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## Vaccination with human tyrosinase DNA induces antibody responses in dogs with advanced melanoma

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### Abstract

Antitumor immune responses can be elicited in preclinical mouse melanoma models using plasmid DNA vaccines encoding xenogeneic melanosomal differentiation antigens. We previously reported on a phase I clinical trial of human tyrosinase (huTyr) DNA vaccination of 9 dogs with advanced malignant melanoma (World Health Organization stages II-IV), in which we demonstrated the safety of the treatment and the prolongation of the expected survival time (ST) of subjects as compared to historical, stage-matched controls. As a secondary goal of the same study, we report here on the induction of tyrosinase-specific antibody responses in three of the nine dogs vaccinated with huTyr DNA. The antibodies in two of the three responders cross-react with syngeneic canine tyrosinase, demonstrating the ability of this vaccine to overcome host immune tolerance and/or ignorance to or of “self” antigens. Most interestingly, the onset of antibody induction in these three dogs coincides with observed clinical responses and may suggest a means to account for their long-term tumor control and survival.

### Keywords

dogs; melanoma; DNA vaccine; xenogeneic; humoral immunity

### Introduction

Canine malignant melanoma (CMM) of the oral cavity, nail bed, foot pad, and mucocutaneous junction is a spontaneous, highly aggressive neoplasm that can readily metastasize to the lymph nodes, liver, lung, and kidney (1,2,3,4,5,6). Prognosis is often dependent on the clinical staging of the tumor; canine patients diagnosed with World Health Organization stage I oral CMM have a reported median ST of 1 yr with carboplatin chemotherapy and hypofractionated radiation (1,7). Patients with advanced disease (World Health Organization stages II, III, or IV) have a reported median ST of less than 5 mo with surgical resection of the local tumor (2,3,4). Overall response rates of canine melanoma patients to chemotherapy are low, with little evidence that treatment improves ST (8). This

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is likely due to subsequent metastasis and resistance of residual tumor cells, despite aggressive therapies. Targeted treatment of microscopic disease should be considered a critical component in the overall management of CMM, in conjunction with current modalities for local/regional disease control.

Numerous immunologic strategies in preclinical models and clinical settings have been described as eliciting antitumor responses capable of tumor regression and rejection (9). Previous attempts in both human and veterinary oncology include the use of autologous tumor cells and/or cell extracts (with or without bacterial adjuvant) (10,11,12,13), gene-modified tumor cells (14,15,16,17,18,19,20,21), heat-shock proteins (22,23), or tumor-specific peptides (24) as vaccines to activate humoral and/or T-cell immunity. Other approaches involve the induction of apoptosis by intratumoral Fas ligand (FasL) DNA injection (25) or vaccination with autologous DCs expressing xenogeneic tumor antigens in order to elicit antitumor responses (26). The utility of autologous tumor vaccines is often limited by the availability of tumor cell lines specifically established for each patient. Similarly, peptide-based vaccines can only be applied to discrete patient populations due to MHC haplotype restrictions. The main challenge in active immunization against malignant melanoma, nevertheless, is to selectively activate the immune repertoire that can recognize and target antigens on melanoma. The best-characterized antigens on melanoma include the melanosomal membrane proteins, which are also expressed on normal melanocytes.

Preclinical mouse models of melanoma demonstrate that mice vaccinated with xenogeneic (human) cDNAs encoding different melanosomal differentiation antigens (that is, TRP1/gp75, TRP2/DCT, pMel17/gp100, or tyrosinase) can generate specific antibody and T-cell responses against syngeneic tumor cells that protect against subsequent tumor challenge, whereas plasmid DNA encoding mouse antigens induces no tumor immunity (27,28,29,30,31). Hence, effective antitumor immunity can be induced *in vivo* when host immune tolerance/ignorance to/of the “self” differentiation antigens is overcome.

We have chosen to investigate xenogeneic DNA vaccines encoding tyrosinase as a means to induce immune responses in CMM patients. Tyrosinase is normally expressed in melanocytes to catalyze the rate-limiting step of melanin biosynthesis from tyrosine (32,33). Tyrosinase, as well as other related glycoproteins, is a suitable target for CMM immunotherapy because of its restricted, tissue-specific expression. Full-length canine tyrosinase (NCBI protein database accession no. AAQ17535) also shares significant homology with that of human (NCBI accession no. AAA61242) and mouse (NCBI accession no. BAA00341) tyrosinase at 87.5% and 84.4% amino acid identity, respectively (Figure 1). Injections of xenogeneic tyrosinase DNA may therefore be a means to overcome canine immune tolerance to the self-tyrosinase because of differences in the sequence that improve epitope recognition by MHC class I or the T-cell receptor. Sequence differences may also create class II-restricted helper epitopes and induce antityrosinase immune responses when expressed *in vivo* in dogs with CMM. We previously reported on the safety and prolonged survival of CMM patients immunized with xenogeneic huTyr DNA in a phase I, single-arm clinical trial (34).

To validate the observed clinical efficacy of xenogeneic DNA vaccination as a therapeutic modality for CMM, the present study examines the humoral immune responses of the same three cohorts of dogs vaccinated with escalating doses (100 µg, 500 µg, and 1500 µg) of huTyr cDNA. Three of nine dogs have tyrosinase-specific antibodies induced after vaccination, with antibody titers as high as 1:1280 for one of the three dogs, compared to its preimmune serum and to the sera of normal, healthy dogs. The specificity of the antibodies generated is confirmed by the ability of the canine postimmune sera to detect both endogenous human and, more importantly, canine tyrosinase in cultured melanoma cell lines

derived from both species. Temporal measures of the serum antibody level further indicate that the induced antibody response to the human antigen can be sustained for 3 to 9 months after the 4 biweekly immunizations. Most interestingly, these 3 dogs exhibited clinical responses with long-term tumor control; 1 of these dogs remains alive as of publication (for approximately 4 years) with an unchanged, cytologically-confirmed pulmonary metastasis. The induction of antibodies by the xenogeneic huTyr DNA vaccine, concurrent with observed antitumor responses in these CMM study subjects, supports the therapeutic feasibility of this treatment in preventing tumor dissemination, possibly through antibody-mediated immune responses. Nevertheless, further evaluation is warranted to fully elucidate its efficacy and the immunologic mechanisms of its action in an outbred, genetically heterogeneous population of large animals with spontaneous cancer. Other, related studies currently in progress are investigating the potential activation of T-cell responses by the huTyr vaccine, as well as other xenogeneic DNA vaccines that may contribute to the overall tumor regression/control of CMM.

## Results

### Measurement of the humoral response induced by vaccination

To measure the humoral immune response induced by the xenogeneic DNA vaccine, we analyzed canine sera for tyrosinase-specific antibodies by indirect ELISA. Of the 9 vaccinated dogs in the study, 3 had a measurable increase in postvaccine serum antibody binding to the mammalian-expressed, recombinant huTyr (Figure 2), but not to a nonspecific substrate, myelin-basic protein (data not shown). The level of antibody response, measured spectrophotometrically to reflect tyrosinase-specific antibody binding to the target substrate, ranged from two- to four-fold higher in the postvaccination sera than in the preimmune sera (Figure 2) or in the serum of a normal, healthy dog used as a control (data not shown). For dog A, initially diagnosed at stage IV with visible pulmonary metastases on thoracic radiographs, the onset of the antibody response was after the second vaccination (data not shown) with the 100- $\mu$ g dose regimen. The induction of response, however, was not maintained by subsequent vaccinations, consistent with dog A's poor clinical prognosis and with the progression of pulmonary metastases. Interestingly, 2 weeks after the fourth vaccination, at a time when antibodies to tyrosinase were again detected in the sera (Figure 2a), this patient experienced a long-term remission with complete disappearance of the existing pulmonary metastases. As previously reported, dog A had a ST of 389 days and was euthanized due to complications from an acute septic episode. Subsequent gross and histopathological necropsy revealed a recurrent 2-cm malignant melanoma caudal to the site of the original oral primary malignant melanoma, but with no evidence of the previously documented pulmonary metastases (34). Evaluations of postvaccination sera taken at 2 weeks (recheck 1) and at 9 months (recheck 2) after the final vaccination indicated an antibody titer of 1:320 at recheck 1 and 1:1280 at recheck 2 (Figure 2a), which correlate with the positive clinical outcome after the completion of the vaccination series.

A similar induction of antibody response was detected for dog B, diagnosed with stage III melanoma, which had a reported ST of 496 d with no evidence of any gross or histopathological melanoma on necropsy (34). Consistent with the immune response in dog A, immunization of dog B at the same dose was sufficient to elicit tyrosinase-specific antibody production (Figure 2b), although the initial onset of the immune response occurred subsequent to the third vaccination. Despite this initial two-fold increase in antibody binding to the substrate, the highest level of response was not detected until 9 months after the final vaccination at recheck 2, with a titer of 1:640.

Although some of the dogs in the other cohorts (500  $\mu$ g and 1500  $\mu$ g doses) clearly experienced prolonged survival (34), we did not detect any measurable difference in

tyrosinase-specific antibody titers between their pre- and postvaccination sera (data not shown). Of particular interest was dog C, initially diagnosed at stage IV with a solitary, cytologically confirmed 4 cm pulmonary metastasis (34). This dog developed an antibody response to the 1500  $\mu$ g dose of DNA vaccine. Consistent with dogs A and B, the antityrosinase antibody response was detected for dog C after the third vaccination, and reached a maximum level 3 months after the final boost, at recheck 2 (Figure 2c). The measured antibody titer, however, was comparatively lower at 1:320, and gradually decreased to a level comparable to that of the preimmune and normal canine sera (Figure 2c and data not shown); this suggests that further vaccine boosts may be needed in order to maintain, if not increase, tyrosinase-specific antibodies. After the completion of the huTyr DNA vaccination program, dog C experienced minor progression of disease and received additional xenogeneic DNA vaccines (mouse tyrosinase-related protein 1/gp75 and, later, mouse tyrosinase). Studies to determine the immunologic response(s) to the multiple DNA vaccine regimens encoding different xenogeneic melanoma-associated antigens are ongoing, as dog C remains alive as of this publication, with a minimally changed pulmonary lesion since trial entry in 2000.

### Confirmation of antityrosinase antibodies by flow cytometry

To confirm the induction of tyrosinase-specific antibodies capable of recognizing native tyrosinase in response to the DNA vaccination, a flow cytometry-based assay with cultured human SK-MEL-188 melanoma cells was used. Cells were incubated with diluted postvaccination or preimmune sera after cellular permeabilization and fixation and analyzed for recognition of endogenous intracellular tyrosinase by flow cytometry. Consistent with the detection of the tyrosinase substrate by ELISA, the postvaccination sera from dogs A, B, and C contained specific antibodies capable of binding endogenous huTyr, as shown by the increase in mean fluorescence intensity of the stained SK-MEL-188 cells (Figure 3 a–c, top panels) as compared to cells incubated with either preimmune sera or FITC-conjugated rabbit antidog secondary antibodies. Given the different experimental conditions and biological context of this methodology in detecting protein-protein interactions, serum samples from the other vaccinated patients were also analyzed for antityrosinase antibodies. There was no detectable difference in SK-MEL-188 binding between the pre- and postvaccination sera from dogs with no response by ELISA (data not shown), thereby demonstrating concordance between the ELISA and the flow cytometry assays.

### Detection of vaccination-induced autoantibodies to canine tyrosinase

Since the prior assays utilized xenogeneic (human) tyrosinase as a target, we were interested in determining whether the antibodies induced by DNA immunization could also recognize syngeneic canine tyrosinase. Canine melanoma cells CML 83-2, which express levels of tyrosinase mRNA comparable to those expressed by human SK-MEL-188 cells (Figure 4), were treated and analyzed by flow cytometry to assess the ability of vaccine-induced antibodies in dogs A, B, and C to bind endogenous canine tyrosinase. Consistent with the stained SK-MEL-188, we observed a modest increase in mean fluorescence intensity for CML 83-2 cells incubated with the same postvaccination sera from dogs A and B as compared to their preimmune sera (Figure 3 a and b, middle panels). In contrast, we did not detect any measurable difference in mean fluorescence intensity when CML 83-2 cells were incubated with either preimmune or postvaccination sera from dog C (Figure 3c, middle panel), despite the development and presence of antibodies that recognized the huTyr in this patient (Figure 3c, top panel). To further ensure that the antibody responses detected in these dogs are tyrosinase-specific, we included a canine MDCK kidney-derived epithelial cell line (a kind gift from Dr. A. Reilein) that does not express tyrosinase as a negative control for the flow-cytometric analyses. For dogs A and C (Figure 3 a and c, bottom panels), we did not observe any measurable change in fluorescence between MDCK cells stained with

preimmune and postvaccinate serum samples. However, we detected a very slight increase in the mean fluorescence intensity for MDCK cells incubated with recheck serum 2 from dog B (Figure 3b, bottom panel). Taken together, these data demonstrate that xenogeneic huTyr DNA vaccination can induce antibodies capable of recognizing both xenogeneic and syngeneic tyrosinase.

### Association of vaccine-induced humoral responses with patient ST

Kaplan-Meier survival analysis for patients A, B, and C ( $n = 3$ ), when compared to the remaining population ( $n = 6$ ) of humoral nonresponders (Figure 5), suggested an association between long-term survival (measured in days) and positive antibody responses. Given the small sample size, however, the association and/or effect of antibody production with overall survival did not reach statistical significance (log-rank  $P = 0.148$  and Cox Proportional Hazards  $P = 0.183$ ).

## Discussion

In our previously published single-arm clinical trial, 9 dogs with advanced CMM (World Health Organization stages II-IV) were vaccinated with a xenogeneic DNA plasmid expressing huTyr, a melanosomal differentiation antigen. The trial demonstrated that the DNA vaccine is safe and efficacious for the vaccinated dogs, which had an increased median ST of 389 d (34), compared to historical controls of 1–5 months with conventional therapies (3,4,35). The results of the present study document an induction of antibody responses in 3/9 of the vaccinated CMM patients, a response rate comparable to other human DNA vaccine programs for malaria (36) and HIV (37). Consistent with preclinical mouse models, the induction of canine humoral responses by the orthologous human antigen to recognize self tyrosinase suggests the overcoming of canine immune ignorance or tolerance, as the tyrosinase-specific antibodies developed in response to the huTyr DNA vaccine are capable of cross-reacting with canine tyrosinase. Unlike some of the mouse melanoma models in which autoimmune coat hypopigmentation associated with xenogeneic DNA vaccines encoding various melanosomal differentiation antigens was seen (25,26,27,28,29), we did not observe any clinical signs of hypopigmentation in any of the dogs under current study. However, we have a single documented case of foot pad depigmentation in a dog vaccinated with DNA vaccine encoding murine tyrosinase (unpublished observation). The generation of canine tyrosinase, unavailable at present, would ultimately allow us to test the potential cross-reactivity of induced antibodies by the human antigen to recognize and target the specific self protein *in vitro*, as opposed to the xenogeneic antigen evaluated in the ELISA here.

With the initial priming followed by 3 biweekly boosts of DNA vaccine, the induced humoral responses were sustained for a relatively long period after the final vaccination. Positive responders had increased levels of antibody 3–9 months after the completion of the vaccination program at recheck 2 (Figure 2), which was associated with long-term tumor control. This study demonstrates the immunogenicity of huTyr DNA and suggests that the resulting humoral responses may persist long-term in some patients. Moreover, our findings are consistent with the temporal model of response for other cancer vaccines, which can take approximately 4 to 8 weeks, if not longer, to elicit specific humoral and/or cellular immune responses, and months for clinical antitumor responses (38,39).

Current models suggest that xenogeneic DNA vaccines may exploit a preexisting repertoire of autoreactive B and T cells typically not responsive to tumor cells, which arise from self tissues and express self antigens. These autoreactive cells have the potential to target the same tumor cells when activated appropriately by orthologous antigens expressing subtle but distinct epitopes (40,41,42). The utility of this therapeutic modality in mounting antibody



and/or T-cell-mediated immunity to tumor antigens has been clearly demonstrated in numerous preclinical mouse tumor models of the skin (25,26,27,28,29), lung (43), breast (43), and prostate (44,45,46,47). Our findings are consistent with these models in that antitumor autoimmunity can be elicited in CMM patients in response to an orthologous antigen expressed *in vivo* from a plasmid DNA expression construct. We speculate that existing differences in protein sequences between human and canine tyrosinase may provide higher-affinity MHC class II-restricted peptides from the human protein to activate autoreactive CD4+ T helper cells with intermediate- or low-affinity receptors for this otherwise poorly immunogenic self antigen and may help generate antibodies against self tyrosinase. For our study, full-length cDNA encoding the huTyr was subcloned into a bacterial expression vector, pING, and constitutively expressed under the control of a CMV promoter (34). Insertion of the antigen encoding sequence in a bacterial expression vector provides the vaccine with an endogenous adjuvant in the form of unmethylated CpG nucleotide sequences, which activate APCs through toll-like receptor 9 (TLR9) (48,49,50,51,52,53).

While the autoantibodies generated in dogs A–C are likely to have induced tumor regression and/or inhibited further metastases, the exact mechanism(s) of antibody-mediated tumor rejection in the canine system will require further elucidation. In particular, it remains unclear how tyrosinase, an intracellular melanosomal glycoprotein, can be recognized and targeted by antibodies in intact tumor cells. We speculate that a low-level, cell-surface expression of canine tyrosinase is the target for the autoantibodies, as detection and modulation of gp75/Trp1 (a tyrosinase-related melanosomal glycoprotein and a well-characterized melanoma antigen) expression in the presence of IFN- $\gamma$  (54) has previously been described on the tumor cell surface of mouse melanoma models. The involvement of gp75-specific antibodies, transferred passively (54,55,56) or induced by active vaccination (27,56), in mediating antitumor responses is directly linked to the host effectors (that is, NK1.1+ or macrophages) expressing activating Fc receptors (Fc $\gamma$ R type I and III). Activated NK1.1+ cells may be directly involved in antibody-dependent cellular cytotoxicity or may modulate the activities of other effector-cell components (that is, macrophages or monocytes) for tumor cytotoxicity. Given the critical role for FcRs (and the inflammatory effectors) for effective antibody-dependent tumor cytotoxicity *in vivo*, a similar cascade of antibody-mediated Fc $\gamma$ R engagement and activation of host effectors is likely conserved in the dog to mediate the observed clinical outcomes in response to the huTyr-induced autoantibodies.

Based on our studies, xenogeneic DNA vaccination should be considered as an adjunctive therapy for CMM patients with minimal residual disease and/or low tumor burden. Ultimately, long-term clinical efficacy with complete control, if not elimination, of microscopic tumor dissemination can only be achieved when the following have been optimized: (i) efficient uptake of the antigen, (ii) efficient antigen processing by APCs with migration to draining lymph nodes, (iii) precise antigen presentation by the APCs (that is, DCs), (iv) induction of appropriate helper and cytotoxic T cells systemically to target tumor cells, and (v) a persistent pool of memory helper and/or effector cells capable of challenging any subsequent tumor growth over time (40). We demonstrate here that xenogeneic DNA vaccine targeting tyrosinase can fulfill some of the above prerequisites to induce antigen-specific antibody responses in large, outbred animals with spontaneously occurring malignant melanoma.

The variability in the measured antibody responses among the vaccinated dogs likely reflects the need for further optimization of all the critical steps involved in vaccine-induced antitumor responses, as dogs A and B clearly developed cross-reactive autoantibodies to tyrosinase while the same humoral response to syngeneic tyrosinase was absent in dog C. It

is equally plausible that the absence of detectable antityrosinase humoral responses in the other vaccinated patients with and/or without positive clinical outcomes may be attributable to the challenges we have discussed. It would be more informative if autologous melanoma tumor cells from each patient were readily available and accessible for us to determine the individual expression level/profile of endogenous tyrosinase and to conduct similar comparative analyses, as discussed earlier. Likewise, functional measures of antibody-mediated cytotoxicity would better substantiate our observations and should be incorporated into future studies of DNA vaccine programs.

Equally important, although the present study demonstrates that induction of specific (auto)antibodies to an intracellular tumor antigen correlates with positive clinical outcome in some patients, we recognize that other components of the immune system, such as CD8+ CTLs, may become activated and contribute to the overall antitumor responses against CMM. To address this, we are currently engaged in the development of an assay to measure antigen-specific T-cell responses in these outbred dogs. Quantitative measures of potential cytotoxic T-cell responses should provide an additional, or alternative, mechanism to explain the positive antitumor effects in the CMM patients lacking detectable antibody response to the vaccine.

## Materials and methods

Patient characteristics, vaccination protocols, vaccine development, and clinical outcomes of the canine patients in the xenogeneic huTyr DNA vaccine trial have been described in an earlier work (34).

### Tissue culture

Human and canine melanoma cell lines SK-MEL-188 and CML 83-2 (kind gift of Dr. L.G. Wolfe, Auburn University, Auburn, AL, USA), respectively, as well as canine MDCK cell line (kind gift of Dr. A. Reilein, Weill Medical College, Cornell University, New York, NY, USA) were grown in RPMI 1640 (Mediatech, Inc., Herndon, VA, USA) supplemented with 7.5% heat-inactivated FCS, 1% nonessential amino acids, 1% L-glutamine, and 1% penicillin/streptomycin and maintained at 37°C with 5% CO<sub>2</sub> until harvested.

### RNA extraction and RT-PCR

Total RNA from SK-MEL-188 and CML 83-2 were isolated using the SV Total RNA Isolation System (Promega, Madison, WI, USA), following the manufacturer's instructions. Tyrosinase mRNA expression in each cell line was analyzed by RT-PCR. Briefly, tyrosinase mRNAs from each cell line were reverse transcribed from the isolated total RNA using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA), followed by amplification of the tyrosinase cDNA from each first-strand cDNA synthesis reaction using conserved primer sets for both human and canine: Primer 3 (reverse): 5'-TGGCAGCTTTATCCATG GAA-3', Primer 4 (forward): 5'-AATGGATCAACACCCATGTT-3', and Primer 5 (forward): 5'-GGAGTCCCTGTGGCCAGCTT-3'. For cDNA amplification, the following thermal cycling conditions were used: denaturation at 95°C for 3 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 1 min, and elongation at 72°C for 30 s, and a final extension at 72°C for 10 min. Amplified cDNA from each cell line was resolved and visualized on 1% agarose gel stained with ethidium bromide.

### Antibody response

Canine sera were collected before the start of trial and after each vaccination. As previously described (34), approximately 2–4 ml of canine whole blood was collected in a

VACUTAINER SST Gel & Clot Activator tube (Becton Dickinson, Franklin Lakes, NJ, USA) and spun for 15 min at 2000 rpm. Each serum was aliquoted and cryopreserved at  $-70^{\circ}\text{C}$  until analysis.

Sera were analyzed by solid phase ELISA for reactivity against mammalian-expressed, recombinant huTyr protein as the specific target substrate. Briefly, high-affinity binding, 96-well plates (Sigma, St. Louis, MO, USA) were coated with recombinant huTyr expressed as secreted (and soluble) protein in CHO-easyC protein-free spinner culture (kind gift of C. Andreoni and Dr. J-C. Audonnet, Merial, Inc., Lyon, France), or purified myelin-basic protein (kind gift of Dr. R.P. Fisher, MSKCC) as negative control, overnight at  $4^{\circ}\text{C}$ . Coated plates were washed twice with 1x PBS (pH 7.4) and blocked with 3% BSA in 1x PBS (pH 7.4) for at least 1 h at room temperature. Plates were washed twice with 1x PBS after blocking and incubated with canine sera serially diluted in 1% BSA (in 1x PBS, pH 7.4) for 3 h at room temperature. To remove any nonspecific antibody or excess serum protein, plates were subsequently washed three times with 1x PBS containing 0.05% TWEEN-20 (PBST) and three times with 1x PBS. Alkaline phosphatase-conjugated antidog IgG antibody (Sigma) was diluted in 1% BSA (1:2500) and added to each well for 1 h incubation at room temperature. Plates were washed as described above to remove any unbound secondary antibody, followed by the addition/incubation of chromogenic substrate, p-nitrophenyl phosphate (Sigma). The colorimetric absorbance of each well was measured spectrophotometrically at 405 nm on the VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

### Flow cytometry

Human SK-MEL-188 and canine CML 83-2 and MDCK cells were trypsinized, counted, and resuspended in FACs<sup>®</sup> buffer (1x PBS supplemented with 1% heat-inactivated serum). For each staining and analysis,  $1-3 \times 10^5$  cells were fixed with prechilled methanol for 30 min on ice, washed once with FACs<sup>®</sup> buffer, and then permeabilized with 0.1% triton X-100 (in 1x PBS) for 30 min at room temperature. Treated cells were washed twice with FACs<sup>®</sup> buffer and incubated with 50  $\mu\text{l}$  diluted (1:500) canine sera for 1 h on ice. Stained cells were washed twice with FACs<sup>®</sup> buffer, followed by incubation with 50  $\mu\text{l}$  diluted (1:500) FITC-conjugated rabbit antidog IgG antibody (Jackson ImmunoResearch, West Grove, PA, USA) on ice for 45 min. Stained cells were washed twice, resuspended in 120  $\mu\text{l}$  FACs<sup>®</sup> buffer, and 10,000 events were counted using a FACscan flow cytometer (Becton Dickinson, San Jose, CA, USA). FloJo software (Tree Star Inc., Ashland, OR, USA) was used to analyze the changes in mean fluorescence intensity after vaccination. An increase (or positive shift) was interpreted as a measure of serum antibody-intracellular tyrosinase interaction.

### Statistical analysis

ST, defined as the time from receiving the first xenogeneic huTyr DNA vaccination until death, was calculated using Kaplan-Meier life table analysis for patients with or without detectable humoral responses. For delineation of statistical significance between the two subgroups on survival, log-rank (Mantel-Cox) test was used;  $P < 0.05$  was considered statistically significant. The Cox proportional hazards ratio was also calculated to evaluate whether the variable (antibody response) was associated with ST. Patients were censored upon death unrelated to melanoma or if still alive at the end of the study period, as previously reported (34).

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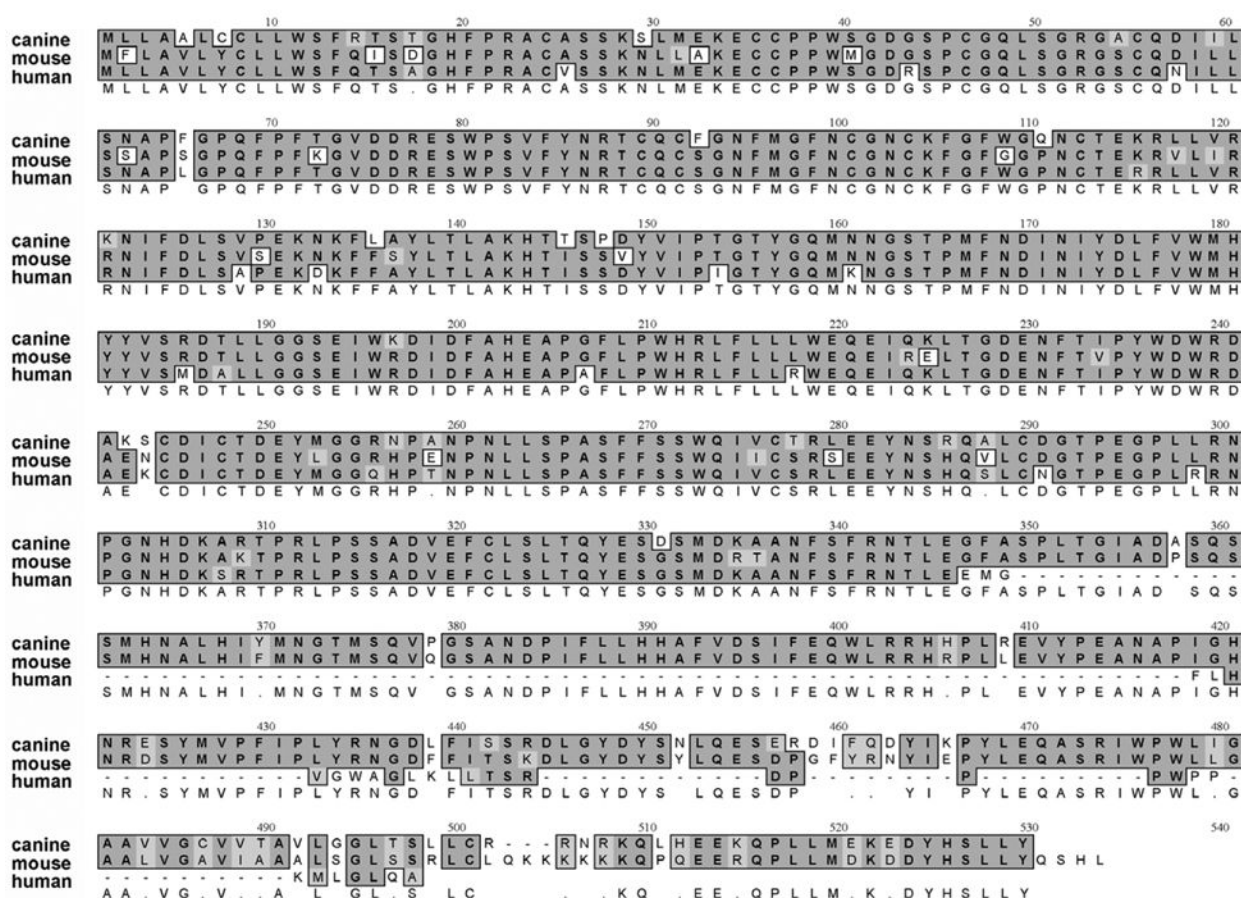
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## Abbreviations

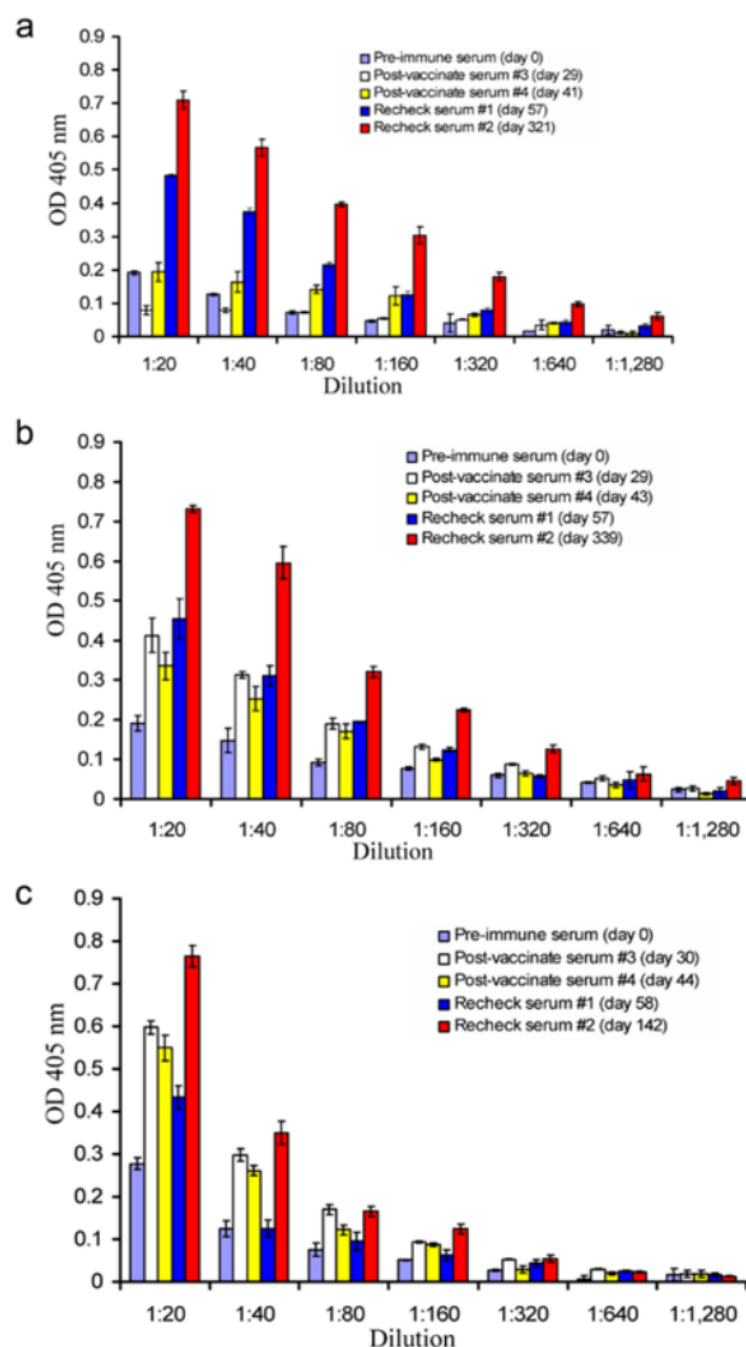
<b>CMM</b>	canine malignant melanoma
<b>huTyr</b>	human tyrosinase
<b>ST</b>	survival time



**Figure 1. Tyrosinase is conserved from dog to mouse to man**

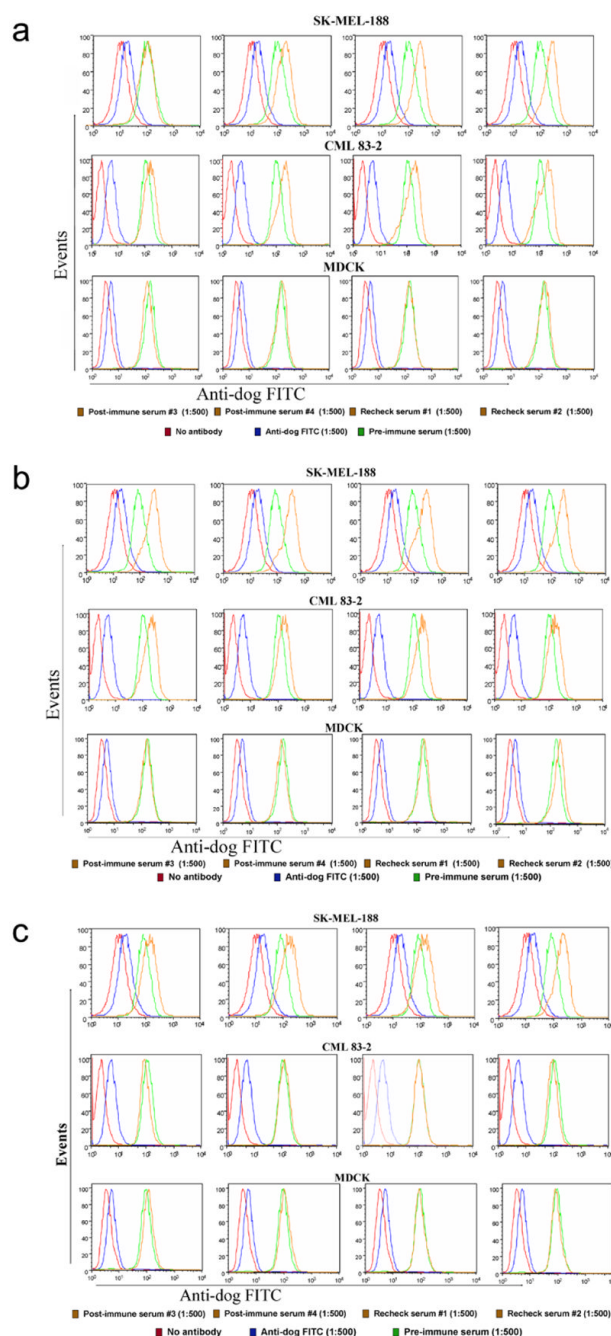
Sequence comparison of tyrosinase from dog, mouse, and human shows a high degree of homology at the amino acid level. The calculated sequence identity by Vector NTI between canine tyrosinase and that of mouse and human is 84.4% and 87.5%, respectively, indicating evolutionary conservation of this protein. Dark gray highlights identical residues and light gray highlights conservative amino acid changes.



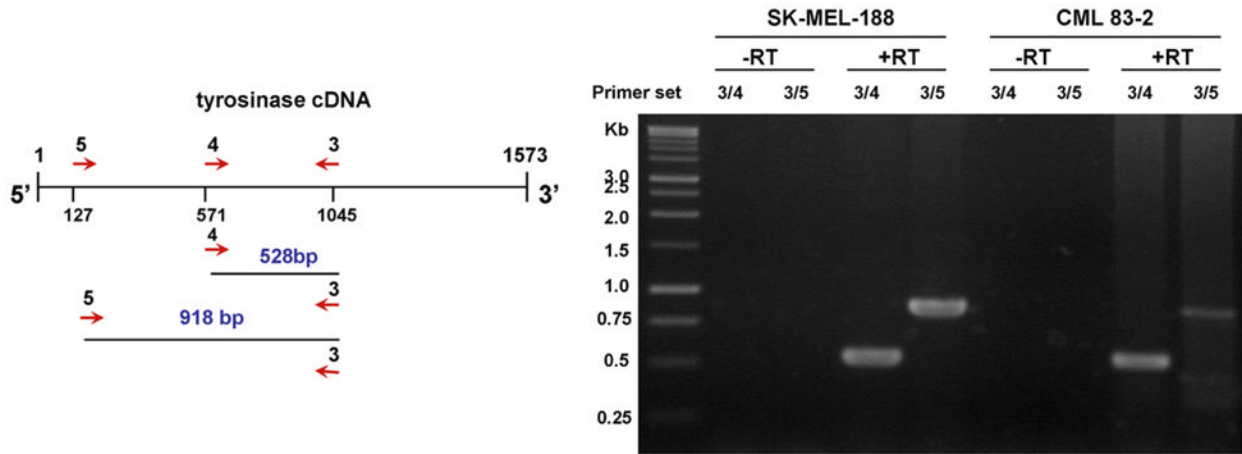


**Figure 2. Detection of tyrosinase-specific antibodies by ELISA**

Representative results of three independent experiments for each patient--canine sera from patients A (panel a; 100  $\mu$ g huTyr DNA dose), B (b; 100  $\mu$ g huTyr DNA dose), and C (c; 1500  $\mu$ g huTyr DNA dose)--were serially diluted at 1:20, 1:40, 1:80, 1:160, and 1:320, 1:640, and 1:1280. Each serum dilution was added in triplicate to individual wells (50  $\mu$ l/well) containing recombinant huTyr (a-c) or myelin-basic protein, a nonspecific substrate (data not shown). The peak increase in tyrosinase-specific titer, compared to the preimmune serum, was detected for all patients at recheck 2: patient A at 1:1280, patient B at 1:640, and patient C at 1:320.

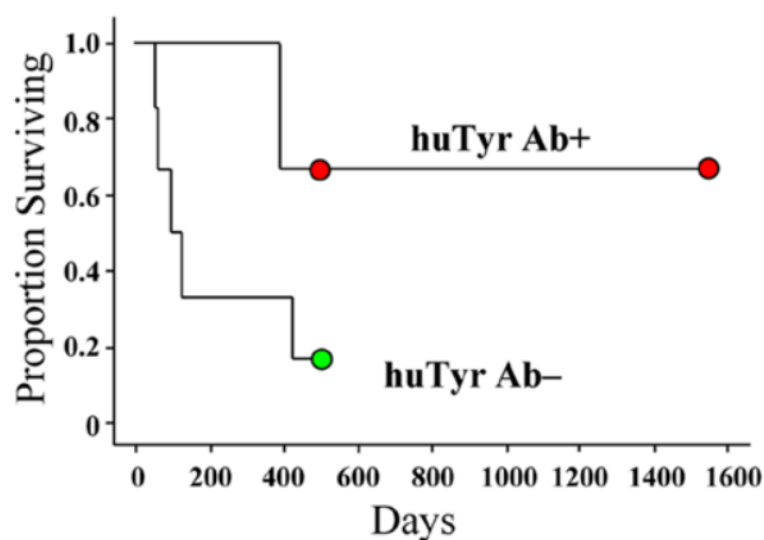


**Figure 3. Reactivity of canine postvaccinate sera to endogenous human and canine tyrosinase** Cultured human melanoma cells SK-MEL-188, canine melanoma cells CML 83-2, and canine MDCK cells were permeabilized and incubated with sera (diluted 1:500) from patients A (panel a; 100  $\mu$ g huTyr DNA dose), B (b; 100  $\mu$ g huTyr DNA dose), and C (c; 1500  $\mu$ g huTyr DNA dose). Flow cytometry analysis showed an increase in mean fluorescence intensity for melanoma cells (but not canine MDCK cells) stained by the postvaccinate and recheck sera, and not by preimmune sera or secondary antibodies. This indicates specific recognition of cells expressing human or canine tyrosinase by the antibodies induced in the postvaccinate canine sera. Data shown are representative of two independent flow analyses for each patient.



**Figure 4. Human and canine mRNA expression evaluated by RT-PCR**

Expression of tyrosinase mRNA by human (SK-MEL-188) and canine (CML 83-2) melanoma cell lines was examined by RT-PCR. Conserved, tyrosinase-specific primer sets, 3/4 and 3/5 (schematic diagram), were used to amplify a 528 bp and an overlapping 918 bp cDNA fragment, respectively, indicating the expression of tyrosinase genes in both cell lines.



**Figure 5. Kaplan-Meier survival curves for canine patients with ( $n = 3$ ; huTyr Ab+) and without ( $n = 6$ ; huTyr Ab-) positive humoral responses to the xenogeneic huTyr DNA vaccination**  
Dots denote patients that were censored from the analysis. The difference in ST between the two groups was not statistically significant ( $P = 0.148$ ; log-rank analysis), possibly due to the small patient group size.