A chimeric human/dog-DNA vaccine against CSPG4 induces immunity with therapeutic potential in comparative preclinical models of osteosarcoma

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Short title. CSPG4 DNA vaccine for osteosarcoma treatment

Abstract

The high mortality rate of osteosarcoma (OSA) patients highlights the requirement of alternative strategies. The young age of patients, the rarity and aggressiveness of the disease limits opportunities for the robust testing of novel therapies, suggesting the need for valuable preclinical systems. Having previously shown the overexpression of the chondroitin sulfate proteoglycan (CSPG)4 in OSA, herein the functional consequences of its downmodulation in human OSA cells were evaluated *in vitro*, with a significant impairment of cell proliferation, migration and osteosphere generation. The potential of a chimeric human/dog (HuDo)-CSPG4 DNA vaccine was explored in translational comparative OSA models, including human xenograft mouse models and canine patients affected by spontaneous OSA. The adoptive transfer of HuDo-CSPG4-vaccine-induced $CD8^+$ T cells and sera in immunodeficient human-OSA-bearing mice delayed tumor growth and metastasis development. HuDo-CSPG4 vaccination resulted safe and effective in inducing anti-CSPG4 immunity in OSA-affected dogs, which displayed prolonged survival as compared to controls. Finally, HuDo-CSPG4 was also able to induce a cytotoxic response in a human surrogate setting *in vitro*. On the basis of these results and the high predictive value of spontaneous OSA in dogs, this study paves the way for a possible translation of this approach to humans.

Introduction

Osteosarcoma (OSA) is a rare pediatric cancer of mesenchymal origin that accounts for 56% of bone tumors in the childhood population.[1] In the case of localized disease, the surgical excision of the primary tumor and the adoption of multi-agent neoadjuvant/adjuvant chemotherapy is curative in up to 70% of patients.[2] However, OSA has a high tendency to recur and metastasize, mainly to the lungs, despite these treatments.[2] Patients that bear undetectable micrometastases at diagnosis or develop metastatic lesions after standard treatment are generally characterized by chemotherapy resistance and treatment failure. Advanced disease is usually incurable in these cases, and the only therapeutic option that remains is palliation, with 5-year survival rates dropping to 20-30%.[3-5]

Immune checkpoint inhibitors (ICI) have revolutionized the management of cancer patients affected by highly immunogenic solid tumors. Unfortunately, OSA is a cold tumor for which ICI have limited clinical activity.[4,6] Therefore, additional therapeutic options to be used alone or in combinatorial approaches, must be urgently identified.

Both the rarity of the disease and the young age of patients limit opportunities to robustly test new therapeutic approaches, and comparative oncology has recently gained significant prominence in the OSA panorama also for this reason.[7,8] In dogs, OSA represents 85-90% of all primary malignant bone tumors and, interestingly, has an incidence that is 27-times higher than in humans.[9,10] Human and canine OSA display common molecular alterations and signaling pathway dysregulations, resulting in similar, aggressive clinical behavior with superimposable responses to the same conventional therapies (i.e. surgery and chemotherapy).[8,11] It can therefore be stated that canine OSA is now widely recognized as a good and reliable model for testing innovative therapeutic approaches, with a strong translational perspective.

Of the tumor-associated antigens (TAA) that are shared by human and canine OSA, we herein focus on the chondroitin sulfate proteoglycan (CSPG)4, a cell surface proteoglycan with a demonstrated oncogenic role in several tumor histotypes[12,13] and that has recently been proposed as a potential clinically relevant target in OSA.[14,15] Indeed, we have recently demonstrated that CSPG4 is highly

expressed in OSA cell lines and in their derived osteospheres, which are enriched in cancer stem cells (CSC), while it is not expressed in osteoblasts or in other normal tissues.[15-17] Moreover, we found that CSPG4 overexpression has been related to worse prognosis in human OSA patients.[15] These features and its cell-surface localization make CSPG4 an ideal target for anti-cancer immunotherapy, as $CSPG4^+$ cancer cells could be simultaneously targeted by CSPG4-specific humoral and cellular immunity.[18,19] Moreover, we have previously shown that CSPG4 overexpression in spontaneous canine OSA is correlated with a poor survival,[15] laying the basis for advancing investigations on the potential of anti-CSPG4 immunotherapy in OSA-bearing dogs, to finally establish the rational for its translation to humans.

Herein, the safety and immunogenicity data concerning the evaluation of a chimeric human/dog CSPG4-targeting DNA vaccine (HuDo-CSPG4)[19] in human OSA xenograft mouse models and in a pilot veterinary trial enrolling client-owned OSA-affected dogs are reported. To the best of our knowledge, this is the first study that investigates the effects of CSPG4 targeting for the treatment of OSA *in vivo*. The results confirm that CSPG4 is an appealing comparative immunotherapeutic target and that anti-CSPG4 vaccination might cooperate with standard chemotherapeutic treatments to improve clinical outcomes of OSA patients. These positive outcomes might prompt a further evaluation of HuDo-CSPG4 vaccination as a possible novel option for human OSA treatment.

Results

CSPG4 silencing in human OSA cell lines results in significant losses in proliferative, migratory, and osteosphere-generation capacity

CSPG4 was silenced in human U2OS OSA cells by means of human (h)CSPG4-siRNA to directly demonstrate its involvement in sustaining several tumor-related processes in OSA. Silencing efficiency was monitored at three different time points (24h, 48h and 72h) using RT-PCR and Western Blot analyses. Decreased levels of CSPG4 mRNA (**Figure 1A**) and protein expression (**Figure 1B**) were confirmed from 24h post transfection and were still constant at 72h. U2OS cells silenced for CSPG4 expression showed significantly decreased proliferative ability (**Figure 1C**) and migratory potential (**Figure 1D**), compared to cells treated with the scramble siRNA. CSC-enriched osteospheres were then generated (**Figure 1E**). CSPG4-silenced U2OS cells displayed a lower ability to generate osteospheres than cells transfected with the scramble siRNA (**Figure 1F, G**). These results were validated in another human OSA cell line, SaOS2 (**Figure S1**). Overall, these data highlight the functional role that CSPG4 plays in the biological behavior of human OSA cells, indicating that CSPG4 targeting may be a relevant option for the treatment of $CSPG4^+$ OSA.

HuDo-CSPG4 DNA vaccination controls OSA growth and metastatic spread in human xenograft mouse models

The anti-tumor potential of HuDo-CSPG4 vaccination was explored in a xenotransplant mouse model of human OSA. U2OS OSA cells were injected subcutaneously into NOD/SCID/$\gamma c^{-/-}$ (NSG) mice and, once the tumors had reached a volume of 0.5 mm^3, $CD8^+$ T cells that had been isolated from the spleens of either empty vector- or HuDo-CSPG4-immunized HLA-A2.1 matched transgenic (Tg) mice were adoptively transferred intravenously, and tumor growth was monitored (**Figure 2A**). OSA-bearing NSG mice that received $CD8^+$ T cells from HuDo-CSPG4 vaccinated animals displayed a significant slowdown in tumor growth compared to those that received $CD8^+$ T cells from controls, which were immunized with the empty pCDNA3.1 plasmid (**Figure 2B; Figure S2A, B**). Only one

of the 6 (17%) mice in the HuDo-CSPG4 vaccinated group showed delayed, but however progressive, tumor growth; the stabilization of tumor growth was observed in 4 mice (67%), while the last mouse displayed consistent tumor regression (**Figure S2B**). Interestingly, the residual lesion collected from this mouse revealed no CSPG4 expression when tested by Western Blot analyses (HuDo#1), while the other tumors collected from mice that underwent HuDo-CSPG4-$CD8^+$ T cell transfer displayed lower CSPG4 expression, despite their growth, than the growing tumors collected from control mice (**Figure S2C**), suggesting that CSPG4-expressing tumor cells were being killed. An IHC analysis of explanted tumors revealed that transferred T lymphocytes derived from HuDo-CSPG4-vaccinated mice infiltrated the tumor mass at different rates, with higher lymphocyte infiltration evident in the more responsive tumors (**Figure 2C** and **Figure S2D**). On the other hand, T cells derived from the control group resided in the periphery rather than in the tumor core (**Figure 2C** and **Figure S2D**), while higher levels of circulating, rather than tumor-infiltrating, $CD8^+$ T cells were observed in mice with larger tumors (**Figure S2D, E**). Overall, these results suggest that $CD8^+$ T cells can hamper tumor growth when they infiltrate the mass, even achieving tumor regression when massive infiltration into the tumor core occurs.

To discover the potential mechanism by which some tumors may grow despite anti-CSPG4-$CD8^+$ T cell adoptive transfer, the expression of PD-L1 and B7-H3 checkpoint molecules was analyzed. While PD-L1 expression was quite stable in the OSA of the control group, its levels were inversely related to tumor volumes in the HuDo-CSPG4 group, with higher PD-L1 expression in smaller and highly infiltrated tumors. Conversely, B7-H3 expression was higher in larger and less-infiltrated tumors, compared to smaller and highly infiltrated ones (**Figure S2C**), suggesting that B7-H3 may possibly play a prominent role in hampering T cell infiltration and tumor regression.

The lungs explanted from mice bearing a tumor of similar volume were then analyzed. Animals that received $CD8^+$ T cells from HuDo-CSPG4 vaccinated mice were found to be free from metastases, while lungs collected from the controls showed $CSPG4^+$ pre-metastatic/metastatic pulmonary lesions (**Figure 2C, D** and **Figure S2D**). This evidence suggests the possibility of HuDo-CSPG4 induced

134 $CD8^+$ T cells playing a role in hindering the primary tumors, and in halting the metastatic spreading
135 of OSA cells.
136 Subsequently, NSG mice carrying a U2OS-derived subcutaneous tumor of 0.5 mm^3 in volume were
137 passively transferred with sera collected from either empty vector- or HuDo-CSPG4 immunized mice
138 (**Figure 2E**) to evaluate the anti-tumor potential of vaccine-induced antibodies. According to the
139 literature,[20] flow cytometry analyses have revealed that Balb/c mice vaccinated with a HuDo-CSPG4
140 plasmid developed a higher antibody response against Hu-CSPG4 than C57/BL6 mice (**Figure S2F**);
141 Balb/c mice were therefore chosen for this experimental vaccination setting. Two weeks after the last
142 immunization, sera were collected and pooled together for intraperitoneal injection into tumor-
143 bearing NSG mice (**Figure 2E**). Although no significant differences were observed, a trend of
144 reduced tumor growth was noted in mice that were treated with sera from HuDo-CSPG4-vaccinated,
145 rather than control mice (**Figure 2F** and **Figure S2G, H**). Lower CSPG4 expression was observed in
146 primary tumors after treatment with sera derived from HuDo-CSPG4 vaccinated mice, than in
147 volume-matched control tumors (**Figure S2I**). Interestingly, mice that received sera from HuDo-
148 CSPG4-vaccinated animals displayed metastasis-free lungs, while control mice displayed pulmonary
149 CSPG4-positive metastases (**Figure 2G, H** and **Figure S2L**). These results indicate that anti-CSPG4
150 antibodies contribute to a potential benefit against tumor growth and metastasis.
151
152 **Pilot veterinary study**
153 The enrolled population included 12 dogs affected by histological grade I-III $CSPG4^+$ appendicular
154 OSA. The main patient characteristics are listed in **Table 1**. All eligible dogs received standard of
155 care treatment. Dogs free of metastasis at restaging (chest radiographs/abdominal ultrasound and/or
156 total body CT scan) at the end of the chemotherapeutic protocol (4-6 cycles of adjuvant carboplatin)
157 were enrolled in the study and underwent to the adjuvant vaccination protocol, with the written
158 consent signed by the owners. Dogs were adjuvantly treated with the HuDo-CSPG4 vaccine (**Figure**
159 **S3A**), as previously described[19,21,22] and assessed for clinical signs of toxicity (i.e., temperature, body

weight, lethargy, lameness, etc.) and clinicopathologic parameters (i.e., complete blood count and/or biochemistry panel and/or urinalysis, when clinically indicated). No toxicity, as defined by VCOG-CTCAE V.2., was observed over the entire vaccination period.

HuDo-CSPG4 vaccination is immunogenic in canine OSA patients

Humoral response

The vaccine-induced antibody response was evaluated in the sera of vaccinated dogs, collected before the first (Pre-Vax) and after the fourth (Post-Vax) vaccination. Sera were tested by ELISA for the presence of IgG against three different domains (D1, D2, D3)[19] of Do-CSPG4 (**Figure 3A; Table S1; Figure S4**). A spontaneous and detectable anti-CSPG4 antibody response was present in most $CSPG4^+$ OSA patients even before vaccination, probably because of the chemotherapeutic treatment (**Figure S3B**).

An increase in IgG response following HuDo-CSPG4 vaccination against at least one of the Do-CSPG4 domains was observed in 5 out of 12 vaccinated dogs (41.66%) (**Figure 3A, B; Table S1; Figure S4**). Of these 5 responding dogs, 2 (16.66% of the vaccinees) displayed an increased IgG response against all the Do-CSPG4 domains in the Post-Vax as compared to Pre-Vax sera. In the Post-Vax sera of the other 3 dogs (23.07%) increased IgG binding was detected only against the D2 domain (partial response, **Figure 3A; Table S1; Figure S4**). The sera from the 7 non-responding dogs (**Figure 3A; Table S1; Figure S4**) were tested in a chaotropic ELISA assay against the Do-D2, to evaluate their binding affinity. After treatment with the chaotropic agent at 5M, a higher percentage (fold change>1) of IgG binding was detected in the Post-Vax than in the Pre-Vax sera of 4 out of 7 dogs (**Figure 3C; Table S1; Figure S4A**). Post-Vax sera were then tested for their ability to bind the Do-CSPG4 as it is overexpressed in its natural conformation on canine OSA cells (Penny). In 6 out of 12 (50%) vaccinated dogs, the Post-Vax sera showed a higher capacity to stain Penny than the corresponding Pre-Vax sera (**Figure 3D; Figure S5A**). Moreover, the antibody level against Penny was also persistent in sera collected at later time points (after the fifth and the sixth immunizations,

Figure S5A). A vaccine-induced anti-Hu-CSPG4 antibody response was detected in 9 out of 12 (75%) immunized dogs after the fourth vaccination (**Figure S5B**) and this modestly increased at sequential time points (**Figure S5C**), as demonstrated by the ability of sera to stain the human U2OS cell line, which naturally overexpresses CSPG4.

To explore the possible mechanisms of action of vaccine-induced antibodies against OSA, Pre-Vax and Post-Vax sera pools were generated and added to the Penny-cell culture medium. The Post-Vax pool displayed a higher ability to inhibit canine OSA-cell proliferation (**Figure 3E**) and migration (**Figure 3F**) *in vitro* than the Pre-Vax pool.

Cellular response

An immunophenotyping analysis of patient-derived PBMC collected before the first (Pre-Vax) and after the fourth (Post-Vax) immunization was performed in 10 vaccinated dogs to evaluate any possible variation in immune cells populations in the circulation. While no significant increase in the number of B and $CD4^+$ T cells was observed, a decrease in the number of myeloid-derived suppressor cells (MDSC) and/or T regulatory cells (Treg) and/or an increase in $CD8^+$ T cells was observed in some of the analyzed dogs (**Figure 4A; Table S1; Figure S4**). In detail, 8 out of 10 (80%) dogs displayed an increase in the number of $CD8^+$ T cells after vaccination (**Figure 4B; Table S1; Figure S4**). Three of these (30% of the analyzed dogs) also showed a decrease in MDSC and Treg percentage, whereas another 3 (30%) only showed a decrease in MDSC, and 1 (10%) only showed a decrease in Treg. Of the two dogs that did not display an increase in $CD8^+$ T cell number, 1 (10%) displayed a decrease in both MDSC and Treg cells, while the other (10%) only showed a reduction in the Treg population (**Table S1; Figure S4)**. The release of IFN-γ was then analyzed in some patients-derived PBMC, in which an increase of $CD8^+$ T cells was observed, after *in vitro* re-stimulation with specific Do-CSPG4 peptides (**Figure 4C**). In 3 out of 4 (75%) dogs an increase of IFN-γ released in the supernatant of re-stimulated Post-Vax PBMC as compared to Pre-Vax PBCM was observed.

Then, the anti-tumor effect potentially mediated by the vaccine-induced cellular response was

assessed by means of an *in vitro* cytotoxicity assay, for the 8 dogs in which an increase in $CD8^+$ T cells was observed. A canine OSA cell line that is naturally negative for CSPG4 expression (D22) was stably transfected to overexpress the Do-CSPG4 (D22 Do-CSPG4; **Figure S6A, B**), which was used as a target. The killing of CFSE-labeled D22 Do-CSPG4 cells was found to be significantly higher after 48h of incubation with Post-Vax PBMC than with Pre-Vax ones (**Figure 4D; Table S1; Figure S4**). The cytotoxic effect was mainly directed against CSPG4, as significantly fewer CSPG4-negative D22 cells were killed (**Figure 4E)**).

Clinical outcome following HuDo-CSPG4 vaccination in canine OSA patients

The overall survival (OS) and disease-free interval (DFI) of HuDo-CSPG4-adjuvantly-immunized (Vax) dogs were evaluated for all patients. OS and DFI of a historical control (Ctrl) group of 13 dogs affected by $CSPG4^+$ appendicular OSA, treated with conventional therapies alone, was retrospectively evaluated. Control dogs had undergone amputation and chemotherapy and were confirmed to be free of pulmonary metastasis at restaging, following the chemotherapy cycles. No statistical differences regarding age, weight, sex, CSPG4 expression and the histological grade of the disease were observed between the two groups.

The clinical information for each dog included in the study is reported in **Figure 5A**. A statistically meaningful 7.8-month improvement in the median survival times (MST) was achieved in the vaccinated group with respect to the control group (438 days versus 202 days, respectively, **Figure 5B**). In particular, a significantly longer OS was observed in the vaccinated dogs as compared to historical Ctrl group one year after surgery (**Figure 5B**), with the OS rates being 66.66% and 30.76%, respectively. Two years after surgery, the benefit of anti-CSPG4 vaccination became more modest, with similar OS rates in the two groups (20% vs 15.38% for Vax and historical Ctrl dogs, respectively). Moreover, the median time to disease progression increased of about 2.7 months in the vaccinated arm as compared to the control arms (DFI of 242.5 versus 160 days, respectively, **Figure 5C**). At 6 months after surgery, only 25% of vaccinated dogs developed metastasis as compared to

75% of controls; the improvement of disease progression was observed also at 1 year from diagnosis, with 61.53% versus 76.92% of vaccinated and historical control dogs who developed metastases, respectively.

Overall, these results suggest that HuDo-CSPG4 vaccination may provide a clinical benefit in initially prolonging OS and delaying metastasis development.

HuDo-CSPG4 is effective in a human surrogate setting

Pilot investigations in human surrogate models were carried out to assess the potential of translating this chimeric vaccination strategy to a human setting. mDC generated from healthy donors were transfected with either the chimeric HuDo-CSPG4 (HuDo-CSPG4-DC) or fully Hu-CSPG4 (Hu-CSPG4-DC) plasmid to evaluate and compare the potential of chimeric and homologous vaccines in inducing an anti-CSPG4 response in a human system that simulates *in vitro* DNA vaccination (**Figure 6A**). mDC transfected with the empty plasmid (empty-DC) were used as a control. Autologous T cells that were pre-activated by HuDo-CSPG4-DC were more effective in killing the human HLA-A2-matched $CSPG4^+$ U2OS OSA cell line than those pre-activated by Hu-CSPG4-DC and empty-DC in an *in-vitro* cytotoxicity assay (**Figure 6B**). These data suggest that the chimeric vaccine may be able to break immune tolerance against the self CSPG4 antigen and induce a cytotoxic response against $CSPG4^+$ OSA cells.

Discussion

High rates of recurrence, metastasis and poor prognosis mean that OSA is still a critical issue in pediatric oncology. The standard of care for OSA patients has remained mostly unchanged since the 1970s, and the introduction of neoadjuvant/adjuvant aggressive chemotherapy, which improves the outcomes of patients with localized tumors, is barely effective in cases of recurrent or advanced disease.[23] Therefore, the development of novel therapies is still an unmet clinical need. Significant limitations in the advancement of OSA management include the rarity of the disease, the very young age of patients and the lack of defined common targetable oncogenic drivers. The considerable and

recognized similarity between human and canine OSA[7-9,24] has driven interest in using pet dogs with spontaneously occurring OSA to test new therapeutic options, including immune-based therapies, with the final aim of informing human clinical trials [8,25]. Indeed, human and canine OSA share many biological and clinical similarities, as they are both highly metastatic and resistant to conventional treatments. Recent examples of relevant comparative oncology studies in the field include a Listeria monocytogenes (Lm)-based vaccine that expresses a chimeric human epidermal growth factor receptor (HER)2 fusion protein; positive results in a phase I veterinary clinical trial led to the issuing of a conditional license by the United States Department of Agriculture (USDA) in 2017 for the adjuvant treatment of dogs with OSA.[26,27] The promising results obtained in canine patients led to a clinical trial in adult patients with $HER2^+$ tumors (NCT02386501) and to a license for its development in the pediatric OSA setting.[28] However, the occurrence of adverse effects, due to Listeria infections reported in some dogs and the potential hazard of the zoonotic spread of the disease in humans, led to some concerns regarding the safety of this strategy.[29,30] Nevertheless, this study has highlighted the potential value of comparative studies for the human clinic.

Of the different immunotherapeutic strategies available, DNA plasmid-based vaccines own the advantages of being stable, easy to manufacture and cost-efficient, as well as safe and effective in stimulating both immune arms of a patient's own immune system, as demonstrated in several clinical trials.[31] In this line, here the immunogenicity and consequent potential clinical benefit of an immunization strategy against CSPG4 by using a chimeric human/dog (HuDo)-CSPG4 DNA vaccine have been exploited.[19] The use of a hybrid DNA plasmid, coding for chimeric CSPG4 protein, was guided by the need of i) ensuring the specificity of the immune response through the homologous domain; ii) breaking immune tolerance against the self-molecule though the heterologous domain; and iii) inducing a high-affinity immune response in vaccinated patients.[19,32] Xenogeneic vaccines have, indeed, demonstrated to induce a low-affinity immune response against the self-TAA, that might be translated in a limited clinical efficacy. CSPG4 has come to prominence as an appealing immunotherapeutic target in the melanoma setting, as demonstrated in human[33,34] and veterinary

clinical trials.[19,21,22] As a co-receptor/plasma membrane scaffold, CSPG4 can enhance, when over-expressed, the signal intensity and duration of multiple oncogenic pathways that sustain the pro-tumoral attitude of malignant cells.[12,13,35,36]

We have previously demonstrated that human and canine OSA cells overexpress CSPG4 and that this overexpression negatively impacts the prognoses of both human and canine OSA patients.[15] In addition, we have shown that CSPG4 is overexpressed on OSA-derived CSC,[15] considered responsible for chemoresistance and favoring relapse and metastasis in OSA.[37] To functionally support these findings, the present study has induced the transient silencing of CSPG4 in human OSA cell lines, resulting in an impairment of their malignant behavior. The anti-tumor impact of CSPG4 down-regulation was independent of the mutational status of OSA cells, with similar effects observed in U2OS and SaOS2 cells (i.e., p53 and Rb wild-type vs. p53 null and Rb mutated, respectively).

Before the in-dog studies, a preclinical evaluation of HuDo-CSPG4 vaccine anti-tumor potential in adoptive-transfer experiments in a human xenotransplant model was performed. Given its recognized higher metastatic potential compared to SaOS2 cells, the U2OS cell line was selected for these experiments as we aimed to investigate the ability of HuDo-CSPG4 to counteract primary OSA growth and lung dissemination.[38,39] For these experiments, HLA-A2.1 Tg mice, expressing the human HLA-A2.1 allele, and matching with the human U2OS OSA cells were exploited. Adoptively transferred HuDo-CSPG4-induced $CD8^+$ T cells significantly hampered human $CSPG4^+$ OSA growth in NSG mice. However, some mice showed slowed, but continuous, tumor growth. In an attempt to better characterize $CD8^+$ T-cell activity against established U2OS tumors, the levels of infiltrating lymphocytes and the expression of PD-L1 and B7-H3 checkpoint molecules in tumors were analyzed, in view of their suggested role in mediating immune evasion in several cancer models.[40,41] Firstly, in a comparison with controls, only HuDo-CSPG4-induced $CD8^+$ T cells were able to infiltrate the primary tumor core, and this infiltration seemed to be related to better tumor rejection (Figure 2C). Furthermore, higher PD-L1 expression was found in smaller, highly infiltrated tumors. This result fits with previous findings in human patients in which PD-L1 expression was associated with the

316 presence of multiple tumor-infiltrating immune cells, with tumor size being larger in PD-L1-negative
317 cases than in PD-L1-positive ones, and negatively correlating to outcomes.[6,42,43] Conversely,
318 increased B7-H3 expression correlated with larger, low-infiltrated tumors, suggesting that the up-
319 regulation of B7-H3 by human OSA cells may be a mechanism of tumor escape, in accordance with
320 recent results in human OSA biopsies.[44] In line with these findings, the use of Atezolizumab (anti-
321 PD-L1 mAb) as a single agent[6,45] was ineffective in pediatric OSA patients, while Enoblituzumab
322 (anti-B7-H3) is promising (NCT02982941). These results suggest that anti-CSPG4 vaccination may
323 be used in combination with B7-H3 immune checkpoint blockade to prime T cells against CSPG4,
324 while increasing their activity and infiltration into tumors.
325 To investigate the contribution of vaccine-induced antibodies, U2OS-bearing NSG mice were
326 adoptively transferred with sera derived from HuDo-CSPG4 immunized mice. Although no
327 significant differences were observed, a trend of tumor-growth reduction was noted (Figure 2F). It
328 must be mentioned that NSG mice lack NK cells, meaning that antibody-dependent cellular
329 cytotoxicity, one of the relevant mechanisms by which the anti-CSPG4 antibody is effective in cancer
330 cell elimination,[19] is defective in these mice. This may lead to an underestimation of the efficacy of
331 anti-CSPG4 antibodies in an immune-competent system. However, a significant impairment of
332 metastasis development was observed compared to controls, both in mice adoptively transferred with
333 immune sera and those with anti-CSPG4 T cells (Figure 2D, H). This result supports the previously
334 hypothesized direct contribution of both the cellular and antibody immune responses in hindering
335 tumor progression.[15,19]
336 These promising data, together with the need to test HuDo-CSPG4 vaccination in OSA
337 immunocompetent models, prompted us to exploit dogs that spontaneously develop CSPG4^{+}
338 appendicular OSA and that have completed the standard therapeutic protocol. In these dogs, HuDo-
339 CSPG4 vaccination was effective in overcoming immune tolerance to the self (canine) CSPG4
340 molecule. A significant increase in the antibody level against Do-CSPG4 was observed following
341 vaccination in the sera of about 42% of dogs, where an increased IgG titer mainly against the Do-D2

342 domain was detected (Figure 3A, B). A high spontaneous humoral response against a CSPG4-
343 overexpressing tumor could be present and amplified following tumor surgical resection and
344 chemotherapy.[19,46] This could lead to an underestimation of the percentage of dogs that respond to
345 HuDo-CSPG4 vaccination in evaluations based on a higher anti-CSPG4 antibody response in post-
346 compared to pre-immunization sera. Indeed, dogs that were classified as non-responders in this way
347 showed an increase in the avidity of antibodies against Do-D2 following vaccination (Figure 3C).
348 The D2 domain constitutes the core of the CSPG4 protein extracellular portion and is the putative
349 mediator of ligand/ECM binding, promoting pro-tumor signals.[35] Interestingly, vaccine-induced
350 antibodies in the sera of immunized dogs exerted a mechanistic effect on the canine CSPG4-
351 expressing OSA cells, Penny, in terms of the increased inhibition of both tumor cell proliferation and
352 migration compared to sera collected before vaccination (Figure 3D, E). These results hint at a
353 possible mechanism of action for vaccine-induced antibodies *in vivo*, and highlight the low
354 effectiveness of spontaneous low-avidity autoantibodies in counteracting growing tumors, which is
355 in line with our recent findings in a veterinary trial in which HuDo-CSPG4 vaccination was applied
356 for the adjuvant treatment of melanoma-affected dogs.[19] High CSPG4 expression in tumors has been
357 found to be directly correlated with a lower tumor infiltration and enhanced immune-suppression.[47]
358 MDSC are the predominant cells observed within the tumor microenvironment in both human[48] and
359 canine[49] OSA, while high Treg levels have been correlated with worse prognosis,[50] with both these
360 issues contributing to immune evasion. Although variation in the percentage of immune cells within
361 circulation is not necessarily correlated with the development of an antigen-specific immune
362 response, the evaluation of peripheral blood immune cells dynamics might reflect immune-
363 modulation and response to therapy during treatment.[51,52] Here, decreased levels of MDSC and Treg
364 were observed in most samples collected after the HuDo-CSPG4 vaccination, compared to those
365 collected before the immunization cycles (Figure 4A). In most cases, an increase in $CD8^+$ T cells, that
366 were able to selectively exert an anti-CSPG4 response was also observed (Figure 4B-E). These results
367 suggest that anti-CSPG4 vaccination can hamper the immune suppressive mechanisms that persist in

368 canine patients with minimal residual disease, together with the induction of CSPG4-specific cellular
369 immunity.
370 Even though this study was designed to explore the immunogenicity of HuDo-CSPG4 vaccination in
371 dogs affected by a poorly immunogenic tumor, such as OSA,[53] also the clinical data were collected,
372 highlighting the potential therapeutic benefit of the adjuvant HuDo-CSPG4 vaccination on survival.
373 We examined the 1-year survival probability as the first clinically relevant time point and dogs who
374 received the adjuvant HuDo-CSPG4 vaccine experienced a significant OS advantage, as compared
375 to the historical control group. Indeed, we observed a meaningful separation of the Kaplan-Meier
376 curves from about three to twenty months from the surgical removal of the primary tumor. Two years
377 after surgery, the effect of adjuvant HuDo-CSPG4 became more modest, but still significantly
378 improved with respect to standard therapies alone (Figure 5B). The OS rate at 2 years is in line with
379 that previously reported for OSA-bearing dogs receiving standard therapies and is a consequence of
380 the development of metastatic disease. The Authors understand that, because of how this study has
381 been designed, an analysis of DFI has intrinsic limits; it may be affected by timing and, potentially,
382 by the different diagnostic imaging procedures adopted in the two groups. It must be underlined,
383 however, that historical controls were treated when HuDo-CSPG4 vaccination was still not intended
384 for OSA treatment; therefore, follow-up was assessed following standard diagnostic procedures[54] and
385 in these cases three-view radiographs scan would have given to a delay and an underestimation of the
386 metastasis in the controls.
387 A separation of the Kaplan-Meier curves from about two to ten months starting from the surgery can
388 be noted, and this corresponds to median DFI of 242 days for vaccinated dogs and 160 days for the
389 controls (Figure 5C). We can therefore speculate that vaccine-induced antibodies and T cells may be
390 able to impair the beginning of the metastatic cascade, mirroring observations in preclinical mouse
391 models.
392 However, the real potential of vaccination when metastases have already been established is still
393 unexplored. Canine patients with distant metastases should be included in a future trial. To further

394 improve the clinical potential of adjuvant vaccination, the immunization protocol should start earlier,
395 before or between chemotherapeutic cycles, to achieve a better curative effect in the first therapeutic
396 window.
397 One of the limitations of this pilot veterinary study is the use of historical controls and the small
398 sample size. As the safety and the induction of an anti-CSPG4 immune response were the main
399 objectives of the present study, a larger randomized controlled study will be necessary to draw
400 conclusions regarding the effective clinical response to the adjuvant vaccine in OSA canine patients
401 (only envisaged here). However, it is also to be considered that veterinary medicine is still running
402 behind the human counterpart, with most of randomized trials conducted thanks to substantial
403 funding. Especially in the veterinary field, many non-randomized studies have been published with
404 relevant informative results.[25,55-57] Non-randomized studies are designed to evaluate the feasibility
405 and the worth to establish the rational for future randomized clinical trials. Indeed, pilot non-
406 randomized studies might play an important role in the preliminary planning of a proposed full-size
407 randomized clinical trial, clarifying potential issues that could be encountered.[58-60]
408 Finally, we have demonstrated that differentiated DC from healthy human donors that were
409 electroporated with the chimeric HuDo-CSPG4 plasmid were better able to activate autologous T
410 cells towards the killing of human CSPG4^{+} OSA cells than DC transfected with fully xenogeneic Hu-
411 CSPG4[21] or the empty vector (Figure 6B). These results may predict the ability of the chimeric
412 vaccine to break immune tolerance against the (human) self-antigen and to also mount an effective
413 *in vivo* cytotoxic response in a human setting.
414 Overall, this study demonstrates that CSPG4 could be a relevant comparative target for OSA
415 treatment through chimeric DNA vaccination. Considering the high translational value of
416 spontaneous canine tumors, these promising results will likely promote the translation of this novel
417 immunotherapeutic approach to a human setting, eventually improving the life expectancy of OSA
418 patients that cannot benefit from present therapies.
419

Material and Methods

Cell lines

Human OSA cell lines (U2OS and SaOS2) were obtained from the American Type Culture Collection (ATCC; HTB-96, RRID:CVCL_0042, and HTB-85, RRID:CVCL_0548, respectively, Manassas, Virginia, USA) and were cultured in RPMI (Sigma-Aldrich, St. Louis, Missouri, USA) supplemented with 20% fetal bovine serum (FBS, Sigma Aldrich). Penny cells, derived from a biopsy of a primary grade III canine OSA tumor,[15,61] were grown in ISCOVE Modified Dulbecco's Medium (Sigma-Aldrich) supplemented with 10% FBS. The canine OSA cell line D22 was obtained from ATCC (ATCC; CRL-6250, RRID:CVCL_3458) and cultured in DMEM F12 (Sigma-Aldrich) supplemented with 20% FBS. The D22 cell line overexpressing dog CSPG4 (D22 Do-CSPG4) was generated by transfecting D22 cells (ATCC; CRL-6250, RRID:CVCL_3458), which are naturally negative for CSPG4 expression, with the canine CSPG4-coding plasmid (obtained from GenScript, Piscataway, New Jersey, USA) using Lipofectamine 2000 (Thermo-Fisher Scientific, Waltham, Massachusetts, USA), according to the manufacturer's instructions. Stable transfected clones were maintained in DMEM F12 supplemented with 20% FBS and 1.5 mg/ml Geneticin (G418, Gibco, Thermo-Fisher Scientific). CSPG4 expression was confirmed by western blot analysis and flow cytometry using an anti-CSPG4 mAb (TP-49; generated and provided by Prof. S. Ferrone[15]). Total IgG binding was evaluated using a PE-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Labs, West Grove, Pennsylvania, USA, Cat# 115-116-072, RRID:AB_2338627) secondary antibody, while samples were acquired using a BD FACSVerse (BD BioScience, Franklin lakes, New Jersey, USA) and analyzed using FlowJO V.10.5.3 (RRID:SCR_008520). All cell lines were grown in medium that was supplemented with penicillin/streptomycin (Sigma-Aldrich) and maintained at 37°C and 5% carbon dioxide (CO_2) in a humidified incubator. The cell lines were regularly monitored for mycoplasma contamination using a commercially available assay kit (Mycoalert from Lonza Inc., Basel, Switzerland).

siRNA transfection

Small interfering RNA (siRNA), specific for human CSPG4 (hCSPG4-siRNA), and the negative control siRNA (Scramble) were purchased from Ambion (Austin, Texas, USA). Human OSA cells were transfected with siRNA using Lipofectamine 2000 transfection reagent (Thermo-Fisher Scientific), in accordance with the manufacturer's instructions. Cells were harvested 24h, 48h and 72h post-transfection to verify CSPG4 down-regulation by Real-Time PCR and Western Blot analyses. For functional studies, cells were detached 24h post-transfection for plating in proliferation, migration, osteosphere-formation and viability assays.

Real-Time (RT) PCR

Total RNA was isolated from human OSA cells and treated with either hCSPG4-siRNA or the Scramble negative control using the TriZol reagent (Thermo-Fisher Scientific), according to the manufacturer's instructions. Genomic DNA contamination was removed from the RNA samples using the Ambion® DNA-free kit (Thermo-Fisher Scientific). RNA concentration and quality were estimated using a NanoDROP 2000 Spectrophotometer (Thermo-Fisher Scientific) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California, USA), respectively. DNase-treated RNA (1 μg) was retrotranscribed with RETROscript reagents (Ambion), and RT-PCR was carried out using gene-specific primers (QuantiTect Primer Assay), SYBR green and a 7900HT RT-PCR system (Applied Biosystems, Waltham, Massachusetts, USA). Applied Biosystems SDS Software Version 1.3.1 was used to analyze data. Quantitative normalization was performed on the expression of the housekeeping glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene. Relative gene expression levels were calculated using the comparative ΔCt method.[62]

MTT cell-proliferation assay

3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazol (MTT; Merck Millipore, Burlington, Massachusetts, USA) was used to assess the proliferation of human OSA cells following siRNA transfection. Briefly,

24h post-transfection, the cells were seeded in triplicate in 96-well plates (5 x 10^3 cells/100µl well) in serum-free medium and allowed to adhere overnight. MTT solution (5 mg/ml) was added to each well, in accordance with the manufacturer's instructions, at different time points (24h, 48h, and 72h). After 4h incubation at 37°C, 100µl of dimethyl sulfoxide (DMSO, Sigma Aldrich) were added to dissolve formazan crystals, and absorbance was measured using an enzyme-linked immunosorbent assay (ELISA) plate reader (Bio-Rad, Hercules, California, USA) at a wavelength of 570 nm.
Canine OSA Penny cells (5 x 10^3 cells/well) were starved for 4h in 96-well plates. Pooled canine sera (1:50) from vaccinated dogs were then added and incubation was continued for 24, 48 and 72 h. Cell viability was then evaluated using the MTT assay, as previously described.

Migration assay

Scramble- and hCSPG4-siRNA-transfected human OSA cells (2 x 10^4 cells/100µl well) were both added to the top chamber of a Transwell insert (8 µm, Corning, Corning, New York, USA) in serum-free medium, while the bottom chambers were filled with complete medium. Penny cells were pre-incubated with a pool of canine sera, collected before the first immunization (Pre-Vax) and after the fourth (Post-Vax), for 1h at 37 °C before being transferred to the top chamber of a Transwell insert. Cells were cultured for 48h at 37°C in a 5% CO_2 atmosphere. The non-migrating cells in the upper chamber were removed using a cotton swab and the migrated cells on the bottom side of the insert were fixed with 2.5% Glutaraldehyde (Sigma-Aldrich) and stained with 0.2% Crystal Violet (Sigma-Aldrich). The migrated cells of four randomly selected fields per well were imaged using an Olympus BX41 microscope (Olympus Corp., Tokyo, Japan) and analyzed using Fiji (RRID:SCR_002285) and ImageJ (RRID:SCR_003070; Rasband, W.S., ImageJ, US National Institutes of Health, Bethesda, Maryland, USA) softwares.

Sphere-generation assay

Human osteospheres were generated according to the protocol previously described.[15,63] Briefly, human OSA cells, 24h post-transfection with Scramble- or hCSPG4-siRNA, were harvested and plated (6 x 10^4 cells/ml) in ultra-low-attachment 75 cm^2 flasks (Sigma Aldrich) in serum-free DMEM F12, supplemented with 0.4% bovine serum albumin (BSA), 20 ng/ml basic fibroblast growth factor (bFGF), 20 ng/ml epidermal growth factor (EGF) and 5 μg/ml insulin, all from Sigma-Aldrich. Non-adherent spherical-cell clusters, named osteospheres, were collected after 5 days for further analysis. Photographs of osteospheres were taken using a CCD-300-RC camera, and images were processed using Fiji Software (RRID:SCR_002285; Rasband, W.S., ImageJ, US National Institutes of Health,) and PowerPoint (Microsoft, Redmond, Washington, USA).

To assess osteospheres viability, MTT solution was added to each well and incubated overnight at 37°C, and formazan crystals were dissolved by adding 100μl isopropanol with HCl 0.04N to each well. Optical density was measured using an ELISA plate reader with a test wavelength of 570 nm and a reference wavelength of 655 nm. The difference between the 570 nm and 630 nm readings represents the output value.

Mice and DNA immunization

Female C57BL/6 HLA-A2.1 Transgenic (Tg) mice (RRID:IMSR_JAX:003475; from The Jackson Laboratory, Bar Harbor, Maine, USA) and Balb/c mice (RRID:IMSR_APB:4790; from Charles River Laboratory, Wilmington, Massachusetts, USA) were maintained at the Molecular Biotechnology Center, University of Turin (Turin, Italy) and treated in accordance with the University's Ethical Committee and European guidelines under Directive 2010/63. All animal studies were approved by the Italian Ministry of Health (Authorization N° 29/2021-PR). Six-weeks-old C57BL/6 HLA-A2.1 Tg and Balb/c mice were vaccinated with HuDo-CSPG4 plasmids, generated as previously described.[19] Briefly, the plasmid is a pcDNA3.1 (Cat# V79020; Invitrogen, Waltham, Massachusetts, USA) backbone and includes the cDNA sequence covering the N-terminal portion of Hu- and the C-terminal portion of Do-CSPG4.[19] Large-scale plasmid preparation was carried out using EndoFree

Plasmid Giga kits (Qiagen, Hilden, Germany), according to Good Laboratory Practice. Mice were anesthetized and then immunized intramuscularly with either 50 μg of the pcDNA3.1 empty vector or the HuDo-CSPG4 plasmid diluted in 20 μL of saline solution. Immediately after injection, two 25-ms transcutaneous low-voltage electric pulses (amplitude 150 V; interval 300 ms) were administered at the injection site via a multiple-needle electrode connected to a Cliniporator^{TM} (IGEA, Carpi, Italy).[64] Mice were immunized twice, at a two-week interval, and were sacrificed two weeks after the second immunization to collect their spleens and blood for the adoptive-transfer experiment.

Female NOD/SCID/$\gamma c^{-/-}$ (NSG; RRID:BCBC_1262) mice (Charles River Laboratory) were used for the adoptive transfer experiment. Mice were injected subcutaneously with 1×10^6 of the HLA-A2.01-positive U2OS cell line in the right flank, and tumor growth was monitored twice a week using a caliper. NSG mice were adoptively transferred, with either $CD8^+$ T cells or sera derived from previously vaccinated immunocompetent mice, when a tumor volume of 0.5 mm^3 was reached. Mice were assigned to treatment groups by simple random sampling and adoptively transferred with $CD8^+$ T cells or sera derived from previously vaccinated immunocompetent mice. Tumor volume was calculated using the following formula: $V = ½ (Length \times Width^2)$.

Adoptive transfer

$CD8^+$ T cells were isolated, under sterile conditions, from the spleens of C57BL/6 HLA-A2.1 Tg mice (RRID:IMSR_JAX:003475; from The Jackson Laboratory) that were either vaccinated with the pcDNA3.1 empty vector or HuDo-CSPG4. Individual spleens were homogenized in RPMI medium supplemented with 10% FBS. Cell suspensions were centrifuged for 10 min at 1100 rpm and room temperature (RT), and were resuspended in red blood cell lysis buffer for 10 min RT. The single-cell splenocyte suspensions were pooled and $CD8^+$ T cells were isolated using magnetic cell sorting via negative selection ($CD8a^+$ T Cell Isolation Kit, Teterow, Germany), according to manufacturer's instructions. Three x 10^6 $CD8^+$ T cells were adoptively transferred via tail-vein injection into OSA-bearing NSG mice.

549 For the adoptive transfer of sera, blood was collected, via intracardiac sampling, from Balb/c mice
550 two weeks after the second immunization. Blood was centrifuged (3000 *g* for 10 min at 4°C) and the
551 serum was isolated. Sera were pooled and stored at -20°C until use. At the time of adoptive-serum
552 transfer, the recipient OSA-bearing NSG mice received 150 µl of serum intraperitoneally once a week
553 for 5 weeks. The mice were sacrificed at the end of the experiment, and the tumors and lungs were
554 explanted for further analysis.
555
556 **Western Blot analysis**
557 Human and murine OSA cell lines were incubated in RIPA Lysis buffer (150 mM sodium chloride,
558 1.0% NP-40, 50 mM Tris pH 8.0, 1 mM Na_3VO_4, 1 mM NaF, and protease inhibitors, all from Sigma
559 Aldrich). All samples were placed on ice for 30 min and then centrifuged at 12,000 *g* for 5 min at
560 4°C, and the supernatant was collected. The total protein concentration was quantified using the
561 Pierce™ BCA Protein Assay Kit (Thermo-Fisher Scientific). Equal amounts of protein (50 µg) were
562 separated by electrophoresis in a 4-20% Mini-Protean TGX precast gel (Bio-Rad). Western blotting
563 for the detection of CSPG4,[15] Stro-1 (diluted 1:250; Thermo Fisher Scientific Cat# 39-8401,
564 RRID:AB_2533437) and CD133 (diluted 1:1000; Proteintech, Rosemont, Illinois, USA, Cat# 18470-
565 1-AP, RRID:AB_2172859) was performed as previously described.[15] β-Actin (diluted 1:500; Santa
566 Cruz Biotechnology, Dallas, Texas, USA, Cat# sc-69879, RRID:AB_1119529) and GAPDH (diluted
567 1:500; Santa Cruz Biotechnology Cat# sc-32233, RRID:AB_627679) were used as the loading
568 control. Images were acquired using a BioRad ChemiDoc imaging system.
569 **Canine-patient enrolment, eligibility criteria and clinical procedures**
570 According to the study protocol approved by the Italian Ministry of Health (0026167-14/10/2019-
571 DGSAF-MDS-P), canine patients that had been diagnosed with appendicular OSA at the Veterinary
572 Teaching Hospital, University of Turin, Grugliasco (Italy) and at the Tyrus Veterinary Clinic, Terni
573 (Italy), over the period from 2011 to 2021, were considered eligible; the study was performed in
574 accordance with CONSORT guidelines. Inclusion criteria were: (1) confirmed histological grade I-

III, CSPG4 overexpressing appendicular OSA; (2) absence of detectable distant metastasis at presentation and restaging after standard treatments; (3) local tumor control via limb amputation; (4) completion of standard chemotherapy based on 4-6 cycles of adjuvant carboplatin i.v. administrations at 3-week intervals (300 mg/m^2); (5) absence of concurrent life-threatening diseases; and (6) written informed consent signed by the owners.

Full pre-treatment tumor staging included a thorough clinical examination, laboratory tests (complete blood count, extensive biochemical profile and urinalysis), and cardiac function examination, clinically assessed by auscultation and electrocardiography. Either cytology or histology after fine-needle aspiration or the incisional biopsy of the primary tumor, fine-needle aspiration of any enlarged regional lymph node, and an examination for metastasis using total body Computed Tomography (CT) scan were performed.

Postoperative tumor samples were immunohistochemically tested for CSPG4 expression as previously described. Only OSA with a CSPG4 expression score ≥ 3 were included.[15,19]

After completing the chemotherapy cycles, all dogs were subjected to tumor restaging using CT scans to confirm absence of distant metastatic lesions. Twelve client-owned dogs were enrolled in the vaccination protocol. Dogs were adjuvantly immunized with the HuDo-CSPG4 plasmid as previously described,[19] starting from 2 weeks after the last chemotherapy cycle. Briefly, 500 µg of HuDo-CSPG4 plasmid in 200 µl of 0.03% NaCl solution were injected into the muscle of the healthy leg. Two minutes after plasmid injection, 1 high voltage pulse (amplitude 450 V, length 50 µs, frequency 3 HZ) and, after 1 s of pause, 8 low-voltage pulses (amplitude 110 V, length 20 ms, pause 300 ms) were applied to the injection site using the Cliniporator (Igea).

Immunization was repeated monthly, for a minimum of 4 and a maximum of 24 cycles. At each vaccination cycle full clinical examinations and CT scans were performed, and, in addition, sera and peripheral blood mononuclear cells (PBMC) were collected, whenever possible. Blood workup and/or urinalysis was performed if clinically indicated.

600 The primary objectives of this study were safety and immunogenicity. The Veterinary Cooperative
601 Oncology Group Common Terminology Criteria for Adverse Events V.1.1[24] was used to classify the
602 adverse events. The secondary objective was the clinical monitoring of disease progression, with
603 overall survival and disease-free interval being considered. A historical group of $CSPG4^+$ OSA-
604 affected dogs (n = 13) fulfilling the inclusion criteria was retrospectively evaluated and used as
605 control. Historical controls received standard treatments only (limb-sparing and 4-6 chemotherapy
606 cycles). Following the completion of chemotherapy, dogs were monitored via three-view chest
607 radiographs and/or CT scans, and abdominal ultrasound if clinically indicated, every 3 months for
608 the first post-operative year, every 6 months in the second year post-surgery, and then once a year, as
609 indicated by standard guidelines.
610
611 **Histological and immunohistochemical analyses**
612 The tumors and lungs collected from NSG mice that were challenged with U2OS cells (ATCC; HTB-
613 96, RRID:CVCL_0042), and tissue samples from the 25 cases of spontaneous canine appendicular
614 OSA, collected via routine care at the Veterinary Teaching Hospital and the Tyrus Veterinary Clinic,
615 were examined in this study. Sections from mouse primary tumors and lungs were stained with
616 hematoxylin and eosin (H&E) for histological tumor evaluation and the presence of metastatic
617 lesions. Sections from canine primary tumors were stained with H&E for histological tumor
618 evaluation. The histological diagnosis and grading of canine appendicular OSA were defined
619 according to Loukopoulos and Robinson (2007). Canine tumors were histologically classified as
620 grade I (low), II (intermediate) and III (high). Immunohistochemical (IHC) analyses were performed
621 on collected samples, which were fixed in 4% formalin and embedded in paraffin, and then sectioned
622 at 4 μm, as described previously.[15,19] IHC staining for CSPG4 was performed on paraffin sections
623 with a primary anti-CSPG4 antibody (MBS716314, diluted 1:40, MyBiosource, San Diego,
624 California, USA), and the CSPG4 semi-quantitative scoring system was adopted as previously
625 described.[15,19] A CSPG4 total score, ranging from 0 to 8, was assigned to each OSA sample in

accordance with 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). IHC staining for T lymphocytes in mouse tumors was performed using a primary anti-CD3 antibody (SP7, diluted 1:500; Abcam, Cambridge, UK, Cat# ab16669, RRID:AB_443425).

ELISA assay

Sera from healthy dogs were obtained from the blood withdrawn for routine blood tests required for anesthesia before a spaying procedure. The sera collected from vaccinated dogs before the first (Pre-Vax) and after the fourth, fifth and sixth (Post-Vax) immunizations were used for ELISA tests, which were performed as described previously.[19] Briefly, thawed sera (dilution 1:100) were incubated in 96-well plates that were previously coated with different recombinant canine-CSPG4 domains (D1, D2, D3; 50 ng/well; GenScript). An anti-CSPG4 IgG response requires activation of CSPG4-specific helper T cells to promote isotype switching from IgM to IgG, therefore this is an indirect measurement of T cell immunity. An anti-CSPG4 IgG ratio > 1 (Pre-Vax/Post-Vax) was defined as an immune response.

In order to test the avidity of the anti-CSPG4 antibodies, a chaotropic ELISA assay was performed by adding the chaotropic Sodium Thiocyanate agent (Sigma Aldrich) to incubated sera at 5M for 15 min at RT, as previously described.[19] Antibody binding was detected using horseradish peroxidase–conjugated goat anti-dog-IgG (diluted 1:10000; Abcam Cat# ab112852; RRID:AB_2927648) on an ELISA Microplate Reader at a wavelength of 470 nm.

For IFN- γ release ELISA assay, $5x10^5$ PBMC collected before (PRE-Vax) and after (POST-Vax) the IV immunization were plated in a 48-well plate in RPMI 10% FBS in the presence of either Concavalin A (ConA; Cat# 11028-71-0, Sigma Aldrich), dog (Do)-CSPG4 peptides (6 pools of 15-mer, with 11 aa overlapping, covering the entire protein), or without any stimulus, and incubated for 72h. Canine IFN-γ release was measured using Canine-IFN-γ ELISA development kit (HRP, 3113-1H-6, Mabtech, Nacka Strand, Sweden).

652

653 **Flow-cytometry analysis**

654 For the flow cytometric analysis of the vaccine-induced antibodies, human U2OS (ATCC; HTB-96,
655 RRID:CVCL_0042) and Penny cells were incubated with sera (1:40 dilution), collected from
656 immunized dogs, for 30 min at 4°C in PBS (Sigma-Aldrich). Total IgG binding was evaluated as
657 previously described.[19] Samples were acquired using a BD FACSVerse (BD BioScience) and
658 analyzed using FlowJO V.10.5.3 (RRID:SCR_008520).

659 The PBMC that were separated from vaccinated dogs before the first (Pre-Vax) and after the fourth
660 (Post-Vax) immunization were used for cytofluorimetric analyses.[19] For Treg cell detection, thawed
661 PBMC were incubated with dog IgG (Thermo-Fisher Scientific) to block the Fc receptor, and were
662 then stained with LIVE/DEAD Fixable Red Dye (Invitrogen) for 30 min at 4°C. After washing, cells
663 were stained with the following mAbs: rat anti-canine CD4-RPE-cy7 (YKIX302.9, MCA1038GA,
664 AbD Serotec, Kidlington, UK) and mouse anti-dog CD25-FITC (P4A10, 11-0250-042, Thermo-
665 Fisher Scientific). Cells were then fixed/permeabilized and stained with anti-mouse FoxP3-APC
666 (FJK-16s, 14-5773-82, eBiosciences, San Diego, California, USA). Samples were acquired using a
667 BD FACSVerse (BD BioScience) and analyzed with FlowJO V.10.5.3 (RRID:SCR_008520).

668

669 **Human specimens and lymphocyte activation**

670 Human peripheral blood leukocytes (PBL) were isolated via Ficoll-Hypaque (Lonza) gradient
671 centrifugation from the heparinized venous blood of healthy subjects (n = 8) that was provided by the
672 local Blood Bank (Turin, Italy). To determine human leukocyte antigen (HLA)-A2 positivity, the
673 PBL were incubated with anti-HLA-A2-PE mAb (clone BB7.2, BD Pharmingen, Franklin Lakes,
674 New Jersey, USA), and expression was evaluated by flow cytometry. Four healthy donors were found
675 to be HLA-A2 positive and were used for further assays.

676 The generation of monocyte-derived dendritic cells (DC) was conducted as previously described.[65]
677 Briefly, monocytes were isolated from PBL using CD14 MicroBeads (Miltenyi) and were

subsequently cultured with IL-4 and GM-CSF, both from PeproTech (Cranbury, New Jersey, USA), to generate immature DC. TNF-α (50 ng/mL) and IL-1ß (50 ng/mL), both from PeproTech, were added for the final 24 h to induce DC maturation. CD14-depleted PBL were stored in liquid nitrogen until use.

Mature DC (mDC) were resuspended in 100 µL of electroporation buffer (DC transfection kit, Amaxa, Lonza) and mixed with either 5 µg of HuDo-CSPG4, Hu-CSPG4 or empty pCDNA3.1 DNA plasmids. Electroporation was performed using a Nucleofector program U-002 (Amaxa, Lonza). After electroporation, cells were transferred into complete media and co-cultured with thawed lymphocytes for 7 days in RPMI-1640 medium with 10% heat-inactivated human serum AB (Lonza) at 2 x 10^6/mL. Pre-activated lymphocytes were then collected for the cytotoxicity assays using U2OS as the target cells, as previously described.[19]

Cytotoxicity assay

Canine D22, canine D22 Do-CSPG4 and human U2OS target cells were labeled with 2µM of carboxyfluorescein succinimidyl ester (CFSE, Molecular Probes, Eugene, Oregon, USA). Canine OSA cells were then cultured with thawed Pre-Vax and Post-Vax PBMC from dogs included in the vaccination group, based on sample accessibility. Human OSA cells were incubated with pre-activated lymphocytes from healthy donors at an effector:target (E:T) ratio of either 50:1 or 25:1 for 48h at 37°C in a 5% CO_2 atmosphere. After staining with 1µg/mL 7-Amino-ActinomycinD (7-AAD, RRID:AB_2869266; BD BioSciences), cells were acquired using a BD FACSVerse and analyzed with FlowJO V.10.5.3 (RRID:SCR_008520). The percentage of killing was obtained by back-gating the $CFSE^+$ targets and measuring the percentage of dead 7-AAD^+ cells, as previously described.[19] Spontaneous death was obtained by culturing target cells without PBMC, and maximal cell death was obtained after treatment with 1% saponin. The percentage of specific lysis was calculated using the following formula: ((dead targets in sample (%) − spontaneously dead targets (%))/(dead target maximum (%) − spontaneously dead targets (%)) × 100.

Statistical analysis

Normal distribution was calculated through the Shapiro-Wilk test. Two-tailed paired and unpaired, parametric and non-parametric, Student's t-tests and one-way ANOVA were used to perform the statistical analyses for normally and not normally distributed data, respectively. The Kaplan-Meier method was used to estimate the overall survival (OS) and disease-free interval (DFI) of dogs enrolled in the study. The OS of dogs was calculated as the number of days from surgery to death. The DFI was calculated as the number of days from the date of surgery to the date in which metastases were first detected. Differences in survival times were analyzed using the Gehan-Breslow-Wilcoxon test and the Log-Rank test. Statistical significance was evaluated using GraphPad V.9 software (GraphPad; RRID:SCR_002798) and values of $p \leq 0.05$ were considered significant**.**

Data Availability Statement: The data generated in this study are available upon request from the corresponding authors.

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Author Contributions

L Tarone: Conceptualization, data curation, formal analysis, investigation, methodology, validation, visualization, writing-original draft, writing-review & editing; **D Giacobino:** Data curation, formal analysis, investigation, writing-review & editing; **MT Camerino:** Data curation, investigation; **L Maniscalco:** Formal analysis, methodology, writing-review & editing; **S Iussich:** Formal analysis, methodology, writing-review & editing; **L Parisi:** Data curation, formal analysis; **G Giovannini**: Data curation, investigation; **A Dentini:** Data curation, investigation; **E Bolli:** Methodology; **E Quaglino:** Methodology; **IF Merighi:** Methodology; **E Morello:** Conceptualization, data curation, investigation, writing-review & editing; **P Buracco:** Conceptualization, data curation, funding acquisition, investigation, project administration, resources, writing-review & editing; **F Riccardo:** Conceptualization, data curation, formal analysis, investigation, methodology, validation, visualization, project administration, resources, writing-original draft, writing-review & editing; **F Cavallo:** Conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, validation, visualization, project administration, resources, writing-original draft, writing-review & editing.

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983 **Table 1.** Characteristics of OSA-bearing dogs included in the study in the period June 2011-
984 November-2021

#Dog	Breed	Age (years)	Weight (kg)	Histological Grade	CSPG4 score	Chemotherapy cycles	Vaccination cycles
#1	**Leonberger**	6	50	III	8	5	7
#2	**Corso**	9	60	II	4	6	30
#3	**Mixed Breed**	9	32.5	II	7	4	10
#4	**Leonberger**	6	68.5	II	7	6	8
#5	**Mixed Breed**	13	33.6	III	8	4	12
#6	**Rottweiler**	8	44	I	3	5	9
#7	**Mixed Breed**	11	33	I	7	6	5
#8	**Shnauzer**	6	17	II	4	4	6
#9	**Mixed Breed**	11	22	I	6	4	4
#10	**Mixed Breed**	9	40	I	6	4	13
#11	**Great dane**	5.5	60	I	5	4	7
#12	**Mixed Breed**	10	6	I	3	4	10
#13	**Mixed Breed**	9	22	III	8	4	-
#14	**Mixed Breed**	9	32	II	6	4	-
#15	**German Sheperd**	7	30	II	5	5	-
#16	**Czechoslovakian Wolfdog**	3	29	II	5	4	-
#17	**Mixed Breed**	7	55	II	4	5	-
#18	**Mixed Breed**	13	28	II	6	5	-
#19	**Mixed Breed**	8	40	I	5	6	-
#20	**Rottweiler**	10	45	III	5	5	-
#21	**Mixed Breed**	6	32	II	6	6	-
#22	**Rottweiler**	9	29	I	4	6	-

#23	**Czechoslovakian Wolfdog**	9	32	II	8	6	-
#24	**Mixed Breed**	13	34	II	7	4	-
#25	**Mixed Breed**	n/a	8	I	7	6	-

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List of Figure Captions

Figure 1. Functional consequences of CSPG4 silencing in human the U2OS OSA cell line. (**A**) Semi-quantitative RT-PCR of CSPG4 mRNA expression in U2OS OSA cells. Results are calculated using the $2^{-\Delta\Delta Ct}$ method and by considering the difference between the Ct of CSPG4 mRNA and the matched Ct of the internal control gene GADPH mRNA, and by then comparing cells treated with scrambled (Scrmbl) siRNA (black) with hCSPG4-siRNA (red). (**B**) Immunoblot of CSPG4 protein expression in U2OS OSA cells, comparing lysates from cells treated with the scramble (Scrmbl) siRNA with those from cells treated with hCSPG4-siRNA. β-actin was used as the protein-loading control. CSPG4 mRNA (**A**) and protein (**B**) expression levels were evaluated 24, 48 and 72h after siRNA transfection. (**C**) U2OS cell proliferation was assessed using the MTT assay, and the results of three biological replicates are expressed as the percentage (mean value ± SEM) of cell viability at different time points, using the optical density, measured at 570 nm, of cells treated with scramble (Scrmbl) siRNA as 100%. Student's t test, **** $p < 0.0001$. (**D**) U2OS cell migratory ability was assessed using the Transwell migration assay. OSA cells treated with either scramble (Scrmbl) or hCSPG4-siRNA were placed in the upper chamber and incubated for the indicated time points. Cells that migrated to the lower surface of the membrane were stained with crystal violet for microscopic observation. Representative images of one of at least three experiments, showing Scrmbl- and hCSPG4-siRNA-migrating cells at 24h, 48h and 72h (upper panels). The percentage (mean ± SEM) of the area covered by migrated cells in five different fields are reported in the graphs (lower panels, one of at least three experiments). Student's t test, **** $p < 0.0001$. (**E**) Western blot analysis of CD133 and Stro-1 CSC markers in U2OS epithelial cells (left panel) and derived osteospheres (right panel), comparing lysates from cells treated with scrambled (Scrmbl) siRNA with those treated with hCSPG4-siRNA. GAPDH was used as the protein-loading control. (**F**) Representative images showing osteospheres derived from scramble (Scrmbl)- and hCSPG4-siRNA-treated U2OS OSA cells. (**G**) Number of osteospheres derived from U2OS cells that were previously treated with scramble (Scrmbl)- or hCSPG4-siRNA, counted in five random fields/well. Results are expressed as

1012 the number of spheres/1000 plated cells and are representative of three replicate experiments.
1013 Student's t-test, * $p < 0.0238$.

1014 **Figure 2. HuDo-CSPG4 vaccination delays human OSA tumor growth and impairs metastatic**
1015 **dissemination *in vivo***. (**A, E**) Experimental protocols of adoptive transfer of isolated $CD8^+$ T cells
1016 (**A**) and passive transfer of sera (**E**) in OSA-bearing NSG mice. HLA-A2.1 Tg mice (**A**) and Balb/c
1017 mice (**E**) were immunized twice, at a two-week interval, with either the pcDNA3.1 empty vector or
1018 HuDo-CSPG4 coding plasmids. Two weeks after the last administration, spleens were harvested and
1019 $CD8^+$ T cells were isolated (**A**) and sera were collected (**E**). $CD8^+$ T cells and sera were injected into
1020 the tail vein of NSG mice previously challenged with 1 x 10^6 U2OS OSA cell line that displayed a
1021 palpable tumor. Illustrations were created using BioRender.com. (**B**, **F**) Tumor growth curves
1022 following the adoptive transfer of $CD8^+$ T cells (**B**) and passive transfer of sera (**F**) collected from
1023 pcDNA3.1-empty-vector- (black line) and HuDo-CSPG4- (blue line) vaccinated mice. Subcutaneous
1024 tumor volumes (mm^3) were measured at the indicated time points. Student's t-test, * $p<0.03$; **
1025 $p<0.0080$. (**C**) IHC analysis of U2OS-derived tumors (upper and middle panels) and lungs (lower
1026 panels) explanted from animals transferred with $CD8^+$ T cells collected from pcDNA3.1 and HuDo-
1027 CSPG4 vaccinated mice**.** Representative IHC analysis of T lymphocytes infiltrating the tumors and
1028 CSPG4 expression in lung lesions. Images were acquired using a Leica DM750 microscope and an
1029 ICC50 camera. (**D**, **H**) Representative H&E images of metastases in the lungs explanted from NSG-
1030 OSA-bearing mice treated with $CD8^+$ T cells (**D**) or sera (**H**) derived from empty pcDNA3.1- or
1031 HuDo-CSPG4-vaccinated animals. (**G**) Representative H&E (upper panels) and CSPG4 IHC (lower
1032 panels) analyses of lungs explanted from animals transferred with sera collected from pcDNA3.1 or
1033 HuDo-CSPG4 vaccinated mice**.** Images were acquired using a Leica DM750 microscope and an
1034 ICC50 camera.

1035 **Figure 3**. **HuDo-CSPG4 vaccination induces a specific anti-canine CSPG4 antibody response in**
1036 **vaccinated OSA-affected dogs**. (**A**) Analysis, by means of ELISA assay, of the presence of IgG
1037 against the D1, D2 and D3 domains of the canine CSPG4 protein in the sera of dogs before the first

1038 immunization (Pre-Vax) and after the fourth (Post-Vax). Results express the optical density (O.D.)
1039 at the absorbance measured at 450 nm. The increase of the O.D. observed in the Post-Vax compared
1040 to Pre-Vax sera (response), is indicated by color codes. Green Boxes indicate a complete response
1041 (against all tested domains), yellow boxes indicate a partial response (against at least one domain)
1042 and red boxes indicate the absence of a response. (**B**) ELISA assay evaluating the presence of IgG
1043 against Do-D2 in the sera of dogs before the first immunization (Pre-Vax) and after the fourth (Post-
1044 Vax), measured in dogs' sera showing both partial and no response (**A**). Results are expressed as the
1045 ratio (fold change) between the O.D. (measured at 450 nm) of Post-Vax and Pre-Vax sera. A fold
1046 change between the Post-Vax O.D./Pre-Vax O.D. > 1 is considered as a vaccine-induced IgG
1047 response. (**C**) Chaotropic ELISA assay measuring avidity against the Do-D2 domain of the vaccine-
1048 induced antibodies in the sera of non-responder (red boxes in panel **A**) dogs immunized with the
1049 HuDo-CSPG4 vaccine. Results are expressed as the percentage (%) of antibodies (Ab) that remain
1050 bound after treatment with the chaotropic agent at a 5M concentration in Post-Vax sera, compared to
1051 Pre-Vax sera**.** (**D**) Flow cytometry analysis of canine $CSPG4^+$ OSA cells (Penny) incubated with sera
1052 of dogs before immunization and after the fourth HuDo-CSPG4 vaccination. Total IgG binding was
1053 evaluated using a FITC-conjugated goat anti-dog IgG secondary antibody. Results are expressed as
1054 the ratio (fold change) between the percentages (%) of stained cells incubated with the Post-Vax/Pre-
1055 Vax sera. (**E**) The proliferation of canine OSA $CSPG4^+$ Penny cells was assessed using the MTT
1056 assay and the results (from two independent experiments, each including triplicate technical
1057 replicates) are expressed as optical density (O.D.; mean value ± SEM) measured at 570 nm. Cells
1058 were incubated for 24-48-72h with pooled Pre-Vax or Post-Vax sera collected from canine OSA
1059 patients. Student's t-test, ** $p < 0.0066$. (**F**) Penny-cell migratory ability was assessed using the
1060 Transwell migration assay. OSA cells treated with pooled Pre-Vax or Post-Vax sera from canine OSA
1061 patients were placed in the upper chamber and incubated for the indicated time periods. Cells that
1062 migrated to the lower surface of the membrane were stained with crystal violet for microscopic
1063 observation. The number (mean ± SEM) of migrated cells in five different fields, representative of

four independent experiments, are reported in the graph. Student's t-test, ** $p < 0.0039$. Representative images showing migrating cells at 48h and 72h were included (right panels).

Figure 4. HuDo-CSPG4 induces a specific anti-CSPG4 cellular immune response in vaccinated dogs. (**A, B**) Flow cytometry analysis of the frequencies of circulating MDSC, gated on $CD11b^+/CD14^-MHCII^-$, T regulatory (Treg) cells, gated on $CD4^+/FoxP3^+$ $CD25^+$ cells (**A**), and $CD8^+$ T lymphocytes, gated on $CD5^+$ cells (**B**). Graphs show the percentage of MDSC, Treg and $CD8^+$ T cells circulating in canine patients before (Pre-Vax) and after the fourth HuDo-CSPG4 vaccination (Post-Vax). (**C**) ELISA quantification of IFN-γ released by canine PBMC upon stimulation with Do-CSPG4 peptides. **(D, E**) Cytotoxicity assays to quantify (**D**) the ability of PBMC to kill D22 Do-CSPG4 cells before (Pre-Vax, light blue bar) and after HuDo-CSPG4 vaccination (Post-Vax, blue bar) and (**E**) the ability of Post-Vax PBMC to specifically kill CSPG4-expressing D22 canine OSA cells (blue bar) compared to the CSPG4-negative D22 counterpart (red bar). Results are shown as the % of lysis of CFSE-labeled tumor cells after 48h of incubation with Post-Vax and Pre-Vax PBMC. Student's t-test, **p = 0.0078; *p = 0.0156.

Figure 5. HuDo-CSPG4-vaccinated dogs showed prolonged overall survival (OS) and disease free interval (DFI) compared to conventionally treated controls. (**A**) Swimmer plot graph of canine OSA patients included in the study. Historical controls are indicated as grey bars, and vaccinated dogs are indicated as blue bars. The survival (in months) and clinical information, including time of surgery, chemotherapy cycles and vaccination treatments are depicted in the graph. First clinical manifestation of recurrence/metastasis is reported in the graph. Arrows indicate that the patients are still alive at the time of publication. The 1-year and 2-year post-surgery time points have been indicated by a dotted vertical line. (**B**) Kaplan-Meier curve comparing the OS (in days) of HuDo-CSPG4 vaccinated (blue line) and control (black line) dogs, updated to April 2023. OS rate (percentage) and median survival time (in days) are indicated in the graph. 1-year OS, Gehan-Breslow-Wilcoxon test, * p = 0.0195, Log-rank test, * p = 0.0346; 2-year OS, Gehan-Breslow-Wilcoxon test, * p = 0.0450, Log-rank test, p = 0.1398. (**C**) Kaplan-Meier curve comparing the 1-

year DFI (in days) of HuDo-CSPG4-vaccinated (blue line) and control (black line) dogs, updated to November 2022. Gehan-Breslow-Wilcoxon test, $p = 0.2871$, Log-rank test, $p = 0.5459$.

Figure 6. HuDo-CSPG4-stimulated PBL from healthy subjects show potential anti-CSPG4 cytotoxic activity. (A) Schematic representation of human surrogate assay protocol. Created with Biorender.com. **(B)** Cytotoxicity assay performed with healthy-donor PBMC (N = 4) recovered after 7 days of co-culture with autologous mDC transfected with either the pcDNA3.1 empty vector, Hu-CSPG4 or HuDo-CSPG4 plasmids. Pre-activated PBMC were incubated for 48h at 37°C with CFSE-labeled $CSPG4^+$ U2OS human OSA cells, at different effector:target (E:T) ratios. Results show the fold change between the percentage ($\pm$ SEM) of CFSE-labeled tumor cells lysed by HuDo- and Hu-CSPG4-pre-activated PBMC/pcDNA3.1-pre-activated PBMC. One-way ANOVA test, $p = 0.0660$.

Figure 1

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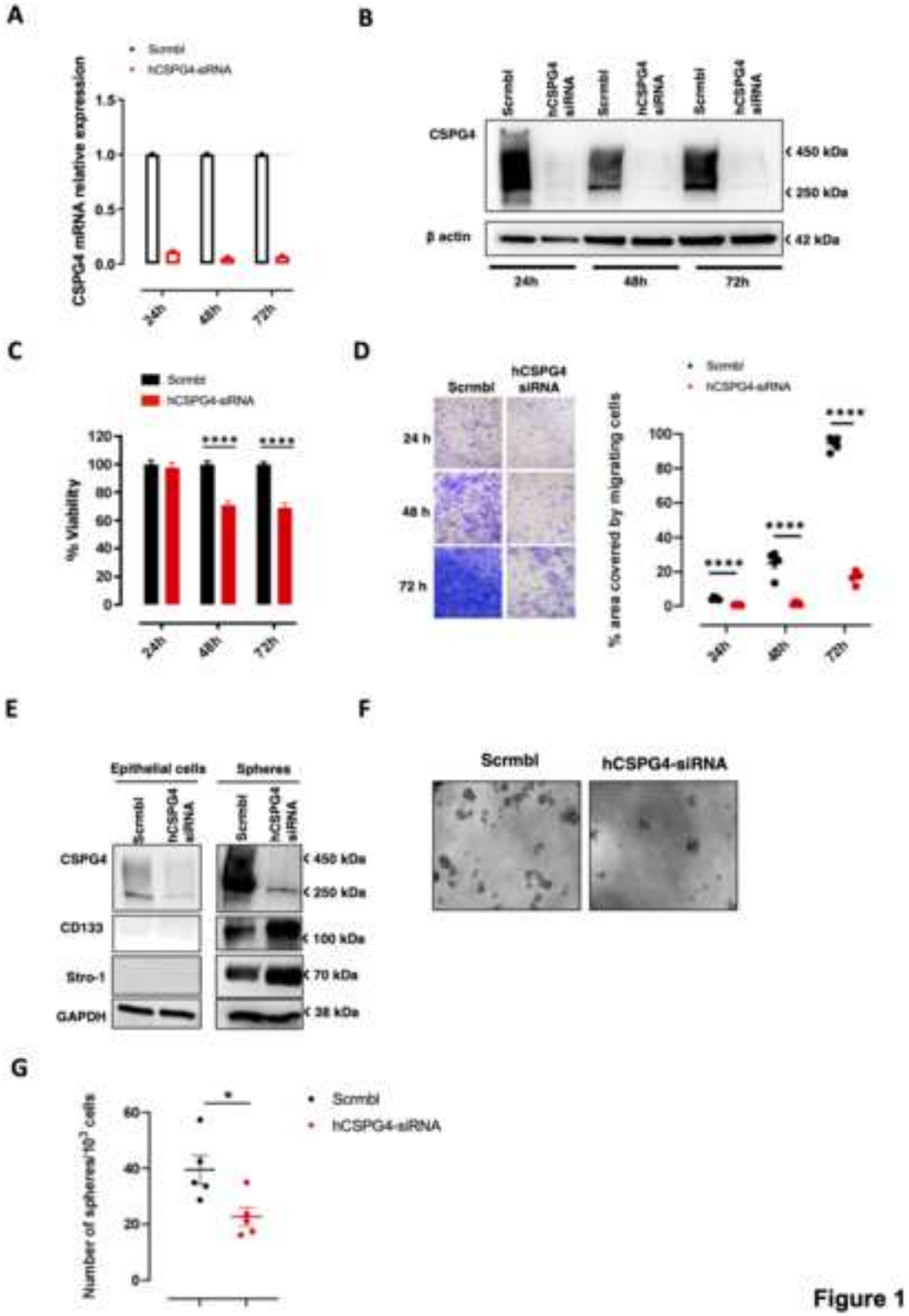

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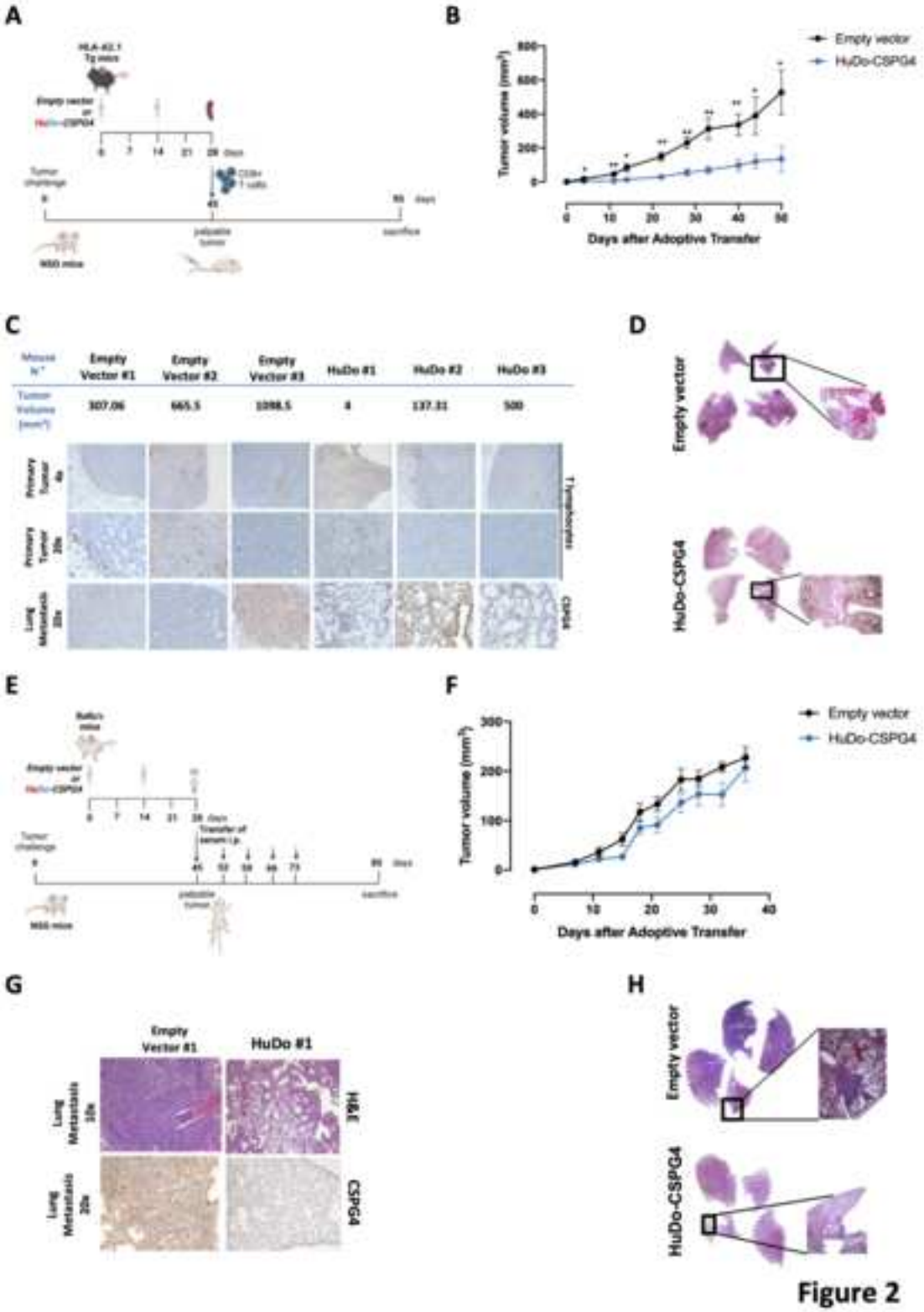

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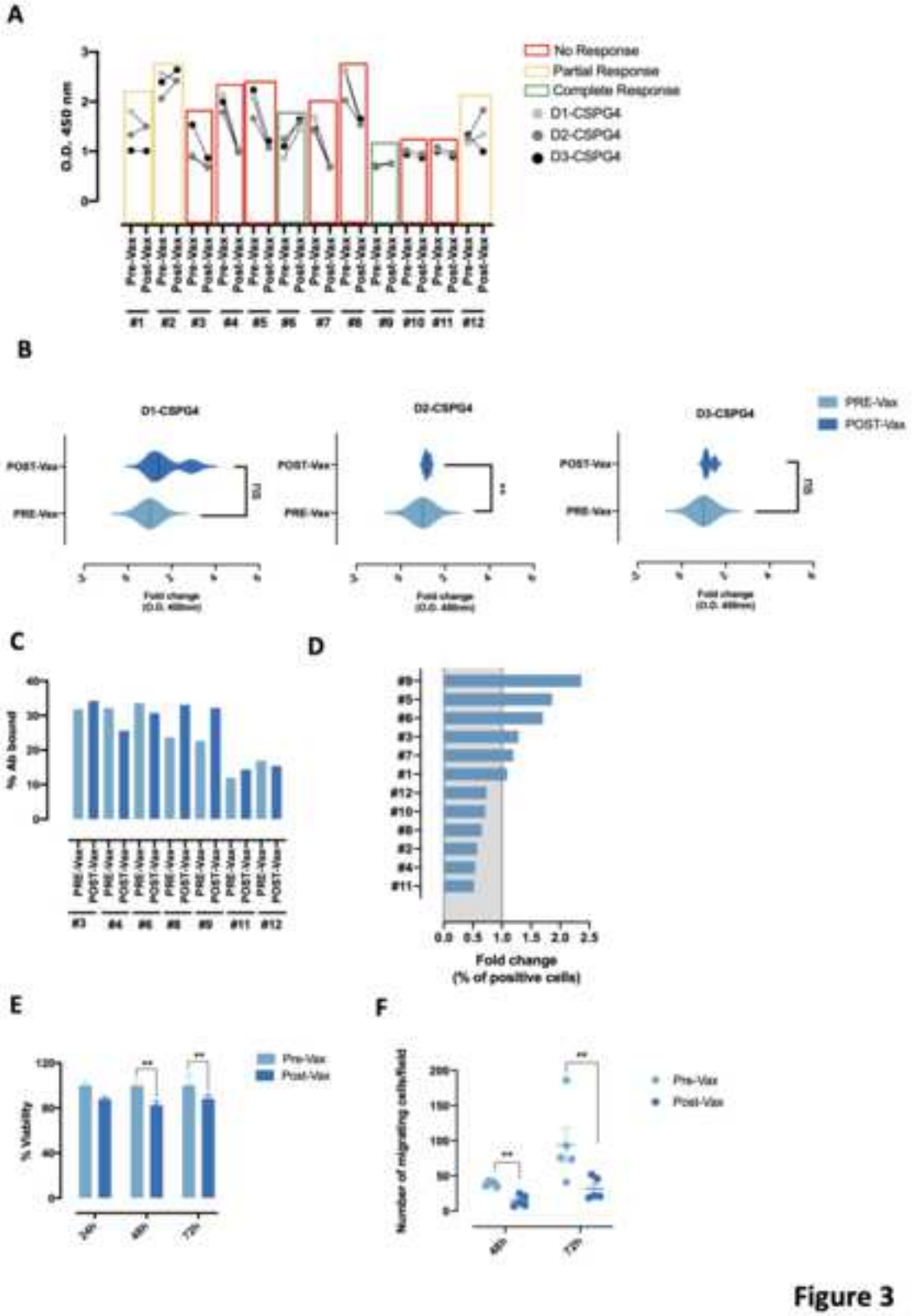

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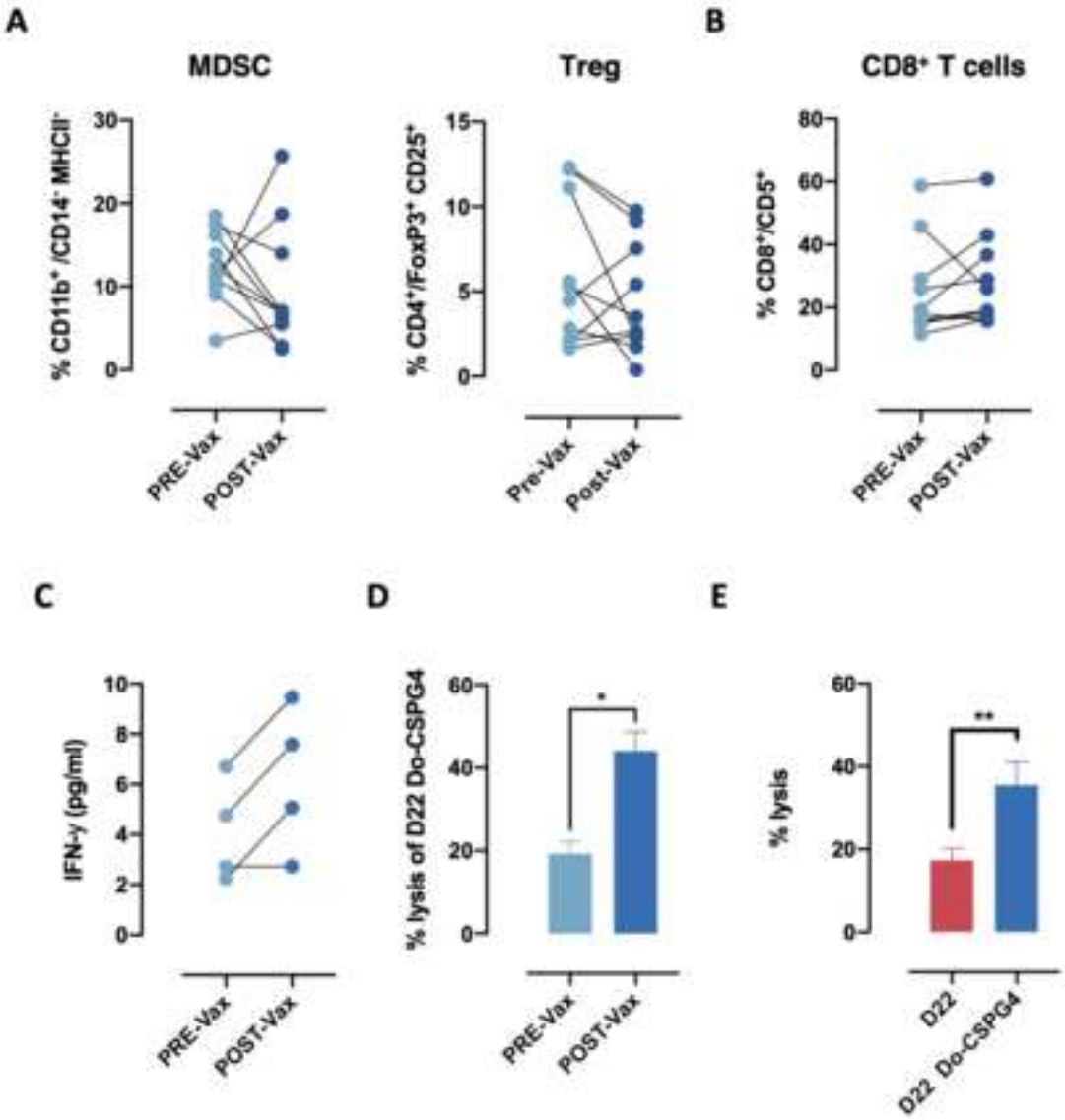

Figure 4

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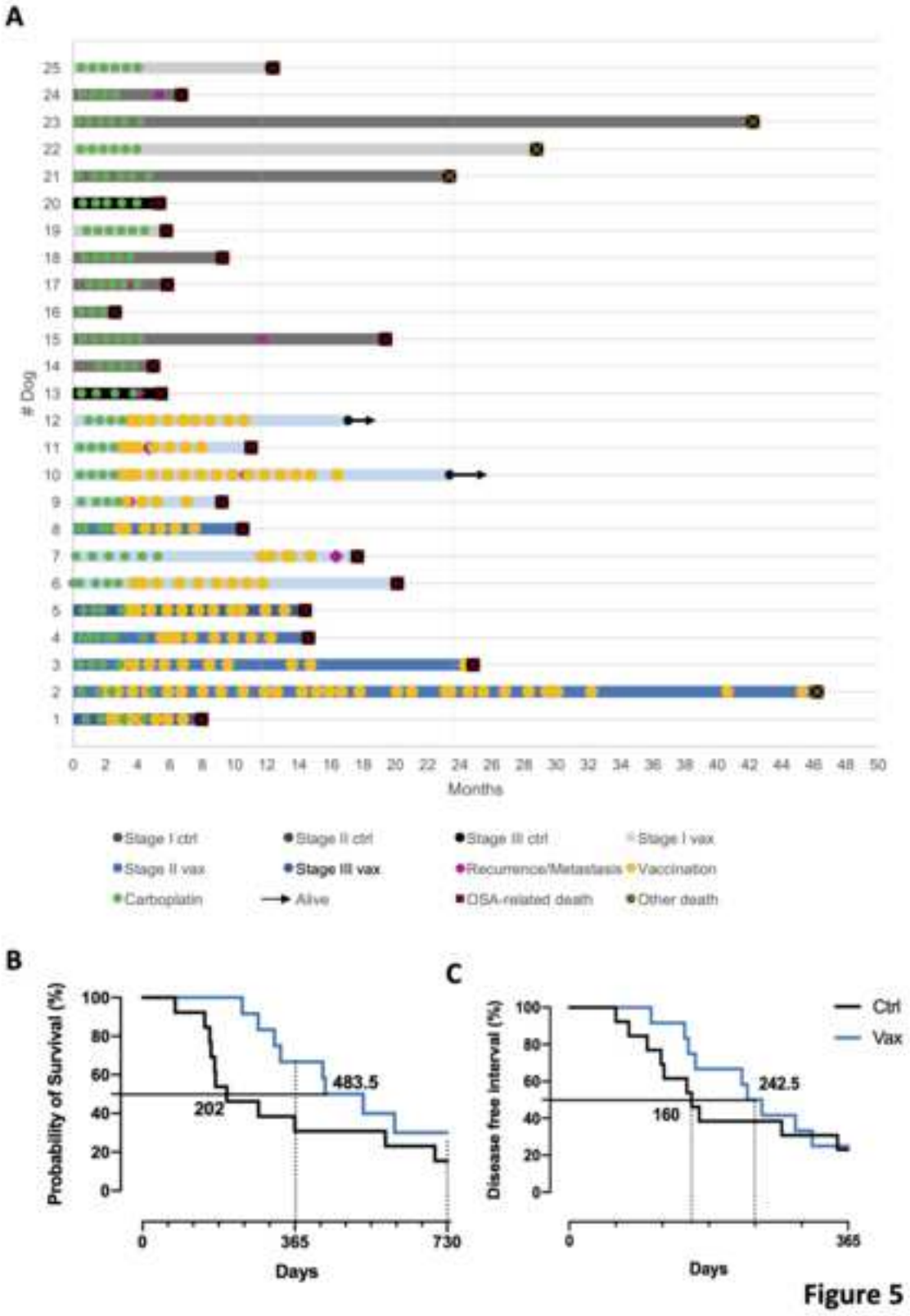

Figure 5

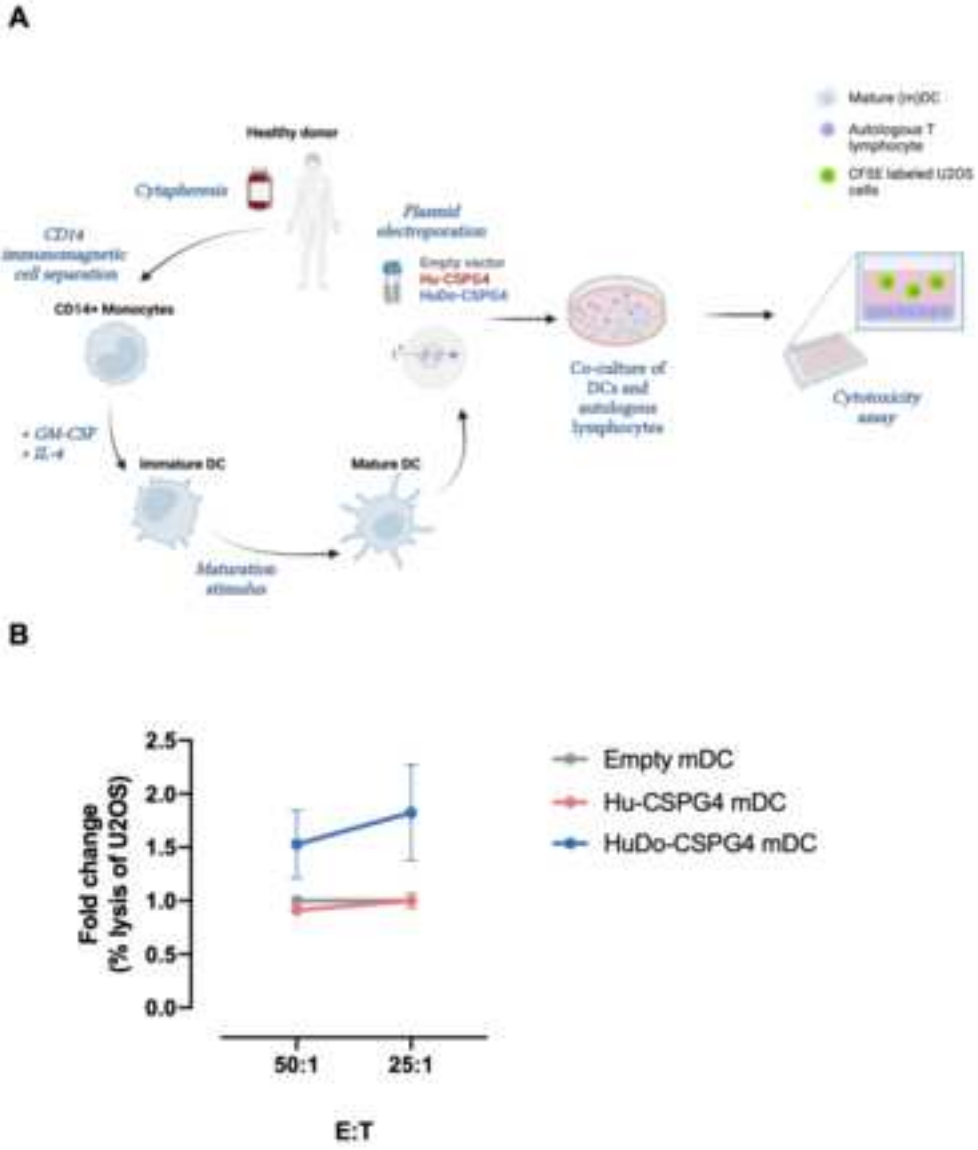

Figure 6

Table S1. Induction of humoral (upper panel) and cellular (low panel) immune responses in vaccinated dogs. The arrows indicate an increase or decrease in the percentage of circulating immune cells (myeloid derived suppressor cells, MDSC; T regulatory cells, Treg; and $CD8^+$ T cells). n/d is specified when the analysis was not performed for the indicated sample.

Humoral response						
Patient	**Do-D1 ELISA**	**Do-D2 ELISA**	**Do-D3 ELISA**	**Do-D2 Chaotropic ELISA**	**Binding to CSPG4+ canine OSA cells**	**Binding to CSPG4+ human OSA cells**
#1	**no**	**yes**	**no**	**n/d**	**yes**	**yes**
#2	**no**	**yes**	**yes**	**n/d**	**no**	**yes**
#3	**no**	**no**	**no**	**yes**	**yes**	**yes**
#4	**no**	**no**	**no**	**no**	**no**	**yes**
#5	**yes**	**yes**	**no**	**n/d**	**yes**	**no**
#6	**no**	**no**	**no**	**no**	**yes**	**yes**
#7	**yes**	**yes**	**yes**	**n/d**	**yes**	**yes**
#8	**no**	**no**	**no**	**yes**	**no**	**yes**
#9	**n/d**	**n/d**	**n/d**	**n/d**	**n/d**	**yes**
#10	**yes**	**yes**	**yes**	**n/d**	**no**	**no**
#11	**no**	**no**	**no**	**yes**	**no**	**yes**
#12	**no**	**no**	**no**	**n/d**	**no**	**no**

Cellular response					
Patient	**MDSC**	**Treg**	**$CD8^+$ T cells**	**In vitro Cytotoxicity (POST-Vax)**	**In vitro Cytotoxicity (PRE-Vax vs POST-Vax)**
#1	↑	↓	↓	**n/d**	**n/d**
#2	↓	↓	↑	**yes**	**yes**
#3	↓	↑	↑	**no**	**yes**
#4	↓	↓	↑	**yes**	**no**
#5	↓	↓	↓	**yes**	**n/d**
#6	↓	↑	↑	**yes**	**yes**
#7	↓	↑	↑	**yes**	**yes**

#8	**n/d**	**n/d**	**n/d**	**n/d**	**n/d**
#9	**n/d**	**n/d**	**n/d**	**n/d**	**n/d**
#10	↑	↓	↑	**yes**	**yes**
#11	↓	↓	↑	**yes**	**yes**
#12	↑	↑	↑	**yes**	**yes**

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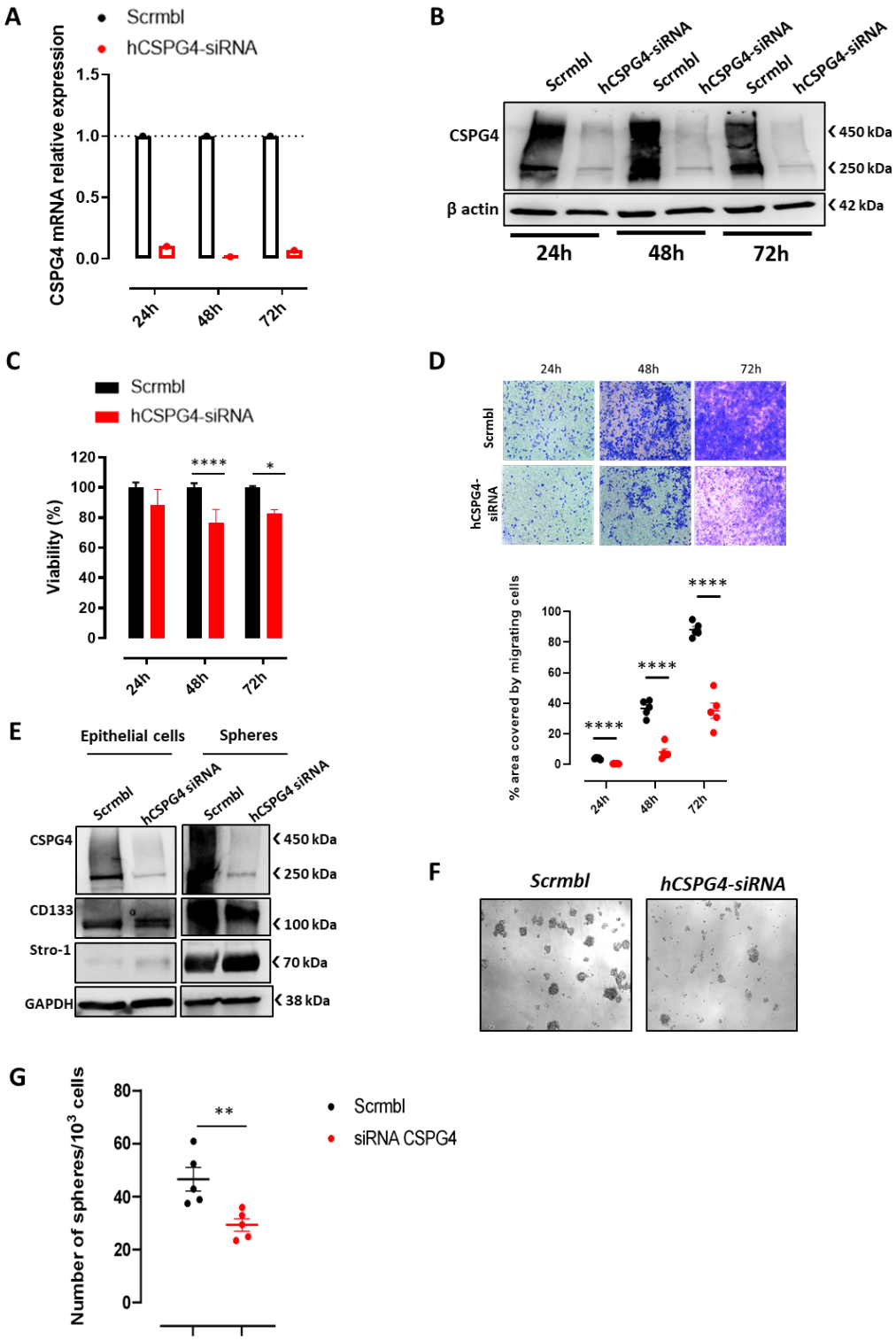

Figure S1. Functional consequences of CSPG4 silencing in human SaOS2 OSA cell lines. (**A**) Semi-quantitative RT-PCR of CSPG4 mRNA expression in SaOS2 OSA cells. Results are calculated using the 2^(-ΔΔCt) method by considering the difference between the Ct of CSPG4 mRNA and the matched Ct of the internal control gene GADPH mRNA, and then by comparing cells treated with either scrambled (Scrmbl) siRNA (black) or hCSPG4 siRNA (red). (**B**) Immunoblot of CSPG4-

protein expression in SaOS2 OSA cell lines, comparing lysates from cells treated with Scrmbl siRNA with those treated with hCSPG4-siRNA. β-actin was used as the protein-loading control. CSPG4 mRNA (**A**) and protein (**B**) expression were evaluated 24h, 48h and 72h post siRNA transfection. (**C**) SaOS2 cell proliferation was assessed using the MTT assay and results of three biological replicates are expressed as the percentage (mean value ± SEM) of cell viability at different time points, considering the optical density, measured at 570 nm, of cells treated with scramble (Scrmbl) siRNA as 100%. Student's t test, **** $p < 0.0001$; * $p = 0.0351$. (**D**) SaOS2 cell migratory ability was assessed using the Transwell migration assay. OSA cells treated with Scrmbl or hCSPG4 siRNA were placed in the upper chamber and incubated for the indicated time points. Cells that migrated to the lower surface of the membrane were stained with crystal violet for microscopic observation. Representative images of one of at least three experiments, showing Scrmbl- and hCSPG4-siRNA-migrating cells at 24h, 48h and 72h (upper panels). The percentage (mean ± SEM) of the area covered by the migrated cells in five different fields are reported in the graphs (lower panels, one of at least three experiments). Student's t test, **** $p < 0.0001$. (**E**) Western blot analysis of CD133 and Stro-1 CSC markers in SaOS2 epithelial cells (left panel) and derived osteospheres (right panel), comparing lysates from cells treated with scrambled (Scrmbl) siRNA with those treated with hCSPG4-siRNA. GAPDH was used as the protein-loading control. (**F**) Representative images showing osteospheres derived from Scrmbl- and hCSPG4-siRNA-treated SaOS2 OSA cells. (**G**) Number of osteospheres derived from SaOS2 cells that were previously treated with Scrmbl- or hCSPG4-siRNA, counted in five random fields/well. Results are expressed as number of spheres/1000 plated cells and are representative of three replicate experiments. Student's t test, ** $p < 0.0091$.

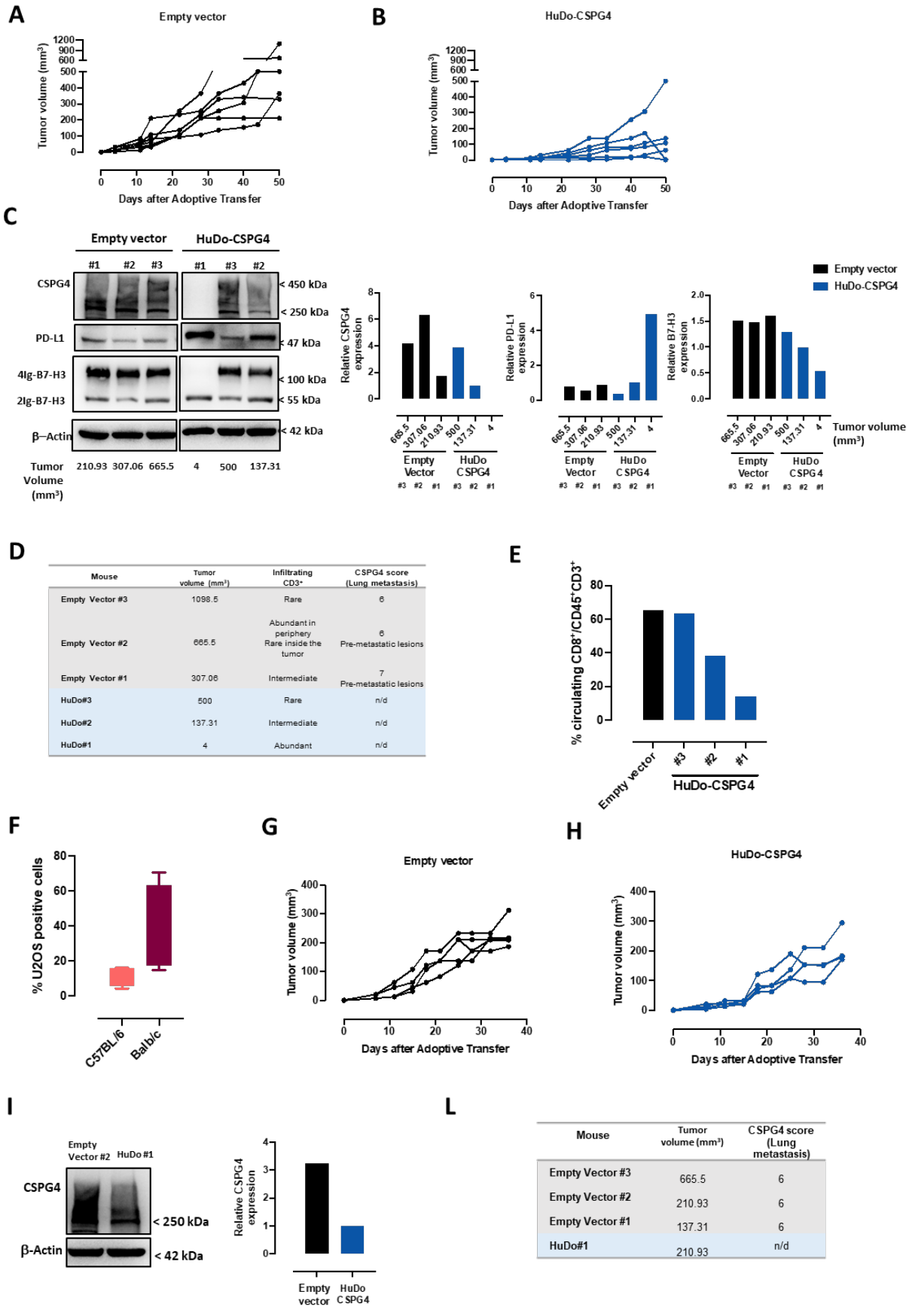

Figure S2. Impairment of human OSA tumor growth may be related to CSPG4 downregulation and high CD8⁺ infiltration. (**A, B**) Tumor growth of U2OS cells injected into NSG mice after

adoptive transfer with $CD8^+$ T cells derived from pcDNA3 (**A**, Empty vector, N = 6 mice) and HuDo-CSPG4 (**B**, N = 6 mice) vaccinated mice. Each line represents the growth of a single tumor. (**C**) Immunoblot depicting CSPG4, PD-L1 and B7-H3 protein-expression levels in tumors explanted from representative NSG mice following adoptive transfer with $CD8^+$ T cells derived from pcDNA3.1 or HuDo-CSPG4 immunized mice. β-actin was used as the loading control. (**D**) Table summarizing the percentage of $CD3^+$ T cells infiltrating the tumors and the CSPG4 score of expression in lung metastases collected from NSG mice transferred with $CD8^+$ T cells from control or HuDo-CSPG4 immunized mice, considering tumor volume. (**E**) Flow cytometry analysis, showing percentage (%) of $CD8^+$ T cells gated on $CD45^+CD3^+$ cells circulating in the blood of adoptively transferred NSG mice on day 50 after the transfer. (**F**) Flow cytometry analysis of U2OS cells incubated with sera of either C57/BL6 HLA-A2.1 Tg mice or Balb/c mice immunized with the HuDo-CSPG4 plasmid. Total IgG binding was evaluated using a FITC-conjugated goat anti-mouse IgG secondary antibody. Results are expressed as the percentage $\pm$ SEM of CSPG4-positive cells. (**G, H**) Tumor growth of U2OS cells injected into NSG mice and adoptively transferred with pooled sera derived from pcDNA3.1 (**F**, Empty vector, N = 5 mice) and HuDo-CSPG4 (**G**, N = 4 mice) vaccinated mice. Each line represents the growth of a single tumor. (**I**) Representative immunoblot showing CSPG4 protein-expression levels in matched-paired tumors explanted from representative NSG mice following adoptive transfer with sera derived from pcDNA3.1 or HuDo-CSPG4 immunized mice. β-actin was used as the loading control.

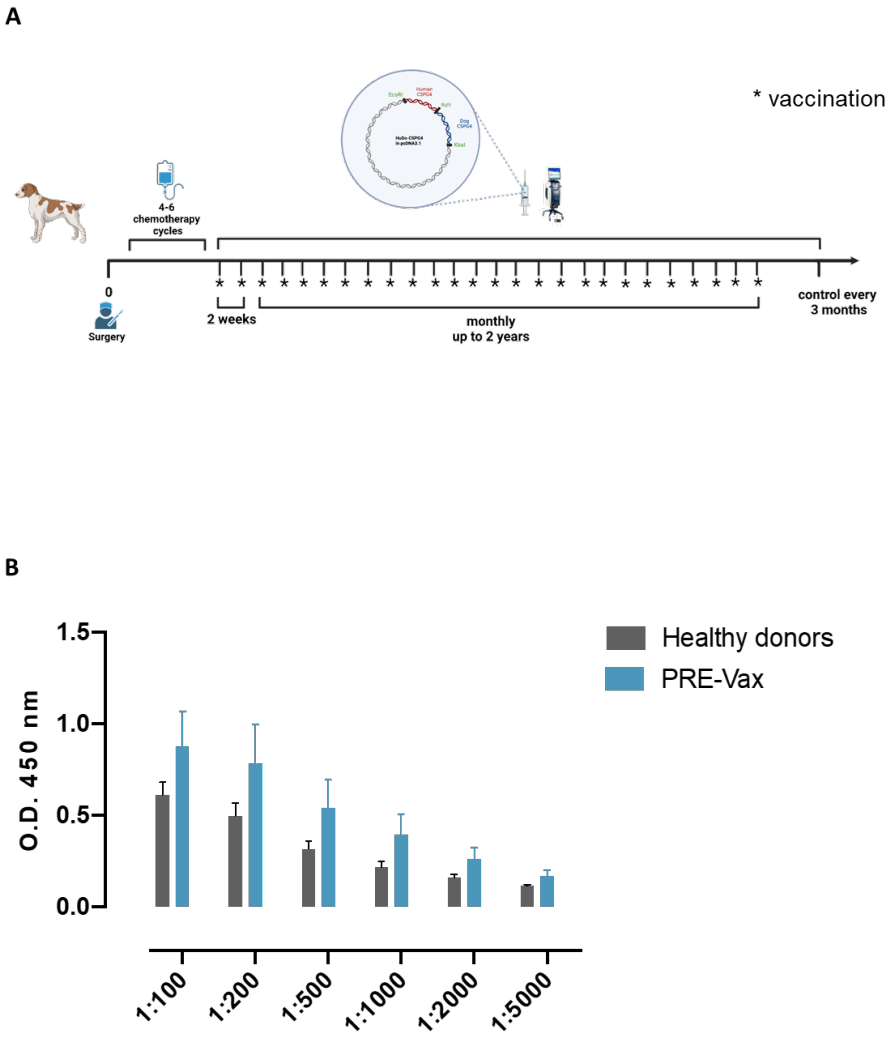

Figure S3. Vaccination protocol for OSA-bearing canine patients included in the study. (A) Schematic representation of the study design of adjuvant HuDo-CSPG4 immunization. Illustration was created using BioRender.com. **(B)** Analysis, by means of ELISA assay, of the presence of spontaneous anti-CSPG4 IgG against the D2 of the canine CSPG4 protein in the sera of healthy dogs or in the sera collected from CSPG4^{+} OSA canine patients after the chemotherapeutic protocol with carboplatin and before the starting of the immunization protocol. Sera were analyzed in sequential dilutions, from 1:100 to 1:5000, and results are expressed as optical density (O.D.) measured at 450 nm.

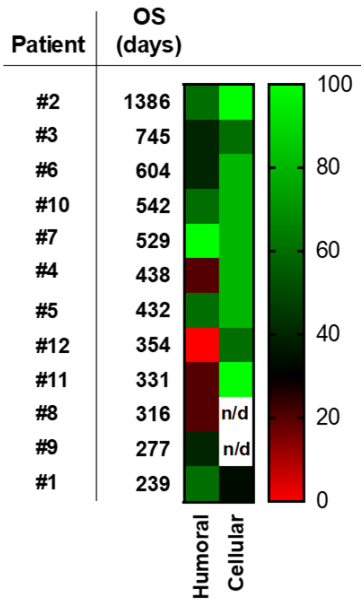

Figure S4. HuDo-CSPG4-vaccine-induced immune response. An immune-response score (between 0 - 100%) has been assigned to each vaccinated canine patient, with 0 indicating the absence of a response and 100 a complete response to the parameters analyzed for both humoral and cellular immunity. The heat map shows the "immune-score" ordered according to the overall survival of vaccinated dogs.

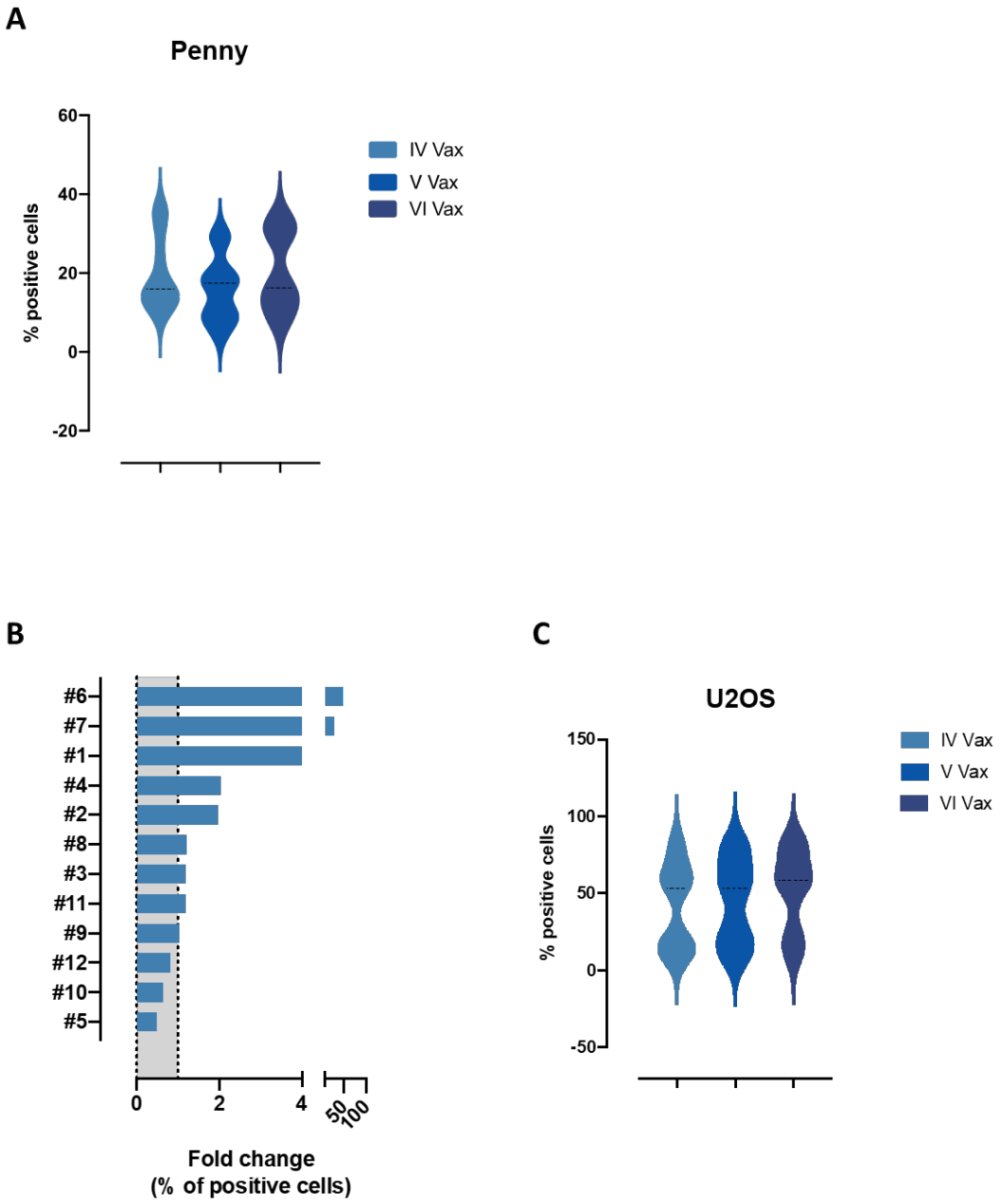

Figure S5. HuDo-CSPG4 boosting in dogs induces antibodies that bind the Do- and Hu-CSPG4 proteins. (**A**) Flow cytometry analysis of canine CSPG4^{+} Penny cells incubated with sera collected after the fourth, fifth and sixth HuDo-CSPG4 vaccinations (IV, V, VI Vax). (**B**, **C**) Flow cytometry analysis of human CSPG4^{+} U2OS cells incubated with sera collected before vaccination (**B**) and after the fourth (**B**), fifth and sixth (**C**) HuDo-CSPG4 vaccinations (IV, V, VI Vax). Total IgG binding was evaluated using a FITC-conjugated goat anti-dog IgG secondary antibody. Results are expressed as the percentage (%) of positive cells (**A**, **C**) and as the ratio (fold change) of the % of stained cells incubated with the Post-Vax (IV)/Pre-Vax sera (**B**).

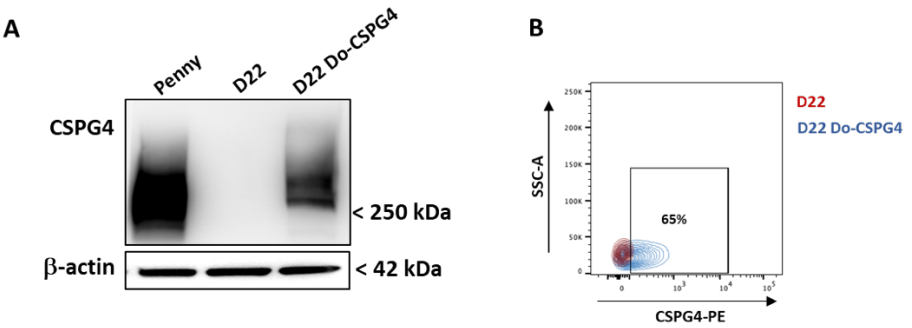

Figure S6. Generation of a canine OSA cell line that stably over-expresses Do-CSPG4. (**A**, **B**) Canine CSPG4$^-$ D22 OSA cells were stably transfected with the Do-CSPG4-coding plasmid and resultant CSPG4 over-expression was confirmed by western blot (**A**) and flow cytometry analyses (**B**). (**A**) For western blot assays, naturally CSPG4-over-expressing canine Penny cells were used as the positive control and β-actin was used as the protein loading control. (**B**) D22 (red) and D22 Do-CSPG4 (blue) cells were incubated with anti-CSPG4 mAb (TP-49). Total IgG binding was evaluated using a PE-conjugated goat anti-mouse IgG secondary antibody. Flow cytometry analysis was performed using a FACS Verse and results were analyzed with FlowJo software. A representative plot is shown and the percentages (%) of D22 Do-CSPG4 stained cells are indicated.

eTOC Synopsis

Human pre-clinical models and canine patients affected by spontaneous osteosarcoma have been exploited to demonstrate that anti-CSPG4 vaccination using a chimeric human/dog plasmid is safe and immunogenic, being able to counteract tumor progression. The herein observed results suggest a possible evaluation of this treatment in the human clinical setting.