Remission of Spontaneous Canine Tumors After Systemic Cellular Viroimmunotherapy

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TITLE: Remissions of spontaneous canine tumors after systemic cellular viroimmunotherapy

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One Sentence Summary: Mesenchymal stem cells carrying oncolytic viruses achieve clinical efficacy in dogs.

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ABSTRACT

Dogs with spontaneous tumors treated in veterinary hospitals offer an excellent opportunity for studying immunotherapies, including oncolytic viruses. Oncolytic viruses have advanced into the clinic as an intratumorally administered therapeutic; however, intravenous delivery has been hindered by neutralization in the blood. To circumvent this hurdle, mesenchymal stem cells have been used as a "Trojan horse". Here we present the treatment of 27 canine cancer patients with canine mesenchymal stem cells infected with ICOCAV17, a canine oncolytic adenovirus. No significant adverse effects were found. The response rate was 74%, with 14.8% showing complete responses, including total remissions of lung metastasis. We detected virus infection, stromal degeneration and immune cell infiltration in tumor biopsies after four weeks of treatment. The increased presence of anti-adenoviral antibodies in the peripheral blood of treated dogs did not appear to prevent the clinical benefit of this therapy. Our data indicate that oncolytic viruses loaded in mesenchymal stem cells represent an effective cancer immunotherapy.

INTRODUCTION

Oncolytic viruses have shown great potential as anticancer therapies with the recent FDA and EMA approvals of Amgen's Imlygic as an intralesional treatment for melanoma patients (1). However, systemic administration of oncolytic viruses would be preferred in many cases because solid tumors are not always accessible. In fact, oncolytic viruses can be intravenously delivered safely with limited toxicity, but also with limited efficacy (2). As a therapeutic alternative, the use of cells that function as a "Trojan horse" delivery vehicle has been proposed. Carrier cells are infected *in vitro* and injected systemically to home into tumor beds and release oncolytic viruses (3). We have developed this strategy for pediatric solid tumors (4) using human mesenchymal stem cells (MSCs) infected with ICOVIR-5, a human oncolytic adenovirus, which we have called Celyvir (5). We reported our initial human clinical results on the use of Celyvir in advanced neuroblastoma patients where we described an excellent toxicity profile and several clinical responses, including two complete remissions out of twelve patients (6,7). This supported an ongoing Phase I clinical trial for pediatric and adult tumors (ClinicalTrials.gov Identifier: NCT01844661). In this study, we improve upon our antitumoral therapy as a method to treat spontaneously occurring tumors in canine patients. Clinical trials conducted in client-owned dogs are very useful for developing new anticancer therapeutics (8,9) for human and veterinary medicine. Histological and genetic molecular alterations that correlate with cancers in dogs and humans are analogous (10,11), and surgery or oncology treatments for humans and dogs are nearly identical.

We sought to determine the safety and potential efficacy of systemic injections in dog patients of an upgraded canine version of Celyvir (dCelyvir), using dog MSCs (dMSCs) and ICOCAV17, a new canine oncolytic adenovirus (12). Previously, we used this virus via intratumoral administration to treat six dogs with different tumor types inducing partial responses and disease stabilization (12). Now, in addition to using MSCs as carriers, we have

focused mainly on canine patients with sarcomas and CNS tumors, since our primary objective is pediatric cancer and carcinomas are infrequent in childhood. Although some subtypes of human soft tissue sarcoma are sensitive to chemotherapeutic agents, the outcome of chemotherapy is unsatisfactory rendering overall response rates of about 25% as a first line treatment (13,14). The potential for immunotherapy in the treatment of sarcomas is beginning to be explored (15), and oncolytic viruses have recently been used for sarcoma treatments with encouraging results (16,17).

Here, in a veterinary trial with dCelyvir in 27 canine patients, we observed an excellent toxicity profile as well as a clinical benefit in 74% of patients, including 14.8% showing complete remissions. Our results also show microenvironment alterations and immune cell infiltration in tumors after dCelyvir treatment, suggesting the activation of antitumor immune responses. We believe that the immune-related response of MSCs when infected with oncolytic adenoviruses has an important role in observed clinical benefit.

MATERIAL AND METHODS

Clinical study and canine patients

We enrolled 27 canine patients between March 2013 and March 2016, for treatment with dCelyvir. The clinical study was approved by the Veterinary Hospital Ethics Committee and all patient owners gave written informed consent. Inclusion criteria were: owner refusal or disease progression to standard treatment (chemotherapy/surgery), absence of severe undercurrent disease, and docile character for easy treatment without sedation. The study included different dog breeds. The diagnosis was performed analyzing tumor biopsies. In osteosarcoma patients a cytology sample was used for diagnosis. Patients with CNS tumors were diagnosed at necropsies for being intracranial non-operable tumors. In some of the cases we did not have access to biopsies due to a difficult localization of tumors.

For treatment with dCelyvir, canine MSC from healthy donors were infected with ICOCAV17 at a multiplicity of infection (MOI) of 1 infectious particle per cell during one hour. Infected cells were washed three times, filtered and resuspended in saline buffer. Prior to dCelyvir infusion, canine patients were treated i.v. with metilprednisone (1mg/kg), metamizol (30mg/kg), and difenhidramine (0.5mg/kg). dCelyvir was administered over 45 min through a peripheral or central venous line (cephalic/safen preferably) at doses of $0.5x10^6$ cells/Kg dog body weight. During first administrations, patients were kept in the hospital for 6 hours with constant monitorization. Treatment was repeated once a week during 4 weeks. After these four administrations, the veterinary board evaluated each case and decided how to continue the treatment. Now and henceforth, when we refer to post-treatment, if not otherwise specified, we are referring to the time after the fourth dose.

During treatment, canine patients were closely followed up after dCelyvir infusions and blood analyses were done every week in order to analyze hematological, renal, and liver functions. Briefly, the veterinary RECIST response characterization is as follows: (i) complete response (CR): disappearance of all target lesions; pathologic LNs <10mm short axis; (ii) partial response (PR): at least 30% reduction in the sum of diameters of target lesions, taking

as a reference the baseline sum; (iii) progressive disease (PD): either the appearance of one or more new lesions or at least a 20% increase in the sum of diameters of target lesions, taking as a reference the smallest sum during the study; the sum must also show an absolute increase of 5 mm; (iv) stable disease (SD): less than 30% reduction or 20% increase in the sum of diameters of target lesions, taking as a reference the smallest sum of diameters during the study. A quality of life test(18) was performed after treatment based on input from the owners.

Mouse model study

The preclinical studies in mice were approved by the "Animal Ethics Committee" of ISCIII in compliance with European Union directives. For *in vivo* imaging of dMSCs and dCelyvir homing into canine tumors, we established subcutaneous tumors in the flanks of 6-8 weeks old NOD.CB17-Prkdc^{scid}/J (NOD-SCID) mice. Tumors were grown for 10-12 days. dMSCs or dCelyvir were incubated with 13.8 μg/mL DiR buffer for 30 min at 37°C according to the protocol of XenoLight DiR (Caliper Lifesciences, Hopkinton, MA). Then DiR-labeled dMSCs and dCelyvir were washed twice with PBS and intraperitoneally injected in dog tumor-bearing mice (1x10^6 cells/mouse). Fluorescence imaging analysis was conducted with the IVIS 200 *in vivo* imaging system (Caliper) 24h after the injection. For the efficacy assays, we injected dMSCs or dCelyvir in PBS (1x10^6 cells/mouse) intraperitoneally in the same immunodeficient model. The first dose was 5 days after tumor inoculation and a total of three doses were administered. Tumors were measured periodically with a caliper, and volume was calculated as (length x width^2)π/6. At day 20, mice tumors were removed and processed for flow cytometry and histology.

Cell Culture

Cells were cultured in Dulbecco's Modified Eagle's Media (DMEM), supplemented with 10% fetal bovine serum, 1% glutamine, streptomycin (100 mg/mL) and penicillin (100 U/mL), at 37°C in a humidified atmosphere with 5%CO_2. Adipose tissue from healthy dog donors was digested with collagenase IV (Sigma-Aldrich), filtered through a sterile 70μM nylon mesh cell strainer (Fisher Scientific) and cultured in DMEM supplemented as above. Non-adherent cells

were discarded through subsequent culture passages, and finally we obtained a homogenous dMSC culture. Bone marrow derived human MSCs were purchased from Lonza. The dMSCs/hMSC multilineage differentiation potential for adipogenic, osteogenic and chondrogenic phenotypes was assessed by the culture of dMSCs in specific cell culture media (Cell Applications) or hMSC specific culture media (Lonza) stained with lipidic drop (oil red O), bone ECM (alizarin red) and chondrogenic glycosaminoglycans (alcian blue), respectively. DKcre cells, a renal tumoral embrionary nonmalignant canine cell type, were a kind gift of Dr Eric Kremer (Institut de Génétique Moléculaire, Montpellier). The Abrams canine osteosarcoma cell line was provided by Dr David Vail (School of Veterinary Medicine, University of Wisconsin-Madison, Wisconsin). The murine non-small-cell lung carcinoma cell line CMT64 was kindly gifted by Dr. Sthepan Kubicka (Hannover Medical School, Germany) and the CMT64-6 clone was selected based on its high human adenovirus replication susceptibility (19). Dog tumoral non-immortalized cells from primary tumors were obtained after mechanical disaggregation followed by the same protocol detailed above for obtaining dMSCs. Primary cells were used after four to five passages. Mycoplasma infection was routinely checked by luminescence using MycoAlert™ Mycoplasma Detection Kit (Lonza).

Viruses

We have previously described ICOCAV17 (12). Briefly, CAV2RGD is based on CAV2, the canine wild-type virus serotype 2, with an RGD motif inserted at in the HI-loop of the CAV2 fiber. ICOCAV17 is a canine conditionally replicative adenovirus, based on CAV2RGD, in which the endogenous E1a promoter has been modified by inserting four palindromic E2F-binding sites and 21 base pairs have been deleted of the E1a pRB-binding domain (E1aΔ21) homologous to Δ24 deletion performed in human oncolytic adenoviruses. ICOCAV17 is armed with the human PH20 hyaluronidase (PH20) gene inserted after the fiber under the control of the canine IIIa protein splicing acceptor (IIIaSA).

For Infectivity assays, dMSCs were seeded in 96-well plates. After 24 hours, the cells were infected in triplicate with serial dilutions of ICOCAV17. After 24 hours, medium was removed

and immune staining against hexon protein was performed according to an anti-hexon staining based method (20).

Cytotoxity assays were performed by seeding dMSCs in 96-well plates in DMEM with 10% fetal bovine serum. The cells were infected with serial dilutions of CAV2 or CAV2RGD starting with 1,000 TU/cell. Six to eight days post-infection, plates were washed with PBS and stained for total protein content through absorbance measurements. For virus production, dMSCs were infected and after 4hours, medium was removed and cells were washed twice with PBS and incubated with fresh medium. Cells and medium (cell extract) were harvested 48 hours post-infection and subjected to three rounds of freeze-thaw lysis. Viral titers of cell extracts were determined in triplicate according to an anti-hexon staining-based method in DKCre cells.

Cytokine analysis

Blood samples were collected each week before treatment, and serum was obtained and frozen. Analysis of GM-CSF, IFN-g, IL-2, IL-6, IL-7, IP-10, MCP-1, TNF-a levels was performed with the CCYTOMAG-90K, Milliplex MAP Canine Cytokine/Chemokine Magnetic Bead Panel. Other cytokines not mentioned were below the lower limits of detection.

qPCR analysis

Whole blood samples were stored at -20°C until processing. DNA was extracted from 200μL of whole blood and was eluted with 50 μL of distilled water according to the QIAamp DNA Blood Mini Kit (Qiagen) instructions. Tumor and liver tissues were stored under different conditions; extraction of each sample was performed following different protocols. Tissue samples that were frozen at -80°C were introduced into a metal cylinder submerged in liquid nitrogen and were ground thoroughly with a pestle until they were completely pulverized. Thirty to forty mg from each sample was used to extract DNA using the QIAamp DNA Mini Kit (Qiagen) according the manufacturer's instructions. For samples stored at -80°C with TRIzol reagent, the tissues were thawed, vortexed during 2 minutes and homogenized using a potter-elvehjem PTFE pestle with a glass tube. One mL of each sample was processed to extract DNA following the TRIzol reagent's instructions. In formalin-fixed paraffin-embedded tissues, DNA was extracted from 6 sections of 10μm for each sample and was processed

using the Cobas DNA Sample Preparation Kit (Roche) according to manufacturer's intructions. DNA quantification and purity (A260/280 and A260/230) were analyzed with Nanodrop 2000 spectrophotometer (ThermoScientific).

A standard curve was prepared based on serial dilutions of ICOCAV17 from 10^8 vp/mL to 10^3 vp/mL using blood from a healthy dog as diluent. DNA extraction was performed as described above for whole blood samples. Purified DNA samples were analyzed in triplicate by the quantitative real-time PCR system 7500 Fast (Applied Biosystems) using the Premix Ex Taq (Clontech), with forward primer (0.5µM) 5'-TGTGGGCCTGTGTGATTCCT-3', reverse primer (0.5µM) 5'-CCAGAATCAGCCTCAGTGCTC-3', and 10pmol of Taqman probe FAM-CTCGAATCAGTGTCAGGCTCCGCA-TAMRA, which identify E1A region. qPCR conditions were: holding stage 10 minutes at 95ºC and cycling stage 15 seconds at 95ºC followed by 1 minute at 60ºC repeated 40 times. Analysis was performed using the 7500 Software v2.0.6 (Applied Biosystems). Positive results were confirmed by sequencing the products of the qPCR. qPCR products were treated with ExoSAP-IT (Affymetrix) following manufacturer's instructions and were sequenced with primer at 0.6 µM using the capillary automatic sequencer DNA Analyzer 3730xl (Applied Biosystems). Sequences resulting from the sequencing process were analyzed with the basic local alignment search tool (BLAST) from the NCBI, and those that produced significant alignments were considered to be positive results.

Western blot analysis

Total protein was extracted with SDS sample buffer (62.5 mM Tris pH 6.8, 2% SDS, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride [PMSF], 5 mM NaF, 20 mM β-glycerophosphate, 0.1 mM Na3VO4, and 1:100 protease inhibitor cocktail from Sigma). Then, samples were boiled at 95°C for 5 min and sonicated (three pulses of 30 seconds on ice). Protein concentrations were determined using the DC Protein Assay (Bio-Rad).

Finally, DTT and bromophenol blue were added to the samples to a final concentration of 100 mM and 0.1%, respectively, and then they were boiled at 95°C for 5 min. Proteins were separated by 10% SDS–PAGE and blotted onto PVDF membranes (Bio-Rad). Antibodies used

are described in Supplementary Table 1. Signal was detected using the Immobilon Western chemiluminescent HRP substrate (Millipore).

Migration assays

Transwell plates (8 µm pore filters, BD Biosciences) were coated with 0.1% gelatin (Sigma) and $5x10^4$ dMSCs were plated on the upper chambers. Cells were incubated in the presence of CMT64-6 or the dog tumoral cells UAX-12 or UAX-15 in the bottom chamber for 24 hours without FBS. A negative control assay was also performed by incubating the cells with only DMEM in the bottom chamber. Medium with 30% FBS was used as a positive control. Migrated cells were fixed, stained with crystal violet and manually counted using 10 high-power fields (HPFs). Experiments were repeated three times.

Flow Cytometry

Cells were incubated with FcR blocking. For murine cells we used mFcR blocking (Miltenyi), and for canine cells we used hFcR Blocking (Miltenyi). We then incubated suspensions for 20 min at 4°C with directly conjugated antibodies diluted in PBS containing 1% FBS. For the study of cell subsets in dog peripheral blood, we dispensed 30µl of blood per tube and lysed the erythrocytes with Quicklisis buffer (Cytognos). Tumor suspensions were obtained with mechanical disaggregation followed by enzymatic disaggregation with collagenase D at 1 mg/ml (Roche), in HBSS with Ca^{2+} and Mg^{2+} (Lonza), 2% FBS, 1h at 37°C. The cellular suspensions were filtered through 70 µM nylon mesh cell strainer (Fisher Scientific, Waltham, MA) and the erythrocytes lysed with Quicklysis buffer (Cytognos). The antibodies used are described in Supplementary Table 1. The analysis was conducted with a Macsquant10 flow Cytometer (Miltenyi).

Immunohistochemistry

Canine biopsies were fixed in 10% formalin overnight. After that they were washed in PBS and dehydrated by immersion in graded alcohol and xylene baths. Then they were embedded in paraffin. After cooling the wax block, 5 µm thick sections were cut in a microtome and were rehydrated (xilol, ethanol 100%, ethanol 96%, tap water) for Hematoxilin-Eosin staining or immunostaining. Before immunostaining we performed antigen retrieval with citrate buffer

(10mM pH 6) in a pressure cooker (2 min at the highest pressure). Endogenous peroxidase inhibition and blocking with normal horse serum was also performed before the overnight incubation with primary antibody at 4°C. Then after washing, the sections were incubated with biotinylated secondary antibody (RTU-Vectastain Kit) for 15 min, incubated with SAV-HRP during 10 min and after that with DAB for 3 min. For ICOCAV17 detection, we used the polyclonal primary antibody Anti-Adenovirus 5 antibody (abcam), followed by a secondary anti-rabbit antibody (Invitrogen). The antibodies used are described in Supplementary Table 1.

Neutralizing antibodies

To determine neutralizing antibodies (IgG) against canine adenovirus (CAV), an enzyme labeled dot assay was performed (ImmunoComb Canine VacciCheck, Biogal Galed Laboratories Acs.). Serum or whole blood samples from either pre-treatment or different post-treatment time points were stored at -20°C until analysis. Serum (5µL) or whole blood (10µL) samples were dispended in the developing plate. The assay was performed at room temperature according to the manufacturer's instructions. ImmunoComb images were digitalized and spot densities were quantified using Image Lab™ 5.0 Software (Bio-Rad). Arbitrary units were calculated as follows: (sample spot intensity – sample mean background intensity) - (positive reference spot intensity – positive reference spot mean background intensity).

Statistical Analysis

Data were graphed and analyzed using GraphPad Prism (GraphPad Software, La Jolla, CA). All experiments were performed in a blinded manner and repeated independently under identical conditions. Data distribution was examined first and transformations were applied as appropriate. To analyze the results, the Shapiro–Wilk test was performed first to find out whether values followed a normal distribution. Then, comparisons between quantitative variables were done using the Student's t-test for samples with normal distribution, or the Wilcoxon's rank-sum test (Mann–Whitney statistic), for samples with non-normal distribution. Differences were considered significant with a p value below 5%. STATA software 11.0

(StataCorp, College Station, TX) was used. Figures represent mean ± standard error of the mean.

RESULTS

dMSCs can be infected with ICOCAV17 and show tumoral homing and anti-tumor efficacy.

We treated dogs suffering from naturally occurring tumors with dCelyvir therapy consisting of dMSCs infected with a canine oncolytic virus. dMSCs were obtained from adipose tissue from healthy donors. The cells were characterized for their capability to differentiate into the three main mesodermal lineages, adipocyte, osteoblast and chondrocyte, by studying the expression profile of MSC markers via flow cytometry (Suppl. Figure S1a and S1b). We assessed the expression of CD90, CD29, CD73 and CD44 molecules and the absence of expression of the markers MHCII, CD3, CD11 and CD45 (Suppl. Figure S1a). The canine oncolytic virus we used was ICOCAV17, a CAV2-based, pRb-responsive, RGD-modified and hyaluronidase-armed canine oncolytic adenovirus, which has been previously tested in dogs by direct intratumoral inoculation (12). ICOCAV17 was able to infect dMSCs in a dose-dependent manner (Suppl. Figure S1c). Compared to the wild type virus CAV2, RGD-modified adenovirus infection resulted in higher cytotoxicity (Suppl. Figure S1d). Production of CAVRGD in DKcre cells was consistently comparable to CAV2 productions (Suppl. Figure S1e). Adenoviral replication in dMSCs was confirmed by immunostaining of late viral proteins (Figure 1a) and viral protein expression quantitation by Western blot (Figure 1b). Additionally, adenoviral genome copies were quantified by qPCR at different time points after ICOCAV17 infection showing a maximum production at 48h (Figure 1c). In order to assess whether dMSCs or dCelyvir home into canine tumors, we performed *in vitro* transwell migration assays. Both dMSCs and dCelyvir migrated efficiently towards tumor cells (Figure 1d).

To confirm this tropism i*n vivo*, we subcutaneously inoculated canine tumors into the flanks of immunodeficient NOD-SCID mice and imaged the fluorescent signal of DIR-stained dMSCs 24h after intraperitoneal administration. Fluorescent signal from the DIR-stained dMSCs was detected in the area of the tumors, and no differences between dCelyvir or

dMSC delivery were found (Figure 1e). Overall, these results indicate that ICOCAV17 efficiently infects and replicates in dMSCs, and that these infected dMSCs (dCelyvir) reach tumors, an essential requirement for them to be used as antitumoral cellular carriers.

In order to assess the anti-tumoral efficacy of this approach, canine tumor-bearing NOD-SCID mice were treated with dCelyvir, dMSCs or PBS starting 5 days after tumor inoculation with the administration of three doses (Figure 1f). Significant differences in tumor volume between dCelyvir- and PBS-treated mice were found (Figure 1g). Tumors treated with dMSCs showed no differences in tumor volume compared to PBS-treated ones.

Canine patients and dCelyvir treatment

We enrolled 27 canine patients for dCelyvir therapy. The patients' clinical characteristics are shown in Table 1. The trial consisted of a repeated weekly administration of intravenous dCelyvir either alone or in combination with additional therapies according to veterinarian board choice (Table 1). A tumor biopsy was collected after the fourth dose, and a specific therapeutic regimen was designed for each dog based on an interim report on clinical benefit. Most dogs (59%) received dCelyvir as the only treatment, while the rest of the dogs were treated with combined therapies, including conventional chemotherapy in 3 dogs (three cycles of doxorubicin during one week plus dCelyvir for two weeks), tyrosine kinase inhibitors (TKIs) in 7 dogs (TKIs every day plus dCelyvir once a week), or cyclophosphamide therapy in one dog (cyclophosphamide every day plus dCelyvir once a week). A group of patients with mainly suffering CNS-derived tumors and mastocytomas were also treated with corticoids (Prednisone).

Outcome assessment of dCelyvir treatment

The veterinary oncologist board evaluated the clinical responses following the four doses of dCelyvir using the veterinary Response Evaluation Criteria in Solid Tumors (RECIST) V1.1 guidelines similar to human RECIST. Responses were classified as complete response (CR),

partial response (PR), stable disease (SD) and progressive disease (PD) (Table 1). Seventy-four percent of the canine patients showed a clinical benefit to dCelyvir therapy with 14.8% of the cases achieving a complete remission (Figure 2a). Twelve out of 16 patients treated only with dCelyvir showed a clinical benefit (2 CR, 3 PR, 7 SD), and 8 out of 11 patients that received combined therapy showed clinical benefit (2CR, 6 SD). Moreover, 2 out of the 5 canine patients with pulmonary metastasis at the time of diagnosis (UAX-05, UAX-08, UAX-23, UAX-24, UAX-26) had a complete response (Figure 2b and Suppl. Figure S2a). Patient UAX-24 initially received combined therapy (dCelyvir plus doxorubicin) and switched to dCelyvir only after 9 weeks, improving the clinical outcome from partial response (week 15) to complete response (week 40, Figure 2b).

Anatomical pathology studies were done on tumor biopsies obtained prior to dCelyvir treatment (pre-treatment, n=14) and after the fourth dCelyvir dose (post-treatment, n=22) (Figure 2c). Tumor biopsies showed a significant increase in fibrosis, hemorrhages, and extracellular matrix (ECM) degeneration after treatment. Necrosis evaluation showed the same tendency (Figure 2d). Hyaluronic acid detection was performed by immunohistochemistry in pre- and post-treatment biopsies without conclusive results due to the heterogeneous patterns found within each individual sample (Suppl. Figure S3a). Immune population analysis showed no differences of immune cell infiltration in pre- and post-treatment biopsies (Figure 2c and 2e); neither when considering "responder" (CR, PR, SD) and "non-responder" (PD) groups together nor separately (Suppl. Figure S2c). However, in post-treatment samples, we observed a trend of increased MAC387^{+} cells, including neutrophils and macrophages (Figure 2e). Interestingly, in several cases we observed that MAC387^{+} cells were localized inside of blood vessels in pre-treatment biopsies, but that they infiltrated the tumors after treatment (Suppl. Figure S2d). In this regard, it should be noted that in some patients (UAX-03, UAX-09, UAX-10) during the first week after the first dose of dCelyvir we observed an evident edema in the tumor and a transient increase in tumor volume followed by a shrinkage of the tumor before the second dose, suggesting a temporary local tumor inflammation or pseudoprogression.

We also studied the immune cells by flow cytometry and cytokines by Multiples Cytokine Panel in the peripheral blood of canine patients during the first 4 weeks (Suppl. Figure S4). The cell numbers of neutrophils, T cells (CD3+, CD4+, CD8+), macrophages, NKs, and T regs tended to increase after each dose, but only an increase in T cell numbers after the first dose was statistically significant (Figure 3a). We did not find significant changes in cytokine levels, although we observed a slight increase in MCP-1 and IL6 following the third dose (Figure 3b). To measure ICOCAV17 replication in tumors after dCelyvir treatment, we analyzed samples by qPCR from tumor biopsies from 12 dogs (pre- and post-treatment), liver samples from 3 dogs (post-treatment), and peripheral blood from 15 dogs (pre- and post-treatment). All pre-treatment samples and all post-treatment tumor biopsies were negative, but ICOCAV17 was detected in 2 liver biopsies and 1 peripheral blood sample. The immunohistochemistry detection of ICOCAV17 in tumor biopsies from 15 patients (pre- and post-treatment) showed clusters of adenovirus-positive cells in 4 of them (Figure 3c). Interestingly, we detected ICOCAV17 in tumors despite most patients (77.7%) having been previously vaccinated against canine adenoviruses (Table 1). Measurements of anti-CAV2 neutralizing antibodies in sera indicated that these dogs had neutralizing antibodies against CAV2 prior to dCelyvir treatment. During treatment, antibody levels increased initially and tended to stabilize (Figure 3d). It is important to highlight that high neutralizing antibody levels before and after treatment did not prevent the anti-tumoral effects of dCelyvir.

Safety and toxicity of dCelyvir treatment

Treatment with dCelyvir was well tolerated. We evaluated the quality of life of the canine patients and 73% of them showed a good quality of life during the treatment (Suppl. Table 2). Most of the patients with bad quality of life presented osteosarcomas or soft tissue sarcomas that affected the legs causing restricted mobility and pain. Adverse events related to dCelyvir treatment were registered following the criteria of the veterinary cooperative oncology group for common terminology criteria of adverse events in dogs and cats (VCOG-CTCAE). Among the 27 patients, clinical adverse events were documented for only

4 of them. Two showed skin alterations and one digestive symptoms, but these were patients that were all concomitantly treated with corticoids. Another patient suffered an orchitis (Table 2). None of these clinical adverse events were classified as severe or changed the quality of life status, and all were transient. Serum alanine transaminase (ALT) levels increased (grade 1/2) in 10 patients, but only one showed concomitantly high aspartate transaminase (AST) levels; an occasional decrease of phosphorus concentration was detected in eight dogs (Suppl. Figure S5a). In addition, there were no significant changes in the peripheral blood cell counts, neither in total leukocyte number nor in hematopoietic subpopulations (Suppl. Figure S5b). Only two patients showed a reduced number of neutrophils, one of which was grade 2 that subsequently recovered upon TKI removal. Of note, no significant differences between responder and non-responder groups were observed for any of the analyzed parameters. Only a slight trend of higher ALT concentrations was observed in the responder group (Suppl. Figure S5a). In summary, dCelyvir was a safe and well tolerated treatment.

DISCUSSION

Dogs with spontaneously arising cancers are an excellent model for the study of human immunotherapies and provide the opportunity to develop new veterinary oncology treatments. Oncolytic viruses represent a new class of immunotherapeutic agents that induce systemic antitumoral activity (1). In order to improve delivery of the oncolytic virus to the tumors, we used MSCs as carrier cells due to their tumor tropism. Migration of MSCs to tumors is thought to be due to inflammatory signaling in the microenvironment resembling that of an unresolved wound (21). However, the complete MSC homing mechanism remains to be elucidated (22), and tumor homing is not absolutely specific, as infused MSCs have been detected in other organs (23). Several strategies have been proposed to increase the tumor homing capability of stem cells in order for them to function as carriers (24,25). Although theoretically an *in vivo* distribution of oncolytic virus-infected MSCs could produce a disseminated viral infection, we have not found significant toxicity using Celyvir in mice (19), dogs (this manuscript), or humans (7,26). Also, we have never detected adenoviral genomes in peripheral blood, except at a peak of 72 hours after the first dCelyvir infusion. Both observations indicate effective immune neutralizing activity in peripheral organs. In this regard, almost all treated dogs were previously vaccinated with a CAV-2-based vaccine and showed high levels of neutralizing antibodies, which increased quickly after dCelyvir administration until reaching a plateau. This is in agreement with the antibody kinetics shown in human patients infused with other oncolytic viruses (27-29). However, these high antibody levels did not preclude achieving a high rate of clinical benefit (74%), including 14.8% achieving complete responses that were maintained for years.

Although different types of tumors were included in the study, most of them were soft tissue sarcomas (STS). Surgery remains the main treatment for STS due to its biological behavior and its poor response to chemotherapy and radiotherapy. Recurrence following surgical resection is the main reason for treatment failure (30-32). This is probably due to small buds of sarcoma cells regularly extruding through the reactive zone to form small isolated nodules (satellite

nodules or skip metastases) (32,33). Tumor recurrence is 10 times more likely in dogs with incomplete margins with a recurrence rate of 17-28% (34,35). In our study, we obtained a longer disease-free survival time suggestive of an anti-tumoral adaptative immune activation, which is supported by the lymphocyte infiltration observed in the tumor samples analyzed. A trend of increased $CD3^+$ cell infiltration was seen in non-responders compared to responders (Suppl. Figure S2c). We observed similar effects in the context of the compassionate use of Celyvir in humans. In contrast to responders, non-responder patients showed increased peripheral blood cell counts and increases in the proportions of terminally differentiated effector T lymphocytes during Celyvir treatment (7). These results may be indicative of an anti-adenoviral response. Viral antigens usually contain immunodominant epitopes that elicit strong antiviral immune responses which may limit the development of robust anti-tumor immunity (36). Therefore, we hypothesize that a less active anti-adenoviral response in responder patients allows them to develop a better antitumor response. Moreover, cellular vehicles can serve to evade antiviral mechanisms encountered in the bloodstream, prevent uptake by off-target tissues and favor their homing to tumor beds where they can produce thousands of oncolytic viruses. After adenoviral secretion and tumor infection, an immune response would be activated involving the activation of immunostimulatory genes, including those implicated in chemotaxis, inflammation, T-cell regulation and antigen presentation. This infiltrationf immune cells might favorably change the immunosuppressive status of the tumor microenvironment and increase cell infiltration. In the present study, we also observed that the number of $CD3^+$ and $MAC387^+$ cells in the patients' tumor biopsies tended to increase with the treatment.

ICOCAV17 expresses hyaluronidase, an enzyme that hydrolyzes hyaluronan, a primary macromolecular component of the ECM of solid malignancies. This enzyme has antitumoral properties as a single-agent. Hyaluronidase inhibited tumor growth in xenograft models, and it has been used in early clinical trials (37). However, a recent phase II clinical trial (NCT01839487) testing hyaluronidase in combination with chemotherapy was transiently halted because of associated toxicity. In our approach, dCelyvir should restrict hyaluronidase

expression to tumor sites and, consequently, limit systemic side effects. It is probably responsible for the increased ECM disaggregation we observed in post-treatment tumor biopsies, thereby enhancing virus spread and immune cells infiltration throughout the tumor. However, our data on this respect are not conclusive.

In summary, dCelyvir has shown clinical efficacy with very low toxicity. dCelyvir has also shown the capacity to transform the tumor microenvironment towards more susceptibility to immune activation. This could benefit it as a candidate for combination therapy with immune checkpoint inhibitors.

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Table 1 Patients´ characteristics and treatment outcome

Patients	Tumor type	Stage	P/R/Met	Diameter (cm)	Race	Gender	Age (years)	CAV vaccine	Previous lines of therapy	Therapies combination	Celyvir doses	Clinical Benefit
UAX-01	Mastocytoma (low and high grade)	3A	R	5	Boxer	M	6	Y	CT	dCELYVIR	22	CR
UAX-02	Rhabdomyosarcoma	2	R	2.3	Golden retriever	M	5	Y	Mn	dCELYVIR	11	PR
UAX-03	Hemangiopericytoma	3	R	12	Siberian husky	F	15	N	Mn	dCELYVIR	3	PR
UAX-04	Osteosarcoma (osteoblastic)	ND	P	ND	Mixed	F	10	ND	CT	CT+ dCELYVIR	6	SD
UAX-05	Osteosarcoma (chondroblastic)	4	P/Met	3.6	Labrador retriever	F	10	Y	CT	dCELYVIR	3	PD
UAX-06	Hemangiopericytoma	2	R	3	German shepard	F	7	Y	-	dCELYVIR	4	PR
UAX-07	Nephroblastoma	ND	R	8.3	Labrador retriever	M	5	N	-	dCELYVIR	7	SD
UAX-08	Osteosarcoma (chondroblastic)	4	P/Met	3	Mastiff	F	12	Y	CT	CT+ dCELYVIR	3	PD
UAX-09	Osteosarcoma (chondroblastic)	3	P	7.8	Leonberguer	F	5	Y	-	dCELYVIR	7	PD
UAX-10	Schwannoma	3	P	5.4	Golden retriever	M	11	Y	Mn	Mn+ dCELYVIR	7	PD
UAX-11	Osteosarcoma (chondroblastic)	2	P	1.3	Border Collie	F	2	N	-	dCELYVIR	4	PD
UAX-12	Hemangiopericytoma	1	P	1	Mixed	F	16	N	Mn	dCELYVIR	14	SD
UAX-13	Fibrosarcoma	1	P	7.5	Bouceron	M	10	Y	-	TKI + dCELYVIR	3	SD
UAX-14	Osteosarcoma (chondroblastic)	3	P	31	Mixed	F	8	Y	CT+TKI	TKI + dCELYVIR	6	SD
UAX-15	Mastocytoma (high grade)	ND	R	8.5	American Standford	M	7	Y	TKI	TKI + dCELYVIR	3	PD
UAX-16	Melanoma	2	R	3	Teckel	M	13	Y	Mn+TKI	TKI + dCELYVIR	28	SD
UAX-17	Rhabdomyosarcoma	3	P	11	Labrador retriever	F	7	Y	-	dCELYVIR	13	SD
UAX-18	Fibrosarcoma	3	P	14	Mixed	M	10	Y	-	dCELYVIR	4	SD
UAX-19	Oligodendroglioma	ND	P	1	French bulldog	M	11	Y	Mn+TKI	TKI + dCELYVIR	13	SD
UAX-20	Oligodendroglioma	ND	P	1	Mixed	F	9	Y	TKI	TKI + dCELYVIR	30	CR
UAX-21	Pituitary adenoma	ND	P	3	Rottweiller	F	1	Y	-	dCELYVIR	15	SD
UAX-22	Oligodendroglioma	ND	P	1.3	French bulldog	F	8	Y	TKI	TKI + dCELYVIR	27	SD
UAX-23	Schwannoma	4	Met	7	Mixed	F	15	Y	-	dCELYVIR	10	CR
UAX-24	Hemangiosarcoma	4	Met	5.4	Greyhound	F	8	Y	CT	CT+ dCELYVIR	23	CR
UAX-25	Undifferentiated pleomorphic sarcoma	3	P	3.2	French bulldog	M	5	Y	CT	dCELYVIR	11	SD
UAX-26	Hemangiosarcoma	4	Met	2.1	Greyhound	M	10	Y	CT+Mn	dCELYVIR	5	PD
UAX-27	Undifferentiated pleomorphic sarcoma	2	P	5.1	Bullmastiff	M	7	ND	CT+Mn	dCELYVIR	4	SD

Table 2. Adverse events.

Adverse events	Grade	
	1 and 2	3 and 4
General		
Fever	0	0
Chills	0	0
Fatigue	0	0
Myalgia	0	0
Asthenia	0	0
Sweating	0	0
Dehydration	0	0
Pain	0	0
Infection	0	0
Cachexia	0	0
Dermatological		
Rash	1	0
Skin ulceration	0	0
Alopecia	1	0
Cardiovascular		
Hypotension	0	0
Hypertension	0	0
Hemorrhage	0	0
Gastrointestinal		
Anorexia	0	0
Dyspepsia	0	0
Nausea	0	0
Diarrhea	1	0
Vomiting	0	0
Respiratory system		
Dyspnea	0	0
Pneumonia	0	0
Apnea	0	0
Hepatic		
Liver dysfunction	0	0
Renal		
Renal dysfunction	0	0
Metabolic		
ALT increased	10	1
AST increased	1	0
Hypophosphatemia	8	0
Fibrinogen increased	0	0
Hematological		
Leucocytes increased	0	0
Platelets decreased	0	0
Neutrophils decreased	2	0
Other		
Orchitis	1	0

FIGURE LEGENDS

Figure 1. Canine mesenchymal stem cells infected by canine adenoviral ICOCAV17, showing tumoral homing and anti-tumor efficacy in immunodeficient mice. (a) Adenovirus detection (green) in ICOCAV17-infected dMSC by immunocytochemistry. Cultures were counterstained with DAPI (blue). Representative images from three different assays performed are shown. Scale bar, 50µm. **(b)** Western blot of adenoviral protein in dMSC at 24h after ICOCAV17 infection. β-actin was measured as a loading control. **(c)** Viral load was determined by qPCR in dMSC after ICOCAV17 infection (1=MOI 1; 10=MOI 10) and in mock-infected cells (M). The graph shows fold change (mean + SD) of two independent assays. **(d)** Cell migration assays of dMSCs or dCelyvir were performed using murine tumoral CMT64-6 cell line or canine primary tumor cultures. dMSCs or dCelyvir were transferred to the upper chambers and incubated in the presence of cell culture medium (negative control), medium including FBS (positive control), CMT64-6, or primary tumor cultures in the bottom chamber for 24 hours. Migrated cells were fixed, stained with violet crystal and counted. Bars represent the mean of triplicates + SD from three independent assays. All of them migrated significantly compared to the negative control ($p<0.005$). **(e)** Primary canine tumor cells were implanted in the flanks of NOD-SCID mice. After 15 days, DiR-stained dMSCs or Dir-stained Celyvir were i.p. infused and analyzed after 24h using an *in vivo* imaging system. As negative control for fluorescence we used PBS-infused mice. Representative *ex vivo* fluorescence images of tumors are shown. **(f)** Treatment schedule used in murine models. **(g)** Tumor volumes in treated immunodeficient NOD-SCID mice are represented as mean + SEM.

Figure 2. Clinical and anatomo-pathological results in canine patients treated with dCelyvir. (a) Summary of clinical benefit following veterinary Response Evaluation in Solid Tumors (RECIST) V1.1. **(b)** X-ray images of a canine patient during dCelyvir treatment showing pulmonary metastasis evolution. Arrows show different metastatic nodules. Upper images correspond to ventrodorsal views and lower images are lateral right views of the same dog on the same date (PRE: pretreatment; 15w and 40w: 15 and 40 weeks post-treatment). **(c)**

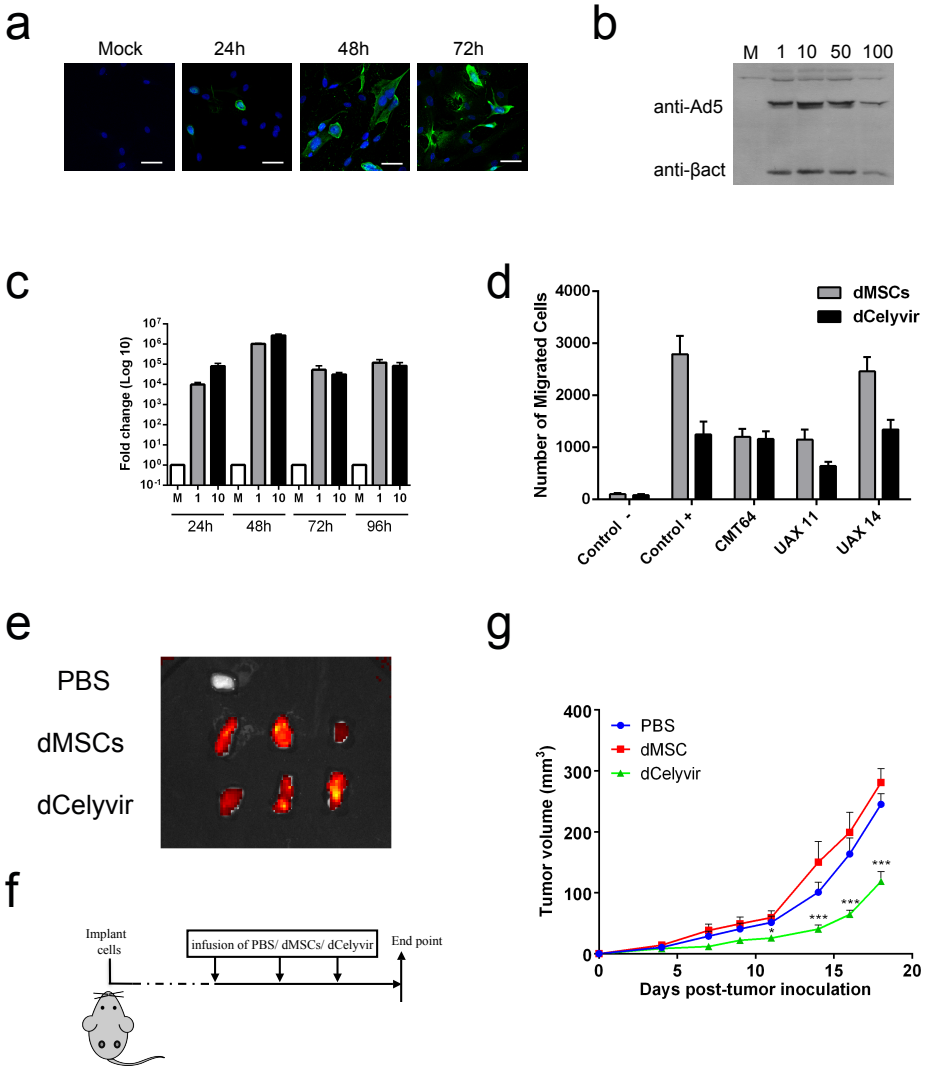
Representative hematoxylin and eosin (HE) staining and Immunohistochemistry images from biopsies taken from tumors prior to treatment (PRE) and after the fourth dCelyvir dose (POST). Images show a representative case (UAX-06) from 20 cases analyzed. Asterisk, region with ECM degeneration; arrow, hemorrhagic area. Scale bar: 200 µm in HE, 100 µm in immunohistochemistry. **(d)** Graphs show semiquantitative evaluation by anatomical pathologists of structural tumor biopsies. Mean (bars) and individual values (dots) are shown. Arbitrary units were assigned by anatomical pathologists: 0, absence; 1, presence; 2, medium; and 3, abundant. Paired T-test was performed (n=20) (* $p<0.05$, **$p<0.005$). **(e)** Quantification of immune cells by immunohistochemistry is shown. Mean (bars) and individual values (dots) are shown.

Figure 3. Peripheral blood immune response and adenovirus detection. (a) Total number of peripheral blood leukocytes in canine patients previous to treatment and during the first doses. Paired T test *$p<0.05$ (n=15). **(b)** Cytokines in peripheral blood of canine patients are represented (n=10). **(c)** Representative images showing adenovirus-positive cells on FFPE biopsies (brown) and cryosections (green), including DAPI (blue) counterstaining. Scale bars: 50 µm. **(d)** Anti-adenoviral immunoglobulins present in serum of canine patients during dCelyvir treatment. The graph shows mean ± SEM. All levels of anti-adenoviral immunoglobulins were statistically higher compared to pre-treatment values. Wilcoxon test was performed (n=5-10; $p<0.005$).

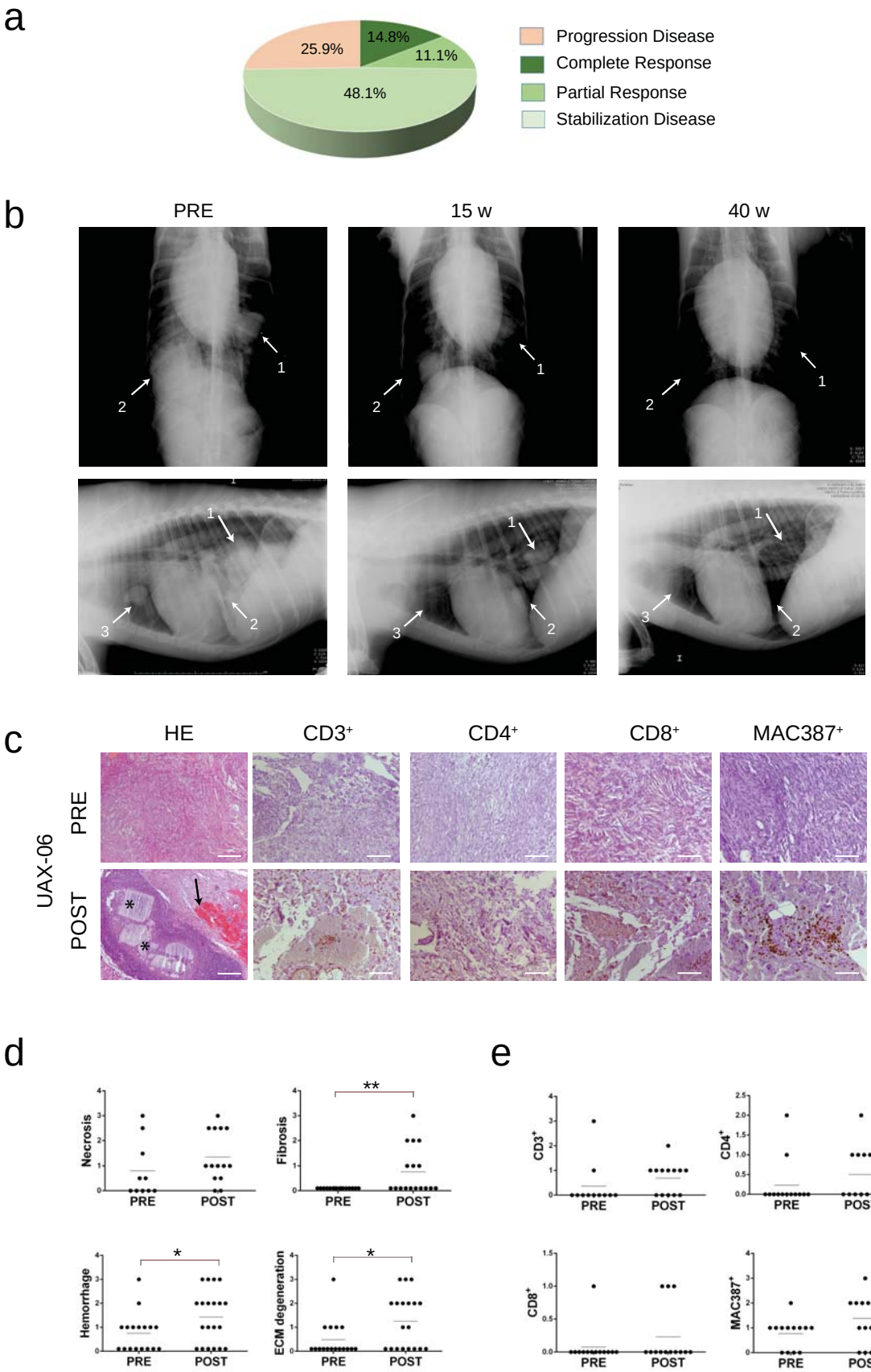
Table 1. Patient characteristics and treatment outcome. Stage: ND, not determined; P/R/Met, primary tumor, recurrent tumor, metastasis. The patients that presented only metastasis were cases in which primary tumors had been removed by surgery. CAV vaccine: Y, vaccinated; N, non-vaccinated, ND, not determined; Previous lines of therapy: CT, conventional chemotherapy (doxorubicin); Mn, metronomic chemotherapy (ciclophosphamide); NT, no treatment; TKI, tyrosin kinase inhibitors. RECIST= Response Evaluation Criteria in Solid Tumors: CR, complete Response; PR, partial response; SD, stable disease; PD, progressive disease.

Table 2. Adverse events in dCelyvir-treated patients. Seven of the dogs who suffered ALT increases were being treated with corticoids and 3 of them showed hepatic illness prior to treatment. Neutropenia was documented in two cases that were receiving concomitant treatment with doxorubicin. Events and grades have been classified following the VCOG-CTCAE v1.1.

Cejalvo et al Figure 1

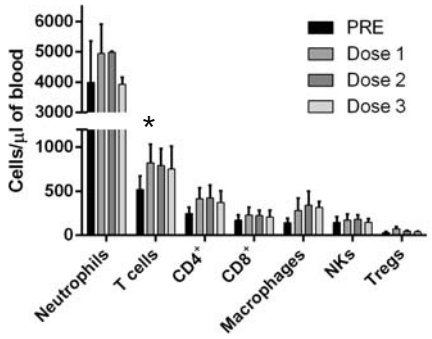


Cejalvo et al Figure 2



Cejalvo et al Figure 3

a



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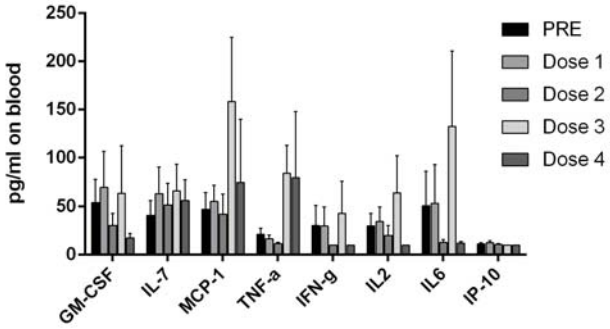

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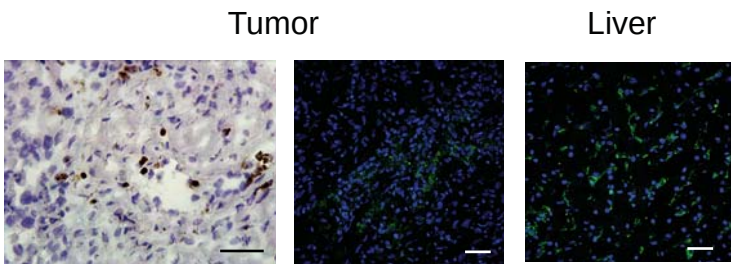

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