

Comparison of Dendritic Cell-Mediated Immune Responses among Canine Malignant Cells

Kyoichi TAMURA¹⁾, Hiroyoshi ARAI¹⁾, Emi UENO¹⁾, Chie SAITO¹⁾, Hiroko YAGIHARA¹⁾, Mayu ISOTANI¹⁾, Kenichiro ONO²⁾, Tsukimi WASHIZU¹⁾ and Makoto BONKOBARA^{1)*}

¹⁾Department of Veterinary Clinical Pathology, Nippon Veterinary and Life Science University, 1-7-1 Kyonan-cho, Musashino-shi, Tokyo 180-8602 and ²⁾Department of Veterinary Clinical Pathobiology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

(Received 8 February 2007/Accepted 18 May 2007)

ABSTRACT. Dendritic cell (DC) vaccination is one of the most attractive immunotherapies for malignancies in dogs. To examine the differences in DC-mediated immune responses from different types of malignancies in dogs, we vaccinated dogs using autologous DCs pulsed with keyhole limpet hemocyanin (KLH) and cell lysate prepared from squamous cell carcinoma SCC2/88 (SCC-KLH-DC), histiocytic sarcoma CHS-5 (CHS-KLH-DC), or B cell leukemia GL-1 (GL-KLH-DC) *in vitro*. *In vivo* inductions of immune responses against these tumor cells were compared by the delayed-type hypersensitivity (DTH) skin test. The DTH response against SCC2/88 cells were observed in dogs vaccinated with autologous SCC-KLH-DC, while the response was undetectable against CHS-5 and GL-1 cells in dogs vaccinated with autologous CHS-KLH-DC and GL-KLH-DC. Skin biopsies taken from DTH challenge sites were then examined for immunohistochemistry, and recruitment of CD8 and CD4 T cells was detected at the site where SCC2/88 cells were inoculated in dogs vaccinated with SCC-KLH-DC. By contrast, neither CD8 nor CD4 T cell infiltration was found at the DTH challenge site in the dogs vaccinated with CHS-KLH-DC or GL-KLH-DC. These findings may reflect that the efficacy of immune induction by DC vaccination varies among tumor types and that immune responses could be inducible in squamous cell carcinoma. Our results encouraged further investigation of therapeutic vaccination for dogs with advanced squamous cell carcinoma in clinical trials.

KEY WORDS: canine, delayed-type hypersensitivity, dendritic cell, malignancies, vaccination.

J. Vet. Med. Sci. 69(9): 925–930, 2007

Dendritic cells (DCs) are the most potent antigen-presenting cells for initiating cellular immune responses through the activation of naïve T lymphocytes [3, 20], making them the most attractive immunoregulatory cells for tumor immunotherapy. Recently, many clinical trials have been conducted on human patients with various types of tumors to evaluate the feasibility of immunization with DC vaccination, in which autologous DCs are loaded with defined tumor antigen such as synthetic peptide [19] and nucleotide-encoding tumor antigen [10] or whole antigenic preparations such as tumor cell lysate [1]. These DC vaccinations have been proven to be effective for the induction of anti-tumor immunities and clinical responses in patients with several types of malignancies such as lymphoma [13, 22], renal and prostate carcinoma [5, 12], and especially melanoma [4, 11]; however, the effectiveness of DC vaccination varies among tumor types. For example, some types of malignancies such as gastrointestinal malignancies, including colorectal, gastric, and hepatocellular carcinoma, are still not sufficient to show anti-tumor therapeutic effect [6, 17]; nevertheless, the protocol of DC vaccination, including preparation of DC, loading antigen(s) to DC, and route of DC administration, are not yet well standardized to compare with other trials in different types of tumor.

We have previously reported the *in vivo* induction of immune responses against canine malignant melanoma cell CMM2, including delayed-type hypersensitivity (DTH) responses to the cells and recruitment of CD8 and CD4 T cells at the site of the DTH skin test, in dogs vaccinated with autologous bone marrow-derived DCs pulsed with lysate prepared from the cells *in vitro* [21]. The data suggested the potential utility of DC-based tumor vaccination in dogs; however, the differential immune responses by the DC vaccination are postulated among different types of tumors as has been shown in human clinical cases.

To establish the basis for the use of DC vaccination in the treatment of canine malignancies, it is important to examine the differences in DC-mediated immune responses from different types of malignancies. In the present study, we compared the *in vivo* inductions of DC-mediated immune response among three different types (solid, solid hematologic, and hematologic tumor) of canine malignant cell lines, including squamous cell carcinoma, histiocytic sarcoma, and B cell leukemia, by using bone marrow-derived autologous DCs pulsed with lysate prepared from these cells *in vitro*.

MATERIALS AND METHODS

Animals: Beagles (1–6 years old) were housed and cared for in the facility of the Institutional Life Care Use Center at Nippon Veterinary and Life Science University and all the experiments were conducted in accordance with the guide-

* CORRESPONDENCE TO: BONKOBARA, M., Department of Veterinary Clinical Pathology, Nippon Veterinary and Life Science University, 1-7-1 Kyonan-cho, Musashino-shi, Tokyo 180-8602, Japan.
e-mail: bonkobara@nvlu.ac.jp

lines of the university.

Cell lines: The canine squamous cell carcinoma cell line SCC2/88 was purchased from CELLnTEC Advanced Cell Systems (Bern, Switzerland). The canine B cell lymphoma cell line GL-1 [18] was kindly provided by Dr. Munekazu Nakaichi, Yamaguchi University, Japan. The canine histiocytic sarcoma cell line CHS-5 was established previously in our laboratory [2]. The SCC2/88 and CHS-5 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) and GL-1 cells were maintained in RPMI-1640 medium (Nissui Pharmaceutical, Tokyo, Japan). Both media were supplemented with 10% fetal calf serum (Nippon Bio-supply Center, Tokyo, Japan) and 1% penicillin-streptomycin-glutamine (Invitrogen).

Generation of DCs: Dendritic cells were generated from bone marrow cells as described previously [9] with minor modifications. Briefly, after the dogs were anesthetized with medetomidine, an approximate total volume of 0.5 ml bone marrow from the ilium was obtained by aspiration with a syringe containing 2 ml of 16 mM EDTA-2Na/phosphate-buffered saline. Mononuclear cells were isolated from bone marrow aspirations by density gradient centrifugation using Histopaque-1077 (Sigma-Aldrich, St. Louis, MO) spun at $500 \times g$ at 22°C for 30 min. After washing with phosphate-buffered saline (PBS), the cells were seeded in RPMI-1640 culture medium (Nissui Pharmaceutical) supplemented with 10% fetal calf serum (Nippon Bio-supply Center) and 1% penicillin-streptomycin-glutamine (Invitrogen) at a density of 4.5×10^7 cells per T-150 flask (30 ml) and cultured for 7 days in the presence of recombinant canine GM-CSF and IL-4 (R&D Systems, Minneapolis, MN) at a dose of 20 ng/ml each.

Preparation of tumor cell lysate: Tumor cell lines (3×10^7 cells) were washed and resuspended in PBS, and underwent six cycles of freeze-thawing using liquid nitrogen, followed by sonication. After cell debris was removed by centrifugation at $2,000 \times g$ for 30 min, the protein concentration of the lysate was determined using a Bio-Rad Bradford protein assay kit (Bio-Rad, Richmond, CA).

Pulsing of DCs and vaccination: On Day 6 of culturing, DCs were pulsed with tumor cell lysate (100 $\mu\text{g}/\text{ml}$) prepared from SCC2/88, CHS-5, or GL-1 or the same volume of PBS in the presence of keyhole limpet hemocyanin (KLH, 50 $\mu\text{g}/\text{ml}$; Sigma-Aldrich) to augment DC-mediated T cell response [16]. After 24 hr of pulsing, DCs were harvested, washed, and resuspended in PBS. Vaccination was performed by subcutaneous injection of 1 ml PBS containing $1.4\text{--}3.3 \times 10^7$ of autologous DCs pulsed with KLH and cell lysate prepared from SCC2/88 (SCC-KLH-DC), CHS-5 (CHS-KLH-DC), or GL-1 (GL-KLH-DC) or KLH alone (KLH-DC) four times at intervals of 2 weeks. Three dogs each received SCC-KLH-DC, CHS-KLH-DC, or GL-KLH-DC, and nine other dogs received KLH-DC (Table 1).

Flow cytometric analysis: Before vaccination, antigen-pulsed DCs were stained with mouse anti-canine CD11c (Serotec, Oxford, UK) or isotype-matched control IgG. A fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Cappel, Aurora, OH) was used as a second antibody and the fluorescence intensity was analyzed by a flow cytometer (FACSCalibur; Becton Dickinson, Franklin Lakes, NJ).

Delayed-type hypersensitivity skin test: Seven days after each of the DC vaccinations, delayed-type hypersensitivity (DTH) skin tests were performed on eighteen dogs (Table

Table 1. DTH responses in dogs vaccinated with DCs

Tumor cells	Dog Nos.	Vaccinations	DTH responses ^{a)} against:									
			DC doses ($\times 10^7$)				Corresponding tumor cells				KLH	
			1st	2nd	3rd	4th	1st	2nd	3rd	4th	1-4th	1-4th
SCC2/88	1	SCC-KLH-DC	3.0	3.2	2.9	2.2	10	15	16	20	10-15	0
	2	SCC-KLH-DC	2.2	2.0	1.5	1.4	5	12	20	21	7-13	0
	3	SCC-KLH-DC	2.5	3.2	2.0	1.8	8	16	18	24	8-12	0
	4	KLH-DC	1.9	2.5	2.4	2.0	0	0	0	0	6-9	0
	5	KLH-DC	3.4	3.0	2.8	2.9	0	0	0	0	4-7	0
	6	KLH-DC	2.7	2.7	2.1	1.5	0	0	0	0	4-8	0
CHS-5	7	CHS-KLH-DC	2.8	2.8	2.5	2.4	0	0	0	0	6-8	0
	8	CHS-KLH-DC	3.0	2.9	3.0	2.7	0	0	0	0	7-10	0
	9	CHS-KLH-DC	2.1	2.0	1.5	1.4	0	0	0	0	4-6	0
	10	KLH-DC	3.3	3.0	2.9	2.6	0	0	0	0	3-6	0
	11	KLH-DC	3.0	2.5	2.7	2.4	0	0	0	0	5-7	0
	12	KLH-DC	2.4	2.2	1.8	1.5	0	0	0	0	4-6	0
GL-1	13	GL-KLH-DC	2.2	2.0	2.1	1.9	0	0	0	0	5-10	0
	14	GL-KLH-DC	2.6	2.5	1.9	1.5	0	0	0	0	6-8	0
	15	GL-KLH-DC	3.3	3.0	2.7	2.0	0	0	0	0	4-7	0
	16	KLH-DC	3.1	3.0	2.8	2.8	0	0	0	0	6-10	0
	17	KLH-DC	2.9	2.5	2.0	1.8	0	0	0	0	5-7	0
	18	KLH-DC	2.5	2.6	2.3	2.2	0	0	0	0	5-8	0

a) DTH responses were expressed by the diameter of erythema (mm).

1). PBS (100 μ l) containing SCC2/88, CHS-5, or GL-1 (1×10^6 cells each) was intradermally injected into three dogs vaccinated with SCC-KLH-DC, CHS-KLH-DC, or GL-KLH-DC, respectively. Nine dogs that were vaccinated with KLH-DC were divided into three groups (three dogs each), and each group was injected with SCC2/88, CHS-5, or GL-1 intradermally by the same way. PBS (100 μ l) containing KLH (20 μ g) and that containing autologous peripheral blood mononuclear cells (PBMC) (1×10^6 cells) were also intradermally injected into all 18 dogs as positive and negative controls, respectively. All intradermal injections were given in sites different from the vaccination sites and previous DTH tested sites. Responses to the DTH skin test were evaluated 48 hr after each injection as a diameter of erythema (mm).

Immunohistochemistry: Skin biopsies were taken from the fourth sites challenged by tumor cells in dogs vaccinated with SCC-KLH-DC, CHS-KLH-DC, GL-KLH-DC, or KLH-DC. Ten- μ m cryosections were prepared from the biopsies and stained with mouse anti-canine CD4 (Serotec, Oxford, UK), mouse anti-canine CD8 α (Serotec), or each isotype-matched control IgG, then stained with FITC-conjugated goat anti-mouse IgG, and visualized with a confocal scanning laser microscope (Leica, Wetzlar, Germany).

RESULTS

To confirm the purity of DCs for vaccination, surface expression of CD11c, a marker for DCs, was examined on antigen-pulsed DCs just before each injection. Figure 1 shows representative data of the expression of CD11c on DCs. All DCs pulsed with antigens (SCC-KLH-DC, CHS-KLH-DC, GL-KLH-DC, and KLH-DC) highly expressed CD11c on the surface at the same levels, indicating that the purity of DCs does not vary among vaccinations.

In vivo induction of immune responses to tumor cells was examined by a DTH skin test. Table 1 summarizes the results of DTH responses against tumor cells. Positive DTH

responses against SCC2/88 cells were observed from the first vaccination in all three dogs (Nos. 1–3) that received autologous SCC-KLH-DC, and the strength of the response (diameter of erythema) tended to increase according to the number of vaccinations. Meanwhile, the response against SCC2/88 cells was not observed in dogs that received autologous KLH-DC (Nos. 4–6) through the first to fourth vaccinations, suggesting specific DTH responses against SCC2/88 cells by SCC-KLH-DC vaccination. In contrast to the successful induction of DTH responses against SCC2/88 cells by SCC-KLH-DC vaccination, undetectable DTH responses against CHS-5 and GL-1 cells were observed in dogs that received autologous CHS-KLH-DC (Nos. 7–9) and GL-KLH-DC (Nos. 13–15) from the first to fourth challenges. The dogs vaccinated with autologous KLH-DC also presented negative DTH responses against intradermal injections of CHS-5 cells (Nos. 10–12) and GL-1 cells (Nos. 16–18). Positive and negative reactions were detected in all dogs after each injection of KLH and autologous PBMC, respectively, from the first to fourth intradermal injections. Representative results of the DTH skin test challenged by SCC2/88 cells in dogs vaccinated with SCC-KLH-DC (No. 2) and KLH-DC (No. 4) and of that challenged by CHS-5 and GL-1 cells in CHS-KLH-DC (No. 7) and GL-KLH-DC (No. 13), respectively, are shown in Fig. 2. The erythema of the DTH skin test challenged by SCC2/88 cells in dogs vaccinated with SCC-KLH-DC tended to become more distinct in its color with its diameter enlarging with increasing numbers of vaccinations.

To examine the recruitment of effector lymphocytes to the DTH challenge site, skin biopsies taken from the fourth challenged sites in dogs vaccinated with SCC-KLH-DC, CHS-KLH-DC, GL-KLH-DC, and KLH-DC were examined for immunohistochemistry using antibodies to canine CD8 and CD4. Representative results of dogs vaccinated with SCC-KLH-DC (No. 2), CHS-KLH-DC (No. 7), and GL-KLH-DC (No. 13) are shown in Fig. 3. Infiltration by numerous CD8 $^+$ and CD4 $^+$ T cells at the DTH challenge site

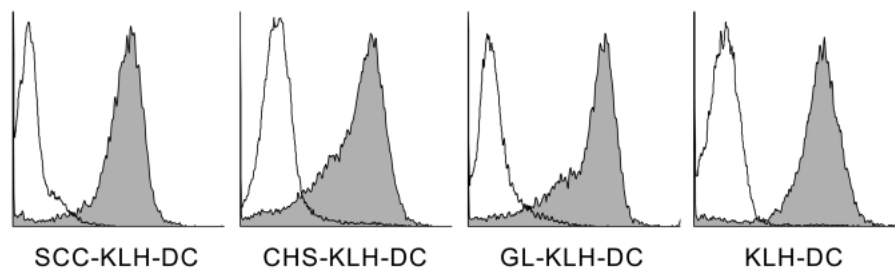


Fig. 1. Surface expression of CD11c by dendritic cells used for vaccinations. Dendritic cells pulsed with keyhole limpet haemocyanin (KLH) and cell lysate prepared from SCC2/88 (SCC-KLH-DC), CHS-5 (CHS-KLH-DC), or GL-1 (GL-KLH-DC) and KLH alone (KLH-DC) were stained with mouse anti-canine CD11c (shaded histogram) or isotype-matched control IgG (open histogram) just before vaccinations (24 hr after pulsing). A fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG was used as a second antibody and the fluorescence intensity was analyzed by a flow cytometer. All dendritic cells used for vaccinations highly expressed CD11c on the surface (more than 90%) and the expression levels did not vary among cell lines pulsed. Data shown are representative results of flow cytometric analyses.

















Vaccinations	Challenges	DTH responses			
		1st	2nd	3rd	4th
SCC-KLH-DC (Dog No. 2)	SCC2/88				
KLH-DC (Dog No. 4)	SCC2/88				
CHS-KLH-DC (Dog No. 7)	CHS-5				
GL-KLH-DC (Dog No. 13)	GL-1				

Fig. 2. Delayed-type hypersensitivity (DTH) skin test. The representative DTH responses challenged by SCC2/88 in dogs vaccinated with autologous SCC-KLH-DC (No. 2) and KLH-DC (No. 4) are shown in the upper two panels. The DTH responses challenged by CHS-5 and GL-1 cells in dogs vaccinated with autologous CHS-KLH-DC (No. 7) and GL-KLH-DC (No. 13), respectively, are shown in the lower two panels. The DTH responses are shown from the first to fourth challenges and white lined circles on the skin indicate the challenge sites. Positive DTH responses were observed as erythema in the dog vaccinated with autologous SCC-KLH-DC from the first to fourth challenges by SCC2/88 cells, and the erythema tended to increase in diameter and become more distinct in its color with increasing numbers of vaccinations. In contrast, no DTH response was observed by challenge with SCC2/88 cells in the dog vaccinated with autologous KLH-DC. Undetectable DTH response was observed in the dog vaccinated with autologous GL-KLH-DC and CHS-KLH-DC by challenge with corresponding tumor cells.

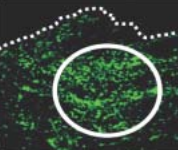

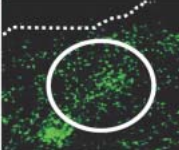
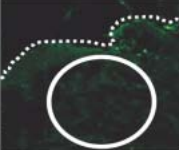


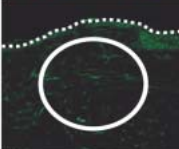
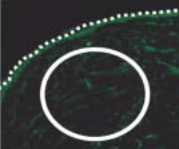




Vaccinations	Challenges	Anti-CD8 α	IgG2a	Anti-CD4	IgG1
SCC-KLH-DC (Dog No. 2)	SCC2/88				
CHS-KLH-DC (Dog No. 7)	CHS-5				
GL-KLH-DC (Dog No. 13)	GL-1				

Fig. 3. Immunohistochemical examination of T cell recruitment into a DTH challenge site. Cryosections of the skin biopsies taken from challenge sites were stained with mouse anti-canine CD8 α , mouse anti-canine CD4, or a control for each isotype IgG (IgG2a and IgG1, respectively), combined with fluorescein isothiocyanate-conjugated goat anti-mouse IgG as a second antibody. The data shown are representative results of dogs Nos. 2, 7, and 13. The white lined circles indicate the points where the tumor cells were inoculated. The epidermal layer is indicated by a dotted white line. Infiltration of CD8 and CD4 T cells into the dermis was observed around SCC2/88 cell-inoculated sites in dogs vaccinated with autologous SCC-KLH-DC. Infiltration of CD8 and CD4 T cells was scarcely detected around corresponding tumor cell-inoculated sites in dogs vaccinated with autologous GL-KLH-DC and CHS-KLH-DC.

of SCC2/88 cells was observed in the dermis of the dogs vaccinated with SCC-KLH-DC (Fig. 3, upper panel), while it was not detectable in dogs vaccinated with KLH-DC (data not shown), suggesting that vaccination by SCC-KLH-DC induces DTH response by eliciting T cell-mediated immunity toward SCC2/88 cells. In contrast, neither CD8⁺ nor CD4⁺ T cell infiltration was present at the DTH challenge site of CHS-5 cells in the dogs vaccinated with CHS-KLH-DC (Fig. 3, middle panel) as well as the site of GL-1 cells in the dogs vaccinated with GL-KLH-DC (Fig. 3, lower panel). No T cell infiltration was found at the DTH challenge sites of CHS-5 or GL-1 cells in dogs vaccinated with KLH-DC (data not shown).

DISCUSSION

In the present study, we showed that DCs loaded with SCC2/88 cell lysate *in vitro* elicit and boost T cell-mediated immunity against SCC2/88 cells *in vivo*. In contrast to SCC2/88 cells, no detectable DC-mediated immune response was elicited against CHS-5 and GL-1 cells *in vivo* despite corresponding cells being used for DC pulsing. The data may suggest that the efficacy of immune induction by DC vaccination is different among tumor types. It has been considered that certain types of tumor cells such as lymphoma, prostate carcinoma, and melanoma are usually antigenic, possibly by expressing highly immunogenic tumor-associated antigens that can be recognized by T cells (e.g., lymphoma, idiotype antigen; prostate carcinoma, PAP and PSMA; and melanoma, MAGE1, tyrosinase, Melan A/MART-1, and gp100/Pmel17) [6]. Conversely, colorectal cancer, which is poorly responsive to immunotherapy, had not been identified with such a highly immunogenic antigen. Although carcinoembryonic antigen, a tumor-associated antigen, is highly expressed by colorectal cancer cells [15], it is known to have poor immunogenicity for inducing potent immune responses [14]. Tumor-associated antigens have been scarcely identified and characterized in canine tumors; however, the different DC-mediated immune responses among canine cell lines examined may reflect such a differential antigenicity and/or expression level of putative tumor-associated antigens expressed by the tumor cells.

Dendritic cell-based immunotherapy applied to patients with SCC is quite uncommon in humans; DC vaccination is performed in patients with cervical cancer (including SCC) by targeting papilloma virus oncoprotein E7 as a tumor-specific antigen [7]. There have been no reports of DC-based immunotherapy in dogs. Because canine SCC has not been found to have viral antigens and other tumor-associated antigens, vaccination with DCs pulsed with whole tumor lysate is feasible for canine SCC cases, and our results suggested that immune responses could be inducible by DC vaccination for tumor cases without identifying particular tumor-associated antigens. Immunogenicity of established cell lines does not always reflect that of spontaneously developed tumors and the same type of tumor may show

varying immunogenicity in different individuals due to the expression of different levels of immunogenic antigens; nevertheless, our results encouraged further investigation of therapeutic vaccination for advanced canine SCC in clinical trials.

In contrast, B cell lymphoma is able to elicit specific immune response and clinical remissions can be induced or maintained by DC vaccination in humans [8, 22], but the induction of immune response totally failed in the B cell line GL-1 in dogs. In these human clinical studies, idiotype protein was used as a source of antigen in DC vaccination. It is considered that B cell whole cell lysate does not contain sufficient amount of idiotype protein to elicit immune responses, and thus, targeting idiotype protein could be required for DC vaccination against canine B cell lymphoma. Regarding histiocytic sarcoma cell line CHS-5, cell lysate used for DC pulsing considered containing insufficient amount and/or antigenicity of putative antigens for priming of DC, and thus pulsing DC with antigenic peptide could be required for priming of DC. Because a candidate for a specific antigen for DC vaccination has not been identified by the tumor, it would be necessary to find immunogenic target antigens and validate its efficacy to elicit immunity before considering clinical trials.

Although it is still necessary to investigate DC vaccination by using other cell lines of the same tumors and examine amount and loading protocols (e.g., fusion of DC with tumor cells) of antigens for the vaccination, our present study may suggest that efficacy of immune induction by DC vaccination varies among tumor types and squamous cell carcinoma is possible target for DC vaccination coupled with pulsing by tumor cell lysate.

ACKNOWLEDGEMENTS. This research was supported partially by a Grant-in-Aid for Scientific Research (No. 18580323) and "Academic Frontier" Project for Private Universities: matching fund subsidy (2005–2009) from Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT).

REFERENCES

1. Ackermann, B., Troger, A., Glouchkova, L., Korholz, D., Gobel, U. and Dilloo, D. 2004. Characterization of CD34⁺ progenitor-derived dendritic cells pulsed with tumor cell lysate for a vaccination strategy in children with malignant solid tumors and a poor prognosis. *Klin. Padiatr.* **216**: 176–182.
2. Azakami, D., Bonkobara, M., Washizu, T., Iida, A., Kondo, M., Kato, R., Niikura, Y., Iwaki, S., Tamahara, S., Matsuki, N. and Ono, K. 2006. Establishment and biological characterization of canine histiocytic sarcoma cell lines. *J. Vet. Med. Sci.* **68**: 1343–1346.
3. Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, Y. J., Pulendran, B. and Palucka, K. 2000. Immunobiology of dendritic cells. *Annu. Rev. Immunol.* **18**: 767–811.
4. Banchereau, J., Ueno, H., Dhodapkar, M., Connolly, J., Finholt, J. P., Klechevsky, E., Blanck, J. P., Johnston, D. A., Palucka, A. K. and Fay, J. 2005. Immune and clinical outcomes in patients with stage IV melanoma vaccinated with peptide-

- pulsed dendritic cells derived from CD34⁺ progenitors and activated with type I interferon. *J. Immunother.* **28**: 505–516.
5. Berntsen, A., Geertsen, P. F. and Svane, I. M. 2006. Therapeutic dendritic cell vaccination of patients with renal cell carcinoma. *Eur. Urol.* **50**: 34–43.
 6. Cranmer, L. D., Trevor, K. T. and Hersh, E. M. 2004. Clinical applications of dendritic cell vaccination in the treatment of cancer. *Cancer Immunol. Immunother.* **53**: 275–306.
 7. Ferrara, A., Nonn, M., Sehr, P., Schreckenberger, C., Pawlita, M., Durst, M., Schneider, A. and Kaufmann, A. M. 2003. Dendritic cell-based tumor vaccine for cervical cancer II: results of a clinical pilot study in 15 individual patients. *J. Cancer Res. Clin. Oncol.* **129**: 521–530.
 8. Hsu, F. J., Benike, C., Fagnoni, F., Liles, T. M., Czerwinski, D., Taidi, B., Engleman, E. G. and Levy, R. 1996. Vaccination of patients with B-cell lymphoma using autologous antigen-pulsed dendritic cells. *Nat. Med.* **2**: 52–58.
 9. Isotani, M., Katsuma, K., Tamura, K., Yamada, M., Yagihara, H., Azakami, D., Ono, K., Washizu, T. and Bonkobara, M. 2006. Efficient generation of canine bone marrow-derived dendritic cells. *J. Vet. Med. Sci.* **68**: 809–814.
 10. Koido, S., Kashiwaba, M., Chen, D., Gendler, S., Kufe, D. and Gong, J. 2000. Induction of antitumor immunity by vaccination of dendritic cells transfected with MUC1 RNA. *J. Immunol.* **165**: 5713–5719.
 11. Lesterhuis, W. J., de Vries, I. J., Adema, G. J. and Punt, C. J. 2004. Dendritic cell-based vaccines in cancer immunotherapy: an update on clinical and immunological results. *Ann. Oncol.* **15** (Suppl. 4): 145–151.
 12. Lodge, P. A., Jones, L. A., Bader, R. A., Murphy, G. P. and Salgaller, M. L. 2000. Dendritic cell-based immunotherapy of prostate cancer: immune monitoring of a phase II clinical trial. *Cancer Res.* **60**: 829–833.
 13. Maier, T., Tun-Kyi, A., Tassis, A., Jungius, K. P., Burg, G., Dummer, R. and Nestle, F. O. 2003. Vaccination of patients with cutaneous T-cell lymphoma using intranodal injection of autologous tumor-lysate-pulsed dendritic cells. *Blood* **102**: 2338–2344.
 14. Marshall, J. 2003. Carcinoembryonic antigen-based vaccines. *Semin. Oncol.* **30** (Suppl. 8): 30–36.
 15. Midgley, R. and Kerr, D. 1999. Colorectal cancer. *Lancet* **353**: 391–399.
 16. Millard, A. L., Ittelet, D., Schooneman, F. and Bernard, J. 2003. Dendritic cell KLH loading requirements for efficient CD4⁺ T-cell priming and help to peptide-specific cytotoxic T-cell response, in view of potential use in cancer vaccines. *Vaccine* **21**: 869–876.
 17. Morse, M. A., Deng, Y., Coleman, D., Hull, S., Kitrell-Fisher, E., Nair, S., Schlom, J., Ryback, M. E. and Lysterly, H. K. 1999. A Phase I study of active immunotherapy with carcinoembryonic antigen peptide (CAP-1)-pulsed, autologous human cultured dendritic cells in patients with metastatic malignancies expressing carcinoembryonic antigen. *Clin. Cancer Res.* **5**: 1331–1338.
 18. Nakaichi, M., Taura, Y., Kanki, M., Mamba, K., Momoi, Y., Tsujimoto, H. and Nakama, S. 1996. Establishment and characterization of a new canine B-cell leukemia cell line. *J. Vet. Med. Sci.* **58**: 469–471.
 19. Slingluff, C. L. Jr., Petroni, G. R., Yamshchikov, G. V., Barnd, D. L., Eastham, S., Galavotti, H., Patterson, J. W., Deacon, D. H., Hibbits, S., Teates, D., Neese, P. Y., Grosh, W. W., Chianese-Bullock, K. A., Woodson, E. M., Wiernasz, C. J., Merrill, P., Gibson, J., Ross, M. and Engelhard, V. H. 2003. Clinical and immunologic results of a randomized phase II trial of vaccination using four melanoma peptides either administered in adjuvant or pulsed on dendritic cells. *J. Clin. Oncol.* **21**: 4016–4026.
 20. Steinman, R. M. 1991. The dendritic cell system and its role in immunogenicity. *Annu. Rev. Immunol.* **9**: 271–296.
 21. Tamura, K., Yamada, M., Isotani, M., Arai, H., Yagihara, H., Ono, K., Washizu, T. and Bonkobara, M. 2007. Induction of dendritic cell-mediated immune responses against canine malignant melanoma cells. *Vet. J.* (in press; doi:10.1016/j.tvjl.2006.11.005).
 22. Timmerman, J. M., Czerwinski, D. K., Davis, T. A., Hsu, F. J., Benike, C., Hao, Z. M., Taidi, B., Rajapaksa, R., Caspar, C. B., Okada, C. Y., van Beckhoven, A., Liles, T. M., Engleman, E. G. and Levy, R. 2002. Idiotypic-pulsed dendritic cell vaccination for B-cell lymphoma: clinical and immune responses in 35 patients. *Blood* **99**: 1517–1526.