IFN\(\gamma\) Markedly Cooperates with Intratumoral Dendritic Cell Vaccine in Dog Tumor Models

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Abstract

Dendritic cell (DC)–based immunotherapy can trigger effective immune responses against cancer in human patients. Although accompanied by little toxicity, further improvements are needed to optimize immune responses for fully satisfactory clinical outcomes. IFN\(\gamma\), a potent inducer of T helper type 1 immune responses, is considered an important tool to realize improvements. In this study, we sought to clarify the effect of IFN\(\gamma\) on the maturation and activation of DCs and the clinical outcome of DC-based cancer therapy in dogs. In vitro experiments indicated that IFN\(\gamma\) significantly enhanced the expression of immune stimulatory molecules and interleukin-12 by DCs derived from canine monocytes. IFN\(\gamma\) also significantly strengthened DC-mediated growth suppression against tumor cell lines. DC inoculation with concomitant delivery of IFN\(\gamma\) into primary or recurrent tumors elicited significant clinical responses, including four complete responses and two partial responses against malignant tumors, also eliciting partial responses against benign but actively growing tumors. Together, our results indicate that combining IFN\(\gamma\) and DCs could induce strong immune responses against tumors, significantly improving clinical outcomes. The present study of dogs bearing common types of cancer in humans offers a unique line of support for the development of human cancer therapies. Cancer Res; 70(18): 7093–101. ©2010 AACR.

Introduction

Because of their high efficiency in initiating and activating immune responses, dendritic cells (DC) have shed light on their therapeutic potential as adjuvants for tumor immunity. In the last decade, there has been an increase in clinical trials against malignant tumors using DCs loading tumor antigens (1–6) or DCs fused with tumor cells (7), which were induced in vitro from peripheral monocytes or bone marrow precursors by defined cytokines, including granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-4, and tumor necrosis factor-\(\alpha\) (TNF\(\alpha\)). These DC treatments have been performed against a variety of malignant tumors including melanoma (1), myeloma (6), and carcinoma of thyroid (2), hepatic (3) pancreatic (5), or renal (4, 7) cells. Regardless of the origin of the tumor, the DC treatments induced tumor-specific immune responses and showed almost no toxicity in patients. Nevertheless, clinical responses to the treatment were not so successful; tumor growth was observed in more than half of the patients treated (1–7). It seems that more potent immune stimulation against tumor is necessary to elicit adequate clinical responses. Inefficiency in immune stimulation may be due to the degradation of DCs after their inoculation into the body of patients in which the specified cytokines abundantly supplied in vitro were deficient (8, 9). In addition, some tumors, such as melanoma (10) and transmissible venereal tumor (11), inhibit DC function. It is therefore vital to obtain a clinical response that prevents DC degradation, and is important to promote the maturation and activation of DCs at the site of DC inoculation.

IFN\(\gamma\) is a cytokine that is known to activate T helper type 1 and natural killer cells, and is produced in response to tumors. IFN\(\gamma\) also directly acts on DCs to promote maturation (12, 13), prevent a reduction in activity (14, 15), and enhance suppression activity against tumor growth (16). Thus, immunity against tumors in vivo may be efficiently augmented to improve the clinical response, provided that IFN\(\gamma\) is maintained in the environment where DCs are involved.

Dogs suffer from common human tumor types, including carcinoma of the mammary gland, lymphoma, osteosarcoma, and soft tissue sarcoma (17). They have also been extensively...
studied for the diagnosis, therapy, and monitoring of such tumors (18). Success in DC therapy against canine tumors consequently provides convincing information that advances DC therapy in humans. In the present study, we clarified the effect of IFNγ on the maturation and activation of DCs derived from dog peripheral monocytes in an in vitro study, then examined the clinical response of canine tumors to therapy using DCs and IFNγ. These results point toward a promising method for cancer therapy.

Materials and Methods

Animals
For the in vitro study, laboratory-bred female beagles, 4 to 8 years of age, were housed and used in accordance with NIH guidelines and with the regulations of the local Institutional Animal Care and Use. The study protocol was also approved by the animal experiment committee of the Graduate School of Life and Environmental Sciences of Osaka Prefecture University. Each separate experiment was carried out using different animals. For the clinical study, seven dogs (5–17 years old) with malignant or benign tumors, which had been admitted to Shin-Kanaoka Animal Hospital (Sakai City, Japan), were treated. Tumors were diagnosed by histologic or cyto logic examination on specimens obtained by biopsy or by surgical removal. Written consent was obtained from all dog owners prior to the start of the study.

Generation and treatment of DCs
DCs were induced from peripheral blood monocytes isolated using anti-human CD14 monoclonal antibodies (mAb; culture supernatants of clone 3C10; American Type Culture Collection) and goat anti-mouse IgG microbeads (Milenyi Biotec GmbH) as described previously (19). After the isolation procedure, 98% of the resultant cells showed DC14 positivities. To induce differentiation into DCs, isolated monocytes were suspended at a concentration of 1 × 106/mL in a culture medium consisting of RPMI 1640 supplemented with 10% fetal bovine serum (Bio-Solutions Japan, Inc.), and were incubated with canine GM-CSF (40 ng/mL) and canine IL-4 (10% w/v) at 37°C for 7 days.

In the in vitro study, the DCs generated were further incubated in the culture medium at various concentrations of recombinant canine IFNγ (kindly provided by Toray, Tokyo, Japan) for 5 days, to examine the morphology, surface antigen implying maturation, and the production of IL-12. They were also incubated for 48 hours to examine tumor growth inhibition, and the expression of TNFα and TNF-related apoptosis-inducing ligand (TRAIL).

In the clinical study, the DCs were washed and further incubated with 50 μg/mL of keyhole limpet hemocyanin (KLH; Sigma) in RPMI 1640 for 3 hours. After incubation, the cells were harvested, washed, and inoculated into the patient dog from which the cells originated.

Examination of morphology and surface antigens
For morphologic examination, samples were prepared by cytocentrifuge using Cytospin 4 (Thermo Shandon), and were stained with a quick staining kit, Hemacolor (Merck). For surface antigen examination, cells were incubated with the following mAbs against surface markers indicating DC maturation (20, 21): FITC-conjugated mouse anti-human CD1a mAb (clone NA1/34-HLK; Serotec), rat anti-canine MHC class II mAb (clone YKIX.334.2; Serotec), PE-Cy5-conjugated mouse anti-human CD14 mAb (clone TÜK4; Serotec), biotin-conjugated rat anti-mouse CD80 mAb (clone 1G10; Becton Dickinson Biosciences), and mouse anti-human CD86 mAb (clone FUN-1; Becton Dickinson Biosciences). The cross-reactivity of these antibodies to dog antigens was confirmed in previous reports (21, 22) or by the manufacturers. The incubation procedure with mAbs took place at 4°C for 30 minutes using 1 × 106 cells suspended in 50 μL of PBS and 2% fetal bovine serum. Incubation with biotin anti-CD80 mAb was followed by incubation with Streptavidin PE-Cy5 (Becton Dickinson Biosciences). Before use in incubation, anti-CD86 mAb was labeled by PE-conjugated goat anti-mouse IgG (Molecular Probe). Excess PE-goat antimmouse IgG was neutralized by excess purified mouse IgG (Sigma). The mean expression intensity of the cell surface molecules was quantified by the mean fluorescence intensity using appropriate software (CellQuest, Becton Dickinson Biosciences) after the assay.

Assay for IL-12 production and TNFα and TRAIL expression
Production of IL-12 (p70) from DCs (1 × 105) was detected by measuring IL-12 present in the culture supernatant, using a kit of ELISA for canine IL-12p40 (DuoSet ELISA Development System; R&D Systems). The mRNA expression of canine TNFα and TRAIL was examined by RT-PCR using the following primers: TNFα sense, 5′-TCAGGCTTTCCTGCTCTTC-3′; TRAIL antisense, 5′-ACATGAGCTATGGCATTGTGC-3′; TRAIL sense, 5′-TGAAGAGGATGATGAACACC-3′; TRAIL antisense, 5′-GAATGAAACACATGCTTC-3′. As a control, a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase cDNA, was examined.

Assay of tumor growth inhibition
A canine thyroid adenocarcinoma line CTAC and a canine osteosarcoma line D17 were maintained as previously described (23, 24). Inhibition of tumor growth by DCs was examined as described by Chapoval and colleagues (16). In summary, CTAC or D17 cells (104) were incubated with DCs (5 × 105) in 200 μL of culture medium containing various concentrations of canine IFNγ at 37°C for 48 hours in a 96-well plate. Proliferation of tumor cells was evaluated by the radioactivity of [3H]thymidine (92.5 kBq/mL; PerkinElmer Life and Analytical Sciences) incorporated into DNA during an additional 6-hour incubation. A control, tumor cells were incubated with the same concentration of IFNγ but without DCs. Results are quoted as the percentage of inhibition, calculated according to the formula: % inhibition = (1 – test cpm/control cpm) × 100%. In some experiments, cell-to-cell contact between DCs and tumor cells was interrupted by a 1.0-μm-pore culture insert (Becton Dickinson). In these experiments, the culture inserts
were placed in the wells of a 24-well plate. Tumor cells (10^5) in the 1 mL medium were added to the well, and DCs (5 x 10^5) in the 1 mL medium containing 0 or 10^3 units/mL of IFNγ were added to the culture insert. After 48 hours, the inserts were removed, and the proliferation of tumor cells was evaluated as described above. Apoptosis was also evaluated by incubating tumor cells with Hoechst 33342 (2 μg/mL; EMD Chemicals) for 30 minutes at room temperature.

**Clinical treatment of patient dogs**

Complete treatment comprised a single injection of DCs combined with three injections of IFNγ (Interdog; Toray) at weekly intervals. Eight cycles of the complete treatment were performed for each patient. In each cycle, autologous DCs were injected directly into tumor tissue along with canine IFNγ (10^5 units/kg), followed by the injection of IFNγ (10^5 units/kg) into the same site on the second and fifth days after DC injection (summarized in Supplemental Fig. S1). After eight cycles of the complete treatment, treatment was performed without the subsequent injection of IFNγ on the second and fifth days. One patient dog received DCs without IFNγ at weekly intervals for 42 days before beginning the DC plus IFNγ treatment (case no. 1 in Table 1). Another dog received IFNγ by itself every other day for 10 days before beginning the DC plus IFNγ treatment (case no. 2 in Table 1).

**Preparation of tumor antigen**

Lysates from tumor tissues were used as the source of tumor antigen and were prepared by repeating a freeze–thaw cycle as described by Geiger and colleagues (25). The protein concentration was adjusted to 1 mg/mL and stored at −80°C before use.

**Assessment of clinical and immunologic responses**

Clinical responses to treatment were evaluated according to WHO criteria. Complete response was defined as the complete disappearance of all clinically detectable disease; partial response was defined as a ≥50% decrease in the sum of the two longest perpendicular diameters of all measurable lesions without the appearance of new lesions; stable disease was defined as a <25% increase or a <50% decrease in tumor size; and progressive disease was defined as a ≥25% increase in existing lesions, or the appearance of new metastasis. Survival time was calculated from the date of the first treatment until the time of death.

To examine whether the inoculated DCs exert their role of priming antigens, the responses of blood mononuclear cells to KLH and tumor lysate were evaluated as described by Geiger and colleagues (25), with minor modifications. In summary, peripheral blood mononuclear cells were collected from animals before treatment, and after the eighth treatment. The peripheral blood mononuclear cells (2.5 x 10^5) were incubated with various concentrations of KLH or lysate in 200 μL of culture medium at 37°C for 5 days. Proliferation of peripheral blood mononuclear cells was quantified by the radioactivity of the [3H]thymidine incorporated into DNA.

**Statistical analysis**

Experimental groups were compared using ANOVA followed by Fisher’s protected–least significance–post hoc

### Table 1. Patient dog characteristics and clinical outcome posttreatment

<table>
<thead>
<tr>
<th>Case</th>
<th>Breed</th>
<th>Age</th>
<th>Sex</th>
<th>Tumor Site Cell type</th>
<th>Treatment</th>
<th>Period of treatment</th>
<th>Clinical response</th>
<th>Survival time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Shih Tzu</td>
<td>17</td>
<td>F</td>
<td>Footpad Sebaceous epithelioma</td>
<td>+ −</td>
<td>42 d</td>
<td>SD</td>
<td>157 d</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>91 d</td>
<td>PR (30% &gt;)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+ +</td>
<td>56 d</td>
<td>PR (necrosis of &gt;90% area) at 28 d</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Beagle</td>
<td>14</td>
<td>F</td>
<td>Intraoral submaxilla Fibrosarcoma</td>
<td>− +</td>
<td>10 d</td>
<td>PD</td>
<td>69 d</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+ +</td>
<td>56 d</td>
<td>PR (necrosis of &gt;90% area) at 28 d</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Yorkshire terrier</td>
<td>11</td>
<td>F</td>
<td>Breast Mammary gland carcinoma</td>
<td>+ +</td>
<td>140 d</td>
<td>CR at 56 d</td>
<td>&gt;800 d</td>
</tr>
<tr>
<td>4</td>
<td>Yorkshire terrier</td>
<td>13</td>
<td>F</td>
<td>Breast Mammary gland carcinoma</td>
<td>+ +</td>
<td>56 d</td>
<td>CR at 35 d</td>
<td>&gt;550 d</td>
</tr>
<tr>
<td>5</td>
<td>Yorkshire terrier</td>
<td>5</td>
<td>F</td>
<td>Breast Mammary gland carcinoma</td>
<td>+ +</td>
<td>56 d</td>
<td>CR at 56 d</td>
<td>&gt;650 d</td>
</tr>
<tr>
<td>6</td>
<td>Beagle</td>
<td>7</td>
<td>F</td>
<td>Breast Mammary gland carcinoma</td>
<td>+ +</td>
<td>189 d</td>
<td>PR (necrosis of &gt;90% area) at 195 d</td>
<td>&gt;450 d</td>
</tr>
<tr>
<td>7</td>
<td>Beagle</td>
<td>12</td>
<td>MN</td>
<td>Rectum Adenocarcinoma</td>
<td>+ +</td>
<td>245 d</td>
<td>CR at 154 d</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; MN, male neutered.
analysis, implemented using Stat-View software (Hulinks, Inc.). The significance level was set at $P < 0.05$.

Results

Effect of IFNγ on maturation and function of DCs

DCs differentiated from monocytes by 7-day incubation with GM-CSF and IL-4 were further incubated for 5 days with various concentrations of IFNγ. The number of resultant cells was not affected by the concentration of IFNγ; $0.8 \times 10^5$ to $1.2 \times 10^5$ cells were generated after the 5-day incubation of $3 \times 10^5$ DCs. Figure 1A shows the morphology of cultured cells. After 7 days of incubation with GM-CSF and IL-4, the size of the cultured cells increased significantly ($P > 0.001$). The cells were round-shaped with a round nucleus. Most of the cells had granules in the cytoplasm, and a veil-like structure or short processus on the surface. A further 5-day incubation without cytokine led to cell enlargement ($P > 0.05$), but the veil-like structure or processus disappeared, or degraded. In contrast, the veil-like structure and processus continued to develop after an additional 5-day incubation with IFNγ. As shown in Fig. 1B, the expression of CD80, an immune stimulatory molecule of DCs, was significantly increased by incubation with GM-CSF and IL-4. The expression of CD80 was significantly downregulated by withdrawing cytokines. However, IFNγ prevented the downregulation and further increased CD80 expression in a dose-dependent manner up to 1,000 units/mL. Expression of the other immune stimulatory molecule, CD86, and molecules for antigen presentation, MHC class II and CD1a, also significantly increased upon incubation with GM-CSF and IL-4. The increase in expression was enhanced by further incubation with $\geq 100$ units/mL of IFNγ. In contrast, expression of CD14, which is a monocyte marker, did not increase either by incubation with GM-CSF and IL-4 or by further incubation with IFNγ (data not shown).

As shown in Fig. 1C, production of IL-12 from cultured cells was significantly enhanced by further incubation with IFNγ.
in a dose-dependent manner. The production of IL-12 from cells further incubated with GM-CSF and IL-4, instead of IFNγ, was comparable to that from cells incubated with 10 units/mL of IFNγ (data not shown).

Consistent with the results of Chapoval and colleagues for human monocyte–derived DCs (16), the canine monocyte–derived DCs successfully inhibited the growth of the canine tumor cell line, and IFNγ significantly enhanced the effect (Fig. 2A). As shown in Fig. 2B, 50% of the inhibitory effect was observed even when direct contact between DCs and tumor cells was interrupted; the effect was also enhanced by IFNγ. These results suggest that soluble factors produced by DCs also inhibit tumor growth, as well as membrane-bound molecules on DCs. Moreover, DCs induced apoptosis of tumor cells, and this ability was significantly enhanced by IFNγ (Fig. 2C and D). As shown in Fig. 3, IFNγ significantly enhanced the expression of TNFα and TRAIL mRNA by DC. Both TNFα and TRAIL induce apoptosis, and TRAIL could be membrane-bound or soluble. These findings together suggest that induction of apoptosis by DC is a mechanism underlying the inhibition of tumor growth.

**Clinical outcomes**

Characteristics of patient dogs, treatments, and clinical outcomes are summarized in Table 1. Case no. 1 was a 17-year-old female Shih Tzu. The dog had a golf ball–sized (37 × 32 × 24 mm) tumor on the left foot pad (Fig. 4A). The tumor was histologically diagnosed as a sebaceous epithelioma (Fig. 4B). The dog also had multiple soy bean–sized papillomatous nodules throughout her entire body. The papillomatous nodules were also diagnosed as sebaceous.
epithelioma. Results from the blood cell counts were within the reference range. In biochemical analyses, a slightly raised level of blood urea nitrogen (63 mg/dL; reference range, 4.8–31.4 mg/dL) and creatinine (2.3 mg/dL; reference range, 0.2–1.6 mg/dL) was detected, suggesting chronic renal disorder which occurs due to aging in dogs and cats. To treat the golf ball–sized tumor, DCs (0.6 × 10^6 to 3.3 × 10^6) were injected into the tumor on six occasions, 7 days apart. The tumor did not decrease in size, but increased slightly (38 × 37 × 28 mm; Fig. 4C). The complete treatment as described in Materials and Methods was then performed. The number of DCs injected on each occasion was between 0.5 and 3.4 million cells. As shown in Fig. 4D, the tumor had significantly reduced in size (5 × 9 × 4 mm) by 56 days (eight cycles) from the beginning of the complete treatment. The papillomatous nodules over the entire body were not directly treated but also diminished in size or disappeared during the treatment (data not shown). After eight cycles of the DC plus IFNγ treatment, the second- and fifth-day injections of IFNγ were omitted. The tumor nevertheless continued to shrink. Levels of blood urea nitrogen and creatinine, however, reached 143 and 4.3 mg/dL, respectively, and the dog died on day 157.

Case no. 2 had a tumor on the left mandibular site in the oral cavity (Supplemental Fig. S2A). X-ray examination revealed that the tumor had infiltrated into the mandible. The tumor in the oral area was surgically removed, but removal of the infiltrated left mandible was not permitted by the owner. The removed tumor was diagnosed by histologic analysis as fibrosarcoma (Supplemental Fig. S2B). To inhibit the growth of residual tumor cells, IFNγ (10^4 units/kg) was injected every other day. Tumor growth was not affected by the IFNγ treatment, however, and it took only 10 days until the tumor had grown back to half the size of the original tumor that was removed (65 × 20 × 22 mm; Supplemental Fig. S2C). The complete treatment was then undertaken beginning on day 17. The number of DCs injected in this case was 3.1 to 8.2 million cells at each time of injection. After four cycles of treatment, most of the tumor changed to necrotic tissue (Supplemental Fig. S2D). Thereafter, the necrotic tissue was carefully removed at every visit for treatment. At 69 days from the start of the treatment, the dog died due to systemic circular disorder caused by disseminated intra-vascular coagulation.

Case no. 3 had a tumor on the left breast (over the second nipple), 50 × 40 mm in size, with an ulcerative appearance (Supplemental Fig. S3A). From histologic analysis, the tumor was diagnosed as carcinoma of the mammary gland (Supplemental Fig. S3B). No metastasis was observed in the lymph node (LN) or lung. Because the tumor had directly extended to the skin, it was considered to be clinical stage IIIB based on tumor-node-metastasis classification. The complete treatment was performed from the beginning of treatment for this tumor. The number of DCs injected was 0.4 to 1.2 million cells at each time of injection. The tumor was half its original size after 28 days (Supplemental Fig. S3C). After 56 days, the tumor disappeared, and only scars remained (Supplemental Fig. S3D). We found no malignant cells upon examining a biopsy sample. We also found no metastasis in the LN or lung upon an X-ray and ultrasound examination. Treatment was performed without injection of IFNγ on the second and fifth days, and continued to day 140. The dog was alive >800 days later without clinical signs of tumor relapse or metastasis.

Case nos. 4 and 5 had breast tumors, with sizes 25 × 25 mm and 55 × 35 mm, respectively, according to ultrasound examination, considered to be stage IIA and IIB. Upon histologic analysis, the tumors were diagnosed as carcinomas. In neither case did the tumor extend to the skin or breast wall, and no regional LN metastasis was found. The tumors were not detectable by day 35 or 56 after the complete treatment began, based on biopsy and ultrasound examination. These dogs were alive (after >550 and >650 days) without clinical signs of tumor relapse or metastasis.

Case no. 6 had a breast tumor that was 110 × 115 × 72 mm in size. Based on histologic analysis, the tumor was diagnosed as a carcinoma. Metastasis was found in the regional axillary and inguinal LNs but not in the lung or liver; this was considered to be stage IIIA disease. The complete treatment

Figure 3. IFNγ enhances mRNA expression of TNFα and TRAIL of DCs. After 48 h of incubation with the indicated concentrations of IFNγ, the expression of TNFα and TRAIL was examined in RT-PCR. A, a typical photographic record of TNFα-, TRAIL-, and glyceraldehyde-3-phosphate hydrogenase (control)-specific PCR products. B, relative intensity of TNFα or TRAIL expression versus control. Three experiments were performed independently using three dogs.

Control

Control

IFNγ (units/mL)

IFNγ (units/mL)

TNFα

TRAIL

0

10^3

0

0.2

0.4

0.6

0.8

1.0

P>0.05

P>0.005

IFNγ (units/mL)

TNFα/Cont

TRAIL/Cont

0

0.2

0.4

0.6

0.8

1.0

P>0.05

P>0.005
was performed, followed by 19 cycles of the weekly treatment with DCs plus IFN\(\gamma\). On each occasion, 1.9 to 4.8 million DCs were injected. The tumor was vulnerable to necrosis and lysis during the treatment. After treatment, the tumor was less than half (50 × 45 × 15 mm) of its original size. However, the dog exhibited cachexia, lost weight significantly, then died on day 195. Autopsy found that most of the tumor area was necrotic tissue at the time of death. No metastasis was found in the lungs or any other organ, and the tumor did not extend to the chest wall.

Case no. 7 had several polyps within a 20 × 30 mm area in the rectum. The tumor was diagnosed as adenocarcinoma based on histologic analysis from a biopsy sample. Metastasis was not found in the LNs. The complete treatment was performed, followed by 19 cycles of the weekly treatment with DCs plus IFN\(\gamma\). On day 154, the tumor was not detectable, and had been replaced by fibrous tissue. The dog was alive after >450 days without clinical signs of tumor relapse or metastasis.

As well as the cases described, two dogs with mammary carcinoma were treated with IFN\(\gamma\) alone, and a dog with a benign mammary tumor was treated with DCs alone. No clinical responses were observed.

**Effect of treatment on antigen-specific immune responses in vivo**

In the treatment using DCs plus IFN\(\gamma\), DCs were pulsed with KLH prior to injection into the tumor. To examine whether the injected DCs presented KLH and tumor antigens to the immune system, the responses of peripheral lymphocytes against KLH or tumor lysate before and after the treatment were compared. Lymphocytes from the peripheral blood of patient dogs did not respond against KLH or tumor lysate before the treatment. However, responses against these antigens were significantly augmented after eight cycles of the complete treatment (Fig. 5).

**Discussion**

We first clarified the effect of IFN\(\gamma\) on canine DCs generated from peripheral blood monocytes. An increase in the expression of immune stimulatory molecules, such as CD80, CD83, or CD86, and an increase in the production of IL-12, is considered to constitute evidence of DC maturation in the human (20, 26), mouse (27), and dog (28). In the present study, we found that DCs induced from dog monocytes increased the expression of the immune stimulatory molecules and IL-12 by IFN\(\gamma\). These results are in agreement with previous studies using myeloid DC precursors in human blood (12) and a cell line of mouse DC precursor (13). Furthermore, as shown by Chapoval and colleagues in a human study (16), canine monocyte-derived DCs inhibited the growth of a canine tumor line, and inhibition was significantly enhanced by IFN\(\gamma\). In contrast, the results from DCs incubated without cytokine show that canine DCs significantly reduced immune activity in an environment lacking stimulation for maturation, as
reported in humans (8, 9). Overall, these results suggest that IFNγ rescues the downregulation and promotes maturation and activation of DCs following differentiation. Because DC maturation is significant for cancer immunotherapy (29, 30), IFNγ should be useful in DC-based immunotherapy.

As the in vitro data indicate, IFNγ ameliorates the activity of DCs in vivo, and improves clinical responses in DC treatment against tumors. All dog patients treated showed clinical responses and significantly increased their immune responses against KLH and tumor antigens. Because dogs suffer from common human tumor types (17), these results suggest that the use of DC plus IFNγ should be valuable in human cancer therapy. In this study, we did not load any antigen or epitope peptide on the DCs to be inoculated. Because DCs generated in the 7-day culture were not fully mature and still had phagocytic activity (19), it was natural to consider that the DCs injected into the tumor took up antigen from tumor cells and matured and activated in situ upon stimulation by IFNγ. The mature DCs may have presented tumor antigens in situ rather than in LNs, and activated the immune system mainly at the effector phase because IFNγ reportedly hinders the expression of CCR7 on DCs, which is necessary for migration into the LNs (31). However, immune response or immune memory against tumor antigens might have later arisen systemically because the disappearance or shrinkage in size of tumors was found at areas distant from the treated tumor, and no metastasis in any other organs was found. Other cytokines which have been used together with DCs in clinical treatment are IL-2 and IL-12 (32–35). Like IFNγ, these cytokines also polarize immune responses to T helper type 1, but their effect on clinical response and immune responses against tumors was less clear than that of IFNγ found in the present study. However, treatment with IFNγ but without DCs did not improve the clinical or immunologic response in case no. 2 or in the study of Apte and colleagues (36). Taking all evidence together, we conclude that concomitant inoculation of IFNγ with DCs strongly induces immune responses against tumors, ultimately leading to significant improvement in clinical response.

The dog in case no. 1 died due to renal failure during treatment, although significant clinical response was obtained. The dog was 17 years old, corresponding to 84 years of age in humans. Because geriatric dogs generally suffer from renal disorder (37), and no other patients showed renal failure, the renal failure is not believed to be a side effect of IFNγ. A serious problem observed in the DC plus IFNγ treatment was disseminated intravascular coagulation or cachexia due to acute necrosis of a large amount of tumor cells. A solution is to surgically remove as much tumor as possible before the DC plus IFNγ treatment. We are now seeking to establish a protocol for the treatment of cancer in internal organs or metastasis using DC plus IFNγ in dogs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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