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TITLE: Adoptive Immunotherapy Combined with Hematopoietic Cell Transplantation  
as a Therapeutic Treatment of Prostate Cancer

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<b>14. ABSTRACT</b> We determined that a prostate cell lysate prepared from canine prostate tissue was immunogenic when injected in female dogs. In addition to the known prostate antigen, canine prostate specific esterase (CPSE), we identified by molecular weight several other proteins against which the dog made IgG antibodies. Using a mixed lymphocyte reaction, we found that a cellular response was generated against the prostate cell lysate after the female dog was injected 3 times with antigen. Sensitization of female dogs with prostate antigen suspended in incomplete Freund's adjuvant appeared to be superior to prostate lysate antigen loaded canine autologous dendritic cells when both were injected subcutaneously near the popliteal lymph node. Peripheral blood lymphocytes and lymph node derived lymphocytes reacted to prostate lysate in the mixed lymphocyte reaction. An immune response to prostate lysate not significantly detected by a delayed-type hypersensitivity reaction 24 to 48 hours after the last of three subcutaneous injections of antigen. One of four male dogs transplanted with dog leukocyte identical female bone marrow engrafted was stably engrafted. Injection of prostate antigen-sensitized female peripheral blood mononuclear cells failed to induce prostatitis, conversion of mixed to 100% hematopoietic chimerism or initiate graft versus host disease in the male recipient. Based on a single example, these results suggest that induction of an inflammatory response against normal tissue following injection of antigen sensitized lymphocytes may require additional perturbation to the recipient's targeted tissue.					
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## **Introduction**

Allogeneic hematopoietic cell transplantation (HCT), or the transplantation of a hematopoietic system from a tissue matched normal donor, has developed into a highly effective therapy for the treatment of hematological malignancies. The canine model has played a major role in the development of these HCT therapies, to the extent that regimens for successful allogeneic HCT in the dog have been directly translated to the clinic (1-3). We and others have shown that the donor cytotoxic lymphocytes mediate an attack against the host hematopoietic system including often successful elimination of the underlying hematopoietic tumor. This has been termed the “graft-versus-tumor effect”.

Oncologists have recently attempted to apply this remarkably efficient form of cancer therapy to solid tumors. Treatment of renal (4), breast (5) and ovarian (6) cancer has met with only limited success in part due to the inability to separate graft versus host disease (GvHD) from anti-tumor effects. What is evidently needed is a method wherein the host lymphocytes are sensitized against tumor or tissue antigens specific to the tumor to improve anti-tumor efficacy.

Prostate cancer is an ideal candidate for HCT therapy as co-elimination of this organ with the cancer is not life-threatening to the patient. In addition to tumor antigens, several male androgen sensitive antigens exist which are essentially foreign to a female immune response and if expressed on the tumor and presented by the major histocompatibility complex (MHC) could be targeted by the female donor prostate antigen-sensitized lymphocytes after infusion into the male recipient. The concept of this grant has been to test the hypothesis that immunotherapy can be combined with allo-HCT to treat prostate cancer. In this model, HCT is required as a platform for adoptive immunotherapy. In this capacity, female donor lymphocytes, sensitized against prostate antigens, are expected to survive in the chimeric environment which by definition is tolerant to them. We hypothesize that the sensitized donor lymphocyte infusion will provide the anti-tumor activity.

## **Body**

The study design was divided into two aims. The first was to identify the better of two methods for immunizing a female HCT donor against prostate antigens. One way was to sensitize female dogs with three injections of a mixture of prostate cell lysate and mollusk hemocyanin antigen in Incomplete Freund’s Adjuvant (IFA). For the other, we cultured female canine bone marrow derived dendritic cells in the presence of the same antigen mixture, and administered three injections of these cells within two centimeters of the popliteal lymph nodes in 2 dogs. The second aim of this study was to establish mixed hematopoietic chimerism in a male recipient using marrow from a female dog leukocyte antigen (DLA)-identical littermate. Next, we sensitized the female donor using the optimum method identified in Aim 1. Finally, we performed an infusion of sensitized donor lymphocytes into the male recipient. At this point, we monitored hematopoietic chimerism, prostate inflammation, serum levels of canine prostate specific esterase (CPSE), and GvHD. Because dog assignments to this study were slow due to other competing studies and limitations of space in our facility, we requested and were granted an extension for the grant in order to complete Aim 2. During this time a total of 4 dogs were transplanted. However, only one of the 4 male dogs established long-term

chimerism and was an evaluable candidate for adoptive immunotherapy with prostate antigen-sensitized female donor lymphocytes.

**Aim 1. Establish methods to induce immune responses to prostate lysate in female dogs.**

We successfully generated prostate antigen using a freeze/thaw method that produced milligram quantities of sterile antigen. Briefly, prostates were removed from cadaverous dogs and frozen at  $-70^{\circ}\text{C}$  until processing. Once 6 prostates were collected, the tissue was minced by scalpel and refrozen in dry ice and ethanol. After repeated freeze/thaw cycles, the tissue was sonicated and then the tissue debris was separated from the solution by centrifugation at  $10,000 \times g$ . Protein concentration was determined using a protein colorimetric assay (BioRad).

Five dogs were vaccinated with three injections of prostate lysate-hemocyanin, 10 days apart, within 2 cm of the popliteal lymph node. Ten days after the third injection, a skin test was performed for delayed type hypersensitivity response against 1) prostate lysate, 2) hemocyanin, 3) Freund's incomplete adjuvant (IFA), 4) a lysate of the canine kidney cell line, Madin-Darby canine kidney cells (MDCK; negative control), and 5) PBS.

An example of the skin test is shown in Figure 1 of dog G768, 48 hours after a subcutaneous injection of the prostate lysate, hemocyanin and the controls of IFA, MDCK lysate and PBS. The results of this assay were not remarkable at the 24 and 48 hour periods, where only very mild erythema was noted for the prostate lysate (0.5 cm in diameter) and hemocyanin (0.5 cm) injection sites as well as the IFA (1.0 cm) injection site. No notable erythema was seen at the MDCK or PBS injection site. Similar findings were observed in the other dogs tested in this manner.

Mixed leukocyte reactions were done to determine whether autologous dendritic cells (DC) could present antigen to sensitized lymphocytes collected either from peripheral blood or from popliteal lymph nodes draining the injection sites. This was an empirical procedure, where we tested different methods of assessment in order to determine effective sensitization. In dog G651 a single stimulation or primary mixed leukocyte reaction (MLR) was used. For this study, bone marrow cells were collected by aspiration of the head of the humerus, red blood cells were lysed, and the CD34+ cells labeled with the monoclonal antibody (mAb) (2E9). Next rat anti-mouse IgG1 magnetic beads were added and the labeled cells collected by magnetic column chromatography (Milteny Automacs). CD34 + cells were cultured for 7 days in medium containing GM-CSF, Flt3-L, and TNF $\alpha$  according to established procedures (2). The cultured cells were harvested and added to 96-well microtiter plates containing PBL or lymph node derived lymphocytes and prostate lysate (0.1 to 10  $\mu\text{g}/\text{ml}$ ) hemocyanin (0.1 to 1  $\mu\text{g}/\text{ml}$ ) MDCK (1  $\mu\text{g}/\text{ml}$ ) or canine vaccine (Parvo, rabies, and distemper at a 1:500 dilution of vaccine). Concanavalin A (Con A) (5  $\mu\text{g}/\text{ml}$ ) was added on day 3 and  $^3\text{H}$ -thymidine added on day 6. Plates were harvested and counted on day 7.

Shown in Figure 2 are the results of a MLR using both peripheral blood leukocytes (PBL) and lymph node derived lymphocytes as responder cells and autologous DC plus antigen as stimulator cells. Although there was no reaction to prostate lysate there was a strong response to hemocyanin and to a lesser extent the

canine vaccine by the PBL. We also observed a response to these antigens by the lymph node lymphocytes. These data indicated that the method of immunization was effective, however we did not detect at this stage of investigation an anti-prostate antigen immune response.

At this point we questioned whether a double stimulation in vitro of PBL or lymph node derived lymphocytes with autologous DC might show better responses to the prostate lysate. Independent studies showed there might be a predominate CD4 T-cell response in a primary MLR but a CD8 T cell response appeared to dominate a secondary MLR.

Therefore, for dog G768 and G149 we set up both a primary MLR as described above and additional secondary MLR in which lymphocytes were cultured 7 days in the presence of autologous DC plus antigen, harvested and incubated an additional 7 days with antigen and additional DC. Here the results for the primary MLR were essentially the same as shown in Figure 2. But as shown in Figure 3, we were able to observe an immune response against prostate lysate and canine prostate specific esterase, one of the major prostate antigens expected in the prostate lysate. We postulated that the number of prostate antigen-specific lymphocytes either in circulation or in the draining lymph node were few in number and sufficiently expanded only after a second in vitro stimulation detected by the MLR method.

A third dog, G149 was vaccinated using IFA and 10 days after the third injection evaluated in a primary and secondary MLR. Again, after the second stimulation, we observed an increased response in PBL stimulated with autologous DC pulsed with CPSE at 10 ug/ml relative to the cells stimulated with autologous DC alone (data not shown).

We then entered into the second phase of Aim 1 which was to vaccinate dogs with autologous DC pulsed in vitro with a mix of prostate lysate, CPSE and hemocyanin. For these studies, on 3 occasions, DC were cultured from female bone marrow CD34+ cells as described above. Two days before injection, DC were pulsed with antigen in vitro. On the day of injection, DC were harvested from culture, counted, and injected within 1-2 cm of the popliteal lymph node according to the injection schedule in Table 1. Due to the limited number of DC collected on the 3<sup>rd</sup> harvest for dog G703, we elected to collect a fourth bone marrow sample and culture DC for an additional immunization of DC pulsed with antigen.

Using this approach for in vivo sensitization, for dog G703 we failed to observe a significant response to prostate lysate, CPSE or hemocyanin after primary or secondary MLR, perhaps in part owing to the high background response of PBL cultured with DC alone. For dog G809, there was a modest (two-fold over background) response in PBL stimulated with DC pulsed with hemocyanin for the primary and secondary MLR. The second dog, G703 showed only a modest response against the viral vaccine in the primary MLR while any positive response to antigens was masked by an abnormally high background response of the PBL to autologous DC alone (data not shown). Overall, these results were less impressive than those obtained with the IFA vaccine method and resulted in our continuing evaluating two additional dogs using the vaccine technique for further validation.

Dogs H072 and H073 were injected with prostate lysate/hemocyanin in IFA as described above. Ten days after the third vaccination, PBL and popliteal lymph node

cells were collected and cultured in 96 well round bottom plates with cultured autologous DC pulsed with antigen. One week later, these cells were collected and stimulated again with fresh DC and antigen for a secondary MLR. Compared to DC stimulators alone, there were modest primary responses to prostate lysate and hemocyanin for dog H073 but not H072 (data not shown). In the secondary immune response, the lymph node cells from H072 responded modestly to prostate lysate and CPSE relative to cells stimulated by DC alone, but in the case of dog H073 no stimulation index was observed owing to the high background of the lymph node cells stimulated by DC alone (Figure 4).

These data, together with the studies outlined above, suggested that there was variability among the dogs tested as to their response to prostate antigens and hemocyanin determined either by a primary or secondary MLR. Such variability may require a large number (10 or more) of dogs to statistically delineate an anti-prostate immune response in chimeric dogs after injection of sensitized lymphocytes.

In addition to MLR as a read-out for anti-prostate immune responses, we collected sera from H072 and H073 and evaluated prostate lysate, CPSE and hemocyanin by sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) followed by a Western Blot using the dogs' immune sera. As shown in Figure 5, several bands were indicated for the prostate lysate (lane 2) in addition to strong bands for both CPSE (lane 3) and hemocyanin (lane 4). Antibody responses were also detectable against the antigens of the control virus vaccine the dogs received as pups (lane 5). These results indicated that prostate antigens are immunogenic in female dogs as demonstrated by IgG antibody responses. In addition, CPSE is a major antigen found in the prostate lysate. Future studies will include identifying the proteins of approximately 55 kDa and 62 kDa found in the prostate lysate that induced antibody responses.

## **Aim 2. Induction of prostatitis in female to male chimeric dogs.**

The goal of this Aim was to demonstrate that a prostate antigen-sensitized donor lymphocyte infusion (DLI) from a female marrow donor into a DLA-identical male chimeric recipient could lead to an anti-prostate immune response. For this aim, we transplanted four dogs each with marrow from a DLA-identical female littermate into male recipients after 2 Gy total body irradiation (TBI) followed by postgrafting immunosuppression with mycophenolate mofetil (MMF) and cyclosporin A (CSP) for 28 and 35 days respectively. All dogs engrafted with one remaining a stable chimera (H008) while the other dogs rejected their grafts between weeks 11 and 22 (Figure 6). Unfortunately, this was a very unexpected finding, as generally in other studies we see a high rate of engraftment using this regimen. For example in one study, we found only 1 of 8 dogs rejected two grafts when given 200 cGy TBI followed by hematopoietic cell transplantation and postgrafting immunosuppression of MMF and CSP (11). We now believe this problem was due to our replacing an aged 4 meV linear accelerator (Linac) with a newer 6 meV that is used for TBI conditioning. After a great deal of investigation, we determined the dosimetry to the animal to be off slightly due to an unrealized phenomenon of energy build-up associated with a higher energy output source. Additional studies have shown that the 200 cGy dose is apparently a barely acceptable minimal dose for nonmyeloablative conditioning and only a slight variation of this dose

may result in higher rejection rates. Thus only one dog, H008, was suitable for evaluating the anti-prostate effects of sensitized lymphocytes.

For these studies *dog G999, the DLA-identical female marrow donor for H008*, was injected 3 times with 5 mg/kg (50 mg total) of prostate lysate and 2.5 ug/kg (25 µg total) of hemocyanin within 3 cm of the popliteal lymph nodes for both hind limbs. Ten days after the last injection, the dog was injected with 100 ul each of PBS, 4 mg of prostate lysate, 200 ug of hemocyanin, and 600 µg of Madin-Darby Canine Kidney cell lysate (a negative control) at 4 different sites on the abdomen of the dog. (All test antigens were suspended/diluted in PBS.) After 24 and 48 hours the dog was examined for delayed-type hypersensitivity (DTH) reactions to these antigens. As shown in figure 7 there was a strong reaction to hemocyanin at both 24 and 48 hours whereas the response to prostate lysate was modest at best at the 24 hour time point relative to the negative controls PBS and MDCK cell lysate. These data suggest that the method of sensitization was successful however the immune response to prostate lysate was significantly less relative to that of hemocyanin.

In addition to DTH responses, we also measured antigen-specific cell proliferative responses in vitro by mixed leukocyte reactions of the female donor pre- and post-sensitization with prostate lysate and hemocyanin. For these studies, as described above, autologous bone marrow cells were collected, CD34+ cells purified by antibody and selection using magnetic beads. The results of the CD34+ cell purification method are shown in Figure 8. Next DC were generated from CD34+ cells cultured in medium containing GM-CSF, Flt3L, and TNF alpha according to established methods (2). DC were pulsed with antigen on day 5 and harvested on day 7, and added to autologous G999 peripheral blood mononuclear cells (PBMC). After 7 days of culture the stimulated PBMC were collected from culture and stimulated a second time with antigen pulsed DC's (each treatment group was set up in quadruplicate wells). Cells were pulsed with <sup>3</sup>H-Thy on day 6 and harvested on day 7. Figure 9 shows the in vitro proliferative reactions of PBMC harvested from dog G999 pre- and post- immunization with prostate lysate and hemocyanin following stimulation in vitro with antigen pulsed DC. Relative to the control DC which were not pulsed with antigen, there was no response to prostate lysate using PBMC prior to sensitization. However, post sensitized PBMC responded well to their autologous DC pulsed with prostate lysate after sensitization. The response to the hemocyanin antigen was also greater relative to the control post sensitization.

Similar to studies done under Aim 1, we evaluated the antibody response to prostate lysate, CPSE, hemocyanin, and the negative control MDCK-cell lysate by Western Blot analysis. As shown in Figure 10, a majority of the antigen contained in prostate lysate appears to be of the same molecular weight as CPSE. The Western Blot indicates a modest response to a CPSE as well as a stronger response to a protein of a molecular weight of approximately 148 kDa. Either of these proteins may be potential candidate antigens to be used in an antigen-specific manner to sensitize a female dog against the prostate.

The effects of adoptive transfer of sensitized donor PBMC from G999 to the DLA-identical chimeric recipient male H008 are first evaluated in Figure 6 bottom right panel. Hematopoietic chimerism was approximately 80% donor for lymphocytes and approximately 90 to 95% donor for granulocytes before DLI. At week 55, a DLI of  $8 \times 10^8$  leukapheresed blood cells plus an infusion of  $3.3 \times 10^7$  lymphocytes isolated from a



popliteal lymph node (obtained near the site of injection of sensitizing antigen) was given to the marrow recipient dog, H008. Blood cells were collected for 5 weeks after DLI and analyzed by VNTR-PCR for chimerism. As shown on the graph labeled H008 (Figure 6), chimerism did not change after DLI, suggesting either the PBMC and lymph node cells were not sufficiently activated to break tolerance against the remaining cells of the recipient hematopoietic system or the shared minor antigens associated with the prostate cell lysate were not also well expressed on cells of the recipient hematopoietic system. In addition, the dog was examined daily for over 90 days and there was no clinical sign of cutaneous GVHD. In a previous study in which minor antigen sensitized lymphocytes were transfused into a chimeric recipient, a shift from mixed to 100% donor chimerism was observed within a median of 14 days and GVHD was seen in less than 30 days (12).

We also examined serum CPSE levels by ELISA by obtaining blood samples from the recipient before DLI and on days 10 and 14 after DLI. An ELISA assay was run in which a plate was coated with 2 ug/ml of anti-CPSE mAb (IgG2a), washed and dilutions of H008 serum, pre and post DLI was added to the plate for 1 hr. After washing, a second anti-CPSE mAb (IgG1) that recognizes a different epitope on CPSE was added for 1 h. After a third wash, goat anti-dog IgG1-HRP was added and binding was determined by the addition of substrate (ABTS). A CPSE standard (obtained from seminal fluid from a breeder dog and used to generate the monoclonal antibodies) was diluted and used to determine absolute levels of CPSE in the serum. At days 10 and 14 there was no increase in CPSE levels in serum relative to the levels before DLI (data not shown).

Inducing benign prostate hyperplasia (BPH) after transfer of prostate antigen sensitized lymphocytes to the chimeric recipient was our primary objective in these studies. Evidence of BPH can be demonstrated by either diagnostic ultrasound or rectal palpation. We employed both of these techniques, performed with the aid of a veterinarian, to determine whether BPH was evident after a DLI of sensitized lymphocytes. As shown in Figure 12, there did not appear to be a significant change in prostate size up to 21 days after DLI. H008 had a small prostate and we found it difficult to obtain clear measurements. This was not the case in practice runs with other dogs in which we felt reasonably certain of the boundaries of the prostate obtained by ultrasound techniques. In addition, prostate size was assessed to be within normal limits by a veterinarian on day 21 and 47 by rectal palpation. Together, these data suggest there was no indication of BPH based on prostate enlargement.

## **Key Research Accomplishments**

1) We have demonstrated that of the two possible methods for inducing an immune response to prostate antigen in female dogs, soluble antigen in IFA gives more reproducible results than antigen-pulsed cultured autologous canine DC. Although DC have been used in other systems to induce immune responses in vivo (for review 9,10), at this time we have not hit upon an effective method of inducing adequate antigen uptake or maturation of canine DC to effectively present prostate antigens to naïve female T cells in vivo. Recent studies in our lab suggest canine B-cells may be superior antigen presenting cells based on our ability to culture orders of magnitude more B cells than DC and their ability to induce an allogeneic immune response in an MLR assay.

- 2). A two step or secondary MLR provided more reproducible stimulation as measured by <sup>3</sup>H-Thymidine uptake than did a primary MLR.
- 3). Lymph node cells obtained from the popliteal lymph node responded to the antigen mix and can be added to the donor lymphocyte infusion from the apheresed prostate antigen sensitized donor dogs.
- 4). We were able to detect antibody responses to prostate lysate, CPSE, and hemocyanin.
- 5). Several proteins were detected by Western blot in the prostate lysate, one of which was CPSE. Identification of the higher molecular weight proteins in the prostate lysate by mass spectrum analysis antigens may provide candidate antigens for future studies.
- 6). Our study of adoptive immunotherapy using prostate antigen sensitized female lymphocytes did not meet expectations. Despite showing an immune response against prostate antigens by a variety of methods before injection of the sensitized lymphocytes into the male recipient, no anti-prostate immune response was detected. This suggests that an additional step(s) is required; for example improving trafficking donor lymphocytes to the target tissue.

## **Reportable Outcomes**

We expect to report in a publication once additional dogs can be enrolled through other funding sources that canine prostate tissue lysate contains antigens that are immunogenic to female dogs when administered in Freund's incomplete adjuvant. One of these antigens appears to be CPSE. These antigens can be presented by autologous marrow derived DC and a response determined by a MLR. Due to the fact that only one dog had stable long-term engraftment and met the requirements for adoptive transfer of sensitized female donor PBMC, we are unable at this time to draw a conclusion as to the acceptance of our study's hypothesis. If the lack of response against the prostate by prostate antigen-sensitized lymphocytes is repeated in at least 2 additional dogs, we are left with the conclusion that either better sensitization of female lymphocytes is required or that method(s) have to be discovered that promote target-specific cytotoxicity.

## **Conclusions**

We conclude at this stage that a female dog can be sensitized against a mixture of prostate antigens. However, there is variability in this response that is presumably MHC class I or class II antigen dependent. Adoptive transfer of sensitized lymphocytes into a toleragenic environment of mixed hematopoietic chimerism did not result in an anti-prostate immune response. Despite strong evidence that we induced an immune response against prostate lysate antigens in the marrow donor, we nonetheless failed to see signs of GVHD, a shift in mixed to complete donor chimerism or an anti-prostate immune response in a male recipient after a DLI from the antigen sensitized DLA-identical marrow donor. This finding needs to be verified with two additional dogs in the near future under other funding sources.

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## Appendices

### Abbreviations

ABTS	2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]
CPSE	canine prostate specific esterase
CSP	cyclosporine A
DC	dendritic cells
DLA	dog leukocyte antigen
GvHD	graft versus host disease
Hem	hemocyanin
HCT	hematopoietic cell transplantation
IFA	incomplete Freund's adjuvant
kDa	kilodaltons
MHC	major histocompatibility complex
MDCK	Madin-Darby canine kidney
MLR	mixed lymphocyte reaction
MMF	mycophenolate mofetil
PBL	peripheral blood lymphocytes
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
SDS-PAGE	sodium dodecyl polyacrylamide gel electrophoresis
VNTR-PCR	variable number tandem repeat-polymerase chain reaction

## Supporting Data



Figure 1. Skin test of dog G768 48 hours after injection of antigen. Ten days after the third injection of prostate lysate in adjuvant, dogs received 100 ul injection of antigen intradermally. In the left hand photograph, the circled areas indicate injection sites for prostate lysate (left), hemocyanin (middle), IFA (right). In the right hand photograph, the circled areas indicate injection sites for MDCK lysate (left) and PBS (right). Only mild erythema was noted for prostate lysate, hemocyanin, and IFA .

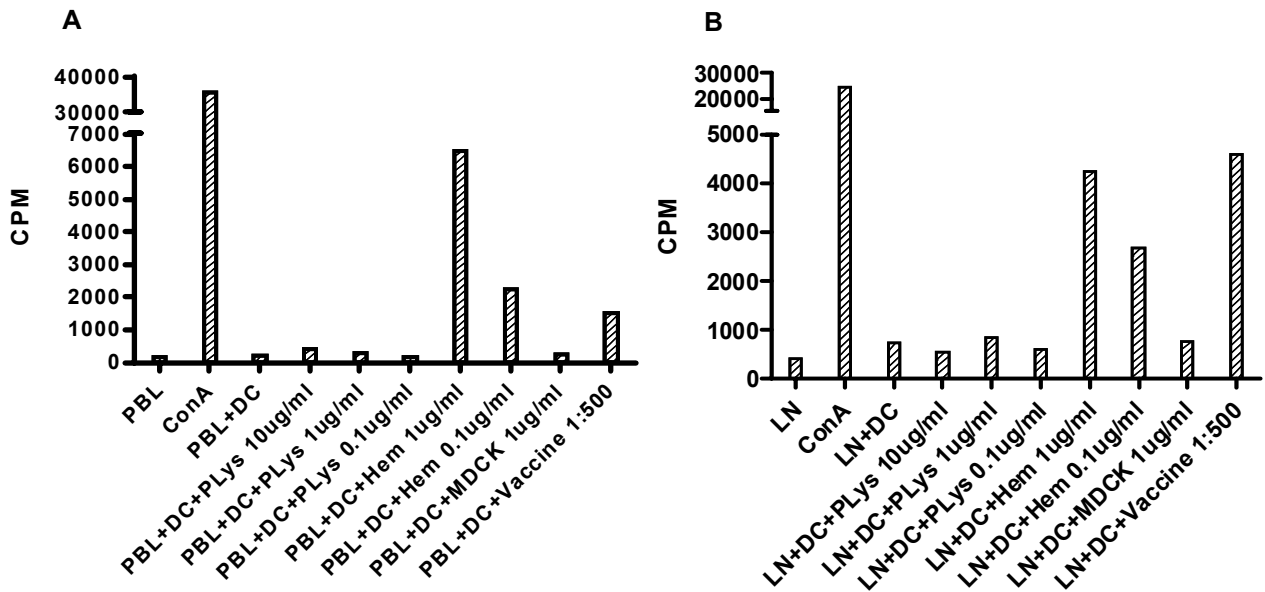


Figure 2. Mixed lymphocyte reaction of antigen pulsed autologous dendritic cells (DC) and PBL (A) or lymph node lymphocytes (LN) (B) for dog G651. Cells were pulsed with antigen prostate lysate (PLys), hemocyanin (Hem), Madin-Darby canine kidney cells (MDCK), vaccine (Parvo virus, Coronavirus at 1:500 dilution) at the concentrations indicated for 7 days. Concanavalin A (ConA) was added on day 3 at 5 ug /ml as a positive control for the reaction.

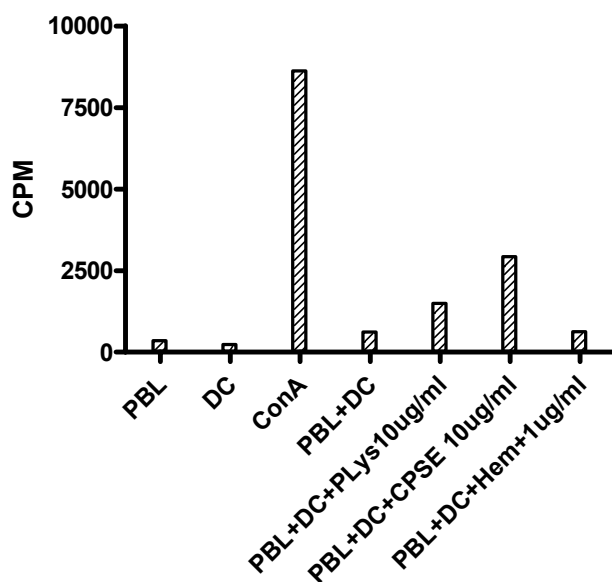


Figure 3. Secondary MLR of dog PBL cultured with autologous DC and pulsed with antigen. PBL from dog G768 were collected 10 days after 3 subcutaneous injections of prostate lysate, CPSE, and hemocyanin in Freund's incomplete adjuvant. PBL were cultured with prostate lysate (PLys), canine prostate specific esterase (CPSE), hemocyanin (Hem) or dendritic cells (DC) alone for 7 days. The cells were collected and cultured for an additional 7 days with DC and the antigens indicated, medium alone or concanavalin A. On day 6 <sup>3</sup>H-Thymidine was added and the cells harvested on day 7. Shown are mean counts per minute (CPM) of triplicate samples.

Table 1. Number of Prostate Lysate/CPSE/hemocyanin Pulsed Dendritic Cells Injected ( $\times 10^7$ )

Injection	Dogs	
	G703	G809
1st	3.5	1.9
2nd	0.19	2.2
3rd	0.025	0.5
4th	1.9	

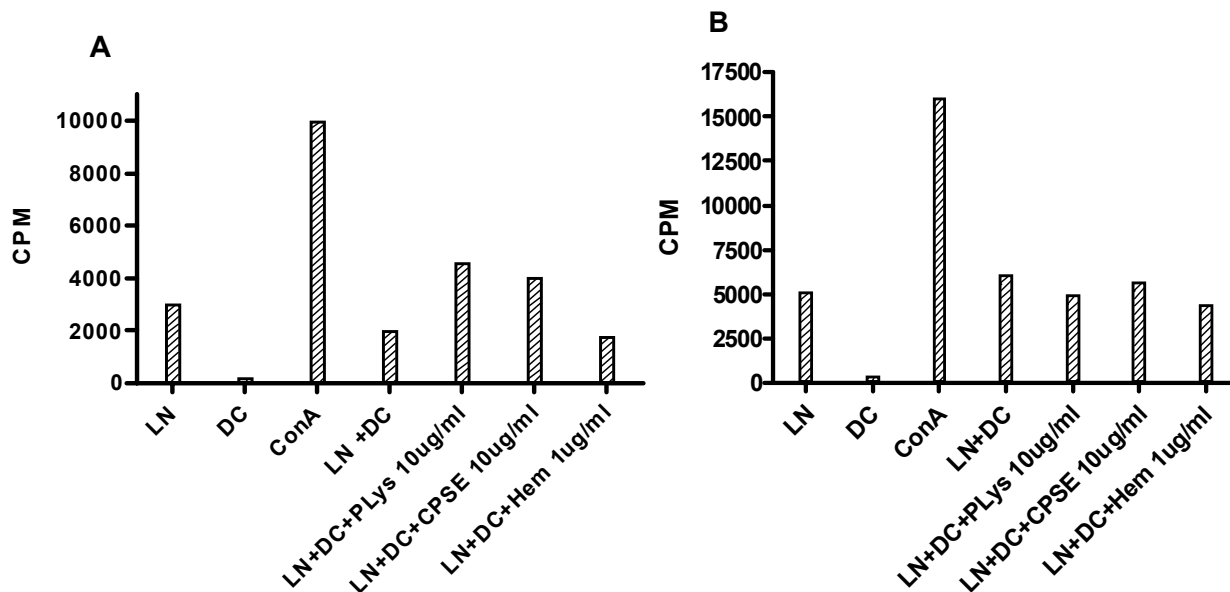


Figure 4. Secondary MLR of lymph node lymphocytes stimulated with autologous DC and antigen. Ten days after a third injection of prostate lysate/hemocyanin antigen, popliteal lymph nodes were collected from two dogs, H072 (A) and H073 (B). The nodes were macerated in PBS, washed and resuspended in culture medium + 10% pooled canine serum and incubated for 7 days in the presence of autologous DC with or without prostate lysate, hemocyanin (Hem), CPSE. Cells were harvested and incubated again in the same manner and pulsed with Con A on day 3 and  $^3\text{H}$ -Thymidine on day 6. Proliferation measured by CPM on Y-axis.

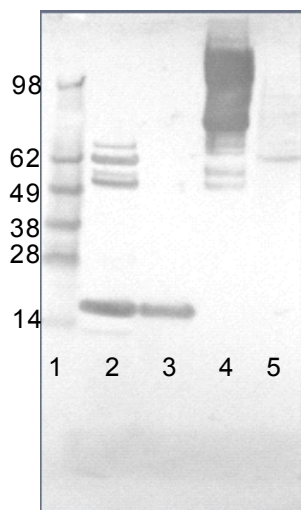


Figure 5. Antibody responses to prostate lysate, CPSE, and hemocyanin in vaccinated dogs. Dogs were vaccinated with prostate lysate/hemocyanin in IFA by 3 injections. Ten days after the third injection, blood was collected for serum. A SDS-PAGE was run with molecular weight markers indicated in kDa (lane 1), prostate lysate (lane 2), CPSE (lane 3), hemocyanin (lane 4), and canine virus vaccine (lane 5). A Western Blot was made of the gel using serum from dog H073 and developed with mouse anti-dog IgG-horse radish peroxidase.

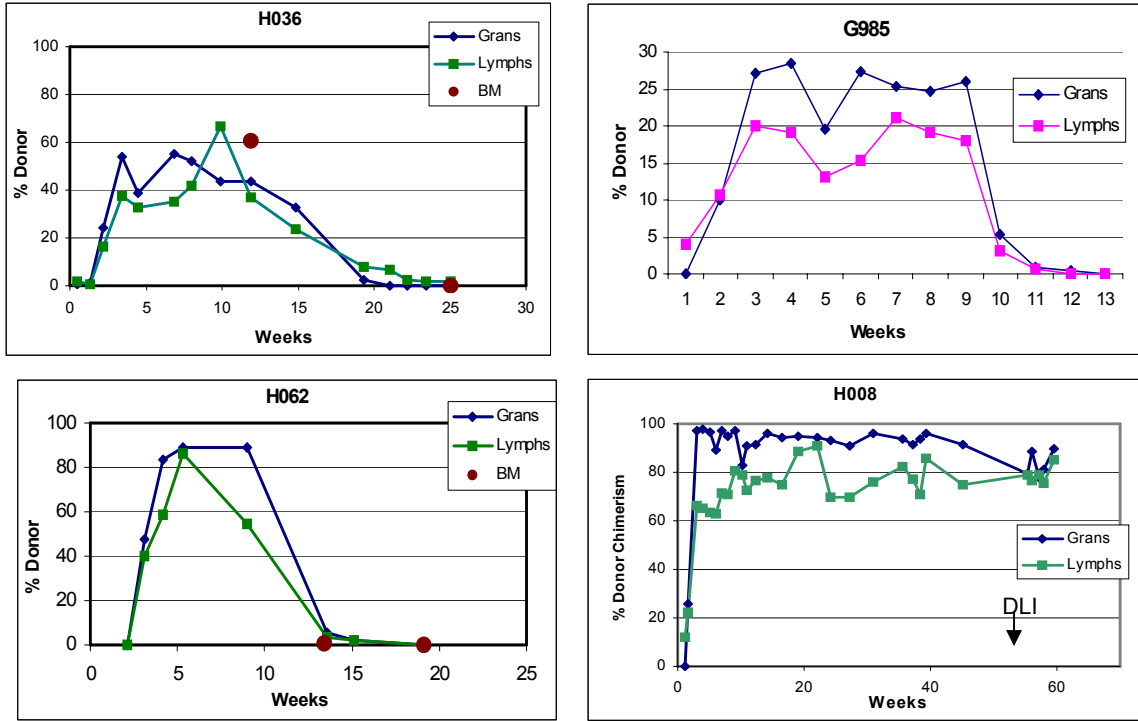


Figure 6. Female to male marrow transplantation for mixed hematopoietic chimerism. Dogs H036, G985, H062 and H008 were given 2 Gy TBI followed by injection of a median of  $3.6$  (range of  $2.8$  to  $3.7$ )  $\times 10^8$  marrow cells/kg on day 0. Cyclosporin A was given on days -1 through 35 and mycophenolate mofetil given on days 0-28. Chimerism was determined by variable number tandem repeat-polymerase chain reaction (VNTR-PCR) analysis (7,8). For chimeric dog H008, a prostate lysate donor lymphocyte infusion was given at week 55 and chimerism followed for an additional 5 weeks.

G999: 24 hour DTH after Antigen Injection SC

G999: 48 hour DTH after Antigen Injection SC

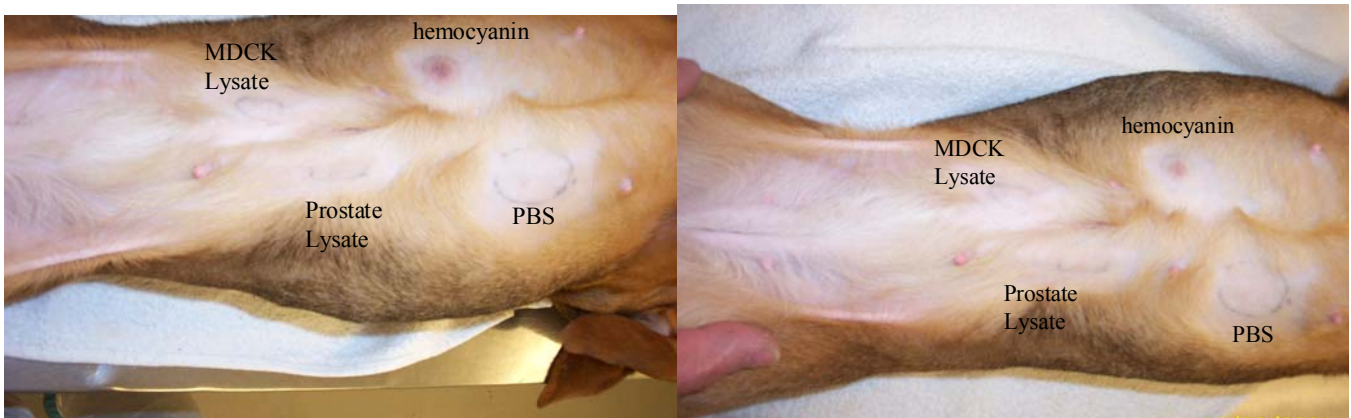


Figure 7. DTH response to PBS, prostate lysate, hemocyanin, MDCK cell lysate in a prostate lysate and hemocyanin-sensitized dog 24 and 48 hours after injection of antigen.



G999: CD34+ Marrow Cells after Miltenyi Bead Purification

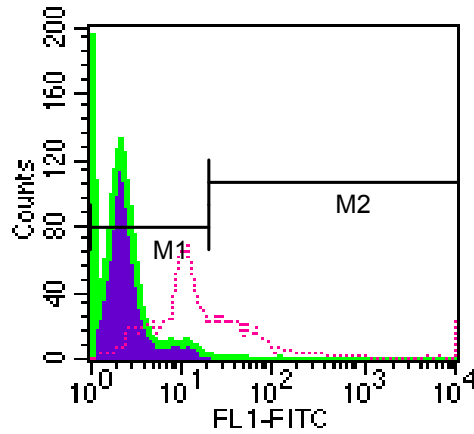


Figure 8. Evaluation of CD34+ bone marrow cells after sorting with magnetic beads. Marrow from dog G999 was treated with ammonium chloride to lyse RBC's, washed and incubated with mouse anti-canine CD34 antibody (1 h). After 3 washes in PBS, the cells were resuspended in Miltenyi buffer (PBS-EDTA with 0.2% bovine serum albumin) and magnetic beads coated with rat anti-mouse IgG1 antibody (Miltenyi Corporation). An aliquot of positive-selected cells and untreated cells were stained with FITC conjugated anti-CD34 antibody or isotype control IgG1-FITC and evaluated by Flow cytometry (Bectin-Dickenson). The dotted red line indicates CD34+ cells compared to the unstained and isotype control samples (green and purple, respectively).

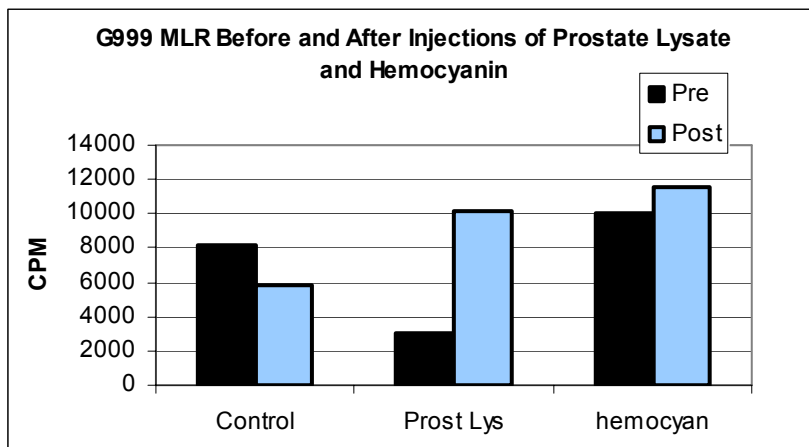


Figure 9. Mixed leukocyte reaction of G999 PBMC, pre- and post-sensitization, against autologous DC pulsed with prostate lysate and hemocyanin.

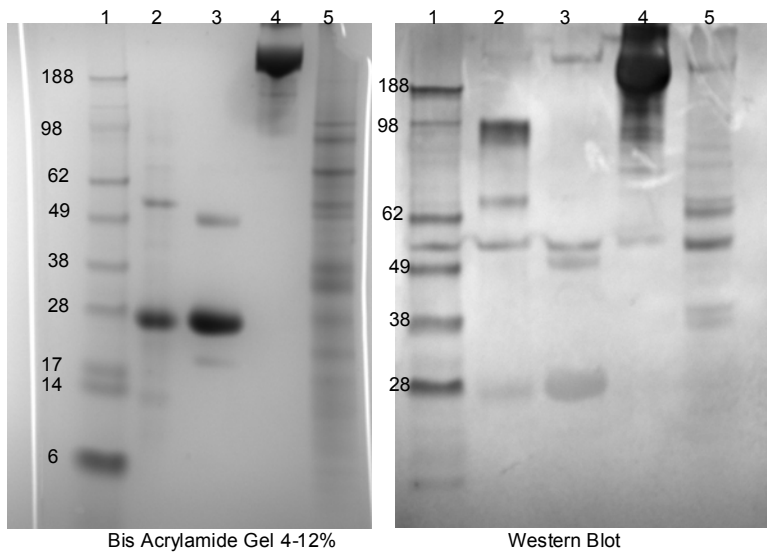


Figure 10 Acrylamide gel and corresponding Western Blot for immune response against prostate lysate, CPSE, hemocyanin and MDCK-cell lysate. Prostate lysate (4 ug, lane 2) CPSE (4 ug, lane 3), hemocyanin (4 ug, lane 4) and MDCK-cell lysate (5 ug, lane 5), and MW standards (See Blue Standard, Invitrogen) were run on a 4-12% Bis-acrylamide gel (Invitrogen). The proteins were transferred to a nitrocellulose membrane and dog G999 serum, 10 day post final sensitization (1:500 dilution) was added and developed using standard Western Blot methods.

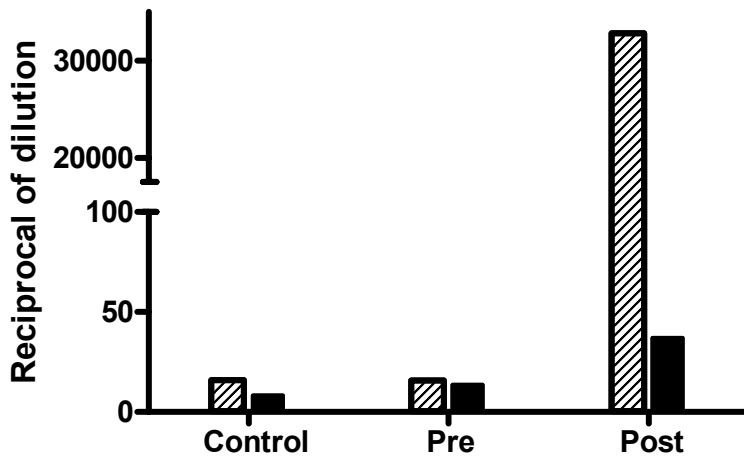


Figure 11. Sensitized female donor dog G999 serum levels of anti-CPSE and anti-hemocyanin antibodies pre- and post-immunization with 3 injections of prostate lysate and hemocyanin. Control serum was made from a pool of 5 unvaccinated dogs. Serum from G999 was collected pre-and post-immunization with 3 injections of prostate lysate and hemocyanin. ELISA plates were coated with either CPSE (solid bars) or hemocyanin (cross hatched bars) at 2 ug/ml overnight and washed. Serum was diluted in PBS-Tween 20 + 5% chicken serum and allowed to react with the antigen for 1 hr at room temperature. After washing, bound canine anti-CPSE/hemocyanin antibodies were detected with goat anti-dog IgG-HRP conjugate antibody (1 hr, room temp.). Substrate (ABTS) was then added after washing unbound conjugate and the results read spectrophotometrically.

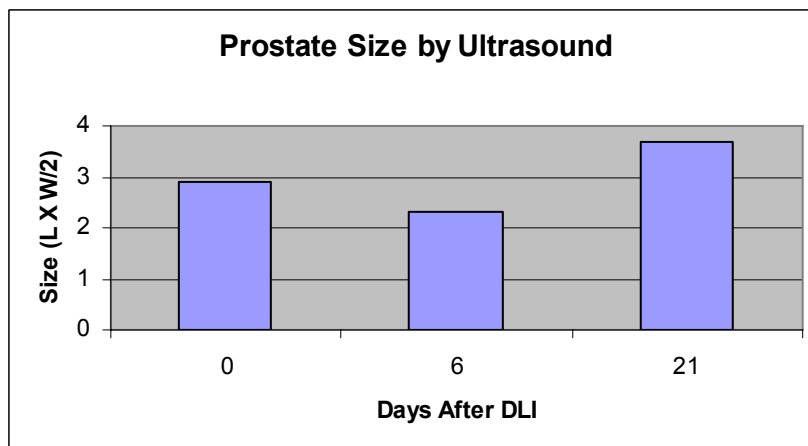


Figure 12. Prostate Size After DLI. Prostate size was determined by diagnostic ultrasound where length and width of the prostate was recorded on day 0, 6, and 21. The formula: length x width/2 was used to create the values shown.