






Antigen mimicry as an effective strategy to induce CSPG4-targeted immunity in dogs with oral melanoma: a veterinary trial

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ABSTRACT

Background Melanoma is the most lethal form of skin cancer in humans. Conventional therapies have limited efficacy, and overall response is still unsatisfactory considering that immune checkpoint inhibitors induce lasting clinical responses only in a low percentage of patients. This has prompted us to develop a vaccination strategy employing the tumor antigen chondroitin sulfate proteoglycan (CSPG)4 as a target.

Methods To overcome the host's unresponsiveness to the self-antigen CSPG4, we have taken advantage of the conservation of CSPG4 sequence through phylogenetic evolution, so we have used a vaccine, based on a chimeric DNA molecule encompassing both human (Hu) and dog (Do) portions of CSPG4 (HuDo-CSPG4). We have tested its safety and immunogenicity (primary objectives), along with its therapeutic efficacy (secondary outcome), in a prospective, non-randomized, veterinary clinical trial enrolling 80 client-owned dogs with surgically resected, CSPG4-positive, stage II–IV oral melanoma.

Results Vaccinated dogs developed anti-Do-CSPG4 and Hu-CSPG4 immune response. Interestingly, the antibody titer in vaccinated dogs was significantly associated with the overall survival. Our data suggest that there may be a contribution of the HuDo-CSPG4 vaccination to the improvement of survival of vaccinated dogs as compared with controls treated with conventional therapies alone.

Conclusions HuDo-CSPG4 adjuvant vaccination was safe and immunogenic in dogs with oral melanoma, with potential beneficial effects on the course of the disease. Thanks to the power of naturally occurring canine tumors as predictive models for cancer immunotherapy response, these data may represent a basis for the translation of this approach to the treatment of human patients with CSPG4-positive melanoma subtypes.

BACKGROUND

Melanoma in humans is the sixth most common cancer in the world, and its incidence has increased over the past 50 years.¹ It can affect multiple anatomical sites, defining four major subtypes, each one with distinct

clinical characteristics: cutaneous melanoma, arising in non-glabrous skin; acral melanoma, that originates in glabrous skin of the palms, soles and nail beds; mucosal melanoma, which arises from melanocytes in the mucosa; and uveal melanoma, which develops from melanocytes in the uveal tract of the eye.² Major progress has been recently made mainly in the treatment of cutaneous melanoma thanks to the introduction of BRAF/MEK-targeted³ and immune checkpoint inhibitor (ICIs)-based therapies.^{4–5} These treatments have induced impressive clinical responses in 20%–50% of patients with melanoma, nevertheless, a still high proportion of patients does not benefit clinically from these therapies.^{4–5} The other melanoma subtypes, including non-ultraviolet (UV)-induced cutaneous, mucosal and uveal melanomas, are rare and less characterized clinical entities with few therapeutic options and a very poor prognosis.^{6–8}

Tumor antigen (TA)-based vaccination strategies, able to stimulate a long-lasting antitumor immune response, could represent an effective therapeutic option for patients with melanoma. In our study, the TA used as a target is the membrane bound chondroitin sulfate proteoglycan (CSPG)4, which is a member of the CSPG family. Members of this family are key bioactive molecules that play a major role in tumor growth, migration, and neoangiogenesis.^{9–12} CSPG4 is an attractive target for antitumor vaccination, since it is highly expressed on melanoma cells in a high percentage of patients with limited inter-lesion and intra-lesion heterogeneity, independently of tumor stage and subtypes, with a restricted expression in normal tissues.^{11–14} However, CSPG4 is a non-mutated self-antigen, and as such it is poorly immunogenic.^{11–15}

In previous studies, anti-idiotypic monoclonal antibodies (mAbs) which mimic CSPG4 have been shown to be able to overcome a host's unresponsiveness to this self TA in patients with melanoma and to induce CSPG4-specific antibodies. This humoral immunity appears to have clinical relevance, since it was associated with patients' survival prolongation. In spite of these encouraging results, the anti-idiotypic mAb approach was abandoned, since the assumed lack of a cellular immune response was thought to be a major deficiency of this type of immunotherapy.¹⁶

Guided by these results, we have taken advantage of the high degree of conservation through phylogenetic evolution of CSPG4 sequence to develop an antigen mimicry DNA-based vaccination strategy. Specifically, we have generated and tested a hybrid plasmid encoding a chimeric CSPG4 protein, partially derived from the human (Hu) and partially from the dog (Do) CSPG4 sequence (HuDo-CSPG4). We have previously demonstrated that plasmids coding for chimeric proteins that include both xenogeneic and autologous domains of the target antigen delivered by *in vivo* electroporation can elicit a humoral and a cellular immune response.^{17–19}

To test the validity of our strategy we have treated dogs affected by spontaneous oral melanoma, since they represent a clinically relevant model of human non-UV-induced and 'triple wild-type' cutaneous, mucosal and uveal melanomas.^{20–22} Canine oral melanoma shares the same aggressive behavior as its human counterpart, with a high propensity to metastasize to lymph nodes and lungs, and has comparable treatment options and clinical responses.^{22–24} Moreover, dogs affected by oral melanoma have been widely used in immunotherapy trials²⁵ and led to the US Department of Agriculture (USDA)-approval of the DNA vaccine ONCEPT (Merial), carrying the sequence of the human tyrosinase, for the treatment of this canine tumor.²⁶ Importantly, a high percentage of oral canine melanomas express the CSPG4 antigen.²⁷ Therefore, our goals were the evaluation of the safety, immunogenicity, and antitumor potential of HuDo-CSPG4 vaccination in the adjuvant setting, in a prospective, multicentric, phase I, non-randomized, veterinary clinical trial enrolling 80 client-owned dogs affected by spontaneous, CSPG4-positive, stage II–IV, oral melanoma, after the surgical removal of the tumor. HuDo-CSPG4 vaccination, used in association with *in vivo* electroporation in 52 out of 80 dogs, was found to be well tolerated and immunogenic. The improvement in the overall survival of vaccinated dogs as compared with controls suggest a potential clinical benefit of adjuvant HuDo-CSPG4 vaccination for the treatment of patients affected by malignant melanoma.

METHODS

Cell lines and reagents

The canine CMM-12 and OLGA cells were derived from a primary oral melanoma²⁸ and from a metastatic

lymph node,¹¹ respectively; the human skin melanoma SK-MEL-28 cells were purchased from the American Type Culture Collection. Cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 20% fetal bovine serum (FBS, Sigma-Aldrich) and penicillin/streptomycin (both from Sigma-Aldrich) and maintained at 37°C in a 5% CO₂ atmosphere. Cell lines were routinely checked for contamination by mycoplasma using the Mycoalert Detection Kit (Lonza). CSPG4 expression by cell lines was assessed as described^{29–31} utilizing western blotting, flow cytometric analysis and immunofluorescence of cells stained with a pool of the mAbs TP32, TP49 and VF20-VT87.41, which recognize distinct and spatially distant CSPG4 epitopes.

Generation of the hybrid human/dog (HuDo)-CSPG4 plasmid

The hybrid HuDo-CSPG4 plasmid (pCDNA3.1 backbone) was generated as described.³⁰ Briefly, the first 3737bp of the Hu-CSPG4 sequence (Gene ID: 1464)²⁹ were ligated to the last 3187bp of the Do-CSPG4 sequence (Gene ID: 487658). The hybrid HuDo-CSPG4 complementary DNA was then cloned into the pCDNA3.1 plasmid and verified by sequencing (BMR Genomics). The large-scale preparation of the plasmids was carried out with EndoFree Plasmid Giga kits (Qiagen) according to *Good Laboratory Practice*. The hybrid HuDo-CSPG4 plasmid encodes for a chimeric protein which includes at the N-terminal portion the domain 1 and part of the domain 2 (amino acid, aa 1–1245) of the Hu-CSPG4 protein and part of domain 2 and the full domain 3 (aa 1246–2307) of the Do-CSPG4 protein at the C-terminal.

Dog enrollment and vaccination

Eighty client-owned dogs were enrolled following owners' informed consent during the period October 1, 2016 to June 30, 2021. The study protocol was approved by the Italian Ministry of Health (0015537-28/06/2017-DGS AF-MDS-P) and conducted at the Veterinary Teaching Hospital, University of Turin, Grugliasco (Turin), Italy, and the Tyrus Veterinary Clinic, Terni, Italy. Dogs without concurrent life-threatening diseases and with stage II (2–4cm diameter, negative lymph nodes (LN)), III (>4cm diameter and negative LN or any tumor size with ipsilateral-positive LN) and IV (any tumor size, with bilateral positive LN without distant metastasis), surgically resected CSPG4-positive oral melanomas, were included in the study. Preoperatively, full tumor staging, defined according to the tumor, node, metastases staging system by Owen,³² included a skull and three-view chest radiography and abdominal ultrasound examination and/or a total body CT. Tumor samples were immunohistochemically tested for CSPG4 expression as previously described.^{29–33} Briefly, a total score ranging from 0 to 8 was assigned to each melanoma sample considering the value assigned to the proportion of CSPG4 positively stained tumor cells (score from 0 to 5) and the average staining intensity of CSPG4-positive tumor cells (score from 0 to

3). Only dogs bearing an oral melanoma with a CSPG4 score ≥ 3 were enrolled in the study.

Dogs included in the vaccination arm were adjuvantly immunized with the HuDo-CSPG4 plasmid as previously described,²⁹ starting 2 weeks after surgery. Briefly, 500 μg of HuDo-CSPG4 plasmid per each dog, diluted in 200 μL of 0.03% NaCl solution, were injected into the muscle of the caudal thigh. Two minutes after plasmid injection, nine electric pulses (1 high voltage, amplitude 450 V, length 50 μs , frequency 3 Hz; 1 s pause; eight low-voltage amplitude 110 V, length 20 ms, pause 300 ms) were applied to the injection site using the CLINIPORATOR (Igea). Immunization was repeated after 2 weeks and then monthly, for a minimum of 4 and a maximum of 24 cycles. Clinical examinations, three-view chest radiographs and/or CT were performed before each vaccination, as well as sera and peripheral blood mononuclear cells (PBMC) were collected, whenever possible.^{29 33} The Veterinary Co-operative Oncology Group-Common Terminology Criteria for Adverse Events V.1.1, (VCOG-CTCAE)³⁴ was used to classify the adverse events.

Antibody binding assays

ELISA was performed as previously described.³⁵ Briefly, 96-well plates (Costar, Sigma-Aldrich) were coated overnight at 4°C with the recombinant D2 (Do–D2) and D3 (Do–D3) domains of the Do-CSPG4 protein (obtained from Genscript), and of the commercially available D3 (Hu-D3) domain of the Hu-CSPG4 protein (R&D Systems) (50 ng/well). Plates were then sequentially incubated with diluted canine sera (1:100) for 2 hours at 37°C and horseradish peroxidase-conjugated anti-dog IgG or IgA xenoantibodies (1:10000; both from R&D system). Plates were washed and chromogenic 3,3',5,5'-tetramethylbenzidine (Sigma-Aldrich) substrate was added. The reaction was stopped by the addition of 2N hydrochloric acid and absorbance was measured at 450 nm on a 680XR microplate reader (Bio-Rad). Vaccinated dogs were considered responders when the fold change between the O.D. at 450 nm of the post-vaccination/pre-vaccination sera was >1.1 . Avidity of anti-CSPG4 antibodies produced by vaccinated dogs was tested in a chaotropic ELISA as previously described.³⁵

For flow cytometric analysis, CMM-12 and SK-MEL-28 melanoma cells were incubated for 30 min at 4°C with canine sera diluted 1:40 in phosphate-buffered saline (PBS; Sigma-Aldrich). Total IgG and IgA binding was evaluated as previously described.²³ Samples were acquired using a BD FACSVerser (BD BioScience) and analyzed with FlowJO V.10.5.3.

Immunofluorescence staining was performed as described²⁹ using 1×10^5 CMM-12 and SK-MEL-28 cells seeded onto glass coverslips. Cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) at room temperature (RT) and blocked with PBS supplemented with 1% bovine serum albumin (Sigma-Aldrich) and 0.1% Tween-20 (Sigma-Aldrich). For internalization analysis, CMM-12 cells were seeded for 3 hours at RT onto glass coverslips

that had been previously coated with fibronectin (5 $\mu\text{g}/\text{mL}$) (Sigma-Aldrich). Cells were then incubated with canine sera (1:10) for 1 hour at 37°C, permeabilized with 0.5% saponin (Fluka), blocked for 1 hour at RT and incubated overnight at 4°C with a pool of the CSPG4-specific mAbs TP32, TP49, VF20-VT87.41 (1:40). Specific antibody binding was revealed with a goat anti-mouse IgG antibody (Alexa Fluor 568; Thermo Fisher Scientific). Nuclei were stained with 4',6-diamidino-2-phenylindole, coverslips were air-dried and mounted. Immunofluorescence images were acquired with an Eclipse 80i-ViCO system (Nikon), using a 60 \times /1.4NA oil immersion objective and analyzed using a Fiji Software (Rasband, W.S., ImageJ, US National Institutes of Health).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

CMM-12 (8×10^3)²⁸ and OLGA (5×10^3)¹¹ cells were starved for 4 hours in 96-well plates. Pooled sera (1:50) from vaccinated dogs were then added and incubation was continued for 48 and 72 hours for CMM-12 and OLGA cells, respectively. Cell viability was then evaluated as previously described.³¹

Cytofluorimetric analysis of circulating leukocytes

Thawed PBMC were incubated with human IgG to block the Fc receptor and then stained with the following mAbs: rat anti-dog CD5-fluorescein isothiocyanate (FITC), rat anti-dog CD4-RPE-cy7, rat anti-dog CD8-Pacific Blue, mouse anti-dog B cells-Alexa Fluor 647, purified mouse anti-dog CD11b, rat anti-dog major histocompatibility complex (MHC) II-FITC and mouse anti-human CD14-Alexa fluor 647 (all from AbD Serotec). To reveal CD11b positivity a secondary PE-conjugated anti-mouse IgG (DakoCytomation) was used. Samples were acquired using a BD FACSVerser (BD BioScience) and analyzed with FlowJO V.10.5.3.

Cytotoxicity assay

CMM-12 target cells (1×10^4) were labeled with 2 μM of carboxyfluorescein succinimidyl ester (CFSE, Molecular Probes) and then cultured with thawed PBMC at the effector:target ratio (E:T) of 50:1 for 48 hours at 37°C in a 5% CO₂ atmosphere. PBMC were from 19 dogs of the vaccination arm, selected based on sample accessibility. Antibody-dependent cell-mediated cytotoxicity (ADCC) was performed by incubating PBMC with CMM-12 cells at the E:T ratio of 50:1 overnight at 37°C in a 5% CO₂ atmosphere in the presence of a 1:50 dilution of canine sera collected before and after the vaccination cycles. After staining with 1 $\mu\text{g}/\text{mL}$ 7-Amino-ActinomycinD (7-AAD, BD BioSciences), cells were acquired using a BD FACSVerser and analyzed using FlowJO V.10.5.3. Percentage of killing was obtained by back-gating on the CFSE⁺ targets and measuring the percentage of 7-AAD⁺ dead cells, as previously described.³⁶ Briefly, percentage of specific lysis was calculated with the formula ((dead targets in sample (%)) – spontaneously dead targets (%)) /

(dead target maximum (%) – spontaneously dead targets (%)) × 100. Spontaneous death was obtained by culturing target cells without PBMC, whereas maximal death was obtained after treatment with 1% saponin.

Western blotting

Western blotting for CSPG4 detection was performed as previously described.^{31 33} β -Actin (Santa Cruz Biotechnology) and Vinculin (Cell Signaling) were used as protein-loading controls.

Cell migration assay

CMM-12 (2×10^4) cells were incubated with pooled sera (1:10 dilution) from vaccinated dogs for 1 hour at 37°C in a 5% CO₂ atmosphere and then seeded into the top chamber of a 24-transwell plate (8 μ m pore size; Corning). Migration assay was performed as previously described.³¹

Statistical analysis

Shapiro-Wilk or Kolmogorov Smirnov test were used to evaluate normal distribution. The non-parametric Mann-Whitney test was used when the distribution was not normal. Two-tailed unpaired and paired Student's t-tests were used to perform the statistical analyses for normally distributed data. The Kaplan-Meier method was used to estimate overall survival and disease-free interval (DFI) of dogs enrolled in the study. Differences in survival times were analyzed using the log-rank test. Pearson's correlation method was used to estimate the correlation between the antibody response determined by ELISA and survival. Statistical significance was evaluated using GraphPad V.9 software (GraphPad) and values of $p < 0.05$ were considered significant.

RESULTS

Molecular and antigenic profile of the HuDo-CSPG4 plasmid used as a vaccine

The hybrid HuDo-CSPG4 plasmid includes the N-terminal portion of the Hu-CSPG4 and the C-terminal portion of the Do-CSPG4 sequences³⁰ (online supplemental figure S1A). The predicted chimeric HuDo-CSPG4 aa sequence (online supplemental figure S1B) has 89.0% identity with the full Hu-CSPG4 sequence and 93.0% with the full Do-CSPG4 sequence.³⁷ National Institute of Health (NIH)/3T3 fibroblasts transfected with the HuDo-CSPG4 plasmid demonstrated the presence of the two CSPG4 components with the molecular weight of 250 kDa and >450 kDa (online supplemental figure S1C) as revealed by western blotting using a pool of three mAbs (the TP32, TP49 and VF20-VT87.41), recognizing distinct Hu-CSPG4 epitopes.^{38–40} Moreover, using this mAb pool, a specific binding on NIH/3T3 fibroblasts transfected with HuDo-CSPG4 was also revealed by flow cytometry analysis (online supplemental figure S1D) and immunofluorescence (online supplemental figure S1E). Overall, these results confirm that the chimeric protein is

correctly coded by the hybrid construct and expressed on the cell membrane.

In addition, sera from C57BL/6 mice vaccinated with the HuDo-CSPG4 plasmid stained murine B16 melanoma cells stably overexpressing either the Hu-CSPG4 (B16-Hu-CSPG4; online supplemental figure S2A) or the Do-CSPG4 (B16-Do-CSPG4; online supplemental figure S2B) proteins. Lastly, a significant delay of the tumor incidence was observed in HuDo-CSPG4 vaccinated mice challenged subcutaneously with either B16-Hu-CSPG4 (online supplemental figure S2C) or B16-Do-CSPG4 (online supplemental figure S2D) cells. These results are compatible with the preservation of the antigenic and immunogenic properties of both the Hu-CSPG4 and the Do-CSPG4 domains encoded by the hybrid construct.

Phase I veterinary clinical trial: eligibility criteria and patient enrollment

Eighty client-owned dogs with oral melanoma were prospectively enrolled in the study. Their principal characteristics are summarized in (online supplemental table S1).

All dogs were treated with an *en-bloc* resection of the primary tumor, with the inclusion, if feasible, of at least 2 cm of macroscopically normal tissue around the tumor, and regional lymphadenectomy. In some cases (10% of vaccinated dogs and 3.6% of controls), adjuvant radiotherapy was given in addition to surgery. Tumor samples were immunohistochemically tested for CSPG4 expression ($n=80$) (online supplemental table S1), Ki67 expression ($n=78$), mitotic index ($n=80$) and nuclear atypia ($n=70$) (online supplemental table S2). Dogs were then assigned to the adjuvant vaccination treatment group or the control one according to the owner's decision. The clinical stage distribution and the CSPG4 expression score²⁷ were similar in the two arms (online supplemental table S1, S3, S4).

HuDo-CSPG4 vaccination is safe and with potential beneficial effects on the overall survival of canine melanoma patients

HuDo-CSPG4 vaccination was started 2 weeks after surgery, repeated 14 days later and then monthly in 52 out of the 80 dogs enrolled in the study (vaccination arm) (figure 1A and online supplemental figure S3). The remaining 28 dogs that did not receive the adjuvant HuDo-CSPG4 vaccination were included in the control arm (online supplemental figure S3).

No significant changes in blood counts, body weight and temperature were detected, as well as no allergic/immunologic events were recorded throughout the entire course of the study. Sixteen out of the 22 dogs with a body weight below 15 kg exhibited transient hind/limb limping after electroporation lasting from some hours to days (grade 1 toxicity, according to the VCOG-CTCAE³⁴). No hospitalization was required for any dog.

Adjuvantly vaccinated dogs exhibited significantly longer overall survival than the control population treated with conventional therapies alone (log-rank test,

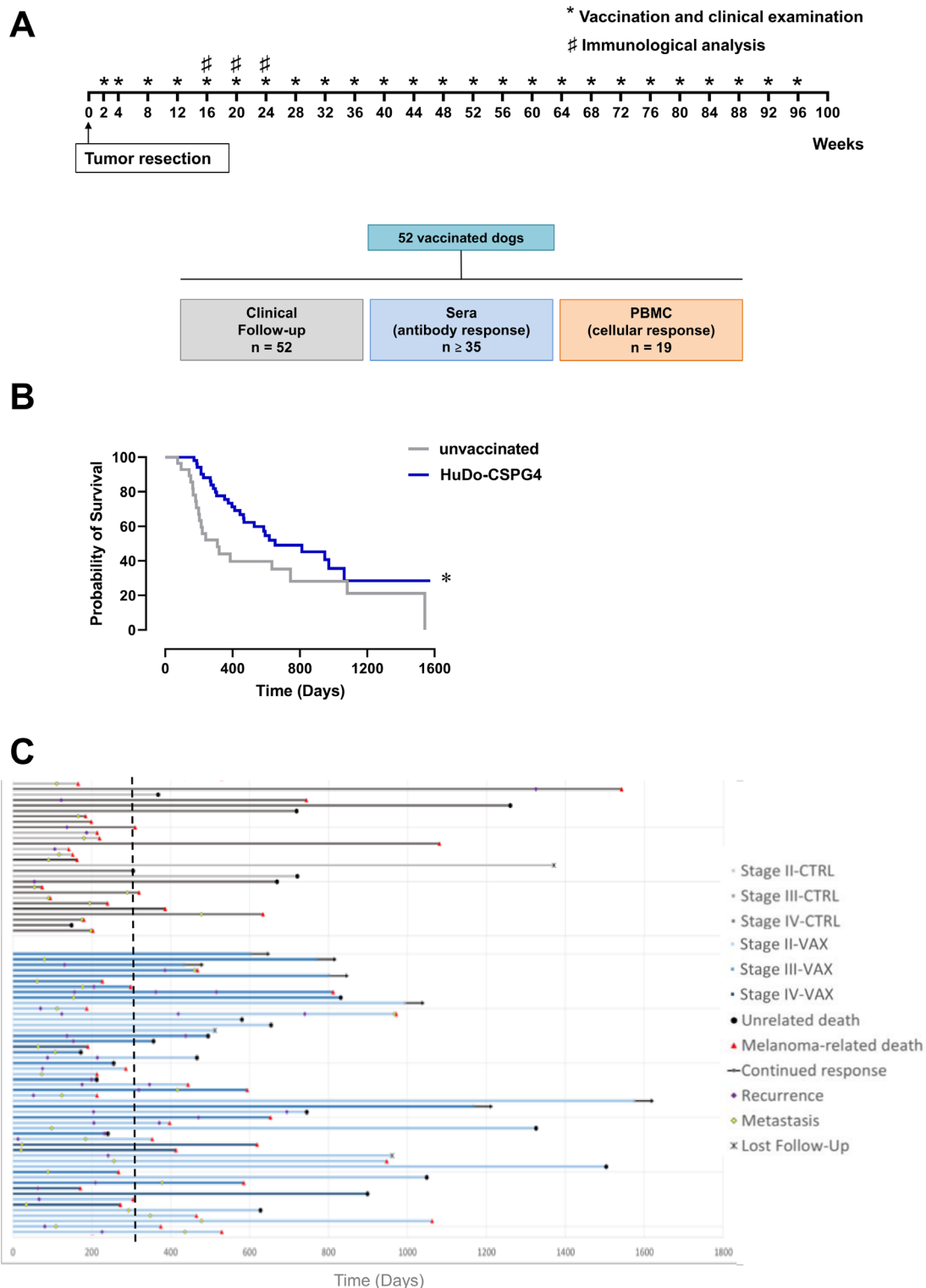


Figure 1 Adjuvant chimeric HuDo-CSPG4 vaccination improves the survival of canine patients affected by CSPG4-positive oral melanomas. (A) Immunization protocol (upper panel) and study design (lower panel). (B) Kaplan-Meier curves comparing the overall survival (in days) of HuDo-CSPG4 vaccinated (blue line) and unvaccinated (gray line) dogs, after the local control of CSPG4-positive oral melanoma, updated to December 2021. Log-rank test, $*p=0.0320$. (C) Swimmer plot depicting the overall survival of canine melanoma patients enrolled in the study. Briefly, the survival (in days) of dogs with surgically resected CSPG4-positive melanoma, either vaccinated (Vax) or non-vaccinated (Ctrl), is reported, considering the day 0 as the moment of the surgery for each dog. Arrows indicate that the patients were still alive at the time of publication (continued response). For each patient, the moment of recurrence or metastasis detection, if any has been indicated. Black dots indicate patients who died because of unrelated reasons, while red triangles indicate patients who died because of melanoma. Dogs lost in the follow-up ($n=3$) were also indicated. The median survival time (310 days) for the control group has been indicated by a dotted vertical line. CSPG4, chondroitin sulfate proteoglycan 4; PBMC, peripheral blood mononuclear cells.

Table 1 Survival times for canine melanoma patients calculated up to December 31, 2021

Groups	MST (days)	Survival (months)			
		6	12	18	24
HuDo-CSPG4 (n = 52)	653	96.15%	69.23%	50.00%	37.25%
Control (n = 28)	310	71.43%	39.29%	32.14%	21.43%

CSPG4, chondroitin sulfate proteoglycan 4 ; Do, dog; Hu, human; MST, median survival time.

* $p=0.0320$) with a median survival time (MST) of 653 and 310 days in the vaccinated and the control group, respectively (figure 1B and C and table 1).

At the end of the observation period (1575 days), 7 out of the 52 (13%) vaccinated dogs were still alive, of which 5 (71%) without evidence of recurrence nor metastasis developed during the study, while in the remaining 2 (29%) dogs we observed the regression of metastasis, and both are currently in remission. Forty-three out of the 52 (83%) vaccinated dogs died during the study and 2 dogs (4%) were lost to follow-up on day 512 and 962, respectively; the latter dog developed both a local recurrence and metastasis on day 214 and 276, respectively. Of the 43 dead dogs, 27 (63%) died because of melanoma and the remaining 16 (37%) succumbed to cancer-unrelated events. In the study period, 40 out of 52 (77%) vaccinated dogs experienced progressive disease; 15 of them (37%) developed local recurrence, 13 (33%) distant metastasis and 12 (30%) both.

In the control arm, 27 out of 28 dogs (96%) were dead at the end of the study and 1 (4%) was lost to follow-up on day 1371, with no recurrence and metastasis developed during the observation period. Out of the 27 deceased dogs, 20 (74%) died because of melanoma and 7 (26%) because of cancer-unrelated events. During the study period 22 out of the 28 (79%) dogs experienced progressive disease; 6 of them (27%) developed local recurrence, 13 (59%) distant metastasis and 3 (14%) both.

HuDo-CSPG4 is immunogenic in canine melanoma patients

Antibody response

Sera collected from HuDo-CSPG4 vaccinated dogs after the fourth immunization were tested in an ELISA to investigate the ability of the HuDo-CSPG4 vaccine to induce an antibody response against the Do-CSPG4 protein. An increased IgG binding to the recombinant Do-D2 and the Do-D3 domains in the post-vaccination as compared with pre-immunization sera was observed in 44% (figure 2A-C) and 46% (figure 2F-H) of dogs, respectively. Interestingly, in the responder dogs, a correlation between an increased IgG binding and the overall survival was observed, with statistical significance for Do-D2 responders (figure 2D,E) and a reliable trend for Do-D3 responders (figure 2I,J). Only a trend was observed between an increased IgG antibody level in responder dogs and the DFI (online supplemental figure S4A, B); although, it must be noted the reduction of the CSPG4

antigen in recurrences for most dogs (online supplemental figure S4C).

When samples were available, the anti-Do-D2 and anti-Do-D3 antibody response was measured also after the fifth and the sixth immunizations (online supplemental figure S5A, C). The percentage of responder dogs to the Do-D2 and the Do-D3 increase from 44% after the fourth vaccination (figure 2B) to 51% (online supplemental figure S5B) and from 46% (figure 2G) to 55% (online supplemental figure S5D), respectively. A significant progressive increase in the antibody levels to the Do-D2 was observed after repeated vaccinations (figure 2C).

Noteworthy, when sera from non-responder dogs were tested against the Do-D2 domain using a chaotropic ELISA, a higher percentage of IgG remained bound to the plate in 70% of the post-vaccination as compared with pre-vaccination sera analyzed (online supplemental figure S6A, B). This increased IgG avidity in the post-vaccination sera suggests that HuDo-CSPG4 vaccination can improve the spontaneous anti-CSPG4 antibody response detectable before vaccination in some of the dogs affected by CSPG4-positive melanoma. However, it must be noted that the spontaneous antibody response to both the Do-D2 and Do-D3 is not predictive of a better patient's survival (online supplemental figure S6C-H), either considering the entire canine population (online supplemental figure S6D, G) or only dogs who were responders (figure 2) to HuDo-CSPG4 vaccination (online supplemental figure S6E, H).

In view of the clinical risk of recurrence in the oral mucosa following local tumor control, we evaluated whether HuDo-CSPG4 vaccination could also induce anti-CSPG4 IgA antibodies. About 36% and 46% of vaccinated dogs developed an IgA response against the Do-D2 (online supplemental figure S7A, B) and Do-D3 (online supplemental figure S7E, F), respectively. In 71% of responder dogs, we observed the development of a local recurrence as compared with 76% in the non-responder group to the Do-D2; while 66% of dogs who respond to the Do-D3 developed a local recurrence as compared with 81% of non-responder dogs. Moreover, a positive trend between an increased antibody level and a prolonged DFI, considering the local recurrences, was found for the responder dogs to Do-D2 (online supplemental figure S7C, D), but not for those to Do-D3 (online supplemental figure S7G, H).

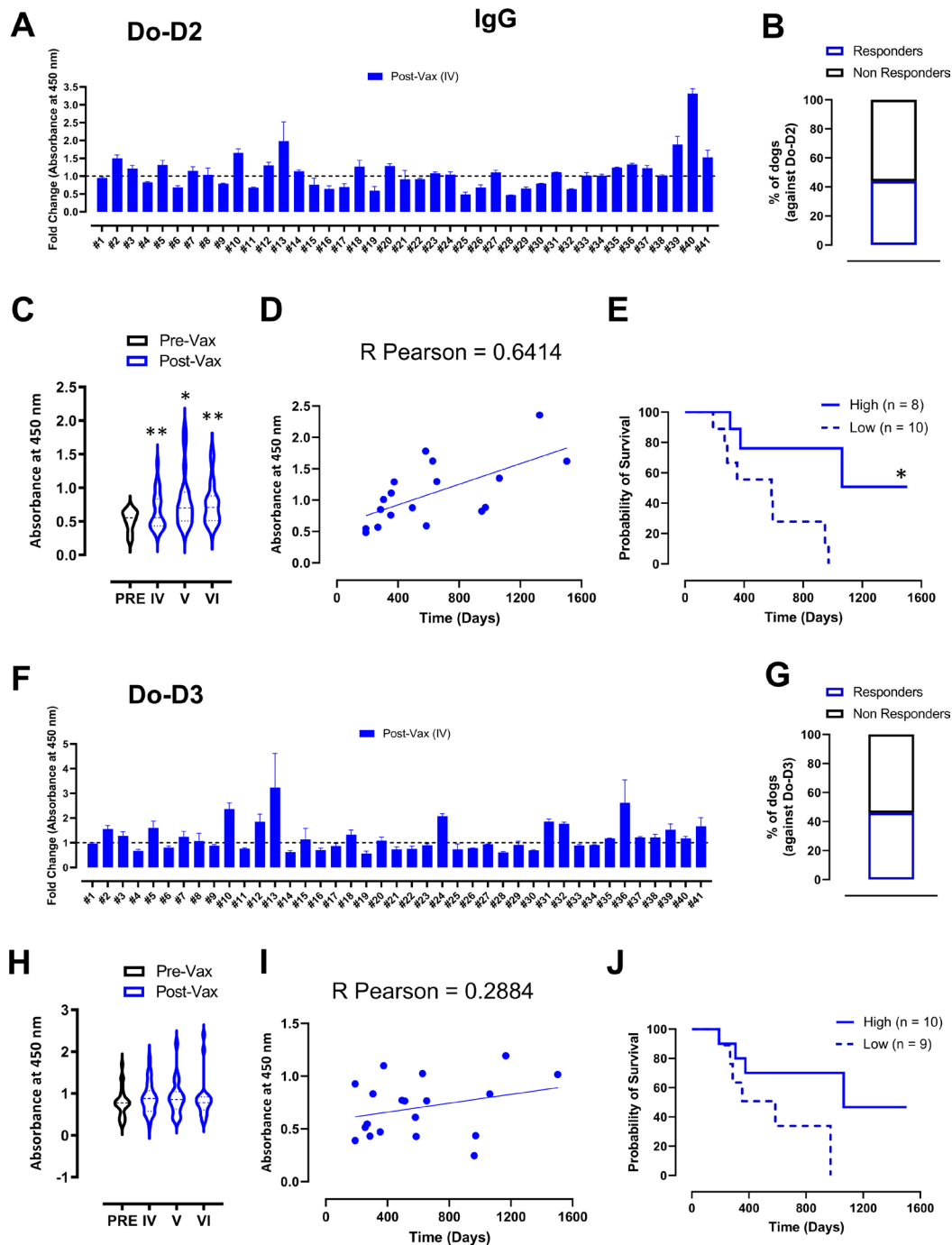


Figure 2 HuDo-CSPG4 vaccination is effective in inducing a specific anti-canine CSPG4 antibody response in dogs. (A) and (F) Analysis of the presence of IgG antibodies against the Do-D2 (A) and Do-D3 (F) domains of the canine CSPG4 protein in the sera of dogs before the first immunization (Pre-Vax, dotted black line) and after the fourth HuDo-CSPG4 vaccination (Post-Vax, blue bars), measured by ELISA. Results are expressed as the ratio (fold change) between the absorbance measured at 450 nm of the Post-Vax and the Pre-Vax sera. Each bar represents a canine patient. (B) and (G) Histograms representing the percentage of responders (blue) and non-responders (black) calculated by enumerating dogs in which sera collected after the fourth immunization displayed an increased ability to bind the Do-D2 (B) and the Do-D3 (G) domains as compared with sera collected before the first immunization. (C) and (H) Violin graphs representing the absorbance measured at 450 nm by ELISA against the Do-D2 (C) and Do-D3 (H) of pre-vaccination and post-vaccination sera from dogs who respond after the fourth, the fifth or the sixth immunizations. Student's t-test, $p^*=0-0108$, $p^{**}<0.0085$. (D) and (I) Correlation between the absorbance values measured at 450 nm by ELISA of the post-vaccination IgG of responder dogs against the Do-D2 (D) and Do-D3 (I) domains and the overall survival. Each dot represents a responder dog. Pearson correlation coefficients (r) are shown. (E) and (J) Kaplan-Meier curves correlating the overall survival of vaccinated dogs who develop (responders) a specific IgG response against the Do-D2 (E) and Do-D3 (J) domains with a high (continuous blue lines) or low (dotted blue lines) antibody level measured in their post-vaccination sera by ELISA, considering as cut-off the mean of the absorbance at 450 nm. Log-rank test, $*p=0.0120$. CSPG4, chondroitin sulfate proteoglycan 4; Do, dog; Hu, human.

Interestingly, sera collected from HuDo-CSPG4 vaccinated dogs after the fourth immunization exhibited an increased ability to stain canine CMM-12 cells, expressing the Do-CSPG4 protein in its natural conformation (online supplemental figure S8A, B), as compared with sera collected before the first vaccination (figure 3A). In 62% of the vaccinated dogs, post-vaccination sera showed a higher binding to CMM-12 cells as compared with the corresponding pre-vaccination sera (figure 3B). The increased binding of post-vaccination sera was validated by testing them with canine CMM-12 cells in immunofluorescence (figure 3C). An increased binding of vaccine-induced IgA to the canine CMM-12 cells was also found in post-vaccination sera (figure 3D).

Moreover, in 33% (online supplemental figure S9A-D) and 51% (online supplemental figure S9E-G) of HuDo-CSPG4 immunized dogs, vaccine-induced IgG and IgA, respectively, bind also the recombinant human CSPG4 D3 domain (Hu-D3), and the detection of this antibody response is associated with an improved overall and disease-free survival (online supplemental figure S9C, D, G). An increased binding of post-vaccination sera as compared with pre-vaccination sera was observed also using the human SK-MEL-28 melanoma cells as targets (figure 3E), naturally overexpressing the Hu-CSPG4 antigen (online supplemental figure S8A, B). Specifically, in 82% of HuDo-CSPG4 vaccinated dogs, post-vaccination sera displayed a higher ability to bind SK-MEL-28 cells as compared with those from the same patients before the vaccination cycle (figure 3E,F). The binding of the sera from HuDo-CSPG4 vaccinated dogs was also confirmed by testing them with SK-MEL-28 cells in immunofluorescence (figure 3G).

Cellular response

An increase in the percentage of both B and CD4⁺ T cells was observed in the peripheral blood of 63% and 53%, respectively, of the 19 analyzed dogs following the fourth HuDo-CSPG4 vaccination, as compared with that in the peripheral blood collected before the first immunization (figure 4A). Moreover, in 53% of the analyzed dogs, PBMC collected after the fourth vaccination displayed an increased percentage of CD8⁺ T cells (figure 4A), and when co-cultured with CMM-12 cells, they were significantly effective in killing CSPG4-positive tumor cells, as compared with those collected before vaccination (figure 4B). Vaccinated dogs that developed an increased cytotoxicity against CSPG4-positive canine melanoma cells (responders) displayed a longer, despite not significant, overall survival as compared with those that did not (figure 4C), with a MST of 972 days for responders as compared with 594 days for non-responder dogs. A slight increase of the DFI in responder dogs was also observed (figure 4D). Lastly, a decrease (fold change Post-Vax/Pre-Vax <1.0) in the percentage of myeloid derived suppressor cells (MDSC) was observed between the pre-vaccination and corresponding post-vaccination samples in 68% of the vaccinated dogs analyzed (figure 4A).

Mechanisms underlying the role of HuDo-CSPG4-vaccine-induced antibodies

The vaccine-induced IgG antibodies recognizing Do-CSPG4 may mediate melanoma cell elimination through an ADCC mechanism. Indeed, in 36% of the sera tested, vaccination-induced antibodies effectively mediated the killing of canine CMM-12 cells (figure 5A). In addition, post-vaccination sera induced Do-CSPG4 internalization (figure 5B) and downregulation (figure 5C), and significantly reduced the proliferative (figure 5D) and migratory (figure 5E) ability of CMM-12 cells. Post-vaccination sera were also able to inhibit the proliferation (figure 6A, left panel) of the canine melanoma cell line OLGA, naturally expressing low levels of CSPG4 (online supplemental figure S8A, B). Interestingly, no inhibition (figure 6A, right panel) was detected when OLGA cells were incubated with dog sera collected after the fourth vaccination with a fully xenogeneic Hu-CSPG4 vaccine, used in previous trials.^{29 33} This difference may reflect the induction of a higher avidity antibody response by HuDo-CSPG4 as compared with Hu-CSPG4 vaccination, as demonstrated by a chaotropic ELISA against the Do-D2 (figure 6B).

These data are supported by the clinical observation that HuDo-CSPG4 vaccination is equally effective for the treatment of melanomas with a low (<5) or high (>5) CSPG4 score (figure 6C), while the antitumor efficacy of Hu-CSPG4 vaccine, was higher for the treatment of melanomas with a CSPG4 score ≥5, weakening its efficacy against oral melanoma with lower CSPG4 positivity.³³ These results emphasize the benefit of using the chimeric HuDo-CSPG4 vaccine over the fully Hu-CSPG4.

DISCUSSION

The CSPG4 antigen is an appealing comparative immunotherapeutic target, highly expressed on melanoma cells in primary and metastatic lesions in both humans and dogs, with a pivotal role for cancer cell malignancy.¹¹ However, being a self, non-mutated tumor associated antigen (TAA), CSPG4 is poorly immunogenic in both species. To overcome this limitation, we revisited the antigen mimicry concept.

Unlike other vaccines based on the use of xenogeneic TAs as a strategy to break immune-tolerance against a self-antigen,¹⁹ including ONCEPT⁴¹⁻⁴³ and our Hu-CSPG4 DNA vaccine,^{29 33} we developed a chimeric CSPG4 DNA vaccine, HuDo-CSPG4, resulting from the fusion of the cell membrane proximal portion of the dog CSPG4 with the cell membrane distal portion of the human CSPG4, taking advantage of the high homology between human and canine CSPG4 sequences. We evaluated, as primary objectives, the safety and the immunogenicity and, as a secondary outcome, the antitumor potential of HuDo-CSPG4 vaccination in a prospective, non-randomized, veterinary clinical trial, enrolling 80 client-owned dogs affected by locally controlled oral, CSPG4-positive, stage II-IV melanoma. Dogs affected by a CSPG4-negative

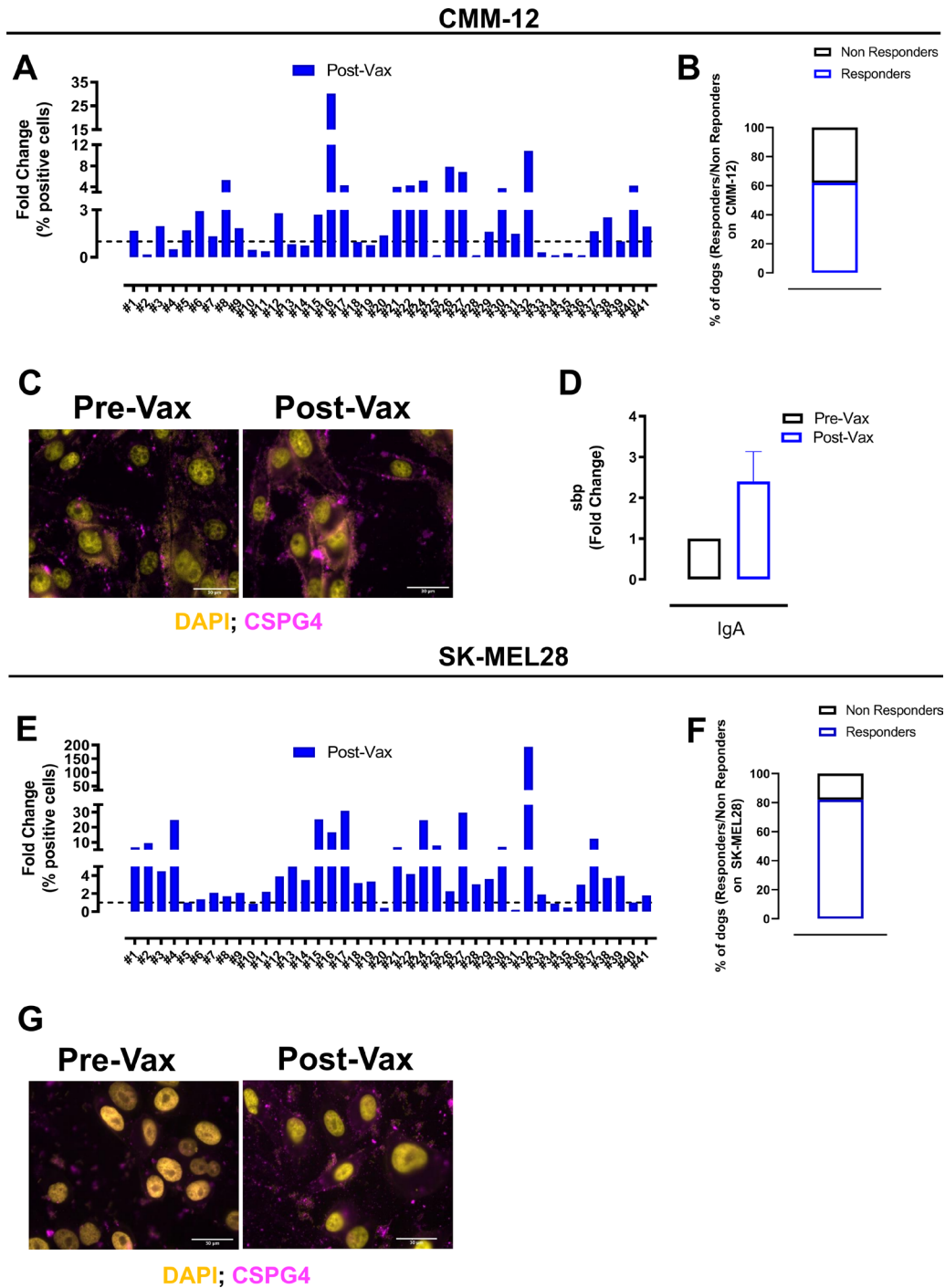


Figure 3 HuDo-CSPG4 vaccine-induced antibodies bind CSPG4-overexpressing canine and human melanoma cells. (A) and (E) Flow cytometry analysis of naturally CSPG4-expressing canine CMM-12 (A) and human SK-MEL-28 (E) melanoma cells incubated with sera from canine patients before the first immunization (Pre-Vax, dotted black line) and after the fourth HuDo-CSPG4 vaccination (Post-Vax, blue bars). Total IgG binding was evaluated using a FITC-conjugated goat anti-dog IgG secondary antibody. Results are expressed as the ratio (fold change) between the percentages (%) of stained cells incubated with the Post-Vax and the Pre-Vax sera. Each bar represents a canine patient. (B) and (F) Histograms representing the percentage of responders (blue) and non-responders (black) calculated by enumerating dogs in which sera collected after the fourth immunization displayed an increased ability (ratio >1.1) to stain the CMM-12 (B) and the SK-MEL-28 (F) melanoma cells as compared with sera collected before the first immunization. (C) and (G) Representative immunofluorescence images (one out of three independent experiments) of canine CMM-12 (C) and human SK-MEL-28 (G) cells stained with Pre-Vax and Post-Vax sera from HuDo-CSPG4 vaccinated dogs. Bound antibodies were revealed using a FITC rabbit anti-dog IgG secondary antibody and nuclei were stained with DAPI. (D) IgA specific binding of Pre-Vax and Post-Vax sera collected from HuDo-CSPG4 vaccinated dogs on canine CMM-12 cells. Results are expressed as the ratio (fold change) between the serum binding potential (sbp) of the Post-Vax and the Pre-Vax sera. CSPG4, chondroitin sulfate proteoglycan 4; DAPI, diamidino-2-phenylindole; Do, dog; Hu, human; FITC, fluorescein isothiocyanate.

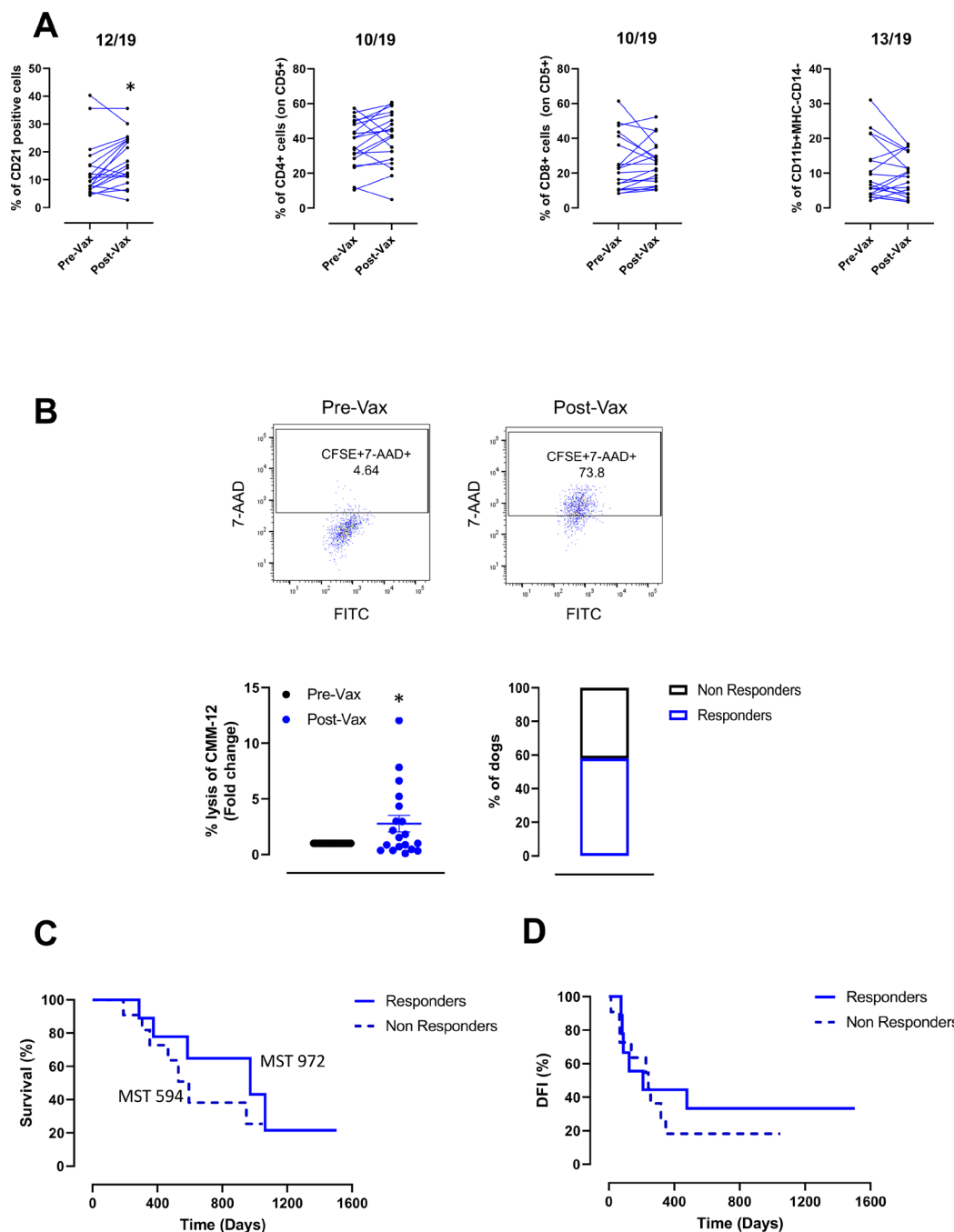


Figure 4 HuDo-CSPG4 vaccination is effective in inducing an anti-CSPG4 cellular immune response in dogs. (A) Flow cytometry analysis of the frequency of circulating B cells, CD4⁺ and CD8⁺ T cells, and MDSC collected from canine melanoma patients before (Pre-Vax) and after the fourth HuDo-CSPG4 vaccination (Post-Vax). Graphs show the percentage of CD21⁺ B cells (gated on live cells), of CD4⁺ and CD8⁺ T cells (gated on CD5⁺ cells) and of MHC-II⁻CD14⁻ (gated on CD11b⁺ cells) cells. The numbers of dogs in which a difference in the frequency (fold change >1.1 or fold change <1.1) of a cell population was observed comparing Pre-Vax and Post-Vax PBMC are indicated above in each graph. Student's t-test, *p=0.0151. (B) Cytotoxic assays to quantify the ability of Pre-Vax and Post-Vax PBMC to kill CSPG4-positive CMM-12 cells. Representative dot plots of one dog analyzed, showing the percentage of 7-AAD⁺ dead cells among CFSE⁺ cells (upper panels) are shown. Results are shown as the fold change between the percentage of CMM-12 cells lysed after incubation with Post-Vax and Pre-Vax PBMC for each dog analyzed (lower, left panel, Student's t-test, *p=0.0260), and as the percentage of dogs of which PBMC induced an increased CMM-12 cell lysis (responders) or not (non responders) (lower, right panel). (C) and (D) Kaplan-Meier curves comparing the overall survival (C) and the disease-free-interval (DFI, (D), in days, of vaccinated dogs who develop (responders, continuous blue line) or not (non responders, dotted blue line) a cytotoxic response against the canine CMM-12 cell line. The median survival times (MST) in days for each group has been reported in the overall survival graph. Log-rank test, p=0.2819. 7-AAD, 7-Amino-ActinomycinD; CSPG4, chondroitin sulfate proteoglycan 4; Do, dog; Hu, human; MDSC, myeloid derived suppressor cells; PBMC, peripheral blood mononuclear cells; FITC, fluorescein isothiocyanate; MHC, major histocompatibility complex; CFSE, carboxyfluorescein succinimidyl ester.

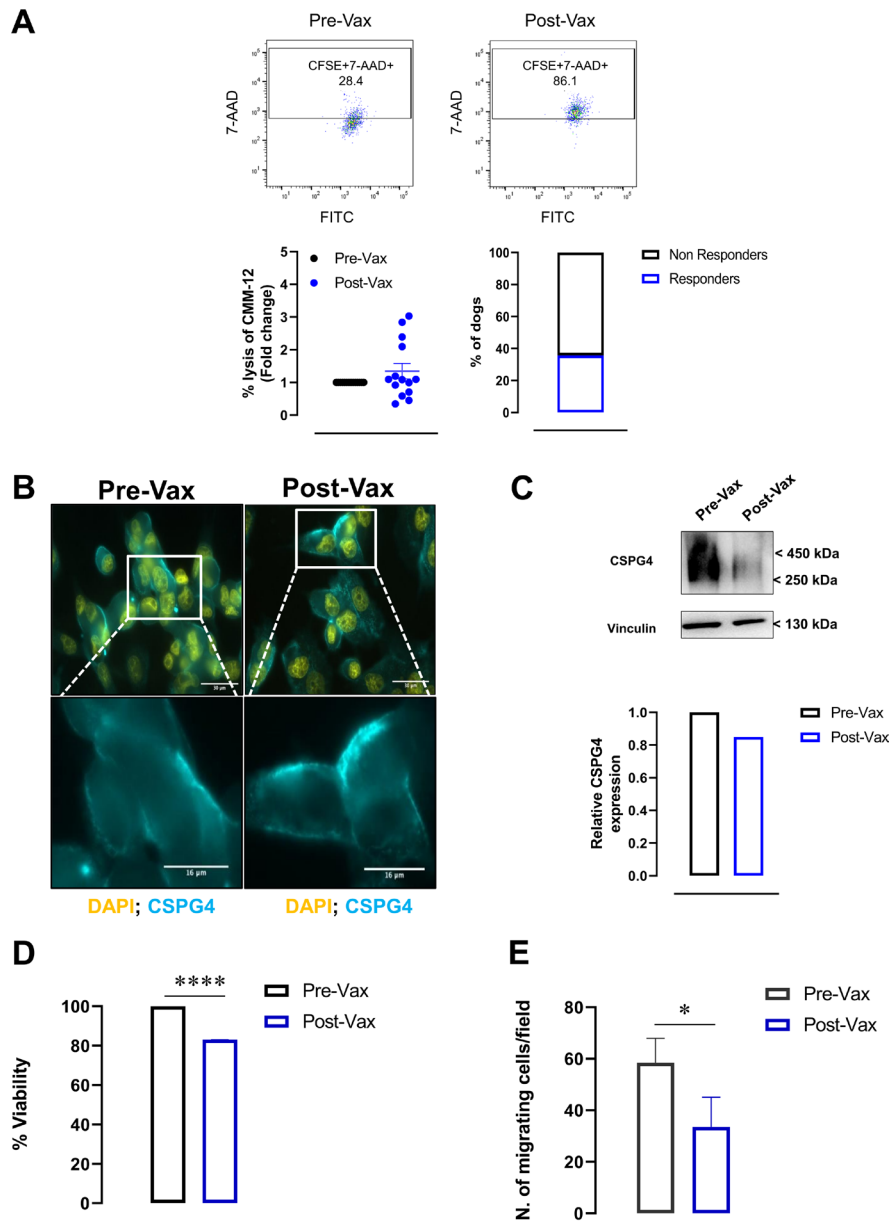


Figure 5 Potential mechanisms of action of HuDo-CSPG4 vaccine-induced antibodies. (A) Cytotoxic assay to quantify the ability of Pre-Vax and Post-Vax sera to induce the killing (ADCC) of CSPG4-positive CMM-12 cells. Representative dot plots of one dog analyzed, showing the percentage of 7-AAD⁺ dead cells among CFSE⁺ cells (upper panels) are shown. Results are reported as the fold change between the percentage of CMM-12 cells lysed after incubation with Post-Vax and Pre-Vax sera for each dog analyzed (lower, left panel), and the percentage of dogs whose sera induced an increased CMM-12 cell lysis (responders) or not (non responders) (lower right panel). (B) Representative immunofluorescence images (one out of three independent experiments) of canine CMM-12 cells incubated at 37°C with pooled sera, collected before (Pre-Vax) and after the fourth HuDo-CSPG4 vaccination (Post-Vax). Anti-CSPG4 IgG binding and localization was detected using a Texas red-conjugated anti-mouse IgG and nuclei were stained with DAPI. (C) Representative Western blot analyses (upper panel) of CSPG4 expression in the lysates of CMM-12 melanoma cells incubated at 37°C for 48 hours with pooled sera collected before (Pre-Vax) and after the fourth HuDo-CSPG4 vaccination (Post-Vax). Relative protein loading was shown using an anti-vinculin antibody. Immunoreactive band density quantification is shown (lower panel); results are reported as relative CSPG4 protein expression, considering Pre-Vax condition as 1. (D) MTT proliferation assay performed on CSPG4-positive canine CMM-12 cells after 48 hours of incubation at 37°C with pool of canine sera collected before (Pre-Vax) or after the fourth HuDo-CSPG4 vaccination (Post-Vax). Results are expressed as the percentage (%) of cell viability, considering Pre-Vax conditions as 100%. Student's t-test, ****p<0.0001. (E) Migratory ability of canine CMM-12 melanoma cells incubated with pool of canine sera collected before (Pre-Vax) or after the fourth HuDo-CSPG4 vaccination (Post-Vax). Results show the number of migrated cells in four randomly selected fields per well. Student's t-test, *p=0.0155. 7-AAD, 7-Amino-ActinomycinD; ADCC, antibody-dependent cell-mediated cytotoxicity; CSPG4, chondroitin sulfate proteoglycan 4; DAPI, diamidino-2-phenylindole; Do, dog; Hu, human; FITC, fluorescein isothiocyanate; MTT, 3-(4, 5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide; CFSE, carboxyfluorescein succinimidyl ester.

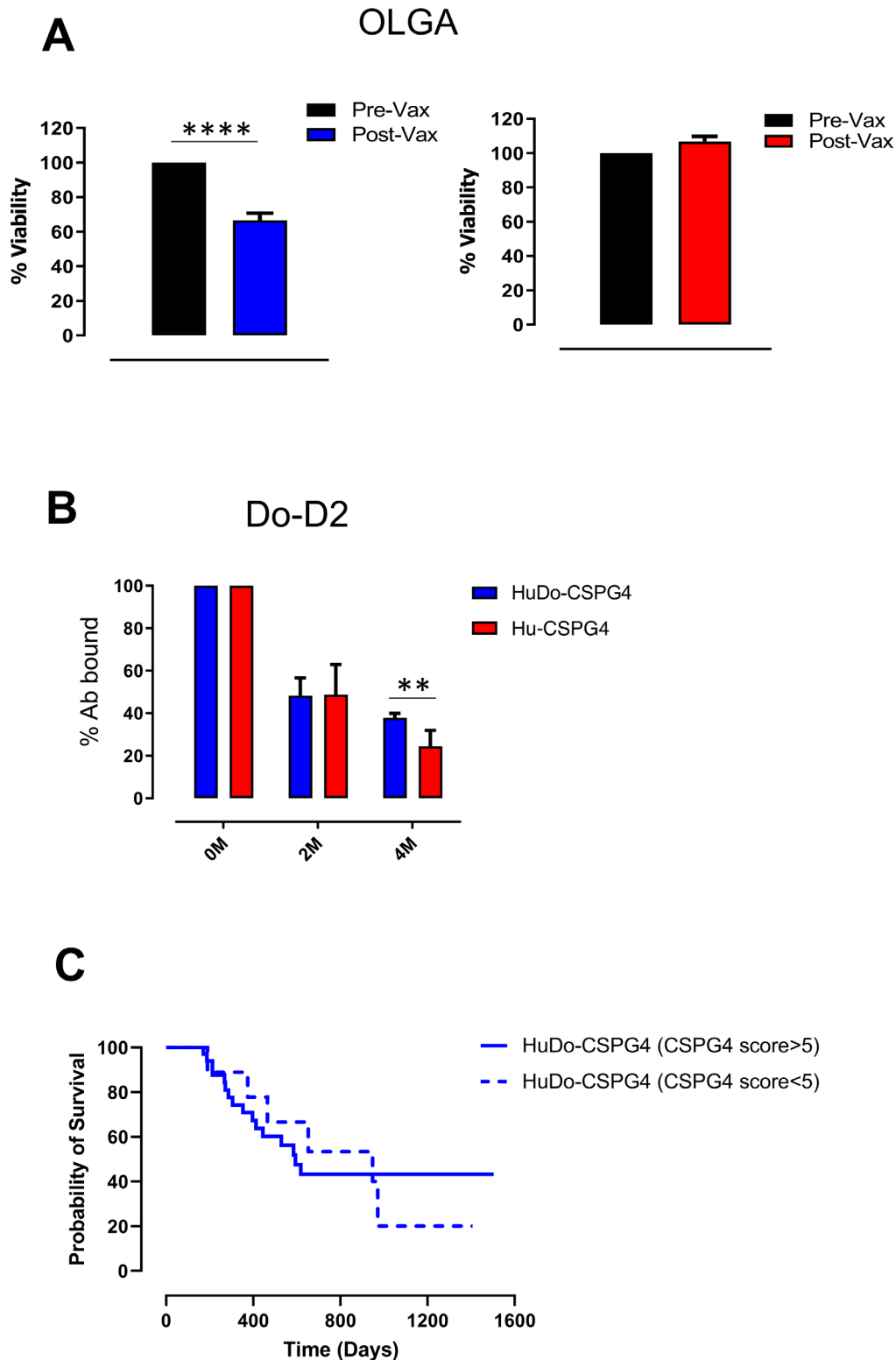


Figure 6 HuDo-CSPG4 vaccination induces a high avidity antibody response. (A) MTT proliferation assay performed on a low CSPG4-expressing canine melanoma cell line (OLGA) incubated for 72 hours at 37°C with pool of canine sera collected before (Pre-Vax) or after the fourth HuDo-CSPG4 (blue bars) or Hu-CSPG4 (red bars) vaccination. Results are expressed as percentage (%) of viability, considering Pre-Vax conditions as 100%. Student's t-test, **** $p=0.0002$. (B) Avidity of anti-Do-D2 vaccine-induced antibodies in the sera of dogs immunized with either the HuDo-CSPG4 (blue bars) or the (Hu)-CSPG4 (red bars) plasmids, evaluated by a chaotropic ELISA. Results are expressed as percentage (%) of antibodies (Ab) that remain bound after the treatment with the chaotropic agent, as compared with the medium alone considered as 100%. Student's t-test, ** $p=0.0050$. (C) Kaplan-Meier curves comparing overall survival of HuDo-CSPG4 vaccinated dogs bearing a melanoma with CSPG4-positivity score <5 (dotted blue line) and ≥ 5 (continuous blue line). CSPG4, chondroitin sulfate proteoglycan 4; Do, dog; Hu, human; MTT, 3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide).

melanoma were not enrolled, since they could not benefit from CSPG4-immune-targeting and display a better prognosis as compared with CSPG4-positive melanoma-affected dogs.²⁹

HuDo-CSPG4 vaccination was effective in inducing an antibody response against the canine CSPG4 protein. After four immunizations, an increased IgG antibody response against both the recombinant D2 and D3 domains of Do-CSPG4 and the native Do-CSPG4 protein was detected in the post-vaccination as compared with pre-vaccination sera in a high percentage of dogs. It must be noted that a spontaneous low affinity anti-CSPG4 antibody response in melanoma-bearing dogs after the local control of the tumor and before vaccination was detected, however this is not predictive for the outcome. This would have resulted in an underestimation of the percentage of responder dogs in our trial. Indeed, spontaneous anticancer autoantibodies are present in patients with a variety of malignancies, including melanoma.^{12 44} However, in dogs with this spontaneous antibody response we observed the production of antibodies with a higher avidity for CSPG4 after immunization, highlighting the benefit of HuDo-CSPG4 vaccination in improving the quality of the antibody response over the quantity. In addition, for most of the dogs, a further increase in vaccine-induced antibodies was detected following subsequent vaccinations (after the fifth and the sixth immunization cycles). Therefore, we cannot exclude the possibility that, for some dogs, other time points than those that we selected^{29 33} would have been more informative as to the development of anti-CSPG4 antibodies, also considering the differences among dogs, including size and age.

However, the finding of a vaccine-induced anti-Do-CSPG4 antibody response is likely to reflect the ability of the antigen mimicry strategy to overcome host's unresponsiveness to the self-CSPG4, thanks to 86% homology in the aa sequence of the human moiety inside the chimeric HuDo-CSPG4 sequence with its dog counterpart. Under our experimental conditions, the immune clones triggered by the Hu-CSPG4 moiety which cross react with the self-antigen may be amplified by the Do-CSPG4 moiety encoded in the chimeric HuDo-CSPG4. This mechanism may account for the herein observed higher avidity and more marked functional effects of the anti-Do-CSPG4 antibodies elicited by the chimeric HuDo-CSPG4 as compared with those elicited in dogs by immunization with the fully Hu-CSPG4 vaccine, used in a previous pilot veterinary trial.^{29 33 45}

The anti-CSPG4 immune response elicited appears to be clinically relevant in the immunized population, since a significant correlation was observed between the vaccine-induced IgG level of responder dogs and the overall survival. In addition, the induction of a mucosal immunity, potentially relevant for the treatment of oral malignancies, was suggested by the detection of anti-CSPG4 IgA in the serum of a high percentage of the vaccinated dogs. Ultimately, these results allow to speculate that the induction of anti-CSPG4 IgA may be partially

protective against local recurrences. Flow cytometry and immunofluorescence analysis demonstrated that vaccine-induced antibodies can bind the Do-CSPG4 antigen over-expressed in its natural conformation on canine CMM-12 melanoma cells, and this is important to assume their effectiveness in mediating antitumor activities *in vivo*. As suggested by our *in vitro* results, anti-CSPG4 antibodies that we have detected in the immunized dogs are likely to mediate multiple mechanisms. They include the elimination of melanoma cells by an ADCC mechanism, CSPG4 downregulation, and inhibition of its role in the biology and functional properties of melanoma cells. Such effective anti-CSPG4 antibody response may overcome the ability of melanoma cells to downregulate MHC-I molecules and escape from T cells.⁴⁶

Finally, an increased percentage of B and CD4⁺ T cells in the PBMC of vaccinated dogs was observed. A cytotoxic activity of PMBC against canine CMM-12 melanoma cells was found in 11 out of 19 vaccinated dogs analyzed. This response in the immunized population is associated with a better overall survival. Moreover, the reduction in circulating MDSC in the majority of analyzed vaccinees, suggest that, following HuDo-CSPG4 vaccination, the immunosuppression that persists after the local control of the tumor can be, at least partially, reduced.

Based on the evidences of a detectable and effective vaccine-induced immune response, the potential clinical consequences of HuDo-CSPG4 vaccination have been evaluated, considering the overall survival as the most objective measure, for both arms, based on the design of the trial.⁴⁷

The DFI instead was not considered as a proper clinical endpoint for this study, since it might be affected by the timing and, potentially, by the different imaging diagnostic procedures adopted. Indeed, both X-rays and CT scan were used for diagnosis, but the different methods depended on the improvement of diagnostic techniques over time, and on the owners' financial resources. While no differences regarding the staging system at baseline were observed between the two arms (ie, vaccinated vs control dogs), in accordance with owners' decision, unvaccinated dogs underwent only a 3–6 monthly check-up, thus potentially limiting the early detection of local recurrence and metastasis as compared with dogs of the vaccinated arm. This makes the evaluation of the overall survival, rather than the DFI, the sole reasonable endpoint for analysis and comparison with the vaccinated dogs in this study.

The adjuvant HuDo-CSPG4 vaccination envisages a potential benefit on the overall survival of immunized dogs as compared with unvaccinated controls, treated with conventional therapies alone (surgery with or without radiotherapy), prompting its more extensive evaluation in a randomized trial.

Beside these promising results, one evidence which is noteworthy for its clinical application is the lack or limited side effects of vaccination observed in HuDo-CSPG4 vaccinated dogs. These data parallel the lack of

toxicity described in human melanoma patients and rats with chemically induced chondrosarcoma immunized with CSPG4 mimics.^{48–51} These results altogether argue against the broad distribution of CSPG4 in normal tissues reported in the Protein Atlas and support the validity of the studies which have shown that the expression of CSPG4 in normal tissues is restricted to activated pericytes in the tumor microenvironment.^{12 14 52}

Some limitations of this study need to be surmised. As mentioned above, these include the lack of randomization, with the inherent potential problems of selection bias and not-blinded outcome evaluation. Unfortunately, a randomization was not possible in this study. In view of the promising results of our previous veterinary trials, using a Hu-CSPG4 vaccine for the treatment of canine melanoma patients, for clinicians it would have been difficult for ethical reasons to randomly assign patients; concurrently, it should be noted that the dog owners always refuse to accept the possibility that their dogs may enter the non-vaccinated arm. Unlike what is expected in the human clinics, the veterinary medicine scenario is different and specifically in this case no dedicated funds covering the expenses in both vaccinated and non-vaccinated arms of dogs were available; thus, the non-vaccinated arm was made up of dogs whose owners were not available to proceed with further therapies other than surgery. Nevertheless, it has to be considered that ONCEPT, the first antitumor vaccine licensed for dogs with locally controlled oral melanoma, was USDA-approved starting from the results of similar non-randomized, retrospective studies.^{42 53} Also, more recent reports on ONCEPT efficacy are non-randomized, uncontrolled, and retrospective studies.^{54–56} Other limitations of our study are the use of a single DNA dose and a single administration procedure as well as the search of anti-CSPG4 IgA in the serum rather than in the mucosal compartment. The small number of enrolled dogs affected by metastatic melanoma is another limitation. Enrollment of other melanoma-bearing dogs with either local or distant metastasis is warranted to evaluate the potential benefit of HuDo-CSPG4 vaccination also in a metastatic setting. Finally, a deeper characterization of circulating and tumor-infiltrating cells at diagnosis and after HuDo-CSPG4 vaccination, including T-regulatory cells, as well as a better dissection of the vaccine-induced cellular immunity, is needed.

By contrast, the relatively high number of dogs enrolled in the prospective study, the long-term follow-up, and the chimeric structure of the proposed HuDo-CSPG4 vaccine that we showed herein to be effective at breaking tolerance to self-CSPG4 in dogs, represent important strengths of this study.

Nevertheless, since disease progression has been observed in some vaccinated dogs, possibly also owed to the escape of CSPG4-negative clones as the result of antigen loss due to the immunological pressure exerted by the vaccine, the identification of other key targetable antigens may be relevant to the design of more effective and multimodal treatments. In addition, since the

expression of programmed cell death 1 and programmed death ligand 1 has also been detected in canine melanoma patients, combinatorial approaches using ICIs plus anti-CSPG4 vaccination in this comparative oncology model should be investigated.^{57 58}

Lastly, it should be noted that no BRAF mutations, which occur in approximately 50% of human cutaneous melanomas,³ have been detected in melanoma-bearing dogs. Still, a significant proportion of human cutaneous melanoma, as well as almost all uveal and mucosal melanomas, do not show any BRAF alterations but overexpress CSPG4.^{22 24}

The results from this veterinary trial suggest that the anti-CSPG4 therapy may represent a new therapeutic possibility for the treatment of these tumor subtypes that behave more aggressively and have less favorable prognosis.

In summary, the application of a novel anti-CSPG4-mimicry strategy, based on the use of a hybrid DNA vaccine coding for a human/dog CSPG4 (HuDo-CSPG4) chimera, resulted safe and immunogenic, displaying a potential clinical benefit in prolonging the survival of CSPG4-positive oral melanoma-affected dogs. Ultimately, thanks to the highly recognized predictive power of comparative veterinary studies and to the structure of the vaccine, these findings justify exploring the possibility to translate the chimeric CSPG4 treatment also to the human clinics. Finally, it should be noted that HuDo-CSPG4 vaccination could be extended to other CSPG4-positive cancers in both canine and human patients.

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