Molecular Therapy
Oncolytics
Original Article

Oncolytic virotherapy with intratumoral injection of vaccinia virus TG6002 and 5-fluorocytosine administration in dogs with malignant tumors

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TG6002 is an oncolytic vaccinia virus expressing FCU1 protein, which converts 5-fluorocytosine into 5-fluorouracil. The study objectives were to assess tolerance, viral replication, 5-fluorouracil synthesis, and tumor microenvironment modifications to treatment in dogs with spontaneous malignant tumors. Thirteen dogs received one to three weekly intratumoral injections of TG6002 and 5-fluorocytosine. The viral genome was assessed in blood and tumor biopsies by qPCR. 5-Fluorouracil concentrations were measured in serum and tumor biopsies by liquid chromatography or high-resolution mass spectrometry. Histological and immunohistochemical analyses were performed. The viral genome was detected in blood (7/13) and tumor biopsies (4/11). Viral replication was suspected in 6/13 dogs. The median intratumoral concentration of 5-fluorouracil was 314 pg/mg. 5-Fluorouracil was not detected in the blood. An increase in necrosis (6/9) and a downregulation of intratumoral regulatory T lymphocytes (6/6) were observed. Viral replication, 5-fluorouracil synthesis, and tumor microenvironment changes were more frequently observed with higher TG6002 doses. This study confirmed the replicative properties, targeted chemotherapy synthesis, and reversion of the immunosuppressive tumor microenvironment in dogs with spontaneous malignant tumors treated with TG6002 and 5-fluorocytosine.

INTRODUCTION

Oncolytic viruses are gaining ground as an alternative therapy in cancer treatment. The oncolytic potencies of oncolytic viruses (OVs) rely on the lytic replication cycle, expression of therapeutic genes, and modifications in the tumor microenvironment. Recently, a genetically engineered herpes simplex virus (Imlygic) was approved by the European Medicines Agency and the US Food and Drug Administration for the local treatment of unresectable melanoma.[1]

TG6002 is an oncolytic vaccinia virus (VACV) developed with deletions of the thymidine kinase (*TK*) and ribonucleotide reductase (*RR*) loci in its genome, resulting in attenuated virulence and enhanced tumor-specific targeting.[2] To enhance its therapeutic efficacy, the *FCU1* gene, which encodes a bifunctional fusion protein combining cytosine deaminase and uracil phosphoribosyltransferase activities, was inserted into the VACV genome. FCU1 converts the nontoxic prodrug 5-fluorocytosine (5-FC) into the chemotherapeutic compound 5-fluorouracil (5-FU) and 5-FU-monophosphate, which inhibit DNA and protein synthesis.[3,4] A study in murine xenograft mice treated with a single intravenous injection of TG6002 followed by oral administration of 5-FC showed significant antitumor efficacy against a large range of human tumors with high levels of intratumoral 5-FU production.[2] A significant decrease in tumor size was observed in canine mammary tumor cells grafted onto mice after intratumoral injection of TG6002 and oral 5-FC administration.[5] Finally, tumor necrosis and conversion of 5-FC into 5-FU have been assessed *in vitro* in canine mammary tumor explants cultured with TG6002 and 5-FC.[5] In addition, a recent study showed that the nonviral FCU1/5-FC system was effective against most of the assayed canine melanoma cell lines.[6]

Preclinical models such as rodents have often been used in drug development. However, rodent models have major limitations, leading to discrepancies between preclinical studies and clinical trials. Spontaneous cancers in dogs are considered relevant models for translational research. Indeed, the clinical presentation, histological features, molecular profiles, and response and resistance to therapy are quite similar.[7–12] Dogs with spontaneous cancers appear to be valuable models to evaluate the efficacy of OVs.[13–17] Therefore, safety and efficacy evaluation of OVs in canine cancers should be considered to assess their potential benefit in human medicine. Safety,

Received 10 April 2023; accepted 17 July 2023;
https://doi.org/10.1016/j.omto.2023.07.005.

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Table 1. Characteristics of study dogs, treatment course, and response to treatment

Cohort	Dog	Age (years)	Diagnosis	Metastasis at diagnosis	Prior therapy	Protocol	Response at day 38 (% of size variation)	PFI (days)	ST (days)	Evolution
1	1	10.5	low-grade STS	no	none	2 injections 5×10^6 PFU/kg	PD (+28%)	38	84	left the study due to PD
	2	12.0	mammary ADK	yes	none	3 injections 5×10^6 PFU/kg	SD (+8%)	38	82	euthanasia due to PD
	3	10.8	intermediate-grade STS	no	none	3 injections 5×10^6 PFU/kg	PD (+32%)	38	147	left the study due to PD
2	4	12.5	mammary sarcoma	no	none	1 injection 5×10^7 PFU/kg	SD (D7) (0%)	N/A	514	euthanasia due to PD
	5	10.0	mammary ADK	no	none	1 injection 5×10^7 PFU/kg	PR (−35%)	32	N/A	lost to follow-up
3	6	12.3	urothelial carcinoma	no	MC	3 injections 5×10^7 PFU/kg	SD (+18%)	44	44	euthanasia due to decreased general conditions
	7	5.0	esophageal sarcoma	no	none	3 injections 5×10^7 PFU/kg	SD (+15%)	21	224	euthanasia due to PD
	8	9.2	colic ADK	no	surgery	3 injections 5×10^7 PFU/kg	SD (+16%)	38	119	euthanasia due to PD
	9	4.6	high-grade STS	yes	surgery	3 injections 5×10^7 PFU/kg	SD (+18%)	28	28	euthanasia
	10	4.6	high-grade STS	yes	RT	3 injections 5×10^7 PFU/kg	N/A	N/A	115	euthanasia due to PD
	11	8.6	high-grade STS	yes	surgery	3 injections 5×10^7 PFU/kg	PD (+35%)	38	93	euthanasia due to PD
	12	12	intermediate-grade STS	no	MC	3 injections 5×10^7 PFU/kg 3 injections 5×10^6 PFU/kg	SD (+12%)	84	481	euthanasia due to PD
	13	10.2	urothelial carcinoma	no	surgery C MC	6 injections 5×10^7 PFU/kg	SD (+7%)	258	500	euthanasia due to PD

ADK, adenocarcinoma; C, maximum tolerated dose chemotherapy; MC, metronomic chemotherapy; N/A, not assessed; PD, progressive disease; PFI, progression-free interval; RT, radiation therapy; SD, stable disease; ST, survival time; STS, soft tissue sarcoma.

biodistribution, and shedding of TG6002 has been established in healthy dogs.[18,19] Three weekly intramuscular injections of 5×10^7 plaque-forming units (PFU)/kg TG6002 did not lead to major adverse events (AEs), and viral shedding was not detected in blood, urine, feces, or saliva.[18] Similar results were reported after intravenous injections of TG6002 (10^7 PFU/kg) in healthy dogs.[19]

The first objective was to evaluate tolerance to intratumoral injections of TG6002 associated with oral administration of 5-FC in dogs diagnosed with spontaneous malignant neoplasia. The second objective was to assess viral replication, intratumoral production of 5-FU, and tumor microenvironment modifications. The third objective was to assess immune response to VACV administration. The fourth objective was to evaluate the response to treatment.

RESULTS

Patient population

Thirteen dogs were enrolled in this study. The trial patients' characteristics are shown in Table 1.

Safety/AEs

Clinical and paraclinical AEs are presented in Table 2.

Twenty-seven clinical AEs (grade 1–2: 9, grade >2: 18) were reported for cohort 1. Five clinical AEs (grade 1–2: 5) were reported for cohort 2. Seventy-four clinical AEs (grade 1–2, 42; grade >2, 32) were reported for cohort 3.

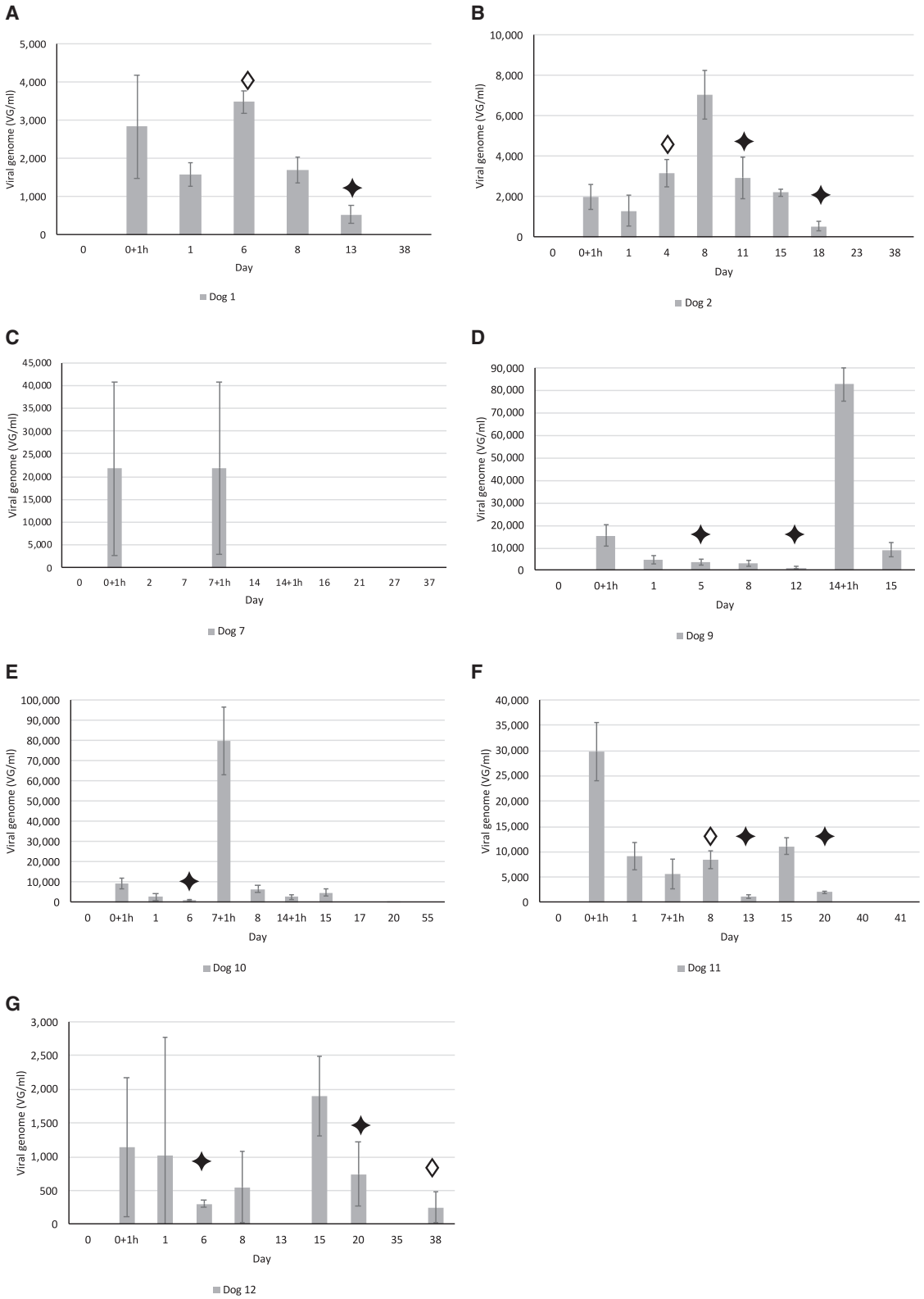
Cutaneous AEs, consisting of depigmentation, ulceration, exudation, and crusts localized on the nasal planum, eyelids, lips, scrotum, and pads, represented the most common AEs (41.5%). Grade 1 to 2 cutaneous AEs accounted for 45.5%, moderate grade 3 AEs accounted for 48%, severe grade 4 AEs accounted for 4.5%, and life-threatening grade 5 AEs accounted for 2%. Histological analyses of five cutaneous lesions diagnosed a cutaneous lupoid drug reaction secondary to 5-FC administration.[20]

AEs at the TG6002 injection site (erythema, swelling, ulceration, and discharge) represented 7.5% of all AEs. Injection site AEs were

Table 2. Clinical, hematological, and biochemical AEs

	Cohort 1					Cohort 2					Cohort 3					
	3 dogs total of 8 injections					2 dogs total of 2 injections					8 dogs total of 30 injections					
Grade	1–2	3	4	5	Total (n =)	1–2	3	4	5	Total (n =)	1–2	3	4	5	Total (n =)	% AEs
AE																
Injection site					**1**					**2**					**5**	**7.5**
Erythema (n =)	0	1	0	0	**1**	0	0	0	0	**0**	1	0	0	0	**1**	
Swelling (n =)	0	0	0	0	**0**	1	0	0	0	**1**	0	0	0	0	**0**	
Ulceration (n =)	0	0	0	0	**0**	1	0	0	0	**1**	0	2	0	0	**2**	
Weeping (n =)	0	0	0	0	**0**	0	0	0	0	**0**	1	1	0	0	**2**	
Gastrointestinal					**8**					**2**					**28**	**36**
Anorexia (n =)	2	3	0	0	**5**	1	0	0	0	**1**	6	2	0	0	**8**	
Constipation (n =)	0	0	0	0	**0**	0	0	0	0	**0**	1	0	0	0	**1**	
Diarrhea (n =)	0	2	0	0	**2**	0	0	0	0	**0**	4	2	0	0	**6**	
Gingivitis (n =)	0	0	0	0	**0**	0	0	0	0	**0**	1	0	0	0	**1**	
Hematochezia (n =)	0	0	0	0	**0**	0	0	0	0	**0**	4	2	0	0	**6**	
Nausea, vomiting (n =)	1	0	0	0	**1**	1	0	0	0	**1**	4	0	0	0	**4**	
Pain (n =)	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**	
Ptyalism (n =)	0	0	0	0	**0**	0	0	0	0	**0**	2	0	0	0	**2**	
General					**4**					**1**					**11**	**15**
Apathy (n =)	1	0	1	0	**2**	1	0	0	0	**1**	3	4	1	0	**8**	
Hyperthermia (n =)	0	1	0	0	**1**	0	0	0	0	**0**	0	1	0	0	**1**	
Weight loss (n =)	0	1	0	0	**1**	0	0	0	0	**0**	0	2	0	0	**2**	
Dermatological					**14**					**0**					**30**	**41.5**
Erythema (n =)	3	2	0	0	**5**	0	0	0	0	**0**	9	3	0	0	**12**	
Pain (n =)	0	0	0	0	**0**	0	0	0	0	**0**	1	4	0	0	**5**	
Pruritus (n =)	0	1	0	0	**1**	0	0	0	0	**0**	3	0	0	0	**3**	
Ulceration (n =)	2	5	0	0	**7**	0	0	0	0	**0**	1	4	1	1	**7**	
Exudation (n =)	0	1	0	0	**1**	0	0	0	0	**0**	1	1	1	0	**3**	
Hematology					**3**					**3**					**30**	**65**
Anemia	1	1	0	0	**2**	1	0	0	0	**1**	7	0	0	0	**7**	
Basophilia	0	0	0	0	**0**	0	0	0	0	**0**	1	0	0	0	**1**	
Eosinopenia	0	0	0	0	**0**	0	0	0	0	**0**	5	0	0	0	**5**	
Eosinophilia	0	0	0	0	**0**	0	0	0	0	**0**	1	0	0	0	**1**	
Lymphopenia	0	0	0	0	**0**	1	0	0	0	**1**	5	0	0	0	**5**	
Monocytopenia	0	0	0	0	**0**	0	0	0	0	**0**	2	0	0	0	**2**	
Monocytosis	0	0	0	0	**0**	0	0	0	0	**0**	4	0	0	0	**4**	
Neutrophilia	0	0	0	0	**0**	1	0	0	0	**1**	3	0	0	0	**3**	
Neutropenia	1	0	0	0	**1**	0	0	0	0	**0**	1	0	1	0	**2**	
Biochemistry					**0**					**0**					**19**	**35**
Hypoalbuminemia	0	0	0	0	**0**	0	0	0	0	**0**	5	0	0	0	**5**	
Hypoproteinemia	0	0	0	0	**0**	0	0	0	0	**0**	6	0	0	0	**6**	
Elevated ALP	0	0	0	0	**0**	0	0	0	0	**0**	0	0	1	0	**1**	
Elevated ALT	0	0	0	0	**0**	0	0	0	0	**0**	0	0	1	0	**1**	
Elevated BUN	0	0	0	0	**0**	0	0	0	0	**0**	6	0	0	0	**6**	

AEs, adverse events; ALP, alkaline phosphatase; ALT, alanine transaminase; BUN, blood urea nitrogen.
Total AEs and % of AEs are in bold.



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observed in one dog in cohort 1, two dogs in cohort 2, and five dogs in cohort 3. Gastrointestinal AEs accounted for 36% of all clinical AEs.

Three hematological AEs were reported in cohorts 1 and 2 and 30 AEs in cohort 3. Hematological AEs consisted of mild anemia (27.7%), mild lymphopenia (16.5%), mild eosinopenia (13.8%), mild neutrophilia (11.1%), severe neutropenia (8.3%), and mild monocytosis (11.1%). One dog (dog 4) experienced grade 4 neutropenia after two administrations of TG6002 at 5×10^7 PFU/kg and two other dogs experienced grade 1–2 neutropenia.

Regarding biochemistry, no AEs were reported in cohorts 1 and 2. Nineteen AEs were reported in cohort 3. Dog 6, treated with three intravesical administrations of 5×10^7 PFU/kg and oral 5-FC, experienced a grade 4 increase in ALP and ALT. Postmortem examination diagnosed a moderate multifocal chronic neutrophilic cholangiohepatitis with vacuolar hepatopathy.

Without taking into account dermatological signs induced by 5-FC, a total of 16 AEs (one of grade 4 or 5) were reported for a total of 8 injections at 5×10^6 PFU/kg. Ninety-three AEs (four of grade 4 or 5) were reported for a total of 32 injections at 5×10^7 PFU/kg. A higher frequency of AEs was not observed with higher dose of TG6002.

Viral shedding and viral replication

Viral genome (VG) was detected in the blood of 7/13 dogs (cohort 1, dogs 1 and 2; cohort 2, dogs 7, 9, 10, 11, and 12) (Figure 1). VG was not detected in the blood of either dog receiving intravesical treatment (dogs 6 and 13). Higher VG copy numbers were observed in dogs receiving multiple injections of TG6002 at 5×10^7 PFU/kg. Based on the increase in VG copy number and the persistence of VG in blood for more than five half-lives, viral replication was suspected for 2/3 dogs of cohort 1 (dogs 1 and 2) and 4/8 dogs of cohort 3 (dogs 9, 10, 11, and 12) (Figure 1). Higher VG titers were detected in the blood of dogs receiving higher doses of TG6002.

VG was detected in the urine at least 2 days after intravesical TG6002 administration but was not detected after 7 days (unpublished observation).

VG was detected in tumor biopsies of 4/11 dogs. VG was not detected at day 38 in tumor biopsies for the three dogs included in cohort 1. For dog 4 (cohort 2), VG was detected at day 7 in tumor biopsies (mean VG [±SD]: 9.38E+03 [±8.25E+02] VG/mg of tumor). Post-treatment biopsies were available for seven dogs of cohort 3 (dogs 6, 9, 11, and 12 at day 38; dog 7 at days 7, 9, and 14; dog 8 at days 7, 28, 38, and 49; and dog 13 at days 87 and 255). VG was identified for three dogs out of seven (dog 7: 5.49 + 05 [±5.23E+04] VG/mg of tumor at day 7, 4.19E+02 [±5.74E+02] VG/mg of tumor at day 14; dog 9: 5.27E+05 [±3.72E+04] VG/mg of tumor at day 38; dog 11: 1.37E+05 [±9.03E+03] VG/mg of tumor at day 38). VG was not detected in tumor biopsies of the two dogs treated with intravesical injection of TG6002. Higher intratumoral VG titers were detected for dogs receiving multiple injection of TG6002 at 5×10^7 PFU/kg.

Cutaneous lesions were observed in 6/13 dogs. qPCR was performed on swabs (muzzle [n = 4/6], lips [n = 3/6], eyelids [n = 4/6], nose [n = 4/6], scrotum [n = 1/6]) and on cutaneous biopsies (n = 2/6). All samples had results below the limit of detection.

5-FC and 5-FU dosages

One hour after oral 5-FC administration, the median 5-FC serum concentration was 24.8 μg/mL (range: 2.2–69.1 μg/mL). Serum 5-FU concentrations were assessed 1 h after oral 5-FC administration. For all dogs, 5-FU serum concentrations were below the limit of detection.

For dogs in cohort 1, the intratumoral 5-FU concentration at day 38 was 72 pg/mg (dog 1), 61 pg/mg (dog 2), and 36 pg/mg (dog 3). For cohort 2, tumor samples were available only for dog 4. Intratumoral 5-FU concentration was 9.032 pg/mg at day 7 for dog 4. For dogs in cohort 3, tumor biopsies were available for three dogs (dog 6, 7, and 12). For dog 7, intratumoral 5-FU concentrations at days 7, 14, and 21 were 314, 2,084, and 795 pg/mg, respectively. For dog 7, 5-FU (216 pg/mg) was also identified in the noninjected site of the tumor at day 21. Intratumoral 5-FU was not detected for dog 6 and 12. Higher concentrations of 5-FU were noticed for dogs treated with higher dose of TG6002.

Histological and immunohistochemical analyses

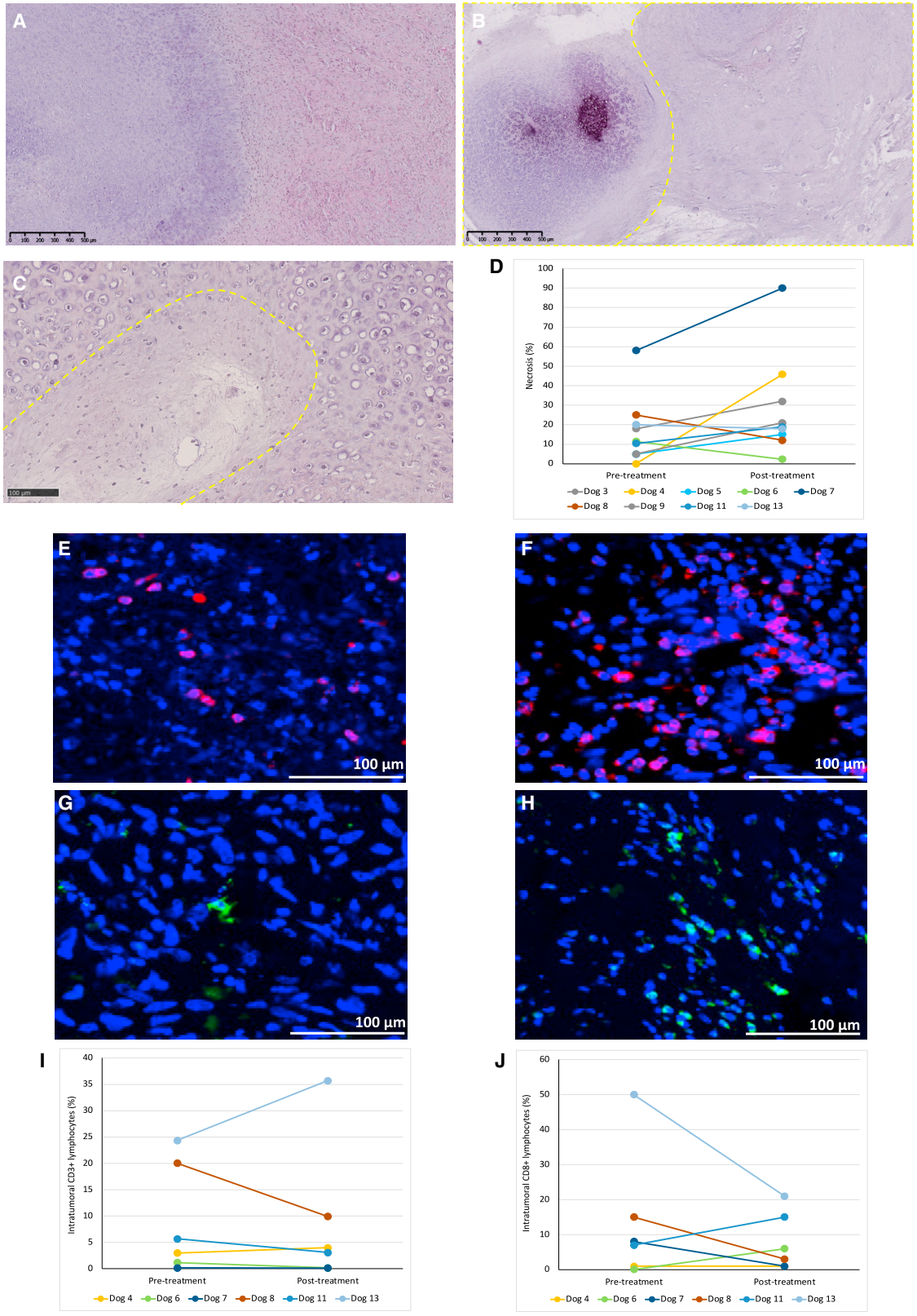
Necrosis was comparatively evaluated between biopsies collected before and after treatment for nine cases. The percentage of necrosis was increased for six dogs out of nine (cohort 1, dog 3; cohort 2, dogs 4 and 5; cohort 3, dogs 7, 9, and 11) after TG6002 administration (Figures 2A–2D). Increases in CD3 lymphocytes (Figures 2E, 2F, and 2I) and CD8 lymphocytes (Figures 2G, 2H, and 2J) in the tumor microenvironment were observed in 2/6 cases (dogs 4, 13) and 2/6 cases (dogs 6, 11), respectively. A decrease in FOXP3 expression in the tumor microenvironment was observed in 6/6 cases (cohort 2, dog 4; cohort 3, dogs 6, 7, 8, 11, and 13) (Figures 3A–3E).

Anti-VACV antibodies and neutralizing antibodies

Increases in anti-VACV antibody and neutralizing antibody titers were observed up to 7 days after TG6002 administration, with maximal values between day 21 and day 35 (Figures 4A and 4B). Neutralizing antibodies were detectable by day 7 for all dogs except for dog 13 treated by intravesical administration (Figure 4B). For

Figure 1. Viral genome measured by qPCR in the blood of dogs after TG6002 injections

(A) Dog 1. (B) Dog 2. (C) Dog 7. (D) Dog 9. (E) Dog 10. (F) Dog 11. (G) Dog 12. qPCR analyses were performed at each time represented on the x axis. (◇) Increase in viral genome copy number, (✦) persistence of viral genome more than five half-lives. The results are presented as the mean of triplicate experiments ±SD.



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dog 12, treated with two sessions of TG6002 at day 230, few anti-VACV antibodies and neutralizing antibodies were observed before the second administration (Figures 4C and 4D). Similar serum antibody profiles were observed independently of TG6002 dose.

Antitumor activity and survival time

Eleven dogs were available for reassessment at day 38. Response to treatment, assessed at day 38, is presented in Figure 5 and Table 1. Following treatment administration, no complete response, one partial response, eight stable diseases, and three progressive diseases were assessed. For cohort 1, a stable disease was observed for dog 2, and a progressive disease was observed for dogs 1 and 3. For cohort 2, a stable disease was observed for dog 4 at day 7, and a partial response was observed for dog 5 at day 38. For cohort 3, a stable disease was observed for 6/8 dogs (dogs 6, 7, 8, 9; 12, and 13) and progressive disease was observed for 1/8 dogs (dog 11). For dogs 4 and 5, the owner elected a surgical approach; therefore, the dogs were not excluded from the survival analysis. The median survival time for all dogs was 115 days (interquartile range: 82–224 days; range: 28–500 days).

DISCUSSION

We previously reported the oncolytic properties of TG6002 in canine mammary tumor explants and canine mammary tumor cells grafted onto mice and demonstrated the safety of intramuscular and intravenous administration in healthy dogs.[5,18,19] In this study, we describe the tolerance and feasibility of intratumoral injections of TG6002 in association with oral 5-FC administration in 13 dogs diagnosed with spontaneous malignant neoplasia. Moreover, we report viral replication, targeted intratumoral 5-FU production, reversion of the immunosuppressive tumor microenvironment, and immune response associated with TG6002 and 5-FC administration.

TG6002 administration in combination with 5-FC was associated with cutaneous AEs, gastrointestinal symptoms, general symptoms, and injection site reaction. A higher dose of TG6002 was not associated with an increased number of grade 3 or higher AEs. Regarding hematological and biochemical AEs, mainly grade 1 to 2 AEs were observed. A higher dose of TG6002 was not associated with increased hematological and biochemical toxicities.

Pustular lesions secondary to VACV infection have been reported in clinical trials using oncolytic VACV administered by intratumoral or intravenous routes.[21–26] Such characteristic lesions were not observed following TG6002 administration. VG was not identified in cutaneous lesions, making viral pock lesions unlikely. Moreover, the histology of cutaneous lesions was consistent with the lupoid drug reaction secondary to 5-FC administration.[20] Intravenous administration of 5-FU has been reported to induce lupoid drug reactions in human patients; however, 5-FU was not found in the blood of the dogs in this study.[27] Therefore, cutaneous lesions were consistent with the 5-FC drug reaction. In human medicine, lupoid drug reactions have not been reported after 5-FC medication. Therefore, a better tolerance is expected. In humans, 5-FC toxicity is known to be related to the maximum plasma concentration, and a threshold value of 100 μg/mL is recommended.[28] For all dogs included in this study, the serum 5-FC concentration was below this threshold. However, 5-FC toxicity was reported at concentrations below 100 μg/mL in healthy dogs.[20] 5-FC is excreted by glomerular filtration; therefore, a higher proportion of AEs will be expected for patients with renal dysfunction. In our study, kidney function assessed by creatinine measurement did not identify renal dysfunction.

Other than the cutaneous lesions induced by 5-FC, mild clinical AEs (grade 1–2), moderate clinical AEs (grade 3), or life-threatening clinical AEs (grade 4) were reported in 58%, 38.7%, and 3.3% of patients, respectively. No death-related clinical AEs (grade 5) were observed in this study. As the cases included in this study had advanced neoplastic processes, we could not exclude the possibility that some of the AEs observed were tumor related. A higher dose of TG6002 was not associated with an increased number of grade 3 or higher AEs. Indeed, nine clinical AEs of grade 3 or more clinical AEs for a total of eight injections at 5×10^6 PFU/kg were recorded compared with 17 clinical AEs of grade 3 or more for a total of 30 injections at 5×10^7 PFU/kg.

Hematological and biochemical AEs were mainly graded as mild (grade 1–2). Only one grade 4 increase in liver parameters was recorded (dog 6). Postmortem histology diagnosed a moderate multifocal chronic neutrophilic cholangiohepatitis and vacuolar hepatopathy. As death-related cutaneous AEs induced by 5-FC were observed for this dog, 5-FC hepatotoxicity was suspected. In human medicine, a dose-dependent hepatotoxicity of 5-FC is reported in 25%–41% of patients.[28,29] Grade 1 neutropenia was reported in two dogs treated with 5×10^6 PFU/kg and one dog treated with 5×10^7 PFU/kg. Only one case of grade 4 neutropenia (dog 8) was observed in our study. For this dog, 5-FU was not detected in the blood and, due to limited material, 5-FU dosage in tumor biopsy could not be performed. VG was not detected in blood or tumor biopsies for this dog. Neutropenia was suspected to be induced by tumor necrosis, as identified on endoscopic examination and histological analysis.

Figure 2. Histological and immunohistochemical analyses following treatment with TG6002 and 5-FC

(A) Histological microphotograph of uninfected mammary sarcoma (dog 4). (B and C) Histological microphotographs of mammary sarcoma (dog 4) 7 days after TG6002 administration; the yellow dashed line demarcates an area of necrosis (B) on the right side of the line and (C) on the left side of the line. (D) Evolution of necrosis before and after treatment. (E) CD3 immunohistochemistry microphotographs of mammary sarcoma (dog 4). (F) CD3 immunohistochemistry microphotographs of dog 4, 7 days after TG6002 administration; note the increase in CD3 expression. (G) CD8 immunohistochemistry microphotographs of high-grade soft tissue sarcoma (dog 11). (H) CD8 immunohistochemistry microphotographs of dog 11, 38 days after administrations of TG6002; note the increase in CD8 expression. (I) Evolution of the proportion of $CD3^+$ T cells before and after treatment. (J) Evolution of the proportion of $CD8^+$ T cells before and after treatment. (A, B, and C) Hematoxylin-eosin-saffron staining. (E, F, G, and H) Cellular DNA was stained in blue with DAPI. (E and F) $CD3^+$ lymphocytes were stained in red/purple. (G and H) $CD8^+$ lymphocytes were stained in green. Scale bars, 100 μm.

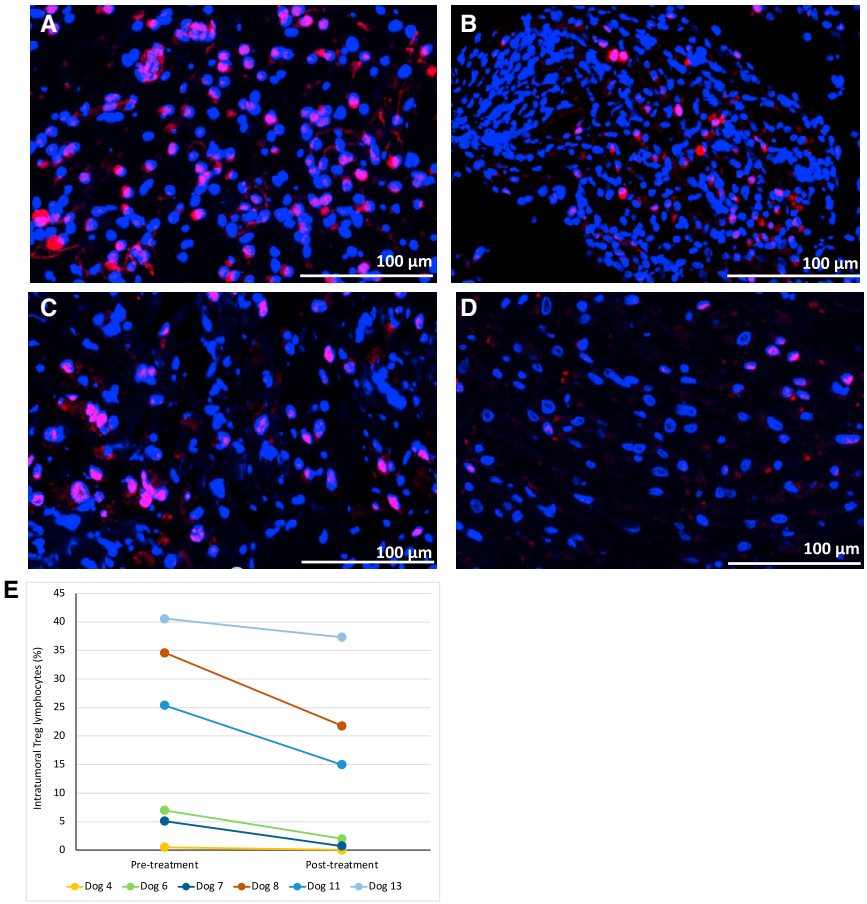

Figure 3. Evolution of intratumoral FOXP3⁺ regulatory T lymphocytes following treatment with TG6002 and 5-FC

(A) FOXP3 immunohistochemistry microphotographs of esophageal sarcoma (dog 7) before treatment. (B) FOXP3 immunohistochemistry microphotographs of dog 7, 38 days after treatment; note the decrease in FOXP3 expression. (C) FOXP3 immunohistochemistry microphotographs of urothelial carcinoma (dog 13) before treatment. (D) FOXP3 immunohistochemistry microphotographs of dog 13, 38 days after treatment, note the decrease in FOXP3 expression. (E) Evolution of the proportion of FOXP3⁺ regulatory T cells before and after treatment assessed by immunohistochemistry. (A–D) Cellular DNA was stained in blue with DAPI, FOXP3⁺ cells were stained in red/purple. Scale bars, 100 µm.

The lytic replication cycle is a mainstay of treatment with OVs. A pharmacokinetics study on healthy beagle dogs showed the absence of VG in the blood after three intramuscular injections of TG6002 at 5×10^7 PFU/kg.[18] Moreover, a clearance less than 24 h was observed after intravenous administration of TG6002 at 10^7 PFU/kg.[19] Therefore, in this study, viral replication was assumed if an increase in the number of VG copies in blood was observed or if VG was identified in the blood of dogs 24 h after TG6002 injection or in tumor biopsy 7 days after TG6002 injection.

Persistence of VG more than 3 days after TG6002 administration for six dogs (dogs 1, 3, 9, 10, 11, and 12) and an increase in VG copy number for four dogs (dogs 1, 3, 11, and 12) were consistent with viral replication. VG was identified in tumor biopsies of two dogs (dogs 9 and 11) at day 38. VG was identified in the tumor biopsies at day 7 for two dogs (dogs 4 and 7) and at day 14 for one dog (dog 7). VG was not identified in the tumor biopsy of the dogs included in cohort 1. In conclusion, replication was suspected for two dogs treated with 5×10^6 PFU/kg and four dogs treated with 5×10^7 PFU/kg. Higher VG copy numbers were observed in the blood of dogs treated with several injections of TG6002 at 5×10^7 PFU/kg. Assessment of infectiveness of viral particles on blood samples and tumor biopsies by plaque assays was not performed but would have been relevant.

Intratumoral synthesis of 5-FU is a second mainstay of treatment with TG6002, which minimizes the systemic toxicity of 5-FU. High intratumoral concentration and prolonged exposure to 5-FU are key mechanisms of 5-FU cytotoxicity. 5-FU was detected in five of the seven available tumor biopsies. Higher concentrations of 5-FU were observed in dogs treated with 5×10^7 PFU/kg.

The 5-FU concentrations observed in tumor biopsies were similar to the concentrations observed in patients treated with 5-FU-based regimens, without related toxicity.[30,31] Tanaka-Nozaki et al. reported a 5-FU intratumoral concentration of 411 ± 381 pg/mg following doxifluridine administration.[32] Moreover, Sadahiro et al. reported a 5-FU intratumoral concentration of 113 ± 45 ng/g 2 h after tegafur administration.[33] Intratumoral production of 5-FU by a nonreplicative modified Vaccinia Ankara encoding the *FCU1* gene (TG4023) has been previously observed in two patients.[34] In our study, 5-FU concentrations exceeded those observed with TG4023 (78.0 and 36.8 pg/mg).[34] This can be explained by the replicative property of TG6002. Prolonged retention of high concentrations of 5-FU in human tumors compared with plasma has been reported previously.[31] Prolonged retention of high concentrations of 5-FU combined to long remanence of TG6002 allows for longer exposure to chemotherapy. Furthermore, the 5-FC plasma half-life is longer (3 h) than the 5-FU plasma half-life (15 min).[20] Thus, the association of TG6002 and 5-FC allows chronic exposure of cancer cells to 5-FU compared with intravenous 5-FU administration. Enhancement of the antitumor activity of TG6002 in association with 5-FC by a bystander killing effect was also observed. Freely diffusible and stable toxic metabolites derived from 5-FC mediating the bystander effect have been reported in culture cells infected with viral vectors armed with the *FCU1* gene.[4] For dog 7, diffusion of 5-FU was noticed in the noninjected part of the tumor, demonstrating a bystander effect. Targeted chemotherapy reduces the risk

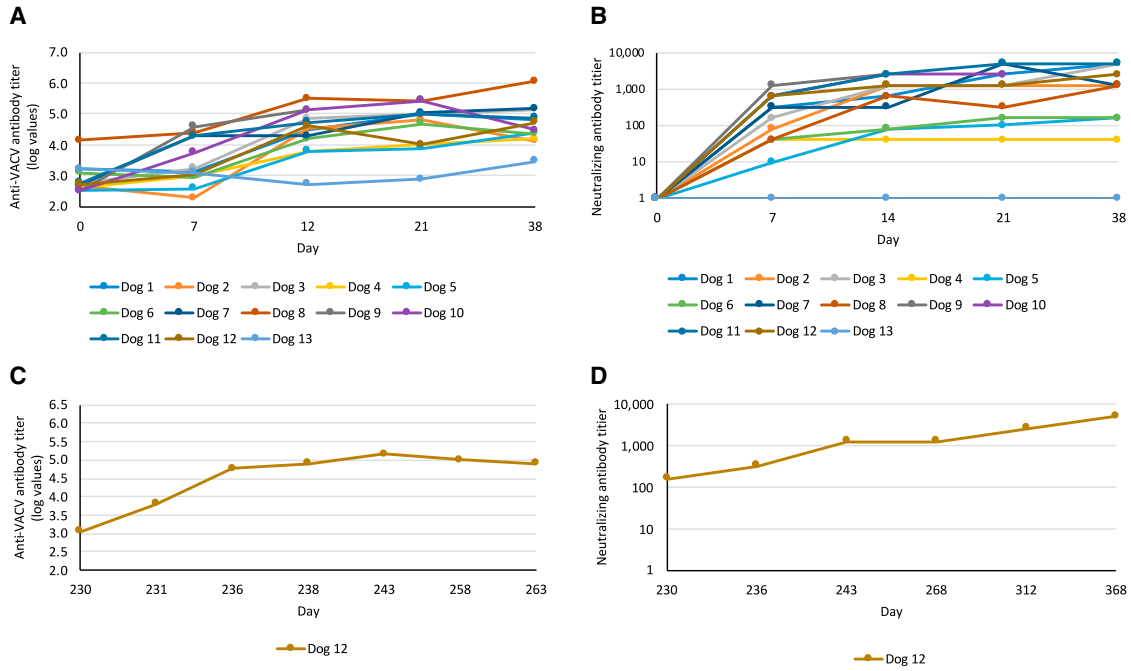

Figure 4. Immune responses of dogs after multiple intratumoral injections of TG6002

(A) Anti-VACV antibody titers after several injections of TG6002. (B) Neutralizing antibody titers after several injections of TG6002. (C) Anti-VACV antibody titers for dog 12 after the second session of treatment. (D) Neutralizing antibody titers for dog 12 after the second session of treatment.

of systemic AEs. Unlike in the study by Husseini et al., 5-FU was not detected in the blood of any dogs, limiting the risk of hematological AEs.[34]

OVs can potentiate antitumor efficacy by remodeling the tumor immune microenvironment. OVs were reported to recruit and activate dendritic cells, T cells, and natural killer cells and to modulate tumor-associated macrophages and myeloid-derived suppressor cells.[35] Systemic administration of a VACV with *TK* and *RR* gene deletion and armed with the *FCU1* gene in an orthotopic model of renal carcinoma resulted in an increase in infiltration of tumors by $CD8^+$ T lymphocytes and a decrease in the proportion of infiltrating regulatory T cells.[36] Our results revealed a decrease in tumor-infiltrating regulatory T cells (n = 6/6). Moreover, an increase in intratumoral $CD3^+$ and $CD8^+$ lymphocytes was observed. Despite the limited number of tumor biopsies, intratumoral injection of TG6002 with 5-FC administration was suspected to reverse the intratumoral immunosuppressive environment.

This study confirms the feasibility and safety of repeated intratumoral injections of TG6002.

The intratumoral route has been favored for a long time in oncolytic virotherapy. However, this route has one major shortcoming in that it focuses on the treatment of nonmetastatic accessible tumors. However, despite local administration, abscopal effects have been reported in both murine models and human patients after intratumoral treatment.[37–39] Abscopal responses have been described with oncolytic VACV in clinical trials,[40,41] and have also been reported in an orthotopic model of fibrosarcoma treated with intratumoral injections of VACV with *TK* and *RR* deletion and armed with *FCU1*.[39] Although VG was identified in the blood of dogs, an abscopal effect was not identified in our study. This can be explained by the reassessment of the dogs on short notice.

The intravesical route was considered for the treatment of bladder neoplasia. Intratumoral ultrasound-guided administration of OVs has been described for urothelial carcinomas.[42] However, this route of administration in veterinary medicine was reported to be associated with a risk of needle track implantation of metastases. Therefore, intravesical instillation was preferred. The urothelium forms an effective barrier in physiologic situations. However, damage to the bladder wall secondary to bladder neoplasia can facilitate intravesical drug delivery. Repeated instillations of 10^7 PFU of oncolytic VACV over 2 h have been described in four patients diagnosed with invasive bladder carcinoma.[43] Histological analyses, performed 3 days after VACV administration was consistent with viral replication.[43] In our study, the absence of viremia and VG in tumor biopsies, and the low level of immune response, were consistent with a low level of infection of bladder carcinoma after intravesical instillation of TG6002. This can be explained by a shorter instillation time in our study, a low permeability of the urothelium to TG6002, and a wash-out of the TG6002. Strategies can be considered to enhance intravesical drug delivery, such as chemical molecules (chitosan, dimethylsulfoxide) that disrupt

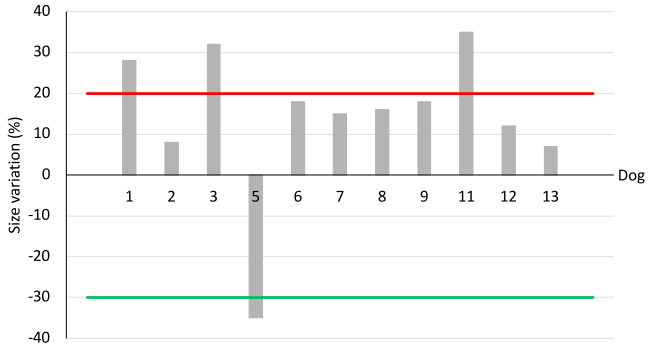

Figure 5. Tumor size variation between day 0 and day 38

Progressive disease is defined by an increase in size over 20% (red line) and partial response is defined by a decrease in size over 30% (green line).

the urothelial barrier, nanocarriers (liposomes), and drug carriers that use mucoadhesive biomaterials that adhere to urothelial cells and prevent wash-out.[44]

To overcome limitations associated with intratumoral administration, the intravenous route can be considered. Intravenous administration of TG6002 has shown a safety profile in healthy dogs.[19] However, the intravenous route may lead to a stronger immune response against OVs, thereby instigating its clearance and limiting its oncolytic activity.[45] In our study, anti-VACV antibodies and neutralizing antibodies were observed from day 7 to 28 after three intratumoral injections of TG6002 and may have limited its activity and potential abscopal effect. However, similar levels of anti-VACV antibodies and neutralizing antibodies were assessed after intravenous injections of TG6002 in healthy dogs.[19] To overcome immune neutralization and to increase intratumoral delivery, pretreatment with cyclophosphamide or ultrasound-mediated cavitation has been shown to be efficient after systemic administration.[46–48] Several studies have confirmed that combining OVs with immune checkpoint inhibitors (ICIs) makes an encouraging efficacy and shows potential for development in further research.[49] Recently, it has been shown that several FDA-approved ICIs can recognize and even block canine PD-1/PD-L1 *in vitro*, showing robust increase in the production of the activation marker IFN-γ.[50] It would therefore be appropriate to combine the administration of TG6002 and ICIs in dogs.

Our study has several limitations including the small patient population; inclusion of dogs with advanced and metastatic neoplasia; heterogeneity of tumor type; the absence or the size of biopsies at day 38 precluding histological, immunohistochemical, and intratumoral dosage of 5-FU; and study endpoint at day 38. Moreover, postmortem examination was only performed for one dog (dog 6), limiting further investigations on long-term influence of TG6002 combined with 5-FC on tumor microenvironment.

Conclusions

In conclusion, this clinical trial demonstrates viral replication, strictly intratumoral 5-FU production, and reversion of the immunosuppressive tumor microenvironment after intratumoral injections of TG6002 and oral 5-FC in dogs with spontaneous malignant neoplasia. A dose-dependent mechanism was assumed. These results support the use of TG6002 in a clinical trial in human medicine. This study strengthens spontaneous canine cancers as an effective model for drug development and emphasizes the importance of a One Health approach in oncology.

MATERIALS AND METHODS

Virus

TG6002 expressing the fusion gene *FCU1* (Δ*I4L*Δ*J2R*/FCU1 VACV) was constructed as characterized previously.[2,5] TG6002 was produced in primary chicken fibroblasts (CEFs) and purified on a sucrose gradient in accordance with standard protocol. Virus stock was titrated on CEFs by plaque assay.

5-FC

5-FC (Toronto Research Chemicals, North York, ON, Canada) was provided as capsules of 500 or 100 mg.

Study design

This study was conducted in accordance with European and French legislations on the protection of animals used for scientific purposes (Directive 2010/63/EU, 2010; Code rural, 2018; Décret no. 2013 − 118, 2013). The clinical trial (2017-12-01) was approved by the Anses/EnvA/UPEC (no. 16) Oncovet Clinical Research (no. 2497) ethical committees. Written informed consent was obtained from the pet owners.

Dogs were prospectively included at the Ecole Nationale Vétérinaire d'Alfort and Oncovet Clinical Research between January 2018 and December 2020. All dogs included in this clinical trial had a histological diagnosis of malignant, solid tumors accessible for intratumoral injections. Dogs were divided into three groups based on dose and number of TG6002 injections. Dogs displaying severe clinical signs or severe decrease in general condition were excluded from the study. Dogs treated with chemotherapy or radiation therapy within 4 weeks, or with a poor general condition or severe concomitant disease, were excluded. The schedule of the study is shown in Figure 6.

Cohort 1 received two to three weekly intratumoral injections of TG6002 at 5×10^6 PFU/kg. Cohort 2 received a single intratumoral injection of TG6002 at 5×10^7 PFU/kg. Cohort 3 received three weekly intratumoral injections of TG6002 at 5×10^7 PFU/kg. Two of these dogs received three further injections of TG6002 at 5×10^6 PFU/kg (dog 12) and 5×10^7 PFU/kg (dog 13) 223 and 186 days, respectively, after the first injection of TG6002.

Intratumoral administration of TG6002 was performed under general anesthesia. Dogs were anesthetized with intravenous administration of 0.2 mg/kg butorphanol (Torbugesic, Zoetis, Malakoff, France), 15 μg/kg medetomidine (Domitor, Orion, Espoo, Finland), 1 mg/kg propofol (Propovet, Zoetis, Malakoff, France), and isoflurane (Vetflurane, Virbac, Carros, France).

Figure 6. Study chart and sample collection
BID, twice daily.

For all tumors, except bladder neoplasia, a non-diluted batch of TG6002 was intratumorally injected. A maximum volume of 0.5 mL TG6002 per site was applied, and multiple injections were performed if the volume was over 0.5 mL TG6002 was injected under ultrasound or endoscopic guidance. For dogs diagnosed with bladder neoplasia, TG6002 was diluted in 5 mL/kg saline solution and instilled after urethral catheterization for 1 h with decubitus changes every 20 min. After 1 h, the bladder was emptied. Four days after each injection of TG6002, 5-FC was orally administered at a dose of 100 mg/kg twice daily for 3 days after the first and second injections and for 20 days after the third injection. Dogs were hospitalized for 3 days after each TG6002 injection.

Clinical toxicity and AEs

Assessment of AEs was performed according to the Veterinary Cooperative Oncology Group Common Terminology Criteria for AEs.[51] AEs were monitored by physical examination, complete blood count, and biochemical analyses at days 0, 7, 14, 21, 28, and 38.

Detection of viral DNA

VG was quantified by qPCR assay on blood, urine, cutaneous lesions, and tumor biopsies. Five milliliters of blood was collected in EDTA tubes at days 0 (before the first injection), 1, 7 (before the second injection), 8, 14 (before the third injection), 15, 20, and 38, and 1 h after each TG6002 administration (days 0, 7, and 14). For dogs diagnosed with bladder neoplasia, VG was quantified in urine at days 0, 0 + 1 h, 1, 2, 7, 7 + 1 h, 8, 9, 14, 14 + 1 h, 15, 16, 21, and 38. Cutaneous lesions were sampled with a swab (Universal Viral Transport Kit, Becton Dickinson, Franklin Lakes, NJ) or 6 mm skin punch biopsy (Skin biopsy punch 273,690, Kruuse, Langeskov, Denmark). Surgical or endoscopic tumor biopsies were performed at inclusion and at days 7, 14, and 38. Samples were stored at −80°C until analysis.

DNA was extracted from 50 µL whole blood, urine or swab using an automatic MagMax96 Deep Well (Life Technologies, Carlsbad, CA). For tumor and cutaneous biopsies, 30 mg tissue were diluted in 600 µL of PBS. qPCR was performed as previously described.[18] Samples were measured in triplicate. The limit of quantification was 15 VG copies/100 µL for whole blood samples, 30 VG copies/100 µL for urine, and 400 VG copies/30 mg for organ samples.

An elimination half-life less than 1 h has been assessed for TG6002.[18,19] Therefore, viral replication was confirmed if an increase

in VG copy number or if VG was detected after five half-lives in the blood or tumor tissue of dogs.

5-FC and 5-FU quantification in blood

5-FC and 5-FU serum concentrations were evaluated prior to 5-FC administration and 1 h after administration on days 7, 14, and 38.

Quantification of 5-FC and 5-FU was performed on serum by high-performance liquid chromatography as described previously.[20] For 5-FC, the limit of detection and the limit of quantitation were 0.39 and 0.45 mg/L, respectively. For 5-FU, the limit of detection and the limit of quantitation were 0.30 and 0.48 mg/L, respectively.

5-FU quantification in tumor biopsies

Intratumoral 5-FU concentration was assessed on tumor biopsies performed at inclusion and, if possible, at days 7, 14, and 38.

Quantification of 5-FU in tumor biopsy was performed using liquid chromatography coupled with high-resolution mass spectrometry. Tumor biopsy samples were ground with 200 µL of phosphate buffer (pH 7.4, 50 mM) in a micro-Potter device. Then, 20 µL of 5-FU $^{15}N_2$ 100 ng/mL was added. The mixture was transferred into an Eppendorf tube, and the micro-Potter device was washed with 200 µL of 1 M zinc sulfate. After 30 s of agitation, 1 mL of an acetonitrile/methanol (95/5, v/v) mixture was added before extraction with 2 mL of a solution containing ethyl acetate/isopropanol (85/15, v/v) and agitated for 10 min. After centrifugation, the organic layer was removed, transferred into a glass vial and evaporated to dryness at 37°C under a stream of nitrogen. The dry residue was reconstituted with 100 µL of water, and 10 µL was injected into the LC-HRMS device. The high-performance liquid chromatographic system consisted of an Ultimate 3000 system (ThermoElectron, San Jose, CA). Samples were separated on a Hypercarb (150 × 2.1 mm, 5 µm (ThermoElectron)) column, maintained at +30°C. Separation was performed in gradient mode using a mixture of water and acetonitrile. The mobile phase was delivered at a flow rate of 200 µL/min. Mass spectrometry detection was performed with a Q Exactive Plus high-resolution mass spectrometer (ThermoElectron). The instrument was operated in negative ion mode with an electrospray (ESI) source. Compounds were quantified using selected ion monitoring mode with a resolution set at 140,000 for ions at (m/z) 129.01058 and 131.00327 for 5-FU and 5-FU-$^{15}N_2$, respectively.

Histological and immunohistochemical analyses

Tumor biopsies were performed at inclusion and, if possible, at days 7, 14, and 38.

Histological analyses were performed on hematoxylin-eosin-saffron-stained tissue sections. Immunohistochemistry was performed using a polyclonal anti-CD3 antibody (dilution: 1:200) (DakoCytomation, Glostrup, Denmark, A0452) to detect T lymphocytes, a polyclonal anti-CD8 antibody (dilution: 1:100) (Abcam, Paris, France, ab4055) to detect cytotoxic T lymphocytes, and a monoclonal anti-Foxp3 antibody (dilution: 1:100) (eBioscience, San Diego, CA, 14-4776) to detect regulatory T lymphocytes. Staining was performed with a Leica BOND RXm Autostainer with the BOND Epitope Retrieval Solution for heat-induced epitope retrieval and Novolink Polymer Detection Systems (Leica Biosystems, Deer Park, IL). Then, TSA solution (Tyramide system amplification, Akoya Biosciences, Marlborough, MA) enabled fluorescent signal detection. Slides were digitalized with a NanoZoomer scanner (Hamamatsu, Massy, France) and digitally quantified with Calopix software (Tribvn, Châtillon, France).

Anti-VACV antibodies and neutralizing antibodies

Anti-VACV antibodies and neutralizing antibodies were assessed at days 0, 7, 14, 21, 28, and 38. Quantification of anti-VACV antibodies and neutralizing antibodies was performed as described previously.[19]

Tumor lesion response

Response to treatment was assessed by computed tomodensitometric examination at day 38.

Computed tomographic scans were performed at inclusion and at day 38. Response to treatment was assessed according to RECIST criteria.[52] Target lesions were defined as measurable lesions at the time of inclusion (minimum $\geq$10 mm). Five target lesions with a maximum of two lesions per organ were selected to characterize the tumor response. Complete response was defined as the disappearance of all target lesions. Partial response was characterized by at least a 30% reduction in the sum of the diameters of the target lesions. Stable disease was defined as a reduction of less than 30% or an increase of less than 20% in the sum of the diameters of the target lesions. Progressive disease was defined by the appearance of one or more new lesions or at least a 20% increase in the sum of the diameters of the target lesions.

The progression-free interval was defined as the time between the first TG6002 injection and the time of progressive disease assessment. Survival time was defined as the time between the first injection of TG6002 and death as a result of any cause.

DATA AND CODE AVAILABILITY

The datasets used and/or analyzed during this study are available from the corresponding author on reasonable request.

ACKNOWLEDGMENTS

This study was conducted in accordance with European and French legislations on the protection of animals used for scientific purposes (Directive 2010/63/EU, 2010; Code rural, 2018; Décret no. 2013 – 118, 2013). The clinical trial (2017-12-01) was approved by the ANSES/EnvA/UPEC (no. 16) and Oncovet Clinical Research (no. 2497) ethical committees. Written informed consent was obtained from the pet owners.

AUTHOR CONTRIBUTIONS

Conceptualization, J.B., D.T., B.K., E.Q., C.M., and P.E.; investigation, J.B., D.T., B.K., and E.Q.; data analysis, J.B., C.M., and P.E.; writing – original draft, J.B.; writing – review & editing, J.B., C.M., and P.E.; approval of final version of manuscript, J.B., B.K., E.Q., C.M., and P.E.; histological and immunohistochemical analyses, E.L. and S.C.; quantification of viral genome, 5-FC and 5-FU blood concentrations, VACV, and neutralizing antibodies, M.G., I.F., C.P. and B.M.; supervision, J.F.; resources, J.-M.B.; quantification of 5-FU in tissue, C.M and J.G.

DECLARATION OF INTERESTS

J.B., S.C., M.G., I.F., C.P., B.M., J.F., J.-M.B., E.Q., and P.E. were employees of Transgene SA when the work was performed. Transgene SA is a publicly traded French biopharmaceutical company, with Institut Mérieux as the major shareholder. The authors declare no other competing interests. J.B. is a recipient of an Industrial Training Convention for Research (CIFRE) doctoral fellowship (2017/0266).

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