Randomized, Placebo-Controlled, Double-Blinded Chemoimmunotherapy Clinical Trial in a Pet Dog Model of Diffuse Large B-cell Lymphoma

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Abstract

Purpose: Active immunotherapy is a promising antitumoral strategy; however its use in combination with chemotherapy in dogs with large B-cell lymphoma (DLBCL) remains largely untested. Heat shock proteins (HSP) bind the small peptides they chaperone (HSPPC), allowing for immunization of the host against a large repertoire of tumor-associated antigens. Hydroxylapatite vehicles HSPPCs and acts as an immunologic adjuvant. The aim of this study was to show that an autologous vaccine with hydroxylapatite and tumor-derived HSPPCs is safe and therapeutically effective in dogs with DLBCL.

Experimental Design: Nineteen dogs with naturally occurring DLBCL were entered into a prospective randomized placebo-controlled double-blinded trial of HSPPCs–hydroxylapatite plus chemotherapy versus chemotherapy alone. Endpoints included time to progression (TTP), lymphoma-specific survival (LSS), and incidence of toxicoses.

Results: Median first TTP after randomization to the vaccine arm was 304 days versus 41 days for the control arm (P = 0.0004). There was also a statistically significant difference in duration of second remission between the two groups (P = 0.02). Median LSS was 505 days for the vaccinated dogs versus 159 days for the unvaccinated dogs (P = 0.0018). Six vaccinated dogs achieved molecular remission, as shown by clonal immunoglobulin H (IgH) rearrangement. Toxicoses were comparable between the two treatment arms.

Conclusions: The results of this trial demonstrate that the autologous vaccine tested here is safe and efficacious in prolonging TTP and LSS in dogs with DLBCL when used in combination with dose-intense chemotherapy. On the basis of these results, additional evaluation of this novel therapeutic strategy is warranted in human DLBCL.

Introduction

Non-Hodgkin lymphoma is the fifth leading cause of cancer-related deaths in people in the United States (1), and for more than 25 years, an anthracycline-based multidrug chemotherapy regimen has been the gold standard for the treatment of diffuse large B-cell lymphoma (DLBCL). Although the addition of rituximab has altered the therapeutic landscape and improved prognosis, approximately one third of patients experience relapse (2). The outlook for this subgroup is dismal, with a median survival time of 6 months or less, indicating that there is a clinical spectrum of sensitivity to the standard treatment (3). Therefore, considerable room for improved outcome remains, including the use of novel strategies acting in concert with cyclophosphamide–adriamycin–vincristine–prednisone (CHOP)–based protocols aimed at providing greater tumor specificity and less nonspecific toxicity (3).

DLBCL is the most common subtype of canine lymphoma (4, 5), and it shares many features with the human counterpart, including clinical presentation, biologic behavior, tumor genetics, and treatment response (6). Constitutive and increased NF-κB activities targeting gene expression were recently detected in primary canine DLBCL tissue, rendering the dog a spontaneous model for activated B cell (ABC) DLBCL in people (7, 8).

Unfortunately, monoclonal antibodies are unavailable in veterinary oncology, and the present standard of care for canine DLBCL includes dose-intense multidrug chemotherapy (9, 10). Although chemotherapy improves the duration of remission and prolongs overall survival, the disease is essentially incurable (11). Similar to the human disease, relapsed lymphomas are refractory to subsequent treatments...
Translational Relevance

Despite the success of rituximab in patients with diffuse large B-cell lymphoma (DLBCL), new therapeutic strategies are needed. In preclinical studies, the most frequently used animal models are engineered mice that overexpress human translocations or oncogenes, but results have been disappointing, and most of the hits generated turned out to be invalid, once tested. To address the urgent need for animal models in preclinical studies, authoritative international institutions have recommended using spontaneous occurring diseases in companion animals. The canine model of DLBCL offers several advantages, including developing spontaneous disease and having a high frequency. Clearly, the immune system has the capacity to recognize and react to lymphoma cells, and recent evidence directed the attention to the importance of mounting immune responses to complement the cytotoxic activity elicited by dose-intensive chemotherapy. This study shows the therapeutic relevance of an autologous vaccine in a canine DLBCL model and has potential translational relevance for the treatment of human DLBCL.

With the initial chemotherapy regimen and can exhibit cross-resistance to a wide variety of anticancer drugs. Ultimately, the emergence of acquired chemoresistance poses a challenge, preventing the successful treatment of DLBCL.

In the last decade, much attention has been paid to immunotherapy, which attempts to direct the protective capacity of the immune system toward eliminating malignant cells (12). Active immunotherapy offers two main advantages: first, it elicits a tumor-specific immune response; second, it potentially establishes long-lasting tumor immunity via the capacity to exhibit memory, thereby limiting the likelihood of relapse (13).

Increasing evidence suggests that the immune system can be manipulated in different ways to recognize and fight cancer cells, and a number of immunotherapy-based strategies are being tested in ongoing clinical studies in human as well as in veterinary oncology (12–16).

The clinical experience of immunotherapy in canine lymphoma is still at an embryonic stage. The first study dates back two decades: an intralymphatic autologous vaccine administered in combination with chemotherapy significantly prolonged first remission compared with dogs receiving only chemotherapy (17).

Later, an autologous tumor cell vaccine administered to dogs with B-cell lymphoma following chemotherapy did not improve the outcome when compared with placebo-matched dogs (18).

More recently, in 14 dogs with B-cell lymphoma, a genetic cancer vaccine targeting telomerase in combination with chemotherapy significantly increased survival time when compared with eight historic controls treated by chemotherapy only (19). In this study, diagnosis was obtained by cytolory; therefore, different disease entities may have been included. Also, chemotherapy and type of vaccination differed among dogs.

In the most recent study, Sorenmo and colleagues demonstrated the immunogenicity of a cell-based vaccine in dogs with lymphoma using CD-40--activated B-cells, which act as antigen-presenting cells (APC; ref. 20). Nineteen dogs in complete remission (CR) after induction dose-intensive chemotherapy were eligible to be vaccinated. Time to progression (TTP) and lymphoma-specific survival (LSS) were not significantly different between vaccinated and nonvaccinated dogs; however, vaccination potentiated the effects of rescue therapy and improved the rate of durable second remissions and LSS, following salvage therapy (20).

Overall, the tolerability and efficacy of the vaccines in these studies were compelling enough to justify the evaluation of alternative vaccines aiming at easier/faster production, better cost effectiveness, or stronger immune response.

Typically, active immunotherapy in patients with human lymphoma consists of vaccines that use the immunoglobulin idiotype as a tumor-specific antigen. However, a possible disadvantage of active immunotherapy is its reliance on the patient’s immune system, which may be compromised or deregulated by the tumor itself or by previous chemotherapy (21, 22). Also, an inefficient presentation of tumor antigens to the host’s immune system may enable cancer cells to evade the immune response, leading to immune tolerance toward the tumor (23). As a consequence, efforts to enhance the efficacy of active immunotherapy are ongoing, with an emphasis on optimization of antigen delivery and presentation, and modulation of the immune system toward counteracting immune suppression.

One promising approach that has emerged is the delivery of an autologous vaccine consisting of hydroxyapatite ceramic powder and proteins purified from the patients’ tumors, such as Heat Shock Proteins (HSP; ref. 24).

HSPs are synthesized under stress situations (including cancer) to protect the cells from damage; among others, HSPs play a key role in bringing tumor-associated antigens (TAA) to professional APC, thereby leading to the cross-priming of antitumor CD8+ and CD4+ T cells through MHC class I and class II molecules (25). Of note, HSPs bind the small proteins and peptides they chaperone, forming HSP–peptide complexes (HSPPC), thereby providing a fingerprint of the tumor peptides, both normal and abnormal. Therefore, if purified from the patient’s own tumor, HSPs may enhance the patient’s immunity by inducing specific and nonspecific cellular immune responses (26).

Hydroxyapatite (Ca10(PO4)6(OH)2) is used in many biotechnology processes to purify proteins from biologic solutions by ion exchange chromatography. Hydroxyapatite nanoparticles showed vaccine adjuvant properties, i.e., making possible to use hydroxyapatite particles loaded with proteins purified by an hydroxyapatite column (24). HSPs, such as gp 96 and HSP 70, showed a particular affinity for the hydroxyapatite surface (24). Indeed, when injected into the dermis or subcutaneous tissue, hydroxyapatite behaves like a foreign body, thereby attracting
monocytes and macrophages to the injection site. Also, if HSSPCs are adsorbed at its surface by means of chromatography, hydroxylapatite also acts as a vehicle of these molecules, which may then be released into APC to be presented to the immune system (24).

In a previous study, an autologous vaccine consisting of hydroxylapatite loaded with HSPs was administered to human patients with various malignancies (27). The vaccine was well tolerated, with only mild local side effects, and showed some antitumoral efficacy.

These results provided the rationale for this randomized placebo-controlled double-blinded trial, with the primary aim of showing that an autologous vaccine with hydroxylapatite and tumor-derived HSSPCs is safe and therapeutically effective in dogs with DLBCL.

Materials and Methods

Dog selection

Dogs with newly diagnosed, previously untreated, multicentric DLBCL of any World Health Organization (WHO) clinical stage admitted to the Centro Oncologico Veterinario between June 2011 and December 2012 were consecutively enrolled.

To be eligible for recruitment, dogs were required to undergo a complete staging work-up, consisting of history and physical examination, complete blood count with differential, serum biochemistry profile, thoracic radiographs and abdominal ultrasound, cytologic evaluation of liver and spleen regardless of the ultrasonographic appearance, and immunophenotype determined by flow cytometry on a lymph node aspirate, peripheral blood and bone marrow aspirate (Supplementary Methods A; refs. 28–30). The cutoff for bone marrow infiltration was set at >3% of CD45+ cells, as it has been shown to negatively affect the outcome in dogs with DLBCL (31).

Before the initiation of therapy (T0), all dogs also were required to undergo lymphadenectomy to confirm pathology, and provide material for the vaccine generation.

Additional entry criteria included an estimated life expectancy of at least 4 weeks and no previous therapy (chemotherapy and/or glucocorticoids). Concurrent serious systemic disorder incompatible with the study was regarded as an exclusion criterion.

Dog owners were required to give written informed consent.

Pathology

The diagnosis of DLBCL was confirmed by a single pathologist (L. Aresu) according to the WHO classification (32). Tissues were processed routinely for paraffin embedding, and stained with hematoxylin and eosin. Immunophenotyping was determined for all cases (Supplementary Methods B).

Vaccine generation

Lymphoma tissue was obtained by lymphadenectomy. A part of the excised lymph node was shipped to the laboratory in a sterile container with dry ice.

The tumor tissue and all material used to prepare the vaccine were handled in sterile conditions under a laminar flow. The frozen tumor tissue (200 mg) was homogenized using a bead tissue homogenizer. One milliliter of sodium carbonate (30 mmol/L, pH 7) was added for 1 mL of homogenate. The resulting homogenate was then centrifuged for 15 minutes to remove all cellular fragments and placed at 4°C.

The supernatant containing the cytoplasmic proteins was used for protein purification by hydroxyapatite column chromatography as follows: precipitations with ammonium sulfate (first at 50%, then at 70%) recovered the pellets. The last pellet was resuspended in 2 mL phosphate buffer (20 mmol/L, pH 7). The column was filled with 0.333 g hydroxylapatite (25–45 μm). The resuspended pellet was then added and the column was washed with a phosphate buffer solution. The powder was then suspended in 5 mL carboxymethylcellulose (CMC) solution (2% in distilled water); 0.5 mL of this solution was used for each vaccine shot.

To obtain the electrophoretic control, 0.2 mL of the previous solution was used. The solution was then centrifuged at 1,000 rpm for 30 seconds. The supernatant was discarded, and the powder in the pellet was washed with 0.1 mL of a 0.5 mol/L NaCl solution. The solution was again centrifuged and the supernatant was used for SDS-PAGE and for protein quantification using UV spectrometer. Ten microliter of the solution was also used for dot blot with anti-HSP70 and anti-gp90 antibodies on a nitrocellulose membrane. For antibody labeling, a WesternBreeze (Invitrogen) kit was used according to the manufacturer’s instructions.

The placebo consisted of the same amount of hydroxylapatite powder in CMC solution without the tumor proteins. The placebo and the vaccine were undistinguishable based on their physical aspect.

Study design

This study was a double-blinded (responsible oncologist, L. Marconato, and owners), centrally randomized, placebo-controlled clinical trial. Dogs were randomized to receive either chemotherapy and an autologous vaccine (group 1) or chemotherapy and a placebo (group 2) in a 2:1 ratio.

Treatment schedule

Dogs in both treatment groups received the same 20-week combination induction chemotherapy, consisting of L-asparaginase, vincristine, cyclophosphamide, doxorubicin, lomustine, and prednisone (Table 1).

Dogs also received either an intradermal injection of 0.5 mL vaccine (group 1) or an equivalent number of 0.5-mL placebo-matched intradermal injections (group 2) on weeks 4, 5, 6, 7, 12, 16, 20, and 24. The injection areas were shaved and aseptically prepared before vaccine or placebo administration.

Safety was assessed at each scheduled treatment session using the Veterinary Co-operative Oncology Group (VCOG) criteria (33). Treatment was delayed for a
maximum of 1 week or dose was decreased by 20% for adverse safety changes. Safety assessments included adverse events, hematology, and clinical chemistry profiles. Concomitant medications, including antibiotics, antiemetic and antidiarrheal, were permitted to manage adverse events.

Response assessment and follow-up
Response was evaluated at each treatment session by one oncologist (L. Marconato) blinded to treatment assignment according to previously published criteria (34). The remission status was assessed on the basis of physical examination and mandatory lymph node cytology at each visit. In doubtful cases, flow cytometry was carried out. Responses were required to last for at least 28 days.

Two weeks after having completed the protocol (T1), all dogs underwent complete end-staging, including flow cytometry and PCR for antigen receptor rearrangement on peripheral blood, bone marrow, and lymph node obtained from a second lymphadenectomy. Dogs were then rechecked through monthly physical examinations and lymph node cytologic samples during the first year, and every other month thereafter.

Relapse was defined as clinical reappearance and cytologic evidence of lymphoma in any anatomic site in dogs having experienced CR, whereas relapse for animals with partial remission (PR) was defined as progression.

Dogs that relapsed during or after the treatment protocol were offered standardized rescue chemotherapy.

Postmortem examinations were performed at the time of death or euthanasia, whenever possible.

Detection of minimal residual disease by PCR for antigen receptor rearrangements
Detection of antigen receptor gene rearrangements was assessed by PCR amplification of the complementarity determining region 3 (CDR3) of the antigen receptor genes, as previously reported (Supplementary Methods C; refs. 35, 36).

In vivo assessment of immune response

In vivo immune responses were documented in all dogs by performing delayed-type hypersensitivity (DTH) skin tests and by evaluating the local inflammatory response generated by vaccination (group 1) and placebo (group 2). All intradermal injections were given in sites different from the vaccination sites before vaccination and 14 days after the last vaccination.

Briefly, the tumor extract to be injected in the dermis for DTH skin test was prepared as follows: after tumor homogenization, 100 μL of the homogenate was diluted in 0.5 mL of a CMC solution (2% in 20 mmol/L NaCl); 0.1 mL of this solution was used for DTH.

Responses to the DTH skin test were evaluated 48 hours after each injection; a diameter of erythema >2 mm was considered a positive response.

Statistical analysis

Documented negative prognostic factors, such as stage, substage, bone marrow infiltration, and weight were analyzed to detect any possible statistically significant difference between the two groups.

The primary endpoint of this study was to determine whether vaccination (group 1) prolonged TTP and LSS compared with placebo (group 2) in dogs with DLBCL receiving the same chemotherapeutic protocol. TTP and LSS were measured from the first day of chemotherapy to the clinical event in both groups (34). The secondary endpoint of this study was to investigate whether the second TTP was longer in vaccinated compared with unvaccinated dogs. All dogs that were randomized were included in remission and survival analysis to fulfill intention-to-treat criteria.

The hypotheses were tested comparing mean and median TTP and LSS between the two groups. Proportional hazards were calculated using Cox regression analysis with entry point at the date of recruitment.

Dogs that were lost to follow-up and dogs that died because of other causes than lymphoma or lymphoma treatment were right-censored at the last date of known status or when they died from other causes, respectively. The Kaplan–Meier product limit method was used to estimate TTP and LSS for both groups.

Mean TTP and LSS were compared with the Student t test, median TTP, and LSS with the Mann–Whitney test, and categoric variables with the χ2 test.

Statistical calculations were performed using STATA (StataCorp. 2007. Stata Statistical Software: Release 10). For all statistical comparisons, significance was set at \( P < 0.05 \).

### Table 1. Chemotherapeutic protocol administered to vaccinated and unvaccinated dogs

<table>
<thead>
<tr>
<th>Drug</th>
<th>Week</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>7</th>
<th>10</th>
<th>13</th>
<th>16</th>
<th>19</th>
</tr>
</thead>
<tbody>
<tr>
<td>l-Asparaginase (400 UI/kg s.c.)</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vincristine (0.75 mg/m2 i.v.)</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclophosphamide (250 mg/m2 p.o.)</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Doxorubicin (30 mg/m2 i.v.)</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lomustine (60 mg/m2 p.o.)</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prednisone (1 mg/kg p.o. until week 4, then 0.5 mg/kg p.o.)</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: p.o., orally; s.c., subcutaneously; i.v., intravenously.
Results

Dog characteristics and treatment administration

Nineteen consecutive treatment-naive dogs with DLBCL were enrolled. Table 1 provides a summary of the characteristics of the dogs entering this trial using known or potential covariates for outcome in dogs with DLBCL. The two treatment arms were well balanced for baseline characteristics and there were no statistically significant differences in prognostic variables between groups 1 and 2 (Table 2).

Twelve dogs were randomly assigned to receive the vaccine (group 1), and 7 to receive the placebo (group 2). Autologous vaccines were successfully produced for all 12 dogs (100%) randomized to receive immunization during chemotherapy. All dogs received the same chemotherapeutic protocol.

All (100%) dogs in group 1 and 4 (57.1%) dogs in group 2 completed the chemotherapy protocol. In group 1, all dogs received the eight intended vaccinations according to schedule, and a total of 96 vaccinations were given within the trial. Conversely, 6 dogs in group 2 did not complete the 8 intended placebo administration because they either relapsed (n = 4) or progressed (n = 2), resulting in death.

Clinical outcome

All 12 vaccinated dogs achieved CR; of the 7 dogs that received the placebo, 5 (71.4%) obtained CR, whereas 2 (28.6%) dogs experienced progressive disease (P = 0.05). Four of the 12 vaccinated dogs in group 1 never relapsed, after 648, 613, 342, and 154 days, respectively. For all 19 randomly assigned dogs, median first TTP was 192 days (mean, 231 d; range, 19–648 d) versus 41 days (mean, 59 d; range, 19–140 d) for the control arm (P = 0.0004). Kaplan–Meier curves for TTP are shown in Fig. 1.

Dogs in group 1 were significantly less likely to experience a relapse compared with dogs in group 2 [HR, 0.1834577; 95% confidence interval (CI), 0.05–0.72; Table 4].

Following progression of disease, 10 dogs randomized to this study received standardized rescue chemotherapy. For all 10 dogs, median second remission was 82 days (mean, 104 d; range, 29–333 d).

Seven of the 12 (58.3%) vaccinated dogs in group 1 that relapsed were treated with salvage chemotherapy, and all of them achieved durable second remission (median, 128 d; mean, 135 d; range, 47–333 d). Two of these 7 vaccinated dogs with a chemotherapy-induced durable second remission are still alive with no evidence of lymphoma at 377 and 581 days, respectively, after the start of the initial chemotherapy. One dog died 443 days after the start of chemotherapy because he was poisoned; there was no evidence of lymphoma at necropsy. The other 4 dogs relapsed during rescue chemotherapy and were euthanized because of their lymphoma.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group 1 (n = 12)</th>
<th>Group 2 (n = 7)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y; median)</td>
<td>7</td>
<td>6</td>
<td>0.6668</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>male</td>
<td>7</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>female</td>
<td>5</td>
<td>2</td>
<td>0.568</td>
</tr>
<tr>
<td>Weight (kg; median)</td>
<td>30, 5</td>
<td>35</td>
<td>0.6121</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I (%)</td>
<td>1 (8.3)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>III (%)</td>
<td>3 (25)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>IV (%)</td>
<td>5 (41.7)</td>
<td>3 (42.9)</td>
<td></td>
</tr>
<tr>
<td>V (%)</td>
<td>3 (25)</td>
<td>4 (57.1)</td>
<td>0.311</td>
</tr>
<tr>
<td>Substage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a</td>
<td>83.3</td>
<td>71.4</td>
<td></td>
</tr>
<tr>
<td>b</td>
<td>16.7</td>
<td>28.57</td>
<td>0.539</td>
</tr>
</tbody>
</table>

Table 2. Baseline characterization of dogs randomized to vaccine (group 1) versus placebo (group 2) for known and potential covariates of outcome in canine lymphoma

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group 1 (n = 12)</th>
<th>Group 2 (n = 7)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>First TTP (mean; d)</td>
<td>332</td>
<td>59</td>
<td>0.0004</td>
</tr>
<tr>
<td>First TTP (median; d)</td>
<td>304</td>
<td>41</td>
<td>0.0004</td>
</tr>
<tr>
<td>LSS (mean; d)</td>
<td>505</td>
<td>159</td>
<td>0.0018</td>
</tr>
<tr>
<td>LSS (median; d)</td>
<td>468</td>
<td>136</td>
<td>0.0001</td>
</tr>
<tr>
<td>Second TTP (mean; d)</td>
<td>140</td>
<td>35</td>
<td>0.10</td>
</tr>
<tr>
<td>Second TTP (median; d)</td>
<td>127</td>
<td>32</td>
<td>0.0167</td>
</tr>
</tbody>
</table>

Table 3. Clinical outcome comparing vaccinated group 1 to unvaccinated group 2

Figure 1. Kaplan–Meier curves comparing vaccinated group 1 to unvaccinated group 2 for first TTP (n = 19).
In the unvaccinated group 2, all 3 dogs that relapsed after the initial chemotherapy received rescue chemotherapy; however, none of them achieved a durable second remission, as they all progressed after 28, 29, and 43 days, respectively.

There was a statistically significant difference in median duration of second remission between the two groups ($P = 0.02$; Fig. 2).

At data analysis closure, 4 (33.3%) dogs in group 1 were still alive with a median follow-up of 598 days (range, 377–648 d). Five dogs had died because of lymphoma, whereas 3 were euthanized for lymphoma-unrelated causes.

All dogs in group 2 had died: only one dog died because of lymphoma-unrelated causes, whereas all the other dogs ($n = 6$) were euthanized because of advanced stage lymphoma.

For all the 19 randomly assigned dogs, median LSS was 342 days (mean 346 d; range, 20–663 d). Median LSS was significantly longer in group 1 when compared with group 2 (Table 2). Median LSS after randomization to the vaccine arm was 505 days (mean, 468 d) versus 159 days (mean, 136 d) for the control arm ($P = 0.0018$). Kaplan–Meier curves for LSS are shown in Fig. 3. Dogs in group 1 were significantly more likely to live longer compared with dogs that received the placebo (HR, 0.043; 95% CI: 0.0049–0.378).

### Table 4. HRs for first TTP, second TTP, both times at once, and LSS in group 1 compared with group 2 estimated by Cox regression

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Group 1 (n)</th>
<th>Group 2 (n)</th>
<th>HR (95% CI)</th>
<th>P</th>
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<tbody>
<tr>
<td>First TTP</td>
<td>12</td>
<td>7</td>
<td>0.18 (0.05–0.72)</td>
<td>0.016</td>
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<tr>
<td>LSS</td>
<td>12</td>
<td>7</td>
<td>0.04 (0.00–0.38)</td>
<td>0.005</td>
</tr>
<tr>
<td>Second TTP</td>
<td>6</td>
<td>3</td>
<td>0.19 (0.02–1.89)</td>
<td>0.158</td>
</tr>
<tr>
<td>First and second TTP</td>
<td>12</td>
<td>7</td>
<td>0.21 (0.07–0.64)</td>
<td>0.006</td>
</tr>
</tbody>
</table>

### Minimal residual disease

Lymph node, peripheral blood, and bone marrow samples were obtained at T0 and T1. At diagnosis, clonal immunoglobulin H (IgH) rearrangement by PCR was found in lymph nodes of all dogs. In peripheral blood as well as in bone marrow, rearrangements were detected in 5 of 19 (26%) dogs, including 2 dogs in group 1 and 3 dogs in group 2. These data were also confirmed by flow cytometry.

At the end of the treatment, 6 dogs in group 1 achieved molecular remission, defined as lymph node negative results for the clonal IgH rearrangement. Three of these 6 dogs never relapsed; at data analysis closure, 2 were alive in CR and 1 dog had died for lymphoma-unrelated causes. One dog relapsed after 423 days; however, at this time point, histopathology showed a T-cell phenotype. On the other hand, all unvaccinated dogs showed lymph node clonality; the presence of neoplastic cells was also confirmed by flow cytometry.

### DTH skin test

After the last injection of vaccine versus placebo, DTH skin tests were performed on all dogs that were still alive at that point to examine in vivo induction of immune responses to tumor cells. Positive DTH responses against tumor cells were observed in all vaccinated dogs (diameter >2 mm). Meanwhile, the response against tumor cells was undetectable in dogs that received the placebo, suggesting...
specific DTH responses against tumor cells by autologous vaccination.

Toxicity
Safety was assessed in all dogs in both the treatment arms. The type, frequency, and severity of treatment-emergent adverse events were comparable between the two treatment arms.

Overall, treatment with the vaccine was found to be safe and well tolerated when combined with dose-intense chemotherapy in dogs with DLBCL. No difference in dose-limiting neutropenia, thrombocytopenia, and gastrointestinal or hepatic toxicity was seen in dogs treated with dose-intense chemotherapy plus placebo versus dose-intense chemotherapy plus vaccine (Table 5). Quality-of-life assessment, defined by a questionnaire completed by pet owners at each visit, did not reveal differences in quality-of-life in dogs receiving dose-intense chemotherapy plus placebo versus dose-intense chemotherapy plus vaccine (data not shown).

Adverse events during treatment cycles, when observed, were manageable, reversible, and dose and regimen dependent. Grade 3 adverse events were similarly reversible and were limited to transient vomiting (4 dogs), febrile neutropenia (1 dog), and elevation of alanine transaminase (1 dog). Grade 4 adverse events were limited to thrombocytopenia and neutropenia (1 dog), which were reversible. There were no vaccine-related deaths.

Discussion
Naturally occurring canine tumors represent valuable tools for studying numerous aspects of human cancer as well as the potential use of this animal model for the development of new therapies (37). In particular, the evaluation of the efficacy of new treatment strategies in the context of a naturally occurring cancer model with phenotypic diversity may provide valuable information, which is currently difficult to obtain from conventional preclinical models or from human clinical trials alone. When considering DLBCL, the translational value of the canine model is further enhanced by the recent recognition of a common dysregulation of the NF-κB pathway (7, 8) which has been linked to the human ABC-DLBCL subtype. It must be stressed that, although gene expression signatures have identified relevant human DLBCL subsets, no successful targeted therapies other than rituximab have been developed yet, and many potential targets have been developed on the basis of preclinical science in cell lines rather than in primary tumor specimens (38). Thus, the molecular classification of DLBCL requires clinical validation, and its role needs to be established within the current treatment paradigm. In this scenario, the dog as a cancer model may accelerate research.

Over the years, one of the more exciting and yet enigmatic concepts recurrent in the development and improvement of antitumoral strategies is the implication that the immune system can be harnessed and directed into a precision attack against neoplastic cells. Therapeutic cancer vaccines target TAAs to induce an active immune response, and 4 early-phase clinical trials in dogs with B-cell lymphoma have provided a strong rationale for this approach (17–20). Nevertheless, immunotherapeutic strategies have to face an important obstacle: the ability of tumor cells to evade the immune attack (23). Indeed, cancer cells may elude the immune surveillance by several mechanisms, including downregulation of MHC Class I molecules from the surface of tumor cells and consequent loss of immunogenicity, increased oxidative stress and recruitment of myeloid-derived suppressor cells and regulatory T-cells with suppressor function, or release of tumor-produced immunosuppressive cytokines (23, 39). Consequently, new strategies are warranted that exploit the immune response in an attempt to increase its strength and specificity to better control the disease.

Building on the premises of the prior Ciocca and colleagues’ study (27), we evaluated an all-biologic, personalized immunotherapeutic approach in canine DLBCL consisting of active immunization with a patient-specific vaccine. It was hypothesized that active immunization would extend the time to disease progression after cytoreduction with a CHOP-based chemotherapy. Indeed, such vaccines could theoretically spare healthy tissues, offer lifetime immunity against cancer, and possibly eradicate all cancer cells from the body.

CHOP-based chemotherapy was chosen for tumor debulking because it is the preferred treatment for dogs with aggressive B-cell lymphoma (9, 10). Although dose-intense chemotherapy can achieve complete responses and even in some cases long-term remission, relapse rates are high.

The vaccine used in this study consisted of hydroxyapatite powder and HSP purified from the dogs’ tumors.

It is well documented that the use of autologous tumor-derived HSSPCs, which function as chaperones of TAAs,
may circumvent the immune evasion caused by cancer heterogeneity by immunizing the host against a large repertoire of individual TAAs (40, 41). By this mechanism, tumor-derived HSPPCs provide protection against tumors derived from the same cancer cells from which the complexes are purified. Indeed, the full repertoire of the TAAs of a single tumor, including the individual strong antigens that make each tumor antigenically different from the other, are presented to and recognized by the patients’ immune system.

To put the immune system into overdrive, thereby exacerbating the immune response, hydroxylapatite was used as an immunologic adjuvant, aiming at activating T cells. It has been previously shown that hydroxylapatite has several advantages, including its ability to purify proteins by means of chromatography, biocompatibility, attraction of monocytes and macrophages to the implantation area, and role as a vehicle to deliver proteins to APCs (27, 42). With respect to vaccine administration, intradermal vaccination was chosen to deliver the antigen to professional APCs of the skin.

Taken together, the above findings, along with the demonstration of anticancer activity of HSPPCs in animal models (41, 43, 44), provided the rationale for HSPPC-based vaccination in dogs with DLBCL.

In the current study, dog characteristics were comparable in the two treatment arms. Treatment was usually well tolerated, and most adverse events were consistent with those expected with dose-intense chemotherapy. The type, incidence, and severity of the adverse events were comparable between the two arms, providing additional assurance that blinding was maintained during the trial. No clinical signs of autoimmunity due to the injection of autologous HSPPCs were observed.

The study did confirm the hypothesized improvement in TTP and LSS with chemoimmunotherapy in randomly assigned dogs receiving the autologous vaccine. There was a significantly increased TTP in the group of dogs receiving chemoimmunotherapy compared with dogs receiving chemotherapy and the placebo.

At a median follow-up of 598 days, 2 of 12 (16.7%) dogs in group 1 have remained in continuous first remission, remaining progression-free for +612 to +646 days. Two (16.7%) dogs had died for lymphoma-unrelated causes, still being in continuous first remission after 154 and 342 days, respectively. The remaining 8 (66.7%) dogs have progressed, with an overall median TTP of 304 days.

In the placebo arm (group 2), the observed median first TTP of 41 days was dramatically inferior to the published studies (9–11). This difference may be attributable to various reasons. Most published studies are retrospective in nature, considered lymphoma as a general entity, and used more dose-intense CHOP-based protocols than the one used here (data not shown). According to a recent prospective study conducted by our research group, dogs with high-grade B-cell lymphoma (in 28% of them, DLBCL was histologically confirmed) receiving a CHOP-based protocol obtained a median TTP of 87 days and a median LSS of 188 days (29). When stratified based on BM infiltration, dogs with a cutoff > 3% obtained a median TTP of 69 days and a median LSS of 155 days (29). Dose intensity, TTP, and LSS were similar to those used or obtained in the current study. Also, 4 out of 7 nonvaccinated dogs had a bone marrow infiltration level > 3%, representing a negative prognostic factor. Finally, another critical point is the assessment of the remission status. In the majority of the published studies using CHOP-based protocols as first-line treatment, the remission status was based on subjective or radiological assessment of lymph node size reduction/enlargement, obviously leading to a great underestimation of relapse/progressive disease. Here, the clinical findings were supported by at least cytologic evaluation of peripheral lymph nodes. This more accurate definition provides a critical view of therapeutic efficacy.

Notably, a primary concern of administering dose-intense chemotherapy is to exert a strong suppressive effect on host immunologic functions, thereby rendering concurrent active immunotherapy pointless. Here, we sought to optimize the immune response by integrating a less dose-intense chemotherapeutic protocol with therapeutic vaccination in schedules that maximized the activity of each modality. Indeed, although a less dose-intense chemotherapeutic approach seems reasonable when concurrent immunotherapy is administered to avoid impairment of the immune responses, and to give time for the dog’s immune system to recover after chemotherapy, the long interval between chemotherapy administrations and the low dose-intensity of the chemotherapeutic regimen used here were deleterious for dogs treated by means of chemotherapy only, leading to a poor outcome.

The median duration of chemotherapy-induced second remission among vaccinated dogs was significantly longer than the median duration of chemotherapy-induced second response in the unvaccinated dogs.

This finding has important clinical implications. Indeed, following standard dose-intense chemotherapy, both relapsed and refractory disease shorten the survival of DLBCL dogs. On the basis of our results, active immunotherapy has the potential to fight residual DLBCL cells, leading to a prolonged second remission and, ultimately, to prolonged LSS.

Interestingly, 50% of the vaccinated dogs achieved molecular remission, as documented by the negative minimal residual disease at the end of treatment. Clinical follow-up supported these results, as 3 of these dogs never relapsed, and 1 developed a T-cell lymphoma after 423 days from the initial diagnosis of DLBCL. In people, disappearance of minimal residual disease after immunization in patients with follicular lymphoma has been reported (45).

The long-term maintenance of antitumor immune responses may maintain the tumor load at an undetectable level. Relapse of lymphoma may well represent disruption of this delicate balance.

Taken together, our results document that active immunization provokes a cognate immune response that engages the adaptive response, leading to the establishment of
immunologic memory. The development of an anamnestic response provides sustained protection and reactivity against any lymphoma recurrence long before a relapse becomes clinically apparent.

A limitation of this study is the small population. Nevertheless, as previously suggested, randomized trials may overcome limitations of small sample size and yield valid conclusions if they are double-blinded and if baseline characteristics are well balanced (46, 47).

Also, as a result of resource constraints, immune assays were not performed, and immune responses were assessed by means of DTH. However, DTH responses have been shown to correlate with protection to a subsequent tumor challenge in an animal model (48). Furthermore, to validate immunogenicity, the production of IFN-γ by cytotoxic T lymphocytes was measured (data not shown). Briefly, BALB/c mice were inoculated with 4T1 tumor cells to generate breast cancer. Tumors were then removed to generate the vaccine as previously described. Murine-derived dendritic cells were matured in vitro with vaccine particles and then cocultured with cytotoxic T lymphocytes derived from mice challenged to 4T1 cells. IFN-γ was produced in response to TAA s, showing that cytotoxic T lymphocytes recognized the TAAs presented at the surface of the dendritic cells after their contact with the vaccine particles.

Finally, another limitation of the HSP vaccination approach consists of the requirement of tumor tissue to be removed by surgery. However, once the lymph node has been removed, the time necessary for vaccine manufacturing is in the order of a few hours, so that immunotherapy can be started without any delay.

In conclusion, the results of this trial indicate feasibility, good tolerability, and potent immunologic activity of the autologous vaccination strategy tested, leading to improved TTP and LSS in dogs with DLBCL. Given the fact that spontaneous DLBCL in dogs shares a wide variety of epidemiologic, biologic, and clinical features with human DLBCL, the acquired information may be applied for clinical applications in human patients with cancer.

Disclosure of Potential Conflicts of Interest
P. Frayssinet and N. Rouquet have ownership interest (including patents) in Urodelia. No potential conflicts of interest were disclosed by the other authors.

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Received August 17, 2013; revised November 8, 2013; accepted November 21, 2013. Published OnlineFirst December 3, 2013.

References

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Clinical Cancer Research

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