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An allogeneic hybrid-cell fusion vaccine against canine mammary cancer

R. Curtis Bird [a,*], Patricia DeInnocentes [a], Steven Lenz [a,1], Erin E. Thacker [c], David T. Curiel [c,d], Bruce F. Smith [a,b]

[a] *Department of Pathobiology, College of Veterinary Medicine, Auburn University, Auburn, AL 36849-5519, United States*
[b] *Scott-Ritchey Research Center, College of Veterinary Medicine, Auburn University, Auburn, AL 36849, United States*
[c] *Division of Human Gene Therapy, Department of Medicine, and the Gene Therapy Center, The University of Alabama at Birmingham, Birmingham, AL 35294, United States*
[d] *Department of Obstetrics and Gynecology, Pathology and Surgery, The University of Alabama at Birmingham, Birmingham, AL 35294, United States*

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Abstract

Mammary cancer is among the most prevalent of canine tumors frequently resulting in death due to metastatic disease. Most tumors fail to raise an effective immune reaction making improving immune recognition a priority. Hybrid-cell fusion strategies have been employed to load dendritic cell populations with tumor cell antigens to stimulate immune recognition; however, recovery, heterogeneity and quality of primary cells from patients present enormous challenges. We employed allogeneic cell lines to develop an improved hybrid-cell fusion strategy and evaluated immune reactions in normal laboratory beagles. Such a strategy relies on enhanced immune recognition of allogeneic tumor cell antigens by antigen presenting cells. Optimized PEG-promoted fusions between uniquely stained canine mammary tumor CMT12 or CMT28 cells and a dendritic cell-like DH82 cell fusion partner resulted in greater than 40% hybrid-cell fusion populations by flow cytometry and fluorescence microscopy. Hybrid-cell fusions were delivered by direct ultrasound guided injection into popliteal lymph nodes of laboratory beagles. Only hybrid-cell fusions provided statistically significant enhancement of cell-mediated immunity (^{51}Cr-release assay) compared to innate reactions in naïve vehicle injected dogs while dogs vaccinated with either single cell component alone did not. Vaccination with hybrid-cell fusions enhanced IFN-γ expression in sorted CD8+ and CD4+ cells but not in CD4−/CD8− cells consistent with a CTL response. Cell-mediated immune assays revealed strong reactions against matched (vaccine component) CMT cells and unmatched CMT cells indicative of an immune response to mammary cancer antigens common to both cell lines. These results provide proof of principle for development of an allogeneic vaccination strategy against canine mammary cancer.

Keywords: Hybrid-cell fusion; Vaccine; Canine mammary cancer; Dendritic cells; Allogeneic

1. Introduction

Mammary cancer is the most common malignancy occurring in unspayed female dogs comprising approximately 52% of all neoplasms (MacEwen and Withrow, 1996). A majority of canine mammary tumors have poor clinical outcomes when metastases occur and

* Corresponding author. Tel.: +1 334 844 2707; fax: +1 334 844 2652.
E-mail address: birdric@vetmed.auburn.edu (R.C. Bird).
[1] Present address: Department of Pathology, Purdue University, West Lafayette, IN 47907, USA.

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are hormone dependent as ovariohysterectomy prior to first estrus results in a risk of 0.05% compared to a risk of 25% if ovariohysterectomy is performed after the fourth estrus cycle (Macewen, 1990; Wolfe et al., 1986; Sartin et al., 1993). Dogs have no known retroviruses and canine mammary cancer does not appear to involve a viral etiology. Dogs with poorly differentiated tumors have increased risk of recurrent or metastatic disease within 2 years following mastectomy with a 90% recurrence rate for the most dedifferentiated tumors. Importantly, dogs with a lymphoid response at the tumor site have about a 45% recurrence rate or develop metastatic disease within 2 years compared to an 83% recurrence rate in dogs with no lymphoid response (Sartin et al., 1993; Macewen, 1990; Rutteman and Misdorp, 1993; MacEwen and Withrow, 1996). Thus, canine mammary cancer presents an important neoplastic disease of dogs and a promising candidate for development of strategies for immune enhancement with the potential to improve disease management.

Because cancer cells can be notoriously variable in susceptibility to treatment and become widely dispersed in metastatic disease, enhancement of natural immunity would be a particularly effective strategy to effect remission. However, the well-documented ability of developing neoplasms to evade immune surveillance has posed a significant barrier. To date, efforts to overcome these barriers in the treatment of malignant melanoma, breast and prostate cancer in humans have shown promise but produced mixed results (Timmerman and Levy, 1998; Schneeberger et al., 2000; Heiser et al., 2001; Avigan et al., 2004; Dees et al., 2004; Svane et al., 2004; Homma et al., 2006). Current enthusiasm for such strategies is based on cellular vaccines in which remission rates are approximately 30% or less. Forced antigen presentation through cell fusion, antigen or mRNA loading, or recombinant modification through transfection of genes encoding antigens, cytokines, co-stimulatory molecules and other immunomodulatory elements have been evaluated.

Such strategies are based on an abundance of data suggesting that tumors express specific antigens recognized by the immune system (Timmerman and Levy, 1998; Nair, 1998) and can cause natural remission (Souberbielle et al., 1998; Avigan et al., 2004; Dees et al., 2004). A therapeutic strategy that could enhance effectiveness and reliability of immune recognition would be of immense value in the treatment of neoplastic disease particularly in metastatic disease. However, naturally occurring immune recognition of tumor cells is, at best, unreliable and, at worst, ineffective in eliminating tumor cells. Escape from immune recognition by tumors is common and many examples of reduction or absence of expression of MHC class I and class II molecules have been observed suggesting that antigen presentation may frequently be defective (Murgia et al., 2006). Defects in antigen presentation and processing appear common in tumor evasion of immune recognition (Dolan et al., 2006).

One approach designed to address defects in immune recognition is based on creation of hybrid-cell vaccines through fusion of antigen presenting cells (APCs) with tumor cells (Gong et al., 2000; Scott-Taylor et al., 2000; Akasaki et al., 2001; Avigan et al., 2004; Homma et al., 2006; Yasuda et al., 2007). Such hybrid-cell fusions express both tumor-specific antigens and the necessary machinery needed for antigen presentation but have been thought to require MHC matching for T cell activation. Among APCs, dendritic cells (DCs) are considered the most effective in antigen presentation and because DCs transport presented antigens to activate effector T cells in lymph nodes they have been suggested as promising candidates for the APC component of such hybrid-cell vaccines (Grabbe et al., 1995; Scott-Taylor et al., 2000; Avigan et al., 2004; Homma et al., 2006; Yasuda et al., 2007). The challenge is that isolation and culture of autologous DC populations from older animal patients or those undergoing chemotherapy or who are immune-suppressed can be difficult and variable. There are also technical difficulties in providing sufficient cells from autologous cancers from patient animals. Successful optimization of fusion for such primary cells for each patient animal can also be time-consuming.

Allogeneic cell lines have been considered ineffective for such strategies because unmatched MHC and lack of appropriate co-stimulatory molecules may preclude appropriate antigen presentation and lymphocyte activation. By taking advantage of cross-presentation of antigens between APCs, an allogeneic hybrid-cell fusion could avoid the presumption that MHC matching is required for effective antigen presentation. Recent evidence suggests that if antigen is presented on unmatched APCs, cross-presentation can allow antigens to be passed on to autologous APCs effectively priming the immune response (Kircheis et al., 2000; Motta et al., 2001; Nelson et al., 2000, 2001; Harshyne et al., 2001; Yasuda et al., 2007) even when membranes separate allogeneic from autologous cells (Chhabra et al., 2004). Allogeneic cancer cells used to immunize mice and humans have been shown to induce measurable cross-presentation providing a mechanism explaining how antigens presented on unmatched APCs become effective immune stimulators (Kircheis et al., 2000; Chhabra et al., 2004; Yasuda et al., 2007).

Canine mammary tumor (CMT) cell lines, derived from cases of malignant spontaneous canine mammary cancer, have been developed and characterized as stable lines of cells with well characterized morphology and genetic changes (Wolfe et al., 1986; Sartin et al., 1993; Ahern et al., 1995; Wang et al., 1995; Migone et al., 2006; DeInnocentes et al., 2006). The purpose of these investigations was to utilize these allogeneic CMT cell lines to develop hybrid-cell fusion strategies to improve immune responses to canine mammary cancer cells by fusion to DC-like DH82 cells to promote an improved immune response.

2. Materials and methods

2.1. Cell culture

CMT12 and CMT28 canine mammary tumor-derived cells were grown under standard conditions in Leibovitz's L-15-medium as previously described (DeInnocentes et al., 2006, Migone et al., 2006). DH82 cells were grown under standard conditions in RPMI-1640 media as previously described (Wellman et al., 1988). In preparation for hybrid-cell fusions, all cells were grown to log phase in 75 cm^2 flasks (<70% confluency), collected by trypsinization, pipetted to a single cell suspension, centrifuged and resuspended in 5 ml Improved MEM (Gibco). Cells were washed 3 times with 1× clear Hanks buffered saline (HBS, Gibco/BRL), centrifuged, resuspended in 10 ml Improved MEM and counted in a hemocytometer.

2.2. rt-PCR analysis of canine CD40, CD209, CD205, L37 and IFN-γ expression

Total RNA was extracted from cells with RNA Stat 60 (Tel-Test, Inc). RNA pellets were resuspended in diethylpyrocarbonate-treated water, concentration determined by absorbance at 260 nm and stored at −80 °C. Specific primers (Omega Biotek) were designed to amplify cDNAs from conserved canine coding regions using Vector NTI software (Informax). Primer pairs for canine CD40 (sense 5′-GTTCTCCTGCCTCTGCGCTGTCTCTT-3′ and antisense 5′-CCAGTTTCTCTCCTGGTGGGCACATA-3′, Genbank AY333789) were amplified at 48 °C for 45 min and 94 °C for 2 min followed by 45 cycles of 94 °C for 1 min, 65 °C for 1 min and 72 °C for 1 min. rt-PCR products were fractionated and analyzed on TBE-buffered agarose gels as previously described (Bird and DeInnocentes, 2004; Migone et al., 2006). Amplicons were cloned, sequenced and compared with sequences in Genbank to ensure identity (DeInnocentes et al., 2006). rt-PCR of canine CD205 (sense 5′-GAAGTGGGTGTCCCAGCATCGGCTCTTTCATTTGCA-3′, antisense 5′-GGTCTGACCATTCCCAGCCTCTAGCA-3′, based on human CD205 Genbank AF011333), canine CD209/DC-SIGN (sense 5′-ATGTGTGACCCCAAGGAG-3′, antisense 5′-GTGGGGTCTGGAGAGCAATG-3′, Genbank XM_542118) and canine ribosomal protein L37 (sense 5′-AAGGGGACGTCATCGTTCGG-3′, antisense 5′-AGGTGCCTCATTCGACCGGT-3′, Genbank XM_844999) were conducted as described above except only 40 cycles were applied at an annealing temperature of 55 °C for CD205 and 62 °C for CD209 and L37. Canine interferon-γ (cIFN-γ) primers (sense 5′-GCAAGTAATCCAGATGTATCG-3′, antisense 5′-TTATCGCCTTGCGCTGGACC-3′, Kaim et al., 2006) were synthesized to amplify cIFN-γ mRNA from peripheral blood mononuclear cells (PBMCs) or ConA-treated PBMC populations (5 μg/ml for 48 h) using the protocol: 48 °C 45 min and 94 °C 2 min followed by 42 cycles of 94 °C 1 min, 56 °C 1 min and 72 °C 1 min.

2.3. Hybrid-cell fusion and vaccine preparation

Vital dyes were employed to differentially stain cell populations. CellTracker Orange CMTMR (5-(and-6)-(((chloromethyl)benzoyl)amino)-tetramethylrhodamine, Molecular Probes—made fresh according to manufacturers direction) was used to stain DH82 cells using a 1:2 dilution of CellTracker Orange CMTMR stock to sterile clear 1× Hanks and then 1 μl of the diluted dye added per 1 × 10^6 cells for 30 min at 37 °C. Cell suspensions were pelleted by centrifugation and resuspended in Improved MEM and incubated for 30 min followed by centrifugation and washing 3 times with Hanks buffered saline. CellTracker Green CMFDA (5-chloromethylfluorescein diacetate, Molecular Probes – made fresh according to manufacturers direction) was used to stain CMT12 and CMT28 cells using a 1:100 dilution of CellTracker Green CMFDA stock to sterile clear 1× Hanks and then 1 μl of diluted dye per 1 × 10^6 cells 15 min at 37 °C. The cell suspension was pelleted by centrifugation, resuspended in Improved MEM and incubated for 15 min followed by centrifugation. The pellet was washed 3 times with Hanks and 5 × 10^4 cells retained for flow cytometry and microscopy.

Sterile solutions of 50% (w/v) PEG (∼3350 MW) in Improved MEM were warmed to 37 °C. Parallel fusions of stained and unstained cells provided stained cells to be analyzed by flow cytometry and unstained cells to be injected into dogs. Cell lines to be fused (optimally 1.5 × 10^7 cells/injection of CMT12/DH82 or CMT28/

DH82 fusion pairs) were combined in a 50 ml tube and collected by centrifugation ($400 \times g$, 7 min). The mixed cell pellet was drained, warmed to 37 °C and fused during the next 8–10 min by addition of 1 ml PEG solution (37 °C) added dropwise over ~90 s to the pellet while tapping the tube lightly to resuspend the cells followed by incubation at 37 °C for 90 s to initiate fusion. An additional one volume of Improved MEM (37 °C) was added to a total of 5 ml (~1 ml/min) with gentle tapping. The volume was diluted to 19 ml total with Improved MEM (37 °C). The cell suspension was collected by centrifugation ($300 \times g$ for 7 min), supernatant discarded and the pellet resuspended in 20 ml complete growth media. The cells were collected by centrifugation and resuspended in 0.3 ml sterile PBS for vaccine preparation. All vaccine injections were diluted with 0.2 ml injection-grade pyrogen-free PBS containing 200 μg/injection CpG-containing phosphorothioate oligonucleotide immunostimulant (oligonucleotide #2007 5′-TCGTCGTTGTCGTTTTGTCGTT-3′, Wernette et al., 2002) to complete each 0.5 ml vaccine dose.

2.4. Flow cytometry and high-speed cell sorting

Flow cytometry was used to analyze stained CMT, DH82 and parallel CMT/DH82 hybrid-cell fusion cell populations prepared alongside those unstained populations used to vaccinate animals on a MoFlo flow cytometer in the green (530 ± 20 nm) and orange (580 ± 15 nm) channels to determine percentages of populations that were either single or dual-stained following fusion.

DH82 cells were separately labeled with monoclonal antibodies recognizing cCD11c (monoclonal mouse anti-canine CD11c, Serotec) or cMHC II (monoclonal mouse anti-canine MHC II, Serotec). DH82 cell suspensions (1×10^6 cells) were recovered by trypsin digestion, resuspended in 1 ml growth medium and incubated (37 °C) 1 h to allow surface antigen regeneration. Cells were brought to room temperature and recovered by centrifugation ($1300 \times g$, 2 min), supernatant removed and cells resuspended in 1 ml flow wash buffer (FWB, HBS/10% fetal bovine serum). The wash was repeated and cells were resuspended in 100 μl FWB. Antibodies recognizing α-cCD11c (10 μl) were added, mixed, and incubated 60 min. Then 2 ml HBS was added and the cells centrifuged ($1300 \times g$, 2 min). The cells were washed twice in HBS and resuspended in 0.1 ml FWB. Secondary antibody was added for cCD11c (2 μl polyclonal goat anti-mouse IgG-Alexa Fluor 660, Molecular Probes), incubated (dark 45 min) and washed as described above. Labeling of α-cMHC II primary antibodies was accomplished using a Zenon labeling reagent (Invitrogen/Molecular Probes, PE/Alexa Fluor 610) and incubating the reaction for >5 min as directed. Labeled α-cMHC II antibodies were added to the cell suspension, incubated and washed as described above. Cell suspensions were filtered (Nylon Mesh 53 μm, Small Parts, Inc.) in a sterile apparatus (assembled from two 1000 μl micropipette tips where the upper tip had been shortened ~1 cm with a layer of filter material between). Flow cytometry was performed on a MoFlo flow cytometer and dot-plots integrating CD11c and MHC II expression profiles were analyzed using Summit 4.3 software (Dako). CMT cell lines were also separately labeled for canine MHC I and II antigen expression with primary antibodies and then labeled with secondary FITC-conjugated secondary antibodies as described above followed by analysis by flow cytometry.

Populations of PBMCs from vaccinated dogs were labeled with directly conjugated monoclonal antibodies against CD4 (FITC-conjugated) and CD8 (RPE-conjugated, Serotec) as described above and sorted into CD4+/CD8−, CD4−/CD8+, CD4−/CD8− and subcellular cell fractions on a MoFlo flow cytometer and high-speed cell sorter (DAKO).

2.5. Animal handling, vaccine injection and sampling

All experiments were conducted under the oversight of the Auburn University IACUC committee in AAALAC approved animal and clinical care facilities. Healthy adult, retired, reproductively intact, unspayed female beagles (6–8 years of age) were held for 2–3 weeks to allow acclimation to kennel facilities. Animals were randomly divided into groups (3 dogs/group: group 1, vaccinated with CMT12/DH82 cell fusions; group 2, vaccinated with CMT28/DH82 hybrid-cell fusions; group 3, vehicle injected control) and the entire experiment was performed twice. Vaccines of 1.5×10^7 cells/injection (5×10^6 DH82 cells fused to 1×10^7 CMT cells at a ratio of 1:2 in 0.5 ml PBS containing CpG oligonucleotide immunostimulant as described above) were prepared for each injection. Hybrid-cell fusions cannot be injected through needles of less than 18-gauge or measurable shearing of the fused cells can be detected by flow cytometry (data not shown). This necessitated mild sedation and local anesthesia at the site of injection. Dogs were sedated with 2.5 mg diazepam (0.19 mg/kg), 2.5 mg butorphanol tartrate (0.19 mg/kg) and 1.0 mg acepromazine

maleate (0.075 mg/kg) injected into the cephalic vein. Local anesthesia was then administered (0.1 ml bupivacaine 5 mg/ml) injected intradermally into tissue overlying the lymph node. Vaccine injections into popliteal lymph nodes were guided by ultrasound to ensure correct injection location at weeks 1, 4 and 14. Blood was collected prior to vaccination and weekly after vaccination from each animal by venipuncture. Each dog was examined daily for physical signs of tumor growth at the injection site or signs of physical distress with regular examinations for acute cytotoxicity and long term effects.

Likelihood of rare events such as tumor growth from vaccine cells was also assessed. At the end of the experiment pathological analysis of each animal was performed by a board certified veterinary pathologist, blinded to experimental groups, to determine if tumor growth had occurred at the primary site of injection or other locations. Tissue specimens from injected and uninjected popliteal lymph nodes, iliac lymph nodes, lung, perinodal adipose tissue, liver, kidney, spleen, intestine, stomach, colon, pancreas, urinary bladder, uterus, ovaries, heart, thymus, cerebrum, cerebellum, brainstem, adrenal glands, thyroid glands, and bone marrow were collected into 10% buffered-neutral formalin, fixed for 24–48 h, processed, sectioned and stained for histopathologic evaluation.

2.6. Determination of MHC I differences in cell lines by DLA88 allele sequencing

Canine MHC I DLA88 sequences were analyzed and differences in alleles determined by PCR and DNA sequencing followed by Blastn analysis against canine DLA88 sequences in Genbank (Altschul et al., 1990) and comparison with MHC I alleles in the MHC sequence database (http://www.ebi.ac.uk/ipd/mhc/dla/align.html, Robinson et al., 2005). Specific primers (Omega Biotek) designed to prime synthesis of DNA from conserved regions of canine MHC class I exon 2 (DLA88 Class I exon 2, Genbank AF218303, forward 5′-ATTGGCGGCCTGTCGGG, reverse 5′-AGGCGAGATCGGGAGGC, Wagner et al., 2000) were used in amplifications as described above using the protocol: 96 °C for 3 min followed by 33 cycles of 96 °C for 1 min, 58 °C for 1 min and 72 °C for 1.25 min including Platinum Taq (Gibco) and 1 mM $MgSO_4$. PCR products were fractionated and analyzed on TBE-buffered agarose gels as described above and amplicons were cloned, sequenced, analyzed and compared with sequences from Genbank to determine if different alleles were evident (DeInnocentes et al., 2006).

2.7. ELISA

Chemiluminescent ELISA assays were developed to evaluate antibody-based immunity against CMT cells. Assays were constructed to quantify canine IgGs bound to CMT cells in an indirect sandwich ELISA (Coligan et al., 1991). CMT12 or CMT28 (5×10^4 cells/well) were attached to 96 well plates as described (DeInnocentes et al., 2006). Plates were centrifuged 10 min ($200 \times g$), media removed and wells washed 3 times with 100 μl PBS. The plate was drained and the cells fixed with 100 μl 0.025% glutaraldehyde (30 min). Plates were washed with PBS and each well was blocked with 200 μl of 10% equine serum (Hyclone) in PBS for 1 h at room temperature and the cells were washed as described above. Primary antibody or serum from vaccinated or control dogs was incubated 1.5 h at 4 °C with shaking every 15 min. The plate was centrifuged, supernatants removed and plates were washed again. Secondary antibody (100 μl of 1:5000 diluted anti-canine IgG conjugated to alkaline phosphatase) was added and incubated 1 h (4 °C) followed by washing as described above. Alkaline phosphatase bound to each well was quantitatively assessed with a chemiluminescence assay kit (Roche) according to direction (100 μl/well), and including one drop levamisole solution per 5 ml alkaline phosphatase substrate buffer (Vector Lab), by incubation (room temperature 10 min) and chemiluminescence determined (in duplicate). Sera from naïve uninjected control dogs and vehicle control dogs served as negative controls. Positive control antibodies (4H6 and 1A10 in 1% FBS/PBS) known to bind CMT cells (Wang et al., 1995) were also included as were primary antibody and secondary antibody alone to control for endogenous alkaline phosphatase activity and adventitious antibody binding.

2.8. Cell-mediated immune assays

Assays of cell-mediated immune (CMI) response were evaluated against CMT cells used to vaccinate and then repeated against the other unmatched CMT cell line as each CMT cell line is unique and derived from an independent mammary cancer. Fresh PBMCs were isolated by centrifugation on Ficoll–Hypaque (Histopaque 1077, Sigma Chemical Co.) from control unvaccinated dogs and each vaccinated animal group 6 and 20 days after vaccination. Target CMT cells (identical to the vaccine CMT cell line or the unmatched CMT cell line) were prepared by loading with ^{51}Cr (200 μCi in 200 μl and 20 μl FBS added directly to

100 μl RPMI-1640 medium containing 2×10^6 target CMT cells for 2 h at 37 °C), washed and post-incubated in RPMI-1640 medium for 45 min at 37 °C, to reduce background, and incubated in 96-well plates (5×10^3 target cells/well) with effector PBMCs (Kircheis et al., 2000). Target cells were incubated with dilutions (as noted) of effector lymphocytes from vaccinated or unvaccinated dogs, or medium without lymphocytes for 16 h to ensure assay reliability as previously described for canine lymphocytes (Helfand et al., 1994, 1999; Khanna et al., 1996). Supernatants were counted for released radioactivity by liquid scintillation counting in triplicate. Corrected percent lysis was calculated to determine relative cytotoxic T-lymphocyte (CTL) activity.

$$\%\text{CMI (specific lysis)} = \frac{\text{experimental isotope release} - \text{spontaneous isotope release}}{\text{maximal isotope release} - \text{spontaneous isotope release}} \times 100$$

experimental isotope release = CPMs released by CTL-specific activity; spontaneous isotope release = CPMs released in the absence of any lysis (negative control); maximal isotope release = CPMs released by 10% Triton X-100 lysis of labeled target cells.

Statistical significance was evaluated by performing an analysis of variance (ANOVA) with least significant difference for all pair-wise comparisons. Alternatively, significance was analyzed by the repeated measures analysis of variance (ANOVA) on ranked data as noted.

3. Results

3.1. Hybrid-cell fusion and analysis

Two independently derived spontaneous CMT cell lines were selected for cell fusion based on culture properties that provided dispersed single cell populations. Cell lines CMT12 and CMT28 were derived from a papillary adenocarcinoma from a poodle and a tubular adenocarcinoma from a mixed breed dog, respectively, and were unlikely to be immunologically matched for MHC with each other or any animal to be vaccinated. Additionally, DH82 cells were selected for the other fusion partner as they have been shown to express characteristics of DC-like origin and thought to represent an early lineage antigen presenting cell (APC) that includes DCs (Wellman et al., 1988). To confirm this characterization, DH82 cells were evaluated for expression of additional DC markers including CD40, CD205 and CD209 (DC-SIGN) by rt-PCR analysis. DH82 cells expressed all three genes consistent with a DC-like character (Fig. 1A). Additionally, like authentic DC cells, DH82 cells also expressed MHC II and CD11c surface antigens detected using canine-specific antibodies and flow cytometry (Fig. 1B). Thus, DH82 cells express many unique or abundant DC-associated genes making them promising candidates for an established allogeneic canine DC-like cell line.

DC-like DH82 cells were combined in pairs that included one of the two CMT cell lines to create two different hybrid-cell fusion vaccine constructs. To evaluate fusion efficiency, DH82 cells were stained with CellTracker Orange CMTMR and CMT cells were stained with CellTracker Green CMFDA. Subsequent staining with trypan blue established that nonviable cells amounted to 1% or less of all cells comparable to pre-fusion levels. Analysis of CMT/DH82 hybrid-cell fusion populations for fusion efficiency was performed by flow cytometry and immunofluorescence microscopy to determine the number of dual-stained cell fusions. Flow cytometry revealed four discrete populations of cells using two-color analysis (Fig. 2A, representative example of CMT12 cells). These included populations of unfused DH82 cells stained with orange fluor (quadrant 1) and unfused CMT12 cells stained with green fluor (quadrant 4) as well as a very small number of cells that remained unstained (quadrant 3). Cell fusions were dual-stained with both fluors (quadrant 2). The total bichromatic fused cell population represented approximately 41.1% of 10,000 cells counted for the experiment shown representative of more than 40 independent fusions (mean %fusion, 49.3%; SD, 16.5; range approximately, 40–70%). CMT28/DH82 cell fusions were essentially identical (data not shown). Immunofluorescent microscopy of dual-stained hybrid-cell fusions of CMT12 and DH82 cells confirmed these results (Fig. 2B). The two stained cell populations could be separately detected in live fused cell populations and, when combined, merged images revealed the presence of dual-stained cells. A phase contrast image of the same field confirmed that most cells were stained and that approximately half of the cells present were stained with both orange and green labels identifying them as hybrid-cell fusions (Fig. 2B, arrows indicate examples of dual-stained hybrid-cell fusions). Cells were combined for fusion in varying ratios in which the proportion of CMT cells was gradually increased to maximize the percentage of hybrid-cell fusions containing a DH82 cell (DH82:CMT cells at 1:1, 1:1.5, 1:2, 1:3, 1:5 and 1:10). Successful fusions were achieved between DH82 and CMT12 or CMT28 cells at all ratios but the maximal

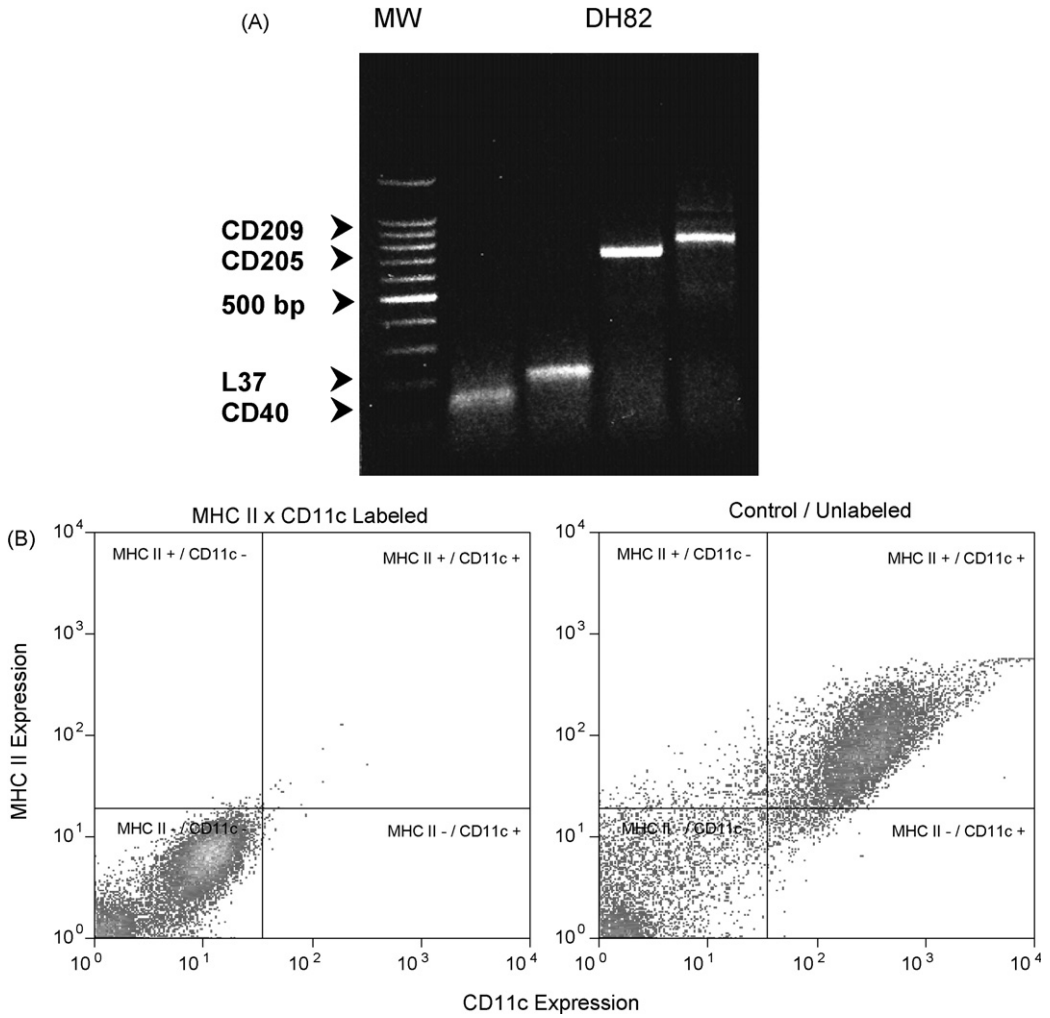

Fig. 1. Characterization of DH82 cell DC antigen expression. The DC-like character of DH82 cells was confirmed by rt-PCR of DC-specific or enriched mRNAs encoding CD40, CD205 and CD209 (DC-SIGN), for which no canine-specific antibodies were available, and by flow cytometry of CD11c and MHC II antigens. (A) rt-PCR assays of total RNA from DH82 cells expressing CD40, CD205 and CD209 mRNAs (amplicons indicated by arrows). rt-PCR assays of L37 large ribosomal subunit protein mRNA expression were included as positive assay controls and controls for RNA integrity (Su and Bird, 1995). rt-PCR amplicons were analyzed by agarose gel electrophoresis and ethidium bromide staining. (B) DH82 cells were labeled with monoclonal antibodies recognizing canine CD11c and MHC II. Anti-cCD11c and secondary antibody were bound (polyclonal goat anti-mouse IgG-Alexa Fluor 660, Molecular Probes) followed by prelabeled α-cMHC II primary antibodies (Zenon reagent, Invitrogen). Flow cytometry was performed on a MoFlo flow cytometer and cell sorter (Dako). Three dimensional dot-plots integrating CD11c and MHC II expression profiles were analyzed using Summit 4.3 software (Dako).

percentage of fused dual-stained cells was achieved at a ratio of 1:2 of DH82:CMT cells for both CMT cell lines.

3.2. Injection of hybrid-cell fusions

Vaccination protocols with hybrid-cell fusions were completed on three groups of laboratory beagles (3 dogs/group: vaccinated with CMT12/DH82 or CMT28/DH82 hybrid-cell fusion or vehicle injected control). In one experiment additional groups were injected with either CMT12 cells alone or DH82 cells alone. The injection regimen consisted of three injections into the popliteal lymph node (Heath and Carbone, 2001) guided and confirmed by ultrasound imaging (Fig. 3). In no case was any sign of toxicity observed due to vaccination beyond mild and transient pyrogenic effects and slight lameness at the site of injection. Symptoms resolved without further treatment within 48 h post-injection. To assess potential of injected hybrid-cell vaccines to establish occult growths, post-mortem analysis was performed on nine animals. Evidence of tumor cell growth was investi-

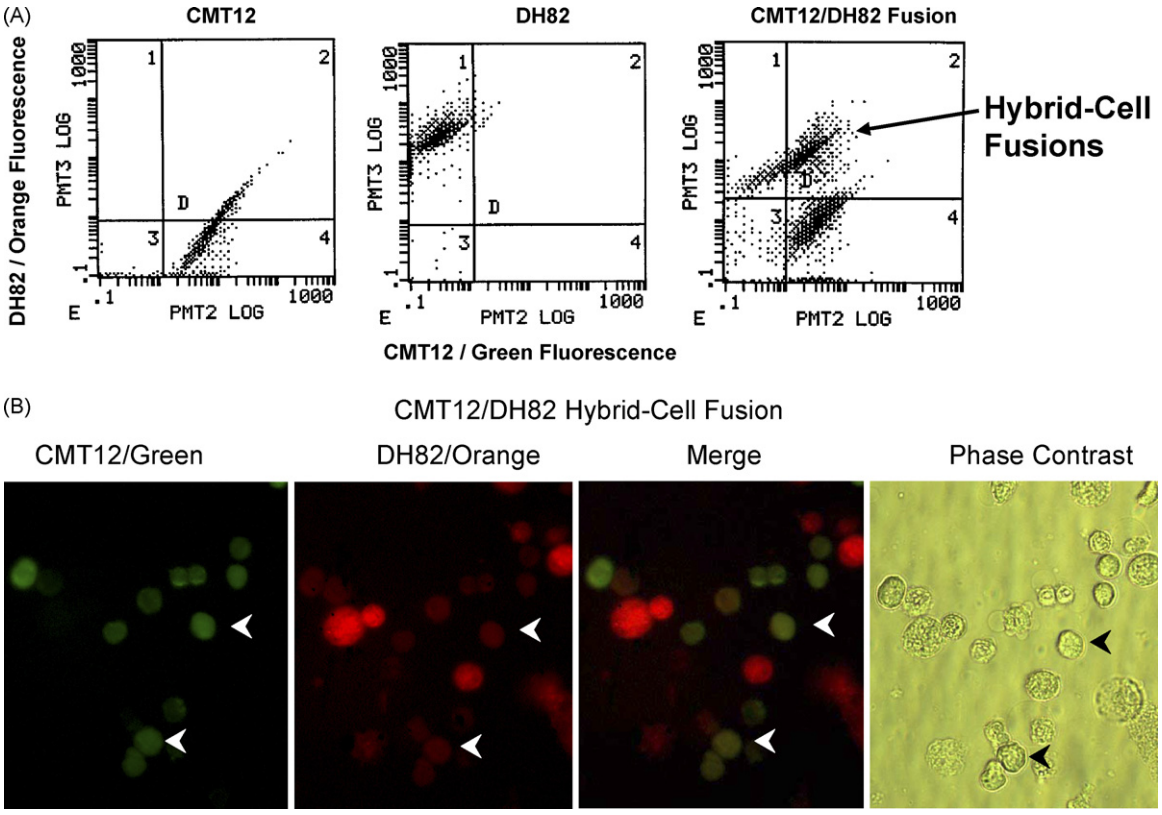

Fig. 2. Flow cytometric and microscopic analysis of two color staining of hybrid-cell fusions of CMT12 and DH82 cell populations. (A) Flow cytometry of dual-stained hybrid-cell fusions of CMT12 and DH82 cells. DH82 cells were stained with CellTracker Orange CMTMR [(5-(and-6)-(((chloromethyl)benzoyl)amino)-tetramethylrhodamine), Molecular Probes] and CMT12 cells were labeled with CellTracker Green CMFDA [5-chloromethylfluorescein diacetate, Molecular Probes] prior to fusion and analysis by flow cytometry. The two cell populations were fused and analyzed by flow cytometry for green fluorescence (PMT2–T12 cells) and orange fluorescence (PMT3–DH82 cells) revealing four cell populations in log plots. Each population of cells was quantified using a 4-quadrant gate including those labeled specifically with orange fluor (quadrant 1: DH82) or green fluor (quadrant 4: CMT12) as well as those cells dual-labeled with both fluors (quadrant 2) – the hybrid-cell fusions – and any cells that remained unlabeled (quadrant 3). The total bichromatic fused cell population was 41.1% of 10,000 cells counted for the experiment shown which was representative of more than 40 independent fusions. (B) Immunofluorescent microscopy of dual-stained hybrid-cell fusions of CMT12 and DH82 cells. The two cell populations were grown, stained and processed for fusion and analyzed by fluorescence microscopy. The identical frame of representative cell fusions is shown detecting green (CMT12) or orange (DH82) fluorescence demonstrating that most cells were dual-labeled (see arrows for examples) as confirmed in the merged image (Merge) as the same cells were colored with a green/orange blend. A phase contrast image of the same field is shown for comparison (phase contrast). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

gated at the site of injection and at other sites, following termination of the experiment, by complete necropsy including both gross and microscopic analysis of all tissues taken revealing no evidence of vaccine cell proliferation.

3.3. Confirmation of unmatched character of cell line MHC I alleles

MHC expression and MHC I allele sequences were determined for all three cell lines. Both CMT12 and CMT28 cells expressed abundant levels of MHC I although only CMT12 cells expressed abundant MHC II (Fig. 4A). MHC class 1 exon 2 (DLA88) sequences for each cell line were determined to verify the unmatched character of each (Fig. 4B and C) as this exon encompasses most of the diversity observed (Wagner et al., 2000). Direct sequencing of DLA88 PCR amplicons encoding canine MHC class I gene sequences revealed distinct alleles for each cell line and this heterogeneity supported the assessment that the cell lines employed were unmatched for MHC class I. Although all variations in CMT MHC I exon 2 (DLA88) amino acid sequences detected have been previously observed among the 48 alleles published, this particular combination of differences from type MHC I sequences does not

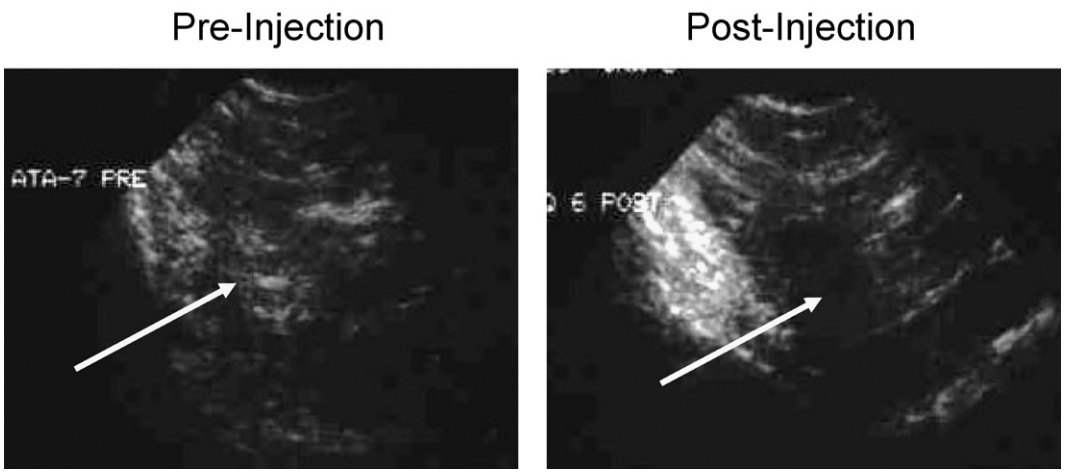

Fig. 3. Ultrasound-guided injection of canine popliteal lymph nodes. Popliteal lymph nodes were located by palpation and verified by ultrasound imaging (arrow left panel: pre-injection). Following injection with hybrid-cell fusions or vehicle controls, imaging of the same node revealed a dark mass of injected fluid (arrow right panel: post-injection) in the lymph node (representative image shown).

appear to have been observed previously (Immuno Polymorphism Database – http://www.ebi.ac.uk/ipd/mhc/dla/align.html? – updated 27 March 2006, Robinson et al., 2005). Likewise, sequencing of the same region of MHC class I in DH82 cells revealed what appeared to be a new allele including at least 2 amino acid substitutions not previously recognized and apparently outside the hyper-variable region. In all, CMT cells each encoded at least 2 amino acids different from each other and only 1 amino acid different from the DLA88 00402 allele, while

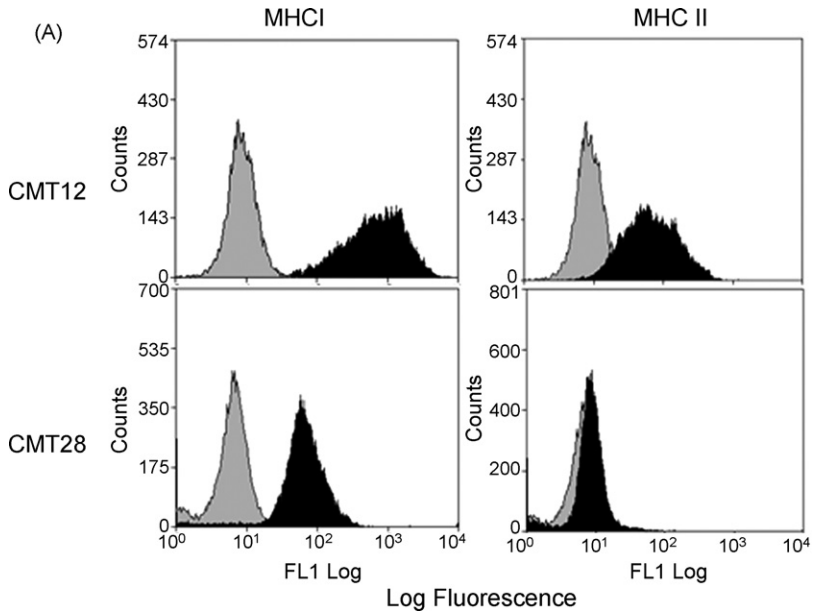

Fig. 4. Expression of MHC class I and II by CMT cells and determination of distinct MHC alleles. (A) CMT12 and CMT28 cells were labeled with antibodies recognizing canine MHC class I or MHC class II antibodies and then reacted with FITC-conjugated secondary antibodies followed by flow cytometry. Control unlabeled populations (grey histograms) and labeled (black histograms) populations are shown. (B) Comparative sequence analysis of the exon 2 of the DLA88 locus in CMT12, CMT28 and DH82 cells compared to similar DLA88 alleles from Genbank (accession numbers as noted). The translation product of allele 12/00402 is noted along with amino acid variations in the other alleles. The hyper-variable region of exon 2 is noted by the solid line. (C) Comparison of inferred amino acid sequences for exon 2 of the DLA88 locus showing differences in sequence between different alleles. Similarities were noted as bases/amino acids conserved among all sequences (black sequence on grey), bases/amino acids conserved among a majority of sequences (white sequence on black) and bases/amino acids unique to one or two sequences (black sequence on white).

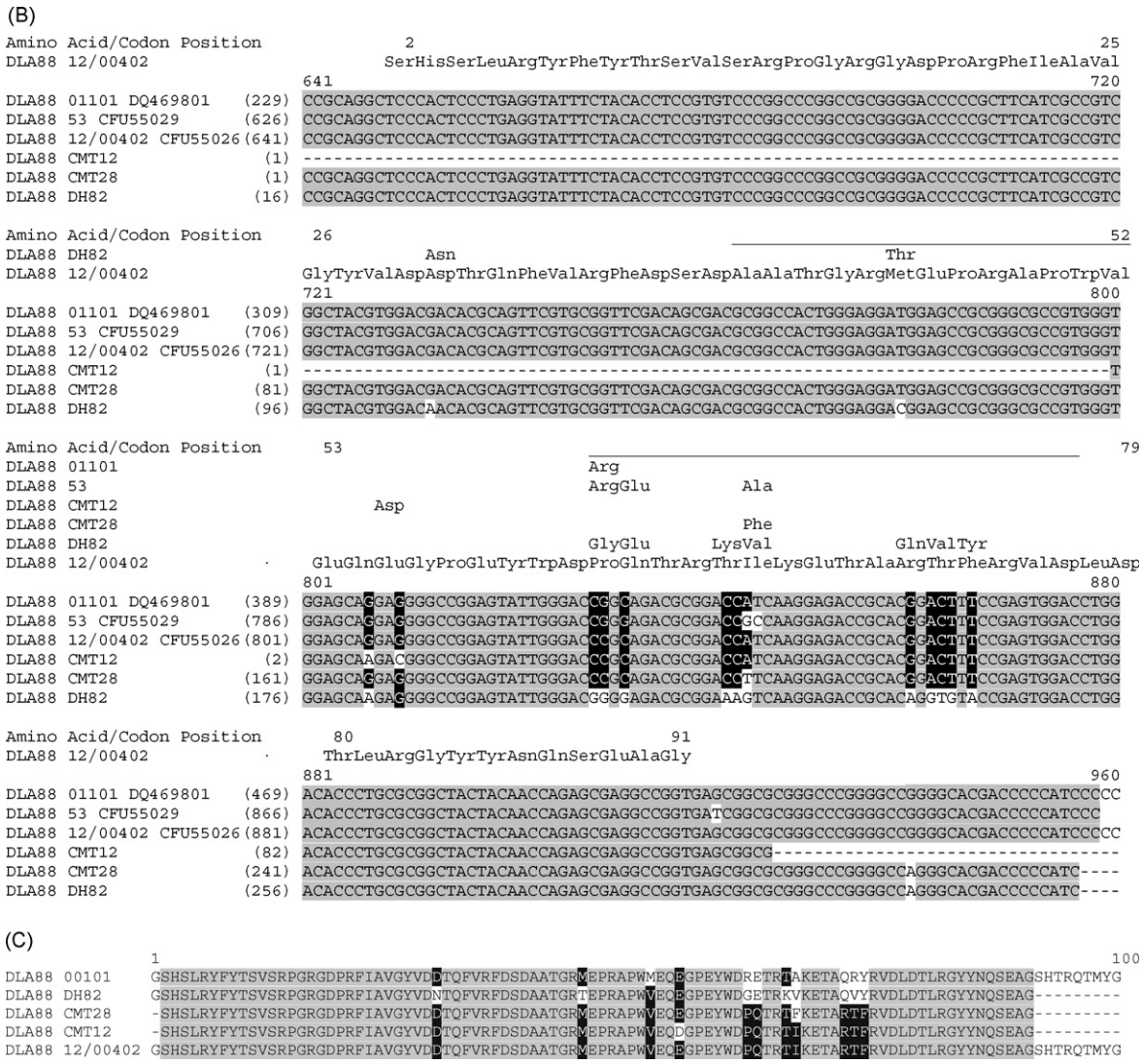

Fig. 4. (*Continued*).

DH82 cells encoded 10 amino acids different from either of the CMT cell lines and 9 amino acids different from the DLA88 00402 allele. Both CMT cell lines and DH82 cells encoded 7 different amino acids when compared to the DLA88 00101 allele. Because unique amino acid sequences were encoded in each canine DLA88 exon 2 analyzed, all 3 cell lines encoded unique alleles for MHC I and were unmatched.

3.4. Immune responses in hybrid-cell fusion vaccinated dogs

Immune responses to antigens common to both CMT cell lines were assessed by comparing immune reactions to CMT cells matched or unmatched for MHC. Such comparisons were used to evaluate recognition of shared CMT cell antigens common to CMT12 and CMT28 cells. Thus, a positive immune reaction with CMT target cells, unmatched for MHC I, would strongly suggest that shared CMT antigens were recognized. Both antibody ELISA and cell-mediated immune (CMI) responses were evaluated. Recognition of common CMT cell antigens was evaluated by assaying sera from CMT12/DH82 fusion injected dogs on matched CMT12 and then unmatched CMT28 cells and vice versa for CMT28/DH82 fusions. Both combinations of sera/cells detected antibody responses, above levels observed with control sera, when reacted with either CMT cell line. However, differences were small and variations large enough in these small groups to preclude statistical significance or

conclusions (data not shown). This suggests that although a portion of the serum immune reaction may be directed against antigens common to unmatched CMT cells the level of reaction did not appear significant.

Cell-mediated immune (CMI) responses were assayed by ^{51}Cr-release from labeled CMT12 cell targets in the presence of fresh canine lymphocytes from vaccinated dogs and vehicle injected control dogs. Potential for antigen cross-presentation or enhanced immune response was evaluated by CMI assays of PBMCs from dogs vaccinated with CMT12/DH82 hybrid-cell fusions, CMT12 cells alone or DH82 cells alone and comparing them to the innate immune response in vehicle control injected dogs (Fig. 5). Only hybrid-cell fusion vaccinated dogs resulted in an immune response that was significantly higher ($p \leq 0.05$) than innate immunity detected in vehicle control injected dogs. Because the CMT12/DH82 hybrid-cell fusions were the only vaccination complex capable of eliciting a significant response, compared to either CMT12 cells or DC-like DH82 cells alone, the enhanced immune response appeared dependent on the presence of both cell types. Although fold enhancement of CMI of vaccinated dogs above control levels was relatively consistent, some variations were detected and were most likely due to differences between groups of dogs injected with the same hybrid-cell fusions, different lots of ^{51}Cr and normal experimental variation.

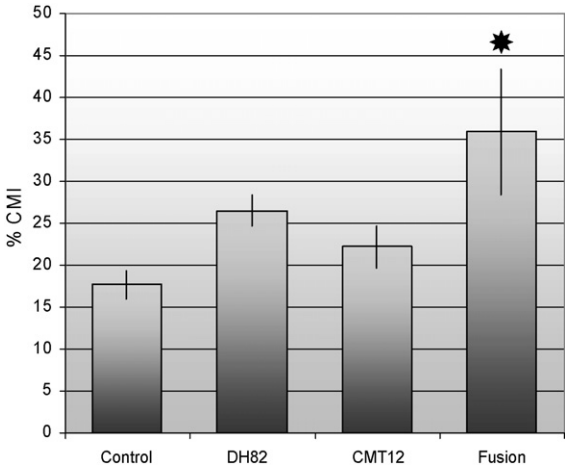

Fig. 5. Comparison of cell-mediated immunity between hybrid-cell fusions, CMT12 and DH82 cells. CMI assays for canine PBMCs were performed where ^{51}Cr-release from labeled target CMT cells was measured in the presence of lymphocytes recovered from immunized or naïve control vehicle injected animals. Lymphocytes from 4 groups of 3 dogs were measured independently in triplicate and the CMI assay calculated as percent specific lysis (% CMI) for PBMC:CMT12 target cell ratios of 50:1. CMI was calculated as a percentage of total releasable isotope available in the assay due to specific lysis (Section 2) for control unvaccinated, as well as DH82, CMT12, and CMT12/DH82 hybrid-cell fusion (fusion) vaccinated dogs. Analysis of variance (ANOVA) with least significant difference for all pair-wise comparisons revealed statistically significant differences between fusion and control values (asterisk, $p \leq 0.05$).

Comparisons of immune responses against vaccine CMT cell and unmatched CMT cell targets were performed to allow comparison of CMI responses to both hybrid-cell fusions as described above. Both CMT12 and CMT28 cells were prepared as ^{51}Cr-labeled targets and cell-mediated immune assays performed with fresh PBMC:CMT cells at ratios of 25:1 and 50:1. For each hybrid-cell fusion vaccinated group (CMT12 or CMT28-based hybrid-cell fusions) PBMCs were evaluated for target cell killing (%CMI) against both CMT12 and CMT28 cell targets 6 and 20 days after the third injection. Comparisons of CMI detected in each of these groups to levels of innate immunity detected in the naïve control vehicle injected group were made (Fig. 6). On day 6 after the third injection, in both CMT12/DH82 and CMT28/DH82 vaccinated dogs, the CMI response detected (%CMI) in vaccinated dogs ranged from 0 to approximately 5.4-fold higher (up to 33% of available isotope released) compared to innate levels of CMI detected in the control group (Fig. 6A and B, compare dark grey CMT28/DH82 and medium grey CMT12/DH82 vaccinated group columns with light grey control vehicle injected columns). Measurable CMI was detected against both matched and unmatched CMT targets in both groups of vaccinated dogs, compared to the innate response in naïve control vehicle injected dogs, at least at ratios of 50:1 of PBMCs to target cells. And, CMT28/DH82 vaccinated animals consistently responded with higher levels of detectable CMI than CMT12/DH82 vaccinated animals (Fig. 6, panels A and B, compare medium grey CMT12/DH82 vaccinated and dark grey CMT28/DH82 vaccinated columns). CMT28 cells provided more sensitive CMI targets resulting in higher levels of %CMI for all assays. Despite the lower numbers of animals utilized, most assays suggested real differences in immune response due to vaccination and results were statistically significant in some cases compared to control unvaccinated animals (Fig. 6, asterisk, $p = 0.0763$). Although PBMCs from both groups of hybrid-cell fusion injected dogs were able to lyse both populations of matched and unmatched target CMT cells at levels in excess of controls (naïve control cells vs. CMT hybrid-cell fusion vaccinated cell levels) by 6 days after the third injection, by 20 days post-injection only CMT28 cell targets still provided comparable measurable levels of CMI response to both vaccine groups (approximately 14–32% CMI, compare Fig. 6,

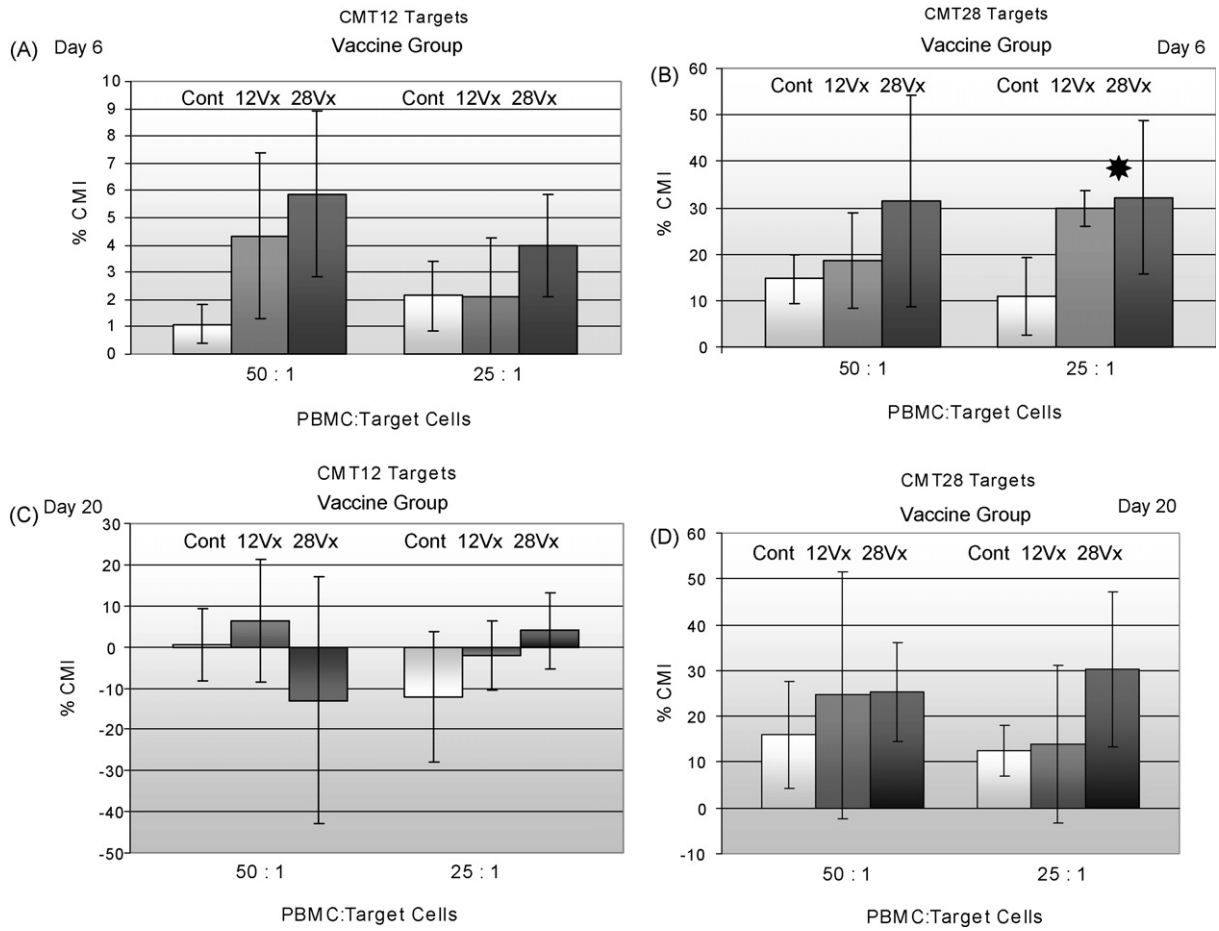

Fig. 6. Cell-mediated immunity of hybrid-cell fusion immunized dogs against matched and unmatched CMT cells. Standard cell-mediated immune (CMI) assays for canine PBMCs were performed where ^{51}Cr-release from labeled target CMT cells was measured in the presence of PBMCs recovered from immunized or naïve control vehicle injected animals. PBMC populations from 3 groups of 3 dogs were measured independently in triplicate and CMI calculated as percent specific lysis (%CMI) for each of 2 PBMC:CMT target cell ratios (50:1 or 25:1) calculated as the percentage of total releasable isotope available due to specific lysis. PBMCs were obtained from dogs 6 days (panels A and B) and 20 days (panels C and D) after the third injection of the hybrid-cell fusion vaccine formulation noted: CMT28/DH82 (dark grey bars), CMT12/DH82 (medium grey bars) or vehicle injected control (light grey bars). CMI assays for two hybrid-cell fusion vaccine groups and control group performed on labeled CMT12 targets (Panels A and C) or CMT28 target cells (Panels B and D). The entire experiment was repeated twice and representative data is shown. Repeated measures analysis of variance (ANOVA) on ranked data revealed statistically significant differences between CMT28/DH82 cell fusions and control values on day 6 (asterisk, $p = 0.0763$).

panels B and D). In contrast, CMI responses measured against CMT12 target cells had diminished to essentially undetectable levels by day 20 (Fig. 6C).

Supporting the observation that enhanced CTL activity was responsible for at least some of the immune response to CMT cell antigens, assays of cIFN-γ in PBMC populations from vaccinated and control dogs detected no cIFN-γ expression in control animals. In contrast, most CMT12/DH82 vaccinated animal PBMCs expressed moderate levels and CMT28/DH82 PBMC populations expressed higher levels of cIFN-γ mRNA comparable to ConA-induced PBMCs (Fig. 7). When lymphocytes from CMT28/DH82 vaccinated dog PBMCs were sorted into CTL (CD8+/CD4−), T-helper (CD4+/CD8−), and other lymphocyte populations including NK cells (CD4−/CD8−) most of the cIFN-γ expression was observed in the CTL population with some detectable expression in T-helper cells. No cIFN-γ expression was detectable in the CD4−/CD8− population (Fig. 7).

4. Discussion

Most tumors express antigens that can generate a cell-mediated immune response and have the potential for effective utilization in vaccine development through

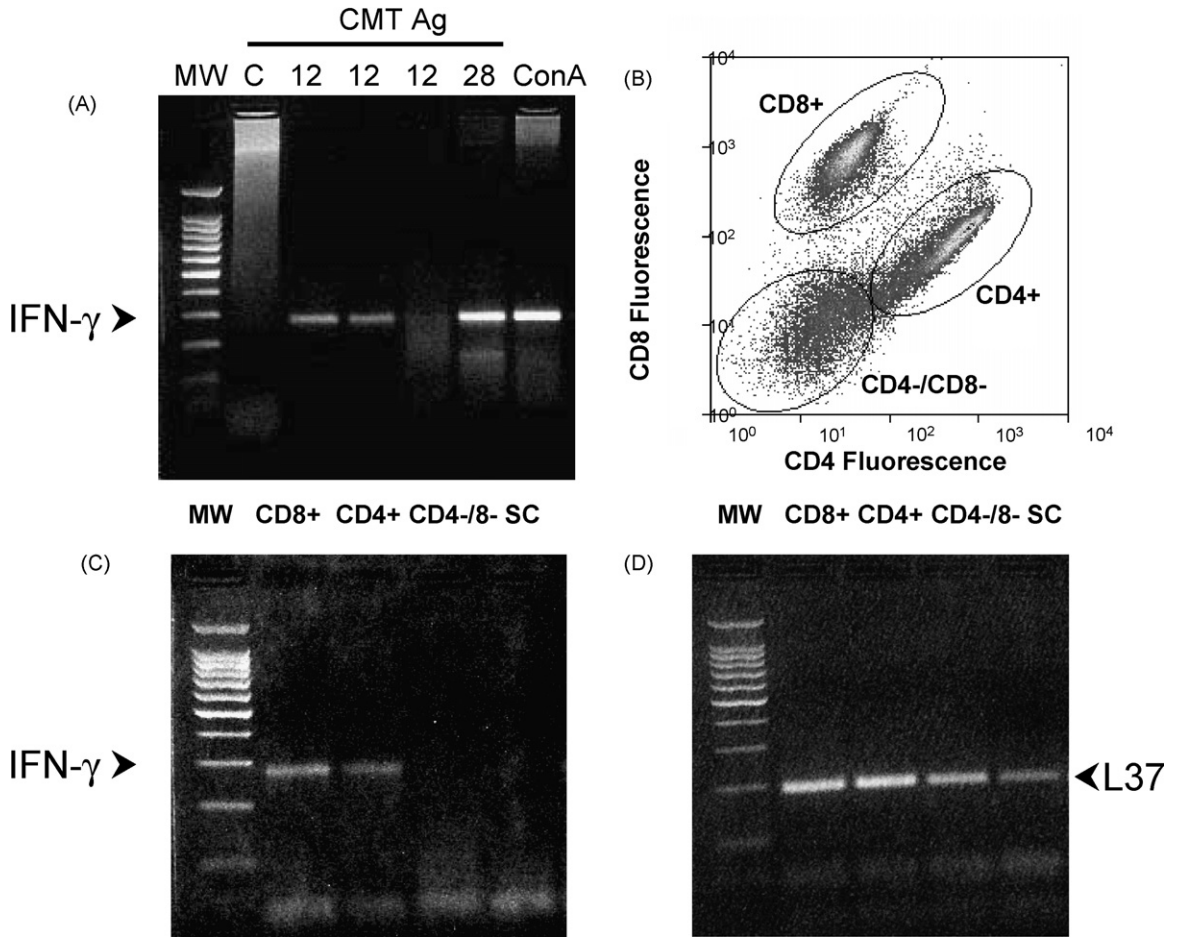

Fig. 7. IFN-γ expression in PBMC populations. Total PBMC and sorted sub-populations from vaccinated and control dogs were assessed for cIFN-γ expression by rt-PCR. Panel A: rt-PCR assays of cIFN-γ expression in total RNA populations from PBMCs from representative dogs vaccinated with CMT12/DH82 fusions (12), CMT28/DH82 fusions (28), vehicle control (C) and Con A treated PBMCs (ConA) as indicated (arrow IFN-γ). Panel B: High-speed cell sorting of labeled PBMCs into CD4+/CD8− (CD4+), CD4−/CD8+ (CD8+) and CD4−/CD8− populations. Non-fluorescent subcellular particles (sc) were also collected (data not shown). Panel C: rt-PCR assays of cIFN-γ expression in total RNA from sorted lymphocyte populations from a representative dog vaccinated with CMT28/DH82 fusions. Panel D: rt-PCR assays of L37 large ribosomal subunit protein mRNA expression (arrow L37) in the same RNAs included as controls for loading and RNA integrity (Su and Bird, 1995). rt-PCR amplicons were analyzed by agarose gel electrophoresis and ethidium bromide staining with 100 bp MW markers (MW).

antigen loading of APCs (Nair, 1998). Such strategies involve potentiation of uptake, processing and presentation of tumor cell antigens by APCs such that there is enhanced antigen recognition (Nair, 1998; Kircheis et al., 2000). Principle APC populations involved appear to include DCs while effector cells following antigen presentation are cytotoxic T cells. However, the limiting step is likely the transfer of antigens to the APC/DC (Nair, 1998). Thus, strategies which target DCs, or which enhance antigen uptake between tumor cells and DCs, have the potential to enhance immune presentation promoting development of cytotoxic T cells. Loading of DCs with antigen can employ different approaches. However, direct fusion of tumor cells to DCs is one of the simplest and one that does not presume to choose effective antigens avoiding the introduction of bias (Kircheis et al., 2000). A theoretical risk of induction of autoimmunity exists using this strategy; however, little evidence of this potential response has been reported (Nair, 1998; Avigan et al., 2004; Homma et al., 2006).

To develop such a strategy for canine mammary cancer, hybrid-cell fusions were created from the DC-like DH82 cell line and canine mammary tumor-derived CMT12 and CMT28 cell populations. DH82 cells were originally isolated from neoplastic progenitors of malignant histiocytosis which were characterized as histiocytic in origin (Wellman et al., 1988). The surface

antigen profile (MHC class II, B7.1, B7.2), their monocyte–macrophage origin and their relatively under differentiated phenotype originally suggested potential as APCs including DC characteristics. We extended and confirmed this characterization as DC-like by demonstrating that DH82 cells also express DC antigens CD40, CD205 and CD209 (DC-SIGN) by rt-PCR analysis. Thus, DH82 cells were considered promising candidates to promote immune recognition by fusion to CMT cells. Originally isolated, cultured and characterized from canine mammary carcinomas, CMT cells represent a series of stable transformed lines derived from independent canine mammary cancer biopsy specimens that have been shown to be very different for both growth and phenotype (Wolfe et al., 1986; Sartin et al., 1993; Ahern et al., 1995; DeInnocentes et al., 2006).

Analysis of cell fusions by flow cytometry successfully discriminated each population of fluorescently stained cells providing a quantitative measure of the efficiency of hybrid-cell fusion. Fusions between DH82 and CMT cells included 40–70% of the cells, almost double the best rates published to date in the more successful attempts in human cells perhaps due to differences in the cell fusion partners selected and/or the method of fusion (Scott-Taylor et al., 2000). CMT/DH82 hybrid-cell fusion vaccination produced a greater CMI response than dogs injected with either CMT cells or DH82 cells alone demonstrating that fusion was successful as only fusions produced significant immune reactions compared to either cell line alone. This, combined with our extended characterization of the DH82 gene expression profile, suggests that DH82 cells may function as DCs to enhance immune recognition of CMT antigens.

Enhanced immune recognition of shared CMT antigens was assessed by comparing the CMI response directed independently against CMT12 and CMT28 target cells. The CMT cell lines were partially typed, along with DH82 cells, for MHC I allele by DNA sequencing to ensure their allogeneic character (Kennedy et al., 2002; Wagner et al., 2000; Hardt et al., 2006). CMI assays directed against matched and unmatched CMT cell lines were used to measure CTL activity in each animal to assess the immune responses to shared CMT antigens (Kircheis et al., 2000; Kruisbeek, 2000; Barratt-Boyes et al., 2000). Immune responses detected using unmatched CMT target cells, in excess of innate immune responses detected in vehicle control dogs, strongly suggested that antigens shared by the two CMT cell lines had been recognized. Although we cannot differentiate between detection of antigens shared by only CMT12 and CMT28 cells and true pan-mammary tumor antigens the latter seems likely since, aside from their origins as mammary carcinomas, the cell lines were otherwise unrelated.

In both vaccine formulations CMI response measured was, in general, proportional to the number of PBMCs added to the assay. However, CMT12 cell targets produced lower CMI responses than CMT28 cells and CMT28 cells proved a more sensitive target population over a longer period post-immunization. Immune responses were detectable and essentially undiminished for at least 20 days using CMT28 cell targets when either CMT cell line was used in the hybrid-cell fusion vaccine. Normal variation within animal groups and the low number of animals used (3/group), typical of larger animals experiments, appear to have reduced statistical significance somewhat, however, measurable statistically significant immunity, above control innate immune levels, was detected against shared CMT determinants in some experiments.

In applying this strategy we hypothesized that enhanced immune recognition of shared CMT cell antigens was the result of enhanced antigen recognition and CTL response. Because immune recognition of shared CMT antigens was observed, enhanced beyond innate control levels, allogeneic CMT cell antigens appear to have been presented by autologous APCs although a contribution by innate NK-activity was not ruled out. Enhanced IFN-γ expression only in CMT/DH82 vaccinated animals, approximately proportional to levels of CMI, supported the conclusion that CTL activity was induced as part of this response. Further, most cIFN-γ expression was confined to sorted CD4−/CD8+ CTL and, to a lesser degree, to CD4+/CD8− T helper cell populations. NK cells were not a major source of cIFN-γ expression and immune recognition as no cIFN-γ expression was detected in canine CD4−/CD8− PBMC populations where NK cells are thought to reside (Knapp et al., 1995; Ghernati et al., 2000).

There is abundant evidence that therapeutic vaccination strategies of this type are more effective if live unirradiated cells are used but this approach poses new problems due to the potential for proliferation of live vaccine cells in vaccinated animals. We have examined this by determining if injections harmed animals either acutely or due to long term vaccine cell survival and growth. To date, among all of the normal beagles we have injected and evaluated using this procedure (21 animals injected 3 times each) we have observed only transient effects on the animals due to injection/vaccination. To assess the potential for occult growth of injected hybrid-cell vaccines, extensive post-mortem

analysis was performed. No evidence of vaccine cell proliferation was detected at the site of injection or other potential sites. Our conclusion is that, beyond slight occasional and transient effects, there were no detectable side effects on the animals after 63 independent injections. Data from human vaccination also suggest that injection of living tumor cells does not result in allogeneic cell colonies/tumors (Salgaller, 2000). This is important as it has been shown that live cells in vaccine formulations provide more effective immunogens eliciting a stronger immune reaction (Souberbielle et al., 1998). Cell vaccines irradiated to ensure no proliferation is possible resulted in significant reduction in immunogenicity (Kircheis et al., 2000; Souberbielle et al., 1998).

We have established that allogeneic vaccination with hybrid-cell fusions has the potential to develop enhanced CMI in dogs and demonstrated the potential of established allogeneic cell lines in promoting simplification of vaccine development. Although immune response levels were modest, these lower levels may well prove ideal if induction of effective immunity is to be achieved and autoimmune reactions are to be avoided.

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