CAPÍTULO 23

TREATMENT OF CANINE OSTEOSARCOMA USING AUTOLOGOUS ACTIVE IMMUNOTHERAPY WITH OR WITHOUT SURGERY

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ABSTRACT: Canine osteosarcoma is a disease having a very poor short term prognosis and is a good model for the study of the human disease. It was demonstrated that both in the human and canine disease heat shock proteins (HSPs) were synthesized by the cancer cells. These molecules are chaperone molecules for the cell peptides and also are involved in their associated peptide presentation to the lymphocytes. We developed a method for immune system stimulation based on the purification of intratumoral autologous heat shock proteins (HSPs) which are then re injected in the subcutaneous tissue with a phosphocalcic particulate adjuvant. We tested this method on 12 dogs and the overall survival (OS) and progression free survival (PFS) were measured. Two groups can be differentiated. A long survival group (n=7) and a short survival group (n=3) in which two dogs were killed for pain while their overall conditions was good. In the long survival group, three dogs were amputated and showed a longer OS than non amputated. No secondary effect was observed following the injections. The OS and PFS were greatly improved compared to the data given in the literature in the long survival group. In conclusions, these results suggest that this protocol could be of interest in human medicine associated to classical radio and chemotherapy.
KEYWORDS: Osteosarcoma, immunotherapy, vaccine.

TRATAMENTO DO OSTEOSSARCOMA CANINO COM IMUNOTERAPIA AUTÓLOGA ATIVA COM OU SEM CIRURGIA

RESUMO : O osteossarcoma canino é uma doença com um prognóstico de curto prazo muito ruim e é um bom modelo para doença humana. Em humanos e cães, as proteínas de choque térmico (HSPs) são sintetizadas por células cancerosas. Essas moléculas, além do papel de chaperonas de peptídeos celulares, desempenham um papel importante na apresentação de antígenos às

células imunocompetentes. Desenvolvemos um método de estimulação do sistema imunológico contra células tumorais por purificação de proteínas autólogas de choque térmico intratumoral e sua administração subcutânea acompanhada por adjuvante fosfocálcico. Testamos este método em uma série de 12 cães com osteossarcomas para os quais a sobrevida global (OS), bem como a sobrevida livre de progressão da doença (PFS) foram medidas. Dois cães se perderam de vista. Nos 10 cães restantes, dois grupos foram individualizados. Um grupo (n = 7) com vida longa e outro (n = 3) com vida curta, no qual dois cães foram sacrificados por um problema de dor quando seu estado geral não justificava a eutanásia. No grupo de cães com longa sobrevida, 3 foram amputados e tiveram uma sobrevida maior do que aqueles sem amputação. Nenhum efeito colateral foi observado em nenhum dos cães. Portanto, parece que esses dois parâmetros foram significativamente aumentados em comparação com os dados da literatura no grupo de sobrevida longa. Em conclusão, esses resultados sugerem que esta técnica de estimulação do sistema imunológico pode ser de interesse em humanos associada ao tratamento quimio / radioterápico.
PALAVRAS-CHAVE: Osteossarcoma, imunoterapia, vacina.

1 | INTRODUCTION

In human medicine, osteosarcoma is the most common, although rare, primary bone tumor. It still has a poor prognosis despite the improvement made in recent years by chemotherapy combined with major surgery. This pathology is all the more worrying as it affects a young population. Canine osteosarcoma shares many points in common with humans and can thus constitute an interesting model. Clinically its presentation and its evolution in particular its capacity to produce metastases are very similar, biologically many alterations of certain cell signaling pathways are found in both cases as well as the overexpression of certain oncogenes [1].

From a veterinary perspective, most dogs are seen at an advanced stage that is already metastatic or micrometastatic, and in daily veterinary practice very few animals undergo chemotherapy. The treatment of choice remains amputation which somewhat improves the prognosis since the median survival of amputated dogs is 5 months [2]. The histological classification makes it possible to distinguish 4 stages of increasing aggressiveness. The overall survival of the dog if the pain does not justify euthanasia remains from a few days to a few weeks [3] without specific treatment.

In recent years, numerous teams have developed techniques for stimulating the immune system in order to fight specifically against tumor cells with few side effects, in particular by preserving rapidly renewing cells which are the preferred target of chemotherapy treatments [4] . Various techniques for activating the immune system have been developed and can be grouped into 2 broad categories.

Passive immunotherapy is widely used today. It uses monoclonal antibodies whether or not associated with immunotoxins or radioelements to destroy the target

cell. These monoclonal antibodies, when not associated with any molecule or toxic element, can be used to block a receptor or an informative molecule such as anti-VEGFs that limit tumor vascularization [5, 6]. There are also techniques using non-specific stimulants such as GM-CSF [7, 8], Il-2 [9], and BCG [10].

On the contrary, active immunotherapy aims to stimulate the patient's immune system and in particular his cellular immunity (CD8) against antigens of tumor cells. Activation of CD8 T lymphocytes against tumor cells takes place through the stimulation and maturation of antigen presenting cells (APCs), mainly macrophages and dendritic cells which will shape tumor antigens and express them on the cell surface associated to MHC class I proteins to present them to CD8s [11, 12]. Stimulation of autologous dendritic cells *in vitro* by tumor antigens followed by their reinjection has been widely used for various types of tumors with varying results [13, 14]. The collection and *in vitro* amplification of T lymphocytes infiltrated into tumors can also be considered for osteosarcomas [15] as well as the injection of NK cells which have the capacity to fight tumor cells without being «educated».

It is known that tumor cells are extremely genetically unstable cells which synthesize many abnormal proteins, several dozens to several hundred. The genetic instability of the cancer cell means that this late abnormal proteins are not the same throughout the course of the tumor and they differ from patient to patient [16, 17]. There are, however, proteins that are abnormal or that are not normally expressed in the cell line from which the cancer cell is derived, which are found almost systematically in a particular pathology. These latter proteins have made it possible to imagine therapeutic monoprotein vaccines that have given poor results in reason of the constant cell mutation.

Vaccines with many antigenic patterns appear to be more interesting because they provide many elements of cellular identity and the recognition of tumor cells by CD8 is less sensitive to point mutations. We have developed a process for the preparation of therapeutic vaccines containing numerous antigenic motifs by isolating stress proteins from osteosarcomas. Gp96 and Hsp70 are indeed found in large quantities in tumor cells, probably due to the cellular suffering induced by the abnormal metabolism of these cells [18]. These are chaperone molecules which are therefore associated with almost all the peptides synthesized by the cell [19, 20, 21, 22]. They are also involved in the presentation of these peptides associated with the surface of APCs and thus participate in monitoring the immune survey of any cell transformation by the immune system. In both dogs and humans, transformed osteosarcoma cells have been shown to produce these proteins in large quantities regardless of the histological type [23, 24, 25].

We selected a series of 12 dogs consulting for appendicular or flat bone osteosarcoma. The stress proteins have been isolated in order to constitute

autologous vaccines. They are purified by adsorption on columns containing micro / nanoparticles of calcium phosphates which are then injected into the same animal to stimulate the immune system. The aim of this study was to investigate the feasibility of the protocol in veterinary practice, to assess the absence of side effects as well as overall survival and the period of absence of disease progression. This study also provides a model of immune stimulation against a solid tumor that can be used in human medicine.

2 I MATERIALS AND METHODS

2.1 Animals treated

The dogs were selected after a consultation motivated by lameness and / or swelling having led to a X rays exploration showing an image of bone proliferation associated with lysis. The inclusion criteria are a general condition preserved suggesting a survival of more than 5 weeks, a count formula within the normal limits, refusal of chemotherapy treatment by the owner, owner able to monitor his dog and bring him back on a fixed date, and signing an informed consent.

A regional extension assessment to search for lymphadenopathy is implemented and a chest x-ray is systematically performed at the first visit. At each visit for an injection of a dose of immunotherapy, the size of the inflammatory area is assessed and noted. An extension assessment is done if clinical signs appear. The animals all received nonsteroidal anti-inflammatory drugs and non-morphine analgesics on demand. Three dogs were amputated at the request of the owner before the beginning of the vaccine protocol without reconstructive surgery or devices.

The progression free survival time (PFS progression free survival) is taken from the date of the first vaccine to a sign of disease progression (tumor growth, metastasis or fracture). A complete response was defined as a disappearance of clinical signs for at least 4 weeks, a partial response as a reduction in the perpendicular diameters of the lesions by more than 50% for 4 weeks, a minor response as a reduction in these same diameters between 25 and 50%, stable disease such as less than 25% reduction without onset of metastasis and progression such as onset of metastasis or growth of diameters greater than 25%

2.2 Dose preparation

Under general anesthesia, a tumor biopsy is taken with a cortical to cortical Michelet trephine. A fragment of the sample is fixed in a formaldehyde solution and sent to the pathological anatomy laboratory. The rest (about 0.5 cm3) is sterilized in a dry tube and frozen at -18 ° C.

After the histological diagnosis is made, the frozen fragment is ground in a ball homogenizer (Setis, France), the ground material is diluted in 2 cm3 of NaHCO3 (30 mM) and centrifuged at 5000 g to remove cell debris. The supernatant is diluted to 50% with a supersaturated sodium nitrate solution, centrifuged at 5000 g. The supernatant is removed, the protein pellet resuspended in a 20 mM phosphate buffer solution, pH 6.8. The solution is then passed through a column containing 200 mg of calcium hydroxyapatite powder. The column is washed with 10 ml of 50 mM phosphate buffer, Ph 6.8. The powder is then suspended in a solution of 2% carboxylmethylcellulose in 20 mM phosphate buffer, pH 6.8 and distributed so as to represent a volume of 0.5 ml per dose in 1 ml syringes.

The doses are injected subcutaneously at a frequency of 1 per week for 4 weeks then once per month for 4 months.

2.3 Dose control

Electrophoresis and Western blotting as well as a dot blot are performed for each tumor.

The electrophoresis is performed on NuPage gels (in vitrogen, France) according to the manufacturer's recommendations (InvitroGen, France). Briefly, the powder residues in the chromatography column are washed in 0.5 ml of 0.5M NaCl. 20 µl are mixed with 10 µl of detergent and 5 µl of reducing agent (in vitroGen, France). 10 µl of this solution are introduced into the wells of the gel and passed through a 200 V field at 120 mA for 30 min. The gels are stained with silver nitrate.

Dot blot: 10 µl of the vaccine solution are applied to a nitrocellulose membrane and left to dry in the open air. Once dry, the membrane is washed with a blocking solution (1% BSA in PBS) then with a solution of Tris buffer (pH 7.4) containing 0.5% of detergent Tween 20. The membrane is then incubated with primary antibody one hour at room temperature (anti-gp96, anti-HSP70, stressgen-US) before being washed in the same buffer. The membrane is incubated for 30 minutes with the secondary antibody labeled with an alkaline phosphatase and then washed with the buffer before adding a developer for alkaline phosphatase.

2.4 Binding of vaccine proteins on cd91

The RAW264 (ATCC) cell line is characterized by the presence of a large number of cd91 receptors on the surface of the cell membrane, these receptors being specific for gp96 and hsp70. For each animal, the vaccine proteins are associated with a peroxidase and RAW264 cells are cultured in the presence of the labeled proteins after having the membrane cd91 blocked or not by an anti cd91 antibody (invitroGen). The cells are cultured in 25cm2 culture dishes (Falcon, France) in a culture medium of DMEM type supplemented with glutamine (Sigma, France) and with 5% fetal calf serum (Sigma, France). They are incubated at 37 °

C, in an atmosphere of 98% humidity and 5% CO2. When they reach 70% of their confluence, the cells are washed in PBS and fixed in ethanol. The labeled proteins are incubated overnight with the cells. The blocking of cd91 is done by incubating the cells for two hours in an antibody solution diluted 1/1000 in a blocking solution (immunocruz, US). In order to be used to label the cells, the vaccine proteins are desorbed from the powders with 0.5M NaCl solution. They are then associated with a biotin using the kit ((Pierce Biotechnology kit, EZ-linkTM sulfo-NHS-Biotinylation kit –thermo scientific) following the manufacturer's instructions. Biotin then reacts with a peroxidase associated with a streptavidin. Peroxidase is then demonstrated by a solution composed of diaminobenzidine (DAB) (0.05% DAB, 0.015% H2O2, 0.01M PBS, pH 7.2) maintained for less than five minutes in contact with the cells. The labeling is visible in the form of a coloration brown cells.

3 I RESULTS

8 dogs were euthanized for reasons related to the tumor (pain, change in tumor size, deterioration in general condition, convulsions), 1 for unrelated reasons and 1 is still alive when the paper was written, 2 were lost to follow-up. Three dogs had 2 to 4 cycles of vaccinations (Table 1). Dogs can be divided into two groups; a long survival group (n = 7, greater than 150 days) and a short survival group (n = 3, less than 150 days). In the latter group, two out of three dogs were euthanized at the owners' request for a pain problem not controlled by analgesics and non-steroidal anti-inflammatory drugs. In this group, euthanasia generally takes place within 45 days of treatment. which does not allow an immune reaction time to have an effect on the tumor.

dog	response			Death cause	side effects	Associated treatment	Vaccination number of cycles	stage
	Minor tumoral regression	OS	PFS					
ost1	yes	310	310	linked (convulsion)	0	analgesic	2	T1N1M0
ost2	n.a.	410	380	linked	0	amputation	1	T2N0M0
ost3	Yes	455	420	linked (dystrophy)	0	analgesic	2	T2N0M0
ost4	Yes	170	170	not linked	0	analgesic	1	T2N0M0
ost5	n.a.	210	200	Linked	0	amputation	1	T2N0M0
ost6	Yes	150	150	Linked	0	analgesic	1	T2N0M0
ost7	No	45	45	linked (pain)	0	analgesic	1	T2N1M0
ost8	n.a.	1015	1015	Not linked (embolism)	0	amputation	4	T2N0M0

ost9	Yes	45	0	linked (pain)	0	analgesic	1	T2N0M0
ost10	No	20	0	linked (convulsion)	0	analgesic	1	T2N1M1
ost11				Lost sight		analgesic	1	T2N1M1
ost12						analgesic	1	T2N1M0

Table 1: characteristics of the treatment of the dogs included in the study and evolution of the parameters measured, OS (overall survival) and PFS (progression free survival - survival without increase in tumor volume

All biopsies contained both gp96 and HSP 70. Few bands are detected by electrophoresis, and they are located in the 90,000 and 70,000 dalton regions (Fig. 1). On the other hand, the dot blots show a significant presence of gp96 and HSP70 on the surface of the HA beads (fig. 2).

Vaccine proteins labeled with peroxidase bind to RAW264 cells and binding was inhibited by anti gp91 (fig. 3).

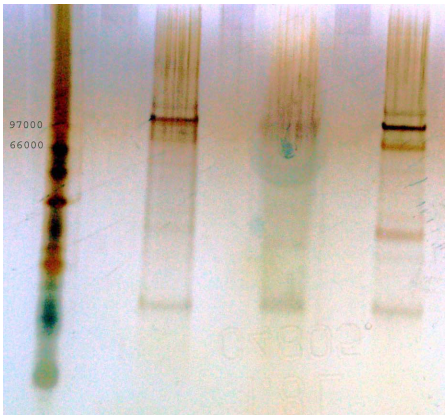

Figure 1 : SDS Page of proteins (dog ost3, fourth column) detached from hydroxyapatite powders by washing in NaCl solutions (0.5M). We see two well-individualized bands at 97 and 70,000 kDa. First column is protein ladder

The average survival of the dogs of the first group (long survival) is 270 days, the median is 260 days. The mean and median of PFS were very close, 260 and 250 days respectively.

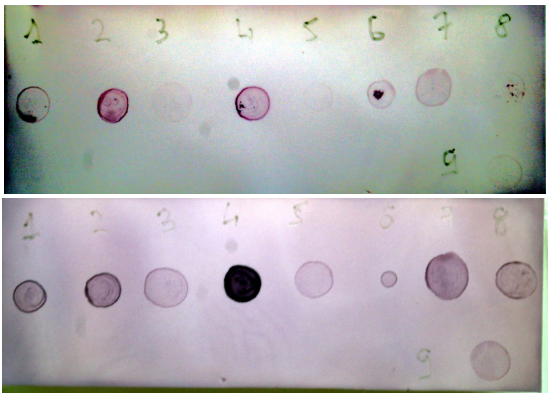

Figure 2 : dot blot of the solutions extracted from powders injected at ost9 and 10 (blots 1 and 2) marked with anti gp96 (top band) and anti HSP70 (bottom band).

Amputated dogs had an overall survival of 531 days while that of non-amputated dogs is 270 days, which was significant (p> 0.005).

All dogs had a stable tumor size (SD) throughout their survival. There was an improvement in the inflammatory state of the tumor site after each injection. Two dogs for which survival was particularly long underwent a second round of vaccination.

Radiologically, the first stages (less than 2 months) of the vaccination are followed by remodeling of the tumor site with periosteal bone formation and densification of the osteolysis zone (Fig. 4).

It should be noted that all long-surviving dogs maintained good general condition with no change in weight for most of their survival.

4 I DISCUSSION

In the long survival group, the results show a much better survival than what is published without treatment or by simple amputation. From a study on 400,000 dogs whose files were extracted from the file of a Swedish insurance company [26], it was shown that the average survival of dogs diagnosed with osteosarcoma is 56 days (table 2). Spotnick et al. [27], from 162 dogs treated by amputation, showed an average survival of 134 days. Lane et al [28] obtained a median survival of 231 days and a progression-free survival of 247 days from 33 amputated dogs and receiving chemotherapy combining doxorubicin and carboplatin. Recently, chemotherapeutic protocols combining amputation and carboplatin or doxorubicin have been published and show an improvement in survival compared to control groups with a median at 307 and 240 days respectively [29, 30]. The combination of the two molecules does not improve the prognosis [31].

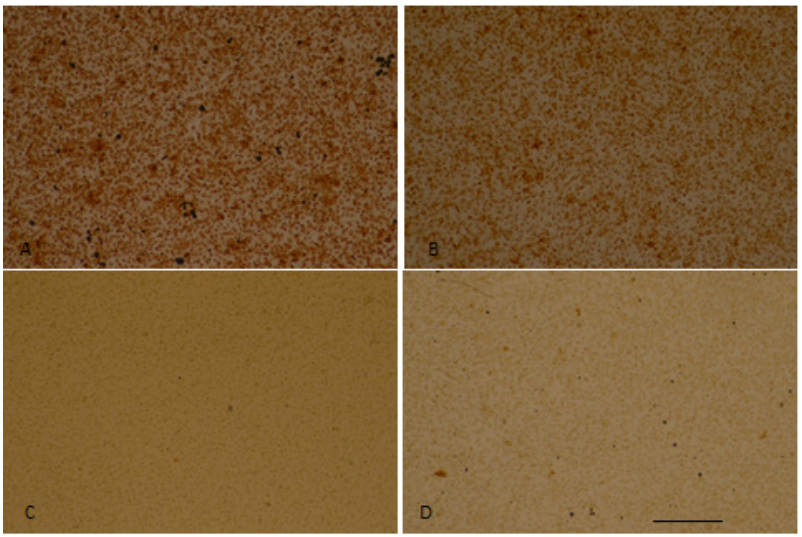

Fig.3 : labeling of the cells with the vaccine proteins associated with a peroxidase before (A) and after (B) the blocking of cd91. Labeling controlled by gp96 before (B) and after (D) blocking of these same receptors. This labeling shows that at least some of the vaccine proteins bind to the cd91 receptors.. bar : 150 μm

The binding of certain vaccine proteins to CD91 shows that the injected doses contained active HSPs, more especially gp 96 and HSP70, since CD91 are specific receptors for these proteins. Protein binding to CD91 can be a test for vaccine activity that can be combined with an elispot-type CD8 stimulation test.

The effect of the injections on the inflammation of the tumor site is relatively reproducible. Nevertheless, at least in 2 cases of non-amputated dogs, pain motivated the request for euthanasia of the animal by its owners very early when the general and local condition did not justify it. Pain control is therefore a very sensitive parameter in non-amputated dogs.

Various techniques can be used *in vitro* to demonstrate the stimulation of CD8 by proteins, in oncology they are not necessarily representative of the effectiveness of vaccination [32]. We have shown that this therapeutic vaccination technique used in Balb / c mice to immunize them against 4T1 tumor cells cross-prime their CD8 against these cells. The latter synthesize IFNγ when brought into contact *in vitro* with antigen-presenting cells stimulated *in vivo* by the vaccine.

The decrease in tumor volume in non-amputated dogs and after injection of the vaccines from the third injection for a period of about twenty days led the veterinarians to carry out several cycles of vaccination when only planned one initially. No side effects have been demonstrated, in particular no unexplained fever, itching or neurological disturbances which could have indicated sensitization to

healthy tissue molecules.

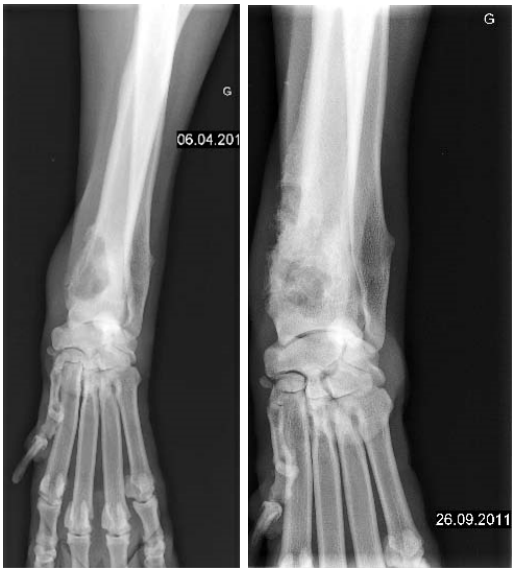

Figure 4 : dog ost3. Radiological changes 170 days apart. There is significant ossification of the area of initial osteolysis

The purification of gp96 and HSP70 is relatively good, but there are contaminating proteins bands at different molecular weights. It is therefore possible that proteins other than heat shock proteins are involved in building an immune response. The presence of gp96 and HSP70 is nevertheless constant. HSP70 is often a membrane protein [33] which is constitutional but which is removed in the purification procedure. There is a cytoplasmic HSP70 which is like gp96 expressed only during cellular stress [34]. The presence of these two stress proteins makes it possible to have in their associated molecules almost all the peptides synthesized by the cancer cell. In addition, these two proteins have a major role in the cross priming of CD8s. Gp96 even has a specific receptor (CD91) on dendritic cells. Srivastava's work has shown the benefits of using purified autologous gp96 in laboratory animals [35].

There are relatively few publications about the treatment of canine osteosarcomas by active immunotherapy. Much more experimentation has been done in the field of passive immunotherapy, in particular an autologous tumor vaccine whose cells express a GM-CSF transgene [36, 37]. Lung metastases from osteosarcoma have also been treated by injection of cationic liposomes containing DNA encoding Il-2 [38]. Similarly, sarcomas have been treated with injections of

irradiated xenogenic cells synthesizing GM-CSF and IL-2 mixed with a lysate of autologous or xenogeneic cells followed by injection of liposomes carrying genes of cIFN –b and HSVtk and ganciclovir [39]. These various and complex cytokine treatments have shown an improvement in the prognosis.

Dog number	traitement	Médian	auteur	stage
162	amputation	134,4	Spodnick GJ et al, J Am Vet Med Assoc. 1992 Apr 1;200(7):995-9	Not given
90	0	3	Boston SE, J Am Vet Med Assoc. 2006 Jun 15;228(12):1905-8.	stade III
	radio/chimio	130		
	amputation	76		
	amputation/chimio	78		
764	0	56	Agneta Egenvalln, Can J Vet Res. 2007 October; 71(4): 292–299	Not given
33	amputation/chimio	247	Lane A, et al. Aust Vet J 2012 Mar;90(3):69-74	Not given
155	Amputation/ Chimio (carboplatine)	307	Philips et al. J Am Anim Hosp Assoc 45(1):33–38	Not given
303	Amputation/ Chimio (doxorubicyn)	240	Moore, AS et al. J Vet Intern Med 21(4):783– 790	Not given

Table 2 : median overall survival in days of published canine osteosarcomas series according to treatment

The complexity of these treatments nevertheless makes them difficult to apply. The treatment of osteosarcomas by immunotherapy by injection of a cytotoxic human T cell line (TALL 104) [40] has given results which appeared interesting with a median survival of 11.5 months but difficult to interpret due to co-treatments by amputation and chemotherapy. Recently Mata et al [41] developed chimeric antigen receptor (CAR) T cells against Her2 receptors in dog osteosarcoma cells. These cells have shown *in vitro* an ability to eliminate canine osteosarcoma cells. The purification of autologous proteins from a lysate seemed to us much simpler and hence less risky. To our knowledge, our publication is the first using an immunotherapy method without association with chemotherapy and even with an unamputated group without backup surgery. However, it seems that amputation before vaccination provides a significant improvement in animal survival.

For technical reasons, we were unable to provide proof of CD8 activation in each dog in this series. On the other hand, the decrease in tumor size observed after

injection from the third in dogs without amputation is in favor of such activation, as has been shown in mice.

HA particles have long been used in adsorption chromatography columns, they have also been used as vaccination adjuvants [42, 43]. When injected subcutaneously, it causes a foreign body reaction leading to an influx of antigen-presenting cells which participate in this adjuvant effect. Placed in the presence of monocytes in culture, they have a very marked activating effect on the inflammasome [44] and also participate in the maturation of dendritic cells with the expression of co-factors necessary for the cross-priming of CD8s (unpublished results) .

5 I CONCLUSIONS

Although this series is short, this method for immune system stimulation appears to improve the prognosis of canine osteosarcoma with or without amputation. We did not note any side effects in particular of an autoimmune nature. The improvement is most noticeable when the dogs are amputated. These results were obtained without associated chemotherapy. We can therefore expect that the combination of cytotoxic treatments during a therapeutic window preserving the immunity of the animal could amplify the improvement in overall survival

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