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Article

Pilot Study of Intratumoral Immunotherapy with Cowpea Mosaic Virus Nanoparticles: Safety in Refractory Canine Oral Tumors

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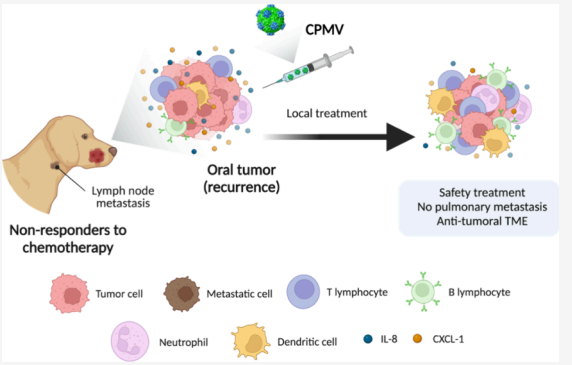
ABSTRACT: Oral tumors (squamous cell carcinoma, malignant melanoma, and fibrosarcoma) represent 6−7% of all canine cancers. Given that these tumors have a high local recurrence rate and metastatic potential, conventional therapies have suboptimal response rates, leading to poor patient outcomes. Here, we report the use of intratumoral virus-like particles from cowpea mosaic virus (CPMV) in four canine patients with recurrent oral malignant tumors and lymph node metastasis. All tumors were nonresponders to chemotherapy and had a mild initial response to CPMV intratumoral immunotherapy without any serious immune-related adverse effects. None of the patients developed pulmonary metastasis during follow-up, although local progression was seen in all the patients. Furthermore, tumor-infiltrated immune T cells increased in number after the intratumoral immunotherapy with CPMV, suggesting activation of the tumor microenvironment. All the patients had a rapid decrease in the tumor-promoting chemokines IL-8 and CXCL1, which could indicate that a decrease in metastatic potential could have been generated by the CPMV immunotherapy. The increased number of infiltrated immune cells, the decrease in some pro-tumoral chemokines, and the absence of adverse effects suggest that CPMV could be a safe treatment and should be further explored as a novel therapy for canine oral tumors.



KEYWORDS: *CPMV, intratumoral immunotherapy, oral canine tumors, virus-like particles, nanoparticles, malignant oral melanoma*

1. INTRODUCTION

Oral malignant tumors represent 6−7% of all canine cancers, with oral malignant melanoma (OMM), nontonsillar squamous cell carcinoma, and oral fibrosarcoma being the most frequent primary tumors.[1,2] If there are no distant metastatic lesions, treatment options for these tumors are based on local control with aggressive surgery, radiotherapy, or electrochemotherapy.[3−6] Although complete excision of oral fibrosarcoma has been associated with improved median survival time, there is a high risk (38−54%) of local recurrence, and 13%−21% of cases will develop metastases, negatively affecting overall survival time.[3,5,7−9] Canine OMM is also a highly invasive neoplastic lesion with a high rate of local recurrence (28−48%) and distant metastasis (26−41%) after complete surgical excision.[4,7−10] These findings suggest that the efficacy of local treatment against these tumor types is limited, and a high percentage of dogs die due to tumor progression. Unfortunately, the efficacy of systemic chemotherapy in recurrent or metastatic fibrosarcoma and OMM is poor, indicating the need of new therapies capable of producing a better therapeutic benefit in canine patients with oral malignant neoplasia.[10−13]

The role of immune control in tumor progression has been well established, and several murine and human immunotherapy trials have shown promising tumor responses against mucosal melanoma and soft tissue sarcoma.[14−16] The use of the immunotherapy Oncept, a bacterial plasmid DNA vaccine encoding the tumor-targeted antigen human tyrosinase, is widely accepted as an adjuvant treatment after surgical excision for canine OMM.[17−19] Despite having shown excellent tolerability, its efficacy has been questioned in some reports, increasing the interest in finding new immunotherapies against OMM.[20] The recent promising results of novel therapies in veterinary medicine with systemic (dCelyvir) or intratumoral

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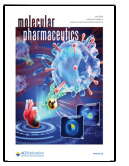

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oncolytic virus, immune checkpoint inhibitors, and nanoparticles support the growing interest in novel immunotherapies.[21−27]

The immune stimulation of the tumor microenvironment after the intratumoral immunotherapy of the cowpea mosaic virus (CPMV) has been extensively demonstrated in murine models[28−30] (Table 1). The intratumoral administration CPMV in murine models has shown excellent clinical efficacy including responses in metastatic pulmonary melanomas, which could potentially improve the overall survival of canine OMM.[31] Adjuvant treatment with CPMV intratumoral immunotherapy has demonstrated excellent tolerability in three canine clinical trials against OMM, mammary carcinoma, and inflammatory mammary carcinoma, and all patients had a measurable response to the treatment[25,27,32,33] (Table 1). In one previous trial, five canine patients with OMM were enrolled; only one patient developed metastatic disease 26 months post-treatment, suggesting that the use of this therapy could prevent the development of metastasis in dogs with OMM[25] (Table 1). However, CPMV has never been used in a canine study as a treatment for refractory oral tumors or with pre-existing metastatic spread to evaluate the possible systemic effect of intratumoral administration of CPMV. In this proof-of-principle study, we evaluated the safety and efficacy of CPMV intratumoral immunotherapy as a third line of treatment for refractory oral tumors with lymph node metastasis.

2. EXPERIMENTAL SECTION

2.1. Study Design. Four dogs with stage III recurrent aggressive oral tumors were enrolled for compassionate CPMV intratumoral immunotherapy at the Alfonso X el Sabio University-Veterinary Teaching Hospital, Madrid, Spain (Figure 1). Empty CPMV to treat dogs were prepared as previously described.[31] The inclusion criteria were owner's rejection to the surgical procedure, inoperable tumors, recurrence of disease after the administration of chemotherapy or surgery, advanced-stage disease with lymph node enlargement, and a docile character to allow follow-up without sedation. Given that the CPMV intratumoral immunotherapy was approved as compassionate treatment for advanced-stage disease, no control group with healthy animals was included in the study. This study was approved by the ethics committee of Alfonso X el Sabio University (2022_03/133). The diagnosis and staging procedure included a head, neck, and chest computed tomography (CT) with iodized contrast (Iohexol, Omnipaque at 2 mL/kg), abdominal ultrasound and a tissue biopsy for histopathological analysis. For the CPMV intratumoral immunotherapy and the tissue biopsies, the patients were sedated with acepromazine (0.03 mg/kg) and methadone (0.1 mg/kg). If the anesthesiologist required an induction of the patient for safety, thiopental (dose effect) and isoflurane (inhalant agent) were used. The veterinary oncologist determined three to five quadrants of the visible tumor to evenly distribute the CPMV diluted in saline solution (100 μL; 100−400 μg). The CPMV intratumoral immunotherapy was repeated every 7 days for 5−6 weeks, depending on the patient's response (Figure 1). Tissue samples were obtained before each CPMV injection for later analysis. All the dogs included in this study had a systemic chemotherapy protocol established by the veterinary oncologist, depending on the tumor type and previous chemotherapy protocols administered. The owner of dog CPMV#2 decided to pursue a

Table 1. Summary of Key References in the Introduction

study	animal model	number of cases	key finding	limitations
Cowpea Mosaic Virus Nanoparticles and Empty Virus-Like Particles Show Distinct but Overlapping Immunostimulatory Properties	murine	5 mice per group	both CPMV and eCPMV significantly reduced tumor burden	small sample size. Durability of the response was not assessed
Virus-like Particle in Situ Vaccine for Intracranial Glioma Immunotherapy	murine	4 mice per group	treated mice experienced a recruitment of innate and adaptive immune cells	small sample size. Only performed in murine models
Cowpea Mosaic Virus Stimulates Antitumor Immunity through Recognition by Multiple MYD88-Dependent Toll-like Receptors	murine	5 mice per group	CPMV induced pro-inflammatory cytokines.	small sample size. Only performed in murine models
Neoadjuvant in Situ Vaccination with Cowpea Mosaic Virus as a Novel Therapy against Canine Inflammatory Mammary Cancer	canine	5 per group	significant improvement of survival time in patients with inflammatory mammary carcinoma.	small sample size, no randomization was performed
Neoadjuvant Intratumoral Immunotherapy with Cowpea Mosaic Virus Induces Local and Systemic Antitumor Efficacy in Canine Mammary Cancer Patients	canine	11 dogs	tumor reduction was seen in primary tumor and in contralateral nodules	small sample size, no long-term efficacy was described
In Situ Vaccination with Cowpea Mosaic Virus Nanoparticles Suppresses Metastatic Cancer	murine	4−10 mice per group	CPMV demonstrated efficacy against lung melanoma, breast cancer, colon cancer and ovarian carcinoma	limited sample sizes and specific cancer models
Treatment of Canine Oral Melanoma with Nanotechnology-Based Immunotherapy and Radiation	canine	5 dogs	combination of CPMV and radiotherapy appears to have good response rates in oral melanoma	limited sample size. No control group with only radiotherapy
In Situ Vaccine Application of Inactivated CPMV Nanoparticles for Cancer Immunotherapy	murine	10 mice per group	treated mice experienced significant tumor regression. CPMV appear to reprogram the tumor microenvironment.	the finding was only performed in murine models

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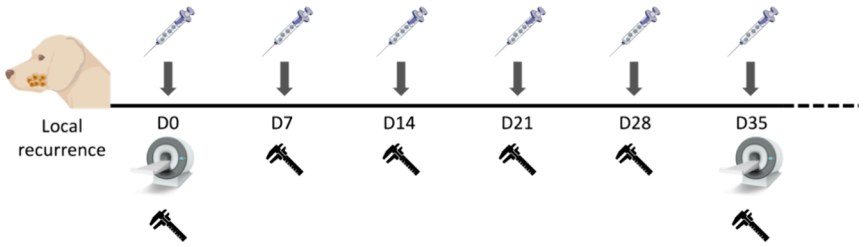

Figure 1. CPMV intratumoral immunotherapy schedule, indicating days (D) of treatment, caliper measurements, and imaging by computed tomography.

hypofractionated radiotherapy protocol on day 64 after progressive disease was shown, with a total dose of 32 Gy (1 fraction of 8 Gy weekly for 4 weeks).

2.2. Safety and Efficacy. To evaluate adverse effects, before each CPMV dose, blood samples were collected in heparin and ethylenediaminetetraacetic acid (EDTA) tubes and processed to obtain plasma and peripheral blood mononuclear cells (PBMCs) for flow cytometry analysis. To ensure the safety of chemotherapy, the collected blood samples were also used for standard hematology and biochemistry assays. The following parameters were evaluated: hematocrit, hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin concentration, mean corpuscular hemoglobin, red blood cell distribution width, reticulocytes, neutrophils, lymphocytes, monocytes, eosinophils, basophils, platelets, mean platelet volume, platelet distribution width, ions (magnesium, sodium, potassium, chloride, calcium, phosphate), renal parameters (urea, blood urea nitrogen, creatinine), hepatic transaminases (alkaline phosphatase, aspartate aminotransferase, alanine transaminase), total proteins (albumin, globulin, albumin/globulin ratio), coagulation times (prothrombin time, activated partial thromboplastin time, fibrinogen), and baseline glucose. At each follow-up visit (time depending on the treatments followed), the patient was evaluated by the oncology specialist to ensure the patient's well-being and detect any possible symptoms due to the treatment or to disease progression. The adverse effects observed were classified following the criteria of the Veterinary Cooperative Oncology Group.[34]

To evaluate the efficacy of CPMV intratumoral immunotherapy, tumor measurements were made at every visit by the members of the oncology team, and a mean was reported. To evaluate the invasion and bone or lymph node involvement, head CT scans were performed on day 0 and day 35. To evaluate the presence of lung metastases, CT scans of the chest or radiography scans in three different views were performed. The metastatic spread to the local lymph nodes was assessed based on lymph node enlargement. Tumor and lymph node measurements were analyzed using Response Evaluation Criteria in Solid Tumors (RECIST). Complete response (CR): disappearance of all target lesions, pathologic lymph nodes <10 mm on the short axis; partial response (PR): at least 30% reduction in the sum of the target lesion diameters, taking as a reference the baseline sum; progressive disease (PD): either the appearance of new lesions or a >20% increase in the sum of the target lesion diameters, taking as a reference the smallest sum in the timeline between doses (the sum must also show an absolute increase of 5 mm); stable disease (SD): less than 30% reduction or 20% increase in the sum of the target lesion diameters, taking as a reference the smallest sum of the diameters during the study.

2.3. Immunohistochemistry. The tumor biopsies were fixed in 10% formalin-buffered solution and embedded in paraffin. Sections of 5 μm were cut with a microtome, dewaxed, and rehydrated using an alcohol battery (xylol 2 × 5 min, ethanol 100% 2 × 5 min, ethanol 96% 1 × 5 min, ethanol 70% 1 × 5 min) for hematoxylin−eosin staining or immunostaining. To perform the immunostaining, a previous antigen retrieval with hot citrate buffer was performed for 3 min. Two washes with H_2O_2 (6%) of 10 min each was followed by a phosphate-buffered saline (PBS) wash with 0.1% Triton, to inhibit the endogenous peroxidase. The immunostaining was performed with the Vector Laboratories VECTASTAIN R.T.U. Kit (Newark, California, USA), using the kit's normal horse serum for blocking. The sample was incubated overnight at 4 °C with the primary antibody anti-CD3 (UCHT1 3 mg/mL; Dako, Santa Clara, California, USA); anti-S100A9 + calprotectin (MAC387 1 μg/mL; Abcam, Cambridge, UK); polyclonal anti-CD20 (0.17 μg/mL; Invitrogen, Waltham, Massachusetts, USA); monoclonal ki67 (sc-101861; Santa Cruz Biotechnology, Heidelberg, Alemania), and PBS + Triton 0.1% + bovine serum albumin 0.2%. After a 5 min wash with PBS and Triton 0.1%, the samples were incubated for 30 min in a wet chamber with biotinylated antirabbit/mouse secondary antibody (VECTOR, R.T.U. VECTASTAIN Kit). Samples were then incubated for 30 min with the kit's ABC reagent, and the DAB Peroxidase Substrate Kit was employed to detect the staining. Counterstaining with hematoxylin (Harris Hematoxylin solution, PanReac AppliChem, Milan, Italy) and dehydration with alcohol (ethanol 50, 70, 96, 100%, and xylol) were performed before mounting the preparations with dibutyl phthalate polystyrene xylene medium. Five representative images from each sample were taken, and the positive cells in the tissue were quantified with ImageJ software. Evaluation of Ki67 immunolabeling was performed following a previously described method: five 40× fields within the areas of heaviest Ki67 labeling were manually assessed, and the Ki67 index was calculated as the mean number of labeled neoplastic cells in the five fields.

2.4. RNA-Seq Library Preparation and Sequencing. Snap-frozen tumor samples were homogenized by bead beating with 2 mm stainless steel beads in Qiagen RLT buffer and RNA extracted using RNeasy Mini kits (Qiagen, Redwood City, California). RNA was quantified by qubit and quality assessed on a Fragment Analyzer instrument (Agilent, Santa Clara, California). All RNA samples used in this study had RNA integrity number score >7. 3′-end RNa-seq libraries were produced from 200 ng of RNA using the QuantSeq Rev chemistry (Lexogen, Greenland, New Hampshire) and pooled for sequencing on an Illumina NextSeq 2000, 1 × 100 bp run, targeting a minimum of 10 M reads per sample.

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2.5. RNA-Seq Analysis. FastQC was used for quality control of raw sequences.[35] Trimming of adapter sequences and poly-A tails was performed with cutadapt as previously described.[33] Trimmed reads were aligned to the *Canis lupus familiariz* genome (RefSeq accession: GCF_000002285.5) using STAR.[36] Raw counts were generated for each gene using the FeatureCounts.[37] Gene expression levels were normalized using transcripts per million (TPM). Immune cell expression was inferred using the average expression of immune cell marker genes from the safeTME R package, normalized by the average expression of six housekeeping genes (ENSCAFG00000018728, ENSCAFG00000006063, ENSCAFG00000005610, ENSCAFG00000015595, ENSCAFG00000008453, and ENSCAFG00000016923). Similarly, the activity of the C5 immunologic gene signature set, obtained from the human MSigDB Collection (v2022.1.Hs), was inferred using the average expression of each signature, normalized by the average expression of the same six housekeeping genes.

2.6. Peripheral Blood Analysis. Blood samples at D0, D7, D14, D21, D28, D34, and D42 were collected in heparin tubes and used to obtain plasma. Plasma was frozen at −80 °C until processed. The MILLIPLEX Canine 13 Cytokine/Chemokine Magnetic Bead Panel was used to measure cytokines in plasma samples as indicated by the manufacturer (Merck Millipore, Burlington, Massachusetts, USA): granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-γ, C-X-C Motif Chemokine Ligand 1 (CXCL1), C-X-C Motif Chemokine Ligand 10 (CXCL10), interleukin (IL)-2, IL-6, IL-7, IL-8, IL-10, IL-15, IL-18, MCP-1 (CCL2), and tumor necrosis factor (TNF)-α.

Blood samples collected on D0, D14, D21, D34, D41, and D46 were used to isolate PBMCs using Ficoll solution 1.077 g/mL (Rafer, Zaragoza, Spain). Isolated PBMCs were transferred to a freezing medium (10% dimethyl sulfoxide in fetal bovine serum), frozen at −80 °C overnight, and transferred into liquid nitrogen according to standard procedures. While samples were shipped on dry ice and with appropriate care, a potential detrimental effect on sample quality cannot be entirely ruled out. After thawing, flow cytometric analysis was performed with a 14-color panel using reagents and procedures as previously described.[38] Stained samples were acquired with an LSR Fortessa II (BD Biosciences, San Jose, California, USA) equipped with 4 lasers and 16 detectors. Flow cytometry analysis was performed with FlowJo software (version 10.7.1; BD Bioscience). The gating strategy to determine cellular populations was previously described.[38] The percentages indicated for monocytes refer to live single cells. The percentage of neutrophils (CD45+, CD14−, CD5−, CD22−, MHCII−, CD4+) refer to the CD45+, CD14−, CD5−, CD22− live single cells. The percentages of B (CD45+, CD14−, CD22+, CD5−) and T lymphocytes (CD45+, CD14−, CD5+) refer to CD45+ CD14− live single leukocytes. The percentages of CD8+ and CD4+ refer to T lymphocytes. The percentage of activated CD8+ T lymphocytes refers to CD8+ cells. The percentage of Tregs refers to CD4+ cells.

3. RESULTS

3.1. Canine Patients and Safety. The study included four oncologic canine patients diagnosed with advanced OMM (n = 3) or oral fibrosarcoma (n = 1). The dog breeds were mixed (n = 2), Labrador Retriever (n = 1), and Shar Pei (n = 1), with an age between 9 and 13 years, including one spayed female and three neutered males (Table 2). All patients included in the study had local lymphadenopathy as assessed by CT, considered to be metastatic at the time of the first CPMV administration. Two patients had previous surgery: patient 2 had surgery at the referral center that recurred 3 months after, when it was included in the study; patient 4 had a debulking surgery at the referral center 1 week before the CPMV intratumoral immunotherapy (Table 2). All the enrolled dogs were receiving a chemotherapy protocol proposed by the oncology team. Patient 1 started with cyclophosphamide (12 mg/m^2) every 24 h; however, progressive disease was reported 20 days after starting chemotherapy and the dog was enrolled in the study while the chemotherapy protocol was continued. Patient 2 started toceranib phosphate (Palladia) therapy (2.5 mg/kg) 3 days per week, 1 week after the surgery; recurrence of the primary lesion was noted 3 months after, and the dog was enrolled in the study while the chemotherapy protocol was continued. After a new progression of the disease, the patient began a combined intravenous protocol of carboplatin (200 mg/m^2) every 21 days, and oral toceranib phosphate 2.5 mg/kg 3 days per week, at the indication of the veterinary oncologist. Due to treatment failure, patient 2 underwent radiotherapy (8 Gy in four doses, total dose 32 Gy), maintaining the previous multidrug protocol. The two remaining patients followed a protocol of single-agent IV carboplatin 250 mg/m^2 every 21 days. Patient 3 was anorexic due to progressive disease and eCPMV was administered as a last-resort treatment. Although slight improvement in appetite was observed, the owners did not feel the quality of life was sufficient and decided to discontinue all treatments, pursuing euthanasia on day 5. Patient 4's owners did not allow any further blood analyses or incisional biopsies during the study. On day 78, they decided to discontinue participation, making further follow-up impossible.

During the follow-up, no abnormalities were detected in the physical examination by the veterinary oncologist. The hematology and biochemistry assays performed at each visit revealed no significant changes pre- and post-CPMV intratumoral immunotherapy (Supplementary Figure 1 and Figure 2). Patient 2 had a significant elevation in creatinine and urea values at day 96, 2 weeks after finishing the radiotherapy course while in treatment with carboplatin and toceranib phosphate (Figure 2). This patient had cardiovascular failure during hospitalization due to acute kidney failure (acute kidney injury grade V).

3.2. Outcome Assessment. Patients 1 and 2 had a partial response after the intratumoral CPMV administration, followed by progressive disease (Figure 3A). Patient 4 had excisional surgery, and the CPMV were then injected into the surgical scar. This dog remained in partial response during the first 2 weeks, until a rapid progression was observed on day 21. Patient 3 was euthanized at the owners' request 5 days post-CPMV intratumoral immunotherapy; at this point, the dog had no adverse reactions. The regional lymph nodes of patients 1 and 2 were measured by CT 36 days after the first dose, and no enlargement was detected (Figure 3B). The overall survival time of the three dogs was 23, 120, and 215 days (Table 1 and Figure 3C), and the specific survival time after CPMV intratumoral immunotherapy was 5, 78, and 96 days (P3, P1, and P2). P1 and P3 were euthanized due to progression of the disease, and P2 had a cardiorespiratory arrest. Owners of patient 4 changed their place of residence after the diagnoses

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Table 2. Canine Patients Enrolled in the Study[a]

patient	age (years)	breed	sex	tumor	surgery	systemic chemotherapy	mitotic count	Ki67 index	doses of CPMV intratumoral immunotherapy	size of the lesion prior to immunotherapy (cm)	lymph node involvement	survival after first intratumoral immunotherapy (days)	cause of death
patient 1	10	mixed	male	high/low fibrosarcoma	yes	yes	>4	0	5	12.56	yes	57	tumor related
patient 2	10	mixed	female	malignant oral melanoma	yes	yes	>4	24	6	20.88	yes	96	renal failure
patient 3	9	Labrador Retriever	male	malignant oral melanoma	no	yes	>4	57	1	2.04	yes	5	tumor related
patient 4	13	Shar Pei	male	malignant oral melanoma	yes	yes	>4	43	4	microscopic	yes	>78	lost to follow-up

[a]Survival time calculated from the first administration of CPMV intratumoral immunotherapy.

due to personal reasons, and we lost the follow-up of this patient 78 days after the first CPMV dose (Figure 3A).

3.3. Immune Response Evaluation. The immune cells infiltrating the tumor tissue were evaluated by immunohistochemistry in the tissue biopsies obtained at D0, D14, and D34 and during the necropsy (Figure 4). Patient 4 was not analyzed because the owners did not authorize us to obtain tumor biopsies and left the study before the necropsy.

Patients 1 and 2 had a longer follow-up period, and both showed an increase of tumor-infiltrating lymphocytes (TILs), including both T (CD3+) and B (CD20+) lymphocytes during the first 40 days (Figure 4A,B). A decrease in the infiltration of CD3+ and CD20+ cells in patient 2 was detected in postmortem tumor tissues (Figure 4B). After the first CPMV intratumoral administration, a rapid decrease of tumor-associated macrophages (TAMs) (MAC387+ cells) was observed in patient 1 and a slight increase in patients 2 and 3 (Figure 4B). Subsequently, disease progression was reported in patients 1 and 2, the percentage of macrophages/monocytes increasing in the tumor tissues of both patients (Figure 4B).

The systemic immune response was analyzed by flow cytometry. Regarding monocytes, patients 2 and 4 showed an increase over the first 30 days after the first dose; however, dog number 2 had a decrease over the following 10 days (Figure 5). Patient 1 had a decrease of monocytes during the first 28 days, with a peak on day 30 and a new drop on day 34. Concerning neutrophils, patient 1 maintained its levels during the first 21 days, followed by a progressive decrease. However, the neutrophil levels of numbers 2 and 4 showed a progressive increase up to day 21, followed by a reduction (Figure 5). The population of B lymphocytes in the patients with OMM showed a progressive decrease during follow-up; however, the patient with an oral fibrosarcoma (patient 1) showed a continuous increase, with a drop at day 34 (Figure 5). Circulating blood T lymphocytes were evaluated, and populations of CD8+, Eomes+ CD8+, CD4+, and T regulatory cells were analyzed. The CD8+ lymphocytes had slight variations during the first 21 days after the first intratumoral administration in patients 1 and 4, with a maintained drop at day 28, whereas patient 2 had two peaks, on days 7 and 35, followed by a continued decrease (Figure 5). The percentage of circulating activated CD8+ lymphocytes (CD8+ Eomes+) increased on day 7 and then continued to decrease throughout the follow-up. The last sample obtained from patient 2 showed an increase of CD8+ activated lymphocytes to the basal levels observed on day 0. Regarding CD4+ lymphocytes, although there was a generalized decrease during follow-up in number 2, there was an increase of circulating regulatory T cells on day 27 in this patient (Figure 5).

The cytokines and chemokines GM-CSF, IFN-γ, CXCL1, CXCL10, IL-2, IL-6, IL-7, IL-8, IL-10, IL-15, IL- 18, CCL2, and TNF-α were quantified in peripheral blood samples during follow-up (Supplementary Figure 2). After receiving the first intratumoral immunotherapy injection, there was a decrease in the chemokines IL-8 and CXCL1 in patients 2 and 3 that was maintained at low levels throughout the follow-up (Figure 6). Patient 1 experienced a similar reduction of both chemokines after a second intratumoral administration CPMV dose. Although a pretreatment sample for patient 4 was not available, serum analyzed at day 7 showed low levels of CXCL1 and IL-8, which were maintained during the study (Figure 6). No significant trends were observed in the

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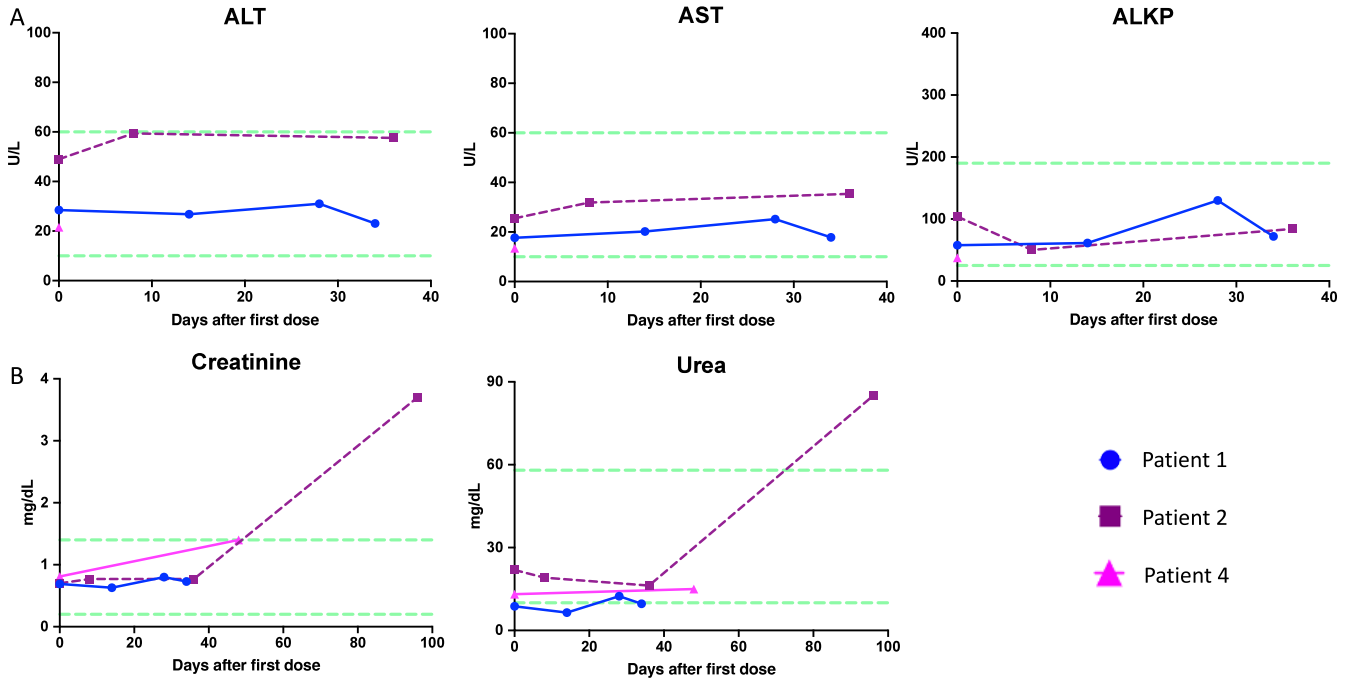

Figure 2. Biochemical analysis of blood samples to assess liver (A) and kidney (B) function. (A) Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALKP) quantification before treatment (day 0) and during follow-up. (B) Creatinine and urea quantified before CPMV treatment (day 0) and during follow-up. Normal ranges: ALT 10−60U/L, AST 10−65, ALKP 25−190, creatinine 0.7−1.4, and urea 4.67−27.1.

remaining cytokines and chemokines analyzed (Supplementary Figure 2).

3.4. CPMV Immunotherapy Induces Significant Changes in the TME. To gain more insights into the potential changes in the TME induced by CPMV, bulk RNA-seq was performed on pretreatment (D0) and post-treatment (D14 and D34 in one dog and D13, D42, and D96 in another dog) tumor samples.

As CPMV immunotherapy stimulates antitumor immune responses,[29,31,39] we used RNa-seq to infer the abundance of several immune cell types known to infiltrate the TME. We found variable changes in the immune infiltration level in the two CPMV-treated dogs. For example, compared to a pretreatment sample (D0), there was an ∼2-fold increase in neutrophils and mDCs by D14 followed by a slight increase in CD8 T cells, CD4 T cells, plasma cells, pDCs, and CD8 T memory cells (Figure 7A and Supplementary Figure 3A). The activation of these cells remains even higher by D34. Of note, Tregs and CD T memory cells showed a slight decrease by D14, but their abundance went up as other immune cells by D34 (Figure 7A and Supplementary Figure 3A). In contrast to patient 1, the contents of the immune system of patient 2 was decreased by D13 (Figure 7B and Supplementary Figure 3B) with a striking fourfold increase in canonic monocytes and ∼3-fold increase in plasma cells, neutrophils, and mDCs by D42 with a subsequent drop to basal or below basal levels by D96 (Figure 7B and Supplementary Figure 3B).

When looking at signaling pathways, the response to chemokines and cytokines increased and remained high during the time of the analysis (Figure 7C and Supplementary Figure 3C, and the NK/T cell activation was upregulated by D14 and remained at this level by D34 (Figure 7C); of note, the T cell-mediated cytotoxicity and antigen processing and presentation had a twofold decreased by D14 but returned to basal levels by D34 (Figure 7C). As with P1, the response to chemokines and NK/T cell activation pathways increased ∼2-fold by D42 with a subsequent drop below basal level (Figure 7D and Supplementary 3D).

4. DISCUSSION

Oral malignant neoplasms such as OMM and oral fibrosarcoma are among the most common in canines.[1,2] Even with aggressive local therapies and achieving sustained local control, the rate of distant metastasis remains high for oral fibrosarcoma and OMM.[4,5,7−11] Terminally ill dogs with tumors often experience significant side effects from the primary disease, chemotherapy, and/or radiation, which can negatively impact their quality of life and contribute to decisions such as euthanasia. Immunotherapy has demonstrated impressive outcomes across various spontaneous canine tumors and is becoming increasingly relevant in cases where conventional treatments fail to provide lasting responses.[21−26] Due to the promising response rates observed in murine models and its role as an adjuvant to radiotherapy in canine OMM and in combination with chemotherapy to canine inflammatory mammary carcinoma, we explored the potential of CPMV-derived VLPs as adjuvant treatment alongside standard chemotherapy for recurrent oral malignant neoplasms.[25,31,32,40]

As in previous canine reports, the tolerability of intratumoral CPMV administration was excellent. None of the patients had acute reactions to the therapy, and no abnormalities in the blood analyses were detected.[25,32] Due to the OMM progression in patient 2 after the first CPMV intratumoral administration, the patient underwent a combination of chemotherapy and radiation. Unfortunately, 2 weeks after finishing radiotherapy, the patient had acute kidney injury and suspected pancreatitis, which caused a disseminated intra-

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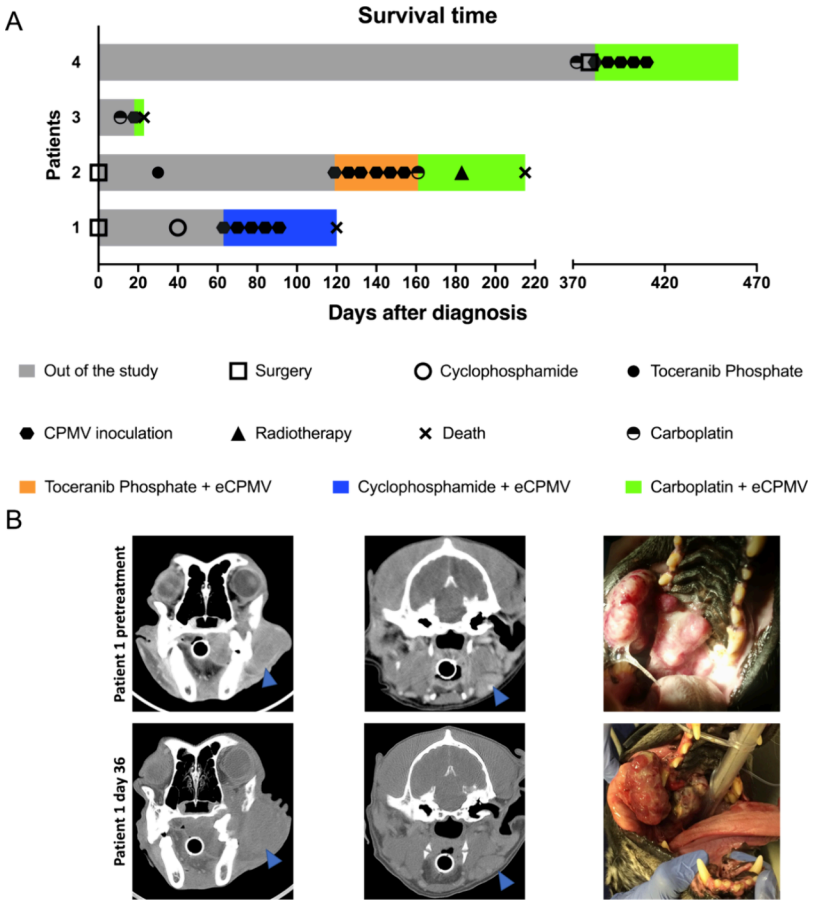

Figure 3. Outcome in dogs treated intratumorally with CPMV. (A) Overall survival time and treatment outcome. The time before enrollment in the trial (dotted gray) and the adjuvant treatments they received are represented for each patient (surgery, chemotherapy, and radiotherapy). The CPMV intratumoral administration (hexagon) and the combination with different treatments (carboplatin, cyclophosphamide, or toceranib) is labeled in different colors. (B) Imaging of patient 1 before and after treatment. CT imaging shows the primary tumor (left images, blue arrow) and the metastatic submandibular lymph node (central images, blue arrow). Macroscopic images taken during anesthesia with measurements by caliper (right images).

vascular coagulation disorder and cardiorespiratory failure during hospitalization. The use of toceranib phosphate or carboplatin in combination with radiotherapy has not shown any grade V acute kidney injury, although a grade III creatinine and urea elevation had been mentioned in one dog receiving radiotherapy with adjuvant toceranib phosphate.[10,41,42] Interestingly, the use of toceranib phosphate in combination with CPMV intratumoral immunotherapy had been proven to be safe for the treatment of canine inflammatory mammary carcinoma, and dogs with OMM receiving radiotherapy with adjuvant CPMV intratumoral immunotherapy had not shown any renal alterations.[25,32] Considering these previous results, it is unlikely that CPMV intratumoral immunotherapy was the cause of patient 2 adverse effects. The combination of carboplatin and toceranib phosphate in 11 dogs showed a grade IV creatine and urea elevation in one of the patients; thus, it is possible that the combination of these two cytotoxic drugs could have been responsible of the kidney failure observed in patient 2.[43]

The patients in which the tumor mass could be measured (P1 and P2) showed partial response after treatment initiation; however, no complete responses were recorded in these patients. Interestingly, the size of the lymph nodes did not change during the full course of the study, and no pulmonary metastatic disease was reported in any of the patients. Even though the sample size was small, our results suggest that intratumoral CPMV injection may reduce the possibility of metastatic disease, which could agree with previous data.[31]

As previously reported in murine models and canine mammary cancer patients, the CPMV nanoparticles induced changes in the TME.[27,30,32,33] Although patient P3 was evaluated over a short period of time, we observed a 2.44-fold increase in T cells and a 1.66-fold increase in macrophages by day 5 postintratumoral immunotherapy. However, the patient was euthanized thereafter. Patients 1 and 2 exhibited a greater increase of infiltrating T cells (4.5-fold and 1.6-fold, respectively) and B lymphocytes (9.82-fold and 8.98-fold, respectively) in biopsies taken on days 35 and 42 after the first intratumoral CPMV dose. Tumor samples obtained at necrosis of patient P2 showed a decrease in both TIL populations; however, patient P1 showed a decrease only in the T-cell population. Although only two patients were evaluated, our results are consistent with previous literature, showing an increase in T-cell infiltration within the TME. As observed in patient P2, administering the full CPMV doses may be necessary to achieve enhanced TME infiltration in some oral tumors.[32]

The infiltration of macrophages in patient 1 showed a significant decrease 14 days after initiating CPMV treatment, which could be related to the decrease in size and the

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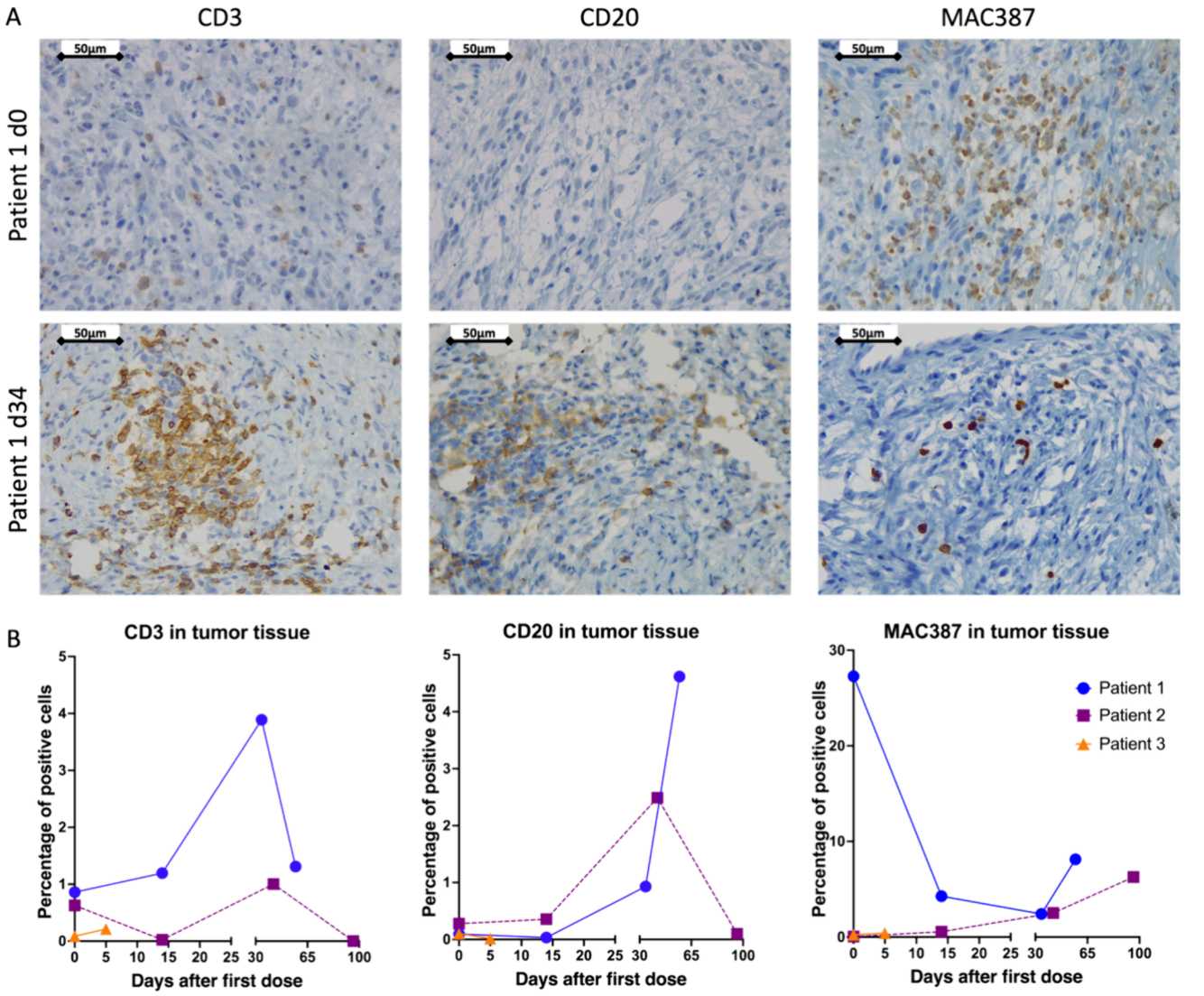

Figure 4. Tumor-infiltrated immune cells by immunohistochemistry in patient 1. (A) T (CD3+) and B lymphocytes (CD20+); and monocyte/macrophages (MAC387+) in pretreatment (d0) and post-treatment (d34) biopsies. (B) Quantification of CD3+, CD20+, and MAC387+ cells in tumor biopsies. Scale bar: 50 μm.

associated infection that typically accompanies large tumor masses in the oral cavity. Patient 2 showed a progressive increase of TAMs, which could be related to the poor clinical response observed and the progression of the disease, accompanied by intratumoral infection. Both tumor samples had a high number of infiltrating macrophages at postmortem evaluation, when the tumor had progressed, which could be related to a concomitant infection, or perhaps a population of anti-inflammatory macrophages was associated with the TME, thus inducing a pro-tumoral immune response.

Although no definitive conclusions could be drawn from the peripheral blood cell populations, most likely due to the small number of patients, there was a novel finding in the blood chemokines that has not been reported in canine OMM. The literature supports that chemokine CXCL1 (originally known as KC) has an important role in the tumorigenesis of melanoma; furthermore, the overexpression of this chemokine immortalizes melanocytes, resulting in their ability to form tumors.[44] CXCL1 can increase its own expression by activation of the nuclear factor kβ (NF-kβ) pathway, the overexpression of which has been described in multiple canine OMMs and fibrosarcomas.[45−47] Previous reports have suggested that canine OMM can express a mutation in p53 protein, which has been associated with an increase in CXCL1 expression.[47,48] In addition to proliferation, CXCL1 has been linked to tumor angiogenesis and inducing cancer cell migration, enabling invasion of lymphatic vessels and lymph node metastasis.[49−51] Although there are multiple pathways that could increase the expression of this chemokine, this is the first time that an elevation in the CXCL1 chemokine has been described in canine patients with oral tumors. The three patients evaluated had a major expression of this chemokine in pretreatment samples, which dropped significantly after the first CPMV intratumoral administration and remained undetectable until the intratumoral treatment was completed. Just as CXCL1, IL-8 is another chemokine that has been strongly associated with NF-kβ in multiple tumors.[52,53] Even though it has not been reported in canine tumors, IL-8 has been associated with angiogenesis and the enhancement of metastatic abilities in some human tumors.[54,55] Interestingly, a similar drop in IL-8 expression was noticed in a series of canine cases treated with CPMV intratumoral immunotherapy.[33] Although only a small

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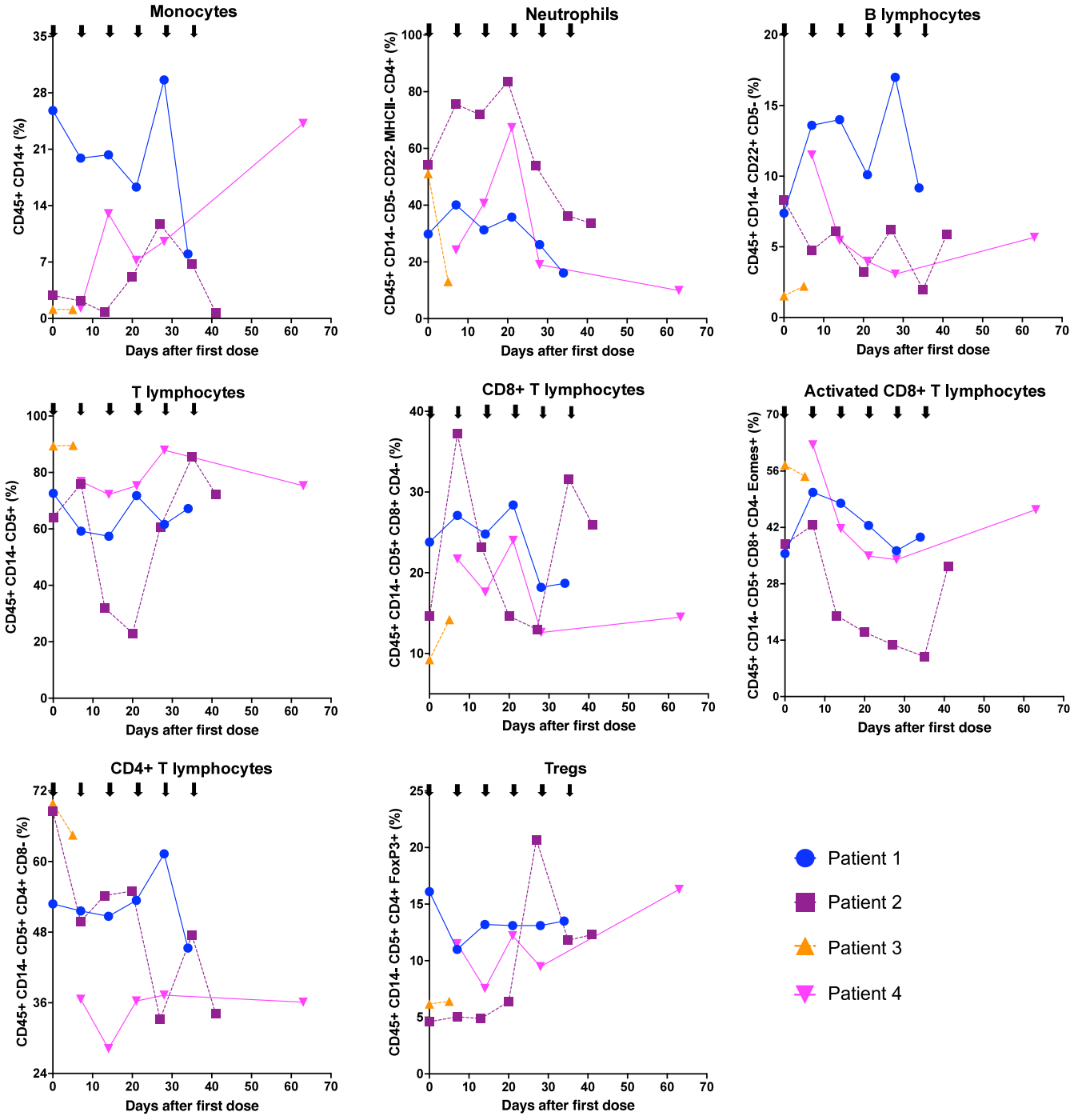

Figure 5. Immune cell populations quantified by flow cytometry after the first CPMV treatment in peripheral blood samples of the four treated patients. The doses of CPMV are marked with black arrows in the top of the graphs; patients 1 and 3 received four doses, patient 2 received six doses, and patient 3 received only one dose.

number of cases were evaluated, all of them showed undetectable or low levels of CXCL1 and IL-8 after initiation of CPMV intratumoral immunotherapy, which could explain the low rate of pulmonary metastasis and supports the results observed in previous studies.[25,39] The downregulation mechanism is still unknown; however, multiple reports suggest that TAMs have an important role in the expression of CXCL1 and IL-8.[50,55] Our results show a reduction in infiltrating macrophages in the TME after the first CPMV intratumoral administration; thus, the treatment could be downregulating the expression of these chemokines by changing the immune populations in the TME.

The results obtained in this study demonstrate that intratumoral administration of CPMV is safe and could have an abscopal effect, maintaining the size of lymph nodes and reducing the rate of metastatic disease. However, it is important to note that while these outcomes are promising, the administration of CPMV did not achieve long-term disease control. These findings highlight the need for further research to optimize the therapeutic regimen, including exploring combination strategies that might enhance efficacy and provide

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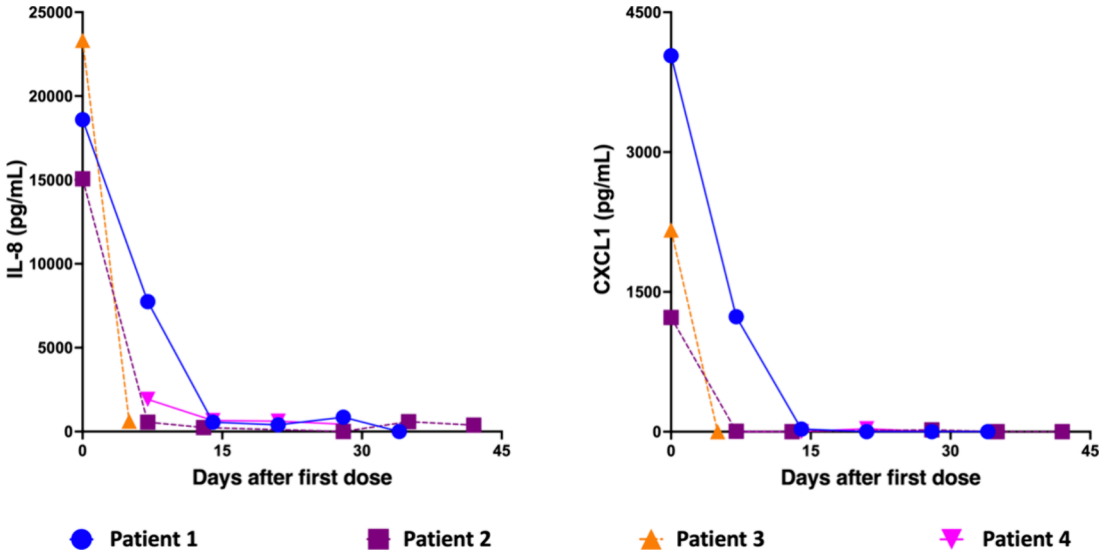

Figure 6. Chemokines quantified in peripheral blood samples of the four treated patients.

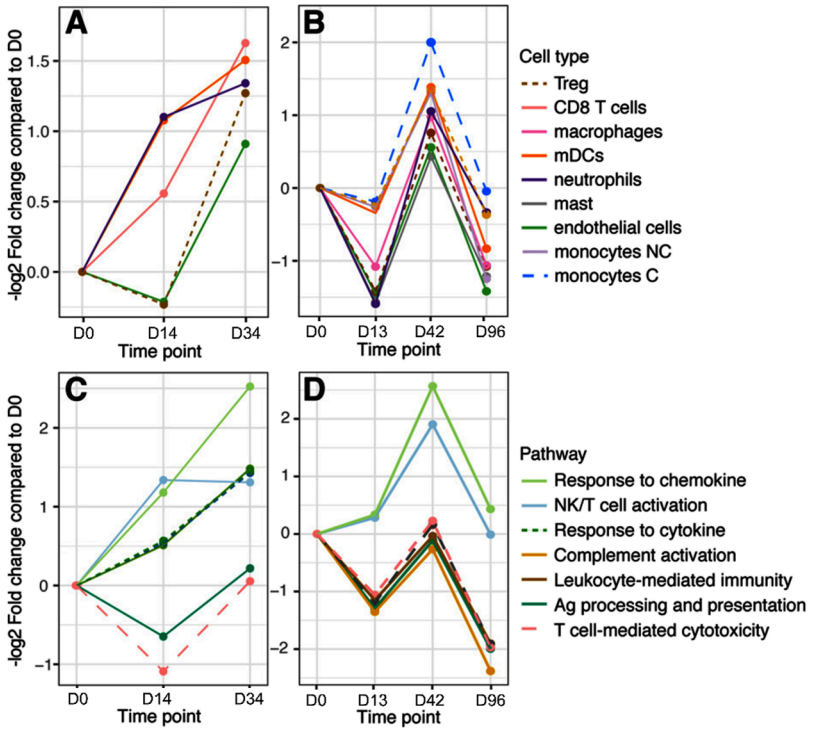

Figure 7. CPMV intratumoral immunotherapy induced changes in immune cell composition and signaling pathways in the TME in patient 1 (A, C) and patient 2 (B, D). CPMV immunotherapy has a variable effect on intratumoral immune cell contents (A, B) and signaling pathways (C, D). Log2 fold changes in gene expression of inferred cell types (A, B) and inferred pathways (C, D) normalized to D0 is on the *y*-axis.

more comprehensive disease control. In addition, our results suggest that CPMV can promote a more antitumoral TME, decreasing the number of TAMs and increasing the infiltration of T lymphocytes. Despite that the role of CXCL1 and IL-8 is not clearly described in canine tumors, CPMV treatment decreases its expression, which could reduce the rate of metastasis in oral tumors. Given that this study was performed including a small number of patients, future studies with a larger population will be needed to assess the effects of CPMV treatment.

Based on the findings presented in this manuscript, future research could focus on optimizing the CPMV particles to enhance their efficacy and therapeutic potential. Investigating modifications to particle structure, surface functionalization, or delivery methods may further improve their ability to control disease progression and achieve better long-term outcomes. Additionally, exploring combination strategies with other therapeutic modalities, such as checkpoint inhibitors or targeted therapies, could maximize their effectiveness. These potential directions provide valuable opportunities for ongoing research and a deeper understanding of the mechanisms underlying the observed effects, ultimately contributing to the development of more effective immunotherapeutic approaches.

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5. CONCLUSIONS

CPMV intratumoral immunotherapy in canine oral tumors is safe and could promote a more antitumoral TME and a decrease in metastatic events. CMPV IT could be a beneficial adjuvant treatment for OMM. Treatment with CPMV also decreases the expression of CXCL1 and IL-8, which could serve as prognostic biomarkers in future studies in these patients.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.molpharmaceut.5c00100.

The rest of the blood biochemistry values of patients who received CPMV as intratumoral immunotherapy, which did not show any abnormalities; changes observed in the rest of cytokines and chemokines quantified in peripheral blood samples during follow-up, as well as the rest of the changes observed in the RNA sequencing (PDF)

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Notes

The authors declare the following competing financial interest(s): N.F.S. and S.F. are co-founders of, equity holders, and have financial interests in Mosaic ImmunoEngineering Inc. SF and NFS are co-founders and equity holders in PlantiosX Inc. N.F.S. is a co-founder and manager of Pokometz Scientific LLC, under which she acts as a paid consultant for Mosaic ImmunoEngineering Inc., Flagship Labs 95 Inc., and Arana Biosciences Inc. N.F.S. and S.F. are co-founders of, equity holders, and have financial interests in PlantiosX Inc.; N.F.S. serves as CEO of PlantiosX Inc. J.v.B. is a co-founder and has a financial interest in InCephalo AG, where he is also a part-time employee. The rest of the authors declare no conflicts of interest.

This study was approved by the ethics committee of Alfonso X el Sabio University (2022_03/133).

Written informed consent has been obtained from the owners of the dogs to participate in the study.

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