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Research paper

Immunopathogenic behaviors of canine transmissible venereal tumor in dogs following an immunotherapy using dendritic/tumor cell hybrid

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ABSTRACT

Canine transmissible venereal tumor (CTVT) is a naturally occurring tumor that can be transmitted between dogs via live tumor cell inoculation. It is also a spontaneous self-regression tumor and its behavior is closely related to host immune responses. Since CTVT had been widely used for tumor models in canine cancers, whether this self-regression may overtake the immunity elicited from an exogenous tumor vaccine remains unclear and certainly worthwhile to be investigated. In this study, we used DCs/tumor hybrids as a tumor vaccine to evaluate the CTVT model. We prepared mature allogeneic dendritic cells from bone marrow and then assessed their phenotype (CD80, CD83, CD86, CD1a, CD11c, CD40 and MHC II), antigen uptake and presenting abilities. Fused dendritic cell/CTVT hybrids were then used as a vaccine, administered three times at two-week intervals via subcutaneous injection near the bilateral auxiliary and inguinal lymph nodes. In comparison with unvaccinated dogs (spontaneous regressed group), within a period of 2.5 months, the vaccinations substantially inhibited tumor progression ($p < 0.05$) and accelerated the rate of regression by a mechanism involving amplification of the host tumor-specific adaptive immune responses and NK cytotoxicity ($p < 0.001$). Pathologic examination revealed early massive lymphocyte infiltration resulting in final tumor necrosis. In addition, there are not any detectable effects on routine physical, body temperature or blood chemistry examinations. In conclusion, our data furnishes a reference value showing that CTVT is a model of potential use for the study of immunity elicited by vaccines against tumors, and also enable early-phase evaluation of the dendritic cell/tumor vaccine in terms of raising host immunity.

Abbreviations: BMDCs, bone marrow derived dendritic cells; CD, cluster of differentiation; CFSE, carboxyfluorescein succinimidyl ester; CMTPX, CellTracker™ Red; CTVT, canine transmissible venereal tumor; CTAC, canine thyroid adenocarcinoma cell line; ELISPOT, enzyme-linked immunosorbent spot; *E*/*T* ratio, effector cell/target cell ration; FACS, fluorescence-activated cell sorting; FSC, forward scatter; GM-CSF, granulocyte-monocyte colony stimulating factor; iBMDCs, immature bone marrow derived dendritic cells; mBMDCs, mature bone marrow derived dendritic cells; PBMCs, peripheral blood mononuclear cells; PEG, polyethylene glycol; SSC, sideward scatter; TAA, tumor associated antigen; TILs, tumor infiltrating lymphocytes.

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1. Introduction

CTVT is a naturally occurring tumor and can be transmitted among dogs by inoculation of viable cells. CTVT cells are round to ovoid in shape with a large nucleolus and prominent vacuolated cytoplasm. Some unique characteristics of CTVT cells are as follows: first, CTVT can only be transplanted by viable tumor cells in experimental studies (Kennedy et al., 1977); however, CTVT cells may soon be induced to undergo apoptosis under *ex vivo* conditions, and the time of implantation will then be critical in ensuring successful passage in canines. Second, insertion of a long interspersed nuclear element (LINE-1) near c-myc can be used as a specific marker to diagnose CTVT (Liao et al., 2003). Third, CTVT is of clonal origin and has been identified as having evolved from a wolf or an East Asian breed of dog by somatic cell mutation (Murgia et al., 2006).

After transmission to a susceptible host, CTVT undergoes a progression phase, which may last for several months, then regresses spontaneously (Yang, 1988). During the progression phase, only a very small proportion (2–5%) of tumor cells express MHC molecules, as compared with up to 40% of cells during the regression phase (Hsiao et al., 2002). Easily transferred, CTVT can be transplanted into other dogs by subcutaneous or intra-organ injection, and has been widely used as an experimental model in pioneer studies of many cancer therapies (Ahrar et al., 2010; Chou et al., 2009; Chuang et al., 2009; Kangasniemi et al., 2004; Schwartz et al., 2009).

With respect to tumor immunotherapy, dendritic cells (DCs)/tumor cell fusion hybrids have been shown to be potent vaccines against tumors (Bird et al., 2008). The rationale behind this approach is that the resulting hybridoma could present an almost complete body of tumor-associated antigens and possesses a co-stimulatory capability to elicit immunity against a given tumor (Yasuda et al., 2006, 2007).

For the reasons discussed above, CTVT has the potential for use as a target model in which to study the effectiveness and mechanisms involved in tumor vaccine therapy. However, there exists the difficulty that only limited amounts of monocyte-derived DCs can be generated from peripheral blood (PBDCs) in canines. Another concern is that CTVT may undergo spontaneous regression as a result of endogenous immune responses, which could overtake the immunity boost delivered by the fusion hybrid vaccine. Thus, one of the purposes of this study was to obtain sufficient mature DCs from bone marrow (BMDCs) for the preparation of DCs/CTVT hybrids. Other aims were to test the hypothesis of whether the immunity elicited by vaccine therapy prevails over spontaneously occurring regression in the CTVT model and to attempt to address some of the mechanisms underlying vaccine therapy in this model.

We found that IL-4, GM-CSF and LPS stimulated mature BMDCs (mBMDCs) exhibiting higher antigen presentation in mixed lymphocyte reactions relative to immature BMDCs (iBMDCs). The rate of fusion of mBMDCs and CTVT was approximately 60%, as determined by double staining and flow cytometry. The resulting hybrid vaccine was able to dramatically inhibit tumor growth and promoted early regression. Finally, we showed that the vaccine significantly augmented host tumor-specific adaptive immune responses and NK cytotoxicity in addition to tumor necrosis, which were associated with early and massive infiltration of lymphocytes into the tumor tissues.

In conclusion, vaccine therapy was effective in attenuating tumor growth and activating host immune responses against tumors, even in spontaneously regressive CTVT, which suggests that CTVT is a model of potential use for the study of immunity elicited by vaccines against tumors.

2. Materials and methods

2.1. Animals and generation of PBDCs and BMDCs

The study protocol was approved by the Institutional Animal Care and Use Committee of the National Taiwan University prior to initiation. A total of 13 beagles were used: seven healthy beagles from 1 to 2 years of age of both genders were used as a source for bone marrow aspiration, and six matched controls of similar age and both genders were used for the fusion vaccine experiments. All animals were negative for dirofilaria, Erlichia, and Lyme disease.

Peripheral blood-derived DCs (PBDCs) and BMDCs were generated as described previously (Isotani et al., 2006; Wang et al., 2007a,b). For BMDCs, 10 mL of bone marrow were aspirated from the femoral head of a hind limb and collected into tubes containing 2 mL of 200 U/mL heparin (Becton Dickinson, Franklin Lakes, NJ). Mononuclear cells were isolated by gradient centrifugation using the Ficoll–Hypaque method (density: 1.077). After two washes with RPMI 1640 (Invitrogen, Carlsbad, CA) containing 10% FCS (Perbio/Thermo) and centrifugation at 450 g for 10 min, the cell pellets were re-suspended at a density of 5×10^6/mL in RPMI 1640 containing 10% FCS, 1% PSA (Sigma, St. Louis, MO), 20 ng/mL of IL-4 (R&D Systems, Minneapolis, MN) and 20 ng/mL of GM-CSF (Leucomax; Schering-Plough, Kenilworth, NJ). Two mL of cell suspension were then seeded into each well of a 6-well plate and incubated for 7 days, with 1 mL of medium being replaced with fresh medium on days 3 and 5. On day 7, the cells were harvested as iBMDCs and re-suspended in RPMI 1640 + 10% FCS medium containing 20 ng/mL each of IL-4 and GM-CSF plus 250 ng/mL of LPS and cultured for 3 more days. On day 10, mBMDCs were obtained.

2.2. Flow cytometric analysis of phenotypes

Commercially available monoclonal antibodies (Table 1) were used to analyze canine surface antigens. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG antibodies (Serotec, Oxford, England) were used as the secondary antibody. Briefly, for immunofluorescence analysis, cells were incubated for 30 min with an isotype control or specific antibodies and were then washed and stained for another 30 min with the secondary antibody. Finally, the washed cells were suspended in FACS buffer containing 5 μg/mL of propidium iodide. The surface immunofluorescence of 1×10^4 viable cells was measured using a FACSCalibur flow cytometer (Becton

Table 1
Monoclonal antibodies used for surface phenotype analysis of BMDCs, CTVT, fusion hybrids and TILs.

Specificity	Clone	Immunoglobulin subclass
Human CD1a-FITC	NA1/34[a]	Mouse IgG2a
Canine CD3	CA17.2A12[b]	Mouse IgG1
Canine CD4	CA13.1E4[a]	Mouse IgG1
Canine CD8	CA9.JD3[a]	Mouse IgG2a
Canine CD11c	CA11.6A1[a]	Mouse IgG1
Canine CD21	CA2.1D6[a]	Mouse IgG1
Human CD40-FITC	LOB7/6a[a]	Mouse IgG1
Canine DLA class II	CA2.1C12[a]	Mouse IgG1
Mouse MHC I	2G5[a]	Mouse IgG2a

[a] *Source*: Serotec (Oxford, England).
[b] *Source*: Veterinary Medicine University of California at Davis, CA, USA.

Dickinson, Mountain View, CA, USA) and analyzed using Cell Quest software (Becton Dickinson).

2.3. Quantitative real-time reverse transcriptase-polymerase chain reaction

Total RNA was extracted from 10^6 iBMDCs or mBMDCs using TRIzol (Invitrogen) according to the manufacturer's recommendations and reverse-transcripted into cDNA using SuperScript II RT (Gibco-BRL/Invitrogen) and oligo (dT) primers. Real-time RT-PCR was performed using a Bio-Rad MyiQ Detection System (Bio-Rad, Hercules, CA, USA) with SYBR Green PCR Master Mix according to the manufacturer's instructions. The primer sequences used for CD80, CD83, and CD86 were developed based on our previous research (Wang et al., 2007b) and are listed in Table 2. The housekeeping gene β-actin was used as an internal control for relative determination, and the results were analyzed using IQ5 analysis software provided by Bio-Rad.

2.4. FITC-dextran uptake assay

Endocytic activity was assessed as described previously (Wang et al., 2007a). iBMDCs and mBMDCs were incubated for 2 h with FITC-dextran (100 μg/mL; Sigma) at 4 °C or 37 °C and then washed extensively with PBS before being subjected to flow cytometry at an excitation of 488 nm to determine the percentage of endocytotic cells. Non-specific binding at 4 °C was subtracted from the value at 37 °C.

Table 2
Sequences of primers used for real-time RT-PCR.

Primer	Sequence
CD 80	
Forward	5′-ATGGATTACACAGCGAAGTGGAGAA-3′
Reverse	5′-AGGCGCAGAGCCATAATCACGAT-3′
CD83	
Forward	5′-CAGTCATATAAAAGCTATGGTGAGATGC-3′
Reverse	5′-AGATGAAAAGGCCCTGCTGGGG-3′
CD86	
Forward	5′-ATGTATCTCAGATGCACTATGGAAC-3′
Reverse	5′-TTCTCTTTGCCTCTGTATAGCTCGT-3′
β-Actin	
Forward	5′-GACCCTGAAGTACCCCATTGAG-3′
Reverse	5′-TTGTAGAAGGTGTGGTGCCAGAT-3′

2.5. Allogeneic mixed lymphocyte reaction (MLR)

MLR analysis was conducted according to the manufacturer's instructions (CellTrace™ CFSE Cell Proliferation Kit; Invitrogen). Briefly, a 5 mM stock solution of CFSE was prepared immediately prior to use by dissolving the contents of a vial of Component A in 18 μL of DMSO (Component B). Peripheral blood monocytes (PBMCs) were isolated from heparinized whole blood by standard gradient centrifugation with Ficoll–Hypaque and harvested from the interface, washed twice, resuspended in pre-warmed PBS/0.1% bovine serum albumin (BSA) at a final concentration of 1×10^6 cells/mL, and 1 μL of the 5 mM stock CFSE solution was added to 1 mL of cells. The mixture was incubated at 37 °C for 15 min. The solution was quenched by addition of five volumes of ice-cold culture media to the cells. The washed PBMCs were then cultured for 5 days alone, or with 20 μg/mL of concanavalin A (Con A) or co-cultured with DCs. The decay in CFSE fluorescence was measured using a flow cytometer at an excitation of 488 nm. To test the fusion hybrids, the same experiments were performed in six groups, in which varying additional anti-canine MHC I or -canine MHC II monoclonal antibodies were added.

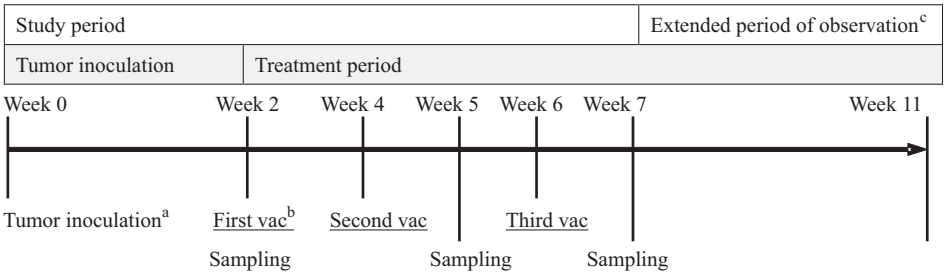
2.6. Preparation of the fusion hybrid vaccine

Tumor cell suspensions were prepared as described previously (Hsiao et al., 2004). The freshly prepared tumor cell suspension was suspended in RPMI 1640 containing 10% FCS and incubated at 37 °C for 2 h prior to fusion, and 5×10^7 tumor cells were then fused with 1.5×10^8 mBMDCs using polyethylene glycol (PEG; m.w. 1450)/50% (w/v) concentration/DMSO solution (Sigma) (Gong et al., 1997). After removal of the medium, the cell pellets were re-suspended by repeated pipetting, and 1 mL of PEG was then slowly added to the pelleted cells while stirring for 2 min. Another 10 mL of serum-free medium were slowly added to the cell suspension over the next 5 min with stirring, and the cells were then centrifuged and re-suspended in RPMI 1640/10% FCS containing 20 ng/mL of IL-4 and GM-CSF and cultured for 3 days. To monitor fusion efficiency, tumor cells were stained with CFSE (5 mM; Invitrogen) and mBMDCs with CMTPX (5 mM; CellTracker™ Red, Invitrogen) before fusion. Fusion rates and phenotypes were determined using flow cytometry. The suspension of fused cells was wet-mounted on slides and examined under a fluorescent microscope (Eclipse TS100, Nikon, Tokyo, Japan).

2.7. Vaccination schedule

The vaccination schedule was as shown in Table 3. Before vaccination, 1×10^8 viable tumor cells were inoculated subcutaneously and bilaterally at each of the six sites on the back to produce tumors in all six dogs, three of which were used as unvaccinated controls. Fusion hybrid vaccines (1×10^8 fusion cells) treated with 15 μg/mL of mitomycin C for 1 h were then washed three times and vaccinated into the three experimental dogs near the bilateral axillary and inguinal lymph nodes at weeks 2, 4, and 6 after tumor inoculation, as described in Table 3.

Table 3
Experimental design of fusion hybrid testing in CTVT beagles.



[a] 10^8 CTVT cells were inoculated bilaterally into the back region at a total of six sites.
[b] 10^8 hybrid cells were injected subcutaneously near the bilateral axillary and inguinal lymph nodes.
[c] A 1-month observation period was completed, during which regular blood and physical examinations were performed in order to evaluate possible toxicity.

Tumor volume was calculated every week after inoculation using the following formula (Hsiao et al., 2004): length × width × height × $\pi/4$. To evaluate the immune response, one tumor was surgically removed from each dog at weeks 2, 5, and 7; the week 2 data were considered as the baseline immune activity. The clinical toxicity of the vaccine was studied by physical and blood examination every week and monitored until week 11.

2.8. Isolation of tumor infiltrating lymphocytes (TILs)

TILs were separated as previously described (Hsiao et al., 2004). Briefly, tumors were mechanically crushed with stainless steel mesh and filtered once through two pieces of gauze (pore size: 190 μm). Then, 8 mL of the cell suspension were overlaid on 4 mL 42% Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden) gradient and centrifuged at 820 g and 4 °C for 25 min, after which TILs deposited at the bottom were collected and washed three times for subsequent FACs staining.

2.9. Nature killer cell activity

Natural killer (NK) cell activity was measured using the CytoTox 96® non-radioactive cytotoxicity assay (Promega, Madison, WI, USA). CTAC target cells (2×10^3 in 50 μL) (European Collection of Cell Cultures, Salisbury, Wiltshire, UK) were seeded into each well of a 96-well flat-bottom plate and incubated overnight at 37 °C, whereupon they were ready for use in the NK cell activity assay. PBMCs isolated from each animal were used as effector cells (*E*) and were co-cultured with target cells (*T*) at *E*/*T* ratios of 50/1, 25/1, 12.5/1, 6.25/1 and 1/1. After 16 h of incubation at 37 °C, culture medium was harvested for analysis of lactate dehydrogenase (LDH) production at an absorbance of 490 nm on an ELISA reader. Percent cytotoxicity was calculated as (experimental – effector spontaneous – target spontaneous)/(target maximum – target spontaneous) × 100%.

2.10. CTL reactions

Isolated PBMCs (3.2×10^6 cells in 1 mL) were added to each well of a 24-well plate and co-cultured for 6 days in RPMI 1640 containing 10% FCS, 20 IU/mL of recombinant human IL-2 (Aldesleukin, Chiron B.V., Amsterdam, The Netherlands), and tumor cells (0.8×10^6 in 1 mL) pretreated with 15 μg/mL of mitomycin C, 1 mL of medium being replaced with 1 mL of fresh medium every day. The cells were then harvested and isolated using the Ficoll–Hypaque method to obtain effector cells. Fresh CTVT cells were used as the target cells and were seeded in 96-well flat-bottom plates at a density of 2×10^3 cells in 50 μL per well. Effector cells were then co-cultured with the target cells at *E*/*T* ratios of 50/1, 25/1, 12.5/1, 6.25/1 and 1/1. After 6 h of incubation, the culture medium was harvested for analysis of LDH production using the CytoTox 96® non-radioactive cytotoxicity assay (Promega) (Huang et al., 2008) as described above. Percent cytotoxicity was calculated as (experimental – effector spontaneous – target spontaneous)/(target maximum – target spontaneous) × 100%.

2.11. Enzyme-linked immunosorbent spot (ELISPOT) assay

ELISPOT assay was performed using a Canine IFN-γ Development Module kit (R&D System) according to the manufacturer's instructions. PBMCs were prepared as described for the CTL reactions. Harvested PBMCs (1×10^5 in 100 μL) were then mixed with 100 μL of tumor lysate (50 μg of protein/mL) in each well of a 96-well filtration plate (Multiscreen™ HTS) previously coated with capture antibodies (1:60 dilution). Medium alone and PBMCs only were used as negative controls and PBMCs plus 20 μg/mL of Con A was used as a positive control. After incubation overnight at 37 °C, color was developed using the streptavidin–alkaline peroxidase and BCIP/NBT provided in the ELISPOT kits. The number of spots was counted manually under dissection microscopy, calculated as test sample spots (PBMCs + tumor lysate) – spots with PBMCs alone – spots with medium alone.

2.12. Statistical analysis

The tumor growth curves, hematological parameters, and expression of MHC I and MHC II at different times in the unvaccinated and vaccinated groups were analyzed by one-way ANOVA and the least significant difference

(LSD) *t*-test. Differences were considered statistically significant at $p < 0.05$. All the other results are expressed as the mean (plus standard deviation) and were analyzed using a two-tailed Student's *t*-test, differences being considered statistically significant at $p < 0.05$.

3. Results

3.1. Preparation of mature bone marrow-derived dendritic cells

We demonstrated that dendritic cells were able to be isolated from bone marrow, with a recovery rate of around 20 times greater than that achieved from peripheral blood (Table 4). As the iBMDCs were matured by stimulation with LPS, the morphologic features of the dendritic processes became longer or more prominent (Fig. 1A). In addition, we determined the essential co-stimulating molecules on the mature BMDCs (mBMDCs) using real-time RT-PCR. Fig. 1B revealed mRNA levels corresponding to CD-80, -83 and -86 that were significantly elevated relative to iBMDCs ($p < 0.01$).

3.2. Phenotype and functional analysis of mature BMDCs

Investigating the phenotype of mature BMDCs, we found that greater than 60% of the mBMDCs expressed CD11c, MHCII, CD1a and CD40 molecules by flow cytometry (Fig. 1C). It was further found by measurement of dextran uptake ability that mBMDCs had a lower antigen uptake ability than iBMDCs (Fig. 1D); however, in mixed lymphocyte reaction experiments, they exhibited a greater antigen-presenting ability relative to iBMDCs (Fig. 1E). These characteristics are consistent with the ideal conditions for the preparation of a hybrid vaccine, in that mature dendritic cells need to maintain a high antigen-presentation ability.

3.3. Characterization of fusion between mature BMDCs and CTVT cells

Next, we conducted fusion between mature mBMDCs containing the red fluorescent dye CMTPX and CTVT cells containing the green fluorescent dye CFSE. After fusion, fluorescent microscopic photographs were taken of the same given area of the fused cell suspension, successful fusion of the hybrids being shown as orange (Fig. 2A, right). The fusion rate, as determined by calculating the percentage of double-positive cells using flow cytometry, reached an average of $60 \pm 5.29\%$ (Fig. 2B). Fusion was also confirmed by phenotyping using flow cytometry, in which the rate of $MHC^+/CD11c^-/CD1a^-/CD40^-$ was intermediate between those of mBMDC ($MHC^{high}/CD11c^+/CD1a^+/CD40^+$) and CTVT cells ($MHC^{lo}/CD11c^-/CD1a^-/CD40^-$) (Fig. 2C).

3.4. Alteration in antigen-presenting ability following fusion

We attempted to define the MHC dependency involved in antigen presentation before and after the fusion process. The results (Fig. 2D) showed that the MLR of the allogeneic fusion hybrids was reduced by addition of anti-MHC I neutralizing antibodies but not by anti-MHC II antibodies. On the contrary, the MLR of the allogeneic mBMDCs not fused with tumor cells was significantly reduced only by the addition of anti-MHC II neutralizing antibodies. These results imply that the ability of allogeneic mBMDCs to present antigen to T lymphocytes is associated with MHC II molecules, whereas, after the fusion process, antigen presentation switched to the MHC I pathway. These findings suggest that cross-priming activity was promoted by the fusion process in the allogeneic fusion hybrids.

3.5. Vaccination of mBMDC/CTVT hybrid suppressed tumor growth

Because CTVT has the ability to spontaneously regress, we employed this model to examine whether the prepared tumor vaccine could prevail over the endogenous immunity. The hybrid vaccine was injected three times at two-week intervals in weeks 2, 4 and 6 into beagles that had been inoculated with CTVT cells at week 0. Average CTVT growth was significantly attenuated ($p < 0.05$) in the vaccinated group as compared with unvaccinated animals, beginning at week 4, upon the 2nd vaccination (Fig. 3A). The tumors in the unvaccinated dogs grew continuously until week 7 before regression, reaching a size of around 174 cm^3, as compared with 52 cm^3 in the vaccinated dogs (Fig. 3B). In the vaccinated group, tumor regression was initiated as early as week 5, 2 weeks earlier than the spontaneously occurring regression in unvaccinated dogs in week 7.

3.6. Enhanced MHC expression of the tumor and histopathologic changes over time following vaccination

As shown in Fig. 3C, the MHC expression of the tumors increased dramatically in the vaccinated group over time in comparison with the unvaccinated beagles. The histopathologic findings in the unvaccinated and vaccinated dogs were characterized. Before vaccination, we observed a high density of sheets of tumor cells in both groups (Fig. 4A, right and left panels). The tumor cells were round to ovoid in shape with a large nucleolus and prominent vacuolated cytoplasm. At week 7 (after the 3rd vaccination), heavy accumulation of TILs (Fig. 4B, upper right panel) and multiple necrotic foci (Fig. 4B, lower right panel) throughout the tumor masses were observed in the vaccinated animals, while in the unvaccinated group these two lesions were much less prominent (Fig. 4B, upper left). We then examined the changes in the subpopulations of TILs, and observed increases in the percentages of $CD3^+$, $CD4^+$, $CD8^+$, and $CD21^+$ cells in the vaccinated group at week 7, while only a low number of TILs were seen in the unvaccinated group ($p < 0.01$; Table 5).

3.7. Induction of adaptive and innate immune responses by vaccination

To investigate the adaptive immune response, CTL and ELISPOT assays were performed, which showed that

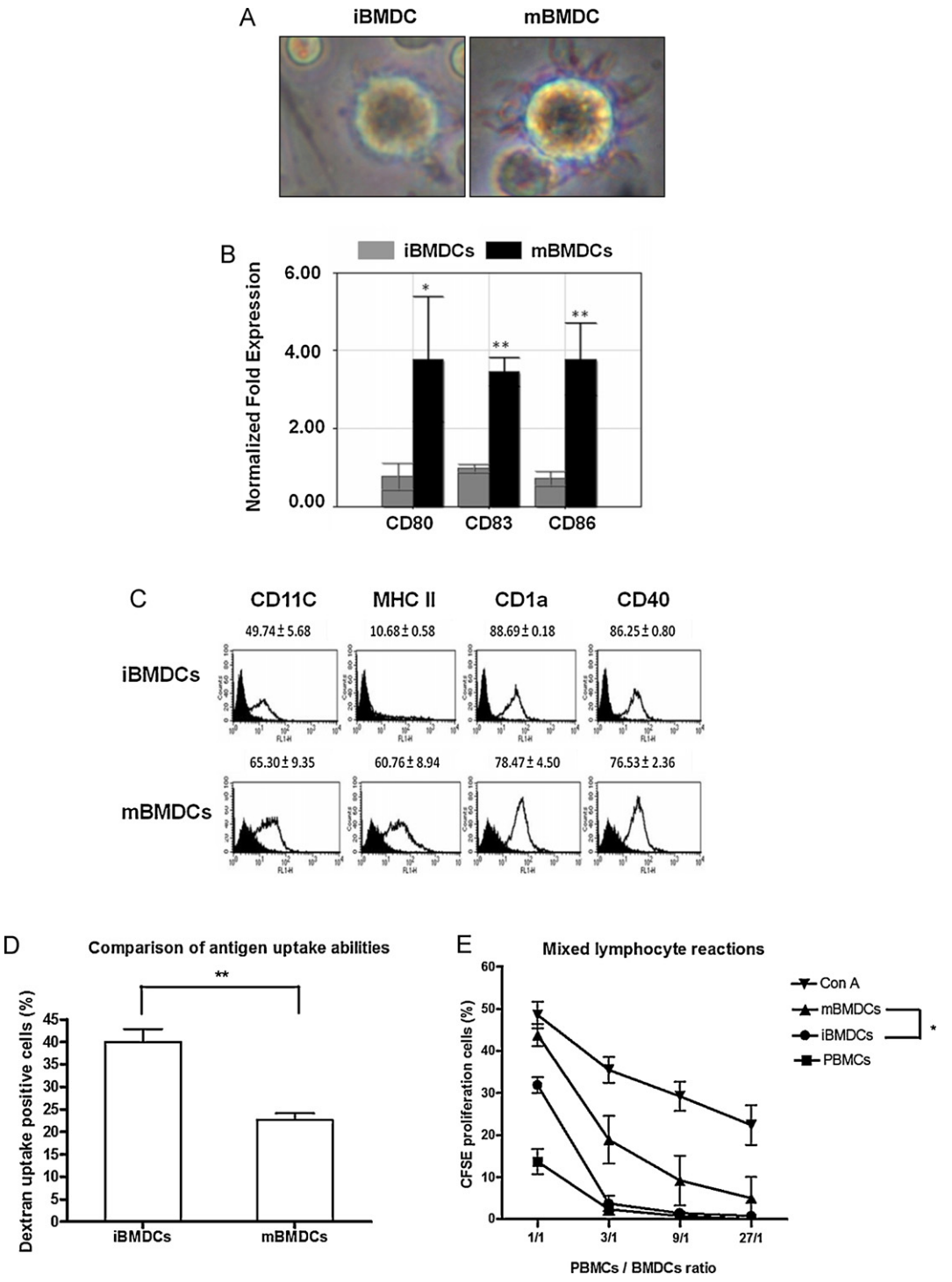


Fig. 1. Generation of bone marrow-derived dendritic cells. Bone marrow-derived dendritic cells (BMDCs) were generated using medium containing 20 ng/mL of IL-4 and GM-CSF. Immature BMDCs (iBMDCs) and mature BMDCs (mBMDCs) were harvested on day 7 and 10, respectively. (A) Phase contrast microscope image showing the morphology of the iBMDCs (left) and mBMDCs (right). (B) Specific primers were used to perform real-time RT-PCR for CD80, CD83, and CD86 in BMDCs. (C) Surface phenotypes were determined using a FACSCalibur^{TM} flow cytometer and the percentage of positive cells were shown. Antibodies against canine CD11c, MHCII, CD1a, or CD40 were used to stain iBMDCs (top row) and mBMDCs (bottom row). (D) Antigen uptake activity was investigated by dextran uptake assay. (E) Allogeneic carboxyfluorescein succinimidyl ester (CFSE)-labeled PBMCs were co-cultured with iBMDCs and mBMDCs for 5 days to evaluate the mixed lymphocyte reaction (MLR). PBMCs incubated alone or with 20 μg/ml of Con A served as the negative and positive control, respectively. The data in B–E represent the means ± SD of four independent cultures; * indicates $p < 0.05$ and ** indicates $p < 0.01$.

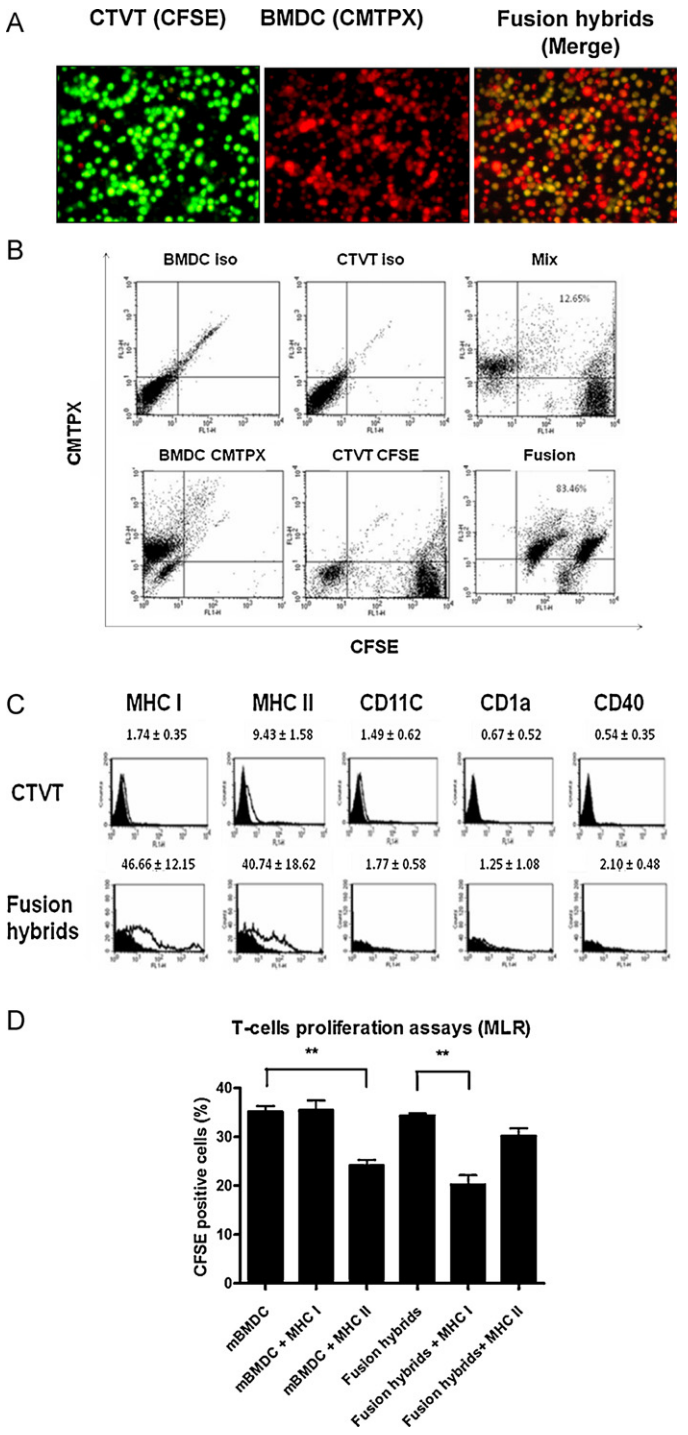


Fig. 2. Fusion rate and functional assay of the fusion hybrids. Freshly isolated tumor cells were fused at a 1:3 ratio with bone marrow derived dendritic cells (BMDCs) using polyethylene glycol, and the fusion cells were then cultured for 3 days. (A) To determine the fusion rate, the tumor cells were labeled with CFSE (green) and the BMDCs with CMTPX (CellTracker™ red). Fused cells then appeared as orange (right panel). The fusion hybrids were examined under different filters on a fluorescent microscope. (B) The fusion rates were determined using a FACSCalibur™ flow cytometer, as shown by the dot plot. (C) The phenotypes of the CTVT cells (top row) and fusion hybrids (bottom row) were determined by staining with a control isotype (black peaks) or antibodies against MHCI, MHCII, CD11c, CD1a, or CD40 (white peaks) and flow cytometry. (D) To verify the presentation of MHC I or MHC II molecules, CFSE-labeled PBMCs were cultured with mBMDCs plus neutralizing anti-MHC II antibody, mBMDCs plus neutralizing anti-MHC I antibody, fusion hybrids plus neutralizing anti-MHC I antibody, and fusion hybrids plus neutralizing anti-MHC II antibody. The data represent the means ± SD of three independent experiments; ** indicates $p < 0.01$.

Table 4
Yield efficiency comparison between bone marrow- and peripheral blood-derived dendritic cells in beagles.

Subject	Spiration (mL)	MNC ($\times 10^7$)	Cells obtained ($\times 10^7$)	Yield efficiency ($\times 10^7$/mL)
BMDCs	10	41.93 (12.28)[a]	41.06 (13.74)[a]	4.1 (1.37)*
PBDCs	10	2	0.08	0.008

BMDCs – bone marrow derived dendritic cells; PBDCs – peripheral blood derived dendritic cells.
[a] Mean % (standard deviation).

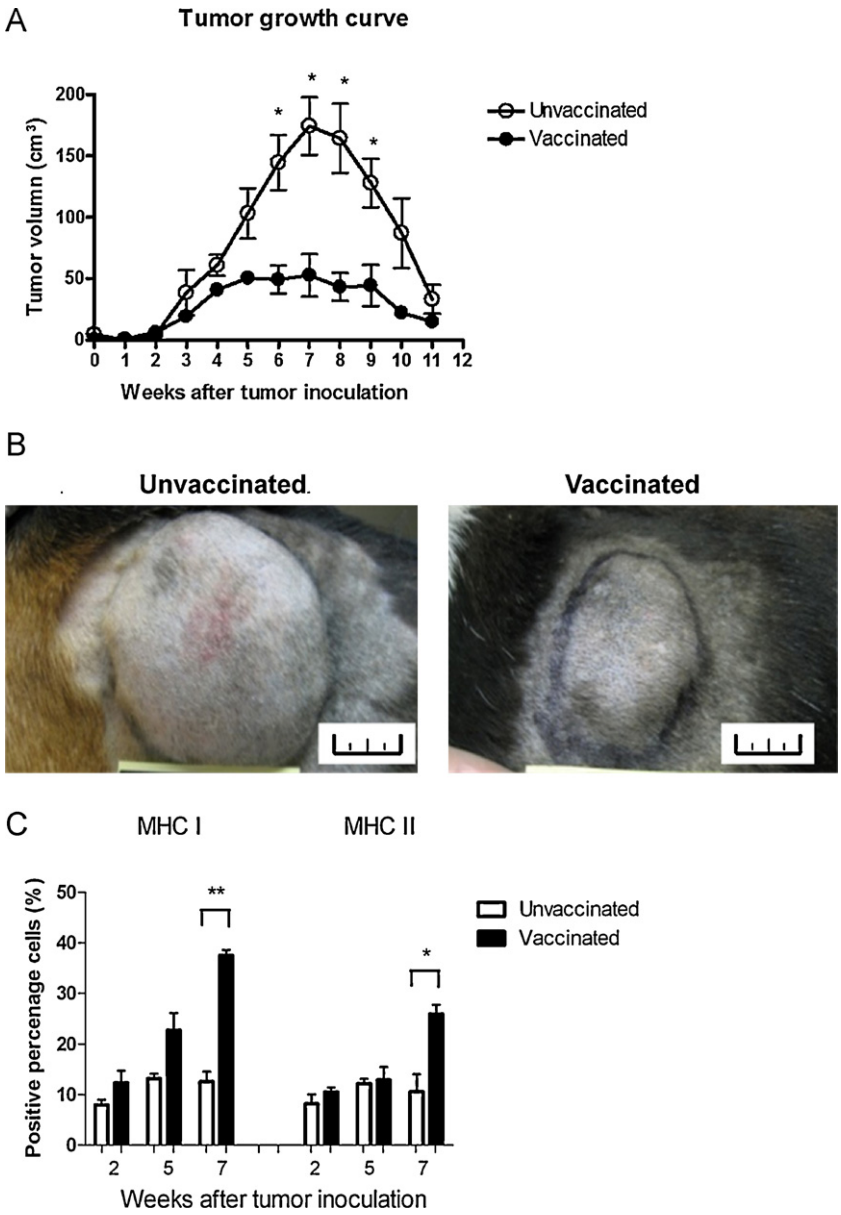


Fig. 3. Clinical evaluation of fusion hybrid vaccinations in the CTVT model. The vaccination schedule is shown in Table 3. Tumor cells were injected bilaterally and subcutaneously into the back of six dogs at six locations. The vaccines were then administered three times at 2-week intervals to three beagles after tumor inoculation. (A) Tumor volume, calculated as $L \times W \times H \times \pi/4$ (L: length; W: width; H: height), was measured each week. The panel shows the tumor growth curves for the vaccinated and unvaccinated dogs. (B) Tumor size at week 7 after tumor inoculation. The original bars represent 2 cm. (C) One tumor was surgically removed from each of the six dogs at week 2, 5, and 7 after tumor inoculation and the MHC expression on the tumor cells was measured as described in Section 2. All results were analyzed by one-way ANOVA and the least significant difference (LSD) t-test; * indicates $p < 0.05$; ** indicates $p < 0.01$.

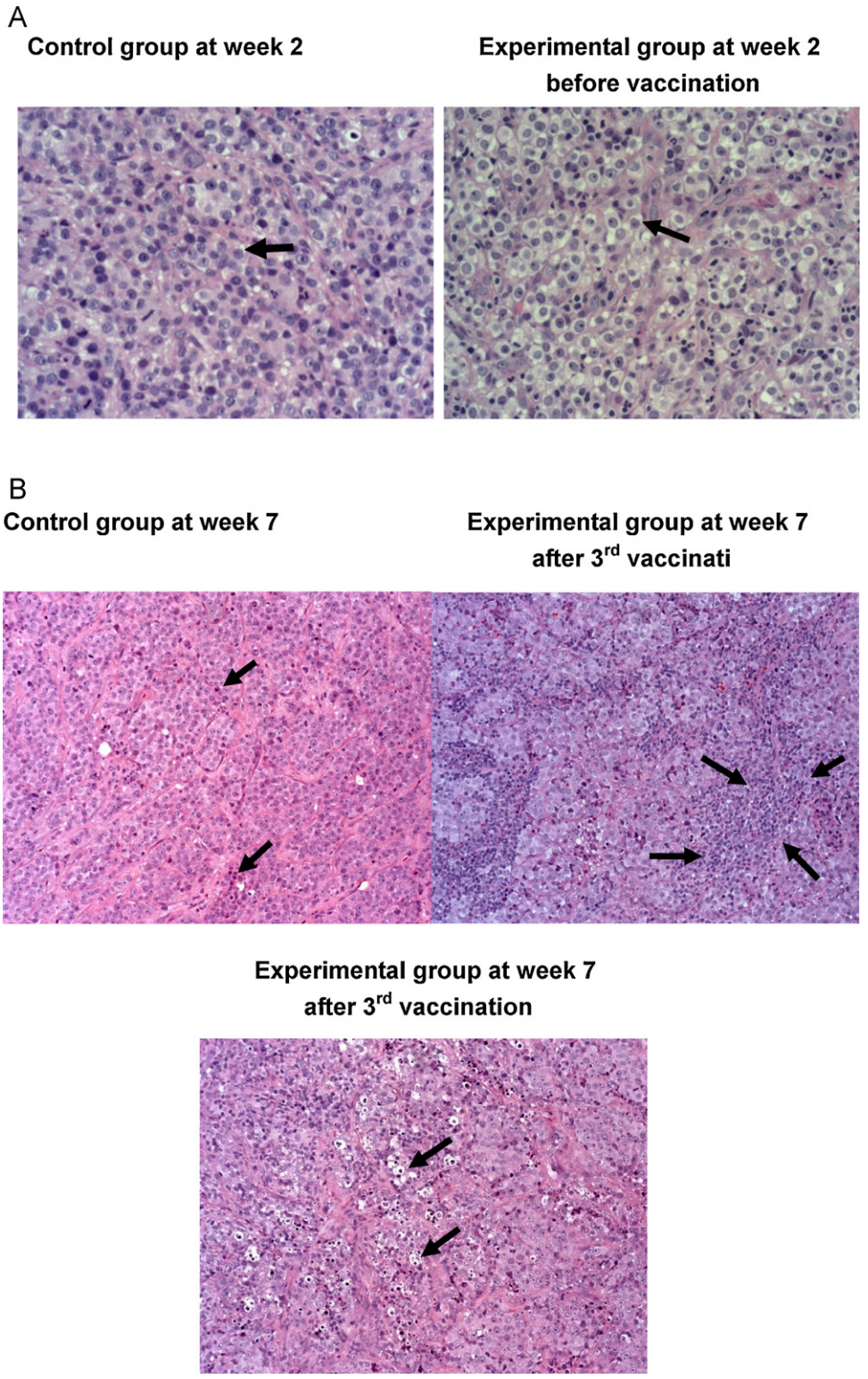


Fig. 4. Histopathologic examination. Formalin-fixed and paraffin-embedded 4-μm tumor sections from unvaccinated and vaccinated dogs. (A) Before vaccination, high densities of tumor cells with large nucleolus and prominent vacuolated cytoplasm were observed in unvaccinated (left, arrow) and vaccinated dogs (right, arrows). (B) After the 3rd vaccination, unvaccinated dogs exhibited a limited number of lymphocytes (upper left, arrow), while in vaccinated dogs, focal aggregations of lymphocytes (upper right, arrows) and focal necrotic foci (lower, arrows) were observed at week 7. H&E stain, 200× and 400×.

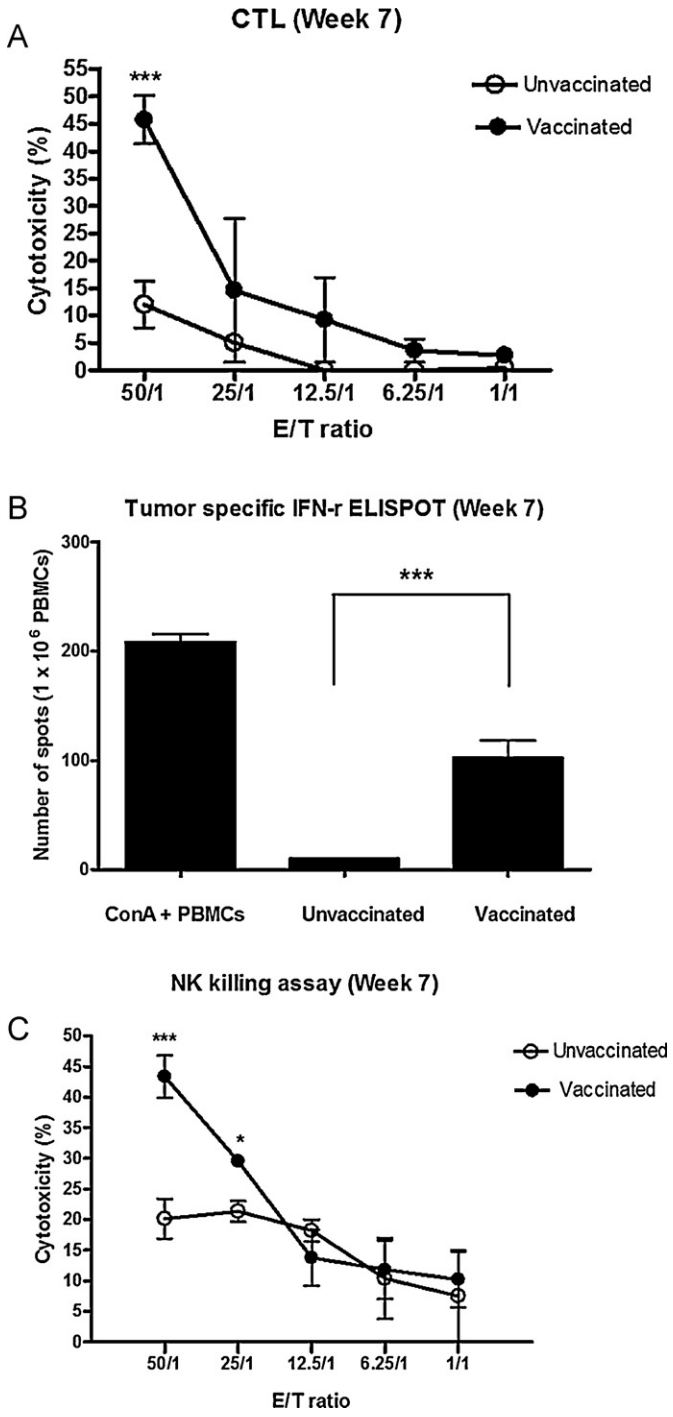


Fig. 5. Immune responses after mBMDC/CTVT fusion hybrid vaccination. The innate and adaptive immune responses were evaluated at week 2, 5, and 7 after tumor inoculation. The data presented are those obtained in the 7th week after tumor inoculation. To monitor adaptive immunity, isolated PBMCs were cultured with 15 μg/ml mitomycin C-treated tumor cells in medium containing 20 IU/ml of IL-2 for 6 days, then the tumor cells were removed using Ficoll and centrifugation. (A) A cytotoxic T lymphocyte (CTL) assay was then performed using a CytoTox 96® Non-radioactive Cytotoxicity assay kit, and (B) ELISPOT assay was performed to detect tumor-specific IFN-γ producing cells. The data were analyzed using a two-tailed Student's *t*-test; * denotes $p < 0.05$ and *** $p < 0.001$ compared with the unvaccinated dogs. (C) Isolated PBMCs were incubated with CTAC cells at 37 °C overnight and NK cell cytotoxicity was measured by LDH release assay using a CytoTox 96® Non-radioactive Cytotoxicity assay kit. PBMCs – peripheral blood monocytes; CTAC – canine thyroid adenocarcinoma cell line.

Table 5
Surface-marker staining of tumor infiltrating lymphocytes.

Surface marker	Non-vaccinated (%)	Vaccinated (%)
CD3	0.19 ± 0.05	16.12 ± 3.99
CD4	0.07 ± 0.03	13.55 ± 3.35
CD8	0.08 ± 0.02	8.9 ± 2.25
CD21	0	3.88 ± 0.75

There are highly significant differences ($p < 0.01$) in all markers (except CD21) between two groups.

the vaccine was able to induce a potent CTVT-specific CTL response (Fig. 5A). It was also demonstrated that vaccination increased the number of tumor-specific IFN-γ-secreting lymphocytes (Fig. 5B). Interestingly, less than 40% of the tumor cells expressed MHC I molecules after the vaccination program, suggesting that the remaining MHC I-negative tumor cells might have been targeted by NK cells. To investigate this possibility, we measured the NK cell activity and found it to have been stimulated significantly by the vaccinations (Fig. 5C). Together, in this experimental CTVT model, the allogeneic mBMDC fusion vaccine was efficient in activating both adaptive and innate immune responses, and the activated immune responses that correlated with tumor growth suppression probably played essential roles in expediting the regression process after tumor inoculation.

3.8. Adverse effects and hematologic toxicity

General physical and blood examinations were performed every week over the experimental period. All unvaccinated and vaccinated dogs appeared to be in normal health with a good appetite. As for the hematologic toxicities (Table 6), there is a significant difference in the ALT ($p < 0.05$); however, the numbers remain within the normal range, with no differences in the other parameters.

4. Discussion

Immunosurveillance is one of the important factors in host inhibition of cancer development and subsequent progressive growth (Dunn et al., 2002). Immunotherapy is considered as a useful adjunct therapy for cancers. Small experimental rodents are by far the most commonly used animal models for vaccine therapy; however, the results are not commonly applicable to humans, and an acceptable outcome in a rodent model is not necessarily indicative of clinical success of vaccination therapy in humans (Casal and Haskins, 2006). Thus, a model of another animal species with a similar genomic background to humans could enable the acquisition of useful reference values with respect to immunotherapy. Canine species have a genomic makeup much closer to that of humans than mice (Joy et al., 2006; Tsai et al., 2007; Uva et al., 2009). In addition, during their life span, dogs can develop many types of cancers that are similar to those that affect human beings (Bronden et al., 2009; Clemente et al., 2010; Ferreira et al., 2010). Therefore, the establishment of a spontaneous canine tumor model has become essential in order to develop a potential tumor immunotherapy. Owing to the unique property of transferability, CTVT can be injected into canines via a

Table 6
Hematological toxicity evaluation.

Parameter	Unvaccinated					Vaccinated					Reference
	Week 0	Week 2	Week 5	Week 7	Week 11	Week 0	Week 2	Week 5	Week 7	Week 11	
BT (°C)	38.6 ± 0.2	38.73 ± 0.15	38.7 ± 0.2	38.8 ± 0.1	38.73 ± 0.15	38.66 ± 0.2	38.73 ± 0.15	38.63 ± 0.05	38.77 ± 0.15	38.77 ± .15	38.5–39.5
Hemoglobin (g/dL)	14.8 ± 0.85	14.1 ± 2.36	14.43 ± 1.85	13.03 ± 3.08	15 ± 0.95	15.2 ± 3.07	15.53 ± 2.23	15.9 ± 1.3	16.63 ± 1.26	16.9 ± 2.09	12–18
WBC ($10^3/\mu L$)	13666.66 ± 5310	12066.66 ± 6924	12233.33 ± 3592	17350 ± 4737	14300 ± 6841	10933.3 ± 3707	8933.33 ± 1331	9433.33 ± 2218	8866.67 ± 1026	10333.33 ± 1450	6000–17,000
ALT (U/L)*	33.33 ± 8.5	35 ± 5	28.33 ± 2.08	31 ± 3.60	28.3 ± 3.78	44 ± 7.93	33 ± 13.52	26.33 ± 10.06	16.67 ± 2.88	27.33 ± 10.96	4–66
BUN (mg/dL)	15.33 ± 7.76	10.66 ± 2.88	11.66 ± 3.78	11 ± 1	15 ± 3.60	12.67 ± 3.21	15.33 ± 2.88	16.33 ± 4.93	20 ± 7	17.33 ± 5.5	5–28
Albumin (g/dL)	3.46 ± 0.05	3.33 ± 0.45	3.4 ± 0.73	3.23 ± 0.66	3.267 ± 0.2	3.4 ± 0.1	3.33 ± 0.15	3.367 ± 0.05	3.1 ± 0.346	3.33 ± 0.2	2.3–3.9
Glucose (mg/dL)	95.66 ± 12.09	95.66 ± 5.13	82.33 ± 5.03	86.33 ± 4.50	102.67 ± 7.37	91.67 ± 11.54	88.33 ± 15.5	87 ± 6.08000	89.67 ± 14.57	87 ± 4.58	67–147

Week 0 was regarded as the baseline hematologic profile.
All the data was collected from three unvaccinated and vaccinated dogs, representing the means ± SD.
The data was calculated by one-way ANOVA between unvaccinated and vaccinated dogs at different weeks and there is a significant difference in the parameter of ALT.

subcutaneous or intra-organ route to allow evaluation of a given tumor therapy (Ahrar et al., 2010; Chou et al., 2009; Chuang et al., 2009; Schwartz et al., 2009). The immunologic interactions between host and CTVT involving TGF-β, IL-6, IFN-γ, MHC expression and DC activities render this tumor a reasonable model for study of the immunologic interaction between host and cancers (Hsiao et al., 2004; Liu et al., 2008). However, the characteristic of spontaneous regression is a critical issue that affects whether this model is informative in terms of evaluating immunotherapy. As mentioned above, the host immune response in the CTVT model is associated with self-regression (Hsiao et al., 2004, 2008), but it may be difficult to distinguish whether the resulting regression is derived from a spontaneous host immune response or from vaccination during vaccine therapy.

Recently, a DC-based tumor vaccine has been widely studied and is believed to have a great potential for use in tumor treatment. The peripheral blood-derived DC/tumor cell vaccine production strategy has been used in human trials in phase I/II studies (Avigan et al., 2004, 2007; Haenssle et al., 2004); however, the problem of the limited amount of DCs able to be generated from peripheral blood in canines must be resolved. Therefore, in this study, we prepared DCs from bone marrow rather than peripheral blood. Multiple vaccinations are usually needed in DC vaccine protocols in order to elicit or boost high immune responses, and the large quantity of healthy BMDCs obtained in this study was suitable for clinical application, providing a solution to this issue. In comparison with iBMDCs, the mature BMDCs (MHC $II^+/CD11c^+/CD40^+/CD1a^+$ DCs) prepared in this study displayed a greater antigen-presenting ability and increased T-lymphocyte proliferation.

After fusion with CTVT cells, three injections were administered at two-week intervals, and the vaccine successfully inhibited tumor growth and enhanced anti-tumor immunity, including adaptive and innate immunities. Further, the fusion process have caused the switch of antigen presentation from MHC II-dependence to an MHC I pathway. Cellular immunity, including increased tumor-specific CTL and IFN-γ producing cells, was greatly enhanced by the vaccination protocol (Fig. 5). In addition to the elevated NK cytotoxicity and MHC expression, the tumor was forced to enter regression much earlier than in spontaneous immunity. The results indicate that this vaccine therapy exhibited an ability to promote host immune responses and prevail over the spontaneous host immune effect on CTVT, and also suggested that CTVT is a useful model for evaluating the potential of tumor immunotherapy.

To further discuss the treatment immune response, we need to clarify the possible immunity trigger by allogeneic DC/tumor hybrids. Because a high degree of MHC homozygosity and restricted representation of DLA alleles are present in dogs (Kennedy et al., 2002; Wagner et al., 2002), the allogeneic source of DCs from healthy donors and tumor-bearing recipients may still share some syngeneic membrane recognition motifs. Thus, this fusion vaccine might confer the following advantages: (a) in terms of allogeneity, the presumably enhanced recognition of TAA (tumor associated antigen) is due to alloreactive T cells, which recognize similar MHC molecules (molecular mimicry) on mBMDCs (Archbold et al., 2008) and can activate the corresponding CD4 or CD8 T lymphocytes. This allogeneic recognition can induce a cytokine storm and instigate the release of high levels of pro-inflammatory cytokines, such as IL-1β, TNF-α, IL-6, and IFN-γ, thereby pushing immunity towards Th1 responses (Laurin et al., 2004). (b) In terms of syngeneity, the donor DCs were able to recognize the self-motif on the recipient's T cells; thus, the TAA and allogeneic peptides presented by the MHC molecules on the donor DCs should be recognized by host T cells as foreign antigens and elicit immune responses. All of the above-described immune reactions might facilitate T cell activation and restore immune surveillance, leading to specific killing of the tumor cells, as demonstrated in this work. This suggests that even though the regression of CTVT is highly related to the host immune response, we are nevertheless able to observe the effects of vaccine treatment via the activation of immunity.

Another feature of CTVT is that its MHC expression may correspond to tumor growth. Lowering the expression of MHC molecules is one of key mechanisms that CTVT cells employ to evade immune surveillance. In our model, fewer than 5% of the progressive tumor cells were MHC I molecule-positive, while up to 40% of the cells expressed MHC molecules as the tumor reached regression. This up-regulation of MHC molecules usually appears a few weeks before tumor regression (Hsiao et al., 2002). Our previous study using the IL-6 gene in *pIL-6*/*pIL-15* gene therapy also supports the idea that MHC expression is essential for tumor suppression (Chou et al., 2009). The mBMDC/CTVT hybrid vaccinations significantly increased the expression of MHC I molecules, from less than 12 to 38%, which should be an important factor in suppressing the tumor growth. However, the remaining MHC I-negative tumor cells must also be killed in order to efficiently inhibit tumor growth, the best candidate for which is NK cell cytotoxicity (Terme et al., 2008). The increased NK activity caused by the hybrid vaccinations as demonstrated in this study also provides a possible explanation. The characteristics of tumor growth corresponding to MHC expression may be important indicators to consider when evaluating therapies in this model.

In conclusion, we have demonstrated that although CTVT can spontaneously regress, it is nevertheless still a suitable model in which to evaluate immunotherapies in canine tumors. As there is an increasing requirement for a canine model between human and mouse clinical investigations, the regimen used in this study may provide a feasible and potential model for the study of tumor vaccines.

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