide (Sigma) was added and cultured for 4 h. DMSO (Sigma) solubilized crystals and cells. Absorbance (595 nm) was measured (EnSpire Multimode Plate Reader, PerkinElmer). Monoclonal lines were generated from selected clones by limiting dilution.

### 2.2 | Preparing canine PBMCs

PBMCs were isolated by diluting whole blood in PBS containing 2 mM EDTA (Sigma-Aldrich, USA), followed by density gradient (Ficoll-Paque Plus, GE Healthcare, USA) centrifugation at 400 *g*, 40 min, with slow acceleration and no brake. PBMCs collected at the interface were washed in PBS/EDTA by centrifugation at 400 *g* for 10 min. A second centrifugation at 100 *g*, 10 min ensured platelet removal. Cell pellets were resuspended in complete medium (Gibco RPMI 1640 medium supplemented with 100 U/ml penicillin–streptomycin, 1× GlutaMAX (all from Thermo Fisher Scientific), 10% Hyclone foetal calf serum (FCS) (Cytiva, USA), and 0.05 mM 2-mercaptoethanol (Sigma-Aldrich, Sydney, Australia).

### 2.3 | Antibodies

Goat-anti-mouse immunoglobulin (Ig)-BV421 (BD); rat-anti-canine CD3-FITC (clone CA17.2A12) and mouse-anti-canine CD21-PE (clone CA2.1D6) both from Bio-Rad, USA; rat-anti-canine CD4-PE-Cy7 (clone YKIX302.9), rat-anti-canine CD8a-PerCP-eFluor710 (clone YCATE55.9) and mouse-anti-canine CD25-eFluor710 (clone P4A10), all from Thermo Fisher Scientific. Zombie Aqua (BioLegend, USA) identified viable cells. Unstained cells were used to determine the optimal photomultiplier tube (PMT) voltages for each fluorochrome. Single-stained cells and BD CompBeads (BD) were compensation controls. Fluorescence minus one controls were used to gate positive cells.

### 2.4 | Identifying hybridomas that bind CD40 on canine PBMCs

After centrifugation, PBMCs ($2 \times 10^5$ per well) in Falcon 96-well U bottom plates (Corning, USA) were resuspended in 20 μl mouse hybridoma supernatant for 30 min. The negative control was FACS buffer (PBS with 5% heat inactivated Gibco goat serum, Thermo Fisher Scientific), 1% BSA (Sigma-Aldrich) and 0.01% w/v sodium azide (Sigma). All incubations were on ice in the dark. Cells washed twice with FACS buffer were resuspended in anti-mouse Ig-BV421 in FACS buffer and incubated for 30 min before washing with FACS buffer then PBS and re-suspension in 1% formaldehyde in PBS. Twenty minutes later cells were washed twice and resuspended in FACS buffer for acquisition on a FACSCanto II using FACSDiva software (BD) and analysis using FlowJo software (BD).

### 2.5 | Identifying agonist anti-CD40 antibodies

PBMCs were labelled with 5-(and-6)-carboxyfluorescein-diacetate-succinimidyl ester (CFSE; Thermo Fisher Scientific), a fluorescent dye that permanently binds intracellular proteins.[18,19] As cells proliferate, CFSE segregates equally between each daughter population.

Two $\times 10^7$ PBMCs/ml in PBS were stained with 2.5 μM CFSE/ml for 10 min at RT. Cells washed three times in medium containing 10% FCS by centrifugation through an FCS underlay were resuspended in complete media and $5 \times 10^4$ cells added to Nunc 96-well flat bottom tissue culture plates plus hybridoma supernatant, or medium for the negative control. Positive controls included PBMCs cultured with

1 μg/ml LPS (Sigma-Aldrich) or 20 ng/ml canine IL-2 (Kingfisher Biotech, USA).

After 7 days in culture at 37°C with 5% $CO_2$, CFSE-labelled PBMCs were pelleted and resuspended in FACS buffer with 5% normal dog plasma and incubated for 30 min. PBMCs were washed twice with FACS buffer, resuspended in anti-canine CD21-PE in FACS buffer, incubated for 30 min, followed by two washes with PBS, resuspension in Zombie Aqua for 15 min, sequential washes with FACS buffer and PBS, before resuspension in 1% formaldehyde in PBS for 20 min, and two washes in preparation for flow cytometric analysis.

### 2.6 | Phase I clinical trial in pet dogs

The trial evaluated safety as a primary endpoint and efficacy as a secondary endpoint using a 3 + 3 dose escalation protocol, which starts with a group of 3 dogs. If no dogs experienced a dose-limiting toxicity (DLT), then the next 3 dogs were treated with the next dose. A DLT was defined as an adverse event graded ≥3 that was believed to be treatment induced. This dose escalation pattern was repeated until one or more dog experienced a DLT. If one of the dogs experienced a DLT, 3 more dogs were treated with the same dose. If ≥2 dogs experienced a DLT, the maximally tolerated dose (MTD) was considered to have been exceeded.Participants were privately owned dogs with spontaneous peripheral soft tissue sarcomas. Owners could withdraw their dogs from the trial at any time. Dogs that presented to, or were referred to, PVS with a confirmed histopathological diagnosis of peripheral soft tissue sarcomas by a veterinary pathologist were assessed for suitability based on physical examination, a review of clinical findings and inclusion/exclusion criteria. Signed owner informed consent was obtained before study procedures commenced.

### 2.7 | Immunotherapy regimen and dose escalation protocol

Dogs received six intratumoral injections of recombinant canine interleukin-2 (IL-2, Kingfisher Biotech) and purified anti-CD40 antibody combined in PBS over 2-weeks. Dogs presented for review for up to 1 year with blood and needle core tumour biopsies collected

The starting dose for canine IL-2 and anti-canine CD40 antibody was based on their bioactivity on healthy dog PBMCs relative to the IL-2 and anti-CD40 antibody bioactivity used in our murine studies on mouse PBMCs. No adjustments were made according to body weight, as local concentration of the agents in tumours was considered a critical factor. Both agents were escalated simultaneously at each dose step, maintaining the relative proportion of each agent (Table S1).

### 2.8 | Toxicology

Blood biochemistry and haematology (Vetpath Laboratory Services, WA), plus weight, temperature and tumour size were measured. Owners monitored their dogs and reported signs of discomfort, swelling, inappetence, fatigue and other changes to daily living activities.

### 2.9 | Adverse events and tumour response classification

Adverse events were graded according to the Veterinary Co-operative Oncology Group Common Terminology Criteria for Adverse Events, v.1.0 (VCOG-CTCAE).[20]

Tumour size refers to the product (area) of the two largest perpendicular diameters (measured using callipers), as per immune-related response criteria (irRC).[21] Responses, based on irRC classifications[21] were: complete remission for disappearance of evidence of cancer at all sites; partial remission (PR) was ≤50% decrease in the size of all tumours; stable disease (SD) was tumour size between <50% decrease and <25% increase for a minimum of 50 days. Progressive disease (PD) was ≥25% increase in tumour size.

## 3 | RESULTS

### 3.1 | Developing and selecting hybridomas that produce agonist anti-canine CD40 antibodies

Of the approximately 1380 mouse-anti-canine hybridomas generated, 13 produced canine-CD40-specific antibodies measured by ELISA (not shown), with six hybridomas containing antibodies recognizing CD40 epitopes expressed by dog lymphocytes (Figure S1 and Figure 1A–D), likely B cells (Figure 1E). Those with weak staining (MFI) (Figure 1D) were not pursued. Promising hybridomas were tested in up to four different dogs (Figure 1F). Although there was variable CD40 staining intensities between individual dogs, the trends were similar.CD40-activated B cells respond by proliferating. Therefore, the next aim was to identify hybridomas that produce anti-CD40 antibodies with agonist activity using B cell proliferation as a measure. Four hybridomas induced a strong proliferative response in at least one dog (Figure 2B), for example, StP1.RH2 had five peaks, meaning some daughter cells had undergone five divisions, whilst StP1.RF3 did not induce any cell division as there is only the parental peak. Two antibodies showed more consistent responses between donor dogs (Figure 2A–C).

Three cell-lines were selected for subcloning, two with agonist activity (StP1.RE2 and StP1.RD1) and one (StP1.RF3) that did not induce proliferation and was intended as a negative control. After a second round of subcloning, StP1.RE2 and StP1.RD1 were deemed fully monoclonal. However, StP1.RF3 failed subcloning. Screening for agonist potential by measuring proliferating PBMCs revealed StP1.RD1 as the lead candidate based on its more consistent performance between dogs (data for StP1.RE2 not shown due to variation between dogs), even though including a third dog revealed variable responses to different doses (Figure 2D,E). B cell responses to increasing doses are shown

from one dog (Figure 2E). Doses 0.1–1 μg/ml barely induced proliferation. Proliferative responses increased with increasing doses, reaching 63% of B cells proliferating at the highest dose of 100 μg/ml.

### 3.2 | The phase I trial using canine IL-2 and the lead agonist anti-canine CD40 antibody

Thirty-nine dogs were recruited, with 27 meeting the inclusion criteria (Figure 3). These dogs were treated in groups of 3 covering 9 dose levels (Figures 3A,B and 4A).

### 3.3 | Safety and toxicology

The IL-2/CD40 immunotherapy was generally well tolerated with only a few issues attributable to treatment. Only dogs of concern outside the acceptable range are identified, for example, body weight changed in three dogs, with one (D1.1 = dose-1/dog #1) experiencing a grade 2 adverse event. (Figure 4B). D1.1 was given the lowest dose yet lost >10% body weight likely due to an oral tumour. D1.1 also experienced elevated body temperature during treatment (Figure 4C).

The ALT liver enzyme was transiently elevated to a grade 3 adverse event in one dog (D9.2) given the highest dose (Figure 4D). No dogs reached a grade 3 elevated AST adverse event (Figure 4E).

Whilst the RBC compartment was not affected, three dogs demonstrated a transient drop in platelets, with two (D9.1 and D9.2) given the highest dose (Figure 4F,G).

No treatment effect on total WBC count was seen. One dog (D1.2) had a high WBC count composed of neutrophils before treatment commenced (Figure 5A–C). D1.1 showed a transient increase in total WBCs and neutrophils during treatment; a similar smaller response was seen in D2.2. D8.3 demonstrated a slow increase in total WBC and neutrophils 3 weeks after treatment ceased. The proportion of neutrophils of WBCs did not change at any dose.

A prolonged decline in circulating lymphocytes was seen in all three dogs given the highest dose (Figure 5D,E). Five dogs (D1.2, D2.2, D4.3, D6.2 and D6.3) given other doses also demonstrated decreasing lymphocytes. Monocytes were mostly not perturbed (Figure 5F,G) although D1.1 demonstrated elevated monocytes from the start of treatment. Two dogs (D1.2 and D8.3) given two different doses showed elevated monocytes after treatment ceased.

The most common side effects were: grade 1 lethargy/fatigue (12 dogs) and grade 1 inappetence (Table S2). These effects occurred at all dose levels.

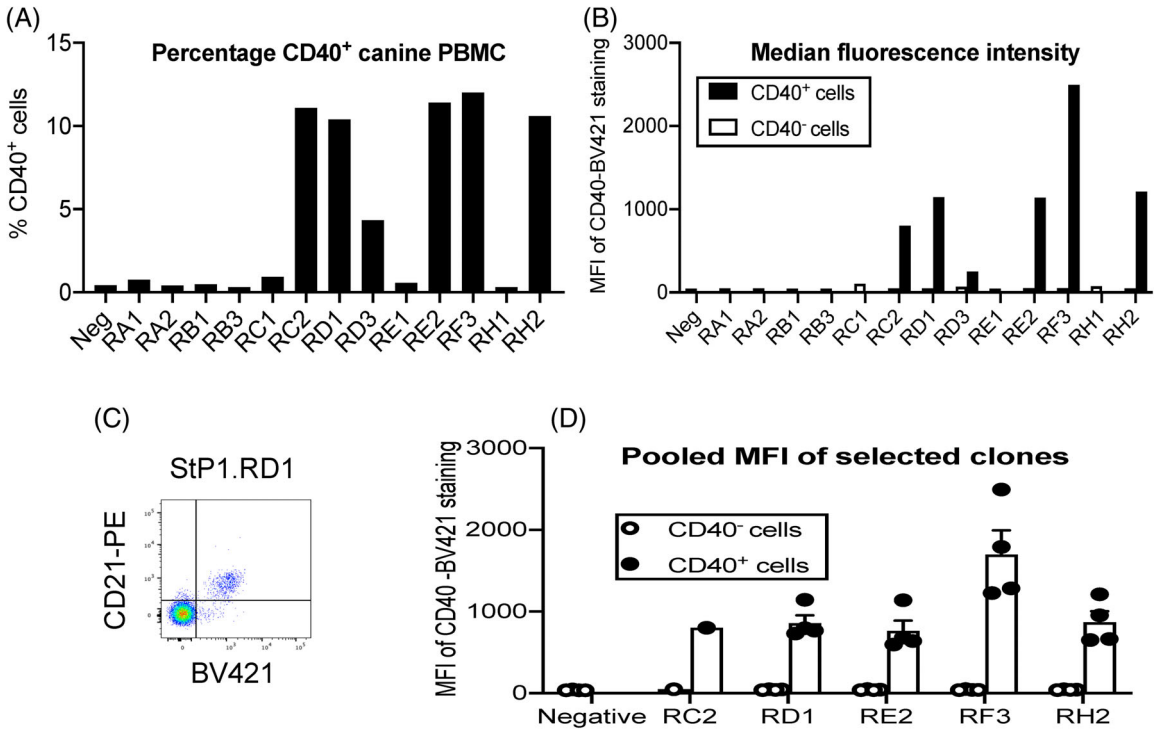


**FIGURE 1** Six hybridomas produce anti-CD40 antibodies that bind canine lymphocytes. Mouse anti-canine CD40 antibodies attached to CD40 on canine PBMCs were detected as per Figure S1. One dog was used to test all clones with staining shown as percentage of $CD40^+$ cells (A) and CD40 median fluorescence intensity (MFI) (B). Dual-staining with CD19-PE and BV421 confirmed B cells expressed CD40 (C). Selected supernatants tested on three dogs; staining shown as mean MFI ± SEM (D). PBMCs, peripheral blood mononuclear cells

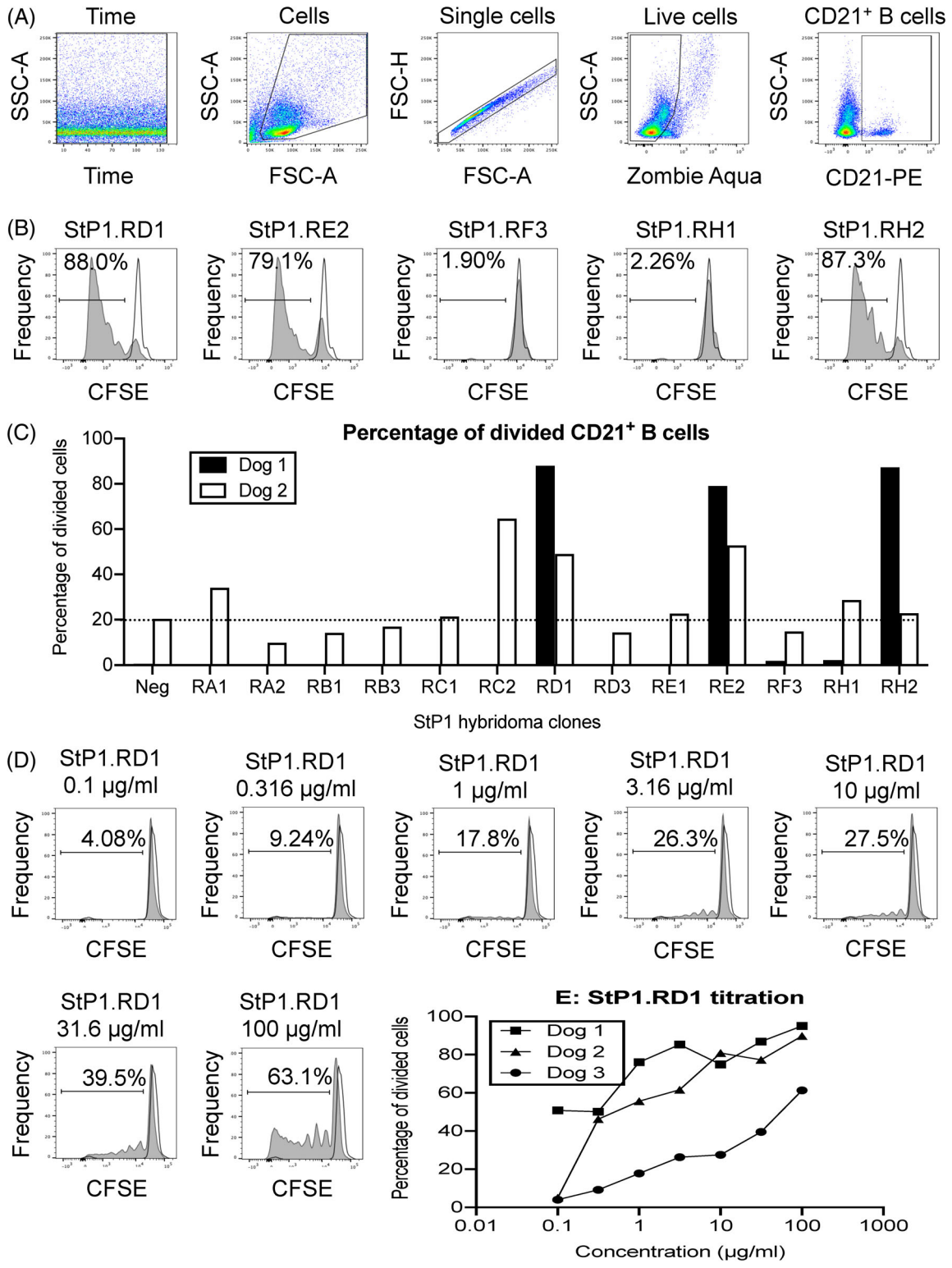


**FIGURE 2** Selecting the lead antibody candidate. PBMC were incubated with hybridoma supernatants before identifying proliferating $CFSE^{+}CD21^{+}$ B cells. Gating started with time, debris exclusion and single cell selection, leaving viable $CD21^{+}$ B cells (A). CFSE binds intracellular proteins and dilutes by 50% with each division. Cells in the brightest peak in the histogram are undivided parental cells, cells in the second peak have divided once, those in the third peak have divided twice, and so forth. $CFSE^{+}$ B cells proliferating in response to selected hybridomas shown as grey overlayed on clear unstimulated control histograms (B). The percentage of dividing cells responding to hybridomas ($n = 1/2$ dogs) (C). The lead hybridoma was titrated, shown as representative histograms in one dog (D), and graphically in three dogs (E). PBMC, peripheral blood mononuclear cell

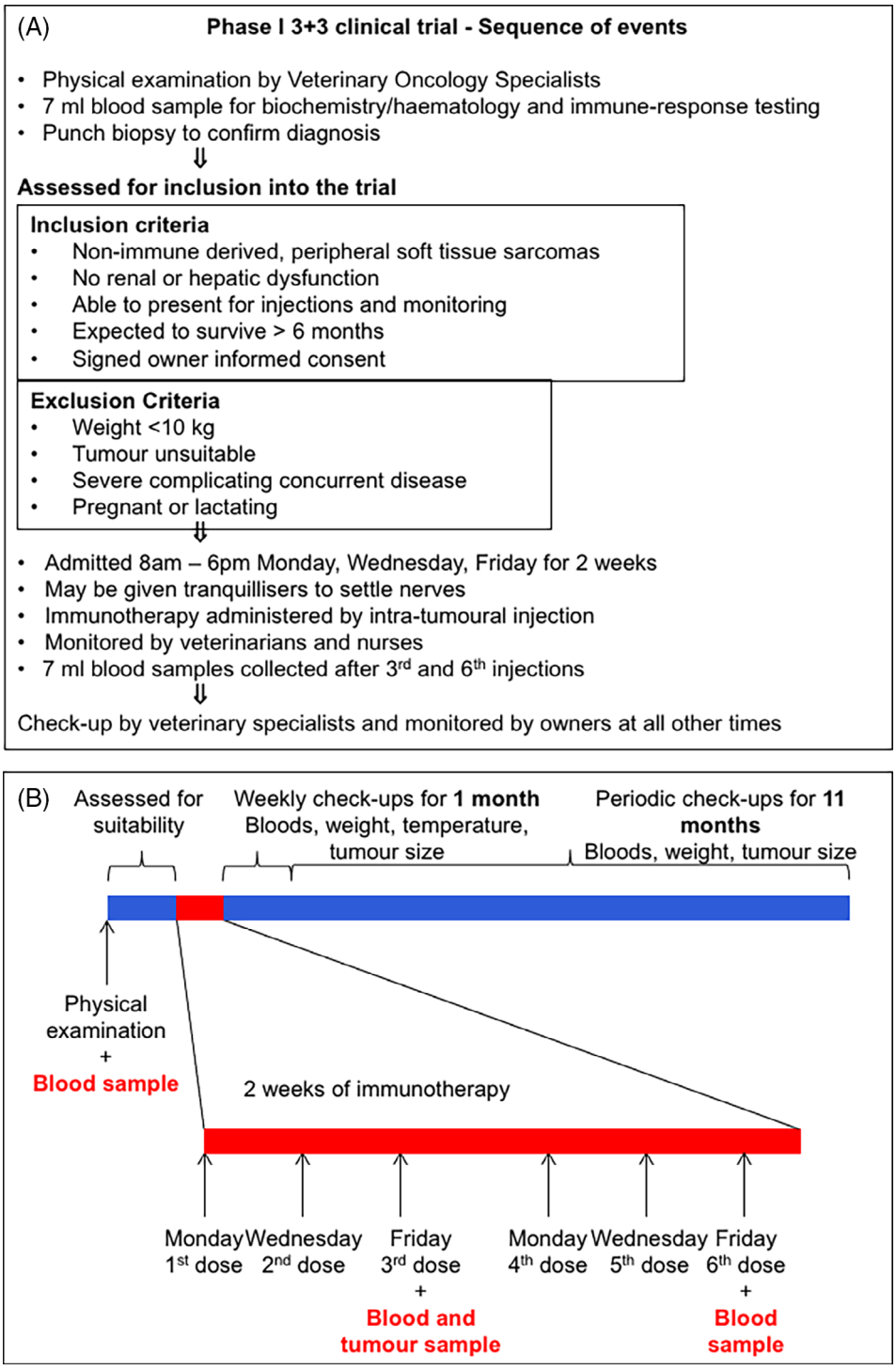


**FIGURE 3** The phase I clinical trial design. The sequence of events and inclusion/exclusion criteria (A), as well as the treatment protocol and follow up (B) are shown

The MTD was dose level 8 because 2/3 dogs experienced a grade 3 motor neuropathy (dropped jaw, presumptive inflammatory trigeminal neuritis) that interfered with daily living at dose level 9. This condition rapidly resolved following treatment cessation. One dog at dose 9 experienced a grade 3 elevated ALT along with a decreased platelet count and albumin/globulin (A/G) ratio (not shown); all classed as a DLT.

### 3.4 | Tumour responses to IL-2/CD40 immunotherapy

Meaningful tumour response data were collected from 19 dogs at the 1-month timepoint because four dogs exited the study, another owner chose to euthanize their dog before treatment effects could be

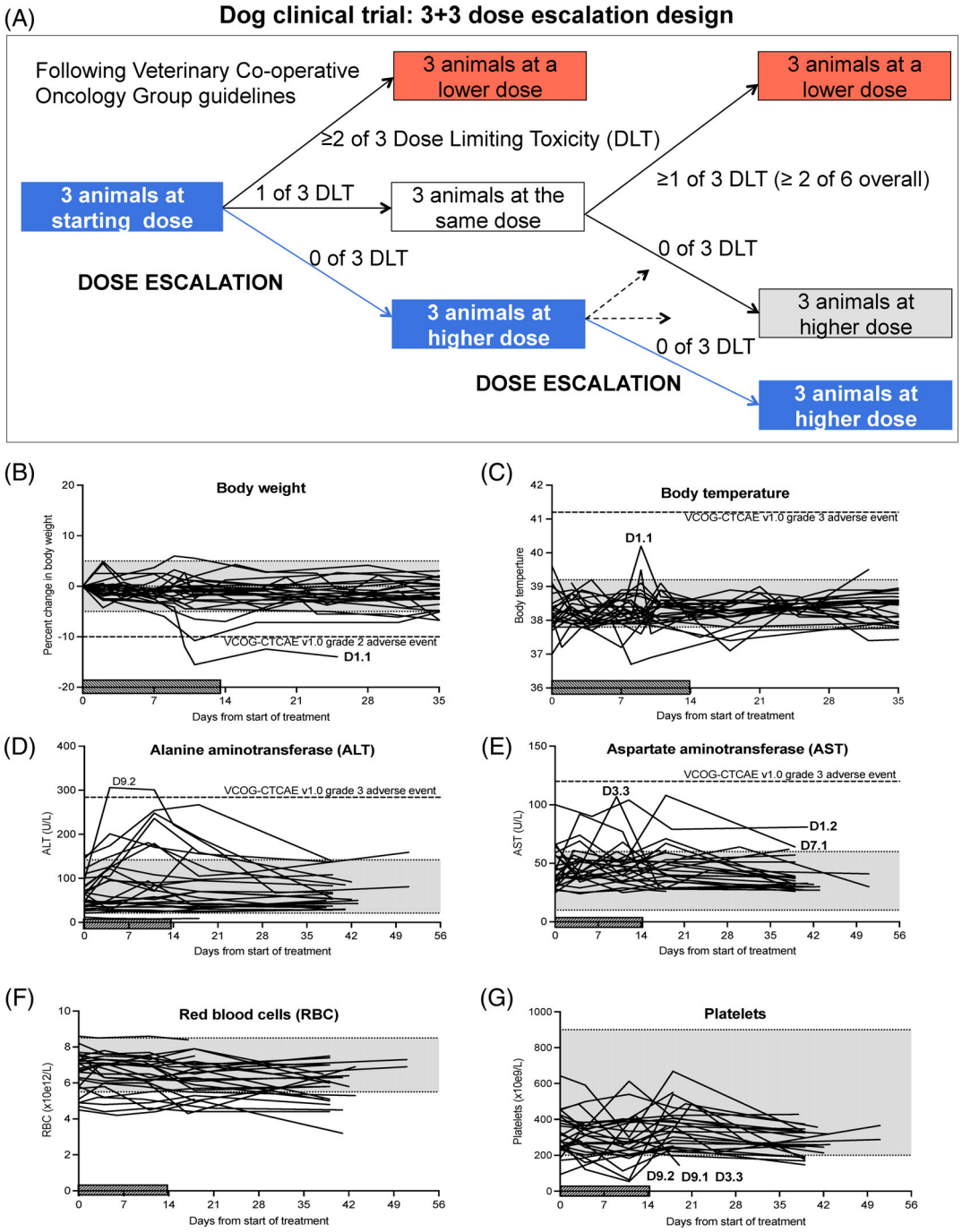


**FIGURE 4** Identifying the maximum tolerated dose. A 3 + 3 clinical trial design was used (A). Changes to weight (B), temperature (C) liver enzymes (D and E), RBCs (F) and platelets (G) were measured over time. Each line represents an individual dog. The shaded area represents the normal range. Dashed lines show the VCOG-CTCAE minimum reference for grade 2/3 adverse events. Dogs outside of the normal range that could be experiencing a toxic effect are identified; for example, D1.1 is the first dog given dose level 1

assessed, one dog was lost to follow up, and two others were not brought in for the 1-month visit, all classified as participant dropouts (PDOs; Table S3).

The clinical benefit rate at 1 month was 68.4% (Figure 6B) including tumour reduction in 13 dogs, of these, 7 dogs given doses 3–9 experienced SD with durable responses for at least 50 days, one lasted 9 months, plus PR was seen in two dogs, with one tumour almost completely clearing (90%) (Figure 6A,C).

## 4 | DISCUSSION

Human sarcomas represent a complex group of rare difficult-to-manage mesenchymal tumours that affect all age groups.[22,23] Up to 40% of patients with localized sarcomas fail to respond to treatment, and 30%–40% develop metastases.[24] Outcomes for dogs with sarcoma vary with grade but can be similar, demonstrating an urgent need for improved therapies for canine and human sarcoma. Soft

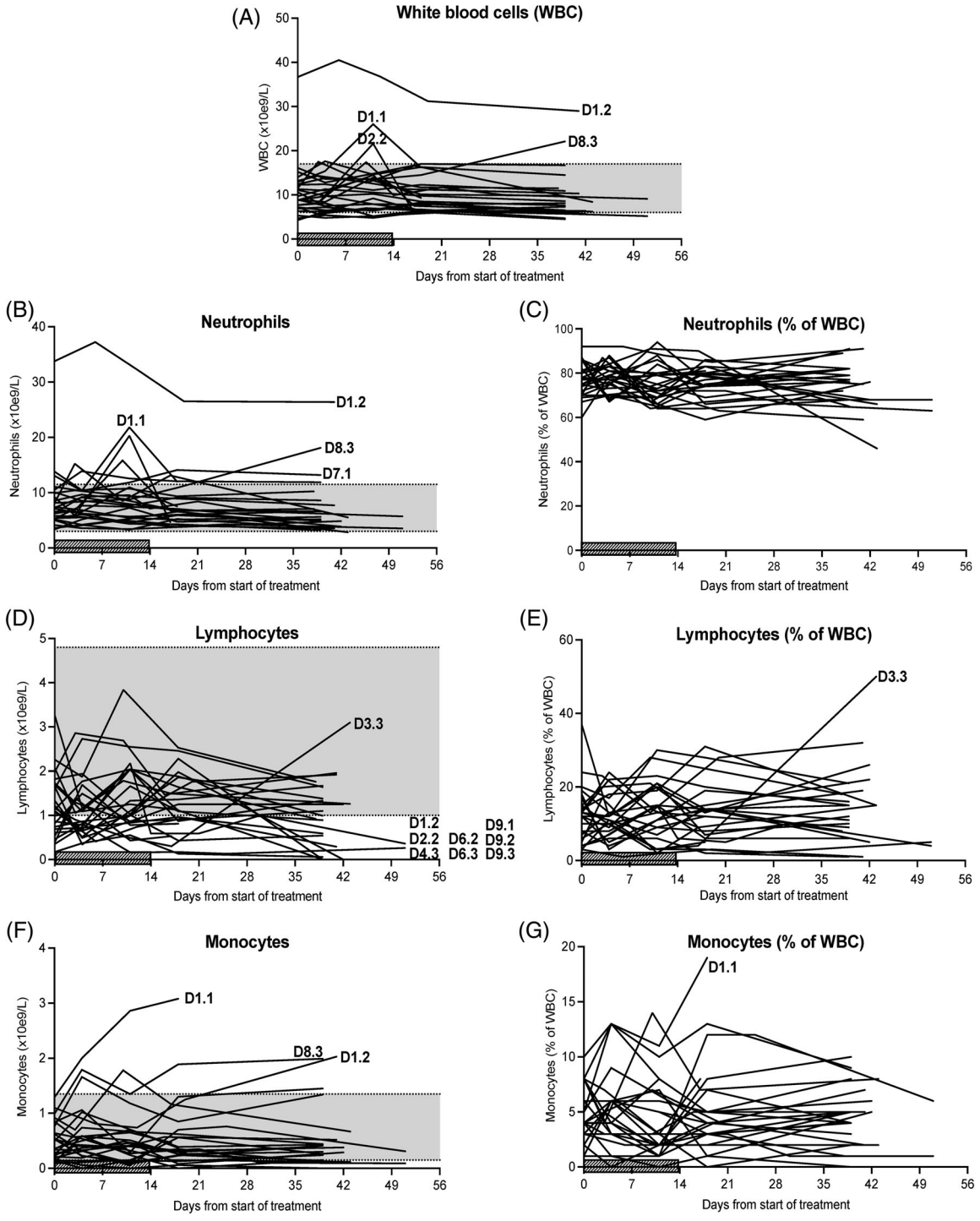


**FIGURE 5** Monitoring effects on WBCs. Changes to WBC numbers (A), numbers and percentages of neutrophils (B and C), lymphocytes (D and E), monocytes (F and G) and eosinophils (H and I) were measured over time. Only dogs with a potential toxic effect are identified, as per Figure 4

tissue sarcomas are relatively common in dogs (>27 000 dogs diagnosed annually in the US[25]). Pet dogs represent an opportunity to identify new therapies for soft tissue sarcoma. Dogs with soft tissue sarcoma are treated similarly to humans (surgery, radiation and sometimes chemotherapy), although the primary treatment modality in dogs is surgery; often because of their invasive nature, curative-intent surgeries can be radical, for example, limb removal.

A drawback of mouse studies is that use of inbred mouse strains and cloned tumour cells results in the lack of genetic diversity seen in larger mammals. We used dogs with naturally-occurring cancer to develop a dog therapeutic and help bridge the gap between mouse and human studies. Although our data are preliminary we will continue this approach, as companion dogs share the human environment, have an unmodified immune system, possess the full genetic

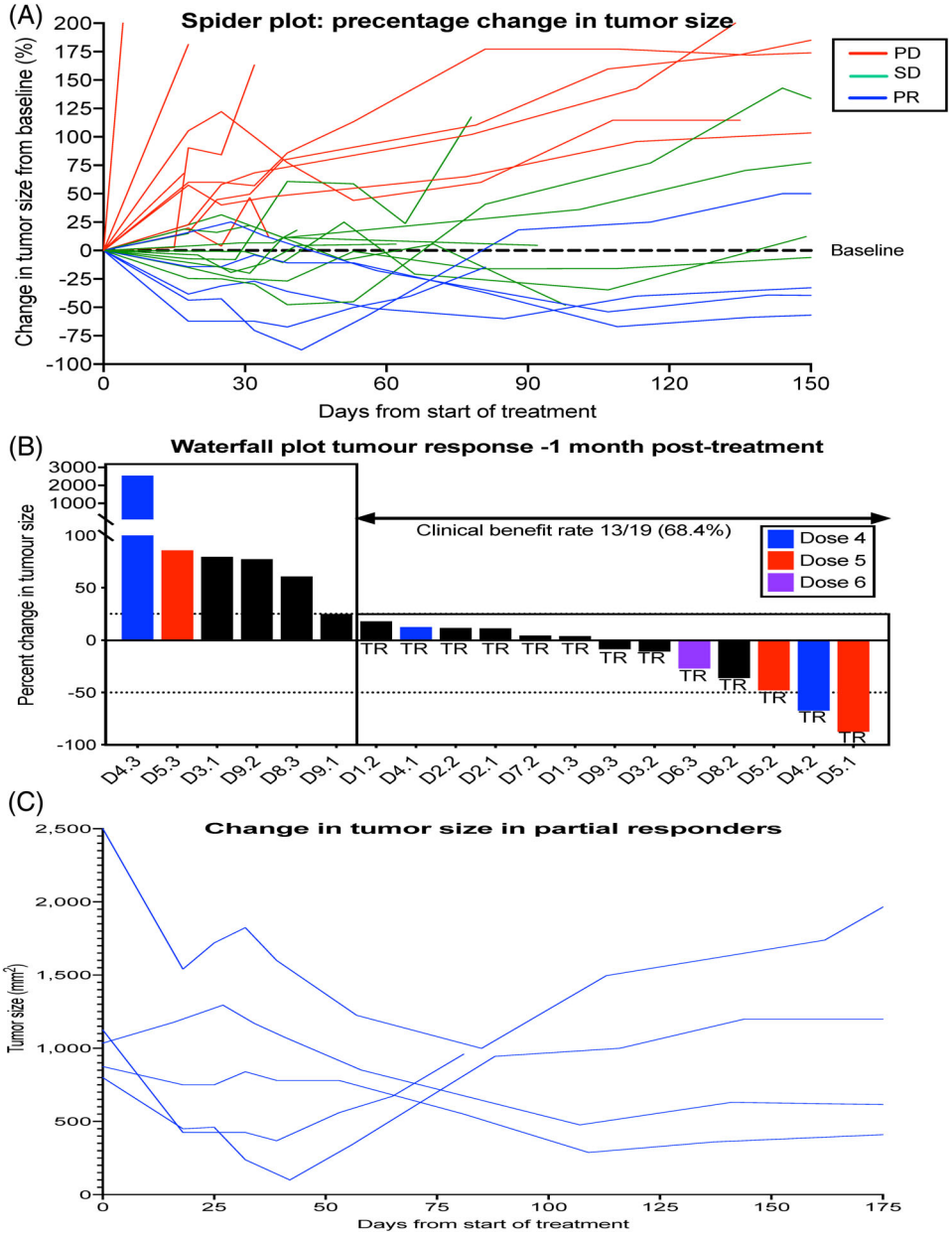


**FIGURE 6** Tumour responses to IL-2/CD40. The percentage change in tumour size over time (A) in individual dogs with progressive disease (red), stable disease (green) and partial responses (blue) shown as a spider plot. The waterfall plot (B) ($n = 19$) shows individual responses to doses 4 (blue), 5 (red) and 6 (purple), plus the other doses combined (black) at 1 month, tumour reduction includes 50% or greater tumour decrease and <25% increase). Changes to tumour size in dogs with partial remission shown as a spider plot (C), starting sizes shown at day 0

heterogeneity/complexity of a natural tumour and show similar spontaneous clinical presentation and pathophysiology to human cancer equivalents.[13,15,25–29] Reassuringly, genomic analyses, although in their infancy in canines, show shared features such as the same cancer-associated genes between dogs and humans.[14,16,30,31] This is also true for sarcoma.[32–34] Furthermore, dogs age five to eight times faster than humans, meaning we can measure treatment efficacy and side effects in a shorter time relative to human trials.

Here, we confirm that mouse pre-clinical studies are crucial for decision-making regarding future treatment options for larger mammals with cancer. Our murine studies using inbred strains and syngeneic transplantable cancer cell lines[1–3,12] provided translatable baseline data for larger, genetically diverse mammals with a different tumour, as >60% of dogs with soft tissue sarcomas responded to the same treatment regimen given to mice.

Another criticism of mouse studies is that they rarely involve old age, even though most cancers affect the elderly. We showed that our immunotherapy can be used in elderly mice with mesothelioma, although the cure rate dropped from 80%–100% in 3-month old mice (18 year old human equivalent) to 45% in elderly (22–24 months old, equivalent to 60–65 year old humans)[4]; nonetheless, this is still a respectable cure rate. Similar to humans, we saw a wide age range of dogs with soft tissue sarcoma in the clinical trial. While the median age was 11 years, that is, mostly elderly, the range was 4–14 years old.

The IL-2/ immunotherapy was well tolerated, regardless of age, with only a few issues attributed to treatment, below the highest dose tested. The most common side effects were grade 1 lethargy/fatigue (in 44% dogs) and grade 1 inappetence (in 33% dogs), these effects occurred at all dose levels. This contrasts greatly to the significant

toxicity seen during and after systemic administration of IL-2 and anti-CD40 Ab as mono- or combination therapies.[35,36]

We did not differentiate between sarcoma subtypes, which may be a limitation of our study; this decision was based on recruitment capacity. We also did not limit our study to age, gender or breed, however the primary endpoint was toxicology, with the secondary endpoint being tumour response.

Given the genetic differences between hosts and cancer types in our murine versus canine studies (mesothelioma, lung cancer in mice[3] and sarcoma in dogs), as well as differences in tumour sizes between mice and dogs, it is likely that improved outcomes for dogs with sarcoma will be achieved by modifying the treatment regimen; this will be examined in future studies. The starting tumour size range in dogs was 80–22 500 $mm^2$. Our murine studies showed that smaller tumours (<25 $mm^2$) respond better and that more resistant, often larger, tumours respond to a prolonged treatment regimen.[1,2,12] Therefore, we were not expecting dramatic tumour responses as we were treating larger tumour masses with exactly the same regimen we had given mice with relatively small tumours. Nonetheless, we saw clinical benefit in a significant number of dogs for greater than 50 days. Seven dogs given the three promising doses demonstrated a durable response for at least 50 days, with the most durable response lasting 9 months, and one dog almost completely cleared their tumour.

We do not believe we have identified the ideal treatment regimen for dogs with soft tissue sarcoma yet. The most promising tumour responses were seen at dose levels 4 and 5 with each group of three containing one dog with PR and one dog with SD for at least 1 month. Moreover, no toxicity was noted at these dose levels. It is possible that that the higher dose over-activated the immune system leading to immune exhaustion and diminished function concomitant with unwanted side effects. Future trials will examine: (i) dose levels 4, 5 and 6 in at least 12 dogs/dose, and (ii) refine the treatment so that extended treatment be administered.

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## CONFLICT OF INTEREST

Stephen Proksch is a consultant for Selvax, the funding body. Delia Nelson and Stephen Francis Proksch are SVX-2001 patent holders.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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