Effects of adoptive immunotherapy using lymphokine-activated T killer cells on peripheral blood lymphocytes of tumor-bearing dogs

大阪府立大学博士（獣医学）学位論文

Effects of adoptive immunotherapy using lymphokine-activated T killer cells on peripheral blood lymphocytes of tumor-bearing dogs

（腫瘍性疾患犬の末梢血リンパ球における活性化自己リンパ球療法の効果に関する研究）

三重　慧一郎

2016

List of abbreviations

CD	cluster of differentiation
cDNA	complementary DNA
CRP	C-reactive protein
DEPC	diethylpyocarbonate
D-PBS	Dulbecco's phosphate buffered physiological saline
EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
FITC	fluorescent isothiocyanate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
IFN	interferon
IL	interleukin
MDSC	myeloid derived suppressor cells
mRNA	messenger RNA
PBL	peripheral blood lymphocytes
PBN	peripheral blood neutrophils
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered physiological saline
PCR	polymerase chain reaction
QOL	quality of life
rhIL-2	recombinant human interleukin-2
R-PE	R-phycoerythrin
TGF	transforming growth factor

T-LAK	lymphokine-activated T killer cells
Treg	regulatory T cells
WBC	white blood cell

Contents

Introduction

The progression of malignant tumors is commonly associated with condition of immune response (Teicher, 2007; Alshaker and Matalka, 2011; Fridman *et al.*, 2012; Olson *et al.*, 2012). Among immune cells, T lymphocytes, which play the central role in adaptive immune response, are important for anti-tumor immune response against the progression of malignant tumors (Koebel *et al.*, 2007, Carvalho *et al.*, 2014). However, malignant tumors suppress T lymphocytes by various ways (Whiteside, 2004; Critchley-Thorne *et al*., 2007), and this suppression of T lymphocytes especially results in depression of cellular immune response. Depression of cellular immune response caused by suppression of T lymphocytes allows promoting the progression of malignant tumors. Therefore, enhancement of T lymphocyte for activation of cellular immune response is important for stopping progression of malignant tumors.

Immunotherapy is an attractive approach for enhancing T lymphocytes suppressed by malignant tumors. Adoptive immunotherapy using lymphokine-activated T killer cells (T-LAK) is one of the immunotherapies applied both in dogs (Itoh *et al.*, 2003; Hoshino *et al.*, 2008) and humans (Takayama *et al*., 2000; Ebina *et al*., 2003). T-LAK are generated from patient's own peripheral blood lymphocytes (PBL) by ex vivo activating with solid phase anti-cluster of differentiation (CD)3 antibody and interleukin (IL)-2 (Sekine *et al.*, 1993; Itoh *et al.*, 2003; Hoshino *et al.*, 2008). Therapeutic effect of T-LAK therapy is expected by following two pathways; (1) cytotoxic activity of T-LAK against tumor cells (Sekine *et al.*, 1993), and (2) activation of anti-tumor immune response by various cytokines secreted from T-LAK (Kwak *et al*., 2000; Ishikawa *et al.*, 2011). In human medicine, in addition to clinical effects including decreasing of postoperative recurrence (Takayama *et al*., 2000; Xie *et al*., 2012; Zhong

et al., 2012), improvement of quality of life (QOL) (Ebina *et al*., 2003; Iwai *et al*., 2012), and life-prolong effect (Ebina *et al*., 2003; Iwai *et al*., 2012; Zhang *et al*., 2015), improvement of proportion and cytokine production in autologous T lymphocytes has been reported (Hirokawa *et al*., 2009; Ishikawa *et al*., 2011; Sun *et al*., 2011; Noguchi *et al*., 2014). Because of difficulty to cure tumors completely by only T-LAK therapy, T-LAK therapy has often combined with traditional therapies such as surgery, chemotherapy, and radiotherapy for treatment of tumors. Immunoenhancing effects of T-LAK therapy is expected to augment therapeutic effects of these traditional therapies.

Although immunoenhancing effects of T-LAK therapy have been reported in healthy dogs in veterinary medicine (Itoh *et al*., 2003, Hoshino *et al*., 2008), immunological effects of T-LAK therapy in tumor-bearing dogs have not yet been demonstrated. Immunological effects of T-LAK therapy have been studied through change of PBL in human medicine. The aim of this study was to evaluate effects of T-LAK therapy on PBL of tumor-bearing dogs for demonstration of immunoenhancing effects of T-LAK therapy. In chapter 1, the relationship between overall survival and change of PBL count was assessed after administration of T-LAK in tumor-bearing dogs. In chapter 2, change of PBL was evaluated after midline laparotomy with or without T-LAK therapy on healthy beagles. And in chapter 3, change of PBL was evaluated in tumor-bearing dogs received T-LAK therapy combined with palliative resection of tumors.

Chapter 1: Relationship between change of PBL count and prognosis in tumor-bearing dogs received T-LAK therapy

Life-prolong effect is one of the clinical effects of T-LAK therapy reported in patients with tumors (Ebina *et al*., 2003; Iwai *et al*., 2012; Zhang *et al*., 2015). Immunoenhancing effects of T-LAK therapy have been reported through evaluation of change of PBL, and some studies have shown that cytokine production and T lymphocyte subsets in PBL are improved by T-LAK therapy on patients with tumors (Hirokawa *et al*., 2009; Ishikawa *et al*., 2011; Sun *et al*., 2011; Noguchi *et al*., 2014). Previous study has reported that these changes of PBL relate to favorable prognosis in patients received T-LAK therapy (Ishikawa *et al*., 2011).

Similarly in humans, T-LAK therapy has tried to apply for tumor-bearing dogs in veterinary medicine. As immunoenhancing effects of T-LAK therapy, increasing of PBL count (Itoh *et al*., 2003) and elevation of plasma interferon (IFN)-γ concentration (Hoshino *et al*., 2008) have been reported in healthy dogs. However, there are few studies about prognosis of tumor-bearing dogs received T-LAK therapy, and relationship between change of PBL and prognosis has still unknown in tumor-bearing dogs received T-LAK therapy.

PBL count is expected as a possible prognostic indicator in tumor-bearing dogs received T-LAK therapy. PBL count may reflect the immune status and has been reported to increase in healthy dogs received T-LAK therapy (Itoh *et al*., 2003). In addition, some studies have shown that PBL count is decreased by diverse tumors (Watabe *et al*., 2011) and associates with prognosis of tumor-bearing dogs (Sottnik *et al*., 2010) similarly to previous studies in humans (Kobayashi *et al*., 2012; Milne *et al*., 2012; Li *et al*., 2013; Zhang *et al*., 2013). It is also thought that PBL count is a suitable

because PBL count could be measured easily in clinical practice. Therefore, the relationship between overall survival and change of PBL count was assessed after administration of T-LAK in tumor-bearing dogs in this chapter.

Section 1: Materials and Methods

Patients

Twenty-four tumor-bearing dogs received T-LAK therapy at Veterinary Teaching Hospital of Osaka Prefecture University from 2012 to 2014 were enrolled in this retrospective study after sufficient informed consent. Peripheral venous blood samples were collected before and about 2 weeks after the first administration of T-LAK. Hematological examination was performed with automated blood cell counting system (pocH-100iV Diff; Sysmex, Hyogo, Japan) and blood smear stained by rapid stain kit (Hemacolor; Merck, Darmstadt, Germany). The dogs received T-LAK therapy at 2 to 4-week intervals until they were unable to receive further treatment due to either poor general condition or if the owners refused any further treatment. On the basis of change of PBL count after the first administration of T-LAK, the dogs were divided into two groups, the increased group (n=15) and the decreased group (n=9).

Preparation of T-LAK

T-LAK were generated from autologous peripheral blood mononuclear cells (PBMC) by culture with recombinant human IL-2 (rhIL-2) and solid phase anti-canine CD3 antibody (Itoh *et al.*, 2003; Hoshino *et al.*, 2008). In brief, 5 to 20 ml of peripheral venous blood was obtained from a tumor-bearing dog and heparinized blood was diluted to twice their original volume with Dulbecco's phosphate buffered physiological saline (D-PBS; Nacalai Tesque, Kyoto, Japan). PBMC were isolated from the diluted blood sample by gradient centrifugation (2,000 rpm, 45 min, 20 °C) using Lymphocyte Separation Solution (specific gravity: 1.077; Nacalai Tesque). PBMC were suspended with appropriate volume of culture medium containing rhIL-2 (containing 700 IU/ml of rhIL-2, LAM-1; Canine-Lab, Tokyo, Japan) with 2.5 % fetal bovine serum (FBS;

Biowest, Nuaille, France) and cultured in a 50 ml or a 250 ml culture flask (Flask for culture of suspension cells; Sumitomo Bakelite, Tokyo, Japan) with solid phase anti-canine CD3 antibody (1 mg/ml mouse anti-canine CD3; AbD serotec, Oxford, U.K.) at 37 °C under 5 % CO_2. The culture flask with solid phase anti-canine CD3 antibody had been prepared before starting culture by incubation overnight at 4 °C with anti-canine CD3 antibody diluted with appropriate volume of D-PBS (final concentration: 5 μg/ml). Appropriate volume of culture medium containing rhIL-2 (containing 175 IU/ml of rhIL-2, LAM-2; Canine-Lab) was added every about 3 days according to proliferation of cultured cells. If cultured cells proliferated well, the cells were transferred to a new 250 ml culture flask with appropriate volume of LAM-2 or a culture bag containing culture medium with rhIL-2 (containing 175 IU/ml of rhIL-2, LAM-3; Canine-Lab). About 2 weeks after starting culture, the cultured cells were harvested when the cells proliferated enough. The harvested cells were washed and resuspended with appropriate volume of sterile physiological saline solution and then administered intravenously to the tumor-bearing dog. For confirming sterility of the cell product, contaminations with bacteria and fungi were checked before administration.

Statistical analysis

Differences were compared by Mann-Whitney's U test for intergroup comparisons. Overall survival was calculated from the day starting the first culture of T-LAK until 1 year later. Overall survival rates were analyzed using Kaplan-Meier curves and the log-rank test. P-values of <0.05 were considered statistically significant. All statistical analyses were performed using Statcel 3, a Microsoft Excel plug-in (OMS publishing, Saitama, Japan).

Section 2: Results

In this chapter, tumor-bearing dogs were divided into two groups by change of PBL count after the first administration of T-LAK because prior study was assessed change of PBL count after once administration of T-LAK (Itoh *et al*., 2003). Median change ratios of PBL counts were 1.29 (range 1.05 to 2.16) in the increased group and 0.68 (range 0.05 to 0.94) in the decreased group.

Characteristics of dogs in this study are listed in Table 1. Median age of the dogs was 10 years (range 6 to 17 years) in the increased group and 9 years (range 7 to 15 years) in the decreased group. There was no significant difference between the two groups in age. The types and stages of tumors varied in both groups, as shown in Table 1. Most of the dogs in both groups underwent treatments including surgery before starting T-LAK therapy.

The PBL counts before T-LAK therapy and the first administered T-LAK counts were also checked between the two groups. PBL counts before T-LAK therapy were 1,344 cells/μl (range 903 to 2,940 cells/μl) in the increased group and 1,485 cells/μl (range 994 to 2,567 cells/μl) in the decreased group. The first administered T-LAK counts were 230 $\times 10^6$ cells (range 2.5 to 3,440 $\times 10^6$ cells) in the increased group and 217 $\times 10^6$ cells (range 1.5 to 1,110 $\times 10^6$ cells) in the decreased group. There were no significant differences between the two groups in PBL counts before T-LAK therapy and the first administered T-LAK counts.

Overall survival rates were compared between the increased group and the decreased group (Fig. 1). One year survival rates were 51.3 % in the increased group and 12.7 % in the decreased group. Overall survival of the dogs in the increased group was significantly longer than the decreased group (log-rank test P=0.04).

Section 3: Discussion

The result in this chapter suggested that increased PBL count was a favorable factor for prognosis of tumor-bearing dogs received T-LAK therapy (Fig. 1). In human medicine, some studies have shown that higher PBL counts after surgery (Lee *et al*., 2012) or chemotherapy (Moore *et al*., 2010; Vicente Conesa *et al*., 2012) may be favorable factor for prognosis of patients with tumors. These studies have also suggested that change of PBL count after treatment associate with the treatment-induced immune modulation, which is relevant for prognosis. Therefore, increased PBL count in tumor-bearing dogs received T-LAK therapy might associate with treatment-induced immune enhancement, which contributed to good prognosis.

Because there were no significant differences of PBL counts before starting T-LAK therapy and the first administered T-LAK counts between the two groups, administered T-LAK could cause change of PBL count after the first administration. Similarly in humans, it is reported that the culture method of T-LAK in this study can obtain many functional lymphocytes effectively in dogs (Itoh *et al*., 2003; Hoshino *et al*., 2008). Hence, administered lymphocytes might supplement PBL counts of the tumor-bearing dogs in the increased group. Additionally, previous studies have reported that T-LAK therapy can improve immune status of T lymphocytes suppressed by tumors (Hirokawa *et al*., 2009; Ishikawa *et al*., 2011; Sun *et al*., 2011; Noguchi *et al*., 2014). T lymphocytes are one of the capital types of lymphocytes and its immune status has been indicated to be suppressed heavily by tumors (Hirokawa *et al*., 2009; Noguchi *et al*., 2014). Thus, improvement of immune status of T lymphocytes by T-LAK therapy might also contribute to increase PBL count in the increased group.

In contrast, administered T-LAK could not increase PBL by lymphopenic

activity and T lymphocyte suppression activity of tumors in the decreased group. Although a suitable method had not yet been established, regulatory T cells (Treg) (Biller *et al*., 2007; Horiuchi *et al*., 2009; O'Neill *et al*., 2009; Tominaga *et al*., 2010), myeloid derived suppressor cells (MDSC) (Goulart *et al*., 2012; Sherger *et al*., 2012), and anti-inflammatory cytokines, such as IL-10 (Catchpole *et al*., 2002; de Andrés *et al*., 2013) and transforming growth factor (TGF)-β1 (Catchpole *et al*., 2002), were reported to be possible indicators for evaluation of immunosuppressive state of tumor-bearing dogs. Evaluation of these indicators might be useful for treatment of tumor-bearing dogs with T-LAK therapy.

In conclusion, increased PBL count can be a positive factor for tumor-bearing dogs received T-LAK therapy. Although further studies are needed, I believe that change of PBL is a key indicator in tumor-bearing dogs received T-LAK therapy.

Table 1. Characteristics of the dogs.

	Increase (n=15)	Decrease (n=9)
Age (years)		
median(range)	10 (6-17)	9 (7-15)
Gender		
Male	4	2
Male（castrated)	2	2
Female	2	3
Female（ovariectomized）	7	2
Histology		
Malignant melanoma	5	1
oral	2	1
other	3	0
Squamous cell carcinoma	0	1
Nasal adenocarcinoma	0	1
Lung adenocarcinoma	2	1
Intestinal adenocarcinoma	0	1
Hepatocarcinoma	2	0
Thyroid carcinoma	2	2
Fibrosarcoma	1	0
Hemangiosarcoma	1	0
Other	2	2
Stage		
Without metastasis	8	2
With metastatic nodes	4	1
With distant metastasis	3	6
Treatment before immunotherapy		
No treatment	1	1
Surgery	10	3
Radiation	1	0
Chemotherapy	0	0
Surgery+Radiation	2	1
Surgery+Chemotherapy	0	2
Radiation+Chemotherapy	0	0
Surgery+Radiation+Chemotherapy	1	2

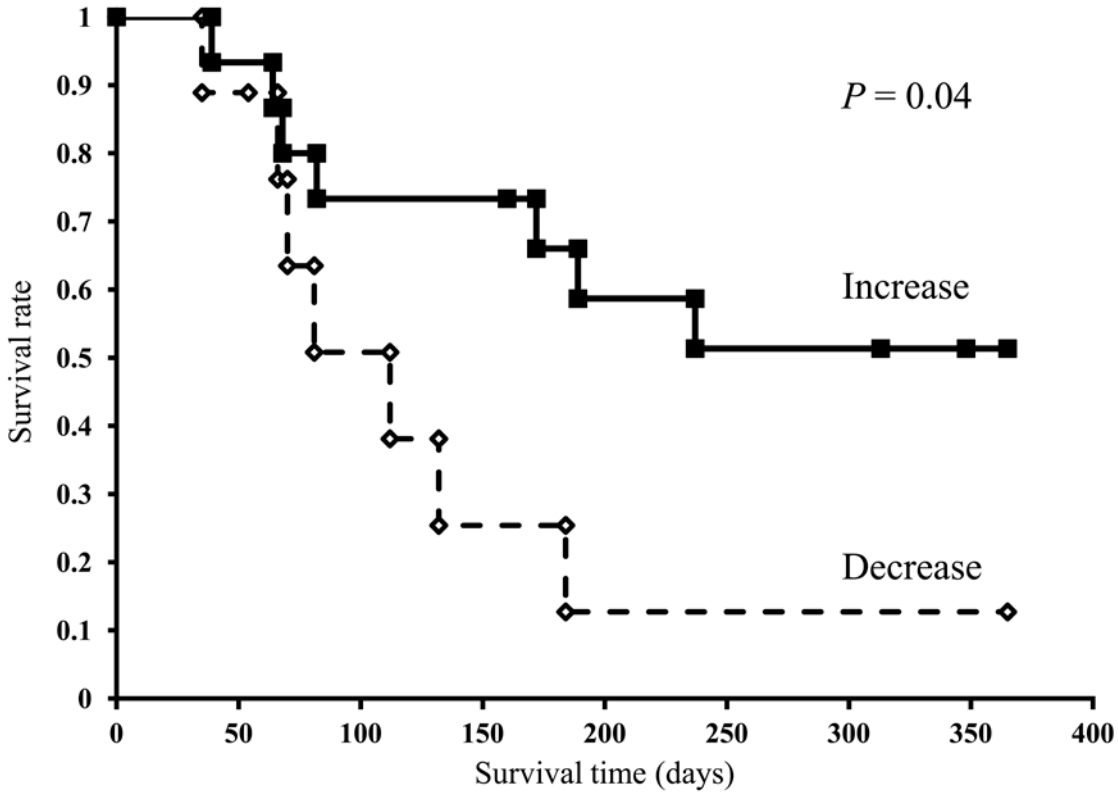

Fig. 1.

Kaplan-Meier survival curves of the increased group (n=15; the black square with solid line) and the decreased group (n=9; the white diamond with dashed line). Overall survival of the dogs in the increased group was significantly longer than the decreased group (log-rank test *P*=0.04).

Chapter 2: Influence of transfusion of T-LAK on PBL in healthy beagles after laparotomy

T-LAK therapy has mainly been applied to tumors. Although T-LAK therapy is expected anti-tumor effect by enhancing anti-tumor immune response, it is difficult to delete the initial heavy load of tumor solely by T-LAK therapy (Sun *et al*., 2011). Hence, T-LAK therapy has generally been applied for patients with tumors in combination with the traditional therapies such as surgery, chemotherapy, and radiotherapy. For applying T-LAK therapy in combination with traditional therapies, it is also suitable that adverse effect of T-LAK therapy has seemed to be slight (Kawamura *et al*., 2000; Ebina *et al*., 2003; Kamigaki *et al*., 2014).

Among traditional therapies for patients with tumors, surgery has been frequently combined with T-LAK therapy because immunotherapy may be most effective for minimal volume of tumors (Takayama *et al*., 2000; Xie *et al*., 2012; Zhong *et al*., 2012). Complete surgical resection of tumors can cure more cancer patients than any other therapies. However, postoperative recurrence and metastasis by residual tumor cells are the most major problems if surgical resection of tumors is incomplete (Aliperti *et al*., 2011). Postoperative immunosuppression is another major problem of surgical resection of tumors (Ni Choileain and Redmond, 2006; Yamaguchi *et al*., 2006; Kimura *et al*., 2010). Postoperatively inflammation is necessary for tissue repair, but an excessive inflammatory response can cause immunosuppression, followed by severe complications such as sepsis and organ failure (Stoecklein *et al*., 2012). Not only increasing risk of postoperative infection and sepsis, postoperative immunosuppression caused by excessive inflammation can also accelerate recurrence and metastasis of tumors (Goldfarb and Ben-Eliyahu, 2006-2007; Gottschalk *et al*., 2010; Predina *et al*.,

2012; Roxburgh *et al*., 2013). Therefore, in human medicine, immunotherapy including T-LAK therapy has been combined with surgery in hopes to prevent postoperative immunosuppression and reactivation of residual tumor cells by enhancing immune response in postoperative period (Kawamura *et al*., 2000; Yamaguchi *et al*., 2006; Judy and Singhal, 2012). Nonetheless, there have been few studies about change of lymphocytes on dogs after surgery with administration of T-LAK.

Postoperative inflammatory response is driven by innate immune system, and immune cells that play central roles on innate immune system are neutrophils and macrophages. In contrast, postoperative anti-inflammatory response, which is necessary response for control of postoperative inflammatory response, is driven by adaptive immune system, and immune cells that play central roles on adaptive immune system are lymphocytes, especially T lymphocytes (Ni Choileain and Redmond, 2006; Stoecklein *et al*., 2012). In veterinary medicine, lymphocytopenia has been reported on healthy beagles after laparotomy (Yamada *et al*., 2002) and postoperative lymphocytopenia may prevent resolution of inflammation after surgery. In chapter 1, it had been shown that increased PBL count could be a positive factor for tumor-bearing dogs received T-LAK therapy. This result suggests that T-LAK therapy can prevent postoperative decrease of PBL and help resolution of postoperative inflammation in dogs after surgery. However, there have been few studies about effects of T-LAK therapy on PBL in dogs after surgery.

The aim of this chapter was to evaluate effects of transfusion of T-LAK on PBL and postoperative inflammatory response in healthy beagles after midline laparotomy.

Section 1: Materials and Methods

Experimental animals

Ten healthy beagle dogs (males, 1 year old) were used in this chapter. The dogs were divided into 2 groups of 5 dogs each. The one underwent laparotomy without postoperative T-LAK therapy (control group) and the other received laparotomy with postoperative T-LAK therapy (T-LAK group). The study was conducted according to the Guidelines of the Experimental Animal Committee of Obihiro University of Agriculture and Veterinary Medicine (Permit number 21-132).

Experimental procedure

The dogs were injected subcutaneously with atropine sulfate (0.025 mg/kg, Fuso Pharmaceutical Industries, Osaka, Japan) and meloxicam (0.2 mg/kg, Metacam; Boehringer Ingelheim Vetmedica Japan, Tokyo, Japan) before anesthesia. General anesthesia was induced by intravenous administration of thiamylal sodium (12.5 mg/kg, Isozol; Nichi-Iko Pharmaceutical, Toyama, Japan) following intravenous injection of midazolam (0.2 mg/kg, Dormicum; Astellas Pharma, Tokyo, Japan) and butorphanol (0.2 mg/kg, Vetorphale; Meiji Seika Pharma, Tokyo, Japan). An endotracheal tube was placed into the trachea to facilitate control of respiration. Anesthesia was maintained with 1.5-2.0% isoflurane (Mylan Seiyaku, Tokyo, Japan) and oxygen. The dogs were placed in ventrodorsal recumbent position. An experimental midline laparotomy from xiphoid to umbilicus was performed. After observation of organs in abdominal cavity, abdominal and skin closure were carried out by routine procedures with synthetic absorbable (Biosyn; Covidien Japan, Tokyo, Japan) and nonabsorbable (Monosof; Covidien Japan) suture materials.

Peripheral blood samples were collected by using venipuncture before (day 0)

and on days 1, 3, 7, 14 and 28 after surgery. Samples were treated with ethylenediaminetetraacetic acid (EDTA) prior to a complete blood count test and heparinized samples were used for isolation of plasma and PBMC.

A complete blood count test was performed with an automatic cell counter (Celltac α; Nihon Kohden, Tokyo, Japan) and microscopic examination of blood smears stained using a rapid stain kit (Hemacolor; Merck, Darmstadt, Germany). Cell counts of peripheral blood neutrophils (PBN) and PBL were calculated from manual white blood cell (WBC) differential and the total WBC count. Plasma C-reactive protein (CRP) level was measured with a Laser CRP-2 (Arrows, Osaka, Japan) according to the manufacturer's instruction.

Isolation of PBMC

PBMC were isolated from heparinized blood by the method described in chapter 1. Isolated PBMC were used immediately in further assays described below.

Flow cytometric analysis of PBMC

T lymphocyte subsets in PBMC were analyzed by flow cytometry (EPICS XL; Beckman Coulter, Miami, USA). A PBMC suspension adjusted at 2×10^6 cells/ml with phosphate buffered physiological saline (PBS) was dispensed by 50 μl to 3 tubes. Ten μl fluorescent isothiocyanate (FITC)-labeled anti-canine CD3 antibody (AbD Serotec) was added to one tube. Ten μl FITC-labeled anti-canine CD4 antibody/ R-phycoerythrin (R-PE)-labeled anti-canine CD8 antibody (AbD Serotec) was added to the second tube. The third tube served as a non-antibody control. The three tubes were incubated for 30 min at 4°C and then the cell suspension in each tube was mixed with an appropriate volume of PBS. The tubes were then centrifuged at 1,600 rpm for 6 min at 20°C. The cell pellets were suspended in 500 μl of sheath solution (IsoFlow sheath fluid; Beckman

Coulter) and were then used for flow cytometric analysis.

Relative quantification of cytokine messenger RNA (mRNA) expression in PBMC

PBMC adjusted at 4×10^6 cells were lysed with 750 μl of ISOGEN (Nippon Gene, Toyama, Japan) and left to stand for 5 min at 20°C. The lysate was applied to a QIA shredder column (Qiagen, Tokyo, Japan) and centrifuged at 12,000 rpm for 2 min at 4°C. The cell lysate was mixed with 200 μl of chloroform, shaken vigorously on a vortex mixer, and then left to stand for 3 min at 20°C. The mixture was then centrifuged at 12,000 rpm for 15 min at 4°C and the aqueous phase was mixed with 500 μl of 2-propanol, and left to stand for 10 min at 20°C. The solution was centrifuged at 12,000 rpm for 10 min at 4°C. The precipitate was washed with 1 ml of 70% ethanol and centrifuged at 7,500 rpm for 5 min at 4°C, and the precipitate was dried after removing the supernatant. The precipitate was dissolved in 10 μl of diethylpyocarbonate (DEPC)-treated water. The total RNA content was quantified by a spectrophotometer (Eppendorf, Hamburg, Germany).

One μg of the extracted total RNA was mixed with 0.5 μl of oligo (dT) 12-18 (Amersham Pharmacia Biotech, Swampscott, USA), 4 μl of 5×buffer for ReverTra Ace, 2 μl of 10 mM PCR nucleotide mix (GE Healthcare, Little Chalfont, UK), and 1 μl of ReverTra Ace (Toyobo, Osaka, Japan), and then the total volume was adjusted to 20 μl by adding DEPC-treated water. The mixed solution was reacted at 30°C for 10 min, at 42°C for 60 min, and at 99°C for 5 min for complementary DNA (cDNA) synthesis.

Real-time polymerase chain reaction (PCR) was performed for relative quantification of IL-2, IL-4, IL-10, IL-12 p35, IL-12 p40, and TGF-β1 mRNA expression in PBMC. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control and relative expressing levels of cytokine mRNA were estimated.

To examine each expression level, the preoperative level was defined as 1. All primer pairs used in this study were designed with an assay design center (Roche Diagnostics, Mannheim, Germany). The primer pairs used in the study are shown in Table 2. Five μl of the synthesized cDNA solution was mixed with 10 μl of water PCR grade, 0.2 μl of the applicable Universal ProbeLibrary probe (Roche Diagnostics), 0.4 μl of 10 μM forward primer, 0.4 μl of 10 μM reverse primer, and 4.0 μl of LightCycler TaqMan Master (Roche Diagnostics). The solution was infused into a LightCycler Capillary (Roche Diagnostics) and centrifuged at 3,000 rpm for 30 sec at 20°C with LC Carousel Centrifuge 2.0 (Roche Diagnostics). After centrifugation, real-time PCR was performed using LightCycler 1.5 (Roche Diagnostics). The reaction conditions were as follows: 10 min at 95°C, followed by 45 cycles each consisting of 10 sec at 95°C and 30 sec at 60°C, and a final 30 sec at 40°C.

T-LAK Therapy

T-LAK were generated by the method described in chapter 1. Total cell counts and percentages of T lymphocyte subsets ($CD3^+$, $CD4^+$, $CD8^+$) in the suspension were investigated as described above before administration to dogs. IFN-γ mRNA expressing level was also evaluated by real-time PCR. The suspension of T-LAK was injected intravenously into each dog in T-LAK group immediately after laparotomy.

Statistical analysis

All results are shown as mean ± standard deviation. A comparison between T-LAK and PBMC was performed with paired *t* test. A comparison between T-LAK group and control group was performed with two-way ANOVA and Tukey-Kramer method. Statistical significance was set at $P<0.05$. All analyses were performed using Statcel 3, a Microsoft Excel plug-in (OMS Publishing).

Section 2: Results

The total cell count administered by T-LAK therapy was 6.4±1.6 $\times 10^8$ cells. Percentages of T lymphocyte subsets of T-LAK were 99±0.4 % in $CD3^+$, 30±9.3 % in $CD4^+$, 69±9.2 % in $CD8^+$, and 24±6.8 % in $CD4^+CD8^+$ cells. The IFN-γ mRNA expressing level in T-LAK on day 14 was significantly higher than that in PBMC before culture (P<0.01, Fig. 2).

PBN counts in T-LAK and control groups increased immediately after laparotomy and reached a maximum on day 1 (Fig. 3-A). PBN count in T-LAK group returned to the preoperative count from day 3, whereas this return did not occur until day 7 in control group. The plasma CRP levels increased immediately after laparotomy and reached a maximum on day 1 in both groups, and were in the normal range from day 3 onwards in T-LAK group and from day 7 onwards in control group (Fig. 3-B).

IL-4 mRNA expressing level tended to decrease after laparotomy, reached a minimum on day 7, and then significantly increased to above the preoperative level on days 14 in T-LAK group (P<0.05, Fig. 4-A). IL-4 mRNA expressing level in control group showed no clear change after laparotomy. Although IL-10 mRNA expressing level in control group showed no change after laparotomy, IL-10 mRNA expressing level increased markedly from day 3 after laparotomy and was significantly higher than all other on day 7 (P<0.05, Fig. 4-B), and then tended to decrease to the preoperative level in T-LAK group. The TGF-β1 mRNA expressing level decreased after laparotomy and reached significantly lower level on day 7 than the preoperative level in T-LAK group (P<0.05, Fig. 4-C). TGF-β1 mRNA expressing level tended to increase on day 28 in both groups.

The PBL cell count increased after laparotomy, remained high on days 3 to 7,

and returned to the preoperative value on day 28 in T-LAK group (Fig. 5-A). The $CD3^+$, $CD4^+$, and $CD8^+$ cell counts in T-LAK group showed similar changes (Fig. 5-B, 5-C, 5-D). The PBL count in control group decreased after laparotomy, showed no change from days 1 to 7 and then returned to the preoperative value on day 28. The $CD3^+$, $CD4^+$, and $CD8^+$ cell counts behaved similarly. Counts of PBL and all T lymphocyte subsets in T-LAK group were significantly higher than those in control group by two-way ANOVA ($P<0.05$).

The expressing level of IL-2 mRNA in both groups tended to decrease after laparotomy and reached a minimum on day 7 (Fig. 6-A). IL-2 mRNA expressing level was significantly higher than the preoperative level on days 14 and 28 in T-LAK group and on day 28 in control group ($P<0.05$). IL-12 p35 mRNA expressing level tended to decrease after laparotomy and reached a minimum on day 7 in T-LAK group and on day 3 in control group (Fig. 6-B). IL-12 p35 mRNA expressing level tended to increase on days 14 and 28 in T-LAK group and on day 28 in control group. IL-12 p40 mRNA expressing level tended to decrease after laparotomy and reached a minimum on day 7, and then increased on days 14 and 28 (Fig. 6-C). IL-12 p40 mRNA expressing level in control group did not change markedly from day 0 to day 28.

Section 3: Discussion

It is a popular method to produce T-LAK using autologous PBL with solid phase anti-CD3 antibody and IL-2 in dogs (Itoh *et al*., 2003; Hoshino *et al*., 2008) and humans (Sekine *et al*., 1993). The count of cultured lymphocytes increased approximately 46-fold and the main subset of cultured lymphocytes were $CD3^+$ cells. In addition, IFN-γ mRNA expressing level of cultured lymphocytes, which was one of the indicator cytokine of T-LAK (Hoshino *et al*., 2008), was significantly higher than PBMC (Fig. 2). Therefore, the cultured lymphocytes in this study are possible to regard as T-LAK.

The PBN count and plasma CRP level are considered markers for the presence and severity of inflammation (Yamashita *et al*., 1994; Nakamura *et al*., 2008). The PBN count and the plasma CRP level in T-LAK group returned to the normal range earlier than in control group (Fig. 3). These results suggested that the postoperative T-LAK therapy might provide earlier resolution of inflammation in dogs after laparotomy.

Resolution of inflammation needs anti-inflammatory cytokines such as IL-4 (Hiroi *et al*., 2013), IL-10 (de Waal Malefyt *et al*., 1991; Moore *et al*., 2001; Pestka *et al*., 2004) and TGF-β (Yoshimura *et al*., 2010), and lymphocytes, especially T lymphocytes, control inflammatory responses by producing these cytokines. Of these anti-inflammatory cytokines, IL-10 is the most important anti-inflammatory cytokine and an initial increase of IL-10 prevents the systemic inflammation and severe postoperative complication (Lin *et al*., 2000; Kimura *et al*., 2010). Although IL-10 mRNA expressing level in control group showed no clear changes after laparotomy, the remarkable increase of the IL-10 mRNA expressing level was observed at the early period after laparotomy, accompanying the decrease of the plasma CRP level to the

normal range in T-LAK group (Fig. 3-B, 4-B). This result indicated that postoperative T-LAK therapy induced production of IL-10 in PBMC, which was closely related to resolution of the early phase of inflammation in the dogs after laparotomy. It is possible to consider that the inflammatory responses in control group might be controlled by local anti-inflammatory responses, whereas the responses in T-LAK group might be resolved by systemic anti-inflammatory responses. There are some studies to report that IL-10 is induced by IFN-γ production of T lymphocytes for prevention of excessive inflammation (Jankovic *et al*., 2010; Cope *et al*., 2011), and the IFN-γ producing T-LAK are thought to be possibly associated with the induction of IL-10 production. On the other hand, the mRNA expressing levels of IL-4 and TGF-β increased after the decrease of the CRP to the normal range in T-LAK group (Fig. 3-B, 4-A, 4-C). These results indicated that IL-4 and TGF-β production was unrelated to control of inflammatory responses at the early phase period after laparotomy. IL-4 and TGF-β promotes wound healing after injury, and increases in IL-4 and TGF-β production by T-LAK therapy are likely to be part of the wound healing response rather than resolution of early phase of postoperative inflammation.

Lymphocytes, especially T lymphocytes, are important in control of inflammation (Ni Choileain *et al*., 2006; Stoecklein *et al*., 2012). Yamada *et al*. (2002) have shown that the numbers of PBL and $CD3^+$ cells decrease immediately in dogs after laparotomy and recover to the preoperative values until 4th week after that. The postoperative decrease of PBL count is thought to be associated with severe complications, such as sepsis and organ failure (Yamaguchi *et al*., 2006; Westerterp *et al*., 2008). Although the reduction of PBL, $CD3^+$, $CD4^+$ and $CD8^+$ lymphocyte counts in control group occurred immediately after laparotomy, there was no decrease in the

counts of PBL and the three subsets were detected after laparotomy in T-LAK group (Fig. 5). It is indicated that postoperative T-LAK therapy could prevent the loss of PBL, $CD3^+$, $CD4^+$ and $CD8^+$ lymphocytes in dogs after laparotomy. The PBL in T-LAK group may be propagated the T lymphocyte subsets producing IL-10 at the early period after laparotomy.

T-LAK therapy is considered activating cell-mediated immune response (Yamaguchi *et al*., 2006; Ishikawa *et al*., 2011). IL-2 and IL-12 are important cytokines for cell-mediated immune responses and surgical trauma has been known to suppress production of these cytokines by T lymphocytes in association with postoperative inflammation (Kimura *et al*., 2010; Stoecklein *et al*., 2012). The expressing level of IL-2 mRNA in T-LAK group increased to significantly higher level, earlier than in control group (Fig. 6-A). The expressing level of IL-12 p35 and IL-12 p40 mRNA in T-LAK group also tended to increase similarly to IL-2 mRNA (Fig. 6-B, 6-C). Interestingly, the increase of mRNA expressing levels of cell-mediated immune cytokines was observed after the resolution of the postoperative inflammation in T-LAK group. It is indicated that T-LAK therapy might induce earlier restoration of cell-mediated immune responses by the resolution of the postoperative inflammation in dogs after laparotomy.

In conclusion, T-LAK therapy in dogs after laparotomy could provide earlier resolution of inflammation, induction of IL-10 production by PBMC in the early phase of postoperative inflammation, and earlier restoration of production of cell-mediated immune cytokines (IL-2 and IL-12) in PBMC in addition to prevention of postoperative loss of PBL. It is indicated that resolution of postoperative inflammation brings about the earlier restoration of cell-mediated immune responses in dogs with T-LAK therapy

after laparotomy. Although further studies including assessment of the protein level of cytokine expression in PBMC are required to evaluate the association between T-LAK therapy and postoperative inflammatory responses in dogs, T-LAK therapy may be effective for resolution of inflammation and restoration of the cell-mediated immune responses in dogs after surgery.

Table 2. List of primer pairs

Primer		Sequence (5'-3')	Length of amplicon
GAPDH	F	ATGATTCTACCCACGGCAAA	76 bp
	R	ATGGACTTCCCGTTGATGAC	
IFN-γ	F	CGGTGGGTCTCTTTTCGTAG	72 bp
	R	TGGCTCTGAATGATTGTTTTGT	
IL-2	F	CAGAGCAACAGATGGAGCAA	61 bp
	R	TTATTAACTCCATTCAAAAGCAACTG	
IL-4	F	CAGATATCTCAGAGGACTCTACAGGA	75 bp
	R	TTCTTGATTTCATTCATAGAACAGGT	
IL-10	F	TGGGAGAGAAGCTCAAGACC	66 bp
	R	TCTCACAGGGCAGAAATCG	
IL-12 p35	F	GGAAAGGCCTCTTTTATGACG	70 bp
	R	CCATCTGGTACATCTTCAAGTCC	
IL-12 p40	F	GCGTCTTCCCTCATGACC	68 bp
	R	GGGTGCCAGTCCAACTCTAC	
TGF-β1	F	TGGCTGTCCTTTGATGTCAC	71 bp
	R	CGAAAGCCCTCGACTTCC	

F: forward primer R: reverse primer

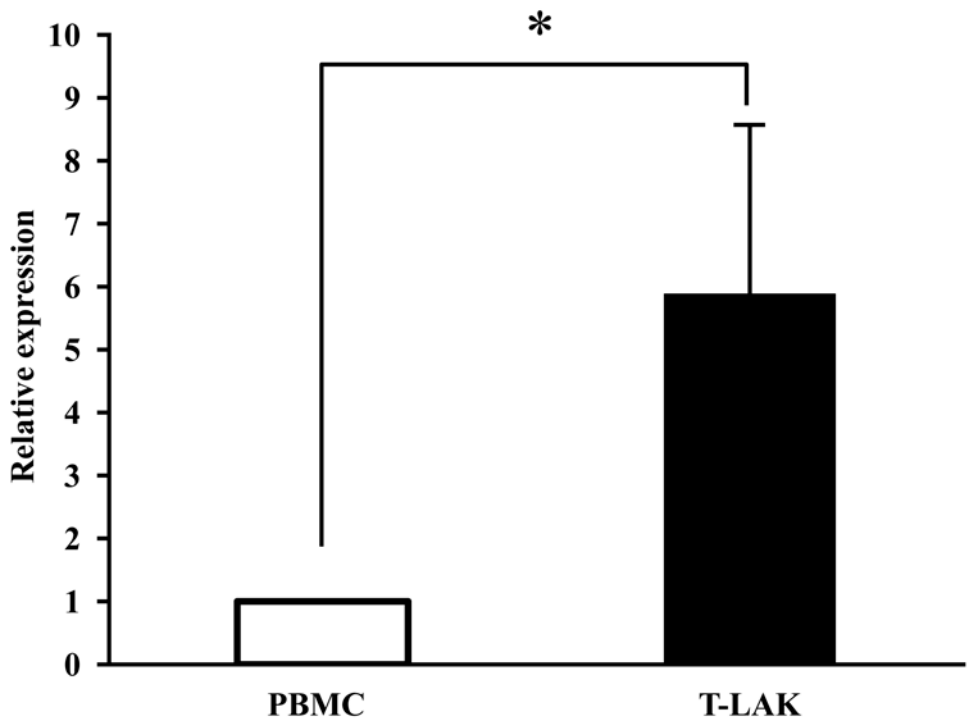

Fig. 2.

IFN-γ mRNA expressing levels in PBMC and T-LAK. The black and white boxes represent T-LAK and control group, respectively. * $P < 0.01$ vs PBMC.

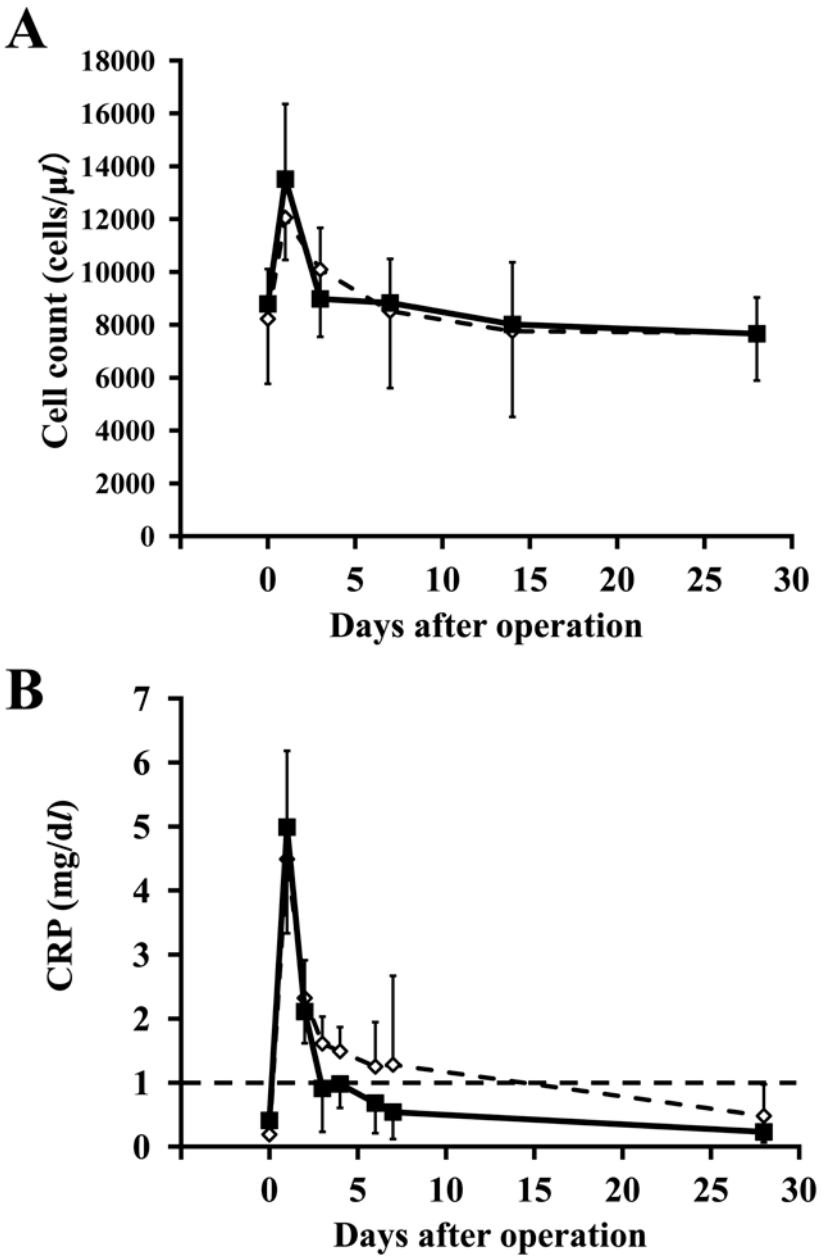

Fig. 3.

Effects of T-LAK therapy on PBN count and plasma CRP level. (A) PBN counts, (B) plasma CRP levels. The black square with solid line represents T-LAK group and the white diamond with dash line represents control group. The normal range of plasma CRP level (<1mg/dl) was shown as dashed line.

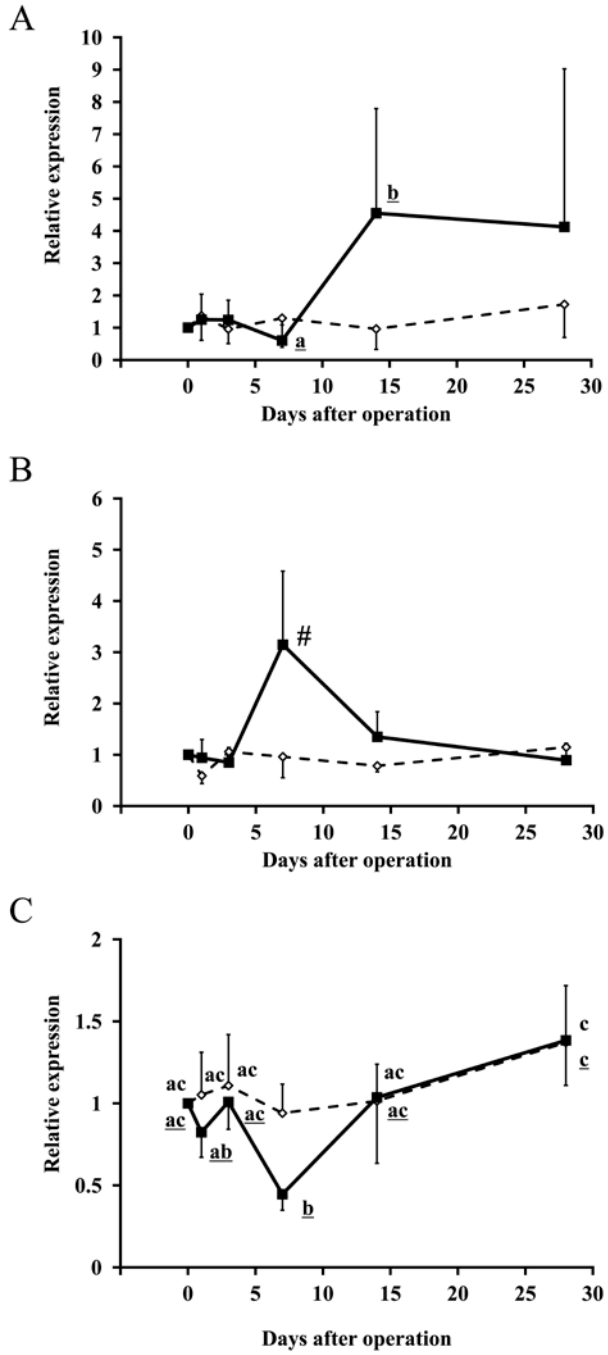

Fig. 4.

Effects of T-LAK therapy on mRNA expression of anti-inflammatory cytokines in PBMC. (A) IL-4, (B) IL-10, (C) TGF-β1. The black square with solid line represents T-LAK group and the white diamond with dash line represents control group. A significant difference ($P<0.05$) is indicated by different lower cases. Lower cases with and without underline indicate the levels in T-LAK and control group, respectively. #$P<0.01$ vs all other.

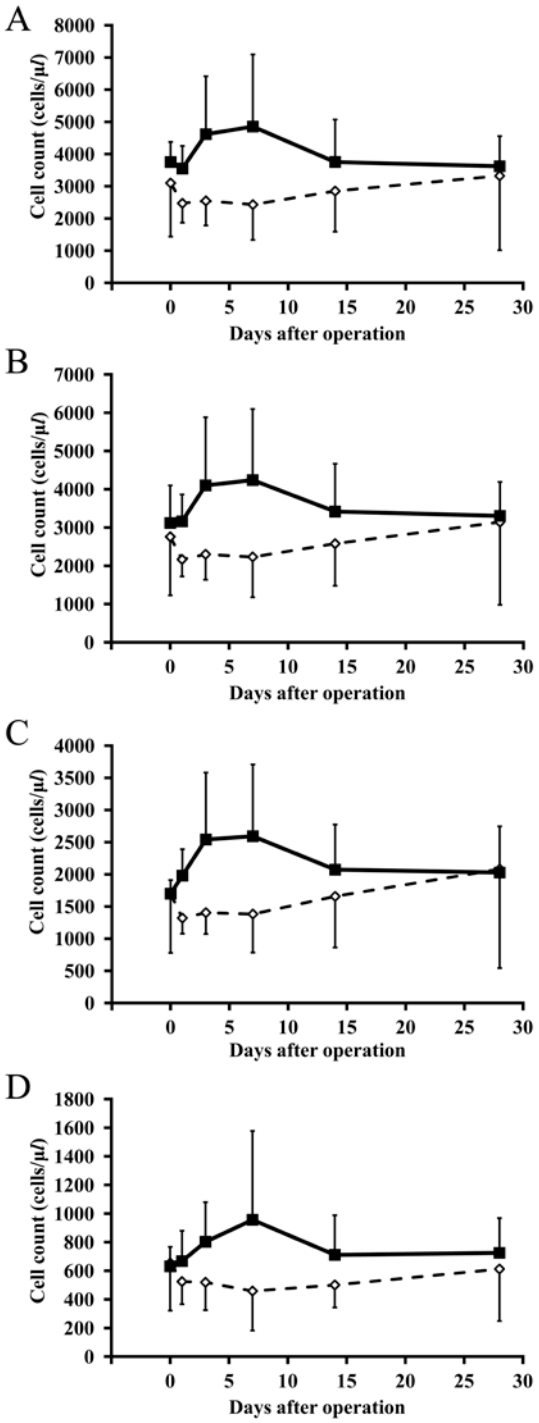

Fig. 5.

Effects of T-LAK therapy on counts of PBL and T lymphocyte subsets. (A) PBL, (B) $CD3^+$, (C) $CD4^+$, (D) $CD8^+$ cells. The black square with solid line represents T-LAK group and the white diamond with dash line represents control group. Counts of PBL and all T lymphocyte subsets in T-LAK group were significantly higher than those in control group by two-way ANOVA ($P<0.05$).

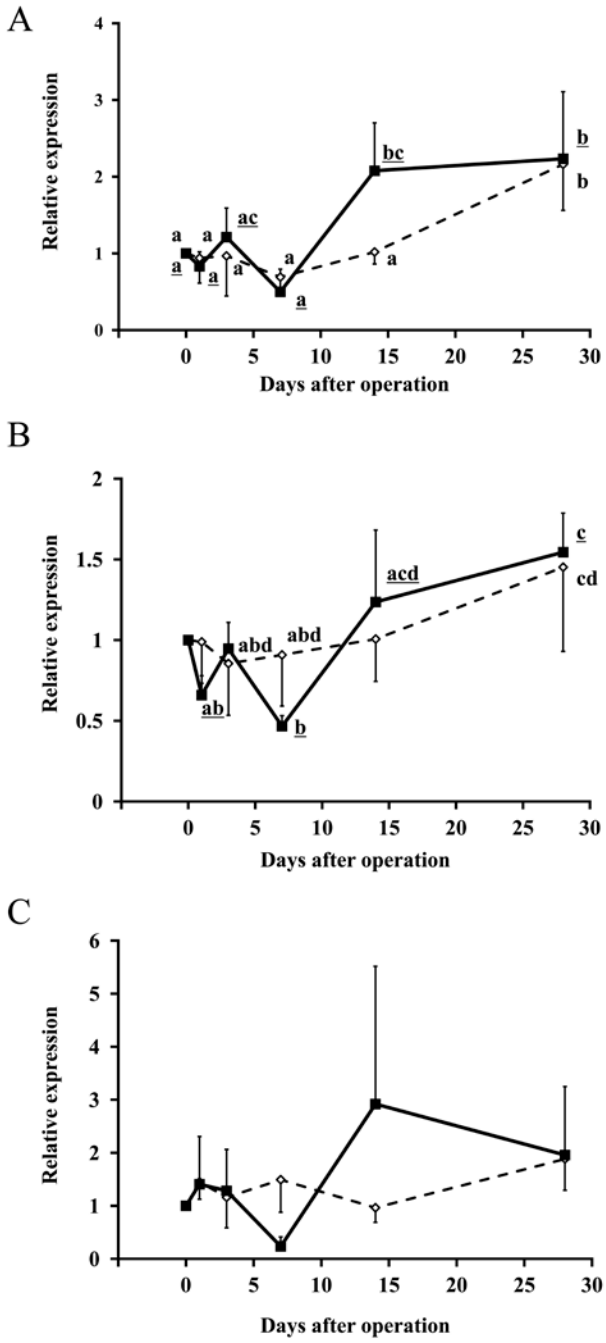

Fig. 6.

Effects of T-LAK therapy on mRNA expression of cell-mediated immune cytokines in PBMC. (A) IL-2, (B) IL-12 p35, (C) IL-12p40. The black square with solid line represents T-LAK group and the white diamond with dash line represents control group. A significant difference (P<0.05) is indicated by different lower cases. Lower cases with and without underline indicate the levels in T-LAK and control group, respectively.

Chapter 3: Change of PBL in dogs received T-LAK therapy combined with palliative resection of tumors

Postoperative inflammation can accelerate postoperative recurrence and metastasis of tumors (Goldfarb and Ben-Eliyahu, 2006-2007; van den Tol *et al*., 2007; Gottschalk *et al*., 2010; Predina *et al*., 2012; Roxburgh *et al*., 2013). Although lymphocytes are vital for resolution of postoperative inflammation, lymphocytes are suppressed by tumors and postoperative inflammation, and this suppression of lymphocytes cannot be resolved by surgical resection of tumors (Romano *et al*., 2003). In chapter 2, it had been shown that T-LAK therapy in healthy dogs after laparotomy could provide earlier resolution of inflammation, induction of IL-10 production by PBMC in the early phase of postoperative inflammation, and earlier restoration of production of cell-mediated immune cytokines in PBMC in addition to prevention of postoperative loss of PBL. Although these effects of T-LAK therapy are expected in tumor-bearing dogs after surgery, there are few studies about change of PBL in tumor-bearing dogs after T-LAK therapy with surgery.

In this chapter, the change of PBL in tumor-bearing dogs received T-LAK therapy combined with palliative resection of tumors was evaluated. The aim of this chapter was to accumulate the basic data for evaluation of immunoenhancing effect of T-LAK therapy combined with surgery in tumor-bearing dogs.

Subchapter 1: Change of PBL counts after single administration of T-LAK

Section 1: Materials and Methods

Patients

Fourteen tumor-bearing dogs received T-LAK therapy at Veterinary Teaching Hospital of Osaka Prefecture University from 2012 to 2014 were enrolled in this retrospective study after sufficient informed consent. The dogs received palliative resection of tumors with or without radiotherapy in combination with T-LAK therapy. Because some dogs were received palliative resection of tumors in other veterinary hospital and then referred our medical center to receive T-LAK therapy, initiation time of T-LAK therapy ranged 3 days before surgery to 5 month after surgery. In 14 tumor-bearing dogs, PBL before and after the first administration of T-LAK were examined for the analysis of change of PBL after single administration of T-LAK.

Analysis of PBL

Peripheral venous blood samples were collected before and about 2 weeks after the first administration of T-LAK. Hematological examination was performed with automated blood cell counting system (pocH-100iV Diff; Sysmex) and blood smear stained by rapid stain kit (Hemacolor; Merck).

Phenotypic analysis of T lymphocyte subsets ($CD3^+$, $CD4^+$ and $CD8^+$ cells) in PBL was performed by flow cytometric analysis. In brief, isolated PBMC were washed with FACS buffer [D-PBS (Nacalai Tesque) with 2 % FBS (Biowest) and 0.01 % NaN_3 (Wako Pure Chemical Industries, Ltd., Osaka, Japan)] and incubated with appropriate volume of FITC-labeled anti-canine CD3 antibody (mouse anti-canine CD3; AbD serotec) or FITC-labeled anti-canine CD4 / R-PE-labeled anti-canine CD8 antibody (rat anti-canine CD4 / CD8; AbD serotec) for 30 min at 4 °C. After incubation, PBMC were

washed and resuspended with 500 μ*l* of FACS buffer. Phenotypic analysis was performed by flow cytometric analysis (BD FACSCalibur; BD biosciences, San Jose, U.S.A.).

T-LAK therapy

T-LAK were generated from autologous PBMC by the culture method described above.

Statistical analysis

All results were represented by box and whisker plots. For statistical analysis, Mann-Whitney's U test was used, and *P*-values of <0.05 were considered statistically significant. All statistical analyses were performed using Statcel 3, a Microsoft Excel plug-in (OMS publishing).

Section 2: Results

Characteristics of 14 tumor-bearing dogs in analysis of change of PBL after single administration of T-LAK are listed in Table 3. Median age of the dogs was 10 years (range 6 to 17 years). The most frequent tumor was malignant melanoma. More than half of the tumor-bearing dogs had metastases.

Count and phenotype of administered T-LAK are listed in Table 4. Median T-LAK count was 223.5 $\times 10^6$ cells (range 5.5 to 3,440 $\times 10^6$ cells). Median proportions of $CD3^+$, $CD4^+$, and $CD8^+$ cells were 73.2 % (range 18.7 to 96.2 %), 38.3 % (range 5.5 to 65.7 %), and 39.8 % (range 7.9 to 79.2 %), respectively.

Changes of PBL and T lymphocyte subsets after single administration of T-LAK are shown in Fig. 7. Counts of PBL and T lymphocyte subsets ($CD3^+$, $CD4^+$, and $CD8^+$ cells) tended to increase after single administration of T-LAK (Fig. 7-A, 7-B, 7-C, 7-D). Especially, $CD8^+$ cells increased significantly ($P<0.05$). In addition, CD4/CD8 ratio tended to decrease after single administration of T-LAK (Fig. 7-E).

Section 3: Discussion

T-LAK therapy has been applied for patients with tumors in combination with surgery to prevent postoperative recurrence and metastasis of tumors by improving the postoperative immune response suppressed by surgical invasion in human medicine (Takayama *et al*., 2000; Xie *et al*., 2012; Zhong *et al*., 2012). In chapter 2, it had been shown that PBL and T lymphocyte phenotypes could be improved by T-LAK therapy combined with surgery in healthy dogs. The results in this subchapter suggested that T-LAK therapy combined with surgery could also improve peripheral blood T lymphocyte phenotypes in tumor-bearing dogs.

The results in this subchapter showed that PBL and T lymphocyte subsets tended to increase after single administration of T-LAK in dogs received palliative resection of tumors (Fig. 7-A, 7-B, 7-C, 7-D). Previous study has reported that counts of PBL and T lymphocyte subsets increased after single administration of T-LAK in healthy dogs (Itoh *et al*., 2003). The result in chapter 2 had also shown increase of PBL and T lymphocyte subsets in healthy dogs administered T-LAK after laparotomy. Hence, as with these findings reported in healthy dogs, the results suggested that T-LAK therapy combined with surgery could increase PBL and T lymphocyte subsets also in tumor-bearing dogs. Notably, peripheral blood $CD8^+$ cells increased significantly and CD4/CD8 ratio tended to decrease after single administration of T-LAK (Fig. 7-D, 7-E). These findings suggested that T-LAK therapy combined with surgery could increase $CD8^+$ cells dominantly in tumor-bearing dogs. Similarly in the results in this subchapter, previous studies in human medicine have also reported that T-LAK therapy could increase peripheral blood $CD8^+$ cells and decrease CD4/CD8 ratio in patients with tumors (Hirokawa *et al*., 2009; Sun *et al*., 2011; Noguchi *et al*., 2014). $CD8^+$ cells,

which are regarded as cytotoxic T cells, have been expected to exert an important effect in anti-tumor immune response (Montes *et al*., 2005; Sun *et al*, 2011). However, the number of $CD8^+$ cells may reduce in dogs with various tumors including malignant melanoma (Watabe *et al*., 2011). Therefore, the results in this subchapter suggested that T-LAK therapy combined with surgery might augment the anti-tumor immune response through increase of cytotoxic T cells in tumor-bearing dogs.

Table 3. Characteristics of the tumor-bearing dogs in analysis of change of PBL after single administration of T-LAK.

Characteristics (n=14)		
Age (years)		
	median(range)	10 (6-17)
Gender		
	Male	1
	Male（castrated)	4
	Female	2
	Female（ovariectomized）	7
Histology		
	Malignant melanoma	7
	oral	3
	other	4
	Hepatocarcinoma	1
	Thyroid carcinoma	3
	Fibrosarcoma	1
	Other	2
Stage		
	Without metastasis	6
	With metastatic nodes	2
	With distant metastasis	6
Combined treatment		
	Palliative surgery	10
	Palliative surgery + radiotherapy	4

Table 4. Count and phenotype of T-LAK in single administration.

Count and phenotype of T-LAK (n=14)		Median	Range
Administered cell count ($\times 10^6$ cells)		223.5	5.5-3,440
Phenotype			
	$CD3^+$ cells (%)	73.2	18.7-96.2
	$CD4^+$ cells (%)	38.3	5.5-65.7
	$CD8^+$ cells (%)	39.8	7.9-79.2

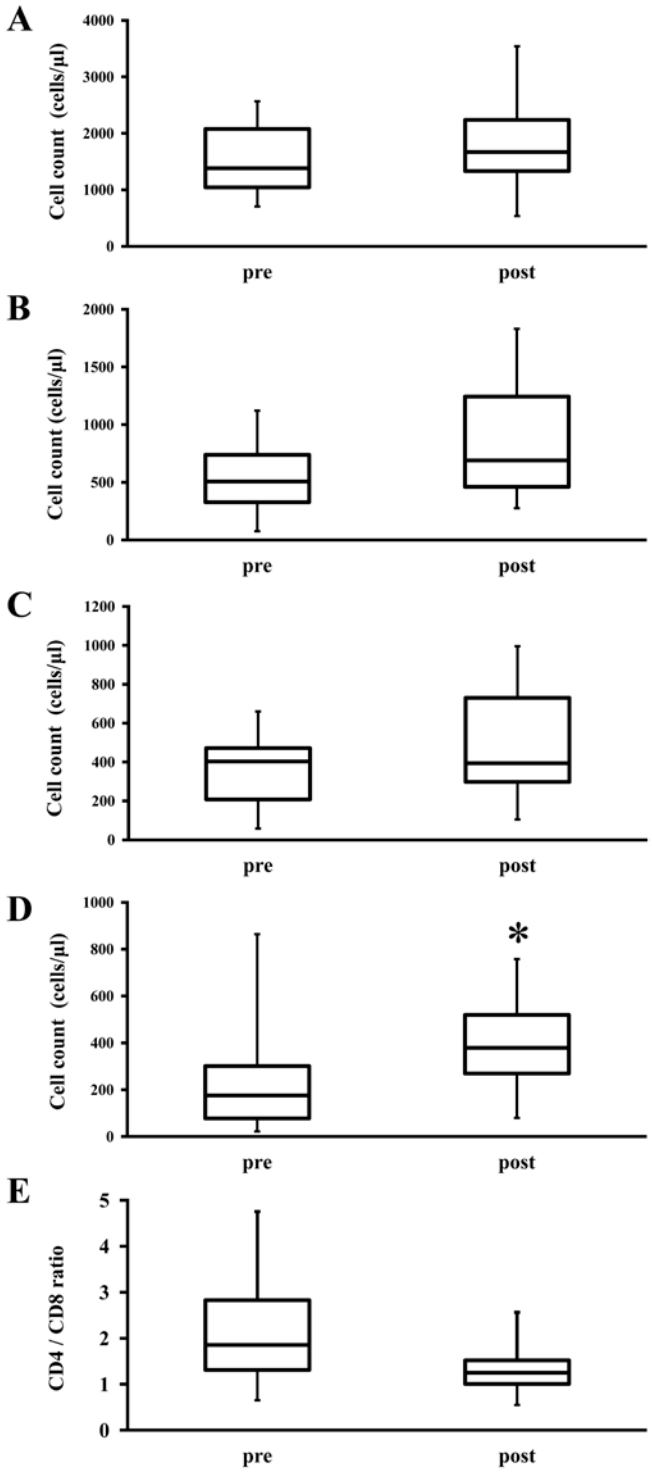

Fig. 7

Changes of PBL and T lymphocyte subsets after single administration of T-LAK. (A) PBL, (B) $CD3^+$, (C) $CD4^+$, (D) $CD8^+$ cells, (E) CD4/CD8 ratio. The data are shown at box and whisker plots. Box and whisker plots represent twenty-fifth/seventy-fifth percentile, median, and range. * $P<0.05$ between pre and post.

Subchapter 2: Change of PBL counts by sequential administration of T-LAK

Section 1: Materials and Methods

Patients

Eight tumor-bearing dogs received T-LAK therapy at Veterinary Teaching Hospital of Osaka Prefecture University from 2012 to 2014 were enrolled in this retrospective study after sufficient informed consent. The dogs received palliative resection of tumors with or without radiotherapy in combination with T-LAK therapy. Because some dogs were received palliative resection of tumors in other veterinary hospital and then referred our medical center to receive T-LAK therapy, initiation time of T-LAK therapy ranged 3 days before surgery to 8 month after surgery. In 8 tumor-bearing dogs received sequential administration of T-LAK, available data of changes of PBL until the fifth administration were analyzed.

Analysis of PBL

In 8 tumor-bearing dogs for the analysis of change of PBL by sequential administration of T-LAK, peripheral venous blood samples were collected before every administration and about 2 weeks after the fifth administration of T-LAK as possible. Hematological examination and phenotypic analysis of T lymphocyte subsets in PBL was performed by same methods described in subchapter 1.

T-LAK therapy

T-LAK were generated from autologous PBMC by the culture method described above. Sequential administration of T-LAK was performed at 2 to 4-week intervals.

Statistical analysis

All results were represented by box and whisker plots. The data were analyzed

by Kuruskal-Wallis test and Steel test in available-case analysis. *P*-values of < 0.05 were considered statistically significant. All statistical analyses were performed using Statcel 3, a Microsoft Excel plug-in (OMS publishing).

Section 2: Results

Characteristics of 8 tumor-bearing dogs are listed in Table 5. Median age of the dogs was 10.5 years (range 7 to 17 years). More than half of the tumor-bearing dogs had no metastases.

Counts and phenotypes of T-LAK from the first to the fifth administration are listed in Table 6. Range of median T-LAK counts was 148.5 to 443.5 $\times 10^6$ cells. Ranges of median proportions of $CD3^+$, $CD4^+$, and $CD8^+$ cells were 53.3 to 79.3 %, 36.0 to 55.2 %, and 23.9 to 41.7 %, respectively.

Changes of PBL and T lymphocyte subsets by five times administration of T-LAK were analyzed statistically by available-case analysis in 8 tumor-bearing dogs. Numbers of available data were 8 cases in pretreatment, 7 cases after the first, 5 cases after the second, 8 cases after the third, 7 cases after the fourth, and 8 cases after the fifth administration of T-LAK, respectively. Changes of PBL and T lymphocyte phenotypes by five times administration of T-LAK are shown in Fig. 8. PBL count tended to increase after the first administration of T-LAK and then returned to the approximate pretreatment value after the second administration (Fig. 8-A). Except after the fourth administration of T-LAK, $CD3^+$ and $CD4^+$ cell counts after T-LAK therapy marked slightly elevated values compared to the pretreatment (Fig. 8-B, 8-C). $CD8^+$ cell count increased after the first administration of T-LAK and kept elevated value after the second administration (Fig. 8-D). Then, $CD8^+$ cell count tended to decrease but marked higher values than the pretreatment from the third to the fifth administration of T-LAK. $CD8^+$ cell counts after the first and second administration of T-LAK were significantly higher than the pretreatment value ($P<0.05$). CD4/CD8 ratio tended to decrease after the first administration of T-LAK and kept decreased values until the fifth administration

(Fig. 8-E).

Section 3: Discussion

Although sequential administration of T-LAK has generally been applied for treatment of tumors, there have been few studies about sequential administration of T-LAK on tumor-bearing dogs. The results in this subchapter showed that $CD8^+$ cells kept higher values than the pretreatment in tumor-bearing dogs received sequential administration of T-LAK in combination with palliative resection of tumors (Fig. 8-D). Although $CD4^+$ cells kept slightly elevated values, CD4/CD8 ratio kept decreased values until the fifth administration of T-LAK (Fig. 8-C, 8-E). These results suggested that sequential administration of T-LAK could keep peripheral blood $CD8^+$ cells more dominant than the pretreatment state in tumor-bearing dogs. Previous study has reported that peripheral blood $CD8^+$ cells decreased and CD4/CD8 ratio increased according to advanced tumor stage in dogs with various tumors including malignant melanoma (Watabe *et al*., 2011). Therefore, sequential administration of T-LAK combined with surgery may recover counts and phenotypes of peripheral blood T lymphocytes from state dysregulated by malignant tumors to nearly healthy state in dogs. Dysregulation of peripheral blood T lymphocytes has possibly been caused by immunosuppressive activity of tumors. Treg, which are subpopulation of $CD4^+$ cells and suppress T lymphocytes mediated immune response, have been reported to increase in various tumors in dogs (Biller *et al*., 2007; Horiuchi *et al*., 2009). Whiteside (2002) has also reported that $CD8^+$ cells are especially sensitive to apoptosis and selective apoptosis induced by malignant tumors may cause depletion of $CD8^+$ cells in patients with tumors. T-LAK therapy has been applied in hopes to prevent such immunosuppressive activities of malignant tumors. In human medicine, T-LAK therapy has been reported to improve T lymphocytes mediated immune response by enhancement of immunostimulatory

cytokine production (such as IFN-γ) of PBL and reduction of peripheral blood Treg in patients with various tumors (Ishikawa *et al*., 2011). Although apoptosis of $CD8^+$ cells and Treg cell counts were not evaluated in this study, immunoenhancing effects of T-LAK might contribute to recovery of counts and phenotypes of peripheral blood T lymphocytes in dogs received palliative resection of tumors.

Surgical invasion also induces postoperative immunosuppression by suppressing T lymphocytes (Ni Choileain *et al*., 2006; Yamaguchi *et al*., 2006; Kimura *et al*., 2010). In subchapter 1 and 2, PBL count, counts of T lymphocyte subsets, and CD4/CD8 ratio before administration of T-LAK marked characteristic values reported in tumor-bearing dogs, which are decrease of T lymphocytes and increase of CD4/CD8 ratio (Watabe *et al*., 2011). Although periods from resection of tumors to initiating T-LAK therapy differed from case to case, the states of PBL and T lymphocyte subsets before administration of T-LAK could not recover to the healthy status despite palliative resection of tumors. In human medicine, Romano *et al*. (2003) indicated the possibility that postoperative immunosuppression could not be resolved over months only by surgical resection of tumors. Therefore, the results suggested that T-LAK therapy might be useful for recovery from postoperative immunosuppression whenever the adoptive immunotherapy combined with surgery.

Interestingly, T lymphocytes did not continue to increase by sequential administration of T-LAK in this study (Fig. 8-B, 8-C, 8-D). One possibility why increase of T lymphocytes did not continue might be migration of T lymphocytes from peripheral blood to tumor environment. Direct contact between activated T lymphocytes and tumor cells has been believed to be necessary for tumor cell lysis (Kjaergaard *et al*., 1998). In fact, increase of tumor-infiltrating $CD8^+$ cells has been reported as a favorable

prognosis factor in patients with tumors (Fridman *et al*., 2012). Although further studies are needed, peripheral blood T lymphocytes, especially $CD8^+$ cells increased by T-LAK therapy, might infiltrate into residual tumor site and thereby peripheral blood T lymphocytes could not continue to increase in dogs received T-LAK therapy combined with palliative resection of tumors. Another possibility might be acceleration of apoptosis of peripheral blood T lymphocytes. Fas mediated activation-induced cell death is one of the biological mechanisms for induction of apoptosis of T lymphocytes to prevent immune disturbance caused by excess activation of T lymphocytes (Maher *et al*., 2002; Maher *et al*., 2005). Because administered T-LAK were activated and cytokines produced by T-LAK could also activate autologous T lymphocytes, T-LAK therapy might trigger Fas mediated activation-induced cell death along with immunoenhancing effect of this adoptive immunotherapy.

In conclusion, T-LAK therapy combined with palliative resection of tumors could increase peripheral blood T lymphocytes, especially $CD8^+$ cells in tumor-bearing dogs. Although further studies are needed, T-LAK therapy combined with surgical resection of tumors might contribute to favorable outcome in tumor-bearing dogs by immunoenhancing effect of the treatment.

Table 5. Characteristics of the tumor-bearing dogs in analysis of Change of PBL by sequential administration of T-LAK.

Characteristics (n=8)	
Age (years)	
median(range)	10.5 (7-17)
Gender	
Male（castrated)	2
Female（ovariectomized）	6
Histology	
Malignant melanoma	3
oral	0
other	3
Hepatocarcinoma	2
Thyroid carcinoma	2
Soft tissue sarcoma	1
Stage	
Without metastasis	5
With distant metastasis	3
Combined treatment	
Palliative surgery	6
Palliative surgery + radiotherapy	2

Table 6. Counts and phenotypes of T-LAK from the first to the fifth administration.

Counts and phenotypes of T-LAK (n=8)		1st	2nd	3rd	4th	5th
Administered cell counts ($\times 10^6$ cells)						
	median	443.5	184.5	310	189.5	148.5
	range	5.5-2410	16-2165	93-1835	126-2365	4-2460
Phenotypes						
$CD3^+$ cells (%)						
	median	79.3	53.3	62.7	71.3	68.6
	range	38.9-91.8	24.5-96.6	32.0-99.6	19.1-95.9	36.7-98.7
$CD4^+$ cells (%)						
	median	40.2	37.4	49.1	55.2	36.0
	range	5.5-65.7	9.4-81.1	23.6-67.8	18.4-67.6	26.3-49.9
$CD8^+$ cells (%)						
	median	33.6	41.0	31.4	23.9	41.7
	range	7.9-78.0	9.1-65.1	10.2-62.3	11.2-74.5	19.7-52.9

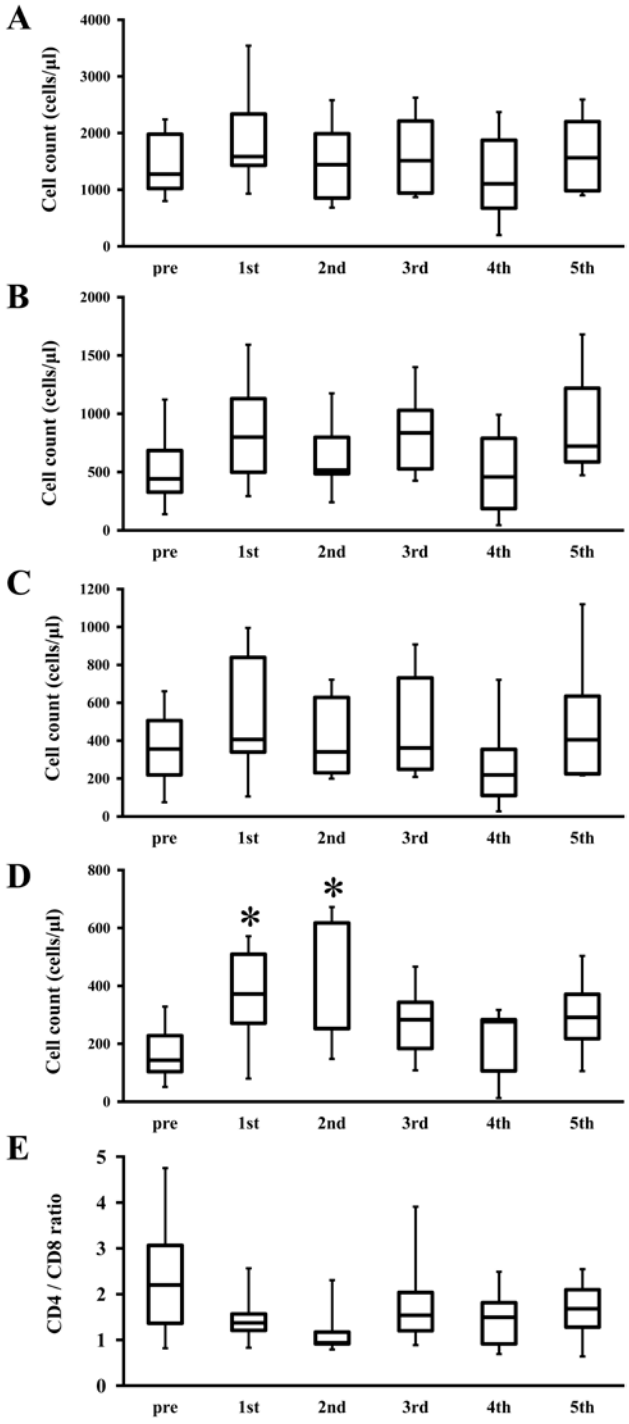

Fig. 8.

Changes of PBL and T lymphocyte subsets by five times administration of T-LAK. (A) PBL, (B) $CD3^+$, (C) $CD4^+$, (D) $CD8^+$ cells, (E) CD4/CD8 ratio. The data are shown at box and whisker plots. Box and whisker plots represent twenty-fifth/seventy-fifth percentile, median, and range. * $P<0.05$ compared with the pretreatment value.

Subchapter 3: Change of Cytokine mRNA Expression of PBL after single administration of T-LAK

Section 1: Materials and Methods

Patients

Four tumor-bearing dogs received T-LAK therapy at Veterinary Teaching Hospital of Osaka Prefecture University from 2012 to 2014 were enrolled in this retrospective study after sufficient informed consent. Dog 1 was a 9-year-old male French bulldog with a hepatocellular carcinoma (T3N0M0). Dog 2 was a 10-years-old spayed female mongrel dog with a thyroid carcinoma (T3N1M0). Dog 3 was a 6-years-old spayed female French bulldog with a fibrosarcoma (T3N0M0). Dog 4 was a 10-years-old spayed female Labrador retriever with a malignant melanoma (T1N0M0). All dogs received palliative resection of tumors with or without radiotherapy in combination with T-LAK therapy, and initiation time of T-LAK therapy ranged 1 week to 3 month after surgery.

Relative quantification of cytokine mRNA expression in PBMC

Peripheral venous blood samples were collected before and about 2 weeks after the first administration of T-LAK, and PBMC were isolated from heparinized blood by the method described above. The total RNA was extracted from PBMC by using Aurum total RNA mini kit (Bio-Rad, Hercules, U.S.A.). The total RNA content was quantified by a spectrophotometer (Eppendorf).

The total RNA was used for cDNA synthesis. 0.5 μg of the extracted total RNA was mixed with 1.25 μl of oligo (dT) 20 (Amersham Pharmacia Biotech, Swampscott, U.S.A.), 10 μl of 5×buffer for ReverTra Ace, 5 μl of dNTPs mixture (Toyobo), and 2.5 μl of ReverTra Ace (Toyobo), and then the total volume was adjusted to 50 μl by adding

DEPC-treated water. The mixed solution was reacted at 30°C for 10 min, at 42°C for 60 min, and at 99°C for 5 min for cDNA synthesis.

Real-time PCR was performed for relative quantification of IL-2 and IL-10 mRNA expression in PBMC. GAPDH was used as an internal control and relative expressing levels of cytokine mRNA were estimated. To examine each expression level, the preoperative level was defined as 1. The primer pairs used in the study are shown in Table 2. Five μl of the synthesized cDNA solution was mixed with 11μl of DEPC-treated water, 2 μl of 10 μM forward primer, 2 μl of 10 μM reverse primer, 5μl of plus solution and 25 μl of SYBR green real-time PCR master mix plus (Toyobo). Real-time PCR was performed using Mini Opticon real-time PCR system (Bio-Rad). The reaction conditions were as follows: 1 min at 95°C, followed by 40 cycles each consisting of 15 sec at 95°C, 15 sec at 60°C, and 45 sec at 72°C.

T-LAK therapy

T-LAK were generated from autologous PBMC by the culture method described above.

Section 2: Results

Counts and phenotypes of T-LAK are listed in Table 7.Changes of PBL and T lymphocyte subsets after T-LAK therapy are shown in Fig. 9. PBL and all T lymphocyte subsets tended to increase after T-LAK therapy in all dogs. Whereas CD4/CD8 ratio of dog 1 did not change markedly, CD4/CD8 ratio of other dogs tended to decrease after T-LAK therapy.

Effect of T-LAK therapy on IL-2 and IL-10 mRNA expression in PBMC of the dogs are shown in Fig. 10. IL-2 mRNA expressing level of dog 1 showed large increase after T-LAK therapy (Fig. 10-A). IL-2 mRNA expressing levels of dog 2 and 3 decreased after T-LAK therapy, whereas the expressing level of dog 4 slightly increased. IL-10 mRNA expressing levels of dog 1, 2, and 3 decreased after T-LAK therapy (Fig. 10-B). Especially, IL-10 mRNA expressing levels of dog 1 and 2 after T-LAK therapy marked below measurable limit. On the other hand, IL-10 mRNA expressing level of dog 4 markedly increased after T-LAK therapy.

Section 3: Discussion

I evaluated changes of IL-2 and IL-10 mRNA expression, which had shown markedly increase in chapter 2 (Fig. 4-B, 6-A), in PBMC of tumor-bearing dogs about 2 weeks after first administration of T-LAK in this subchapter. Although IL-2 mRNA expressing level was significantly increased 2 weeks after administration of T-LAK in healthy dogs with laparotomy in chapter 2, increases of IL-2 mRNA expression were seen in only 2 tumor-bearing dogs in this subchapter (Fig. 10-A). Changes of IL-10 mRNA expressions also did not show homogenous tendency (Fig. 10-B). These results suggest that changes of IL-2 and IL-10 mRNA expressing might not correspond with increases of PBL and T lymphocyte subsets after T-LAK in dogs received palliative resection of tumors (Fig. 9).

Cytokines secreted by lymphocytes are essential for regulation of immune response. Tumors can influence cytokine production of PBL and change of cytokine production of PBL may differ by tumor types and progression (Lippitz, 2013). Cytokine production of PBL is also influenced by degree of surgical invasiveness, which depends mainly on differences of surgical procedure (Brune *et al*., 1999; Yamaguchi *et al*., 2006; Karanika *et al*., 2013) and anesthesia technique (Schneemilch *et al*., 2005; Ionescu *et al*., 2013; Sofra *et al*., 2013). Differences of tumor types and surgical invasiveness might cause the different changes of IL-2 and IL-10 mRNA expressing of PBL in tumor-bearing dogs in this subchapter. It is reported that sequential administration of T-LAK can regulate cytokine production of PBL concurrently with increase of PBL count in patients with tumors (Ishikawa *et al*., 2011; Sun *et al*., 2011; Noguchi *et al*., 2014). Thus, sequential administration of T-LAK may need for improving of cytokine production of PBL in tumor-bearing dogs. A further study of change of cytokine

production of PBL after sequential administration of T-LAK in tumor-bearing dogs should be conducted.

Table 7. Counts and phenotypes of T-LAK

	Administered cell counts ($\times 10^6$ cells)	Phenotypes		
		$CD3^+$ cells (%)	$CD4^+$ cells (%)	$CD8^+$ cells (%)
Dog 1	5.5	79.3	5.5	78.0
Dog 2	171	89.3	13.4	79.2
Dog 3	270	48.7	18.8	64.2
Dog 4	585	80.3	58.2	27.2

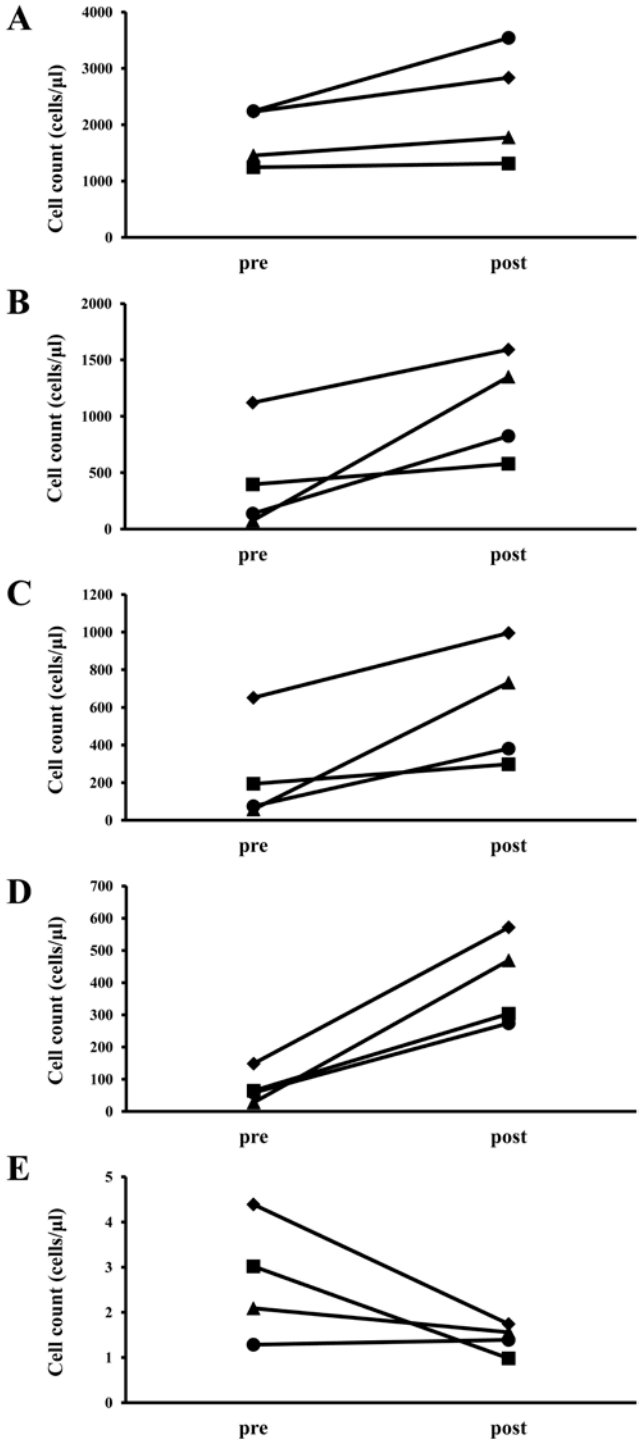

Fig. 9.

Change of PBL and T lymphocyte subsets after T-LAK therapy. (A) PBL, (B) $CD3^+$, (C) $CD4^+$, (D) $CD8^+$ cells, (E) CD4/CD8 ratio. The circle, triangle, square, and diamond represent dog 1, dog 2, dog 3, and dog4, respectively.

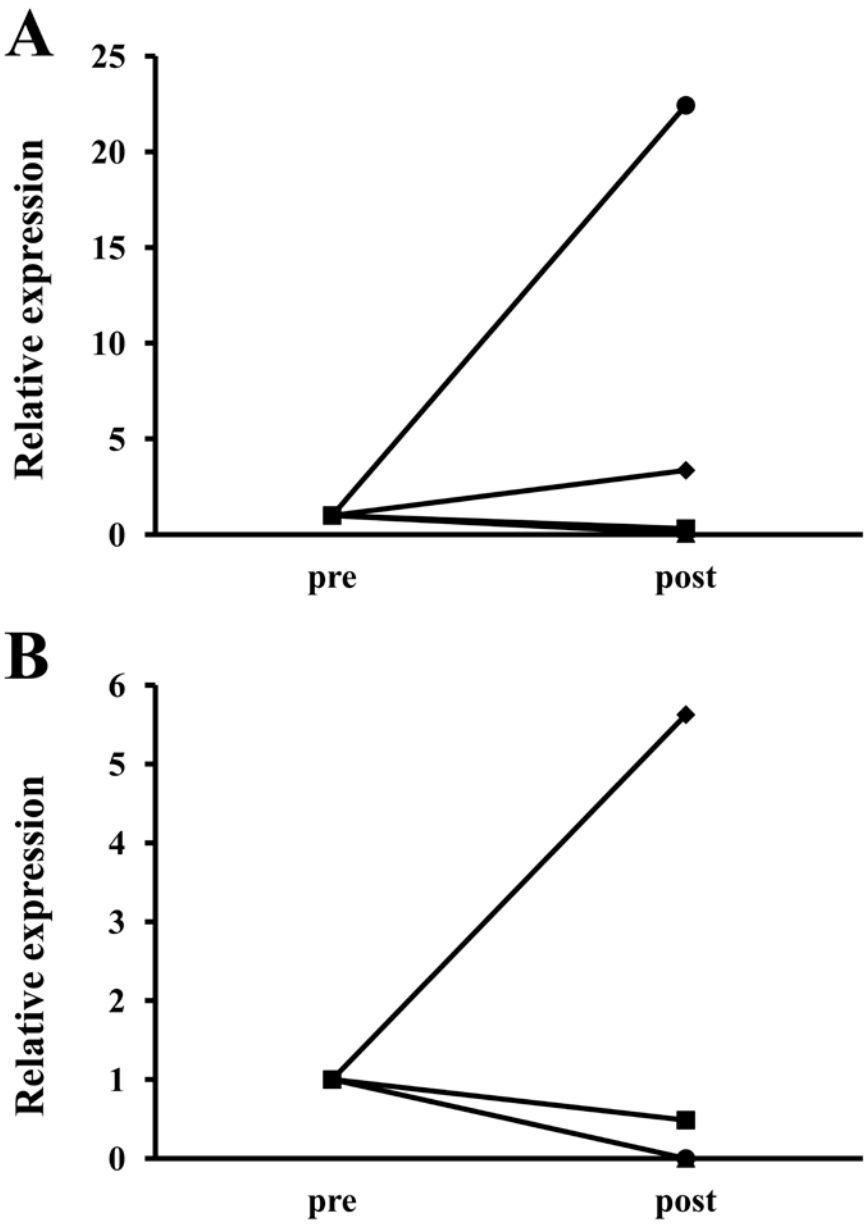

Fig. 10.

Effect of T-LAK therapy on IL-2 and IL-10 mRNA expression in PBMC of tumor-bearing dogs. (A) IL-2, (B) IL-10. The circle, triangle, square, and diamond represent dog 1, dog 2, dog 3, and dog4, respectively.

Subchapter 4: Relationship between change of peripheral blood $CD8^+$ cell count and prognosis in dogs received T-LAK therapy combined with palliative resection of tumors

Section 1: Materials and Methods

Patients

Among 14 tumor-bearing dogs in subchapter 1, 12 dogs were enrolled in this retrospective study after sufficient informed consent. The dogs received T-LAK therapy at 2 to 4-week intervals until they were unable to receive further treatment due to either poor general condition or if the owners refused any further treatment. On the basis of median increase rate of peripheral blood $CD8^+$ cell count after the first administration of T-LAK (median increase rate: 1.7), the dogs were divided into two groups, those with higher increase rate ($1.7\leq$, n=6) and those with lower increase rate (<1.7, n=6).

Statistical analysis

Differences were compared by Mann-Whitney's U test for intergroup comparisons. Overall survival was calculated from the day starting the first culture of T-LAK until 1 year later. Overall survival rates were analyzed using Kaplan-Meier curves and the log-rank test. *P*-values of <0.05 were considered statistically significant. All statistical analyses were performed using Statcel 3, a Microsoft Excel plug-in (OMS publishing).

Section 2: Results

Characteristics of dogs in this study are listed in Table 8. Median age of the dogs was 9.5 years (range 6 to 17 years) in the higher increase rate group and 12 years (range 7 to 15 years) in the lower increase rate group. There was no significant difference between the two groups in age. The types and stages of tumors varied in both groups, as shown in table 8.

Count and phenotype of the first administered T-LAK are listed in Table 9. There were no significant differences between the two groups in count and phenotype of the first administered T-LAK.

Overall survival rates were compared between the higher increase rate group and the lower increase rate group (Fig. 11). One year survival rates were 83.3 % in the higher increase rate group and 20.8 % in the lower increase rate group. Overall survival of the dogs in the higher increase rate group was significantly longer than the lower increase rate group (log-rank test P=0.04).

Section 3: Discussion

The results in subchapter 1, 2 and 3 had indicated that peripheral blood $CD8^+$ cells might change most obviously after T-LAK therapy in dogs received palliative resection of tumors. Hence, the relationship between overall survival and increase rate of peripheral blood $CD8^+$ cell count was assessed after T-LAK therapy in dogs received palliative resection of tumors. There are some studies to report that $CD8^+$ cells are more sensitive to immunosuppressive effect of tumors (Whiteside, 2002). $CD8^+$ cells may also be more sensitive to immunosuppression by surgical invasiveness (Delogu *et al.*, 2000; Yamada *et al.*, 2002). Thus, it is possible to consider that change of $CD8^+$ cells might be suitable for evaluation of immunological state in tumor-bearing dogs received T-LAK therapy with surgery.

In this subchapter, the tumor-bearing dogs were divided into two groups on the basis of median increase rate of peripheral blood $CD8^+$ cell count after the first administration of T-LAK. The result in this subchapter suggested that higher increase rate of $CD8^+$ cells might be a favorable factor for prognosis of dogs received T-LAK therapy combined with surgery (Fig. 11). Increase rate of $CD8^+$ cells was considered not to depend on administered cell count of T-LAK because there were no significant differences of count and phenotype of T-LAK. Thus, it was suggested that increase rate of $CD8^+$ cells might be associated with immunoenhancing effect of administered T-LAK. Assessment of change of immunological function of $CD8^+$ cells is needed for further understanding of therapeutic effect of T-LAK therapy.

In conclusion, higher increase of peripheral blood $CD8^+$ cells can be a positive factor for tumor-bearing dogs received T-LAK therapy combined with surgery.

Although further studies are needed, I believe that $CD8^+$ cells are key immune cells for obtaining good therapeutic effect of T-LAK therapy in tumor-bearing dogs.

Table 8. Characteristics of the tumor-bearing dogs

	1.7≤ (n=6)	<1.7 (n=6)
Age (years)		
median (range)	9.5 (6-17)	12 (7-15)
Gender		
Male	1	0
Male (castrated)	0	2
Female	0	2
Female (ovariectomized)	5	2
Histology		
Malignant melanoma	2	3
oral	0	2
other	2	1
Hepatocarcinoma	1	0
Thyroid carcinoma	2	1
Fibrosarcoma	1	0
Other	0	2
Stage		
Without metastasis	4	1
With metastatic node	1	1
With distant metastasis	1	4
Combined treatment		
Palliative surgery	5	4
Palliative surgery + radiotherapy	1	2

Table 9. Count and phenotype of the first administered T-LAK

		1.7≤ (n=6)	<1.7 (n=6)
Administered cell count ($\times 10^6$ cells)			
	median	250	663.5
	range	5.5-585	142-3440
Phenotype			
$CD3^+$ cells (%)			
	median	79.8	48.3
	range	48.7-91.9	21.9-84.4
$CD4^+$ cells (%)			
	median	29.5	39.0
	range	5.5-58.2	17.5-65.7
$CD8^+$ cells (%)			
	median	48.9	46.2
	range	16.0-79.2	7.9-73.1

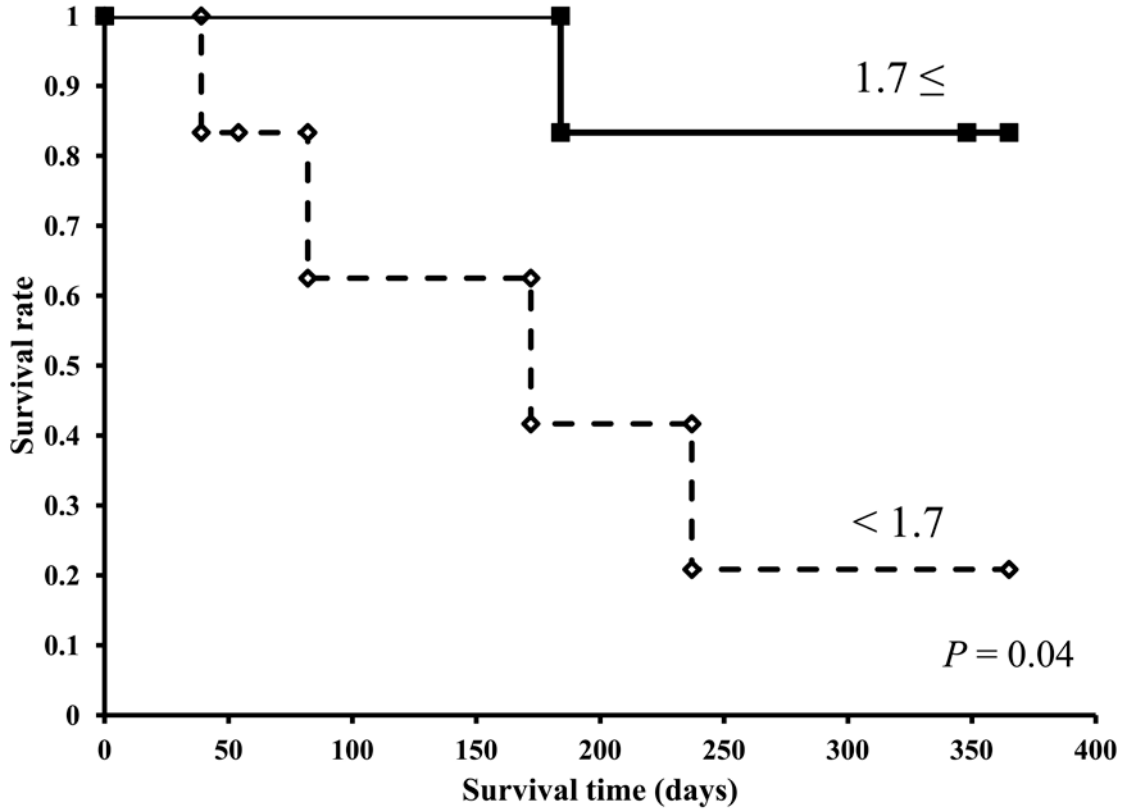

Fig. 11.

Kaplan-Meier survival curves for tumor-bearing dogs with higher $CD8^+$ cell increase rate (1.7≤, n=6; the black square with solid line), versus those with lower $CD8^+$ increase rate (<1.7, n=6; the white diamond with dashed line). The median increase rate of peripheral blood $CD8^+$ cell count after the first administration of T-LAK marked 1.7 times. Overall survival of the tumor-bearing dogs with higher $CD8^+$ cell increase rate was significantly longer than those with lower $CD8^+$ increase rate (log-rank test P=0.04).

Conclusion

The conclusions drawn from these studies are listed as follows:

1. Increased PBL count can be a positive factor for tumor-bearing dogs received T-LAK therapy.
2. T-LAK therapy in healthy dogs after laparotomy can provide earlier resolution of inflammation, induction of IL-10 production by PBMC in the early phase of postoperative inflammation, and earlier restoration of production of cell-mediated immune cytokines (IL-2 and IL-12) in PBMC in addition to prevention of postoperative loss of PBL.
3. T-LAK therapy combined with palliative resection of tumors can increase peripheral blood T lymphocytes, especially $CD8^+$ cells, whereas change of IL-2 and IL-10 production might not correspond with increases of PBL and T lymphocyte subsets in dogs received palliative resection of tumors. Higher increase of peripheral blood $CD8^+$ cells can be a positive factor for tumor-bearing dogs received T-LAK therapy combined with surgery.

These findings suggest that immunoenhancing effects of T-LAK therapy relate strongly to increase of PBL, especially $CD8^+$ cells. It is also indicated that T-LAK therapy combined with surgery can prevent postoperative immunosuppression by increasing PBL and controlling postoperative inflammation. It is hoped that these findings will contribute to better treatment outcome of anti-tumor surgical therapy combined with T-LAK therapy in dogs.

References

Aliperti, L.A., Predina, J.D., Vachani, A. and Singhal, S. (2011) Local and systemic recurrence is the Achilles heel of cancer surgery. *Ann. Surg. Oncol.* **18**: 603-607.

Alshaker, H.A. and Matalka, K.Z. (2011) IFN-γ, IL-17 and TGF-β involvement in shaping the tumor microenvironment: The significance of modulating such cytokines in treating malignant solid tumors. *Cancer Cell Int.* **11**: 33.

Biller, B.J., Elmslie, R.E., Burnett, R.C., Avery, A.C. and Dow, S.W. (2007) Use of FoxP3 expression to identify regulatory T cells in healthy dogs and dogs with cancer. *Vet. Immunol. Immunopathol.* **116**: 69-78.

Brune, I.B., Wilke, W., Hensler, T., Holzmann, B. and Siewert, J.R. (1999) Downregulation of T helper type 1 immune response and altered pro-inflammatory and anti-inflammatory T cell cytokine balance following conventional but not laparoscopic surgery. *Am. J. Surg*. **177**: 55-60.

Carvalho, M.I., Pires, I., Prada, J. and Queiroga, F.L. (2014) A role for T-lymphocytes in human breast cancer and in canine mammary tumors. *Biomed. Res. Int.* **2014**: 130894.

Catchpole, B., Gould, S.M., Kellett-Gregory, L.M. and Dobson, J.M. (2002) Immunosuppressive cytokines in the regional lymph node of a dog suffering from oral malignant melanoma. *J. Small Anim. Pract.* **43**: 464-467.

Cope, A., Le Friec, G., Cardone, J. and Kemper, C. (2011) The Th1 life cycle: molecular control of IFN-γ to IL-10 switching. *Trends. Immunol.* **32**: 278-286.

Critchley-Thorne, R.J., Yan, N., Nacu, S., Weber, J., Holmes, S.P. and Lee, P.P. (2007) Down-regulation of the interferon signaling pathway in T lymphocytes from

patients with metastatic melanoma. *PLoS Med.* **4**: e176.

de Andrés, P.J., Illera, J.C., Cáceres, S., Díez, L., Pérez-Alenza, M.D. and Peña, L. (2013) Increased levels of interleukins 8 and 10 as findings of canine inflammatory mammary cancer. *Vet. Immunol. Immunopathol.* **152**: 245-251.

Delogu, G., Moretti, S., Antonucci, A., Marcellini, S., Masciangelo, R., Famularo, G., Signore, L. and De Simone, C. (2000) Apoptosis and surgical trauma: dysregulated expression of death and survival factors on peripheral lymphocytes. *Arch. Surg.* **135**: 1141-1147.

de Waal Malefyt, R., Abrams, J., Bennett, B., Figdor, C. G. and de Vries, J. E. (1991) Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J. Exp. Med.* **174**: 1209-1220.

Ebina, T., Ogama, N., Shimanuki, H., Kubota, T. and Isono, N. (2003) Life-prolonging effect of immunocell BAK (BRM-activated killer) therapy for advanced solid cancer patients: prognostic significance of serum immunosuppressive acidic protein levels. *Cancer Immunol. Immunother.* **52**: 555-560.

Fridman, W.H., Pagès, F., Sautès-Fridman, C. and Galon, J. (2012) The immune contexture in human tumours: impact on clinical outcome. *Nat. Rev. Cancer.* **12**: 298-306.

Goldfarb, Y. and Ben-Eliyahu, S. (2006-2007) Surgery as a risk factor for breast cancer recurrence and metastasis: mediating mechanisms and clinical prophylactic approaches. *Breast Dis.* **26**: 99-114.

Gottschalk, A., Sharma, S., Ford, J., Durieux, M.E. and Tiouririne, M. (2010) The role of the perioperative period in recurrence after cancer surgery. *Anesth. Analg*.

110: 1636-1643.

Goulart, M.R., Pluhar, G.E. and Ohlfest, J.R. (2012) Identification of myeloid derived suppressor cells in dogs with naturally occurring cancer. *PLoS One.* **7**: e33274.

Hiroi, M., Sakaeda, Y., Yamaguchi, H. and Ohmori, Y. (2013) Anti-inflammatory cytokine interleukin-4 inhibits inducible nitric oxide synthase gene expression in the mouse macrophage cell line RAW264.7 through the repression of octamer-dependent transcription. *Mediators. Inflamm.* **2013**: Article ID 369693.

Hirokawa, K., Utsuyama, M., Ishikawa, T., Kikuchi, Y., Kitagawa, M., Fujii, Y., Nariuchi, H., Uetake, H. and Sugihara, K. (2009) Decline of T cell-related immune functions in cancer patients and an attempt to restore them through infusion of activated autologous T cells. *Mech. Ageing Dev.* **130**: 86-91.

Horiuchi, Y., Tominaga, M., Ichikawa, M., Yamashita, M., Jikumaru, Y., Nariai, Y., Nakajima, Y., Kuwabara, M. and Yukawa, M. (2009) Increase of regulatory T cells in the peripheral blood of dogs with metastatic tumors. *Microbiol. Immunol.* **53**: 468-474.

Hoshino, Y., Takagi, S., Osaki, T., Okumura, M. and Fujinaga, T. (2008) Phenotypic analysis and effects of sequential administration of activated canine lymphocytes on healthy beagles. *J. Vet. Med. Sci.* **70**: 581-588.

Ionescu, D.C., Margarit, S.C., Hadade, A.N., Mocan, T.N., Miron, N.A. and Sessler, D.I. (2013) Choice of anesthetic technique on plasma concentrations of interleukins and cell adhesion molecules. *Perioper. Med.* **2**: 8.

Ishikawa, T., Kokura, S., Sakamoto, N., Matsumoto, T., Funaki, J., Adachi, S., Okayama, T., Uchiyama, K., Handa, O., Takagi, T., Yagi, N., Ando, T., Uno, K., Naito, Y.

and Yoshikawa, T. (2011) Adoptive cellular therapy enhances the helper T cell response and reduces the number of regulatory T cells. *Exp. Ther. Med.* **2**: 737-743.

Itoh, H., Kakuta, T., Kudo, T., Sakonju, I., Hohdatsu, T., Ebina, T. and Takase, K. (2003) Bulk cultures of canine peripheral blood lymphocytes with solid phase anti-CD3 antibody and recombinant interleukin-2 for use in immunotherapy. *J. Vet. Med. Sci.* **65**: 329-333.

Iwai, K., Soejima, K., Kudoh, S., Umezato, Y., Kaneko, T., Yoshimori, K., Tokuda, H., Yamaguchi, T., Mizoo, A., Setoguchi, Y., Kamigaki, T., Fujimoto, K. and Goto, S. (2012) Extended survival observed in adoptive activated T lymphocyte immunotherapy for advanced lung cancer: results of a multicenter historical cohort study. *Cancer Immunol. Immunother.* **61**: 1781-1790.

Jankovic, D., Kugler, D. G. and Sher, A. (2010) IL-10 production by $CD4^+$ effector T cells: a mechanism for self-regulation. *Mucosal. Immunol.* **3**: 239-246.

Judy, B.F. and Singhal, S. (2012) How can cytoreduction surgery improve the prospects for cancer patients receiving immunotherapy? *Immunotherapy.* **4**: 1077-1080.

Kamigaki, T., Matsuda, E., Okada, S., Naitoh, K., Kondo, T., Ibe, H., Maekawa, R. and Goto, S. (2014) Prospective evaluation of safety of immune-cell therapy for patients with various types of advanced cancer. *Anticancer Res.* **34**: 4601-4607.

Karanika, S., Karantanos, T.and Theodoropoulos, G.E. (2013) Immune response after laparoscopic colectomy for cancer: a review. *Gastroenterol. Rep.* **1**: 85-94.

Kawamura, A. Jr., Sekine, T., Sekiguchi, M., Yanoma, S., Kaneko, A., Haneda, T., Moriya, Y., Hayasaka, K. and Kakizoe, T. (2000) Six-year disease-free survival of a patient with metastatic eyelid squamous cell carcinoma and colon

adenocarcinoma after repeated postoperative adoptive immunotherapy. *Jpn. J. Clin. Oncol.* **30**: 267-271.

Kimura, F., Shimizu, H., Yoshidome, H., Ohtsuka, M. and Miyazaki, M. (2010) Immunosuppression following surgical and traumatic injury. Surg. Today. **40**: 793-808.

Kjaergaard, J., Hokland, M., Nannmark, U., Hokland, P. and Basse, P. (1998) Infiltration patterns of short- and long-term cultured A-NK and T-LAK cells following adoptive immunotherapy. *Scand. J. Immunol.* **47**: 532-540.

Kobayashi, N., Usui, S., Kikuchi, S., Goto, Y., Sakai, M., Onizuka, M. and Sato, Y. (2012) Preoperative lymphocyte count is an independent prognostic factor in node-negative non-small cell lung cancer. *Lung Cancer.* **75**: 223-227.

Koebel, C.M., Vermi, W., Swann, J.B., Zerafa, N., Rodig, S.J., Old, L.J., Smyth, M.J. and Schreiber, R.D. (2007) Adaptive immunity maintains occult cancer in an equilibrium state. *Nature.* **450**: 903-907.

Kwak, J.Y., Han, M.K., Choi, K.S., Park, I.H., Park, S.Y., Sohn, M.H., Kim, U.H., McGregor, J.R., Samlowski, W.E. and Yim, C.Y. (2000) Cytokine secreted by lymphokine-activated killer cells induce endogenous nitric oxide synthesis and apoptosis in DLD-1 colon cancer cells. Cell Immunol. **203**: 84-94.

Lee, Y.Y., Choi, C.H., Sung, C.O., Do, I.G., Hub, S.J., Kim, H.J., Kim, T.J., Lee, J.W., Bae, D.S. and Kim, B.G. (2012) Clinical significance of changes in peripheral lymphocyte count after surgery in early cervical cancer. *Gynecol. Oncol.* **127**: 107-113.

Li, J., Jiang, R., Liu, W.S., Liu, Q., Xu, M., Feng, Q.S., Chen, L.Z., Bei, J.X., Chen, M.Y. and Zeng, Y.X. (2013) A large cohort study reveals the association of

elevated peripheral blood lymphocyte-to-monocyte ratio with favorable prognosis in nasopharyngeal carcinoma. *PLoS One.* **8**: e83069.

Lin, E., Calvano, S. E. and Lowry, S. F. (2000) Inflammatory cytokines and cell response in surgery. *Surgery.* **127**: 117-126.

Lippitz, B.E. (2013) Cytokine patterns in patients with cancer: a systematic review. *Lancet Oncol.* **14**: e218-228.

Maher, S., Toomey, D., Condron, C. and Bouchier-Hayes, D. (2002) Activation-induced cell death: the controversial role of Fas and Fas ligand in immune privilege and tumour counterattack. *Immunol. Cell Biol.* **80**: 131-137.

Maher, S.G., Condron, C.E., Bouchier-Hayes, D.J. and Toomey, D.M. (2005) Taurine attenuates CD3/interleukin-2-induced T cell apoptosis in an in vitro model of activation-induced cell death (AICD). *Clin. Exp. Immunol.* **139**: 279-286.

Milne, K., Alexander, C., Webb, J.R., Sun, W., Dillon, K., Kalloger, S.E., Gilks, C.B., Clarke, B., Köbel, M. and Nelson, B.H. (2012) Absolute lymphocyte count is associated with survival in ovarian cancer independent of tumor-infiltrating lymphocytes. *J. Transl. Med.* **10**: 33.

Montes, M., Rufer, N., Appay, V., Reynard, S., Pittet, M.J., Speiser, D.E., Guillaume, P., Cerottini, J.-C., Romero, P. and Leyvraz, S. (2005) Optimum in vitro expansion of human antigen-specific CD8+ T cells for adoptive transfer therapy. *Clin. Exp. Immunol.* **142**: 292-302.

Moore, C., Eslin, D., Levy, A., Roberson, J., Giusti, V. and Sutphin, R. (2010) Prognostic significance of early lymphocyte recovery in pediatric osteosarcoma. *Pediatr. Blood Cancer.* **55**: 1096-1102.

Moore, K. W., de Waal Malefyt, R., Coffman, R. L. and O'Garra, A. (2001)

Interleukin-10 and the interleukin-10 receptor. *Annu. Rev. Immunol.* **19**: 683-765.

Nakamura, M., Takahashi, M., Ohno, K., Koshino, A., Nakashima, K., Setoguchi, A., Fujino, Y. and Tsujimoto, H. (2008) C-reactive protein concentration in dogs with various diseases. *J. Vet. Med. Sci.* **70**: 127-131.

Ni Choileain, N. and Redmond, H.P. (2006) Cell response to surgery. *Arch. Surg.* **141**: 1132-1140.

Noguchi, A., Kaneko, T., Naitoh, K., Saito, M., Iwai, K., Maekawa, R., Kamigaki, T. and Goto, S. (2014) Impaired and imbalanced cellular immunological status assessed in advanced cancer patients and restoration of the T cell immune status by adoptive T-cell immunotherapy. *Int. Immunopharmacol.* **18**: 90-97.

Olson, B.M. and McNeel, D.G. (2012) Antigen loss and tumor-mediated immunosuppression facilitate tumor recurrence. *Expert Rev. Vaccines.* **11**: 1315-1317.

O'Neill, K., Guth, A., Biller, B., Elmslie, R. and Dow, S. (2009) Changes in regulatory T cells in dogs with cancer and associations with tumor type. *J. Vet. Intern. Med.* **23**: 875-881.

Pestka, S., Krause, C. D., Sarkar, D., Walter, M. R., Shi, Y. and Fisher, P. B. (2004) Interleukin-10 and related cytokines and receptors. *Annu. Rev. Immunol.* **22**: 929-979.

Predina, J.D., Judy, B., Kapoor, V., Blouin, A., Aliperti, L.A., Levine, D., Okusanya, O.T., Quatromoni, J., Fridlender, Z.G. and Singhal, S. (2012) Characterization of surgical models of postoperative tumor recurrence for preclinical adjuvant therapy assessment. *Am. J. Transl. Res.* **4**: 206-218.

Romano, F., Caprotti, R., Bravo, A.F., Conti, M., Colombo, G., Piacentini, G., Uggeri, F. Jr. and Uggeri, F. (2003) Radical surgery does not recover immunodeficiency associated with gastric cancer. *J. Exp. Clin. Cancer Res.* **22**: 179-183.

Roxburgh, C.S., Horgan P.G. and McMillan, D.C. (2013) The perioperative immune/inflammatory insult in cancer surgery: Time for intervention? *Oncoimmunology.* **2**: e27324.

Schneemilch, C.E., Ittenson, A., Ansorge, S., Hachenberg, T. and Bank, U. (2005) Effect of 2 anesthetic techniques on the postoperative proinflammatory and anti-inflammatory cytokine response and cellular immune function to minor surgery. *J. Clin. Anesth.* **17**: 517-527.

Sekine, T., Shiraiwa, H., Yamazaki, T., Tobisu, K. and Kakizoe, T. (1993) A feasible method for expansion of peripheral blood lymphocytes by culture with immobilized anti-CD3 monoclonal antibody and interleukin-2 for use in adoptive immunotherapy of cancer patients. *Biomed. Pharmacother.* **47**: 73-78.

Sherger, M., Kisseberth, W., London, C., Olivo-Marston, S. and Papenfuss, T.L. (2012) Identification of myeloid derived suppressor cells in the peripheral blood of tumor bearing dogs. *BMC Vet. Res.* **8**: 209.

Sofra, M., Fei, P.C., Fabrizi, L., Marcelli, M.E., Claroni, C., Gallucci, M., Ensoli, F. and Forastiere, E. (2013) Immunomodulatory effects of total intravenous and balanced inhalation anesthesia in patients with bladder cancer undergoing elective radical cystectomy: preliminary results. J Exp Clin Cancer Res. **32**: 6.

Sottnik, J.L., Rao, S., Lafferty, M.H., Thamm, D.H., Morley, P.S., Withrow, S.J. and Dow, S.W. (2010) Association of blood monocyte and lymphocyte count and disease-free interval in dogs with osteosarcoma. *J. Vet. Intern. Med.* **24**:

1439-1444.

Stoecklein, V. M., Osuka, A. and Lederer, J. A. (2012) Trauma equals danger--damage control by the immune system. *J. Leukoc. Biol.* **92**: 539-551.

Sun, Z., Shi, L., Zhang, H., Shao, Y., Wang, Y., Lin, Y., Li, X. and Bai, C. (2011) Immune modulation and safety profile of adoptive immunotherapy using expanded autologous activated lymphocytes against advanced cancer. *Clin. Immunol.* **138**: 23-32.

Takayama, T., Sekine, T., Makuuchi, M., Yamasaki, S., Kosuge, T., Yamamoto, J., Shimada, K., Sakamoto, M., Hirohashi, S., Ohashi, Y. and Kakizoe, T. (2000) Adoptive immunotherapy to lower postsurgical recurrence rates of hepatocellular carcinoma: a randomised trial. *Lancet.* **356**: 802-807.

Teicher, B.A. (2007) Transforming growth factor-beta and the immune response to malignant disease. *Clin. Cancer Res.* **13**: 6247-6251.

Tominaga, M., Horiuchi, Y., Ichikawa, M., Yamashita, M., Okano, K., Jikumaru, Y., Nariai, Y. and Kadosawa, T. (2010) Flow cytometric analysis of peripheral blood and tumor-infiltrating regulatory T cells in dogs with oral malignant melanoma. *J. Vet. Diagn. Invest.* **22**: 438-441.

van den Tol, M.P., ten Raa, S., van Grevenstein, W.M., van Rossen, M.E., Jeekel, J. and van Eijck, C.H. (2007) The post-surgical inflammatory response provokes enhanced tumour recurrence: a crucial role for neutrophils. *Dig. Surg.* **24**: 388-394.

Vicente Conesa, M.A., Garcia-Martinez, E., Gonzalez Billalabeitia, E., Chaves Benito, A., Garcia Garcia, T., Vicente Garcia, V. and Ayala de la Peña, F. (2012) Predictive value of peripheral blood lymphocyte count in breast cancer patients

treated with primary chemotherapy. *Breast.* **21**: 468-474.

Watabe, A., Fukumoto, S., Komatsu, T., Endo, Y. and Kadosawa, T. (2011) Alterations of lymphocyte subpopulations in healthy dogs with aging and in dogs with cancer. *Vet. Immunol. Immunopathol.* **142**: 189-200.

Westerterp, M., Boermeester, M. A., Omloo, J. M., Hulshof, M. C., Vervenne, W. L., Lutter, R., Out, T. A. and van Lanschot, J. J. (2008) Differential responses of cellular immunity in patients undergoing neoadjuvant therapy followed by surgery for carcinoma of the oesophagus. *Cancer. Immunol. Immunother.* **57**: 1837-1847.

Whiteside, T.L. (2002) Apoptosis of immune cells in the tumor microenvironment and peripheral circulation of patients with cancer: implications for immunotherapy. *Vaccine*. **20**: A46-A51.

Whiteside, T.L. (2004) Down-regulation of zeta-chain expression in T cells: a biomarker of prognosis in cancer? *Cancer Immunol. Immunother.* **53**: 865-878.

Xie, F., Zhang, X., Li, H., Zheng, T., Xu, F., Shen, R., Yan, L., Yang, J. and He, J. (2012) Adoptive immunotherapy in postoperative hepatocellular carcinoma: a systemic review. *PLoS One.* **7**: e42879.

Yamada, R., Tsuchida, S., Hara, Y., Tagawa, M. and Ogawa, R. (2002) Apoptotic lymphocytes induced by surgical trauma in dogs. *J. Anesth.* **16**: 131-137.

Yamaguchi, Y., Hihara, J., Hironaka, K., Ohshita, A., Okita, R., Okawaki, M., Matsuura, K., Nagamine, I., Ikeda, T., Ohara, M. and Hamai, Y. (2006) Postoperative immunosuppression cascade and immunotherapy using lymphokine-activated killer cells for patients with esophageal cancer: possible application for compensatory anti-inflammatory response syndrome. *Oncol. Rep.* **15**: 895-901.

Yamashita, K., Fujinaga, T., Miyamoto, T., Hagio, M., Izumisawa, Y. and Kotani, T. (1994) Canine acute phase response: relationship between serum cytokine activity and acute phase protein in dogs. *J. Vet. Med. Sci.* **56**: 487-492.

Yoshimura, A., Wakabayashi, Y. and Mori, T. (2010) Cellular and molecular basis for the regulation of inflammation by TGF-β. *J. Biochem.* **147**: 781-792.

Zhang, G.Q., Zhao, H., Wu, J.Y., Li, J.Y., Yan, X., Wang, G., Wu, L.L., Zhang, X.G., Shao, Y., Wang, Y. and Jiao, S.C. (2015) Prolonged overall survival in gastric cancer patients after adoptive immunotherapy. *World J. Gastroenterol.* **21**: 2777-2785.

Zhang, J., Huang, S.H., Li, H., Li, Y., Chen, X.L., Zhang, W.Q., Chen, H.G. and Gu, L.J. (2013) Preoperative lymphocyte count is a favorable prognostic factor of disease-free survival in non-small-cell lung cancer. *Med. Oncol.* **30**: 352.

Zhong, J.H., Ma, L., Wu, L.C., Zhao, W., Yuan, W.P., Wu, F.X., Zhang, Z.M., Huang, S., You, X.M. and Li, L.Q. (2012) Adoptive immunotherapy for postoperative hepatocellular carcinoma: a systematic review. *Int. J. Clin. Pract.* **66**: 21-27.

Acknowledgements

First of all, I should like to express my deep gratitude to Dr. Fumihito Ohashi, my supervisor, Professor of Laboratory of Veterinary Surgery, Osaka Prefecture University, for his valuable guidance and supports.

I am grateful to Dr. Kazumi Sasai, Professor of Laboratory of Veterinary Internal Medicine, Dr. Terumasa Shimada, Professor of Veterinary Medical Center, and Dr. Hideo Akiyoshi, Associated Professor of Laboratory of Veterinary Surgery, Osaka Prefecture University, for their valuable advice and helpful comments on this thesis.

I would like to express my deepest appreciation to Mr. Akiyoshi Hayashi, Assistant Professor of Laboratory of Veterinary Surgery, Osaka Prefecture University, for excellent advice and supports.

I would like to offer my special thanks to Dr. Tomohiro Yamaguchi (Canine-Lab) for useful support to culture of T-LAK.

I am also thankful to the members of Laboratory of Veterinary Surgery and the veterinary medical staffs of Veterinary Medical Center, Osaka Prefecture University, for their cooperation and encouragement throughout these studies.

Finally, I would also like to express my gratitude to my family for their moral support and warm encouragements.